SCOPE 52

IPCS Joint Activity 19
SGOMSEC 8

METHODS TO ASSESS DNA DAMAGE AND REPAIR

Interspecies Comparisons

Edited by
Robert G. Tardiff
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Methods to Assess DNA Damage and Repair:
Interspecies Comparisons

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The mandate of SCOPE is to assemble, review, and assess the information available on man-made environmental changes and the effects of these changes on man; to assess and evaluate the methodologies of measurement of environmental parameters; to provide an intelligence service on current research; and by the recruitment of the best available scientific information and constructive thinking to establish itself as a corpus of informed advice for the benefit of centres of fundamental research and of organizations and agencies operationally engaged in studies of the environment.

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Foreword

The development of scientifically sound methodology for the assessment of human health and environmental risks from exposure to chemicals continues to be one of the objectives of the International Programme on Chemical Safety (IPCS). This objective also supports a major IPCS activity, namely: the preparation and dissemination of evaluations of the risks to human health and the environment from exposure to chemicals.

Many chemicals, both man-made and natural, are capable of reacting with DNA, thus having the potential to cause cancer, mutations and adverse reproductive outcomes. The complexity of the field and the rapid development of knowledge related to DNA damage and repair makes it extremely difficult to integrate this information into public health programmes. However, this can be accomplished when research scientists are given an opportunity to discuss their findings with scientists having the responsibility for the assessment and management of human health risks from exposure to genotoxic chemicals. By convening the workshop on "Methods to Assess DNA Damage and Repair: Interspecies Comparison," the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC) has provided such an opportunity.

The in-depth scientific review made by world leaders in the field will provide much needed guidance to those asked to use the results from studies in experimental animals to assess human health risks. Also, the results of this SGOMSEC activity will assist IPCS in developing further its activities related to the role of biomarkers in the overall process of health risk assessment.

Michel J. Mercier
Manager, International
Programme on Chemical Safety

Preface

The Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC) was established in 1979 at the initiative of Professor Norton Nelson from New York University. It is a non-governmental organization sponsored by IPCS (the International Program on Chemical Safety, established within WHO with the cooperation of UNEP and ILO), and the Scientific Committee on Problems of the Environment (SCOPE), itself a body of the International Council of Scientific Unions (ICSU).

The broad objective of SGOMSEC is to contribute to the reduction and prevention of risks caused to humans and non-human targets (ecosystems) by the introduction in the environment in increasing quantities of a large number of natural and man-made chemicals. The specific contribution of SGOMSEC is to assess the methodologies in use for the evaluation of these risks with a view to determine their values, to identify gaps and emerging needs, and to make recommendations for future research. Previous SGOMSEC projects have dealt either with general problems of chemical exposure and effects or with specific issues such as the non-intentional effects of pesticides or the consequences of large chemical accidents.

Volume 8 of SGOMSEC is concerned with a subject basic to chemical risk assessment: DNA damage and repair, taking account of interspecies differences. Many natural and man-made chemicals, as well as some physical agents, may react with DNA and thus produce harmful effect including cancer. However, mammalian species have various defence systems which may overcome the deleterious effects, of these genotoxic agents: metabolic and pharmacokinetic processes, DNA repair mechanisms, immunological processes, etc. This report evaluates current knowledge of the mechanisms of activation/detoxication of DNA damaging agents, organotropic and cell-structure effects, induction and repair of DNA damage, and the molecular and phenotypical analysis of mutation induction This evaluation is presented in seven contributed papers and a joint report which was prepared at a workshop held at the National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, North Carolina, USA in March 1990.

The joint report includes general recommendations aimed at improving research in this field and developing methodologies for genotoxicity testing, taking account of the potentials and limitations of available animal models.

The project was co-chaired by Professors H. M. Lohman of Leiden University and Gerald N. Wogan of MIT. They and the authors of the contributed papers, all experts in their respective fields, are thanked for their participation. We are also most grateful to Robert G. Tardiff (EA Engineering, Science and Technology, Inc., Silver Spring, Maryland) for his part in editing the report.

Special thanks are due to Dr. David Rall, Director of NIEHS, for hosting the

workshop at the US National Institute of Environmental Health Sciences (NIEHS).

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Part 1

JOINT REPORT

1 Introduction, General Conclusions, and Recommendations

1.1 INTRODUCTION

Numerous chemical and physical agents in the environment are capable of reacting with DNA. These potential DNA-damaging substances include not only man-made compounds, but also those of natural origin (e.g., food constituents and ultraviolet radiation). Fortunately, mammalian species possess powerful defence systems to overcome potentially harmful effects of dangerous agents. These systems comprise: (a) metabolic and pharmacokinetic processes that determine the absorption, movement, alteration, and detoxification of xenobiotic chemicals within organs and tissues of the body; (b) cellular DNA repair mechanisms; and (c) immunological and other defence processes for diseases such as cancer.

The complex nature of both the intrinsic and acquired potential for DNA damage from a myriad of chemical and physical agents together with limited knowledge of the organotropic and cellular defence mechanisms makes risk assessment, both qualitatively and quantitatively, of adverse biological responses in exposed humans very difficult. Consequently, due to developmental and economic factors, only limited test systems can be applied in practice. Moreover, tests in humans and even in some experimental animals must be restricted and, therefore, considerably limit confidence in extrapolation of results.

This monograph and the workshop from which it is derived are an attempt to evaluate the current state of knowledge of defence mechanisms and to apply this knowledge to estimate risk to health of humans exposed to substances that alter genetic material. The analysis is meant to be an overview rather than an exhaustive treatment of new and promising approaches. To limit possible bias introduced by a "windowed" view from the use of a particular species, a specific endpoint, or a limited class of DNA-damaging agents, emphasis is placed on integrating information obtained from various disciplines.

This evaluation deals predominantly with mechanisms of activation and detoxification of DNA-damaging agents, organotropic and cell-structure effects, the induction and repair of DNA damage, and the molecular and phenotypical analysis

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of mutation induction. Because DNA damage is the initiation step of diseases of genetic origin, this report highlights cancer as the adverse biological response of primary concern; therefore, the processes influencing promotion and progression of transformed cells are not included. For mechanistic studies relied upon in the current practice of risk assessment, several approaches are considered: (a) DNA adduct formation is viewed as a "key event" in the process of cancer formation; (b) the identification of agent-specific "key mechanisms" involved in the initiation of cancer; and (c) application of experimental tests applied among species to rank carcinogenic potency of chemical carcinogens—but only when the nature of such "key mechanisms" is known (e.g., the relative reactivity of various monoalkylating agents toward different nucleophilic centers in DNA, or the role of recombination events in the working mechanism of bifunctional agents). Two other reviews of this subject are recommended to readers: National Research Council (1989) and Volume **98** of *Environmental Health Perspectives* (1991).

In many cases, however, agent-specific "key mechanisms" are unknown. Risk assessment, in practice, is based on the evaluation of test results from a battery or from tier systems, or through interpretation of the total activity profiles of genetic and related effects (Genetic Activity Profiles [GAP], Waters *et al.*, 1987). Ultimately, ranking chemicals through analysis of the GAPs can be achieved through a consensus approach of ICPEMC (Lohman *et al.*, 1990); however, this ranking will not necessarily reflect the carcinogenic potency of chemicals involved in the study (Waters *et al.*, Chapter 13).

Genotoxicity can be compared qualitatively and quantitatively with the structure of compounds. This approach is called "structure–activity analysis" (SAR), whose aim is to differentiate molecular fragments within chemicals that impart a greater or lesser probability of biological injury for humans. However, limitations in our knowledge greatly restrict SAR to a few biological endpoints.

1.2 CARCINOGENESIS AS A MULTISTEP PROCESS

Carcinogenesis, a complex multistep process, may vary with the type of carcinogen, target site for tumour induction, and species. Although the actual mechanisms responsible for the induction of cancer are unknown, two or more genetic events may be involved, and cell proliferation is required. Genotoxicity can result from point mutations, chromosomal rearrangements, recombination, insertions or deletions of genes, and gene amplification. Cell proliferation is required to convert DNA damage into mutations and for clonal expansion of initiated cells, which also increases the probability of additional genetic events occurring in initiated cell populations.

For the majority of human carcinogens and for many carcinogens in laboratory animals, DNA damage is produced by electrophilic attack by the parent compounds or via their metabolites—hence, use of the term "genotoxic carcinogen." For the remaining carcinogens, their mechanism(s) of action appears not to involve detectable DNA damage—thus, they are referred to as "non-genotoxic carcinogens"

or "tumor promoters."

Major steps in the multistage process of chemical carcinogenesis are summarized as follows. Chemical carcinogens to which exposure takes place through ingestion, inhalation, or dermal contact are absorbed, distributed, metabolized, and excreted with rates that are determined by the chemical properties of the carcinogen and by biochemical and physiological factors of the host. Many biotransformation pathways produce detoxified products that are excreted. Chemically reactive electrophilic derivatives produced through similar metabolic pathways react with available nucleophilic centers in cellular constituents, including DNA, RNA, and proteins. Formation of covalent adducts with nucleophilic sites in DNA is vital to induce mutations which, according to prevailing opinion, are the initiating events in carcinogenesis. Most carcinogens produce a complex spectrum of adducts through electrophilic attack on multiple nucleophilic sites on DNA bases. These adducts vary in structure, stability, and, as a consequence, ability to induce mutations. Both quantitative and qualitative features of the adduct profile for any given carcinogen are known to be determinants of the carcinogenic response. The number of adducts formed in cellular DNA of humans or animals exposed to carcinogens is influenced by: (a) kinetics of absorption, distribution, and excretion of the parent substance; (b) enzymatic competence for metabolic conversion to electrophilic derivatives; and (c) cellular content of protective factors such as glutathione. Each factor is important in the determination of organotropic, interspecies and intraspecies differences in susceptibility.

DNA repair processes also affect both quantitative and qualitative characteristics of carcinogen–DNA adduct profiles. Mechanisms of DNA repair have been characterized extensively in prokaryotic cells. Although the process in higher organisms appears to share some features in common with that in prokaryotes, the process appears more complex in eukaryotic cells. Processes have evolved to more effectively repair damage to functionally important lesions (i.e., in transcriptionally active genes) than untranscribed regions. Several types of alterations in gene structure (namely, point mutations, deletions, sister chromatid exchanges, and chromosomal aberrations) are created by replication or recombination of damaged DNA. Accumulation of these lesions leads to the initial stages of transformation. Certain characteristics of the DNA repair process, such as the influences of adduct structure or transcriptional activity on repair efficiency, may be important influences on mutagenesis, and, therefore, may alter carcinogenic susceptibility.

1.3 REACTION KINETICS AND ADDUCT MONITORING

Sufficient information exists about the causal relationship between DNA damage induced by genotoxic chemicals and the occurrence of chemically induced tumours for DNA adducts and DNA repair to be recognized as sentinels of exposure to chemical carcinogens. Therefore, the following premise is widely supported: A population whose DNA contains adducts derived from a known chemical

carcinogen is likely to develop a higher incidence of tumours than a matched control group having far fewer adducts. The question thus arises: Can individuals in multiple groups be sorted according to levels of adducts and to levels of cancer risk consistent with levels of adducts? Presently, this separation cannot be accomplished for the following reasons:

1. Covalent binding of chemicals to DNA is an imprecise concept. That is, some DNA adducts are of greater significance with respect to carcinogenic outcomes. Specifically, some types or locations of adducts are removed faster than others, while others go unrepaired. Furthermore, the relative level of various adducts will change temporarily according to alternative rates of repair. In some cases, the *presence* of an adduct leads to mutation via miscoding during replication; whereas, in other cases, the error-prone *removal* of adducts leads to mutations. To complicate matters further, each factor varies according to the chemical, species, strain, sex, or tissue under study.
2. DNA adduction, damage, repair, and mutation represent only the initiation steps in the multistep process of carcinogenesis. Thus, the response of an individual to a given initiatory event will vary according to a wide range of influences referred to as toxicity, promotion, progression, immune response, and individual sensitivity.

Consequently, the measurement of adducts, DNA repair, and mutation provides a list of points of departure for complex phenomena whose outcome is certainly to be decided by a variety of additional factors. The absence of a correlation between adducts in tissues and carcinogenicity in organisms may be inevitable, because not all tissues with adducts develop cancer. Nonetheless, in cases where a compound-specific adduct is identified in a tissue that subsequently develops tumours, then positive correlations have been observed between these two parameters.

The previous considerations can have a perverse effect on seeking simple correlations. For instance, although two methylating agents (e.g., MNU and DMS) yield grossly similar levels of DNA methylation, minor O^6guanine (O^6G) methylation products derived from MNU are the determinants of cancer risk. This source of confusion may be magnified when adducts are measured in DNA surrogates such as protein or haemoglobin, leading to the possibility that the greater the extent of protein adduction, the less adduction at critical sites (e.g., O^2thymine (O^2T) or O^6G). This situation requires considerable caution when going from the qualitative assessment of exposure to carcinogens to extrapolating adduct data as a measure of carcinogenic risk. A fertile area of research to overcome such limitations is perhaps prospective epidemiology which measures secondary variables to seek correlations with incidences of cancer that become available in the future.

1.4 GENERAL RECOMMENDATIONS

1. Tolerance mechanisms for unexcised damage to DNA need to be further characterized, because they may play an important role in mutagenesis. Excision–repair is not the only way in which cells handle DNA damage. Tolerance processes (daughter-strand repair) exist whereby cells are able to cope with unexcised damage during DNA replication.
2. Research is needed into the efficiency, heterogeneity, and fidelity of the excision–repair process in different species, and in different tissues and stages of development within an organism. This effort should include purification of excision–repair gene products. Excision–repair in mammals is a highly complex process involving many gene products. More than seven proteins are required to carry out the incision step which can be effected by three polypeptides in *Escherichia coli*. The individual role of these many gene products in this process and their mechanism of action is not yet understood.
3. Research using whole animals is needed to define the extent to which interspecies similarities and differences can be extrapolated from cultured cells. Studies of molecular characterization of excision–repair in mammals, confined largely to cultured cells, have pinpointed some interspecies differences, e.g., the apparent inefficiency of rodent cells in excising cyclobutane dimers in the bulk DNA, when compared with human cells.
4. Relevant animal models should be developed to understand the influences of other factors on the relationships between repair, mutagenesis, and carcinogenesis. Such models are now becoming feasible as a result of measurements of targeted mutagenesis of repair genes and the production of transgenic mice engineered to be deficient in DNA repair. Such repair-deficient mice having heightened sensitivity to cancer induction could also serve in testing chemicals for carcinogenicity. From the xeroderma pigmentosum paradigm, defective repair has been implicated in increased mutagenesis and carcinogenesis; yet this relationship is quite simplistic. In Cockayne's syndrome and trichothiodystrophy, defects in DNA repair result in pathologies in the apparent absence of cancer.
5. The window through which one looks at mutational events should be broadened, for instance by determining mutation spectra in a variety of reporter genes.
6. Unselectable markers should be utilized to investigate mutation induction in transcriptionally inactive loci which might be relatively poorly repaired. Mutational events in such loci might become important when expressed later in life. Presently, mutation spectra are determined only in selectable marker genes.
7. Spectra in repair proficient and deficient conditions should be compared.
8. Investigations should be undertaken of the precise mechanism of mutation fixation. This objective can be achieved by using mutagen-sensitive mutants which are not deficient in DNA repair or via homology with yeast or *E. coli*.
9. Mutational spectra should be determined in whole animals using intrinsic genes, restricting the analysis mainly to one cell type, e.g., T cells or germ cells.
10. Transgenic organisms should be developed to study organ specificity among species and to facilitate risk assessment. While transgenic plant and fish can be

produced, no reporter gene designed for mutation detection has been incorporated in these organisms.

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2 DNA Damage

2.1 MOLECULAR DOSIMETRY

Carcinogen-induced DNA damage involves the formation of specific adducts with DNA and protein. Molecular dosimetry provides the tools to elucidate dose–response relationships for these adducts in carcinogen-exposed humans and laboratory animals.

Compared with measurement of external dose, molecular dosimetry offers the distinct advantage of integrating dose-dependent differences in absorption, distribution, biotransformation, and DNA repair to determine carcinogen dose more accurately. Moreover, molecular dosimetry is capable of determining which events play a critical role in eliciting cancer by examining, for instance, the dose–response relationships between DNA-adduct levels and the incidences of tumours. For example, cell proliferation is required to convert promutagenic DNA adducts to mutations. Since the extent of cell proliferation is known to be dependent on age, tissue, and dose, the impact of each factor on carcinogenesis in a specific population should be examined at the molecular level to improve the certainty in extrapolating a wide array of dose-response relationships for chemical carcinogens among organs and across species.

2.1.1 ABSORPTION AND DISTRIBUTION

Humans are exposed to chemical carcinogens most often by ingestion or inhalation. Consequently, differences in toxicokinetics are usable to anticipate within and among species major differences in the extent to which organs and tissues are exposed directly to a carcinogen. For oral intake, species differences in stomach pH or in metabolism of intestinal flora may affect the availability of a compound to the systemic target organ. Likewise, the formation of carcinogenic metabolites may depend on these conditions, as exemplified by the formation of nitrosamines from nitrite and secondary amines in the acid medium of the stomach. For exposure by inhalation, alveolar ventilation varies greatly among species, as illustrated by the fact that, on a body weight basis, the amount of a chemical inhaled by humans is only 10% of that inhaled by mice, so that for brief times, systemic inhaled doses are usually much less in humans than in mice. By contrast, the rate of elimination in humans is generally slower than in rodents, so that

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accumulation may occur more readily in humans than in mice. Physiologically based pharmacokinetic models can simulate such toxicokinetic behaviour of a compound in various species. Allometric scaling, a mathematical interpolation procedure, enables estimation of physiological parameters such as blood flow in a specified organ of a test species when such a parameter has been characterized for the same organ in some other species. Physiologically based pharmacokinetic models are already proving useful in estimating organ load, internal exposure, and covalent binding of reactive intermediates.

2.1.2 BIOACTIVATION AND DETOXIFICATION

To form DNA adducts, most carcinogens have to be converted by the body into reactive metabolites, usually more reactive than the parent compound. This process, bioactivation, is accomplished by enzymes (sometimes called "drug metabolizing" enzymes) present to varying degrees in numerous tissues of the body. These enzymes are families of isoenzymes having varying composition according to cell types and within and among the same and different species. The result is a difficulty in extrapolating the formation of metabolic by-products from one organ to another in the same species or from one species to another for the same organ. Such extrapolations are further complicated when results are obtained from one component of a cell (e.g., the microsomes), yet other cell components have similar capabilities. Moreover, some of these enzymes require the addition of co-factors to function properly outside the body; failure to do so in *in vitro* preparations results in erroneous conclusions about biotransformation reactions and toxic potential. The cytochrome P_{450} isoenzyme families are frequently recognized for their important role in bioactivation, leading often to overlooking the relatively important role of conjugating enzymes. Therefore, to obtain relevant findings from the measurement of biotransformation reactions, "metabolically competent cells" that contain not only P_{450} but also the full complement of xenobiotic transferases are needed.

These same enzymes can also detoxify carcinogens. Certain low molecular factors in the cell, like glutathione or methionine, may trap reactive intermediates before they can form appreciable levels of DNA adducts. Clearly the extent of adduct formation in a cell depends on the efficiency of detoxification and trapping, relative to bioactivation. The differences in every cell type and in every species of the composition of "drug metabolizing" enzymes are major determinants in tissue or cell specificity for carcinogenicity as well as of species differences. In genetically heterogeneous individuals of the same species, a wide variation in these enzyme activities exist, which may explain much of the interindividual variation in sensitivity to chronic toxicity of chemicals. Widely different DNA-adduct levels in the same tissue between individuals or between species may be correlated to differences in the balance between activation and detoxification. The possibility exists that compounds may be activated not only to DNA-adduct-forming

metabolites but also to metabolites able to function as cancer promoters. Therefore, the prediction is very difficult of the extent of formation of any given reactive metabolite when formed through metabolic pathways in which major cell, tissue, and species heterogeneity is present with respect to enzymatic competence for the reactions involved.

2.1.3 METHODS FOR MOLECULAR DOSIMETRY

Many chemical carcinogens or their metabolites form covalent adducts with nucleophilic sites on nucleic acids. Because formation of such DNA adducts is regarded as a key step in mutation and in the initiation of cancer, their measurements could be useful in estimating cancer risk. Such measurements should take into account inter-individual differences in dose, duration, absorption into susceptible tissues, and biotransformation.

Adduct levels vary among organs and cells. Furthermore, the spectrum of adducts also varies widely among carcinogens, yet only a few adducts—each subject to removal by chemical or enzymatic means—are likely to be critical to the induction of disease. The magnitude of genotoxic injuries are held to be correlated in some way to the persistent residues of critical adducts in target cells. For several carcinogens including aflatoxin B1, a linear relationship exists between administered dose and DNA-adduct concentration in experimental animals, regardless of whether the dosage was singular or repeated. Some non-linear correlations have been obtained in some cases, due perhaps to saturation of activation or detoxification pathways, thereby requiring the exercise of caution when extrapolating from high to low doses.

For such analyses, samples of human target tissues cannot be routinely collected; surrogates are needed. With parallelism between adduct levels in tissues of target and surrogate species, measurements in laboratory animals can be used to estimate those in humans. A linear relationship between adduct levels and external or internal doses of a carcinogen facilitates such extrapolations. Although such parallelisms and linear relationships have been observed between experimental animals and humans, generalizations are not yet possible.

The analysis of DNA adducts *in vivo* is complex because the adducts are formed in relatively small quantities (e.g., typically <100 fmol/μg DNA or <3 adducts per 10^5 nucleotides). Two techniques, immunoassay and ^{32}P-postlabelling, can detect DNA adducts in either experimental animals or humans. The immunological approach requires that monoclonal or polyclonal antibodies be produced against either carcinogen-modified DNA or carcinogen-nucleoside adducts coupled to a protein carrier. The resulting antibodies are then used to quantify specific adducts by either of two methods: radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). The quantity of adduct detectable by immunoassays depends on the specific method, with ELISA generally giving greater sensitivities (~1 adduct per 10^7 nucleotides) than RIA. Both methods are

relatively inexpensive, and can be used to process many samples. However, their sensitivity is generally limited by the amount of DNA that can be analyzed. Furthermore, because an antibody may cross-react with a broad spectrum of adducts, the findings of DNA adducts may be complicated for individuals with unknown exposures to alkylating substances. These limitations can be overcome partially by combining chromatographic purifications (e.g., HPLC) with the immunoassays.

Under appropriate conditions, the ^{32}P-postlabeling method can detect DNA adducts at nearly one adduct in 10^{10} nucleotides, but is limited in its ability to identify adducts. Where carcinogen exposures are known, adducts can be identified by comparing their chromatographic mobilities to standards—an impossibility for unknown substances. Quantification of adducts can be performed reliably by comparison to standards when the adducts have been identified, but not for unknown entities. The shortcomings with immunoassays and ^{32}P-postlabelling emphasize the need for much improved methods to detect DNA adducts, such as the recently developed mass and fluorescent spectrometric techniques.

As an alternative to measuring adducts to DNA, adducts to haemoglobin and serum albumin have been used for molecular dosimetry. Gas chromatographic/mass spectrometric and immunological methods have been developed and applied to these human tissues. Protein adducts are not repaired, and have a relatively slow turnover rate, permitting their accumulation and facilitating their measurement. A decided advantage of protein is its ready availability in relatively larger quantities than for DNA, hence increasing appreciably the sensitivity of such measurements.

2.1.4. EXAMPLES OF CARCINOGEN DOSIMETRY IN HUMANS

Illustrations of the use of molecular dosimetry for four carcinogens are presented below.

Vinyl chloride monomer (VCM) VCM, a recognized human carcinogen, offers an excellent opportunity for cross-species comparisons because: (a) reliable exposure and epidemiological data on VCM are available; (b) it is the only known organic compound that produces liver angiosarcomas in humans; and (c) its mechanism of carcinogenic action and its dosimetry have been investigated in great detail.

Studies in rats have clearly established a dose dependency for VCMs rate of metabolism. A greater percentage of administered dose is metabolized at low versus high doses, leading to a supralinear dose–response for VCM metabolism and carcinogenesis. The carcinogenic effects of VCM appear entirely due to its ultimate metabolite, chloroethylene oxide.

Several DNA adducts are formed in tissues of rats exposed to VCM. The major adduct, 7-(2″oxoethyl)guanine (Oxel-G) causes no miscoding upon replication. Three etheno adducts are formed in tissues exposed to VCM, and each causes base-

pair mismatch on replication. Because the known adducts of VCM and nucleic acid bases involve only N atoms, the genotoxic effects of VCM may result from *N*-alkylation of DNA bases rather than from *O*-alkylation. After cessation of exposure, Oxel-G is removed rapidly from DNA with a half-life in liver of approximately 62 hours; by contrast, the three etheno adducts were stable or poorly repaired, and their levels in the liver were unchanged for up to 14 days.

In neonatal and adult animals, the levels of etheno adducts resulting from exposure to VCM were found to be reliable indicators of tissues at risk of tumour development. The miscoding potential and persistence in DNA of these etheno adducts provide evidence that they may play an important role in VCM-induced carcinogenesis through the production of point mutations. These DNA-etheno adducts, therefore, should be sensitive and reliable markers of molecular dosimetry of VCM-exposed humans and animals. In the same way that the carcinogenic potency of VCM in humans can be estimated from the incidence of liver angiosarcoma in VCM-exposed individuals occupationally, interspecies comparisons made with rats, mice, and hamsters demonstrate that the TD_{50} of VCM was similar, although no correction for differences in metabolism were made.

Dimethylnitrosamine (DMN) Simple methylating agents such as DMN are among the best studied carcinogens for their ability to alkylate DNA. Early investigations demonstrated no correlation between target and non-target tissues for the formation of either 7-methyl-deoxyguanine (7-met-dG), the major adduct formed by DMN, or simple methylating agents such as 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Subsequently, attention was focused on O^6-methylguanine, an adduct predicted to cause base-pair mismatch and which persists in brain, not liver, of exposed rats. The presence of this adduct correlated with the target site for tumor induction by these agents. The dose–response for 7-met-dG was different from that of O^6-met-dG in liver DNA. The greater efficiency for repair of O^6 methylguanine (O^6MetG) at low doses is the result of a saturable DNA repair protein, O^6-metG-DNA-methyltransferase. This highly efficient repair system effectively restores the alkylated base to its normal counterpart, and inactivates the protein preventing repair. For molecular dosimetry, when the DNA repair protein is saturated, the amount of O^6-met-dG present in DNA increases per unit dose of carcinogen, causing a nonlinearity in the dose–response curve, i.e., the slope of the dose–response for O^6-metG increases dramatically above a specified dose range. Such data together with those on cell proliferation appear to be vital to the shape of the dose–response curves for cancer induced by these agents. These data imply that the damage caused by methylating agent-mediated DNA alkylation in target tissues of either humans or laboratory animals is modulated by factors such as tissue distribution, metabolism, DNA repair, and cell replication. Thus, the levels of DNA adducts in a human tissue is likely to be different among individuals exposed to the same dose of methylating agent.

The presence of O^6-met-dG and O^7-metG in human liver DNA was described for the first time in two persons who were poisoned by DMN (estimated exposure of

more than 20 mg DMN/kg bw). More recently, O^6-met-dG was detected in surgical specimens of oesophageal and stomach mucosa taken from patients belonging to populations at very different risk of cancer for these anatomical sites. These patients came from the People's Republic of China or France; the nature of methylating agents to which they were exposed is unknown. O^6-Met-dG was also found in placenta and peripheral lung DNA of smoking and non-smoking individuals and in the oral mucosa of DNA from cigarette smokers.

DMN, a multispecies and multi-organ carcinogen, has been studied extensively for its tissue distribution, metabolism, and DNA repair in rodent and human tissues and cells. The preponderance of evidence indicates that dosimetry data obtained in humans were comparable with those measured in organs of species in which DMN produced tumours, implying that DMN may under high-dose circumstances is a carcinogenic risk to humans. To establish causality, DNA adduct levels in individuals need to be correlated with cancer incidence obtained in molecular epidemiology studies, which to date have not been undertaken.

Aflatoxin B_1 (AFB) Exposure to AFB has been associated to the induction of liver tumours in a wide variety of species including humans. Among experimental animals, the relative sensitivity to AFB in causing hepatocarcinogenesis decreases in the following order: trout > rat >> hamster ≈ mouse ≈ salmon. A comparable dose–response relationship holds for the extent of binding of AFB to liver DNA, as illustrated by the observation that hepatic DNA binding is linear over a 10^5-fold dose range for rats treated with a single dose of AFB. A linear correlation has also been observed for the binding of AFB to plasma proteins. In rats and trout, steady-state hepatic DNA adduct levels were observed at approximately 1 month of continuous administration of AFB. In both species, steady-state adduct concentrations is linearly related to the concentration of AFB administered chronically. Furthermore, if the steady-state adduct levels are compared with the incidence of hepatic tumours, a nearly identical linear relationship is obtained for both species. In humans, a highly significant correlation has been observed between the daily consumption of AFB, the concentrations of serum AFB albumin adducts, and urinary AFB nucleic acid adducts.

Ethene and ethylene oxide Ethylene oxide (EtO) is carcinogenic in rats, and has been linked to an increased incidence of leukaemia in exposed workers. EtO exposure can be monitored by analysis of haemoglobin adducts, and this procedure has been applied to workers whose increased exposure to EtO was found to be mirrored by corresponding increases in adduct concentrations.

Ethene is metabolized to EtO, and thereby gives rise to the same adducts in DNA and haemoglobin. Haemoglobin adducts have been increased in cigarette smokers and in occupationally exposed workers; however, low levels of the same adducts have been detected in presumably unexposed individuals. The level of ethene in urban air and environmental tobacco smoke is considered to be too low to account for the adduct levels detected. The designation of "unexposed" individuals is

complicated by ethene's production metabolically in humans and in experimental animals through lipid peroxidation. This endogenous production of ethene is the most likely source of adducts detected in unexposed individuals.

2.2 CROSS-SPECIES SENSITIVITY IN CARCINOGENIC RESPONSE

Carcinogenicity data for 770 compounds administered by various routes of exposure in approximately 3000 rodent bioassays allow comparisons of cancer rates among animal species. For a carcinogen administered by the same route, either the TD_{50} (in mg/kg per day) or the ratio of minimum TD_{50}s can be used to estimate the relative cancer potency between two species (from among rats, mice, and hamsters). The geometric means of the ratios of minimum TD_{50} for rat/mouse was estimated to be 1:2.2 and 1:3.3 for dietary and gavage exposures, respectively. For many compounds, the minimum TD_{50} for each of the three rodent species is generally within a factor of 100 of one another. Such overall agreement provides justification to extrapolate tumor rates across species including from rodents to humans. Consistently high correlations have also been obtained for the potencies of carcinogens in human and laboratory species, and the potencies are similar to those demonstrated between mouse and rat. Interspecies sensitivities appeared to be less than a ratio of 5:1 for both human/mouse and human/rat. Relative sensitivity is equal to $(\text{potency})^{-1}$, with potency equal to 1 in the mouse.

Correlations among cancer potencies of 23 substances (mostly genotoxic) known to cause cancer in animals and humans were examined using epidemiological data and animal bioassays. Potency was expressed as TD_{25} (in mg/kg bw/day). A log-log plot yielded a statistically significant correlation of $r \approx 0.890$ ($p < 0.001$). Adjustment for metabolic rate was made by assuming the human–animal equivalent daily dose to be proportional to dose absorbed per square meter of surface area. Adjustment is performed by multiplying animal dose by $(Wt_a \text{ and } Wt_h)^{1/3}$, whereby W_a and W_h represent the average body weight of a species of laboratory animal and humans, respectively). Using this adjustment, humans are expected to be six and 14 times more sensitive than rats and mice, respectively. The available data, although limited by the small number of chemicals studied and by the large errors for human risk estimates, justify cautious extrapolations across species of carcinogenic potency of chemicals; however, measurements of DNA or protein adducts are likely to improve such risk estimates for humans. A major source of uncertainty in the epidemiological analyses on which cancer potency estimates are based stems from poor exposure data, providing an incentive for the future application of molecular dosimetry methods in clinical and epidemiological investigations.

2.3 APPROACHES TO PREDICT CANCER ACTIVITY AND POTENCY

2.3.1 STRUCTURE–ACTIVITY RELATIONSHIPS

Some chemicals induce carcinogenic effects in mammals, while others are inactive. Some aspects of chemical structure, therefore, may be causally associated with chemical carcinogenesis, thereby providing an opportunity for the study of structure–activity relationships (SAR). Models for SAR rely on defined toxicity caused by certain molecular fragments or structures to predict carcinogenicity among chemicals for which no carcinogenicity data exist. To reliably estimate which compounds are more likely to cause cancer, the amount of data that must be analyzed is large; therefore, these data must be analyzed using computerized systems, particularly those that rely on artificial intelligence. Meaningful correlations between chemical structure and carcinogenic potency, however, do not imply causality, and, therefore, extrapolations from such correlations are not always possible.

Given those basic premises, the next step is differentiation of information at the chemical and biological levels. Relationships between chemical reactivity (as a surrogate of carcinogenicity) and structure should be established for the most likely chemical classes. This foundation should then be used to synthesize a flexible multiclass generic SAR model.

2.3.1.1 Fragmentation of the chemistry

A chemical is defined by its structure. Yet when a chemical elicits a response in a biological system, one or more of its many physico-chemical attributes is responsible. Understanding the systematic way in which these attributes function in biological systems is the foundation for deriving a causal relationship between structure and activity. Such attributes include:

1. Substructural fragments. The simplest and most often used parameter, key substructures such as $ArNO_2$, CH_2Cl, and ring oxide, are recognized for their high correlation with biological activity. These methods are capable of being self-learning and self-correcting. For example, *p*-chloronitrobenzene will initially be classed both as ArCl and as $ArNO_2$; but if the database is self-learning, it will soon associate mutagenicity with $ArNO_2$ and remove an association for ArCl.
2. Molecular shape. Current computerized correlative SARs methods represent structure in two dimensions; however, for some activities (e.g., a receptor interaction), 3-dimensional molecular shape may be the primary determinant. Furthermore, steric effects can dramatically modulate reactivity; e.g., neopentyl bromide is unreactive due to crowding of its bromomethyl group, and, therefore, is not a simple analog of ethyl bromide.
3. Solubility and partition properties. Absolute solubility and log P, which are essential to determine bioavailability, can be either estimated or measured.
4. Chemical stability. Rates of hydrolysis and degradation of a parent

compound or its metabolite(s) can be a major determinant of biological activity (e.g., local versus systemic effects; effects seen *in vitro* versus *in vivo*).

5. Chemical classes. Chemical classes can be designated by the capacity for certain types of reactions, (electrophiles, alkylating agents, or Michael reactivity); however, not all electrophile–nucleophile pairs are equally reactive. Thus, the Swain–Scott equation provides data on relative O, N, S reactivity via *s* values. Extension to aromatic amine nitrenium ions can be achieved. Acrylamide, a Michael reactive agent, is reactive primarily with protein.
6. Metabolic activation/deactivation. The potential for metabolic activation and deactivation can be critical to SARs, but are difficult to predict.

2.3.1.2 Fragmentation of biological parameters

All tissues of all species are not equally susceptible to carcinogenesis. Some of the attributes include:

1. Species, sex, tissue specific metabolism. As data on these variables accrue, they will enhance databases. Furthermore, differences between these and those from S9 fractions will resolve many inconsistencies and explain the absence of expected correlations.
2. Detoxification. This parameter may have a greater impact *in vivo* than *in vitro*.
3. Response to DNA adducts. Not all DNA adducts lead to the same biological changes. Existing data could refine biological data, such as differences in: (a) key enzymes between tissues or species; (b) DNA repair pathways; (c) rates of repair between tissues or species; and (d) DNA sequence specificity for adduct formation and repair.
4. Tumor classification. If tumors are listed by site, species, and sex, more refined SARs are likely to result.
5. Non-genetic toxicity. Such data are usually available, and may contribute key information to improve or refine correlations. Thus, liver or thyroid toxicants predispose to cancer at those respective sites. The hormonal properties of a chemical or its mitogenicity are also potentially important parameters.
6. Non-genotoxic carcinogenesis. A generally accepted concept worthy of separate treatment from electrophilic carcinogens, non-genotoxic carcinogens (e.g., non-mutagenic mouse liver specific carcinogens) have proven difficult to predict, thereby requiring a different SAR approach than that used for genotoxic carcinogens (e.g., mutagenic epoxide skin carcinogens).
7. Genetic activity profiles. Such profiles can provide a valuable means of biological recognition of unexpected chemical classes or biological patterns.

Consensus correlations Methods exist by which a large volume of data can be

merged so that a chemical with divergent activities can be categorized by consensus to either a probable genotoxicant or a non-genotoxicant, and likewise to a probable carcinogen or non-carcinogen. However, such SARs are less precise than those derived within a specific chemical class and having a documented biological endpoint.

2.4 GENOTOXICITY PROFILES IN EUKARYOTES

Progress in understanding how genotoxicants exert their biological effects in diverse species has been impeded by the lack of a concept by which physicochemical properties are linked to mutation spectra. The "single-endpoint" approach (i.e., relating induced genetic damage to exposure levels) is of limited value to predict relative carcinogenic potency of chemicals in mammals, despite its wide application in more than 150 short-term tests for mutagenicity. For example, attempts to correlate relative carcinogenic potency of chemicals with qualitative mutagenicity data obtained from a single test was an oversimplification of a complex matter, as demonstrated repeatedly for a wide array of genotoxic carcinogens also tested in the *Salmonella* assay.

Multi-endpoint analysis in eukaryotes combined with SAR considerations provides a more useful tool to categorize genotoxicants with known mechanism(s) of adversely affecting DNA. Fundamentally, this approach compares various endpoints with each other (e.g., gene mutation induction with clastogenic effects) and evaluates genotoxicity profiles against the following parameters: (a) nucleophilic selectivity as expressed by Swain–Scott's s values and/or N^7/O^6-alkyl dG ratios; and (b) relative carcinogenic potency (e.g., TD_{50} estimates in mg/kg bw) against nucleophilic selectivity. In addition, either modifying DNA repair conditions or assessing enhanced genotoxicity (e.g., hypermutability) in relation to SAR parameters can provide insight as to the role of various promutagenic lesions and to the significance of repair in mutation fixation.

In particular the Swain–Scott empirical linear relationship has proven valuable in not only understanding but also predicting the great diversity of genotoxicity profiles exhibited by numerous alkylating agents. Two sets of data emerged from multi-endpoint analysis of 60 genotoxic chemicals, mostly alkylating carcinogenic, in rodents and *Drosophila*: (a) carcinogenic potency and hypermutability were correlated linearly with the s value; (b) monofunctional arachidonic acid that can alkylate oxygen atoms had lowest TD_{50} values; and (c) genotoxic agents capable of cross-linking DNA could be separated clearly from those giving the monoadduct. Thus, these multi-endpoint analyses should assist in the quantitative evaluation of risk from genotoxic agents.

3 DNA Repair and Mutagenesis

3.1 STATE OF KNOWLEDGE

Coping with genetic injury caused by environmental agents, all living organisms are equipped with an intricate network of repair processes. The excision–repair pathway serves as a valid model to assess the role of repair systems as general cellular defence mechanisms against cancer, genetic defects, and ageing. As one of the most extensively studied DNA repair processes in both prokaryotes and eukaryotes, nucleotide excision appears to be not only universally present but also of major importance, since it covers a broad range of DNA lesions induced by UV-irradiation or by chemicals causing bulky adducts and cross-links. These conclusions are derived from numerous studies of cellular biochemistry, such as mutants altered by DNA repair, the isolation of genes controlling repair, and functional analysis of encoded proteins. These data have culminated in a detailed understanding about the mechanisms and specificity of this system in the prokaryote *Escherichia coli*. Excision-repair can be effected *in vitro* by six purified gene products from *E. coli*, of which three are involved together in scanning the DNA, initial recognition of the damaged site, and incision of DNA on both sides of the lesion.

Understanding the process in eukaryotes is less advanced. The overall process appears to be similar, but the mechanism much more complex. In yeast, at least 10 genes are involved, many of which have been cloned and have shown little resemblance to the genes involved in *E. coli*. In mammals, excision–repair is delineated by a minimum of nine genetically distinct mutations in humans, which are represented by the human repair disorders xeroderma pigmentosum (XP) and Cockayne's syndrome (CS). How much overlap exists between these two complementation groups is unknown.

Several human repair genes have been cloned. The most crucial finding in the context of interspecies relationships is the high level of sequence conservation between yeast and human genes. Furthermore, cloned human genes are able to correct the defects in rodent mutants. The findings of interspecies functional complementation and sequence conservation suggest that the mechanism of excision repair may be similar between two extreme members of the eukaryotic kingdom. From these observations, the basic mechanism of excision repair is likely to be similar in most eukaryotes. Thus differences in genotoxicity between species are unlikely to be caused by large differences in the mechanism of excision repair. The

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same conclusion may hold for enzymes involved in other repair processes such as uracil glycosylases and methyltransferases. Nevertheless, important differences in the efficiency of excision repair may be present.

3.2 IMPACTS OF PREFERENTIAL REPAIR

Recent indications that some DNA repair processes are not homogeneously distributed over the genome could have major implications for the relationship between DNA damage frequencies and biological endpoints of interest. For CPD induced by UV light, repair of the transcribed strand of several different genes has been demonstrated to be much more rapid than repair of the complementary strand or the genome as a whole. This condition has been found in *E. coli*, a plasmid-borne gene in yeast and cultured from rodent and human cells.

Actively-dividing UV-irradiated rodent cells in culture present an extreme case. These cells remove CPD very poorly from their genomes, yet remove them very efficiently from the transcribed strands of the few genes examined in detail. Should such characteristics hold for animal cells, poorly repaired silent sequences might accumulate DNA damage or unexpressed mutations. This situation could greatly increase the probability of altered expression upon activation, either in a normal developmental manner or in some abnormal way. Unrepaired damage might itself contribute to gene activation by either interfering with normal DNA–protein interactions at specified regulatory sequences or by promoting DNA rearrangements. Such processes might be particularly relevant to activation of some oncogenes. In any event, determination of mutation frequencies in active genes might seriously underestimate frequencies in inactive sequences.

Whether this specific deficiency in CPD repair in non-transcribed sequences in cultured rodent cells reflects the situation in animals is unclear. The meagre evidence available suggests that repair is more efficient in the whole animal; but more analysis is warranted. Presently, no evidence exists to bear on the question of repair heterogeneity, promoting the need to pursue research in this area.

Human cells in culture exhibit "preferential" repair of CPD in transcribed strands, which is observed as an increase in the rate of repair, because the removal of CPD in the genome overall in this case is quite efficient. Although this activity suggests that preferential repair has little effect on long-term endpoints in humans, extreme repair heterogeneity between active and silent sequences might occur at the continuous low background levels of DNA damage. Furthermore, even at damage frequencies used for experiments with cultured cells, chromatin domains may exist whose repair is significantly less efficient than that of the average. Very few data are available on repair in silent sequences to address this possibility, prompting the need for such measurements as well as for those of preferential repair in human tissue, particularly lymphocytes.

For chemical carcinogens, the picture is much more complex. Indirect evidence for the preferential repair and formation of O^6-MetG in transcriptionally active

DNA in rat liver has been presented, yet no technique is available to assay this important DNA adduct in specified sequences. Evidence against the operation of preferential repair of *N*-methylpurines has been presented for rat liver and for CHO cells; likewise, preferential removal of aflatoxin B adducts from an active sequence in Chinese hamster ovary cells has not been observed. However, in cultured human cells, some indication of more rapid removal of AFB adducts from active genes compared with an unexpressed gene has been presented.

The lack of systematic comparisons also plagues any attempt to compare repair of bulky chemical carcinogens by human and rodent cells in culture. To date, rarely have precise measurements been made side-by-side with two cell types. Such comparisons might be useful when attempting to extrapolate to humans from data obtained in rodents.

3.3 MECHANISMS OF MUTAGENESIS

Most chemical mutagens introduce more than one type of DNA adduct. The contribution of each DNA adduct to the mutagenic events caused by a chemical mutagen can vary greatly. The spectrum of mutagenic events in relation to DNA adducts are usually analysed in a selectable reporter gene. Analysis of the spectrum of mutational events provides information about the relative contribution of these DNA adducts to mutation induction in a specific reporter gene. Removal of a particular DNA adduct by DNA repair processes will influence the spectrum of DNA adducts present at the time of DNA replication, and, therefore, will change the spectrum of mutational events observed. Comparison of such spectra in repair-deficient and repair-proficient conditions provides a tool with which to investigate the relative importance of the various DNA adducts.

All methodologies for studying genetic changes, including cytogenetic techniques, give a selective view on the mutational capacity of a chemical. Some reporter genes allow only detection of selectable phenotypic changes after specific changes in the DNA sequence; others are sensitive to a relatively large variety of mutational events. The latter ones will be more informative, for instance, to investigate the capacity of a chemical to introduce genetic changes. To get a full sense of the capabilities of a chemical to cause genetic damage, the effects on several reporter genes should be investigated, i.e., the window through which one looks at mutational events should be as broad as possible.

The process of malignant transformation is currently formulated to incorporate the sequential acquisition of genetic lesions at loci intimately involved in the control of cellular proliferation and differentiation. Mutational events responsible for unleashing the transforming potential of proto-oncogenes and for reducing the ability of other loci to serve as suppressors of unrestrained cellular growth are well documented. However, the spectra and mechanisms of carcinogen-induced oncogene activation have been characterized extensively solely for the *ras* family of proto-oncogenes, which represent a relatively small fraction of suppressor genes.

Furthermore, systematic surveys of mutational events occurring at these loci for particular tumor types have not been reported; therefore, links between an agent, adduct formation, misrepair, and mutation at these critical gene loci remain to be determined.

A major questions remaining is: To what extent do the molecular changes that lead to tumor formation overlap with the molecular changes observed from activity of reporter genes used in mutation assays? Genotoxic carcinogens are likely to show some overlap between these two types of molecular spectra.

4 DNA Repair, Mutagenesis, and Risk Assessment

4.1 INTRODUCTION

The following analysis is based on the premise that improved methodology for risk assessment will issue from improved understanding of the fundamental processes underlying the formation of DNA adducts and their repair, and of mutagenesis, which may result should repair fail. Studies of the mutational specificity of DNA damaging agents can implicate specific DNA adducts as precursors to mutations and perhaps neoplastic changes. Molecular techniques are available by which such causal relationships can be tested. These studies are meant to identify specific DNA adducts that are expected to be the most reliable predictors of genotoxicity, and, hence, for monitoring human populations. Similarly, knowledge of the chemical structures of these adducts that result in genetic changes may provide insight into the exact nature of the metabolites that originally reacted with DNA. These data in turn may be used to identify specific adducts of a chemical and an amino acid within protein; such adducts could prove to be excellent dosimeters in blood proteins.

Several studies showing that differential kinetics of adduct removal in various organs provide a likely explanation for the organotropic effects of a DNA damaging agent. These results demonstrate the importance of studies of the removal of adducts from DNA. This argument is strengthened by the existence of human genetic disease (often associated with a high risk of cancer) associated with deficiencies in DNA repair. This association is especially prevalent for clinical syndromes such as xeroderma pigmentosum, where a defect in repair of UV light-induced damage is responsible for the disease. Speculatively, other sub-populations may exist in which DNA repair capability is diminished but not lacking totally. In principle, individuals in these groups are at elevated risk, and should be identified. However, no methodology exists to achieve this objective. The isolation of the proteins involved in repair and the genes that encode them should provide new tools by which an assessment of the risk of genetic disease can be made.

Finally, the relationship between repair of DNA adducts within specific gene sequences and changes of functional importance in tumor initiation or development is difficult to prove. Although mutational changes within oncogene or tumor suppressor gene sequences are known to occur with varying frequencies in cancers of different types, the molecular precursors to the mutational changes have yet to

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be identified. A possible modulating factor in carcinogenesis, which could explain inter- or intraspecies differences as well as organotropic effects, might be the selective repair of critical oncogenic loci. For example, a proto-oncogene sequence in a transcriptionally active gene might be subject to repair in a species refractory to carcinogenesis; whereas the same locus could be transcriptionally quiescent, and hence unrepaired, in a species sensitive to the carcinogenic regimen. Thus, differential DNA damage or repair as a consequence of differential gene transcription may possibly be contributing factors.

4.2 MONITORING OF HUMAN POPULATIONS

Several markers in humans are available to measure directly genotoxicant exposures and effects. These markers are being proposed to monitor exposures to mutagens and carcinogens. Implicit in proposals for human biomonitoring is that the process is of value to the individuals being studied. Benefits include: (a) identification of exposed individuals; (b) identification of the environmental mutagens/carcinogens; and (c) definition of range of susceptibility among humans to mutagens and carcinogens.

For an individual, the value of biomonitoring relies on the ability to differentiate between exposed persons likely to become ill from those unlikely to do so. This ability rests on the assumption that the markers being measured are valid surrogates of disease-producing events and that the endpoints measured are directly related to disease manifestation. This assumption can be tested directly in humans, providing a fourth advantage for human biomonitoring: Linking quantitatively genotoxicant exposure with disease incidence to estimate risk in other circumstances.

The genotoxicity markers addressed in this report include DNA adducts as markers of critical target tissue, chromosomal, and gene interactions. Damage to genes in somatic cells has also been analyzed at the molecular level to identify the spectrum of mutations that may indicate exposures to specific mutagens or carcinogens. At a less complex level, human population exposures may be described by either ambient monitoring or questionnaire. Finally, individuals can be characterized hypothetically, with respect to their ability to metabolize various classes of mutagens or carcinogens (pharmacogenetic characterization) and to their ability to repair DNA damage (genetic DNA repair deficiency states).

In a human population, the level of intervention justifiable for monitoring is that minimal amount needed to identify individuals with increased health risk due to genotoxicant exposures. However, intervention at this minimal level presupposes that markers of true disease relevance are known. Humans being exposed to genotoxicants are available to make this determination. Malignant diseases are being treated and cured in many individuals; unfortunately, curative therapeutic regimens often involve the use of mutagenic and carcinogenic agents. Exposure to such agents occasionally results in a second malignancy in treated individuals—usually haematological malignancy such as acute non-lymphatic

leukaemia (ANLL). Although relatively high in some patient groups (i.e., ≈ 5%), the risk of ANLL is quite acceptable, given the almost certain death from the original untreated malignancy. However, this situation provides a human population (knowingly exposed to relatively precise doses of specific mutagens and carcinogens) for study with currently available markers of genotoxicant exposure and effect. The findings of such studies would reveal relationships between markers and diseases and the degree of heterogeneity in human susceptibility.

Most current markers of genotoxicant exposures and injuries employ DNA from white blood cells or haemoglobin from red blood cells. ANLL is a disease of haematopoietic stem cells, whereby the tissue being monitored and that of the disease are identical. Although lymphocytes are usually the white blood cells being monitored, recent findings indicate a relationship between these cells and the cells involved in ANLL. ANLL is also a malignancy of short latency, with disease arising from 2 to 7 years after initial exposure to a carcinogen.

Retrospective case–control studies of only individuals who develop ANLL, and exposure-, sex-, and age-matched controls are the most efficient and relevant human population studies, as contrasted with studies using biomarker assays on large numbers of individuals. This conclusion is correct provided that blood samples are obtained for all treated cancer patients at defined times following treatment. Medical and other identifier information would be keyed to these cryopreserved samples, and updated periodically as part of patient follow-up. Virtually all cancer patients treated in medical centers in the USA, Europe, Japan, and Australia collected, stored, and updated this information; therefore, the systems exist for performing such an evaluation.

Blood samples could be cryopreserved periodically from small cohorts known not to be exposed to carcinogens or mutagens. Furthermore, this repository of samples and information could also be used to store samples from humans who have suffered large accidental exposures to mutagens and carcinogens. Some planning is required to rapidly obtain samples from accidentally exposed populations and store these samples, and later update relevant clinical information.

Once sufficient cases of secondary ANLL have been documented, retrospective case–control studies can be conducted, using "blind" study designs, relying on three groups of individuals: (a) treated patients who developed ANLL, (b) treated controls, i.e., those treated patients who did not develop ANLL, and (c) untreated controls. Only those experienced in performing biomarker assays should be relied upon to analyze the samples. Results can be correlated with disease rates to define reliable relative-risks or odds-ratios associated with the presence of a marker and the occurrence of the disease (ANLL).

Despite the limited intent of such study designs, some extensions may be possible. Individuals being studied can also be evaluated for pharmacogenetic or DNA repair characteristics. These latter measures may outweigh the value of predictors of genotoxic risk. No current marker may predict health risk with greater precision than does simple information concerning exposure, which may be useful to design in humans monitoring studies of exposures to mutagens and

carcinogens.

By extension, studies of biomarkers could also be used with rodent cancer bioassays. All markers used in human studies have counterparts in laboratory animals. Such a study would focus on the value of such biomarkers as predictors of the number and kinds of cancers induced in test animals. Such analyses can focus subsequent mechanistic studies in either cell culture or intact animals. In turn, these studies could assist in the design of additional biomarkers for human studies. As a result, delineation of mechanisms of action and the assessments of human risk can proceed as different facets of the same research undertakings.

Part 2

CONTRIBUTED PAPERS

5 DNA Adducts and Their Consequences

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5.1 INTRODUCTION

A central tenet of chemical carcinogenesis is that the covalent binding of carcinogens to DNA is causally related to tumorigenesis. This belief is supported by a number of observations, including the facts that:

1. the majority of carcinogens are also mutagens;
2. the mutagenic and carcinogenic properties of many carcinogens depend upon their conversion to electrophilic derivatives that react with nucleophilic sites within DNA;
3. the extent of DNA adduct formation can often be correlated with the magnitude of mutagenic and carcinogenic responses; and
4. the activation of certain proto-oncogenes can be accomplished through the interaction of carcinogens with DNA.

DNA adducts are typically formed in very low concentrations *in vivo* (<100 fmol/μg DNA; <3 adducts/10^5 nucleotides), which has made their detection difficult; however, with the advent of immunoassays (Poirier, 1984) and ^{32}P-postlabelling (Gupta *et al.*, 1982), their occurrence in exposed human populations has become possible to investigate. In this review, the DNA adducts obtained from four classes of carcinogens for which there is substantial evidence of human exposure are considered: *N*-nitrosamines, aflatoxins, aromatic amines, and polycyclic aromatic hydrocarbons. For each class, the metabolic activation pathways that lead to DNA adduct formation will be discussed briefly. The adducts that have been identified *in vitro* will be compared with those found *in vivo* in experimental animals and humans. Whenever possible, the discussion will include dose–response relationships, the significance of particular adducts in tumorigenesis, the

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heterogeneity of adduct distribution and processing, and the role of the adducts in oncogene activation.

5.2 *N*-NITROSAMINES

Humans are exposed to *N*-nitrosamines from a wide variety of sources, including foods, beverages, tobacco, cosmetics, cutting oils, hydraulic fluids, and rubber products (Preussmann and Eisenbrand, 1984). Over 300 *N*-nitrosamines have been demonstrated to be carcinogenic in experimental animals (Preussmann and Stewart, 1984). As with the majority of chemical carcinogens, these chemically inert compounds are metabolized to reactive electrophiles before binding to cellular macromolecules (Lawley, 1984). This process typically involves the oxidation of the carbon adjacent to the amine nitrogen (α-hydroxylation), as is illustrated by the ubiquitously distributed *N*-nitrosodimethyl amine, and the tobacco-specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Hecht *et al.*, 1986). The resultant α-hydroxy-*N*-nitrosoalkylamines are unstable, and rapidly decompose to produce aldehydes and alkyl diazohydroxides, the latter of which have the ability to alkylate DNA. *N*-Nitrosodimethylamine is a symmetrical *N*-nitrosamine; thus, α-hydroxylation of either carbon will yield the same methylating agent. Since 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is asymmetrical, DNA is methylated or pyridyloxobutylated depending upon the carbon that undergoes α-hydroxylation. To date, the *N*-nitrosamine DNA adducts that have been identified and studied most rigorously result from either methylation or ethylation of DNA.

As noted earlier, *N*-nitrosamines do not react directly with DNA; therefore, to characterize the methylated and ethylated DNA adducts resulting from exposure to these compounds, experiments have been conducted with direct-acting compounds that yield the same electrophilic intermediates. Table 5.1 illustrates the distribution of adducts observed in DNA from *in vitro* reactions with a series of dialkylsulphates, alkyl methanesulphonates, and *N*-alkylnitrosoureas (Singer and Grunberger, 1983). A general rule regarding these alkylating agents is that the potential exists to form adducts with all exocyclic oxygens and ring nitrogens, with the exception of the N^1 site of guanine. Except for *N*-ethylnitrosourea, the primary site of substitution is N^7 of guanine. Ethylating agents bind to a greater extent than methylating agents with phosphodiesters, and exocyclic oxygens are preferentially modified by *N*-alkylnitrosoureas as compared with dialklysulphates and alkyl methanesulphonates.

The distribution of DNA adducts found *in vivo* from direct-acting alkylating agents is similar to that observed *in vitro*, with two notable exceptions: N^3-methyladenine and O^6-methylguanine are present in decreased quantities *in vivo*, suggesting that these adducts are subject to enzymatic repair processes. Since *N*-alkylnitrosoureas are more carcinogenic than dialkylsulphates and alkyl methanesulphonates, a comparison of the distribution of adducts from these agents indicates that substitution of exocyclic oxygens (e.g., O^6 of guanine) is more

important for the induction of tumours than reaction with the ring nitrogens (e.g., N^7 of guanine; Frei and Lawley, 1976; Frei *et al.*, 1978). This interpretation is supported by experiments demonstrating that O^6-alkylguanines and O^4-alkylthymines give rise to base substitution mutations, of which the majority are transitions, whereas alkylation of ring nitrogens does not cause miscoding (Abbott and Saffhill, 1977, 1979; Saffhill and Abbott, 1978).

Table 5.1. Alkylation of DNA *in vitro* and *in vivo*[†]

Alkylating agent (DNA Source)	Percentage of total alkylation											
	Adenine			Guanine			Thymine			Cytosine		Phospho-diester
	N^1	N^3	N^7	N^3	O^6	N^7	O^2	N^3	O^4	O^2	N^3	
Dimethylsulphate												
(*In vitro*)	1.9	18	1.9	1.1	0.2	74	—	—	—	nd	<2.0	1
Diethylsulphate												
(*In vitro*)	2.0	10	1.5	0.9	0.2	67	—	nd	—	—	0.7	16
Methyl methane-sulphonate												
(*in vitro*)	3.8	10	1.8	0.6	0.3	85	nd	0.1	nd	nd	<1.0	1
Ethyl methane-sulphonate												
(*in vitro*)	1.7	4.9	1.1	0.9	2.0	65	nd	nd	nd	nd	0.6	13
(*in vivo*)	—	3.3	—	—	1.5	70	—	—	—	—	—	—
n-Methyl-nitrosourea												
(*in vitro*)	1.3	9.0	1.7	0.8	6.3	67	0.1	0.3	0.4	0.1	0.6	16
(*in vivo*)	—	3.6	—	—	3.6	70	—	—	—	—	—	—
n-Ethyl-nitrosourea												
(*in vitro*)	0.2	4.0	0.3	0.6	7.8	12	7.4	0.8	2.5	3.5	0.2	57
(*in vivo*)	—	4.1	0.6	1.4	7.2	14	7.4	—	2.3	1.3	—	60
n-Nitroso-dimethylamine												
(*in vivo*)	0.8	2.4	1.5	0.6	6.6	69	—	0.4	—	—	0.6	9
n-Nitroso-diethylamine												
(*in vivo*)	—	3.7	—	—	5.6	15	6.0	—	0.7	—	—	—

[†]Data are from Singer and Grunberger (1983). *In vivo* refers to the alkylation pattern in rat liver DNA. A dash indicates that the adduct was not analyzed; "nd" indicates that the adduct was not detected.

The adduct distribution obtained from *N*-nitrosodimethylamine and *N*-nitrosodiethylamine after metabolism *in vivo* is shown in Table 5.1. The pattern is very similar to that observed with the respective *N*-alkylnitrosourea, which supports the proposition that they share a common reactive electrophile. These data are from rat liver; however, the ratio of adducts in other tissues and species appears similar (Lawley, 1976). In general, the administration of direct-acting alkylating agents, such as alkyl methanesulphonates or *N*-alkylnitrosoureas, results in a similar adduct concentration in all tissues. In contrast with *N*-nitrosodialkylamines which require metabolism, the concentration of adducts depends upon the capability of the specific tissue to catalyze α-hydroxylation (liver tissue > kidney tissue > lung tissue; Lawley, 1976). The ability of *N*-nitrosodialkylamines to undergo α-hydroxylation can also vary within cell types of a particular tissue. For example, Belinsky *et al.* (1987) found preferential alkylation of Clara cells as compared with alveolar small cells or type II cells in the lungs of rats administered 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. This trend was more pronounced at low doses of the compound, and was not found with *N*-nitrosodimethylamine, which is weakly carcinogenic for lung tissue.

A non-random distribution of alkylation within DNA exists. With *N*-methylnitrosourea, for instance, the *in vitro* formation of O^6- and N^7-methylguanine in small oligonucleotides is favoured in positions with an adenine or thymine 5′ to the adducted guanine (Dolan *et al.*, 1988). Other alkylating agents, including dimethylsulphate, have been shown to react preferentially *in vitro* with guanine-cytosine-rich sequences in the 5′-flanking region of the c-*Ha-ras* oncogene (Mattes *et al.*, 1988). A similar degree of resolution has not been obtained *in vivo*; nevertheless, using immune electron microscopy, Nehls *et al.* (1984) demonstrated a non-random distribution of O^6-ethylguanine in brain DNA from fetal rats treated with *N*-ethylnitrosourea. Likewise, Ryan *et al.* (1986) found enhanced O^6-methylguanine formation in transcriptionally active chromatin and nuclear matrix-associated DNA as compared with bulk chromatin in livers from rats administered *N*-nitrosodimethylamine. Recently, Milligan and Archer (1988) observed an excess of strand breaks, presumably due to 7-methylguanine and 3-methyladenine, in the transcriptionally active albumin gene as compared with the non-transcribed *IgE* gene in the livers of rats treated with the same carcinogen. The non-random distribution of adducts is also reflected in the observed mutations. In *Escherichia coli* treated with *N*-methylnitrosourea, 95% of the mutations are guanine-to-adenine transitions, and these are 10 times more likely to occur in a 5′-purine-guanine-3′ sequence than in a 5′-pyrimidine-guanine-3′ sequence (Burns *et al.*, 1988). *Ras* oncogene activation in rats (Zarbl *et al.*, 1985) and mice (Belinsky *et al.*, 1989) administered alkylating agents is associated primarily with a guanine-to-adenine transition in a 5′-purine-guanine-3′ sequence.

The kinetics of DNA adduct formation *in vivo* with alkylating agents are dependent upon the treatment regimen. Following the administration of single doses of *N*-ethylnitrosourea or *N*-methylnitrosourea to mice (Frei *et al.*, 1978) or a linear relationship was observed between dose and adduct concentration with the

adduct profile being similar to that shown in Table 5.1. In rats given a single dose of *N*-nitrosodimethylamine, the hepatic concentrations of N^7-methylguanine also increased linearly with dose; however, the amount of O^6-methylguanine increased in a sublinear manner, which was attributed to the saturation of repair of O^6-methylguanine at higher doses of *N*-nitrosodimethylamine (Pegg and Hoi, 1978). A similar sublinear dose–response relationship was found for the formation of O^6-ethylguanine in the livers of rats treated with a single dose of *N*-nitrosodiethylamine (Scherer *et al.*, 1977). In contrast, in the lungs of rats treated multiple times with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, the correlation between administered dose and the concentration of O^6-methylguanine was supralinear (Belinsky *et al.*, 1987). This effect was suggested to result from a decreased rate of metabolism at higher doses of the carcinogen. During continuous administration of *N*-nitrosodiethylamine, an essentially linear relationship between dose and DNA adduct concentration appears to exist (Boucheron *et al.*, 1987); however, the adduct profile can differ markedly from that observed after a single treatment. For example, in hepatic DNA from rats treated once with *N*-nitrosodiethylamine, three to four times more O^6-ethylguanine is formed compared with O^4-ethylthymine, whereas the amount of O^4-ethylthymine produced during continuous administration is 50 times greater than that of O^6-ethylguanine (Richardson *et al.*, 1985). Likewise, following a single dose of the rat hepatocarcinogen, 1,2-dimethylhydrazine, the ratio of O^6-methylguanine to O^4-methylthymine is 100:1, whereas this ratio falls to <2:1 during chronic exposure (Richardson *et al.*, 1985). The change in adduct profiles that occurs during continuous dosing is the result of the preferential repair of O^6-alkylguanines by alkyltransferases. Differences in alkyltransferase activity between liver cell types also may account for the preferential accumulation of O^6-methylguanine in non-parenchymal cells as compared with hepatocytes in rats administered 1,2-dimethylhydrazine (Lewis and Swenberg, 1980).

With the exception of the induction of oral cancer in snuff dippers (Winn, 1986; Montesano *et al.*, 1988), no conclusive epidemiological evidence exists for the carcinogenicity of *N*-nitrosamines in humans. Nevertheless, the types of DNA adducts formed in exposed individuals are similar to those observed in experimental animals. For example, Herron and Shank (1980) found N^7- and O^6-methylguanine in the liver of a victim poisoned with *N*-nitrosodimethylamine. More recently, Umbenhauer *et al.* (1985) detected O^6-methylguanine in stomach and oesophageal tissue and oesophageal tumor DNA from Chinese cancer patients. This adduct was also found at lower levels in the same tissues in Europeans, who are at lower risk for developing oesophageal tumors. Similar results were obtained by Saffhill *et al.* (1988) when comparing individuals in Southeast Asia with those in England. Likewise, Hsieh *et al.* (1988) found higher levels of O^4-ethylthymine in DNA from Japanese cancer patients compared with non-tumor-bearing control patients.

5.3 AFLATOXINS

Humans are exposed to aflatoxins through the consumption of mouldy cereals, grains, and nuts (Busby and Wogan, 1984). Four major naturally occurring aflatoxins, aflatoxin B_1 (AFB_1), aflatoxin B_2, aflatoxin G_1, and aflatoxin G_2, have been characterized. AFB_1 is the most abundant as well as the most carcinogenic. The metabolic activation of AFB_1 involves oxidation of the 8,9-olefinic bond to give AFB_1-8,9-oxide (Baertschi *et al.*, 1988), which reacts with DNA to yield *trans*-8,9-dihydro-8-(deoxyguanosin-7-yl)-9-hydroxy AFB_1 (AFB_1-N7-dG; Essigmann *et al.*, 1977), a structure consistent with *trans* opening of the epoxide ring and simultaneous attack on N^7 of guanine. AFB_1-N7-dG carries a positive charge and is, therefore, unstable. It can undergo depurination to give *trans*-8,9-dihydro-8-(guan-7-yl)-9-hydroxy AFB_1 (AFB_1-N7-Gua) (Essigmann *et al.*, 1977) or base-catalyzed opening of the imidazole ring to yield two pyrimidine adducts: 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy AFB_1 (AFB_1-N7-P4r major) and 8,9-dihydro-8-(2-amino-6-formamido-4-oxo-3,4-dihydropyrimid-5-yl)-9-hydroxy AFB_1 (AFB_1-N7-P4r minor) (Hertzog *et al.*, 1982).

Several investigators have demonstrated that AFB_1-8,9-oxide does not react randomly with DNA *in vitro* (D'Andrea and Haseltine, 1978; Misra *et al.*, 1983; Muench *et al.*, 1983; Marien *et al.*, 1987). This finding has been studied in greatest detail by Benasutti *et al.* (1988) who showed that the reactivity of a particular guanine was markedly affected by the 5′- and 3′- flanking bases. The most reactive sequence was 5′-GGG-3′ followed by 5′-CGG-3′ and 5′-GGT-3′; these were approximately 20-fold more reactive than the least reactive sequence, 5′-TGA-3′. Whether a similar sequence specificity occurs *in vivo* is unknown; however, AFB_1 preferentially binds (a) linker, as compared with core, DNA sequences of nucleosomes in trout liver (Bailey *et al.*, 1980), (b) mitochondrial, as compared with nuclear, DNA in rat liver (Niranjan *et al.*, 1982), and (c) transcriptionally active, as compared with bulk, DNA in rat liver (Irvin and Wogan, 1984).

The hepatocarcinogenicity of AFB_1 varies among species with the relative order of sensitivity being: trout > rat >> hamster ≈ mouse ≈ salmon (Busby and Wogan, 1984; Bailey *et al.*, 1988). A similar relationship appears to hold for the extent of binding to liver DNA; thus, the magnitude of adduct formation correlates with the relative degree of hepatocarcinogenicity (Garner and Wright, 1975; Lutz *et al.*, 1980; Ueno *et al.*, 1980; Croy and Wogan, 1981b; Bailey *et al.*, 1988). Adduct formation is also dose-related; for instance, following a single administration of AFB_1 to rats, the extent of hepatic DNA binding is linear over a 10^5-fold dose range (Appleton *et al.*, 1982; Lutz, 1986; Wild *et al.*, 1986). The analysis of AFB_1 DNA binding is complicated by the instability of AFB_1-N7-dG and the persistence of AFB_1-N7-P4r major and AFB_1-N7-P4r minor. In rat liver, AFB_1-N7-dG has a half-life of 7.5 hours (Croy and Wogan, 1981a), compared with 3 to 4 weeks in trout liver (Goeger *et al.*, 1986). Therefore, DNA binding measured during the chronic administration of AFB_1 reflects a mixture of adducts, which will presumably be species-dependent.

The instability of AFB_1-N7-dG also complicates the analysis of mutations induced by AFB_1. Base-substitution and frameshift mutations have been detected in

Salmonella, and these have been attributed to AFB_1-N7-dG (Stark *et al.*, 1979); whereas the guanine-to-thymine transversions in *E. coli* (Foster *et al.*, 1983) and the mutations induced by AFB_1 in human diploid lymphoblasts (Kaden *et al.*, 1987) have been ascribed to the existence of apurinic sites that result from the loss of AFB_1-N7-Gua. Likewise, the induction of liver tumors in rats by AFB_1 has been associated with a guanine-to-adenine transition at codon 12 of the *ras* oncogene (McMahon *et al.*, 1987); yet whether this effect is due to AFB_1-N7-dG, its imidazole-ring-opened derivatives, or the apurinic site remains unknown. It has been noted that tumor initiation best correlates with initial levels of DNA damage (Kensler *et al.*, 1986; Bailey *et al.*, 1988), suggesting that AFB_1-N7-dG may be the critical lesion.

During the continuous administration of AFB_1, steady-state hepatic DNA-adduct levels are observed after approximately 2 to 6 weeks in rats (Wild *et al.*, 1986; Buss and Lutz, 1988) and after 3 weeks in trout (Bailey *et al.*, 1988). In both species, steady-state adduct concentrations appear to be linearly related to the concentration of AFB_1 administered chronically (Dashwood *et al.*, 1988, 1989; Buss and Lutz, 1988). Furthermore, if steady-state adduct levels are compared with hepatic tumor incidence, a nearly identical linear relationship is observed for both species (Bechtel, 1989).

In humans, a positive correlation exists between the amount of AFB_1 ingested and the incidence of liver cancer (Busby and Wogan, 1984). Furthermore, AFB_1 DNA adducts have been detected in tissues and urine from exposed humans (Autrup *et al.*, 1983; Groopman *et al.*, 1985; Hsieh *et al.*, 1988). Recently, a highly significant correlation was observed between daily consumption of AFB_1 and concentrations of serum adducts of AFB_1 and urinary AFB_1-N7-Gua (Gan *et al.*, 1988; Groopman *et al.*, 1989), representing perhaps steady-state levels.

5.4 AROMATIC AMINES

Human exposure to aromatic amines and amides occurs from a number of sources, including various industrial processes, cigarette smoke, and certain foods (Beland and Kadlubar, 1990). Widespread exposure also exists to nitropolycyclic aromatic hydrocarbons, which are products of incomplete combustion and are converted to aromatic amines by nitroreduction (Tokiwa and Ohnishi, 1986). The initial activation of aromatic amines and amides generally consists of an *N*-oxidation to yield *N*-hydroxy arylamines and *N*-hydroxy arylamides (arylhydroxamic acids), respectively. Similarly, the first step in the activation of nitropolycyclic aromatic hydrocarbons is a nitroreduction to an *N*-hydroxy arylamine. *N*-Hydroxy arylamines can react directly with DNA or be further activated through the formation of acetate and sulphate esters. Arylhydroxamic acids are not directly electrophilic and must be further metabolized to reactive esters (Figure 5.1). Typically, major adducts from these electrophilic intermediates are formed through covalent linkage of the amine or amide nitrogen to the C^8 of guanine, whereas

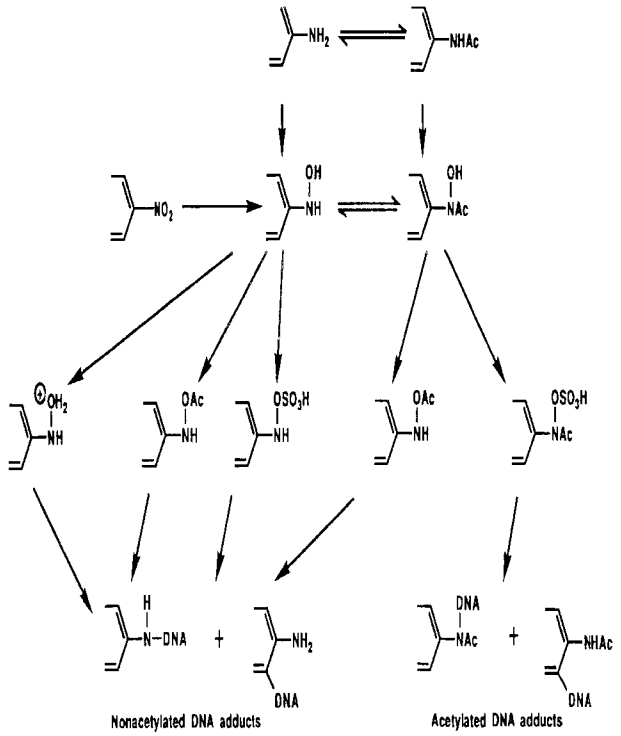

Figure 5.1. Metabolic activation pathways of aromatic amines, aromatic amides, and nitropolycyclic aromatic hydrocarbons

minor adducts arise from reactions between carbons in the *ortho* position in relation to the amine or amide nitrogen and the exocyclic nitrogens and oxygens of guanine and adenine. Non-acetylated C^8-substituted guanine adducts are the predominant products at doses normally used in carcinogenesis experiments, even when aromatic amides or arylhydroxamic acids are administered.

The distribution and mutagenic processing of aromatic amine DNA adducts appear to be non-random and influenced by the nature of the surrounding nucleotides at the binding site of the carcinogen. While this phenomenon has been studied most extensively with the adducts derived from 2-acetylaminofluorene, it applies to other compounds as well. For example, in plasmid DNA reaction with model electrophile *N*-acetoxy-2-acetylaminofluorene, the extent of modification of specific guanines varied 40-fold (Fuchs, 1983). Similar differences in reactivity, although smaller in magnitude, have been observed with *N*-hydroxy-2-aminofluorene (Bichara and Fuchs, 1985), *N*-acetoxy-2-trifluoroacetoxy-2-aminofluorene (Mah *et al.*, 1989), and *N*-hydroxy-1-aminopyrene (Yang *et al.*, 1988). In each case, no obvious reasons for the differences are apparent. A non-random induction of mutation is also found in the modified plasmids; however, the nucleotides most frequently mutated do not always correspond to those containing

the most adducts. Plasmids modified with *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene and transformed into *E. coli* cause frameshift mutations almost exclusively, and these occur in guanine repeatitions or in cytosine-guanine sequences (Koffel-Schwartz *et al.*, 1984). In the same system, the non-acetylated adduct *N*-(deoxyguanosin-8-yl)-2-aminofluorene induces mainly guanine-to-thymine transversions, but with no apparent sequence specificity (Bichara and Fuchs, 1985). Plasmids modified with *N*-(deoxyguanosin-8-yl)-2-aminofluorene or *N*-(deoxyguanosin-8-yl)-1-aminopyrene and transformed into human cells cause primarily guanine-to-thymine mutations in 5′-purine-guanine-purine-3′ sequences (Yang *et al.*, 1988; Mah *et al.*, 1989). Guanine-to-thymine transversions are also observed in Chinese hamster ovary cells exposed to *N*-acetoxy-2-acetylaminofluorene to produce *N*-(deoxyguanosin-8-yl)-2-aminofluorene-modified DNA (Carothers *et al.*, 1989). Similar mutations occur in the c-*Ha-ras* proto-oncogene in the livers of $B_6C_3F_1$ mice administered *N*-hydroxy-2-acetylaminofluorene (Wiseman *et al.*, 1986), presumably through the formation of *N*-(deoxyguanosin-8-yl)-2-aminofluorene (Lai *et al.*, 1985). The reaction of aromatic amine metabolites with DNA is strongly influenced by chromatin structure. In cells treated with *N*-acetoxy-2-acetylaminofluorene, for example, more extensive binding occurs in internucleosomal linker regions as compared with the nucleosome core (Kaneko and Cerutti, 1980; Lang *et al.*, 1982). Likewise, in the livers of rats administered 2-acetylaminofluorene or its arylhydroxamic acid, more extensive binding has been observed in staphylococcal nuclease-sensitive (Metzger *et al.*, 1976) and DNAse I-resistant (Ramanathan *et al.*, 1976; Metzger *et al.*, 1977; Baranyi-Furlong and Goodman, 1984) regions of chromatin, transcriptionally active DNA (Moyer *et al.*, 1977; Schwartz and Goodman, 1979; Walker *et al.*, 1979), and repetitive DNA sequences (Gupta, 1984).

2-Acetylaminofluorene is more hepatocarcinogenic in rats than in mice; in both species, a linear relationship exists between the concentration of a single oral dose of carcinogen and the extent of hepatic DNA binding, with higher binding levels being observed in rats (Pereira *et al.*, 1981). A linear relationship has also been observed between the amount of benzidine administered intraperitoneally to mice and hepatic DNA adduct levels (Talaska *et al.*, 1987). In rats, the highest quantities of aromatic amine DNA adducts are typically found in liver (Neumann, 1983; Gupta *et al.*, 1988, 1989), but while this organ is a target tissue for 2-acetylaminofluorene tumorigenesis, it is refractory to tumor induction by a number of other aromatic amine carcinogens (Garner *et al.*, 1984; Beland and Kadlubar, 1990). Within rat liver, more extensive adduct formation from 2-acetylaminofluorene occurs in hepatocytes, the presumed target cells for this aromatic amide, as compared with non-parenchymal cells (Westra *et al.*, 1983; Swenberg *et al.*, 1983; Poirier *et al.*, 1989).

During the continuous administration of aromatic amine carcinogens to rats or mice, steady-state concentrations of the amine in blood (Jackson *et al.*, 1980; Green *et al.*, 1984) and tissue (Jackson *et al.*, 1980), hepatic DNA adducts (Poirier *et al.*, 1984; Buss and Lutz, 1988; Beland *et al.*, 1990a), and bladder DNA adducts

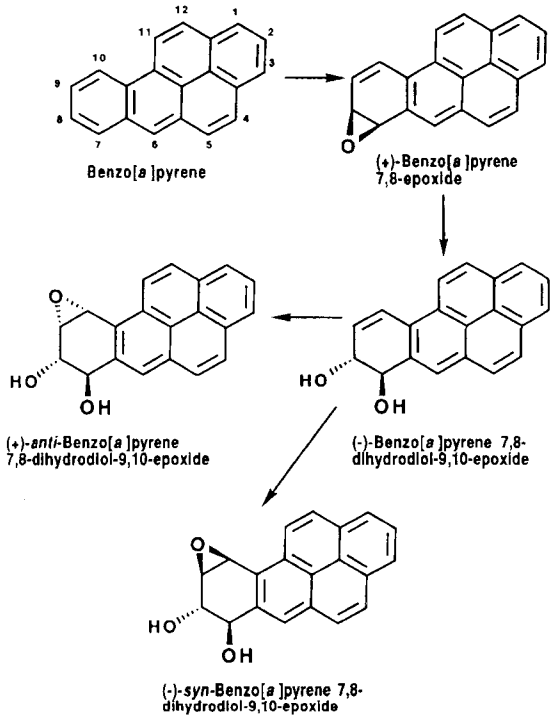

Figure 5.2. Metabolism of benzo[*a*]pyrene to *syn*- and *anti*-benzo[*a*]pyrene 7,8-dihydrodiol-9,10-epoxides

(Beland *et al.*, 1990a; Talaska *et al.*, 1990) appear after approximately 1 month of dosing. These steady-state levels are dose-related (Jackson *et al.*, 1980; Buss and Lutz, 1988; Beland *et al.*, 1990a); and a linear correlation exists between the DNA-adduct concentration and the hepatic tumor incidence in rats (Buss and Lutz, 1988), in mice administered 2-acetylaminofluorene (Beland *et al.*, 1990a), and in mice treated with 4-aminobiphenyl (Beland *et al.*, 1990b). A linear relationship has also been observed between bladder tumor incidence and bladder DNA adduct concentration in male mice given 4-aminobiphenyl (Beland *et al.*, 1990b); however, in female mice fed 2-acetylaminofluorene, the correlation between the occurrence of bladder tumors and DNA-adduct concentration is non-linear (Beland *et al.*, 1990a). Hepatic tumor induction by 2-acetylaminofluorene in mice and rats is sex-related. In mice, tumorigenesis is correlated with the extent of DNA adduct formation; in rats, nearly equal concentrations of adducts are found in both sexes, but males have a much higher tumor yield (Pereira *et al.*, 1981; Beland *et al.*, 1982).

Aromatic amines are clearly implicated in the induction of bladder cancer in humans (Parkes and Evans, 1984). Haemoglobin adducts of 4-aminobiphenyl have been detected in blood samples from humans, and these concentrations are higher

in smokers than in non-smokers (Bryant *et al.*, 1987, 1988), which is consistent with tobacco-related increases in the incidence of bladder cancer (Mommsen and Aagaard, 1983). In other studies, ^{32}P-postlabelling and immunoassays have indicated the presence of 4-aminobiphenyl DNA adducts in human lung and bladder samples (Kadlubar *et al.*, 1988, 1989; Wilson *et al.*, 1989; Talaska *et al.*, 1990), with the concentration in the bladder increasing in the following order: smokers > ex-smokers > non-smokers (Kadlubar *et al.*, 1989).

5.5 POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAHs) are by-products of combustion processes, resulting in ubiquitous human exposure to this class of carcinogens (Dipple, 1985). The initial step in the activation of PAHs (*e.g.*, benzo[*a*]pyrene) typically involves an epoxidation in a terminal benzo ring (*e.g.*, carbons 7, 8, 9, and 10 of benzo[*a*]pyrene; Figure 5.2) (Dipple *et al.*, 1984). This step is followed by hydrolysis of the epoxide to a dihydrodiol and then an additional epoxidation in the same benzo ring to give a vicinal dihydrodiol epoxide. Since PAHs are generally asymmetrical, more than one geometric dihydrodiol epoxide can be formed; for example, the metabolism of benzo[*a*]pyrene can give rise to both a 7,8-dihydrodiol-9,10-epoxide and a 9,10-dihydrodiol-7,8-epoxide. However, tumor data as well as theoretical calculations (Jerina and Daly, 1977) support the concept that the most active derivatives are those in which the epoxide function is pointed toward the angular "bay" region of the PAH (*e.g.*, carbons 9, 10, 11, and 12 in benzo[*a*]pyrene). The metabolism of PAHs is further complicated by the fact that, in addition to geometric isomers, both diastereomeric (*e.g.*, *syn*- and *anti*-benzo[*a*]pyrene 7,8-dihydrodiol-9,10-epoxide) and enantiomeric (*e.g.*, (+)- and (-)-*anti*-benzo[*a*]pyrene 7,8-dihydrodiol-9,10-epoxide) stereoisomers can be formed that have markedly different biological properties. For example, (+)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide is more mutagenic in mammalian cells (Brookes and Osborne, 1982) and tumorigenic in experimental animals (Buening *et al.*, 1978; Slaga *et al.*, 1979) than the (-)-*anti* or (±)-*syn* isomers. Likewise, (-)-*anti*-benzo[*c*]phenanthrene-3,4-dihydrodiol-1,2-epoxide is the most mutagenic (Wood *et al.*, 1983) and tumorigenic (Levin *et al.*, 1986) dihydrodiol epoxide stereoisomer obtained from benzo[*c*]phenanthrene.

DNA adducts of PAH dihydrodiol epoxides have been studied exhaustively for benzo[*a*]pyrene (Baird and Pruess-Schwartz, 1988) and to a lesser extent for benz[*a*]anthracene (Hemminki *et al.*, 1980), chrysene (Hodgson *et al.*, 1983), dibenzo[*a,e*]fluoranthene (Pèrin-Roussel *et al.*, 1984), benzo[*c*]phenanthrene (Agarwal *et al.*, 1987), 7,12-dimethylbenz[*a*]anthracene (Cheng *et al.*, 1988), dibenz[*a,j*]anthracene (Chadha *et al.*, 1989), and fluoranthene (Gorelick and Wogan, 1989). Adduct formation normally involves *cis* or *trans* opening of the epoxide ring with covalent attachment at the benzylic carbon (*e.g.*, carbon 10 of benzo[*a*]pyrene 7,8-dihydrodiol-9,10-epoxide) of the dihydrodiol epoxide. Generally, guanine is the preferred base for reaction; however, depending upon the

PAH, considerable binding can also occur with adenine and cytosine (Table 5.2). Furthermore, the extent of reaction with a particular nucleic acid base will depend upon the particular stereoisomer being considered. For instance, with (+)-*syn*-benzo[*c*]phenanthrene-3,4-dihydrodiol-1,2-epoxide, ≈10% of the binding is to guanine; this binding increases to ≈40% with the (-)-*anti* isomer (Dipple *et al.*, 1987).

As with other carcinogens, PAH dihydrodiol epoxides do not appear to bind randomly to DNA. When studied by a photochemical cutting technique, (±)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide was found to bind preferentially to guanines in 5′-XGG-3′ and 5′-GGX-3′ sequences (Boles and Hogan, 1986), whereas alkali-labile lesions occur mainly in 5′-pyrimidine-guanine-3′ sequences (Lobanenkov *et al.*, 1986). The differences between these results may be due the nature of the adduct being examined, with the former reflecting N^2-deoxyguanosine lesions and the latter indicating the location of N^7-deoxyguanosine adducts.

The mutagenic processing of PAH dihydrodiol epoxide DNA adducts is also nonrandom. Guanine to thymine transversions are the primary mutations found in Chinese hamster ovary cells treated with (±)-*anti*-benzo[*a*]pyrene 7,8-dihydrodiol-9,10-epoxide and these tend to occur in 5′-AG_nA-3′ sequences (Mazur and Glickman, 1988). Similar mutations are observed in human cells treated with an (±)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide-modified shuttle vector, and these are found mainly in runs of guanines (Yang *et al.*, 1987). In the same human cell system, (-)-*anti*-benzo[*c*]phenanthrene-3,4-dihydrodiol-1,2-epoxide causes adenine-to-thymine and guanine-to-cytosine (or cytosine-to-guanine) transversions primarily in 5′-AGA-3′, 5′-AAC-3′, and 5′-GAG-3′ sequences (Bigger *et al.*, 1989). Guanine-to-thymine transversions are associated with the activation of the c-*Ha-ras* proto-oncogene treated with (±)-*anti*-benzo[*a*]pyrene 7,8-dihydrodiol-9,10-epoxide and transvected into NIH 3T3 cells (Vousden *et al.*, 1986). In contrast, the activation of the *ras* proto-oncogene by 7,12-dimethylbenz[α]anthracene appears to involve an adenine-to-thymine transversion (Zarbl *et al.*, 1985; Bizub *et al.*, 1986; Dandekar *et al.*, 1986; Quintanilla *et al.*, 1986).

Higher level chromatin structure also affects the binding of PAH metabolites. In *in vitro* incubations with rat liver or lung tissue, cells, or nuclei, benzo[*a*]pyrene has been found to bind to a greater extent to nuclear matrix associated DNA than bulk chromatin (Blazsek *et al.*, 1979; Hemminki and Vainio, 1979; Ueyama *et al.*, 1981; Mironov *et al.*, 1983; Obi *et al.*, 1986). Similar observations have been made with 7,12-dimethylbenz[α]anthracene and (±)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide in rat liver nuclei (Mironov *et al.*, 1983) and with dibenzo[*a,e*]fluoranthene in mouse fibroblasts (Pèrin-Roussel *et al.*, 1988). In other *in vitro* experiments, benzo[*a*]pyrene and its (±)-*anti*- and (±)-*syn*-7,8-dihydrodiol-9,10 epoxides demonstrated greater binding to linker sequences than to the core sequences of nucleosomes (Koostra and Slaga; 1980; Jack and Brookes, 1981) and to transcribed as compared to nontranscribed regions of DNA (Arrand and Murray, 1982; Obi *et al.*, 1986). Fewer studies have been conducted *in vivo*; however, a single dose of

benzo[*a*]pyrene was found to bind preferentially to the nuclear matrix DNA isolated from lungs and livers of rats (Hemminki and Vaino, 1979).

Table 5.2. Sites of modification in DNA *in vivo* by aromatic amine and nitropolycyclic aromatic hydrocarbon carcinogens

Carcinogen	Nucleic acid base				
	Guanine			Adenine	
	C^8	N^2	O^6	$C8$	N^6
1-Naphthylamine	++				
2-Naphthylamine	++	+			
4-Aminobiphenyl	++	+		+	
4-Acetylaminobiphenyl	++[b]	+			
4-Nitrobiphenyl	++	+		+	
4′-Fluoro-4-acetylaminobiphenyl	++[b]	+			
2-Aminofluorene	++				
2-Acetylaminofluorene	++[b]	+			
Benzidine	++				
NN′-Diacetylbenzidine	++[b]				
4-Aminoazobenzene	++				
N-Methyl-4-aminoazobenzene	++[c]	+		+	
NN-Dimethyl-4-aminoazobenzene	++[c]	+			
2-Acetylaminophenanthrene[d]	++	+			
4-Acetylaminostilbene	++				
1-Nitropyrene	++				
1,6- and 1,8-Dinitropyrene	++				

[a]Data are from Beland and Kadlubar (1985, 1990). Results are from target tissues, typically after the administration of a single dose. For aromatic amines or amides, their *N*-hydroxy derivatives may have been given.
++ = >75% binding; + = <25% binding.
[b]Both *N*-acetylated and non-acetylated adducts are found in a ratio of ≈1:3.
[c]Both *N*-methylated and non-methylated adducts are found.
[d]Data are from Gupta *et al.* (1989).

PAHs induce primarily skin, stomach, lung, and mammary gland tumors in experimental animals (Dipple *et al.*, 1984). Following a single dose, PAH–DNA adducts are formed in both target and nontarget tissues, with the concentration being relatively uniform between tissues (Stowers and Anderson, 1985). A number of dose–response studies have also been conducted with PAHs; in nearly all of these, DNA adducts are linearly related to dose. In mice administered benzo[*a*]pyrene orally over a 10^5-fold range in dose, the extent of DNA binding in liver and stomach increased in a linear fashion (Dunn, 1983). In another study, a decrease in the extent of adduct formation was observed at higher doses of

benzo[*a*]pyrene, which may be due to a saturation of the metabolic activation pathways (Adriaenssens *et al.*, 1983). Supralinear dose–response relationships between cancer incidence and the binding to epidermal DNA have also been observed in mice treated topically with benzo[*a*]pyrene (Pereira *et al.*, 1979; Perera *et al.*, 1982; Nakayama *et al.*, 1984) and 7,12-dimethylbenz[*a*]anthracene (Phillips *et al.*, 1978). The correlation between administered dose and DNA-adduct concentration does not appear to have been examined in the mammary gland; however, an adduct formed by the reaction of (+)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide with N^2 of deoxyguanosine is the major product detected in mammary gland DNA when benzo[*a*]pyrene is administered orally to rats (Seidman *et al.*, 1988). DNA-adduct dose–response experiments have not been reported for the continuous administration of PAHs.

PAHs are probably carcinogenic in humans (IARC, 1983); nevertheless, attempts to detect DNA adducts from these compounds in exposed populations have met with mixed success. Since cigarette smoke contains substantial quantities of PAHs (Surgeon General, 1982), a number of studies have compared DNA adduct concentrations in various tissues from smokers and non-smokers. Immunoassays with antibodies elicited against (+)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide-modified DNA have revealed that smokers generally have slightly higher PAH–DNA adduct levels (termed benzo[*a*]pyrene antigenicity) than non-smokers (Everson *et al.*, 1986, 1988; Perera *et al.*, 1987), although this is not always the case (Perera *et al.*, 1982, 1988; Harris *et al.*, 1985; Shamsuddin *et al.*, 1985; Haugen *et al.*, 1986). More dramatic differences have been found when assays are based upon ^{32}P-postlabelling. Depending upon assay conditions, smokers have had higher levels of discrete adducts (Everson *et al.*, 1986, 1988; Randerath *et al.*, 1986), or a diffuse area of adducts (Phillips *et al.*, 1988; Randerath *et al.*, 1989), than non-smokers. However, none of these adducts has been characterized chemically, and most cannot be attributed to specific PAH DNA adducts. The presence of benzo[*a*]pyrene dihydrodiol epoxide DNA adducts in human placenta has been established by subjecting placental DNA to immunoaffinity chromatography followed by high pressure liquid chromatography and synchronous fluorescence spectroscopy, as well as gas chromatography–mass spectrometry; however, the levels did not appear to be related to smoking (Manchester *et al.*, 1988). Exposure-related increases in benzo[*a*]pyrene antigenicity have been reported in peripheral blood lymphocytes from iron-foundry workers (Perera *et al.*, 1988) and individuals ingesting charcoal-broiled beef (Rothman *et al.*, 1990). Positive results for PAH–DNA adducts (or benzo[*a*]pyrene antigenicity) have also been found in blood samples from coke oven workers (Harris *et al.*, 1985), foundry workers, roofers (Shamsuddin *et al.*, 1985), and fire fighters (Liou *et al.*, 1989).

5.6 SUMMARY

Some interesting similarities and differences observed between the classes of

carcinogen–DNA adducts considered in this review include the following:

1. With alkylating agents, mutations and, presumably, tumors result from minor adducts (*e.g.*, O^6-alkylguanine and O^4-alkylthymine); whereas with the other carcinogens, the biological responses are normally associated with the major forms of DNA damage.
2. The site of substitution for biologically important adducts appear to be chemical-class specific. Mutations and tumor induction from alkylating agents are correlated with O^6-guanine and O^4-thymine substitution. For aflatoxins, these responses are best correlated with reaction at the N^7 site of guanine; with aromatic amines, C^8-guanine substitution generally appears to be the critical lesion; and for PAHs, N^2 of guanine and/or N^6 of adenine appear to be the important sites for substitution.
3. Reactions with DNA are clearly non-random. Sequence specificity for adduct formation has been demonstrated *in vitro* with chemicals of each of class; however, presently not enough information is available to determine if significant differences exist between classes or what factors are critical in determining the sequence specificity for both the formation and processing of the adducts. Furthermore, essentially nothing is known about the sequence specificity of adduct formation and processing *in vivo*.
4. Higher order chromatin structure affects the binding of carcinogens to DNA. With each of the classes, for example, more extensive binding has been found with transcriptionally active DNA, presumably due to its open conformation. Nevertheless, most observations concerning the distribution of DNA adducts in chromatin have been made after single doses of carcinogen, rather than after dosing regimens that give rise to tumors. The distribution of DNA adducts during continuous dosing reflects not only a dynamic process of adduct formation and removal, but also changes in chromatin structure as a function of time. How these factors affect the final distribution of adducts is unknown.
5. For several carcinogens, the relationship between administered dose and DNA-adduct concentrations is linear after both single doses and continuous administration. In certain instances, however, correlations are non-linear, which may be due to saturation of activation or detoxification pathways at high doses of carcinogen. Thus, caution must be exercised in extrapolating from high to low doses.
6. Steady-state DNA-adduct concentrations are obtained during continuous carcinogen administration, and this condition typically occurs after approximately 1 month of chronic dosing. Because steady-state DNA-adduct levels occur, an estimation of the risk for developing a tumor cannot be obtained from the DNA-adduct concentration by itself, but must include both the DNA adduct concentration and the length of carcinogen exposure.
7. DNA adducts have been measured in target tissues following dosing regimens that induce tumors. DNA adduct concentrations necessary to induce a 50%

tumor incidence have been measured. The results are both interesting and troubling. For example, a 10-fold higher concentration of O^6-methylguanine as compared with O^6-ethylguanine is required to induce a 50% incidence of thymic lymphoma. This difference may reflect the fact that the former is repaired more readily than the latter. Likewise, the inability of O^4-ethylguanine to be repaired probably accounts for the even lower concentrations of this adduct necessary to induce the equivalent hepatic tumor incidence. The observation that in two species the identical levels of AFB_1–DNA adducts induce the same incidence of hepatic tumors indicates that making cross-species comparisons may be possible. In addition, the data from mice treated with 2-acetylaminofluorene and 4-aminobiphenyl suggest that structurally similar DNA adducts in different organs are processed in a similar manner. Nonetheless, the apparent 100-fold range in the ability of DNA adducts to induce the same tumor incidence raises concerns about the prospect of being able to predict the tumor induction potential of unknown DNA adducts.

8. DNA adducts can be detected in humans using immunoassays and ^{32}P-postlabelling. In some instances exposure-related increases in DNA-adduct concentrations have been demonstrated. At times, however, the correlation between different types of assays has been unsatisfactory; for example, positive results have been obtained for benzo[*a*]pyrene antigenicity, and yet identifiable PAH–DNA adducts have not been detected in the same samples by ^{32}P-postlabelling. When used alone, neither technique has chemical specificity, but when combined with other adduct-detection procedures (e.g., immunoaffinity chromatography followed by high-pressure liquid chromatography or gas chromatography/mass spectrometry), these methods have allowed the identification of specific DNA adducts in human samples. Such an approach holds promise for much improved sensitivity and specificity.

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6 Nucleotide Excision-Repair Among Species

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6.1 INTRODUCTION

DNA molecules are subject to damage by a wide variety of electrophilic chemicals and by radiation. The damage can consist of alterations to the bases, the deoxyribose sugar, or the phosphodiester backbone. All organisms have evolved a series of complex interrelated enzymatic pathways whereby these different types of damage can be reversed, removed, or tolerated. This monograph addresses one relatively well-characterized pathway: the excision-repair process to remove and replace bulky damage of DNA, with special emphasis on damage produced by UV light. As an introduction, this system is described in the bacterium, *Escherichia coli*, in which the basic steps are now well understood; then the state of knowledge will be presented in detail for eukaryotic organisms, in particular yeast, *Drosophila*, and mammalian cells. The biochemistry of excision-repair *in vivo* is considered together with the properties of repair-deficient mutants, followed by the detailed understanding obtained from cloning DNA repair genes and from the characterization of gene products. A full description of this system has not yet been obtained for any eukaryotic organism. The final section of this monograph highlights interspecies similarities and differences at the levels of gene, protein, and process.

6.2 EXCISION-REPAIR IN *ESCHERICHIA COLI*

Excision-repair of UV damage in *E. coli* was first reported when wild-type cells were shown to be able to remove UV-induced pyrimidine dimers from cellular DNA, and they postulated a four-step pathway of incision, excision, repair synthesis, and resealing. At about the same time, evidence was provided for a

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non-conservative type of repair synthesis in UV-irradiated cells. Boyce and Howard-Flanders isolated mutants in three genes, *uvr A*, *uvr B*, and *uvr C*, which were shown to be deficient in excision-repair of UV damage. Subsequent work from the laboratories of Seeberg, Grossman, Rupp, Sancar and others, culminated in the cloning of the genes and isolation of the gene products involved in this excision-repair process. The enzymology of the pathway is now understood in considerable detail. References to the original work can be found in Friedberg (1985) and in the relevant chapters in Friedberg and Hanawalt (1988).

The mode of action of the *uvr ABC* enzyme is understood in great detail, although certain aspects remain controversial. The work of Orren and Sancar (1989) led to formation of a model. A dimer of the *uvr A* gene product associates with a molecule of *uvr B*, and delivers it to the damaged sites. *Uvr A* then dissociates, and *uvr C* binds to the *uvr B*–DNA complex to form the active nuclease.

The work of Grossman and coworkers (Friedberg and Hanawalt, 1988) suggested that the *uvr A* dimer binds to DNA followed by association of *uvr B*. The ternary complex is then translocated to the damaged site where the *uvr C* product associates to form the active nuclease. Irrespective of the precise mechanism, the net result is the introduction of two incisions, seven nucleotides 5′ to the pyrimidine dimer (in the case of UV damage), and three to four nucleotides 3′ to the damaged site, with a total of 12 nucleotides between the two nicks. Incisions on both sides of the damaged site by the enzyme complex had not been anticipated from *in vivo* studies, and this mechanism was revealed only following isolation and purification of the proteins. The enzyme complex remains bound on the DNA, and it is released, together with the 12-nucleotide fragment, only following the combined action of the helicase II activity encoded by the *uvr D* gene and DNA polymerase I. The latter enzyme fills in the gap left by removal of the damaged 12-nucleotide fragment, and DNA ligase joins the new stretch of DNA to the pre-existing strand.

DNA polymerase I has the ability to extend the excision gap by nick translation; but *in vitro* about 85% of the patches are 12 nucleotides long (the same size as the excised nucleotide fragment), the remaining 15% being somewhat longer. The majority of the patches *in vivo* are about 30 nucleotides in length, which may be within experimental error of the value found *in vitro*.

The *uvr ABC* enzyme has broad substrate specificity, acting primarily on damage which results in bulky distortions in DNA. These include psoralen-induced monoadducts and cross-links, as well as adducts induced by 4-nitroquinoline-1-oxide, *cis*-platinum, acetylaminofluorene, benz[*a*]pyrene-diol-epoxides, ethylating agents, higher alkylating homologs, and mitomycin C. Both principal UV-induced products—the cyclobutane pyrimidine dimer and the (6-4) photoproduct—are substrates. Recently, the enzyme complex was shown at least *in vitro* to be able to remove small lesions, such as thymine glycols and methylated bases. The physiological importance of this property of the *uvr ABC* system is unknown. The principal mechanism for removal of the latter types of DNA injury is via a completely different set of enzymes, the DNA glycosylases, which remove such

damaged bases from the DNA by rupture of the base-sugar glycosylic bonds. These enzymes are not discussed in detail in this paper.

A comparison of the *uvr ABC* enzyme with the UV-endonucleases found in T4 phage-infected *E. coli* and in *Micrococcus luteus* is instructive. The latter are small single polypeptide enzymes able to incise DNA at cyclobutane pyrimidine dimers by a different mechanism. These enzymes contain two activities, a glycosylase activity that disrupts the glycosylic bond of the 5′ pyrimidine of the dimer, and an AP endonuclease activity that breaks the phosphodiester bond between the two pyrimidines of the dimer. The net result is the breakage of the DNA backbone adjacent to 5′ of an unhooked pyrimidine dimer. The mechanism of action is thus completely different from that of the *uvr ABC* excinuclease; furthermore, these small UV-endonucleases are absolutely specific for cyclobutane pyrimidine dimers, and exert no activity on any of the other adducts listed above.

Damage to cellular DNA in *E. coli* results in the induction of the SOS response, a complex system in which at least 15 genes are induced, including both *uvr A* and *uvr B* genes whose expression increases by five- to 10-fold following UV-irradiation.

6.3 EXCISION-REPAIR IN *SACCHAROMYCES CEREVISIAE*

6.3.1 YEAST AS A MODEL SYSTEM

Currently available data support the notion that *S. cerevisiae* represents a convenient model organism for the study of excision-repair in eukaryotes, with considerable relevance for the equivalent process in mammals. As one of the most simple eukaryotes, *S. cerevisiae* has a number of important, basic characteristics that appear to be conserved throughout eukaryotic evolution. These include the main properties of chromatin structure, cell-cycle regulation, transcription, and DNA replication. This conservation is likely to extend to the principal features of DNA repair as well. On the other hand, major differences exist between yeast and mammals in aspects of mitosis, in certain types of modification of DNA (e.g., methylation), and of chromatin (e.g., poly-ADP-ribosylation), as well as in the importance and mechanisms of RNA splicing. *S. cerevisiae* genes generally have few introns.

Specific advantages of the use of this yeast species for the study of repair are:

1. its low genome complexity, being only four- to fivefold greater than that of *E. coli* (an inventory of all transcripts on the physical map of *S. cerevisiae* is expected to be completed in the near future);
2. a versatile and well studied genetic system (including haploid and diploid phases of the life cycle) which is readily amenable to powerful recombinant DNA techniques; and
3. an extensive collection of repair-deficient mutants.

The status of the knowledge on DNA repair in *S. cerevisiae* has been reviewed previously (Haynes and Kunz, 1981; Friedberg, 1988); this chapter focuses on global aspects and recent findings.

6.3.2 REPAIR MUTANTS IN *S. CEREVISIAE*

Approximately 30 different genetic loci (complementation groups) are implicated in cellular resistance to DNA damaging agents (Haynes and Kunz, 1981; Friedberg, 1988). However, the presence of a high proportion of complementation groups represented by a single mutant indicates that the *S. cerevisiae* genome is far from saturated with respect to this type of mutant (Cox and Parry, 1968). Since the more easily mutable genes are likely to be over-represented in the present mutant collection, the actual number of loci may more likely be 50 or more. Studies on the sensitivity of single and double mutants has led to the establishment of three epistasis groups: the *RAD3*, *RAD6*, and *RAD52* groups. Within one epistasis group the sensitivity of a double mutant is no higher than that of the most sensitive parent; whereas, additive or synergistic effects are found for double mutants of which the corresponding single mutants belong to different epistasis groups (Haynes and Kunz, 1981). This behavior is interpreted to mean that each epistasis group represents a distinct and non-overlapping cellular response to DNA injury: the *RAD3* epistasis group—nucleotide excision-repair; the *RAD6* group—mutagenesis and postreplication repair; and the *RAD52* group—recombination processes in response to DNA damage. Whether these groups indeed represent separate pathways and whether this subdivision will hold when knowledge expands about the genes and mutant alleles involved remain to be demonstrated.

6.3.2.1 The *RAD3* epistasis group

The *RAD3* epistasis group consists of at least 10 complementation groups all of which are sensitive to UV and in general display enhanced UV-induced mutagenesis. Five of these (*rads 1*, *2*, *3*, *4*, and *10*) are highly defective in incision of DNA-containing pyrimidine dimers or interstrand cross-links. The others (*rad 7*, *14*, *16*, *23*, and *mms 19*) exhibit a partial defect in incision of DNA after UV-exposure and an intermediate level of UV-sensitivity. Obviously, this defect could be due to leakiness of the mutant alleles. However, for *rad 7* and *rad 23*, even deletion mutations cause only a limited deficiency in repair (Schiestl and Prakash, 1989). A possible interpretation of this finding is that these mutants are involved in a subpathway of the excision-repair process. Together the *RAD3* group reveals a minimum of 10 distinct genes, participating to a greater or lesser extent in nucleotide excision-repair. In view of the likely incompleteness of the collection of yeast repair mutants, the actual number of genes implicated in this system may be even higher.

Table 6.1. Summary of sequenced *S. cerevisiae* genes induced in nucleotide excision

Gene	Chromosomal localization	Protein (predicted)	Human homolog	Remarks
RAD1	XVI	1100 aa[1]	unknown	acidic C-terminus involved in recombination
RAD2	VII	1031 aa	unknown	transcript inducible
RAD3	V	778 aa	*ERCC-2*	nucleotide, DNA binding, 5′→3′ DNA helicase, acidic C-terminus, vital function
RAD4	V	754 aa	unknown	DNA binding?[2], acidic C-terminus
RAD7	X	565 aa	unknown	acidic stretches membrane association? partial excision defect
RAD10	XIII	210 aa	*ERCC-1*	DNA binding?
CHE-3	IX	~765 aa	*ERCC-3*	nucleotide, DNA binding? acidic stretches

[1] aa = amino acids.
[2] ? = function or property postulated based on homology to known functional domains in other proteins; no direct proof at protein level.

The genes for the majority of the existing *RAD3* group mutants have been cloned (and subsequently characterized), most by transfection of yeast genomic libraries into repair deficient mutants followed by selection for UV-resistant transformants and rescue of the correcting gene (see Friedberg, 1988). The *RAD4* and *RAD7* genes were isolated based on available cloned DNA fragments containing other closely linked markers (Fleer *et al.*, 1987; Perozzi and Prakash, 1986). In addition, a yeast gene has been identified recently because of its base sequence homology with a human gene involved in excision-repair. This gene—designated *CHE-3* (for *S. cerevisiae* homologue of *ERCC-3*)—is not identical to any of the sequenced *RAD3* group genes. Table 6.1 summarizes the main features of the *S. cerevisiae* nucleotide excision-repair genes cloned and sequenced thus far (Naumovski *et al.*, 1985; Reynolds *et al.*, 1985a, b, 1987; Madura and Prakash, 1986; Perozzi and

Prakash, 1986; Gietz and Prakash, 1988; Couto and Friedberg, 1989).

The *RAD3* group genes as well as other repair genes are scattered over the yeast genome. None of the excision-repair genes contains introns. With the notable exception of *RAD2*, transcription of the *RAD3* group genes is not substantially enhanced by treatment with DNA damaging agents although a low level of induction cannot be completely excluded at this stage. This observation provides evidence in yeast against the existence of a major SOS-response mechanism that includes the excision-repair system. Furthermore, no clear evidence exists for cell cycle regulated transcription for any gene investigated for this property. Another general characteristic is the low level of expression both at the RNA and at the protein level, as suggested by the translation initiation sequence and codon usage of these genes. Apart from the apparent homologues in higher organisms, the encoded proteins have no extensive similarity to other polypeptides with a known function or involvement in other processes. As shown by Table 6.1, a high proportion of the encoded gene products harbors either acidic regions or an acidic C-terminus, either of which may confer the ability to bind chromatin as mediated by electrostatic interaction with the basic histones. Both *RAD3* and *CHE-3* proteins contain a region that perfectly matches the consensus sequence of the well-characterized nucleotide binding box found in numerous ATPases. Furthermore, *RAD3*, *RAD4*, *RAD10*, and *CHE-3* proteins may have DNA-binding domains as suggested by structural homology to "helix–turn–helix" DNA binding motifs identified in other proteins. However, only in the case of *RAD3* has ATP-dependent DNA binding been demonstrated (Sung *et al.*, 1987a). This gene was shown by Sung *et al.* (1987b) to specify a DNA-helicase capable of unwinding a double-stranded region in a DNA molecule in the 5′ to 3′ direction. In the *RAD3* protein, regions can be recognized with similarity to seven consecutive domains, found to be conserved between two superfamilies of DNA and RNA helicases (Gorbalenya *et al.*, 1989).

Gene disruption studies have disclosed that *RAD3* has a vital function in yeast as well (Higgins *et al.*, 1983; Naumovski and Friedberg, 1983), the nature of which is unknown. Of those genes examined so far, none of the other *RAD3* group genes displays such a property. However, the *RAD1* (in contrast to *rad2*, *rad3*, and *rad4*) gene appears to be involved in mitotic recombination in addition to its function in nucleotide excision (Schiestl and Prakash, 1988). Functional interactions between individual gene products of the *RAD3* group have not been demonstrated directly. Analysis of specific single and double mutants of *rad7* and *rad23* suggest, however, a functional relationship between these genes, namely, that the *RAD23* gene product can substitute for the *N*-terminal part of the *RAD7* protein (Perozzi and Prakash, 1986). Furthermore, the UV-sensitivity of double deletion mutations between *rad7* and *rad6*, and between *rad23* and *rad6* indicate a complex relationship between the *RAD7* and *RAD23* excision-repair functions and that of *RAD6* (Schiestl and Prakash, 1989). The *RAD6* gene specifies a histone 2A, 2B specific ubiquitin-conjugating enzyme (Jentsch *et al.*, 1987). Hence *RAD6* is thought to be implicated in the modulation of chromatin conformation, required for various DNA

metabolizing processes. This observation explains the extremely pleiotropic phenotype characteristic of *rad6* mutants, including defects in postreplication repair, induced mutagenesis, meiotic recombination, and sporulation (Siede, 1988). *Rad7/rad6* and *rad23/rad6* double mutants display an unexpectedly high UV-sensitivity (Schiestl and Prakash, 1989). One possible explanation for this finding is that the *RAD6* gene is also involved in a specific subpathway of excision-repair that is unaffected in the partial *rad7* and *rad23* mutants. This observation implies that *RAD6* is a gene that functions in more than one type of repair system.

6.4 *DROSOPHILA*

6.4.1 *DROSOPHILA* AS A MODEL SYSTEM

The use of the fruit fly *Drosophila melanogaster* as an experimental system offers many advantages and some disadvantages (Rubin, 1988). *Drosophila* is a multicellular highly differentiated, sexually reproducing organism, that can be subjected to detailed genetic analysis of the type to which unicellular organisms are amenable, but which are not possible with mammals. Furthermore, the *Drosophila* genome is some 15-fold smaller than that of mammals, so that, in principle, cloning of *Drosophila* genes should be correspondingly easier. The principal disadvantage is that cell culture systems of *Drosophila*, though successfully practised in several laboratories, are somewhat trickier to handle than mammalian cell culture systems, and they are not widely used on a routine basis. Nevertheless, several groups including those of Boyd and of Smith have been able to isolate many repair-deficient mutants in *Drosophila*, and to characterize the DNA repair systems in this organism. Their work has been reviewed by Boyd *et al.* (1983, 1987).

6.4.2 REPAIR-DEFICIENT MUTANTS

Repair-deficient mutants of *Drosophila* have been isolated by their hypersensitivity to mutagens, and, less directly, as mutants with increased mutation frequencies or decreased recombination. Several mutants with reduced meiotic functions (*mei* mutants) turn out to also be repair-deficient. About 30 complementation groups governing DNA repair have so far been identified. In contrast to bacterial, yeast, and mammalian systems, all the UV-sensitive *Drosophila* mutants are also sensitive to alkylating agents and in some cases to X-rays. One possible reason for this effect is that the *Drosophila* mutants were not isolated by selection for UV sensitivity, since the mutant selection procedure is carried out in whole flies, which do not easily lend themselves to UV-irradiation. Therefore, possibly a class of more specifically UV-sensitive mutants exists, and has yet to be identified.

Table 6.2. Properties of excision-repair-deficient *Drosophila* mutants

	Incision	UV endo Sites	UDS	PRR	Meiotic recombination
mei-9	0	0	---	+++	10%
mus201	0	0	---	+++	100%
mus205 100%	98	47	±		
mus302 100%	113	72	±		
mus304	90	43	±		Reduced
mus306	74	47	±		
mus308	51	24	±		

UDS = unscheduled DNA synthesis
PRR = pyrimidine dimer removal

DNA repair has been examined at the cellular level in cultured *Drosophila* cells. The steps of excision-repair, involving incision, excision, repair synthesis, and ligation are detected using similar techniques to those used for mammalian cells. The properties of some of the mutants are summarized in Table 6.2. Two mutants, *mei-9* and *mus-201*, are totally deficient in excision-repair, as measured by incision breaks, removal of pyrimidine dimers, and unscheduled DNA synthesis. In this respect, they resemble the incision-defective mutants of other species, such as the *uvr ABC* mutants of *E. coli*, the *RAD3* epistasis group of *S. cerevisiae*, at least five of the Chinese hamster complementation groups, and several of the human xeroderma pigmentosum groups. This result implies that the *mei-9* and *mus-201* mutants are defective in the incision step of excision-repair. However, in contrast to the incision-defective mutants of other species, that are only hypersensitive to UV and agents producing bulky adducts but show normal sensitivity to X-rays and alkylating agents, the *mei-9* and *mus-201* mutants of *Drosophila* are also hypersensitive to alkylating agents and, in the case of *mei-9*, to X-rays also.

Mutants at five other loci (*mus-205*, *mus-302*, *mus-304*, *mus-306*, *mus-308*) have a reduced (25 to 70%) ability to excise pyrimidine dimers, although only the *mus-306* and *mus-308* mutants are deficient in the ability to carry out the incision step following UV-irradiation. The other three mutants (*mus-205*, *mus-302*, and *mus-304*) may be defective in a later step.

6.4.3 INTERACTION OF EXCISION-REPAIR ENZYMES WITH OTHER PROCESSES

6.4.3.1 DNA replication on damaged templates

Cells from all species have the ability to tolerate damage persisting in their DNA by using an ill-characterized process termed "daughter-strand," or "postreplication repair." This process enables cells to synthesize high molecular weight intact daughter DNA strands, despite the presence of damaged sites on the parental strands. The totally excision-deficient *mei-9* and *mus-201* strains of *Drosophila* show no deficiency in this process; whereas four of the five mutants with intermediate levels of excision-repair also show a reduced level of daughter-strand repair. These observations suggest that the gene products of these loci may be involved in both repair processes.

6.4.3.2 Recombination

The central role of the *mei-9* gene product in DNA metabolism is indicated by its widely pleiotropic effects. Not only is it involved in the response to nearly all DNA damaging agents, but it also plays an important role in meiotic recombination and gene amplification. Evidence suggests that the *mei-9* product is involved in the actual meiotic exchange event. Crossing over is greatly reduced; whereas, gene conversion is unaffected. *Mei-9* mutants are also deficient in transposition and in ribosomal DNA magnification. The only other gene in any species with such a diverse series of effects is the *recA* gene in *E. coli*, which is involved in the control of the SOS response, and also plays a direct role in genetic recombination and in UV mutagenesis. In contrast to *mei-9*, however, *recA* strains of *E. coli* are relatively proficient in excision-repair of UV damage. The other totally excision-deficient *Drosophila* mutant *mus-201* shows no recombination abnormalities.

6.5 MAMMALIAN CELLS

6.5.1 CELLULAR BIOCHEMISTRY

The basic process of excision-repair of UV damage in mammalian cells is similar to that in other organisms; but the precise details of the mechanism must await the successful cloning of genes and isolation of gene products.

The cellular biochemistry of excision-repair has been studied in great detail. All steps of incision, excision, repair synthesis, and ligation can be demonstrated in cultured cells. The incision step is rate-limiting; therefore, incision breaks can be detected only either using very sensitive techniques or by blocking the subsequent repair synthesis step with inhibitors of DNA polymerases such as aphidicolin or

cytosine arabinoside (Squires *et al.*, 1982). Excision of pyrimidine dimers is effected efficiently, but fairly slowly in human cells, with most of the damage removed in about 24 hours. By contrast, cultured rodent cells are able to remove only a small fraction (10 to 30%) of pyrimidine dimers from their DNA, despite showing more or less the same resistance as human cells to the lethal effects of UV light.

A possible explanation for this paradox was provided in 1985 by the discovery that cultured hamster cells were able to remove efficiently most of the damage from actively transcribed regions of DNA, while excision from the bulk of the DNA not being actively transcribed is very inefficient (Bohr *et al.*, 1985). (This preferential repair of active genes is discussed in detail in Chapter 10 by Mullenders and Smith.) An alternative explanation for the similar sensitivity of rodent and human cells to UV-induced cell killing could be that both cell types are able to remove UV-induced (6-4) photoproducts efficiently from their DNA.

Repair synthesis, unlike replicative DNA synthesis, is not confined to the S phase of the cell cycle and occurs in all phases. This situation provides a method to measure repair synthesis termed unscheduled DNA synthesis (UDS), whereby DNA synthesis following UV-irradiation is measured autoradiographically in non-S phase cells. A further difference between repair and replicative synthesis is the relative resistance, under most conditions, of the former to the inhibitor hydroxyurea which can almost completely abolish replicative DNA synthesis. However, a combination of hydroxyurea and either cytosine arabinoside or aphidicolin can be used to block repair synthesis (Mullinger *et al.*, 1983). Since the latter is a specific inhibitor of DNA polymerase *a* and *d*, but is without effect on DNA polymerase β or γ, the latter two polymerases are not likely to be important contributors to repair synthesis; whereas one or both polymerase *a* and *d* are positively implicated. The average size of the repaired patch in human cells has been estimated to be about 30 nucleotides.

6.5.2 REPAIR-DEFICIENT MUTANTS

6.5.2.1 Human mutants

Xeroderma pigmentosum (*XP*)

The crucial importance of repair-deficient mutants in helping to dissect the DNA repair process in lower organisms is underscored by the findings described in the previous sections. The discovery by Cleaver (1968) that individuals affected with the sun-sensitive cancer-prone genetic disorder, XP were deficient in excision-repair of UV damage provided a major step toward understanding this process in human cells, and indicated the importance of excision-repair in the avoidance of cancer and in the maintenance of a healthy condition in humans. Cell fusion studies on many XP patients have enabled them to be assigned to eight distinct complementation

groups, designated A–G and variant (Lehmann, 1982a; Lehmann and Dean, 1989). Individual members originally assigned to groups H and I are now thought in fact to belong to groups D and C, respectively (Johnson *et al.*, 1989; Bootsma *et al.*, 1989). The XP variants, comprising about 20% of all XPs, are deficient in daughter strand repair, but have normal levels of excision-repair (Lehmann *et al.*, 1975). They will not be discussed further here. The other seven groups are all deficient to a greater or lesser extent in excision-repair of damage produced by UV light and by carcinogens which produce bulky adducts in cellular DNA. This deficiency results in hypersensitivity of XP cells to the lethal effects of these agents. In contrast, XP cells show a normal response to ionizing radiation, methylating agents, and other mutagens which produce only minor distortions in DNA. In this respect, they correspond to the *uvr ABC* mutants of *E. coli*, and the *RAD3* group mutants of *S. cerevisiae*.

Biochemical studies using various techniques have provided convincing evidence that all seven XP groups are deficient in an early step of excision-repair which occurs at or before the incision step (Tanaka *et al.*, 1975; De Jonge *et al.*, 1985). These findings attest to the complexity of this step in human cells. The gene products could be involved either directly in the insertion of one or more breaks near the damaged site, in preparation of the DNA structure for attack by the incision enzyme, or in altering the chromatin structure such that the damaged DNA is rendered accessible to the repair enzymes. The properties of cells in different complementation groups show some differences. Repair synthesis levels in most cells in groups A and G are extremely low; whereas in groups C and D, they are between 10 and 30% of that in normal cells (Lehmann and Dean, 1989). Nevertheless, cells in group D are very sensitive to the lethal effects of UV; whereas those in group C are less sensitive. Preliminary evidence suggests that XP-C cells are able to repair damage in active regions of DNA with some efficiency but are disturbed in the overall genome repair. In contrast, XP-D cells appear to be totally deficient in the removal of cyclobutane dimers. The residual levels of repair synthesis in XP-D strains may be caused by repair of the (6-4) photoproduct. In group F, repair synthesis is relatively slow, but it is maintained for prolonged periods (Fujiwara *et al.*, 1985).

Trichothiodystrophy (TTD)

This genetic disorder is characterized by sulphur-deficient brittle hair, mental and physical retardation, and photosensitivity in some patients. Recently, the majority of TTD patients studied were shown to have deficient excision-repair of UV damage; cell fusion studies have indicated that the defect was in the same gene as XP group D (Stefanini *et al.*, 1986). This discovery has important implications for the relationship between DNA repair and cancer (Lehmann and Norris, 1989).

Cockayne's syndrome (CS)

This genetic disorder is characterised by diverse symptoms including dwarfism, mental and physical retardation, skeletal deformities, and sun-sensitivity. Unlike those with XP, however, patients with CS show no elevated level of sunlight-induced skin cancers. Cultured CS cells are very sensitive to killing by UV light, but there no gross defect occurs in excision-repair.

Nevertheless, following UV-irradiation, RNA synthesis and DNA synthesis fail to recover to normal levels (Mayne and Lehmann, 1982). This observation led to the hypothesis—subsequently confirmed by Mayne *et al.* (1988a)—that CS cells were deficient in the ability to remove damage from active genes, while remaining proficient in excision-repair of damage from bulk DNA. The implication of this finding is that at least some of the gene products involved in preferential repair (i.e., those deficient in CS) are distinct from those involved in bulk repair. Two distinct complementation groups have been identified in CS (Lehmann, 1982b); in addition, a few patients have the symptoms of both CS and XP. One of these forms the single member of XP group B; another has been assigned to group H; recent evidence suggest that the latter cell strain may belong to group D (Johnson *et al.*, 1989).

6.5.2.2 Rodent cell mutants

The human mutants discussed above were isolated by their existence as individuals with particular clinical symptoms. Any mutant identified in this way must clearly be compatible with life in the whole individual. Therefore, mutants in other human DNA repair genes may well exist; but, they would not be compatible with life, and, therefore, could not be identified in this way.

An alternative means of obtaining mammalian cell mutants is by mutagenizing cultured mammalian cells, and selecting for mutants on the basis of their sensitivity to UV light. A major drawback to this procedure is that the mammalian genome is diploid, and the probability of obtaining a cell containing a mutation in both homologs of a particular locus is very low. Parts of the genome of cultured rodent cells are, however, functionally hemizygous, so only one copy of the genes is active in these regions. Consequently, isolation of repair-deficient rodent mutants has been possible, a process particularly useful to clone DNA repair genes (Busch *et al.*, 1989). However, that the mutant selection procedures are strongly biased in favor of mutations occurring in functionally hemizygous genes, so that some loci may not to be identified by these procedures.

Many UV-sensitive rodent cell mutants have been isolated, and is characterized genetically and biochemically. Eight complementation groups have been identified all controlling excision-repair (Zdzienicka *et al.*, 1988; Thompson *et al.*, 1988; Busch *et al.*, 1989). The properties of these mutants are summarized in Table 6.3.

Table 6.3. Properties of UV-sensitive rodent cell mutants

C-Group #	Chr #	Example	S	MMC S	T-T R	6-4 R	UDS/ incision
1	19	UV20	sss	sss	0	0	0
2	19	UV5	sss	s	0	0	0
		V79-VH-1	sss		0	50	50
3	2	UV24	sss	s	0	0	0
4	16	UV41	sss	sss	0	0	0
5	13	UV135	sss	s	0	0	0
6	10	UV61/US46	ss	s	0	100	100
7		V79-VB11	ss	s			30-70
8		L51-US31	ss	ss			

MMC = mitomycin C; UDS = unscheduled DNA synthesis; C-Group # = Complementation Group number; Chr # = Chromosome number; S = sensitivity; R = removal.

In at least five of these groups (1 to 5), the defect appears to lie in the incision step. In this respect, these mutants resemble the XP human mutants; as yet little evidence exists for overlap of mutants between the Chinese hamster and in humans. In particular, mutants in complementation groups 1 and 4 of the hamster are extremely sensitive to mitomycin C (Thompson *et al.*, 1980); whereas none of the XP groups show such sensitivity.

6.5.2.3 Repair of (6-4) photoproducts

For many years, the cyclobutane pyrimidine dimer has been recognized as a quantitatively major photoproduct produced by UV-irradiation, and its biological importance has been proven in many systems. Work carried out in recent years has shown that the (6-4) photoproduct is a minor one of some significance. Studies on mammalian cells have been carried out principally by Mitchell and coworkers, who have developed a sensitive radioimmunoassay for measuring (6-4) photoproducts (Mitchell *et al.*, 1988). Using this assay, they have shown that (a) (6-4) photoproducts are produced in cellular DNA at about 25% the frequency of cyclobutane dimers; and (b) they are removed relatively rapidly from both human and rodent cells. Most of these lesions are removed within three hours after irradiation; whereas very few cyclobutane dimers are removed during this period. The implications of these findings are that measurements of the rates of incision or

repair synthesis, which are nearly always made at early times after irradiation, will include a major contribution from the repair of (6-4) photoproducts, rather than resulting exclusively from repair of cyclobutane dimers as had previously been assumed. Thus, interpretations of some earlier work may need reevaluation.

In most of the repair-deficient mutants of both human and hamster origin, removal of both (6-4) products and cyclobutane dimers is defective (Mitchell *et al.*, 1985, 1988). However, four mutants show differences in the effect of the mutation on repair of cyclobutane dimers and on (6-4) photoproducts. The mutant VH-1 in hamster complementation group 2 is totally unable to repair cyclobutane dimers, as are other members of this group. In contrast, VH-1 is able to remove (6-4) products at about 50% of the normal rate and has a level of 50% of UDS compared with wild-type V79 cells measured during the first 3 hours after irradiation (Mitchell *et al.*, 1989). Similar properties have been found for mutant UV61 in complementation group 6 (Table 6.3). Again repair of cyclobutane dimers is absent, but repair of (6-4) photoproducts is barely affected (Thompson *et al.*, 1989). In human systems, two cell strains from patients with TTD showed opposite properties. Excision of pyrimidine dimers was normal, but repair of (6-4) photoproducts occurred at a reduced rate (Broughton *et al.*, 1990). Finally, in a revertant of a totally deficient XP-A line, repair of (6-4) products was restored; whereas repair of cyclobutane dimers in bulk DNA remained defective (Cleaver *et al.*, 1987). These findings show that, although the overall process for excision-repair of cyclobutane dimers and (6-4) products involves the same gene products, subtle differences exist perhaps in the affinity of the gene products for the two different lesions or in the domains of the proteins used in the repair of the individual lesions. Furthermore, these findings indicate that repair of both (6-4) photoproducts and of cyclobutane dimers in active genes is important for the survival of the cell.

6.5.2.4 Cloning of human repair genes

The general strategy for the isolation of mammalian repair genes is based on transfection of genomic, or cDNA, from a repair competent cell into a repair deficient mutant cell line, followed by selection of primary and secondary transformants that have nearly regained wild-type resistance. Molecular cloning of the sequences can be achieved by standard recombinant DNA techniques using tags provided by the transfected sequences themselves (species-specific dispersed repeats) or by markers linked to exogenous DNA to distinguish it from host chromosomal DNA. Presently, successful cloning of repair genes has been obtained by employing genomic DNA, although in principle full length cDNA should also be a suitable starting material. Possibly, the quality and complexity of available cDNA libraries in mammalian expression vectors were insufficiently high for the genes attempted. Because exogenous DNA is subject to scrambling in the mammalian cell during the transfection process, gene size is a limiting factor for

genomic DNA transfections. Another important parameter influencing the rate of success is the transfection and repair characteristics of a recipient cell. Different cell lines vary dramatically with respect to transfection frequencies (number of transformants per cell) as well as with regard to the average amount and degree of intactness of integrated exogenous DNA (Hoeijmakers *et al.*, 1987; Mayne *et al.*, 1988b). Unfortunately, most human cell lines (mainly SV40 transformed fibroblasts) appear to perform poorly with respect to the latter two criteria, rendering them less suitable for this approach than some rodent lines (e.g., Chinese hamster ovary, CHO) discussed below. Furthermore, the nature and stability of the repair mutation of the recipient cell line is crucially important, particularly with respect to the occurrence of revertants (Hoeijmakers *et al.*, 1988a).

The XP-A correcting (XP-AC) gene

Tanaka and coworkers (1989) succeeded in isolating the mouse and corresponding human gene that substantially and specifically enhances UV-resistance and UDS of XP-A cells. This result was obtained, notwithstanding the inferior transfection properties of the SV40 transformed XPA fibroblasts (compared with some rodent cell lines) and the occurrence of revertants noted by others (Royer-Pokora *et al.*, 1984; Schultz *et al.*, 1985).

The very extensive transfection experiments done by Tanaka *et al.* yielded two primary and one secondary transformants. The gene cloned from the secondary transformant was assigned to chromosome 9q34 (Table 6.2), in accordance with recent cell hybridization studies (e.g., Kaur and Athwal, 1989) and with a very early observation linking an XP defect with blood group markers on this chromosome (Westerveld *et al.*, 1976). The XP-AC gene was found to specify transcripts of 1.1 kb in mouse, and 1.3 kb (major) and 1.1 kb (minor) mRNA's in human cells (Tanaka *et al.*, 1989). The latter result is in good agreement with microneedle injection experiments of size-fractionated poly(A)$^+$RNA of repair competent cells into XP-A fibroblasts by Hoeijmakers *et al.* (1988b, 1990), suggesting that these two independent approaches score for the same gene. Cells of a number of XP-A patients contained either decreased amounts or abnormally sized mRNAs for this gene (Tanaka *et al.*, 1989), further strengthening the correlation between the cloned sequence and the defect in XP-A. Molecular identification of the mutation in one or more XP-A patients would provide definite proof for this proposition. A remarkable finding is that the cloned gene and cDNA correct UV-resistance and UDS only to intermediate levels (Tanaka *et al.*, 1989). This finding contrasts with complete restoration of UDS to wild type levels obtained after microinjection of partially purified XP-A correcting factor from HeLa cell extracts (De Jonge *et al.*, 1983; Vermeulen *et al.*, 1986) or calf thymus (Hoeijmakers *et al.*, 1990) when injected into XP-A fibroblasts. The XP-A correcting activity behaves as a single protein in various purification steps, binds to ss, ds, and UV-irradiated DNA with roughly the same affinity, and has a

molecular weight of 45 kD as determined by sodium dodecyl sulphate gel electrophoresis and gel filtration (Table 6.4; Hoeijmakers *et al.*, 1990). This size is sufficient to be accommodated by a 1.3 kb mRNA. However, direct proof that this protein and mRNA detected by microinjection are derived from the same cloned gene must await sequencing of the cDNA and protein.

Isolation of other XP-correcting genes

Several reports on the cloning of other XP correcting genes by DNA transfection have appeared. Unfortunately, unequivocal evidence that the cloned sequences indeed represent genes that specifically confer repair proficiency to a wild type level to representatives of the respective XP complementation groups has not been presented as yet. One of the human genes correcting a Chinese hamster repair mutant was identified as the XPB correcting gene.

The ERCC *genes*

The superior transfection properties of some rodent excision-repair mutants has permitted the cloning of several human genes by genomic DNA transfection to repair-deficient Chinese hamster lines of different complementation groups (Van Duin and Hoeijmakers, 1989). These genes are designated "*ERCC*" (for excision-repair cross complementing rodent repair deficiency) genes, followed by a number referring to the rodent complementation group. Thus the human *ERCC-1* gene complements the excision-repair defect of rodent mutants of complementation group 1. Mutants of this group are very sensitive to UV-light, carcinogens causing bulky adducts, and even more so to cross-linking agents (Thompson *et al.*, 1981; Hoy *et al.*, 1985; Zdzienicka and Simons, 1986). Transfection of the *ERCC-1* gene fully and specifically compensates for the wide spectrum of impaired repair properties of group 1 mutants (Westerveld *et al.*, 1984; Zdzienicka *et al.*, 1987; Van Duin *et al.*, 1988a). This gene is very likely not involved in any of the known XP and CS complementation groups (Van Duin *et al.*, 1989a; Table 6.4).

The *ERCC-1* gene spans a region of 15 to 17 kb on chromosome 19q13.2, and is composed of 10 exons (Van Duin *et al.*, 1987). *ERCC-1* transcripts are found at a low, basal level in all mouse tissues and in stages of embryogenesis analyzed, and do not seem to be substantially induced in UV-irradiated HeLa cells (van Duin *et al.*, 1987, 1988b). The 1.1 kb *ERCC-1* mRNA encodes a protein of 297 amino acids (Table 6.4). Comparison with consensus sequences of functional protein domains has pointed to the presence of a potential nuclear location signal (NLS) and a "helix–turn–helix" DNA binding motif (Van Duin *et al.*, 1986; Hoeijmakers *et al.*, 1986). Computer comparison of the *ERCC-1* amino acid sequence with known repair proteins of lower organisms revealed striking homology with the predicted amino acid sequence of the yeast excision-repair protein *RAD10* (van

Duin *et al.*, 1986). This finding suggests that *ERCC-1* and *RAD10* are descendants of the same ancestral gene and, hence, have analogous functions. The only major difference between the two proteins is the fact that *ERCC-1* is longer than *RAD10*. At the position where the homology with *RAD10* stops, a stretch of amino acids begins, with significant similarity with part of the *E. coli* excision-repair protein *uvrA* (Hoeijmakers *et al.*, 1986; Table 6.2). Intriguingly, at the point where this homology terminates, yet another region of similarity turns up: this time between the carboxyl terminus of *ERCC-1* and that of the *E. coli uvrC* protein (Doolittle *et al.*, 1986). Another striking similarity between the human *ERCC-1* and yeast *RAD10* genes emerged from detailed analysis of their 3′ regions. Both genes appear to overlap with the 3′ terminus of another gene (Van Duin *et al.*, 1989b). This unusual type of gene configuration seems to be conserved from yeast to humans, suggesting that it has an important biological function. At present, neither this function nor that of the antisense genes is known.

The *ERCC-2* gene cloned by Weber *et al.* (1988) corrects the incision defect and UV-sensitivity of group 2 mutants. The gene, which is approximately 20 kb in size is located on chromosome 19q13.2 in close proximity (within 250 kb) to *ERCC-1*. The cDNA sequence of ≈2.7 kb estimates a protein of 760 amino acids, with putative NLS, DNA binding, and nucleotide binding domains (Table 6.4; Weber *et al.*, 1988). The *ERCC-2* polypeptide harbors extensive homology to the yeast *RAD3* repair helicase (Weber *et al.*, 1990). Comparison of the frequency of rodent *ERCC-2* mutants generated by point mutagens versus agents causing predominantly frameshifts (which in general have a more deleterious effect) suggests that complete inactivation of *ERCC-2* (versus *ERCC-1*) is lethal and, consequently, that this gene may have a vital function in mammals (Busch *et al.*, 1989). This observation strengthens the functional homology with the *RAD3* gene in yeast.

The *ERCC-3* gene corrects the UV-sensitivity and UDS of mutants belonging to group 3 (Weeda *et al.*, 1990). The gene is ≈45 kb in size and is assigned to chromosome 2q21 (Table 6.4). The predicted 782 amino acid *ERCC-3* protein contains putative domains for nucleotide, DNA, and chromatin binding as well as for helicase activity. Its amino acid sequence bears no significant homology to known repair genes of yeast and *E. coli*. However, the gene is very strongly conserved, and recently the yeast cognate CHE-3 has been cloned in one step using human *ERCC-3* probes (Tables 6.1 and 6.4). Microinjection of the *ERCC-3* cDNA in fibroblasts of various XP complementation groups indicated that this gene specifically and completely corrects the UDS defect in XP complementation group B, the single member of which displays the clinical symptoms of both XP and CS. This finding reveals for the first time overlap between ERCC- and XP-genes.

The *ERCC-6* gene was isolated by DNA mediated gene transfer to a UV-sensitive mutant of CHO complementation group 6. The preliminary characterized gene appears to have a size of >100 kb, is located on chromosome 10q1.1, and encodes two poorly expressed mRNAs of 6.5 and 8.5 kb (Table 6.4). Determination of its nucleotide sequence should reveal whether a yeast equivalent can be recognized, and may provide hints as to its function.

Table 6.4. Summary of cloned human genes involved in nucleotide excision-repair

Gene yeast	Size			Chromosome location	Protein homolog
XPAC	~25 kb[1]	9q341[1]	~45 kD2[2]	Unknown	DNA binding[2]
ERCC-1	15-17 kb	19q13.2	297	*RAD10*	DNA binding?[3] Homology to parts of *uvrA*, and *uvrC*
ERCC-2	~20 kb	19q13.2	760	*RAD3*	Nucleotide, DNA binding? DNA-helicase? Vital function?
ERCC-3	~40 kb	2q21	782	*CHE-3*	Nucleotide, DNA binding? Acidic stretches
ERCC-6	~100 kb	10q1.1	>1000	Unknown	Unknown

[1]Data from gene, cloned by DNA transfection and from cell hybridization experiments.
[2]Data from partially purified protein using microinjection; formal proof that protein and gene are the same is lacking.
[3]? = property or function postulated on amino acid sequence homology to known functional domains in other proteins; direct proof at protein level lacking.

6.6 RELATIONSHIP BETWEEN REPAIR SYSTEMS OF DIFFERENT ORGANISMS

With the present state of knowledge about pro- and eukaryotic excision-repair processes, considerations are relevant of the extent to which these systems are comparable and how far extrapolation of the molecular mechanism elucidated for the *E. coli* pathway to eukaryotes is justified.

6.6.1 PROKARYOTES AND EUKARYOTES

In several respects, excision-repair is similar in pro- and eukaryotic organisms. First, the spectrum of sensitivities of excision-deficient mutants in both kingdoms is very alike, ranging from various UV-induced DNA lesions (cyclobutane dimers and (6-4) photoproducts) to bulky DNA adducts and cross-links. This variety indicates that the principal targets for both pathways overlap, and implies that mechanisms for recognition of structural alterations in DNA are likely to be strongly related. Second, several common, basic steps in the excision-repair

process have been shown to occur in both pro- and eukaryotes, although the underlying molecular mechanisms may differ markedly. This effect holds for DNA unwinding by helicases, incision of damaged DNA, repair synthesis, and ligation. However, little is known about the mechanism of incision (single or dual cut, distance from the lesion) in eukaryotes. Third, some similarity exists in the occurrence of preferential repair of the transcribed strand of DNA, found first in mammalian cells (Bohr *et al.*, 1985; Mellon *et al.*, 1987) and recently also in *E. coli* (Mellon and Hanawalt, 1989). This finding suggests that a tight linkage between the transcription process and excision-repair existed before divergence of pro- and eukaryotes and that the elements involved may have been conserved as well. Finally, at the level of the genes and proteins involved the discovery of amino acid sequence homology between part of the human *ERCC-1* and of *uvrA* and *uvrC* discussed above is of great importance. This finding implies that at least some functional aspects are shared between excision-repair proteins of *E. coli* and humans. The region of homology between *ERCC-1* and prokaryotic polypeptides, which seems to be essential for the human gene product is absent in the yeast *RAD10* protein (van Duin *et al.*, 1986). This function in yeast may not be essential, and eventually has been lost, or perhaps other yeast-repair proteins have incorporated these particular domains.

Important differences between pro- and eukaryotic excision-repair have been demonstrated, while others can be anticipated. The fundamental difference in chromatin structure between pro- and eukaryotes is expected to have major consequences for a number of DNA metabolizing cellular processes, including repair. This difference may at least explain in part the failure of *uvrABC(D)* gene products to function in intact mammalian cells (Zwetsloot *et al.*, 1986), whereas they do function in "cell-free" extracts using purified, damaged plasmid DNA as substrate (Hansson *et al.*, 1990). Another important difference, which may be related to chromatin structure, is the number of repair-deficient mutants in eukaryotes compared with those in *E. coli*. Complementation groups have been identified in yeast (10 or more) and in mammals (eight to 15), the majority of which are disturbed at incision or pre-incision steps of the excision process. This result is considerably higher than the number of genes involved in incision in *E. coli*, particularly since the values in yeast and mammals are likely to be underestimates. This observation means that the biochemical complexity, at least in the early steps of excision-repair, is probably much greater than in *E. coli*. Furthermore, except for the C-terminus of *ERCC-1*, none of the other eukaryotic excision-repair genes sequenced to date displays extensive amino acid sequence similarity to *E. coli* proteins. Finally, with respect to excision-repair itself, in none of the eukaryotic systems studied has evidence been obtained that supports the presence of a major SOS response, like in *E. coli*. The yeast RAD2 gene is the only one of the eukaryotic excision-repair genes examined thus far that is substantially induced at the transcription level by UV-irradiation.

Thus, important similarities between the molecular mechanisms of pro- and eukaryotic nucleotide excision-repair exist, perhaps in recognition of structural

aberrations in DNA and in later steps of excision-repair. At the same time, fundamental differences clearly occur, differences that originate at least in part from the principally different structure in which DNA is packaged in the two systems.

6.6.2 YEAST AND HUMANS

Available data point to a very high level of homology between the excision-repair systems of two extremes in the eukaryotic spectrum: *S. cerevisiae* and humans. Mutants from both species resemble each other in phenotype (e.g., sensitivities to various damaging agents, induced mutagenesis, lesion removal). They are also very similar with respect to the number of mutants and assignment of the defect in the majority of the mutants to (pre)incision step(s) of the excision pathway that can be bypassed by introduction of the bacteriophage T4 endonuclease (Tanaka *et al.*, 1975; De Jonge *et al.*, 1985; Valerie *et al.*, 1986). Furthermore, in high and low eukaryotes, the same basic steps in excision-repair have been demonstrated, including incision of damaged DNA, repair synthesis, ligation and preferential repair of pyrimidine dimers in actively transcribed genes. The degree of similarity is most impressive when genes are compared. All mammalian repair genes analyzed to date have a closely related cognate in yeast. This similarity concerns *ERCC-1*, with its yeast equivalent *RAD10* (*ERCC-2* which exhibits extensive amino acid sequence identity with the repair helicase *RAD3*) and *ERCC-3* (that harbors a similar level of homology with the yeast counterpart CHE-3; Tables 6.1 and 6.4). Additional genes need to be analyzed to determine whether this trend continues and whether it is representative of the entire collection of excision-repair genes. For the genes mentioned above, the similarity between the predicted gene products also implies considerable functional resemblance. Whether this degree of similarity is sufficient to permit functional cross-complementation requires thorough investigation. In the case of the couple *RAD10/ERCC-1*, expression of the *RAD10* gene in Chinese hamster mutants of group 1 resulted in a very small increase in survival following UV or MM-C treatment, which appeared independent of dose (Lambert *et al.*, 1988). This result, if significant, is difficult to rationalize with the finding that the C-terminus of the *ERCC-1* protein, which is absent in *RAD10*, is essential for its ability to confer UV and MM-C resistance to mutants of the same complementation group (Van Duin *et al.*, 1988a). Furthermore, introduction of the *ERCC-1* gene in yeast rad 10 mutants induced no significant increase in UV-survival suggesting that the *ERCC-1* protein has diverged too much from its yeast equivalent to substitute for it functionally. Clearly further research is required to see whether this observation holds also for the other combinations: *ERCC-2/RAD3*; *ERCC-3*/CHE-3 which show a considerably higher level of homology than *ERCC-1/RAD10*, and for the remaining repair genes that are and will be in the process of being cloned and analyzed.

Undoubtedly, the near future will witness considerable progress with respect to the isolation of additional genes, their evolutionary conservation, and their

functional analysis. The major challenge will be to disclose the function of each individual component, and to fit these pieces into the complex puzzle of the molecular mechanism of the excision-repair process *in vivo*.

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7 Interspecies Determinants: Bioactivation and Inactivation of Carcinogens

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7.1 INTRODUCTION

Many carcinogens have to be converted into ultimate carcinogenic metabolites by biotransformation before they can induce tumors in animals or humans, a process having many differences among species (Langenbach *et al.*, 1983; Slaga, 1988). For some compounds, such bioactivation is not required (e.g., direct-alkylating agents, or metals), and species differences in their carcinogenic potency may be the consequence of differences in pharmacokinetics or detoxication (i.e., in the rates of absorption via the various portals of contact) and in their distribution *in vivo*. Furthermore, if bioactivation is required, even greater species differences may result due to variations in enzyme activities and availability of co-factors. The effect of these pro-carcinogens will probably be limited to the cells in which they are activated to ultimate carcinogens, because electrophilic metabolites are often too reactive to leave a cell and be taken up by another cell. Differences in carcinogenic responses among species are to be expected, when the tissue or cell type in which tumors arise occurs in one species and not in another. For example, the forestomach and the Zymbal gland are present in rodents but not in humans.

The major factors to consider in species differences in carcinogenic responses are the following:

1. access of a pro-carcinogen to a susceptible tissue, and its absorption into cells of that tissue;
2. presence or absence of bioactivating enzyme(s) required to convert the compound to the ultimate carcinogen(s) in cells in which a pro-carcinogen is absorbed;
3. the efficiency of detoxifying systems either in competing with the bioactivating enzymes or in trapping reactive intermediates before they can reach the DNA.

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7.2 EXPOSURE AND PHARMACOKINETIC SCALING

The most relevant routes of exposure are ingestion and inhalation. Occasionally, test compounds are painted on skin to determine the role of local metabolism. For only very special purposes are other routes (e.g., intraperitoneal injection) used; in that case, the metabolism is expected to be similar to that by oral administration, except for that biotransformation caused by intestinal micro-organisms.

When compounds are administered orally, common laboratory animals and humans perform similar processes. The compounds pass rapidly to the stomach, limiting uptake and metabolism in the oral cavity. However, among species, differences exist in stomach characteristics. For instance, the pH of gastric juice varies from 1 in guinea pig, rabbit, and human to 7 in dog, and the time that a compound remains in the stomach after ingestion may also vary considerably. If a compound is acid-labile, or can react only at a (very) acidic pH (e.g., nitrosamine formation), differences may occur at this level. Otherwise most of the dose of a compound is released unchanged into the gastrointestinal tract. A sufficiently lipid-soluble compound is taken up readily from the gut lumen into the mucosa cells. Unless a compound is a suitable substrate for any of the enzymes (usually catalyzing hydrolytic or reductive metabolism) contained in or released by bacteria, or secreted into the gut lumen by animal cells, it is metabolized little in the gut. However, a suitable substrate may be metabolized to an appreciable extent, and species differences in gut flora may result in pronounced differences in the fate of a compound. For instance, quinic acid is aromatized extensively by the gut flora in humans and Old World monkeys (e.g., baboon), but very little by that in New World monkeys (e.g., capuchin) (Adamson *et al.*, 1970). If uptake occurs, the compounds are subject to extensive (i.e., "first pass") metabolism in the liver. Species differences in first pass metabolism will have a profound impact on overall metabolism of compounds and on their bioavailability in the remainder of the body.

The physiology of the lungs plays an important role during inhalation. For instance, whether a substance is inhaled through the nose only or through both nose and mouth (as for humans), the nasal epithelium is substantially exposed. Because of differences in alveolar ventilation volume between large and small animals, the rate of accumulation may be markedly different. Alveolar ventilation per unit of body weight is much less in humans than in laboratory animals: specifically, 2.8 l/min per kg (human) compared with 15 l/min per kg (rat) or 33 l/min per kg (mouse). Using a physiologically-based pharmacokinetic approach that accounts for differences in many physiological characteristics between animal species (i.e., pharmacokinetic scaling; Mordenti, 1986), a large difference in the blood concentration of styrene exists when rats, mice, or humans are exposed to styrene for a short time (Ramsey and Anderson, 1984). If the solubility of a compound in blood has been determined (e.g., as a partition coefficient between air:blood or blood:tissue) and the extent of absorption in the lung in one species is known, then extrapolation to other species is relatively reliable when relying on parameters of lung physiology and lung blood flow. Even fluctuations in the concentration of a

compound in tissues such as fat can be estimated using physiologically based pharmacokinetics.

Drug metabolism in the lungs is generally unlikely to play a major quantitative role. However, activation in the lung of a compound to an ultimate carcinogen that also causes tumors in the same tissue may be quite important from a public health standpoint. If a carcinogen is a particle (e.g., asbestos) or if chemical carcinogens are present on the surface of a particle, species differences in the way the lungs handle these particles will greatly affect the pathologic outcome, because duration of contact with a particle capable of releasing bound carcinogens locally in the lung influences directly the dose of the toxicant to the proximate tissue.

During inhalation, first pass metabolism in gut and liver (normally seen after oral exposure) is bypassed. Thus, species differences due to the effects of metabolites generated via ingestion will be absent when the same substance is inhaled.

Physiologically based pharmacokinetic models and scaling procedures generally make possible the prediction of body burden and, to some extent, the blood or tissue pharmacokinetics of a compound, provided that data exist in one species. However, if bioactivation is required for a carcinogenic effect, data on the metabolic capacity for that substance are essential for such scaling procedures, particularly when a specific route of bioactivation is considered.

7.3 ACTIVATION OF CARCINOGENS

7.3.1 REACTIVE INTERMEDIATES: RATE OF FORMATION

The present discussion deals only with those carcinogens that form DNA adducts after activation to a reactive intermediate. All those compounds that act as "promoters" are excluded, because promotion by itself is by definition not a cause of cancer. Direct-acting carcinogens (e.g., metals and certain nitrosamines), that do not require bioactivation, can be evaluated by the physiologically based models and scaling procedures noted above.

To make scientifically valid extrapolations across species, the particular activation reaction(s) responsible for the formation of the ultimate carcinogenic metabolite(s) must be known. In many cases, however, the ultimate carcinogen has not been identified with certainty. Furthermore, if a metabolite produces several DNA adducts that may or may not be involved in carcinogenicity, interspecies extrapolation is further complicated. In some cases, the same adduct may be formed by different electrophilic metabolites. For instance, the aminofluorene adduct at C^8 of guanine may be formed from the hydroxylamine and from its sulphate conjugate. This observation implies that two different pathways can yield the same DNA adduct; therefore, species differences in both pathways must be considered. Because such metabolic and mechanistic information is available on so few chemical carcinogens, the following discussion is necessarily theoretical.

If a compound is converted to a reactive intermediate, the chemical structure of

that intermediate determines the degree of its biological reactivity and hence its ability to penetrate cells and to react with vital DNA. For instance, very stable styrene epoxide can reach many organs after inhalation or ingestion. On the other hand, the sulphate conjugate of *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-2AAF) is extremely labile, and is not expected to leave the cell in which it is formed. Whether species differences exist for the same intermediate is unknown but unlikely.

The extent of formation of the reactive intermediate(s) is very important. A factor that may make extrapolation very difficult is that the reactive intermediate that also leads to the ultimate carcinogen represents only a tiny fraction of the administered dose of the parent compound. For instance, one of the bioactivation routes of 2-acetylaminofluorene (2-AAF) requires two subsequent steps: *N*-hydroxylation and subsequent conjugation. However, the bulk of the dose of 2-AAF is excreted as ring-hydroxylated conjugates which have no role in its carcinogenic action (Mohan *et al.*, 1976). Probably an ultimate carcinogen will only rarely be a major metabolite, as occurs after intravenous administration (an impractical route for routine carcinogen testing) of a high dose of *N*-hydroxy-2AAF in the rat. At least 20% of the dose will form the sulphate conjugate which may be responsible for part of the hepatocarcinogenicity of 2AAF (Meerman *et al.*, 1980).

If indeed the reactive electrophilic intermediate represents only a small fraction of the dose, two problems have to be faced. First, its production may be determined by the inefficiency of competing pathways, rather than by the activity of the activating pathway *per se*: Only what is "left over" is available for activation. Second, if the production is very slow, the detoxifying systems may well be able to prevent toxicity from occurring. In only those rats "at risk," the defense systems may fail, resulting in a tumor.

An illustration of such a situation is styrene. The major metabolite is styrene-7,8-oxide, which itself is further metabolized by both glutathione conjugation and epoxide hydrolase activity. Styrene is non-carcinogenic in most animal experiments, after either ingestion or inhalation. As a result, the Dutch Health Council decided to consider it a non-carcinogen. However, its main metabolite, styrene-7,8-epoxide is highly mutagenic, and most likely carcinogenic in animals *in vivo*. Why then does styrene show no carcinogenic action? The most likely reason is that the subsequent metabolism by both glutathione transferase and epoxide hydrolase is so efficient that the epoxide formed is instantaneously detoxified. The result is that the steady-state concentration of the epoxide is so low that it poses virtually no risk to the exposed individuals. Because two extremely efficient enzymes cooperate to remove the carcinogenic metabolite, species differences are not to be expected. That is, if one pathway is low in a species, the other pathway by itself can readily remove the epoxide. Only when a species has very low activity in both enzymes can one expect a carcinogenic effect. Indeed, if the exposure to the epoxide itself is high enough, certainly DNA adducts are formed as observed in cells exposed to the epoxide (Liu *et al.*, 1988). Another possibility that might lead to carcinogenicity by styrene is that glutathione becomes depleted, e.g., as a result of

high styrene exposure. Although epoxide hydrolase may compensate for the loss of glutathione conjugation, the steady-state concentration of the epoxide inside the cell might increase.

The preceding discussion implies that a small amount of a reactive intermediate may be very difficult to estimate through means such as physiologically based models in which metabolism is included when that small amount results from a tiny difference between several major processes. Empirical data are needed to mitigate assumptions about V_{max} and K_m values across species boundaries.

7.3.2 BIOACTIVATION MECHANISMS: IDENTIFICATION OF ULTIMATE CARCINOGENS

Every biotransformation reaction is capable of converting a substrate to a reactive electrophilic metabolite; whether detoxification or toxification is the result depends on the structure of the substrate and its product. Thus, no inherently "detoxifying" enzymes exist. Pronounced species differences have been observed in the activities of biotransformation enzymes.

Most xenobiotic biotransformation enzymes are actually enzyme groups, consisting of several series of isoenzymes that have overlapping substrate specificity, such as the cytochrome P_{450} group of enzymes and the conjugating enzymes. Previously, enzymatic conjugation was perceived to be solely detoxification; however, this situation is definitely not the case, since many examples of activation by conjugation have been identified (Mulder *et al.*, 1986). Admittedly, conjugation reactions play an important role in the detoxification of reactive species. Indeed, cancer may result if a conjugation fails when it should normally remove the reactive intermediate (e.g. styrene oxide or ethylene oxide). Species differences may be related to such differences in detoxification.

The composition of the isoenzymes of a group may demonstrate large species differences, both qualitatively and quantitatively, as illustrated for cytochrome P_{450} by Guengerich *et al.* (1982). An example of conjugation is sulphation which is involved in the activation of *N*-hydroxy-AAF to a reactive sulphate ester. The sulphotransferase involved is only one of a family. This particular isoenzyme is lacking in female rat liver, so that *N*-hydroxy-AAF is not, or very weakly, carcinogenic in the liver of female rats (Miller *et al.*, 1985). Other sulphotransferase isoenzymes show no such sex differences (Meerman *et al.*, 1987). Similar differences in isoenzyme pattern may play a role in species differences in the hepatocarcinogenic action of *N*-hydroxy-AAF, implying that an assay of the overall sulphotransferase activity is insufficient to phenotype the species; rather, the contribution of the separate isoenzymes needs to be considered. While this effect is widely recognized for cytochrome P_{450}, its full impact is much less for conjugation. An example in the cytochrome P_{450} field is the *N*-hydroxylation of 2-AAF, which is the first step towards generation of hepatocarcinogenic metabolites. The cytochrome P_{450} isoenzyme responsible for this *N*-hydroxylation was claimed

to be deficient in the guinea pig, which may explain why 2-AAF was not hepatocarcinogenic in this species.

The only way to avoid misinterpretations is to use the relevant precarcinogenic substance as substrate. However, this situation can only be accomplished if the reactive intermediate responsible for the carcinogenic effects has already been identified—knowledge which is, regrettably, often unavailable. Thus, if the reactive intermediate is known, its precursor should be tested as the substrate for the assessment of the activity of the activating enzymes in the extrapolation from one species to the other. If an alternative substrate is used, one may fail to assay the relevant isoenzyme. When the relevant isoenzyme(s) has not been identified, such extrapolation is unreliable unless one uses the relevant precarcinogen as substrate. For proximate carcinogens, more than one pathway (or reactive intermediate) may be involved. For instance, for *N*-hydroxy-AAF, a number of potential intermediates are known, all (or none) of which may be responsible in a particular tissue or species. Some of them yield the same DNA adducts, which makes it even more difficult to decide which is the ultimate carcinogenic metabolite(s).

7.4 PREDICTION OF METABOLITE PATTERN

A general summary of species differences in xenobiotic metabolism must first consider co-factors in these reactions. Conjugation reactions require co-factors also be used for endogenous processes, such as glutathione (GSH), UDP-glucuronic acid or PAPS (active sulphate). Phase 1 reactions do not need a co-factor other than NADPH, which normally is readily available. Few species differences in the availability of the co-factors have been described. These co-factors are synthesized from precursors which may be in limited supply (Mulder and Krijgsheld, 1984). For instance, glutathione and PAPS synthesis both depend on cysteine and methionine in food as their precursor. If high doses of substrates for sulphation or glutathione conjugation are given, these co-factors may be depleted. Thus, during ethylene oxide inhalation, hepatic GSH decreased to 50% of control (Katoh *et al.*, 1989). A relevant species difference has been reported: namely, in male guinea pigs, the rate of GSH synthesis is very low, so that these males seem highly sensitive to GSH depletion. Such a sex difference is not observed in rat, mouse, or hamster (Ecobichon, 1984).

The genetically determined variation in certain biotransformation (iso-)enzymes are more important than differences in co-factors. Species differences in the metabolism of numerous compounds, presumably based on differences in enzyme capacities, have been detected mainly in *in vivo* experiments (Williams, 1959). Hucker (1983) provides many examples of such species differences. In recent years, biotransformation activities and isoenzyme compositions of tissues in many species have been reported, but the findings are dispersed throughout the literature. Data on several carcinogens can be found in a book by Langenbach *et al.* (1983) on organ and species specificity in chemical carcinogenesis.

In several cases, the lack of a carcinogenic response to a chemical could be traced back to the lack of a particular isoenzyme. For instance, the lack of hepatocarcinogenicity of 2-AAF in the guinea pig was attributed to a lack of the cytochrome P_{450} species that *N*-hydroxylates 2-AAF to *N*-hydroxy-2-AAF, a bioactivation step required first (Miller, 1978). Similarly, sulphation of *N*-hydroxy-AAF has been correlated with hepatocarcinogenicity of this compound. Problems arise when one goes beyond the few extremely well studied model carcinogens, prompting the question: Can generalizations be made about species differences in drug metabolism?

Estimating the possible metabolites for a given compound is relatively simple. However, predicting which of these potential pathways is followed in a particular species at a given dose is far more difficult. Furthermore, to do so quantitatively is virtually impossible, unless many details are known about these reactions in animal and human tissues *in vitro* or *in vivo*.

Of course, some metabolic routes are restricted to a few species. For instance, quaternary glucuronides are formed only in humans and Old World monkeys. In addition, some species lack the capacity to follow certain metabolic routes, or possess them only with extremely low activities. For instance, glucuronidation activity of low molecular weight phenols is very low in the cat, and the dog lacks acetylation activity towards primary amines. Many examples have been reported in both *in vivo* and *in vitro* studies of species differences for particular compounds. Unfortunately, simple rules are not possible to obtain, certainly when several metabolically competitive pathways occur at the same time. A series of amphetamine derivatives best illustrates this concept (Table 7.1). The results for several species show not only the complexity but also that, within a series of analogs, some prediction can be made about species differences. However, if the structure of interest does not belong to a series of analogues for which a large database exists, estimating the outcome of such highly competitive metabolism is difficult. In addition, metabolite patterns often show pronounced changes when dose is varied, if several metabolic reactions, each with its own K_m and V_{max}, are competing with one another. Thus, the metabolism observed at a high, single dose cannot be extrapolated simply to a very low level of exposure. The animal species that seems to be most similar to humans qualitatively is the rhesus monkey (Caldwell, 1981).

The above discussion indicates again that, for extrapolation, the pathway that leads to the ultimate carcinogen must be known. If a particular isoenzyme responsible for activation has been identified, its presence in the other species can be ascertained, as can its ability to convert the substrate to the same ultimate carcinogen. Tissue specificity of carcinogens is highly relevant in this context. In one tissue, one metabolite may be the ultimate carcinogen, whereas another metabolite may have that property in another tissue. Furthermore, the absence of carcinogenicity in one species may be due to extremely efficient detoxification of the ultimate carcinogen in that species, a process that may be deficient in the other.

Table 7.1. Species variations in the metabolism of amphetamine derivatives

		Relative extent of pathway			
Species	*n*	R_x1	R_x2	R_x3	R_x4
Rat	11	++++	+++	+	++
Guinea pig	6	0	++	++++	++
Rabbit	8	+	++	++++	+
Marmoset	4	+		+	++++
Rhesus monkey	4	+++		+++	++
Human	13	++	+	+++	+++

Taken from Caldwell (1981)
+ → ++++ arbitrary quantifications
0 = absent
n = the number of derivatives tested
R_x1 = ring hydroxylation; R_x2 = *N*-dealkylation; R_x3 = deamination (α-carbon); R_x4 = excreted unchanged

Given all these uncertainties, a rational way of extrapolation exists once the ultimate carcinogen and competing metabolic routes are known. An example is methylene chloride (dichloromethane), a simple structure with relatively few metabolites. Methylene chloride shows a high incidence of malignant tumors in lung and liver of mice, whereas hamsters did not develop tumors at similar exposure levels. The rat was in between; it developed no tumors at those sites, but, non-malignant mammary tumors were observed. The findings of various studies suggest that glutathione conjugation is responsible for the ultimate carcinogenic metabolite, while oxidation by the mixed function oxidase system to carbon monoxide represents an alternative pathway, leading to a non-carcinogenic metabolite. Based on experimentally determined enzyme kinetic parameters of these two metabolic pathways as determined in liver preparations from a number of species including humans, the exposure of the liver and lung to the glutathione-derived metabolite in these species could be calculated by a physiologically based pharmacokinetic model (Reitz *et al.*, 1989). The predicted dose levels in liver and lung corresponded to the observed carcinogenicity in the various species. Furthermore, calculations revealed that at the very low levels of environmental methylene chloride exposure in humans, the organ exposure to the reactive glutathione conjugate was extremely low. The major reason was that glutathione conjugation of this substrate is very low in human tissues. The competing oxidative metabolism, that functions as detoxication and has a much lower K_m than glutathione conjugation, prevented the formation of an appreciable amount of the carcinogenic metabolite at low dose levels. The results of Reitz *et al.* (1989) also demonstrate, however, that allometric scaling on enzyme activities (i.e., to convert the V_{max} in one species to a V_{max} in an other species just by correcting for body weight) is dangerous, and may lead to errors of up to one order of magnitude when compared with the real enzyme

activities.

An assumption in the approach outlined above is that the metabolism of the compound takes place only in the tissues considered. Often the liver is assumed to be the sole metabolizing organ—an unproven and in many cases incorrect assumption, particularly at the very low exposure levels that usually occur in real life. In many recent publications, the metabolism of carcinogens is measured in hepatic microsomal fractions or hepatocytes from several species (Degawa *et al.*, 1987; Hsu *et al.*, 1987). However, generalisations presently are impossible from such findings usually obtained in only two or three species. Thus, interspecies extrapolation is impossible unless the ultimate carcinogenic metabolites, and the rate at which they are generated *in vitro* in tissues from the relevant species, are known. In addition, the rate at which these metabolites are detoxified by those tissues also needs to be known.

7.5 PROTECTIVE SYSTEMS

Most cells possess the ability to enzymatically convert a potential carcinogen into non-toxic metabolites. A balance between activating and deactivating reactions is critical in a cell's defense against carcinogens. Often conjugation reactions can detoxify. For example, glutathione conjugates aflatoxin B_1 epoxide that forms DNA adducts, and prevents cancer by aflatoxin B_1. Increasing glutathione conjugation (e.g., by inducing appropriate transferases using phenobarbital pretreatment) decreases DNA binding of aflatoxin B_1 dramatically (Lotlikar *et al.*, 1989); a difference in DNA adduct formation in isolated hepatocytes from rat and hamster can be explained by increased glutathione conjugation in hamster versus rat hepatocytes (Ho *et al.*, 1989). Similarly, the lack of hepatocarcinogenicity of 2-AAF in the guinea pig may be due to a rapid inactivation of the *N*-hydroxy-AAF metabolite rather than a lack of its formation, because the hydroxyl amine appeared to be formed in guinea pig liver microsomes (Takeishi *et al.*, 1979).

Another protective mechanism involves trapping reactive electrophilic intermediates by nucleophiles inside or outside a cell; thiol or amine groups are sufficiently nucleophilic to react rapidly with electrophilic metabolites. The glutathione transferases may increase the reaction rate with the thiol of glutathione. Also the methylthio group (e.g., in methionine) may provide protection against the reactive sulphate ester of *N*-hydroxy-AAF (Van den Goorbergh *et al.*, 1987). Very little research has been done to elucidate such species differences.

Clearly, species differences in these defense mechanisms may be as important as bioactivation reactions in the final outcome of exposure to carcinogens in animals or humans.

7.6 EXTRAPOLATION FROM LABORATORY ANIMALS TO HUMANS

From the above discussion, the conclusion is clear that interspecies extrapolations can be performed in a scientifically valid way only when the mechanism(s) of carcinogenesis such as the bioactivating enzymes, the ultimate carcinogen(s) and protective pathways are known. Then the relevant activities in various species including the human can be assayed. Furthermore, the integrated metabolism of a carcinogen with regard to DNA adduct formation can be measured in intact cells or slices, a procedure that offers a decided advantage over isolated enzymes. Isolated cell preparations from humans may also be available. For such cell systems, however, the results reflect merely the potential of a compound and do not provide a quantitatively reliable response.

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8 Monitoring Cytogenetic Damage *In Vivo*

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8.1 INTRODUCTION

Chromosomal alterations have been used as an important biological endpoint to study the mutagenic effects of ionizing radiation and chemicals. Some basic principles in radiobiology were based on chromosome aberration data obtained in plant cells and *Drosophila* (Lea, 1946).

Structural and numerical chromosomal aberrations are encountered often in human newborns (0.6%) and in spontaneous abortions (30 to 40%). In addition, many tumors are associated with chromosomal aberrations that may be involved in the initiation or promotion stages of carcinogenesis. These facts emphasize the importance of studying chromosome aberrations *per se* as a relevant biological endpoint to assess the risks involved in exposure to mutagenic carcinogens. The origin and significance of chromosomal alterations have been discussed earlier (Natarajan, 1984). The techniques to detect chromosomal changes have been improved constantly to increase the ease and accuracy of recognition of aberrations, and in some cases automation has become available. In this review, the authors summarize some of the recent significant advances in this field.

Biological endpoints to be discussed include chromosomal aberrations, micronuclei, sister chromatid exchanges, and aneuploidy. In *in vivo* studies, only

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Methods to Assess DNA Damage and Repair: Interspecies Comparisons
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some tissues are amenable to chromosomal analysis, the prerequisite being that the cells are capable of proliferation. This requirement limits the number and type of cells that can be studied, and is thus usually confined to bone marrow cells, blood lymphocytes, regenerating liver cells, or proliferating male germ cells. Techniques to study non-dividing cells by premature chromosome condensation have also become possible (Hittelman, 1990).

8.2 CHROMOSOMAL ABERRATIONS

The types and frequencies of induced chromosomal aberrations depend on the mutagen used and the stage of the cell cycle treated. Ionizing radiation and chemicals which directly produce DNA strand breaks induce chromatid aberrations in the G_2 stage and chromosome aberrations in G_1 stage. Short-wave UV, alkylating agents, and most of the other chemical mutagens induce chromatid-type aberrations in G_1 (detected in the following mitosis) and G_2 (detected in the second division following treatment), as the chemically induced lesions require a DNA-synthesis dependent repair process for the formation of aberrations. Experimental protocols should be designed to take into account these points.

The classical method to assess *in vivo* exposure to mutagens in laboratory animals is to study the frequency of chromosomal aberrations in bone marrow cells. Usually several fixation times are used to discern effects on the stages of the cell cycle as well as to compensate for the expected mitotic delay due to treatment with a mutagen. The scoring of aberrations is very time consuming, and requires expertise. This method can easily be replaced by the use of inducer micronuclei in polychromatic erythrocytes. The doubling dose (DD) is calculated from the linear equation

$$y = a + bD \tag{8.1}$$

as the ratio of the spontaneous rate (a) and the linear regression coefficient

$$DD = \frac{b}{a} \tag{8.2}$$

Adler (1990) measured the induction of chromosomal aberrations and micronuclei following treatment with six known clastogens, and found the doubling doses to be very similar.

Chromosomal aberrations in peripheral blood lymphocytes have been used to monitor human exposure to genotoxic agents as well as from patients treated with cytostatic agents. Blood samples are cultured *in vitro* in the presence of a mitogen, such as phytoheamagglutinin, and dividing lymphocytes are evaluated for the presence of chromosomal alterations. In cases of radiation accidents, where no physical dosimetry is feasible, chromosome aberrations (especially the frequencies

of dicentrics and rings) have been used as a biological dosimeter. This technique can evaluate cases such as partial body irradiation and exposure to a mixture of high and low LET radiations (International Atomic Energy Agency, 1986). This technique was found to be very useful in a recent accident involving a ^{137}Cs gamma source resulting in a heavy exposure to several individuals in Goiania, Brazil, in 1987 (Ramalho *et al.*, 1988a).

In cases where chromosomal aberrations have to be determined immediately after exposure using non-proliferating cells, the use of premature chromosome condensation technique (PCC) is feasible. This technique has been exploited to study the induction of breaks and their repair in cells grown *in vitro* (Hittelman, 1990). The technique involves fusing non-dividing treated target cells with mitotic cells of Chinese hamster ovary cells (CHO) or HeLa cells in the presence of inactivated Sendai virus or polyethylene glycol. In the hybrids formed, the mitotic factors present in the donor cell condense the nucleus of the target cell. G_1 chromosomes appear as single chromatin threads; G_2 chromosomes appear as two chromatid threads. Dose–response curves for induction of fragments in lymphocytes of rats irradiated *in vitro* and lymphocytes derived from rats irradiated *in vivo* (assessed by the PCC technique) were very similar, indicating that the response of lymphocytes *in vivo* and *in vitro* is very similar. In acute radiation accidents involving large doses, the PCC technique could be useful to determine quickly whether the exposure is partial or whole body, as such information is necessary when making decisions concerning a possible bone-marrow transplantation to the victims.

Improved staining methods have become available recently, which can increase the resolution power of conventional chromosomal aberration evaluation. Chromosome specific libraries have been generated that can be used to stain specific individual chromosomes by *in situ* hybridization. The technique involves: (a) amplification of chromosome specific probes; (b) nick translating using DNAase, DNA polymerase, and four deoxynucleoside triphosphates of which one is biotinylated; (c) denaturation of the mitotic preparations and renaturing with the labelled probes; and (d) detection of the sites of hybridization with avidin and FITC. When one chromosome is exclusively stained, translocations involving this chromosome can be detected easily and accurately. Using this "chromosome painting" technique, translocations involving chromosome 2 in the lymphocytes of individuals involved in the radiation accident in Goiania, Brazil, have been detected accurately (Natarajan *et al.*, 1991). Another approach using the same technique employs telomeric and centromeric specific probes of a chromosome, such as chromosome 1 region 1q12 and 1p36. Detection of dicentrics and translocations involving this segment of chromosome 1 can be achieved with greater accuracy and ease than by conventional methods of C or G banding to detect dicentrics and translocations, respectively (Lucas *et al.*, 1989).

Automated scoring of chromosomal aberrations has been attempted. The synthesis of automated computerized devices that scan cytological preparations and find metaphases that are well spread was the first step toward this objective. The

frequency of dicentrics scored from selected metaphases does not differ from the frequency obtained by conventional manual selection techniques. In principle, many image analysis systems available for complete karyotyping of metaphases can be used to score chromosomal aberrations. However, this method is not used because of the equipment cost and the long time for an analysis. Rather, systems are being developed to rapidly analyze chromosome spreads to detect dicentrics (Piper *et al.*, 1988; Lorch, *et al.*, 1989).

8.3 SISTER CHROMATID EXCHANGES

Sister chromatid exchange (SCE) is a cytological manifestation of DNA double-strand breaking and rejoining at apparently homologous sites between two chromatids of the same chromosome. The occurrence of SCEs was first detected from the transformation of small ring chromosomes to large ring chromosomes in maize following cell divisions (McClintock, 1938). Using tritiated thymidine as a marker and micro-autoradiography for detection, Taylor *et al.* (1958) demonstrated the presence of SCEs from the silver grain patterns. This method was replaced eventually by cytochemical methods, using 5-bromodeoxyuridine (BrdU) and fluorochrome or fluorochrome plus Giemsa staining (Latt, 1973; Perry and Wolff,1974).

SCEs are induced efficiently by numerous mutagenic or carcinogenic agents, especially those which form covalent adducts to the DNA or interfere directly or indirectly with DNA replication. The induction of SCEs has been correlated with the induction of point mutations (Carrano *et al.*, 1978) and cytotoxicity (Natarajan *et al.*, 1984). While most of the mutagens induce SCEs very efficiently, not all agents which induce SCEs also induce point mutations. Nonmutagens such as inhibitors of DNA synthesis, (e.g., cytosine arabinoside and hydroxyurea) as well as inhibitors of poly (ADP-ribose) synthetase, (3-aminobenzamide) also induce SCEs efficiently (Natarajan *et al.*, 1981).

The frequency of SCEs has been correlated with the extent of BrdU incorporated in DNA, and most spontaneous SCEs occur when the BrdU-containing DNA is used as a template for replication. Attempts have been made to minimize the incorporation of BrdU and to detect the SCEs by reacting with antibodies against BrdU (Pinkel *et al.*, 1985; Natarajan *et al.*, 1986; Tucker *et al.*, 1986). Using this method, the baseline frequency of SCEs averages about two per cell cycle, a value similar to that obtained in systems using ring chromosomes without the incorporation of BrdU (Natarajan *et al.*, 1986).

In animals, the frequencies of SCEs *in vivo* can be measured by injecting or infusing solutions of BrdU or by implanting tablets containing BrdU, and examining dividing bone marrow or spermatogonial cells. The frequency of spontaneous SCEs obtained *in vivo* is usually very low compared with that obtained *in vitro*.

The frequency of SCEs following *in vivo* exposure to genotoxic agents can also be determined by culturing peripheral blood lymphocytes obtained from exposed

animals or humans for two cell cycles in a medium containing BrdU. Numerous studies exist in which human populations exposed to known mutagens or patients undergoing chemotherapy have been studied for the frequencies of SCEs. Several confounding factors may influence the frequency of SCEs in humans, and these have been discussed by Carrano and Natarajan (1988).

8.4 MICRONUCLEI

Micronuclei are formed in anaphase by chromosomal fragments or lagging chromosomes not included in the nucleus of the daughter cells. The presence of micronuclei in eukaryotic cells has been known for over six decades (Wilson, 1925). In the ensuing years, micronuclei have been observed in plants (Evans *et al.*, 1959) and in numerous tissues of many animal species (Heddle *et al.*, 1983). Presently, micronuclei serve as an important endpoint to detect the genetic damage by chemicals or radiation in cultured cells and intact organisms. Compared to more traditional approaches involving the analysis of metaphase chromosomes, micronucleus methods are rapid and easy to learn, and have comparable sensitivity. For these reasons, micronucleus assays are being used with increasing regularity. In this section, various forms of this assay are presented, their uses discussed, and possible future developments raised.

8.4.1 ERYTHROCYTES

8.4.1.1 Bone marrow

The most common form of micronucleus assay uses rodent bone marrow for several reasons:

1. Rodents are frequently used as models for human biological responses. Their small size and ease of handling makes them a natural subject of *in vivo* testing. Mice in particular are favored, primarily because murine bone marrow lacks the leukocytic granules, which can be confused easily with micronuclei found in rat preparations.
2. Bone marrow cells can be removed with little effort, smeared on to slides and then fixed and stained. No tissue culture is necessary, and the slides can be viewed within minutes of obtaining the tissue. The presence of numerous erythrocytes in microlitre volumes of bone marrow and the enucleate nature of these cells facilitate observation of micronuclei, and enhance scoring speed and accuracy.
3. Erythrocyte precursors in bone marrow are constantly undergoing cell division, making them sensitive to the effects of agents that interfere with DNA replication or cell division.

4. As with most procedures examining genetic damage through the use of proper staining techniques, only those cells that were actively growing during the exposure period are to be scored. Hayashi *et al.* (1983) demonstrated that the fluorescent dye acridine orange can distinguish mature, RNA negative "normochromatic erythrocytes" (NCEs) from immature, RNA positive "polychromatic erythrocytes" (PCEs). While earlier Giemsa-based techniques (Schmid, 1975) also make this distinction, acridine orange offers substantially superior contrast. With the proper selection of sampling time (approximately 24 to 48 hours after exposure depending on the agent and dose), the RNA positive erythrocytes are derived from cells undergoing their final division (and enucleation) during the exposure period.

The use of bone marrow erythrocytes as an endpoint for genetic toxicity testing has been well validated. Guidelines for the bone marrow micronucleus test have been published (MacGregor *et al.*, 1987). Heddle (1973) showed that micronuclei could be induced in the mouse bone marrow by X-rays. Hayashi *et al.* (1983) demonstrated that bone marrow micronuclei reflect chromosomal breakage phenomena. The USEPA Gene Tox Program has conducted an extensive review of the literature, and showed this system to be sensitive to chemical clastogens and to spindle disrupting agents (Heddle *et al.*, 1983). A series of publications has shown that micronuclei can be induced efficiently by different exposure routes including oral gavage and intraperitoneal injection (Hayashi *et al.*, 1989); others have shown induction following inhalation (Erexson *et al.*, 1986; Odagiri *et al.*, 1986; Jauhar *et al.*, 1988; Tice *et al.*, 1988) and exposure to 50-Hz electric fields (El Nahas and Oraby, 1989).

Thus, the preponderance of evidence is in agreement that the bone marrow erythrocyte micronucleus assay is a valid and sensitive means to detect genetic damage resulting from many routes of exposure to a wide variety of chemical and physical agents.

8.4.1.2 Peripheral blood

With increasing frequency, peripheral blood is being used to assess frequencies of micronuclei. In rodent studies, the most obvious advantage is that the test animal does not have to be euthanized to obtain blood samples. A simple tail vein nick or puncture of the periorbital sinus is sufficient to obtain the few microlitres of blood needed for a smear. This permits the investigator to obtain multiple sequential samples from the same animal, and enables more accurate determination of the effects of various agents upon the kinetics of erythrocyte and micronucleus development.

In human studies, obtaining bone marrow samples to measure the effects of exposure is generally not practical or ethical, although such sampling has been reported (Hogstedt *et al.*, 1983). Peripheral blood is much easier to obtain. The

disadvantage of using peripheral erythrocytes in humans is that micronuclei are observed only in people who have no functional spleen (Schlegel *et al.*, 1986), making the method generally not useful to monitor exposed populations. In large urban areas, substantial numbers of splenectomized people are present. Everson *et al.* (1988) studied 20 individuals 6 months after splenectomy, and described the effects of folate intervention on a person who had a high spontaneous frequency of micronuclei. Some regimens of cancer therapy, including Hodgkin's disease, involve routine splenectomy prior to radiotherapy. In such populations, monitoring the effects of therapy using peripheral blood micronuclei, and correlating the micronucleus response with other markers of exposure is considered relatively easy to accomplish.

8.4.1.3 Automation

The popularity of the erythrocyte micronucleus assay in bone marrow and peripheral blood has led to efforts to automate the scoring of these preparations using image analysis (Oleson, 1989; Romagna and Staniforth, 1989; Hayashi *et al.*, 1990) and flow cytometry (Hutter and Stohr, 1982; Ishidate *et al.*, 1987; Hayashi *et al.*, 1990; Tometsko and Leary, 1990). Both image analysis and flow cytometric approaches have been successful (Tucker *et al.*, 1989). Several image analysis systems are on the market (Romagna and Staniforth, 1989). Although manual scoring of micronuclei is relatively rapid compared with the analysis of metaphase chromosomes, the advantages of automation are significant, including increased speed of producing results and increased numbers of cells scored per sample enabling detection of lower doses and reliance on smaller cohorts.

One primary difficulty encountered in the early phases of automation was distinguishing erythrocytes from the large number of leucocytes. To solve this problem, Romagna and Staniforth (1989) used a cellulose column to purify erythrocytes, which has the advantage of removing leukocytic granules that are problematic in rat bone marrow, thereby making the rat amenable to erythrocyte micronucleus assays. A limitation of existing image analysis systems is that it is based on Giemsa-stained preparations. Although at least one commercial system is able to distinguish between RNA-positive and RNA-negative cells, the use of acridine orange would almost certainly improve erythrocyte characterization. In flow systems as in manual scoring, the use of acridine orange is relatively easy. In the near future, both flow cytometry and image analysis are expected to be used regularly to assess micronucleus frequencies in human and rodent erythrocytes.

8.4.2 NUCLEATED CELLS

Micronuclei in nucleated cells have been used to measure exposure since Obe and Beek (1975) treated human lymphocytes with radiation and observed a significant

increase. This approach was employed for the next 10 years to assess the effects of various agents, in spite of a fundamental problem: No adequate means existed to determine which cells had undergone division since exposure. Because not all lymphocytes respond to a mitogen, distinguishing between proliferating and non-proliferating cells was impossible. Nevertheless, the procedure was used with considerable success *in vitro* (Countryman and Heddle, 1976; Heddle and Carrano, 1977), and *in vivo* with lymphocytes (Aghamohammadi *et al.*, 1984; Maki-Paakkanen, 1987), buccal mucosa cells (Stich and Rosin, 1983; Sarto *et al.*, 1987), fibroblasts (Rudd *et al.*, 1988), and exfoliated cells (Rosin and German, 1985; Reali *et al.*, 1987; Stich, 1987).

The micronucleus assay, however, did not work with combination protocols (e.g., X-rays plus caffeine; Natarajan *et al.*, 1982). Several years ago, however, two procedures were introduced that offered potential solutions. Pincu *et al.* (1984) used BrdU followed by fluorescence and Giemsa staining to distinguish between proliferating and non-proliferating cells. The second solution, proposed by Fenech and Morley (1985a), used cytochalasin B which arrests cytokinesis yet does not interfere with karyokinesis. The resulting multinucleated cells may be centrifuged directly on to microscope slides and stained with May–Grunwald–Giemsa. With this procedure, only cells with intact membranes are scored. The primary advantage is that each binucleated cell represents the products of a single mitosis. In a direct comparison between the BrdU and cytokinesis block (CB) procedures, Ramalho *et al.* (1988a) concluded that the CB method detected chromosome fragments more efficiently than the BrdU procedure. For these reasons, the use of cytochalasin B is now the preferred method of measuring micronuclei in nucleated cells.

8.4.2.1 Application of the CB procedure *in vitro*

Early work with the CB procedure on human peripheral lymphocytes showed significant yields following radiation exposure (Fenech and Morley, 1985a, 1986). The micronucleus frequencies appeared to be consistent with the production of acentric fragments (Littlefield *et al.*, 1989), although agreement on this issue is incomplete (Prosser *et al.*, 1988). Others have shown dose–response to chemicals (Eastmond and Tucker, 1989a). The CB method has also been applied successfully to established cell lines. Wakata and Sasaki (1987), Eastmond and Tucker (1989b), and Krishna *et al.* (1989) demonstrated the utility of cytochalasin B on Chinese hamster cells for *in vitro* genotoxicity testing. Significant increases in micronuclei have been observed following treatment with clastogenic and spindle disrupting agents. Application of the CB procedure to *in vitro* testing gives every indication of being a rapid and sensitive means of measuring exposure.

8.4.2.2 Application of the CB procedure *in vivo*

The CB procedure has also been used following exposure *in vivo*. Experiments involving mice treated with radiation (Erexson *et al.*, 1989) and chemicals (Erexson *et al.*, 1987) have indicated that this procedure works very well even in small animals where limited amounts of peripheral blood are available for sampling.

In humans, the simplicity and utility of this procedure makes it applicable to monitor exposed populations. Sorsa *et al.* (1988) and Yager *et al.* (1988) examined people exposed to alkylating agents, and found a positive trend between exposure and micronucleus frequencies. Fenech and Morley (1985b, 1986, 1987) demonstrated that the frequency of micronuclei increases significantly with age, being about four times higher in people aged 80 than in newborns. Other confounding variables such as smoking and sex may exist, but have not yet been demonstrated.

8.4.2.3 Automation

Several investigators have attempted to automate the scoring of micronuclei in cytokinesis-blocked cells. The requirements for automating this method are much more complex than for erythrocytes. Such a system should:

1. determine the number of micronuclei in each binucleated cell;
2. distinguish between binucleated cells with an intact cytoplasmic membranes and other artifact cellular and non-cellular objects;
3. distinguish micronuclei from nuclei and nuclear blebs;
4. have the option of determining the average number of nuclei per cell as a means of assessing toxicity; and
5. operate quickly and reliably with a minimum of user interaction.

The need for multiple morphological assessments of each cell indicates that image analysis would be more suitable than flow cytometry, despite of the results of Nusse and Kramer (1984). In preliminary studies using image analysis, Fenech *et al.* (1988) demonstrated the utility of automating the BrdU incorporation method (Pincu *et al.*, 1984). Fenech's procedures to automate the (CB) method have been unsuccessful.

Studies directed towards automated scoring of micronuclei in binucleated lymphocytes using the CB method and the LeyTAS-MIAC system (Leyden Texture Analysis System using the Leitz Modular Image Analysis Computer and a multichannel Leitz Autoplan microscope) have been reported (Tates *et al.*, 1990). These investigators indicated that the requirements for automated detection of micronucleated binucleated cells can be fulfilled, provided more time was invested in fully developing this method.

Microcomputer-based image analysis system with microscope control for slide scanning has been developed as a less costly alternative. Using this system, an image analysis procedure was developed successfully (Tates *et al.*, 1990). This

procedure involves sequential analysis of micronuclei and nuclei (stained with Gallocyanin) and cytoplasm (stained with Naphtol Yellow S). Initial results of validation tests indicated that about 60% of the micronuclei and about 65% of binucleated cells can be detected automatically. The artifact rejection procedure is capable of eliminating most kinds of non-relevant cellular and non-cellular objects present on slides. Nuclear blebs, when present, do not result in false positive micronuclei. The present system still needs further refinement.

8.4.2.4 Aneuploidy

Aneuploidy is a frequent cause of fetal wastage, birth defects, and mental retardation, and also appears to play an important role in the pathogenesis of malignancy; yet, its molecular mechanisms are poorly understood. Until recently, acceptable procedures to measure aneuploidy in mammalian cells were limited to counting metaphase chromosomes (Galloway and Ivett, 1986; Dulout and Natarajan, 1987). This procedure is not only tedious and prone to artifact chromosome loss, but is limited to measuring hyperploidy and not hypoploidy. However, the procedure developed by Dulout and Natarajan (1987), using embryonic diploid fibroblasts of Chinese hamster grown on cover glasses and fixed *in situ*, overcomes these difficulties. Recently, several other excellent reviews of aneuploidy have been published (Cimino *et al.*, 1986; Dellarco *et al.*, 1986; Parry and Parry, 1987; Vig and Sandberg, 1987). Some of these have stressed the need new cytogenetic approaches to measure aneuploidy in mammalian cells. In this section, recent developments are described, and their uses and limitations discussed.

Kinetochore staining in micronuclei

Micronuclei can result from spindle disruption or clastogenic processes. Until recently, the ability to discriminate between these processes was limited to comparing the size of micronuclei induced by various agents (Hogstedt and Karlsson, 1985). This approach works reasonably well when large numbers of micronuclei are examined; yet, it is unable to determine the mechanism of origin for individual micronuclei. Recently several laboratories have begun to use antikinetochore antibodies obtained from the serum of people with the crest form of scleroderma. These antibodies are highly specific for the kinetochore region, and appear to react equally well with human, mouse, and Chinese hamster cells. The use of kinetochore staining in micronucleated cells is based on the assumption that kinetochore-positive and kinetochore-negative micronuclei contain whole chromosome(s) and chromosome fragment(s), respectively. This method has now been applied by several laboratories to measure the effects of exposure *in vitro*. Both human lymphocytes (Thomson and Perry, 1988) and fibroblasts (Hennig *et al.*, 1988) have been used, as well as cultured hamster cells (Degrassi and Tanzarella,

1988). These studies have demonstrated that this procedure discriminates efficiently between aneuploidogenic and clastogenic responses in individual cells. Other studies have combined the use of antikinetochore antibodies with the CB method of obtaining micronuclei (Eastmond and Tucker, 1989a, b; Fenech and Morley, 1989). This combination of techniques is especially powerful because it enables analysis of the products of a single mitosis and avoids the problem of artifact loss of chromosomes. The procedure is only slightly more difficult than the analysis of micronuclei alone, yet it permits the characterization of both aneuploidogenic and clastogenic processes.

The future of kinetochore labelling as an assay for aneuploidy appears promising, especially if coupled with the CB procedure. This method should be of interest to those performing standardized toxicity tests and to regulators in need of a simple and reliable procedure to measure the induction of aneuploidy. The method is also capable of screening populations of exposed individuals.

DNA probes

With *in situ* hybridization using chromosome specific probes, aneuploidy is determined by counting the number of hybridization signals present in each cell. Scoring is rapid, minimal training is needed, and the cells of interest need not be cultured. This latter characteristic has significant implications for solid-tumor cytogeneticists. Cells from solid tumors are difficult to grow; metaphase preparations are difficult to obtain, and are often of poor quality. *In situ* hybridization avoids these limitations; consequently, significant advances are being made in this area (Cremer *et al.*, 1988; Devilee *et al.*, 1988; Hopman *et al.*, 1988).

In spite of such success, several disadvantages remain for this procedure. The number of different fluorophores available for simultaneous use, and investigators can measure aneuploidy for only a few chromosome domains per slide. In addition, hybridization signals in interphase nuclei have a significant chance of overlapping, partly because the three dimensional structure of the nucleus is viewed in only two dimensions, and partly because two separate chromosome domains may truly be adjacent. Consequently, hypoploidy is difficult to detect, and investigations must be limited to examining the effects of hyperploidy.

In the future, fluorescent *in situ* hybridization may be used to answer specific and fundamental questions concerning aneuploidy in both normal and malignant tissues. Monitoring human exposure using sperm to investigate potential reproductive effects and cytokinesis-blocked cells to address the specificity of chromosome loss may also be possible. Most available probes are human in origin; but as material from other species becomes available, animal models to mimic humans may be developed.

Transgenic animals

Transgenic mice carrying large sequences of foreign DNA in one or more chromosomes have been used to study aneuploidy *in vivo* (Natarajan, 1989; Natarajan *et al.*, 1990). In these mice, either multiple copies of lambda sequences and/or c-*myc* sequences have been introduced. The chromosomes carrying these sequences can be detected by *in situ* hybridization in both metaphase and interphase nuclei. Aneuploid events can easily be detected in different organs; therefore, this system offers the advantage of studying organ-specific changes.

Automation

Presently, no automated systems are capable of detecting aneuploidy in mammalian cells. The antikinetochore antibody procedure as applied to binucleated cells could be automated by modifying the micronucleus system to include the ability to detect the presence of kinetochore label. The DNA probe method could be automated by developing an instrument to quantify the number and intensity of chromosome-specific labels in interphase cells. Each approach is probably amenable to image analysis, while a flow system scheme would appear less feasible.

Table 8.1. Spontaneous frequencies of chromosome aberrations in human spermatozoa

Authors	Subjects (*n*)	Sperm (*n*)	Chromosome abnormalities aneuploid	structural	total
Rudak *et al.* (1978)	1	60	3	1	4
Martin *et al.*(1983)	33	1000	52	40	92
Brandriff *et al.*(1985)	11	2468	41	190	231
Martin *et al.*(1982)	18	240	18	7	25
Kamiguchi and Mikamo (1986)	4	1091	10	142	152
Mikamo *et al.* (1989)	26	9280	125	1357	1482
Total	93	14139	249 (2%)	1737 (12%)	1986 (14%)

8.5 SPERM CYTOGENETICS

The ultimate objective of genetic risk assessments is the quantitative estimation of genetic damage transmitted to subsequent generations. Recently, the direct study of chromosomes in human spermatozoa has been made possible by using interspecific *in vitro* fertilization with zona-free golden hamster oocytes (Table 8.1; Rudak *et al.*, 1978; Martin *et al.*, 1983; Brandriff *et al.*, 1984, 1985). With this

technique, the hamster egg unfolds the human sperm chromatin, and processes it into microscopically analyzable mitotic chromosomes amenable to microscopic analysis. Presently, this method is the only one for direct assessment of chromosomal effects of mutagens in human germ-line cells.

Spontaneous frequencies of chromosome aberrations (Table 8.2), particularly structural aberrations, in human spermatozoa are much higher than those in mouse and Chinese hamster. The basis of the high frequency of chromosome aberrations in human sperm is unclear. The possible effect of interspecies cross fertilization should be examined. However, considerable variation among donors suggests involvement of environmental factors such as cigarette smoking. An increase in chromosome aberrations in spermatozoa has been found in cancer patients receiving radiotherapy and chemotherapy (Martin *et al.*, 1986, 1989; Brandriff *et al.*, 1987; Jenderny and Rohrborn, 1987; Genescà *et al.*, 1990).

Table 8.2. Species difference in spontaneous chromosome aberrations in spermatozoa

Species	% Chromosome abnormalities		
	Aneupoloid	Structural	Total
Humans (Table 8.1)	1.8	12.3	14.1
Mouse[1]	0.6	0.9	1.5
Chinese hamster[2]	0.7	1.4	2.1

[1]Fraser and Maudlin (1979)
[2]Mikamo and Kamiguchi (1983)

The frequency of chromosome aberrations in human spermatozoa by *in vitro* exposure to ionizing radiation have been studied by Kamiguchi *et al.* (1987, 1989) and Brandriff *et al.* (1988). The aberrations were mostly chromosome breaks and fragments, and their frequency increased linearly with radiation dose. The interspecies comparison of chromosomal radiosensitivity indicated that the human spermatozoa were highly vulnerable and about three times more radiosensitive than those of mouse (Table 8.3).

Chromosome analysis in spermatozoa can also be used to determine the segregation of translocations in meiotic processes (Martin, 1989; Pellestor *et al.*, 1989). Segregation properties vary among different types of translocations, and thus the analysis of sperm chromosomes provides a useful means to assess risks arising from the induction of translocations in spermatogonial stem cells as well as the heritable translocations.

8.6 INTERSPECIES COMPARISONS

Interspecies comparisons of cytogenetic response to chemical mutagens have been carried out to a limited extent, although data exist for radiation responses. Rodent:human comparisons are difficult for the following reasons:

1. most human data come from studies on resting (G_0) peripheral lymphocytes, whereas rodent data are obtained from proliferating bone marrow cells;
2. karyotypes of rodents and humans are different, and karyotype configurations are known to influence the yield and/or detection of aberrations;
3. the extent and distribution of heterochromatin, which also influences the yield and distribution of aberrations, is different between rodents and humans; and
4. metabolic activation and repair capacities differ between human and rodents.

Table 8.3. Chromosome radiosensitivity of spermatozoa of different species

Species	Relative radiosensitivity
Guinea pig	0.5 (DL)
Rabbit	0.5 (DL)
Golden hamster	2 (DL)
Mouse	1 (SCA)
Chinese hamster	1 (SCA)
Human	3 (SCA)

DL = dominant lethal
SCA = sperm chromosome abnormality

Some interspecies comparative studies have been reported. Following treatment with benzo[*a*]pyrene, for instance, more SCEs were induced in rat compared with mouse for equivalent amounts of adducts (Kligerman *et al.*, 1989). Dichloromethane-induced chromosomal aberrations and SCEs in mice (Allen *et al.*, 1990), but had no effect in rats and Chinese hamsters (Burek *et al.* 1984). Diaziquone induced more SCEs in humans than in mice (Kligerman *et al.*, 1988). More comparative studies of this nature are needed.

8.7 CONCLUSIONS

Chromosomal alterations can be analyzed directly in humans using proliferating bone marrow cells and stimulated peripheral blood lymphocytes. The significance of chromosomal aberrations is twofold: chromosomal rearrangements (a) are associated with cancer and hereditary diseases and (b) may indicate exposure to carcinogenic or mutagenic agents.

Combining molecular biological techniques with cytogenetic methods has led to

easy and rapid detection of chromosomal rearrangements. Classical cytogenetic techniques can be used easily to detect chromosome or chromatid aberrations (unstable aberrations), the frequency of which is a quantitative index of exposure to ionizing radiation and a qualitative index of exposure to chemical mutagens. To detect stable chromosomal rearrangements, however, chromosome painting (*in situ* hybridization with chromosome specific probes) can be used with ease and accuracy. In addition, the PCC technique (hybridization of interphase cells to mitotic cells) allows one to study chromosomal aberrations particularly in non-proliferating cells. Similarly, cross-fertilization by interspecies hybridization *in vitro* (e.g., human sperm in golden hamster egg) enables one to study sperm chromosomes directly. Using this technique, human spermatozoa have been found to have many more background aberrations than those of rodents.

To assess rapidly the frequency of chromosomal fragments, micronucleus assays have been developed. Of special significance is the cytocholasin-B block method of generating binucleated cells, which possesses increased sensitivity and ease of quantification of micronuclei frequency in proliferating cells.

Numerical aberrations (aneuploidy) lead to important genetic consequences of exposure to environmental agents. Direct detection of aneuploid cells in different organs (including germ cells) has become feasible by use of transgenic mice carrying large insertions of foreign DNA. The chromosome carrying such insertions can be detected by *in situ* hybridization with the appropriate probes. In addition to aneuploidy, the recombination events can be detected in transgenic mice. Kinetochore-specific antibodies can be used to determine whether micronuclei contain a centromere (an indicator of a whole chromosome loss), and can be used as a measure of aneuploidy.

Many of the techniques discussed in this chapter can be, or are being, automated. When automation is achieved, large numbers of cells can be scored quickly, enabling detection of effects produced at relatively low doses.

Though a large amount of data are available for interspecies comparison of cytogenetic response to ionizing radiation, the database for chemicals is comparatively small. Perhaps this situation is due to inherent difficulties in comparing experimental animals to humans following chemical exposures. Certainly more effort is needed in this area.

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9 *In Vivo* Somatic Cell Gene Mutations in Humans

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9.1 INTRODUCTION

9.1.1 RATIONALE

In humans, gene mutations in somatic cells probably underlie a variety of developmental and pathological processes. Among the latter are cancer and, possibly, aging. Disease relevance alone provides motivation for undertaking studies to understand mechanisms by which mutations arise, and is a powerful incentive to conduct studies of their *in vivo* occurrence in humans.

In addition, assays for *in vivo* mutations in indicator genes are useful tools in genetic toxicology and risk assessment. Such assays may allow quantitative and qualitative assessments of adverse effects in human populations, and may even enable identification of the nature of toxic exposures. *In vivo* mutagenicity studies may identify heterogeneity of susceptibility among humans for specific or classes of mutagens or carcinogens. Ultimately, relating *in vivo* mutations in indicator genes to occurrences of subsequent genotoxic diseases such as cancer or birth defects may be possible. As this becomes possible, estimates of relative risk may be made for individuals exposed to deleterious environmental agents, providing a basis for medical intervention such as removal from the source, early diagnosis, or, in the case of cancer, application of chemopreventive measures. Somatic mutations arising *in vivo* in humans are, therefore, worthy of study for their biological as well as toxicological significance.

9.1.2 HISTORICAL CONTEXT

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Methods to Assess DNA Damage and Repair: Interspecies Comparisons
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The mouse "spot-test" was the first useful assay to quantify *in vivo* somatic cell gene mutations in mammals (Russell and Major, 1957). Only a year later, a less well-known report described an *in vivo* assay in humans that detected loss of ABO antigens from red blood cells (RBC; Atwood and Scheinberg, 1958). These variant cells were considered to be somatic cell mutants. Two additional types of variant cells containing altered hemoglobin, found at low frequencies in human peripheral blood, were reported over the next 15 years (Sutton, 1972, 1974; Stamatoyannopulos *et al.*, 1975; Wood *et al.*, 1975). Although these assays for variant cells arising *in vivo* were initially proposed as measures of somatic mutations in humans, all were eventually rejected for this purpose, because the cellular changes could never be demonstrated to have been the result of gene mutations. Similar variants could result from non-genetic causes (Atwood and Petter, 1961; Papayannopouou *et al.*, 1977a, 1977b), i.e., each assay was contaminated with "phenocopies" (cells which exhibit a mutant phenotype in the absence of the mutant genotype).

9.1.3 CURRENT ERA

Four assays to detect *in vivo* gene mutations in human somatic cells now exist, and each addresses phenocopies. Results of studies using these assays indicated that they actually defined mutant cells; however, the assays enumerate mutant cells, not the underlying mutational events. For most toxicological evaluations, the mutations themselves are most important. At best, frequencies of mutant cells correlate only imperfectly with frequencies of mutations, because the latter may yield one or more mutants per event, depending on cell division. This effect is compounded when spontaneous mutations may occur preferably in dividing rather than quiescent cells. This issue is considered in detail when discussing each assay.

9.2 CURRENT ASSAYS FOR HUMAN *IN VIVO* SOMATIC CELL MUTATIONS

All four assays currently used for *in vivo* studies of human somatic cell gene mutations measure changes in peripheral blood cells, either RBCs or lymphocytes. Although blood is a useful tissue for human mutagenicity monitoring because of the ease of sample acquisition, exclusive reliance on blood cells for measuring *in vivo* mutation results in serious deficiencies. Most human malignancies, for instance, do not arise in blood cells; the common human cancers are of epithelial origin. For toxicity studies, therefore, information obtained from blood cells must be extrapolated to other tissues; yet no information exists regarding the validity of such extrapolations. Thus, research in this area is of paramount importance.

9.2.1 RBC ASSAYS

RBCs are abundant in human blood, and are easily analyzed for the presence of rare variants. However, since a mature RBC has lost its nucleus and, hence, its DNA, any gene mutation seen in an RBC must have arisen *in vivo* in precursor cells. Of necessity, this implies some distance (in terms of cell divisions) between mutational events and measured variants. Furthermore, the possibility for molecular analyses of variants is eliminated. The RBC assays rely on indirect means to avoid phenocopies and to relate mutant frequencies to underlying mutational events.

9.2.2 HEMOGLOBIN MUTANTS

Of the current human *in vivo* mutation assays, the detection of altered haemoglobin in RBCs was the first to be proposed (Stamatoyannopoulos *et al.*, 1984; Bigbee *et al.*, 1983, 1984). Hemoglobin, a tetrameric protein consisting of two heterodimers, is encoded by genes at two sets of linked loci on chromosomes 11 and 16 (Deisseroth *et al.*, 1977, 1978). Most normal post-natal human haemoglobin is designated haemoglobin A (HBA, α_2; β_2) with a small amount of haemoglobin A_2 (α_2; δ_2).

Mutations of altered haemoglobin polypeptide chains produce specific and unambiguous changes. Such changes cannot be produced as phenocopies. More than 300 such mutations have been defined at the germinal level (Weatherall, 1985), each of which can serve in principle as a marker for *in vivo* somatic cell gene mutation. The best known example is mutation of the β gene (chromosome 11) that produces sickle-cell haemoglobin (HbS). This mutation is an A→T base change that causes the amino acid valine to be substituted for glutamine at position 6 of the β polypeptide. Therefore, although the β gene contains three exons and spans 2 kb, the effective target size for the HbS mutation is a single base pair.

Quantitatively relating RBC haemoglobin variants to underlying somatic mutational events is complex (Stamatoyannopoulos *et al.*, 1984). This degree of complexity holds for all mutations detected in mature RBCs. If the *in vivo* mutations responsible for variant RBCs arise in pluripotent or multipotent stem cells, a factor determining the frequency of variant cells at any time is the number of stem cells generating the RBC population at that time. If this number is small, the many cell divisions between the mutations and the sampled RBCs may produce large interindividual variations in variant frequency (Vf) values. Likewise, for mutations in pluripotent stem cells, large interindividual variations of RBC Vf may exist following identical exposures. Such variability is due to the cell stage at which mutations occur, and will be reduced if somatic mutations occur early in the development of the individual (i.e., before even the entire stem cell complement is generated), and occurs repeatedly as a result of chronic exposure. The advantage to stem cell mutations is memory, i.e., mutant progeny are produced continuously.

On the other hand, if the mutations reflected in RBCs arise *in vivo* in committed RBC precursors (rather than in true stem cells), the relationship between mutations and mutants will be more direct. However, such mutations will produce mutants

only transiently, because these precursor cells themselves are lost with time. The disadvantage of mutation in differentiated cells is loss of memory, while the advantages are close temporal relationships of Vf elevations to mutagen exposures and less variability in Vf values.

Stamatoyannopoulos and colleagues (1984) originally developed specific antibodies to detect HbS-containing RBCs. Their early methods depended on manual reading of slides to detect rare variant RBCs. Recently, a method that treats fixed RBCs on slides with polyclonal anti-HbS antibodies and uses automated image analysis to screen large numbers of cells to detect rare RBCs labelled with anti-HbS fluorescent antibodies has been reported (Verwoerd *et al.*, 1987). Using this technique, Tates and coworkers (Tates *et al.*, 1989) have measured HbS Vf for several groups of individuals: normal non-smoking adults, smokers, xeroderma pigmentosum (XP) and ataxia telangectasia (AT) patients and heterozygotes, and workers exposed to ethylene oxide.

These preliminary results establish the background of HbA → HbS Vf to be approximately 5×10^{-8} for normal adults. Such a low frequency, although expected for the change of only a single base, creates the technical difficulty that extremely large numbers of cells must be analyzed to obtain interpretable data. This difficulty may be mitigated by simultaneous assays for several different mutant hemoglobins using a mixture of several different antibodies (Tates *et al.*, 1989).

9.2.3 GLYCOPHORIN A (GPA) LOSS MUTANTS

GPA is a glycosylated cell surface protein (approximately 10^5 molecules per RBC) that carries the M and N blood group antigens (Furthmayer, 1977; Gahmberg *et al.*, 1979). M and N, which differ by two non-adjacent amino acids, are concomitantly expressed and represent the only two alleles of the GPA locus. The gene for GPA is located on human chromosome 4, has seven exons, and spans more than 44 kb (Kudo and Fukuda, 1989).

Highly specific anti-M and anti-N antibodies linked with green and red fluorophors, respectively, label the wild type RBCs from M/N heterozygous individuals with double fluorescence. Scoring with a fluorescence activated cell sorter permits rapid enumeration of rare RBCs that have lost either antigen. A GPA loss Vf is defined as the number of single colour cells (green or red) divided by the total number of RBCs scored (single plus double fluorescence). In principle, this assay measures large targets, detecting a variety of mutations that inactivate the GPA gene. However, the target size cannot be known precisely because of the protein structure required for antibody binding.

In addition to simple M or N losses, another class of GPA variants found in heterozygous individuals is characterized by the loss of expression of one allele (M or N), with double expression of the other. Such variants, called "homozygous variants" (as opposed to simple loss, or "hemizygous variants") are interpreted as products of somatic crossing-over or gene conversion.

The GPA assay has internal safeguards against the scoring of phenocopies. For a cell to be recorded, one antigen (M or N) must be detected in RBCs from an M/N individual. Non-genetic, or genetic non-GPA, locus events should result in loss of all GPA proteins. Although only M/N heterozygotes can be studied in the GPA assay, the M and N alleles have approximately equal gene frequencies in all populations. Therefore, 50% of individuals are suitable for study.

Most results with the GPA assay are obtained from the Lawrence Livermore National Laboratory, and are based on use of a dual beam flow cytometer (Jensen *et al.*, 1987; Langlois *et al.*, 1989b). GPA results are usually expressed as either hemizygous variants (gene loss) or homozygous variants (recombinants). Mean Vf values for normal non-smoking adults are approximately 10×10^{-6}, with rather wide interindividual (but narrow intraindividual) variation. Mean Vf values in smokers are approximately 30% above those of non-smokers; however, this difference is significant only to the 0.1% statistical level. Hemizygous and homozygous variants occur with equal frequencies in normals. An age effect occurs for GPA Vf values resulting in a linear rise in Vf values of approximately 2% per year, with Vf values at birth (i.e., measured in placental cord blood) falling on the calculated regression line. *In vivo* GPA Vf values have been reported for several DNA-repair-defect syndromes (Langlois *et al.*, 1989a, 1990; Bigbee *et al.*, 1989; Kyoizumi *et al.*, 1989a).

The results with XP patients and heterozygotes may be explained by the site of GPA mutations, i.e., in RBC precursors in bone marrow. Probably little opportunity exists for UV-light to penetrate this body compartment. Alternatively, these results may reflect specific characteristics of the target GPA gene. The 10-fold increase in Vf seen in AT homozygotes and the 100-fold increase seen in Bloom's syndrome (BS) patients parallels their known propensity to show chromosome breakage and translocations. The increase in numbers of homozygous variants in BS patients is believed to be due to *in vivo* somatic gene recombination events reflecting the cancer predisposition of BS patients. None of the heterozygotes for these repair-deficiency syndromes showed elevated Vf values.

Mean GPA Vf values for cancer patients prior to chemo- or radiotherapy showed no differences over comparable controls (Bigbee *et al.*, 1990a). However, longitudinal studies in breast cancer patients receiving adjuvant chemotherapy regimens showed Vf increases after an initial latency of 1 to 3 weeks. At most, these increases approached eight-fold over background, and returned to baseline 120 days after cessation of therapy. This return to baseline presumably indicates that chemically induced GPA variants arise from *in vivo* mutations in committed RBC precursors rather than in pluripotent stem cells.

Several cancer patients receiving a variety of high dose, external beam, localized radiotherapy regimens were studied by GPA assay, and no elevations in Vf have been observed (Mendelsohn, 1991). Perhaps such localized bone marrow radiation produces massive lulling of stem cells, with few survivors for production of mutants. These findings differ from those of atomic bomb survivors studied by GPA assay more than 40 years after exposure; on average, they showed dose-

dependent increases in GPA Vf (approximately 40 variants/10^6 RBCs per Gray). This finding was duplicated by another laboratory in Hiroshima (Kyoizumi *et al.*, 1989b) using a single beam cell sorter. The significance of this finding include not only confirmation of the initial results but also demonstration that this simpler, more widely available instrument is suitable for the GPA assay. As expected, in both Hiroshima studies, large interindividual variations were observed, presumably reflecting: (a) the occurrence of mutations in stem cells; (b) the limited numbers of such cells available for mutations; and (c) cell killing in this limited pool.

A preliminary report of GPA mutations in individuals in the Chernobyl area presented 9 months after the accident revealed a dose-dependent (based on dicentric chromosomes) increase in GPA Vf values (Langlois *et al.*, 1990). The shape of the dose–response curve agreed within a factor of 2 with the Hiroshima data (Mendelsohn, 1991). Wide scatter existed in the data, with the highest Vf being 536×10^{-6}. Most of the increase in variants in Chernobyl individuals was of the hemizygous rather than the homozygous type, suggesting deletions rather than crossing-over events.

These results demonstrated that the GPA assay is operational, should soon be available to study more groups, and will continue to provide information regarding *in vivo* somatic gene mutation in human RBC precursors. The utility of the RBC assays rests on the speed and ease with which many cells may be scored. The lack of genetic material precludes further molecular analyses; recent advances in the use of reticulocytes in this assay may allow molecular analysis of some of the mutations (DuPont *et al.*, 1991; Langlois *et al.*, 1991).

9.2.4 LYMPHOCYTE ASSAYS

T lymphocytes are nucleated cells, also readily available in blood. T lymphocytes can be easily expanded *in vitro* by the use of T-cell growth factors and feeder cells. DNA can be extracted from these cells for molecular studies, including determination of mutational spectra.

9.2.4.1 *hpri* Mutants

Rare 6-thioguanine-resistant (TG) T lymphocytes in peripheral blood arise *in vivo* by mutation at the gene for hypoxanthine-guanine phosphoribosyltransferase (HPRT; *hprt*). HPRT mediates the phosphoribosylation of hypoxanthine and guanine for reutilization of purines. This enzyme is constitutive but non-essential. HPRT can also phosphoribosylate purine analogues such as TG or 8-azaguanine, causing these analogs to become cytotoxic. Thus, loss of HPRT activity can be selected by resistance to such analogs.

The *hprt* gene is located on the X-chromosome (Xq26) (Henderson *et al.*, 1969), and is approximately 44 kb in size and contains nine exons (Patel *et al.*, 1986).

The entire gene region has been sequenced (Edwards *et al.*, 1990). A large background of information exists on *hprt* mutations *in vitro* in human and other mammalian cell systems (Wilson *et al.*, 1986). The *hprt* T-cell assays were the first to become operational for human *in vivo* mutation studies; consequently, much quantitative and molecular information supports the use of this system.

Short-term DNA replication assays

Hprt mutant T cells arising *in vivo* can be detected by two short-term assays. One method uses autoradiography to detect ^{3}H-thymidine incorporation in mutant T cells that are able to overcome TG inhibition of first-round phytohemagglutinin (PHA) stimulated DNA synthesis *in vitro* (Strauss and Albertini, 1979). The original autoradiographic assay allowed pseudoresistant T cells to be scored as mutants (Albertini *et al.*, 1981; Albertini, 1985). These phenocopies appeared to arise because TG cannot totally block the first round of *in vitro* DNA synthesis in actively cycling T lymphocytes, as contrasted to its effect in resting cells. Several ways exist to eliminate this problem (Albertini *et al.*, 1981; Albertini, 1985; Amneus *et al.*, 1982, 1984; Matsson *et al.*, 1985; Zetterberg *et al.*, 1982). All depend to some extent on elimination of *in vivo* dividing T cells from enumeration in the assay. Because of its simplicity, the autoradiographic assay is useful for population screening, is relatively inexpensive, and has the potential for automation (Stark *et al.*, 1984; Amneus *et al.*, 1982, 1984; Matsson *et al.*, 1985; Zetterberg *et al.*, 1982). Equally important, a recently developed short-term method has been developed; it incorporates BrUdR followed either by Hoechst staining (dye #33258) or detection by antibodies to score for TG cells, thereby eliminating the need for autoradiography.

Using autoradiographically determined *hprt*, background Vf values for normal adults have now been reported by several groups using cryopreservation to eliminate the scoring of phenocopies. Ostrosky-Wegman *et al.* (1987) found a mean Vf value of 6.9×10^{-6} for 18 normal adults; Ammenheuser *et al.* (1988) reported a mean *hprt* Vf of 1.9×10^{-6} for eight normal adults; and Albertini (1985) gave 8.7×10^{-6} as a mean value for 82 assays of normal individuals. A sub-group of 26 individuals within this last cohort demonstrated an age effect, with an increase in Vf of 0.26×10^{-6} per year (or approximately 5% per year where the Vf value is 5×10^{-6}).

Amneus and coworkers (Amneus *et al.*, 1982; Amneus and Erikson, 1986) used a cell sorter to enrich for labelled TG T cells in the autoradiographic assay. The window used in sorting cells after short-term culture in TG included nuclei with twice the DNA content. This served to eliminate phenocopies, which usually are able to progress only to early S-phase in the presence of TG. These workers reported a background Vf of 3 to 5×10^{-5} for non-mutagen exposed adults, a value three to 10-fold different from that reported using cryopreservation and slide scoring. The reason for this discrepancy is unknown.

Chemotherapy and radiotherapy in cancer patients have usually resulted in elevated autoradiographically determined *hprt* T-cell Vf values. In multiple sclerosis patients, Ammenheuser *et al.* (1988) reported 14 days after cyclophosphamide treatments an increase in mean Vf from 4.1×10^{-6} to a range of values between 11.6 and 40.3×10^{-6}. However, over 2 to 4 months, these elevated values fell to baseline levels. These declines are consistent with the induction of mutations primarily in mature, differentiated T lymphocytes, and is interpreted as evidence for *in vivo* selection against *hprt* mutant cells. Whether this phenomenon is generalized or the result of the cytotoxic effects of cyclophosphamide treatment is unknown. These workers also reported elevated Vf values after external beam radiotherapy in cancer patients, rising from a mean of 3.2×10^{-6} (n = 7) before treatment to 17.7×10^{-6} four weeks after treatment (Ammenheuser *et al.*, 1989, 1991). Again, increases in Vf value were not seen until 14 days post-treatment. Ostrosky-Wegman *et al.* (1987) reported elevated Vf values in three persons 1 year after exposure to ^{60}Co-external-beam γ-irradiation.

Cloning assay

A disadvantage of the autoradiography assay is that mutant cells cannot be recovered; therefore, the direct cloning assay has been developed to study mutations in T lymphocytes (Albertini *et al.*, 1982; Morley *et al.*, 1983, 1985; Messing and Bradley, 1985; O'Neill *et al.*, 1987; Cole *et al.*, 1988; Hakoda *et al.*, 1988). T lymphocytes are cultured in limiting dilutions in the absence and presence of TG in wells of microlitre plates. To culture medium and TG, wells contain a source of growth factor (interleukin-2; IL-2) and X-irradiated feeder cells. Cloning efficiencies are determined from the Poisson relationship $P_0 = e^{-x}$ where P_0 is the proportion of wells without growing colonies and "x" is the calculated average number of clonable cells per well. The calculated mutant frequency (Mf) is the ratio of the cloning efficiency in the presence to the cloning efficiency in the absence of TG. Growing colonies can be isolated and propagated *in vitro* to sufficient numbers for molecular and other analyses.

Several laboratories have studied background *hprt* Mf in normal subjects using the clonal assay. The results are consistent, with mean values for adults ranging from 3 to 10.1×10^{-6} (mean = 5.3 ($\pm$ 2.7)$\times10^{-6}$. Clearly, an age-related increase in Mf was seen, and was most pronounced for newborns for which background mean *hprt* Mf values were considerably less than expected from simple regression. After the newborn period, Mf values increase 1.7 to 5%.

Chemotherapy in cancer patients also caused significant increases in *hprt* Mf values. Eleven patients receiving chemotherapy alone had elevated Mf compared with 42 healthy controls (19.57 versus 6.72×10^{-6}, respectively), while combination chemotherapy–radiotherapy resulted in a further mean Mf elevation to 34.4×10^{-6} (Dempsey *et al.*, 1985). These mutagenic effects of combined therapy appear to result primarily from the radiotherapy component (Sala-Trepat *et al.*, 1990).

Ionizing radiation also elevated *in vivo* Mf values (Sanderson *et al.*, 1984; Messing *et al.*, 1986, 1989; Seifert *et al.*, 1987). A study of therapeutic external beam, ionizing radiation showed *hprt* mutants to be produced at a rate of 23×10^{-6}/Gray (Sanderson *et al.*, 1984). Patients receiving chronic, total body radioimmunotherapy (RIT) with ^{131}I, ^{90}Y, or a combination of the two as internal emitters also showed elevated *hprt* Mf values (Nicklas *et al.*, 1990). Patients who received ^{90}Y showed Mf values ranging from normal to 42×10^{-6}, while those who received ^{131}I showed elevated Mf values in all cases with a mean for 21 patients of 90.2×10^{-6} (an eight- to 10-fold elevation over normal young adults). The RIT study showed good correlation between Mf values and initial doses of administered radioactivity. However, this correlation of Mf to total exposures was poor after several rounds of treatment. A blind study of the *hprt* mutant frequency from relatively low atmospheric concentrations of ionizing irradiation in the form of household radon exposure has been undertaken recently. Such studies are relevant for human risk assessment, and should be undertaken in a wide range of populations exposed to radon.

A study of atomic bomb survivors conducted 45 years after the exposure showed a significantly elevated *hprt* Mf value in exposed individuals (Hakoda *et al.*, 1989a). Although a dose–response relationship was present between Mf and exposure level, the slope was very shallow (2 to 3 mutants/10^6 cells per Gray) as compared with the responses observed in the GPA assay. This observation most likely reflects the different sites for mutations in RBCs and T lymphocytes with a subsequent loss of *hprt* but not GPA mutants.

Most quantitative studies of induced *in vivo hprt* mutations appear to show a decline in mutant cells with time after treatment. This effect could be due to either of the following: (a) negative *in vivo* selection against mutant cells, possibly as a consequence of cytotoxic treatments resulting in lymphocyte proliferation; or (b) a characteristic of mutations in committed, mature cells. The practical consequence of this observation is that elevated *hprt* T-cell mutant frequencies suggest a recent (i.e., less than 6 months) rather than a distant genotoxic exposure.

Several laboratories are investigating *in vivo hprt* mutations at the DNA level, and a repository for these molecular data is being established (Albertini *et al.*, 1989; Craft *et al.*, 1991). Several reports of analyses at the Southern blot level now exist, with surprising agreement that large alterations account for no more than 15% of background (i.e., spontaneous) *hprt* mutants recovered from adults. Most gross *hprt* alterations are deletions, with some being more numerous than total deletions.

Analyses of gross structural alterations allow inferences regarding the nature of spontaneous *in vivo* deletions. One inference concerns the maximum size deletion that can be tolerated at *hprt*. Twenty-eight simple deletions analyzed by Nicklas *et al.* (1989) showed that the intragenic breakpoints were evenly distributed across the gene. This finding, coupled with the finding that approximately 50% of the breakpoints occurred outside of the gene, suggested that spontaneous *hprt* deletions of at least 94 kb could be recovered. Additional X-linked DNA probes have subsequently been used to define the extent of viable multi locus deletions that may

accompany *in vivo hprt* mutations in T cells. Thus far, losses of linked markers that may map up to 10 cm (~10 Mb) distant from *hprt* by linkage analysis have been detected (Nicklas *et al.*, 1991). This observation requires re-examination of the hypothesis that *hprt* is insensitive to mutational events resulting from large deletions.

Mutations (*hprt*) have been isolated from the human fetus via placental cord blood. McGinniss *et al.* (1989) reported that gross structural alterations were detected in 85% of placental mutant isolates, with most deletion mutations involving loss of *hprt* exons 2 and 3, a finding confirmed in a larger study (Lippert *et al.*, 1990). The spontaneous *hprt* mutations in the fetus have a characteristic molecular spectrum even at this gross level of analysis. These unique intragenic deletions of exons 2 and 3 have been studied further by polymerase chain reaction (PCR) of genomic DNA and sequence analysis. In the 13 mutants analyzed, the deletion breakpoints occurred in the same sequences in introns 1 and 3 of the gene. These sequences contained a consensus heptamer which directs DNA cleavage by the V(D)J recombinase, which directs the T-cell receptor (TCR) and immunoglobulin gene rearrangements. In these mutants, during T-lymphocyte differentiation in the fetus, the recombinase has created this illegitimate deletion of exons 2 and 3 in the *hprt* gene—the first example of developmental mutagenesis in a constitutive gene *in vivo* in any species (Fuscoe *et al.*, 1991a, 1991b).

In adults, however, most background T-cell *hprt* mutants arise from point mutations. The PCR procedure is being used to amplify mutant *hprt* cDNA derived from the mRNA of these point mutation isolates, followed by direct sequencing of the products (Recio *et al.*, 1990; Rossi *et al.*, 1990). Observed changes include base-pair changes (transitions and transversions), multiple base gains and losses, frameshift mutations, and exon losses that probably represent splice-site mutations (Albertini *et al.*, 1990). In some instances, the base change(s) responsible for the splice-site mutations have been localized. Data sufficient to define a meaningful background adult *hprt* mutational spectrum are accumulating rapidly.

Molecular studies of *hprt* mutants arising *in vivo* after ionizing radiations are ongoing. All reports involve only analysis at the Southern blot level. Generally, patients receiving chronic therapeutic ionizing irradiations show an increased proportion of their *hprt* mutants with gross structural alterations, and the maximum size of these *hprt* deletions is larger than seen for background mutants (Hakoda *et al.*, 1989a; Nicklas *et al.*, 1990). Mutants isolated from atomic bomb survivors have produced mixed results (Hakoda *et al.*, 1989a). In one survivor, 26% of the mutant isolates showed gross structural changes of *hprt*; in another, the proportion of mutants with gross *hprt* gene alterations was the same as in normals.

The ability to measure *hprt* mutants *in vivo* permits characterization of the spectrum of mutations in humans. The number of mutational events is the appropriate denominator to describe this range. However, genetic changes themselves are characterized in clones of mutants. In cell populations capable of division and when mutations do not inhibit cell division, the frequencies of mutations and mutant progeny are not necessarily identical. Thus, characterization

of multiple mutants arising from the same mutational event is possible. However, in T lymphocytes, TCR gene rearrangement analyses can establish *in vivo* the relationship among *hprt* mutant isolates. T-cell mutants having identical TCR gene rearrangement patterns are related clonally in that they arose *in vivo* in the same clone of cells. Such mutants are referred to as TCR gene defined mutant sets (TCR sets). If mutants in a TCR set also show the identical *hprt* change, then they arose *in vivo* from the same *hprt* mutational event (i.e., they are siblings). All such mutants represent but a single mutation, and should be scored only once when defining a spectrum of mutations.

Molecular analyses of TCR gene rearrangement patterns in *hprt* T-cell mutants are being used to define relationships between mutants and their underlying *hprt* mutations. TCRs are encoded by TCR genes that undergo conformational rearrangements, in a manner similar to immunoglobulin genes, from a germ line to a functionally rearranged pattern during intra-thymic cellular differentiation. An enormous diversity of TCR gene rearrangement patterns are recognizable on Southern blots, with a given pattern marking a differentiated T cell and its clonal descendants.

Pair-wise comparisons from a single individual of different *hprt* mutant isolates (according to both *hprt* change and TCR gene rearrangements) allow estimations of the origin of mutants from multiple independent *in vivo hprt* mutations and of the differentiation stage *in vivo* at which the *hprt* mutation occurred. The scheme to interpret these comparisons has been described fully (Nicklas *et al.*, 1986; Albertini *et al.*, 1990, 1991). Thus, the approach to RBC mutations is comparable for T lymphocytes: Determination of whether they arise in stem cells or in more differentiated progeny. In T cells, stem cells are of pre-thymic origin; the more differentiated cells are mature, post-thymic T cells. In adults, *in vivo hprt* mutations in T cells occur predominantly in mature, differentiated (post-thymic) cells (Nicklas *et al.*, 1989). By contrast, placental blood *hprt* mutant isolates frequently show a pattern that indicates pre-thymic (stem cell) mutations (McGinniss *et al.*, 1989).

A great diversity of TCR gene rearrangements can be identified by Southern blot analyses. In wild type (non-mutant) T-cell clones, TCR gene rearrangements are very rarely shared. In a study of ~20 wild type clones in each of three individuals, no TCR defined sets were observed (Nicklas *et al.*, 1989); while in a much larger study (339 wild type clones from one individual), only three pairs of clones were seen (99.1% unique clones). By contrast, TCR gene pattern sharing is not uncommon among *hprt* mutant isolates (~10% of mutants from the same individual share patterns). This observation, along with the finding that different *hprt* changes are often seen among mutant isolates with the same TCR gene rearrangement patterns, suggests that background spontaneous *in vivo hprt* mutations in T cells arise preferentially in actively dividing cells (Nicklas *et al.*, 1989).

The finding that 90% of *hprt* mutants suggests unique TCR gene patterns argues that most measured Mfs, at least in normal young men with normal Mf values, closely approximate mutation frequencies. However, individuals with very high

hprt T-cell Mf values may show large discordances between the frequencies of mutants and the frequencies of the underlying mutations. Significantly, TCR gene analyses often show remarkable clonality as defined by TCR gene rearrangements among the mutants. Nicklas *et al.* (1988) reported an Mf value in an otherwise normal individual that had risen progressively over 4 years to approximately 10^{-3} (100-fold above background). More than 98% of these mutant isolates (from hundreds analyzed) showed the same TCR gene rearrangement pattern. Furthermore, molecular analyses of *hprt* revealed that *hprt* mutations had occurred repetitively in this clone, including secondary *hprt* mutations in cells that had already suffered primary *hprt* mutations. This observation of sequential somatic mutations occurring over time in an *in vivo* dividing T-cell clone is the first of its kind. This observation further supports the hypothesis that spontaneous *hprt* mutations arise preferentially *in vivo* in dividing T cells.

Despite abundant information on the *hprt* system, the representative nature of the *hprt* gene for mutational studies has been questioned, because its location on the X-chromosome makes it, therefore, either physically or functionally haploid in all individuals. Detection of gene conversion or homologous crossing-over events at a haploid locus is impossible. Therefore, potentially important events may go undetected, unless *in vivo* mutations can be measured at an autosomal locus.

HLA mutations

The HLA complex includes several linked loci located on the short arm of chromosome 6. These loci contain two classes of genes encoding cell surface recognition (restriction) molecules of importance in immune responses (Bodmer, 1984). The class I loci contain the HLA-A, HLA-B, and HLA-C genes, and a large number of as yet undefined genes. Class R (HLA-DP, HLA-DQ, HLA-DP), and class III loci (complement genes) are also present.

The HLA system is extremely polymorphic. For class I genes, at least 23 alleles of HLA-A, 47 alleles of HLA-B, and eight alleles of HLA-C are known; they are co-dominantly expressed in heterozygous individuals (Bodmer, 1984). Loss due to mutation of an antigen specified by one allele can be easily detected.

Studies of HLA mutations arising in virus transformed B lymphocytes *in vitro* have shown that such mutations occur (Pious *et al.*, 1973; Pious *et al.*, 1977; Kavathas *et al.*, 1980a, 1980b; Gladstone *et al.*, 1982; Nicklas *et al.*, 1984). Detection of mutants involves immunoselection using specific anti-HLA sera or monoclonal antibodies plus complement. This method has been applied to T lymphocytes to study *in vivo* HLA mutation using a cloning assay, similar to that described for studies of *hprt* mutations (Janatipour *et al.*, 1988).

Presently, published reports of *in vivo* HLA mutations have come from a group studying mutations of the class I HLA-A gene (Janatipour *et al.*, 1988; Turner *et al.*, 1988; McCarron *et al.*, 1989). This gene contains seven exons and spans 5 kb. Although initial results suggested that HLA Mfs were different for the two HLA-A

alleles studied (i.e., HLA-A2 and HLA-A3), subsequent improvement in the immunoselection method resolved this discrepancy. Mean Mf values for both alleles for 21 normal adults, aged 18 to 50 years, were essentially the same at 30×10^{-6} ($\pm$ 1 SD = 14.8×10^{-6}). Comparable Mf values for 10 older individuals, aged 70 to 90 years, were significantly higher at 71.6×10^{-6} ($\pm$ 40.6×10^{-6}) (McCarron *et al.*, 1989). Therefore, background HLA Mf values are approximately five- to 10-fold higher than are background GPA and *hprt* Mf values, with an age effect being observed.

HLA mutant T-cell isolates have been studied for DNA changes. Complete deletions of the region of the HLA gene being probed were reported for approximately 30% of the *in vivo* background mutants (Tumer *et al.*, 1988). Some deletions were quite large as demonstrated by the associated loss of the HLA-B allele in the *cis* position to the deleted HLA-A allele (approximately 1000 kb distant). The most recent molecular analyses of *in vivo* derived HLA loss mutants by Southern blot studies show that approximately 30% resulted from mitotic recombination (Morley *et al.*, 1990). Therefore, this important class of mutational events is detectable.

Thus far, the reports of TCR gene rearrangement patterns among HLA mutants from single individuals have been insufficient to determine the T-cell differentiation stage for *in vivo* mutations or clonality, similar to what has been found in the *hprt* system. No HLA studies have been reported in newborns or in individuals with DNA repair deficiency syndromes. However, as this assay becomes operational, further studies are certain to follow.

9.3 OTHER MEASURES OF *IN VIVO* SOMATIC CELL GENE MUTATION

The four assays thus far considered form our current frame of reference regarding *in vivo* somatic cell gene mutations in humans. However, new systems are under development. For example, detection of rare RBCs in peripheral blood that express the Tn antigen has been proposed as a measure of somatic mutation in erythroid precursor cells (Bigbee *et al.*, 1990b). Expression of the Tn antigen apparently results from loss of 3-6-D-galactosyltransferase activity, which normally glycosylates the Tn structure to a product that eventually becomes the M or N blood group antigen (Bigbee *et al.*, 1990b). Rare individuals exist whose blood contains large numbers of Tn RBCs. Some of these individuals also have hemolytic anemia. The disease condition is thought to arise from somatic cell mutation(s) in one or more RBC precursors, with subsequent amplification of these stem cell mutants (Bigbee *et al.*, 1990b). If so, this situation illustrates the complex relationships between mutants and their underlying mutations that will be encountered as each new marker of *in vivo* somatic cell mutations is studied.

New markers may soon be available for the T-cell assays. Diphtheria toxin-resistant mutant T-cells arising *in vivo* in humans have been described, and this

system may be developed for possible use in mutagenicity testing (Albertini, 1982). Losses and alterations of TCR genes, occurring *in vivo* in humans have been described (Kyoizumi *et al.*, 1990). These effects may result from somatic mutations, and might be developed as mutagenicity markers. Also, *in vivo* mutation studies with lymphocytes need not be restricted to T cells. Some investigators have reported *in vivo hprt* mutations in both peripheral blood B lymphocytes and in natural killer (NK) cells in an atomic bomb survivor (Hakoda *et al.*, 1989b). This report illustrates that such systems can probably be developed into useful assays, and is of interest also because it again demonstrates the complex relationship between mutants arising *in vivo* and their underlying mutations. An original *hprt* mutation in a lymphoid stem cell in an atomic bomb survivor was amplified during differentiation, eventually appearing in T lymphocytes (with multiple TCR gene rearrangement patterns), in B lymphocytes (with multiple immunoglobulin gene rearrangement patterns), and in NK cells.

Despite the progress with cell growth mutation systems, such methods have inherent limitations. All methods based on cell growth, such as the cloning assays, employ selective systems to detect and isolate rare mutants. Selection itself may restrict the numbers and kinds of mutants that can be studied (Bradley, 1980, 1983; Worton and Grant, 1985). The lymphocyte assays use selective conditions that kill non-mutant cells, and allow the appropriate mutants to survive. However, mutations that do not alter the mutant molecule sufficiently to resist the selective agent will not allow the cell to survive, thus eliminating this class of mutations from recognition. The red cell assays, while not selecting against non-mutant cells in this way, employ immunological methods for mutant recognition. Therefore, mutations that produce mutant molecules differing from non-mutant molecules at sites other than the antibody recognition site may also be undetected. Since different monoclonal antibodies against the same cell marker may differ with respect to epitope recognition, different spectra of mutations may be recognized when different antibodies are used. Mixtures of monoclonal antibodies, or antisera, may be expected to produce mutational spectra that contain a large proportion of deletions.

Some agreement exists that definitions of *in vivo* mutational spectra at the DNA level will help to define the contribution of environmental mutagens to the overall burden of somatic mutations in humans. Attempts are under way to identify molecular spectra that underlie both the background and induced *in vivo* mutations, with the expectation that at least some mutagens will give characteristic spectra that may serve as signatures for exposures to an agent. The cell-by-cell approach may be inadequate to describe mutational spectra, because the methods lack sensitivity, are laborious, and distort these spectra through selection. Finally, all cell growth based methods require that the cells being analyzed have the ability to propagate *in vitro* in order to provide sufficient DNA or RNA for analysis.

To overcome these limitations, DNA-based methods which rely on denaturing gradient gel electrophoresis and high fidelity DNA amplification using the PCR are being proposed and developed for *in vivo* mutagenicity studies (Cariello *et al.*,

1988; Keohavong and Thily, 1991). The methods envisaged will not employ selection for mutant cells in order to avoid distortions, and will use genetic elements present in multiple copies per cell to achieve high sensitivity. Although only artificial reconstruction experiments have been described thus far, enormous technical difficulties have been overcome (Cariello *et al.*, 1988; Keohavong and Thilly, 1991). Certain technical problems still remain, and some means must be devised to account for clonal amplification of mutants (i.e., the mutant/mutation relationship) when describing mutational spectra. This innovative method clearly addresses limitations of current cell-based systems, but is a system under development.

Although this review is concerned with somatic cell gene mutations *in vivo* in humans, in principle, all markers and their analogs used to assess somatic mutations *in vivo* in humans are available for use in animals. The further development of such *in vivo* mutation assays in animals is important for several reasons. Some basic studies can be done only in animals, and a variety of investigations can be carried out, including those defining dose-response relationships. These are necessary to fully interpret the results of human *in vivo* studies. This area is an important one for further research.

Several groups have now reported results of studies of T-cell *hprt* mutations arising in animals (Gocke *et al.*, 1983; Jones *et al.*, 1985; Inamizu *et al.*, 1986; Dempsey and Morley, 1986; Ward *et al.*, 1989; Ammenheuser *et al.*, 1990; Aidoo *et al.*, 1991; Zimmer *et al.*, 1991). Both a short-term autoradiographic and a cloning assay have been used to study these *in vivo* events. Both are widely used in humans, and a large database has been accumulated for these *in vivo* mutations. The background mutant frequency values have thus far been determined for mouse, rat, and non-human primates, and are essentially the same as for humans. The molecular bases of the *in vivo* mutations in animals are being investigated, in hope that they will be applicable to humans. Results of molecular analyses of the *hprt* changes have already been described for mutations in mouse and monkey (Jones *et al.*, 1987; Burkhart-Schultz *et al.*, 1990; Harback *et al.*, 1991). The sequence analysis of spontaneous and *N*-ethyl-*N*-nitrosourea-induced mutations arising *in vivo* in the cynomologus monkey have been described (Harback *et al.*, 1991); similar studies are under way in the rat. Induced mutations also are being studied in animals. Elevated mutant frequencies have been reported for smoking baboons (Ammenheuser *et al.*, 1990), as has the time course for appearance and persistence of N-ethyl-N-nitrosourea-induced *hprt* mutants in Cynomologus monkeys (Zimmer *et al.*, 1991). The animal systems clearly respond to model environmental mutagens.

An assay described for *in vivo* somatic cell mutations in animals is the granuloma pouch assay in rodents. It allows measurement of *hprt* or other mutations in the fibroblasts of specially developed subcutaneous pouches in rats. Quantitative studies of induced mutation are possible, and mutant cells are available for molecular analyses.

9.4 CONCLUSIONS

This past decade has seen significant advances in our ability to detect and analyze *in vivo* somatic cell gene mutations in humans. The four assays considered here have demonstrated conclusively that such mutations occur; however, the underlying biology of these events has not been fully studied.

One factor of importance for *in vivo* somatic cell gene mutations will probably be the differentiation stage at which they occur. This stage can be determined with current methods. The long memory of the GPA RBC assay for atomic bomb survivors can be contrasted with the short memory of this same assay for cancer patients after chemotherapy. The former presumably indicates *in vivo* mutation in pluripotent stem cells, the latter mutation expressed in more differentiated progeny. The differentiation stage of mutation is being studied by TCR gene probes in lymphocytes. Lymphocyte *hprt* mutations in the human fetus often arise in pre-thymic stem cells, whereas these same mutations appear to arise in differentiated, post-thymic T lymphocytes of adults. Lymphocyte mutations in the fetus, therefore, may have considerably more biological importance than similar mutations in the adult. If multiple somatic hits are important in disorders such as cancer, one way to increase the probability that multiple mutations will eventually converge in a single mutant cell is to have some of these events occur early, when few cells exist.

A mechanism that can result in multiple mutations occurring in a single mutant cell in adults is unremitting *in vivo* cell division, that creates space for sequential gene mutations to accumulate in descendants of the same cell. If this is one route to malignant transformation, perhaps the evolution of cancer can occur by spontaneous mutations with the role of environmental toxicants being primarily to induce this unremitting cell division. This recognition of the role of cell division and the accumulation of multiple mutations in individual mutant cells in T lymphocytes may allow insights into the evolution of a number of somatic genetic disorders. Whether or not this phenomenon is unique to lymphocytes remains to be seen.

Both the GPA and the HLA assays have demonstrated somatic crossing-over or gene conversion *in vivo* in human somatic cells. These processes can now be studied with *in vivo* model systems in humans. An understanding of their role in pathological processes and in differentiation will certainly be facilitated by the availability of these systems.

The final role of human *in vivo* gene mutation assays for toxicology remains to be defined. The low background Vf and Mf values for the four assays permit a baseline for assessing environmental effects. However, the relative rank-order of these values (i.e., HbS Vf < GPA Vf = *hprt* Mf < HLA Mf) is explained only partially. The agreement of GPA Vf with *hprt* Mf might be expected by the similar sizes of these two genes, but not by the different kinetics through which mutants arise. The reasons for the higher HLA Mf values are unknown, but may relate also to different kinetics or lack of *in vivo* selection. However, immunological selection

in vivo might be expected for mutant cells bearing other than null surface marker mutations. Very frequent *in vivo* crossing-over events could explain the relatively high HLA Mf values; but this occurrence should apply also to GPA homozygous variants. The GPA and *hprt* assays, have been used to study individuals with DNA repair defects, and the expected increases in mutants have been observed; yet this effect was not seen in studies using the HbS system.

Three assays have thus far been used to study individuals exposed to a variety of environmental mutagens. Mean elevations in Vf and Mf values have usually been observed, with a good deal of interindividual variability. This phenomenon suggests that the assays may be useful as indicators of population exposures to environmental mutagens, but leaves unresolved the question of their utility for making assessments of exposures and their health consequences. In terms of sensitivity, the GPA and the *hprt* assays have demonstrated their ability to detect the mutagenic effects of smoking; and all of the mutation detection assays clearly record increases in Mf or Vf with increasing age.

Data on which to base a toxicologic evaluation of these *in vivo* mutagenicity assays are rapidly accumulating. However, given the biological considerations, perhaps the effects of mutagen exposures on *in vivo* somatic mutations will not be reflected in terms as simple as quantitative dose–responses. Definition of mutational spectra may be required to define environmental genotoxic effects, at least following acute exposures before cell division confounds the situation. Clearly, much research is still needed, and the tools for this task are now in hand.

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10 DNA Repair in Specific Sequences and Genomic Regions

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10.1 INTRODUCTION

Analysis of repair processes in mammalian cells has been based largely on results obtained with prokaryotes, most notably *Escherichia coli*. In mammalian cells, the main enzymatic pathways for removing various damaged bases and bulky adducts appear to be similar to those in prokaryotes. However, some features that distinguish eukaryotes from prokaryotes are likely to play a role in determining the efficiency and specificity of the mammalian repair systems. The complexity of the substrate for repair enzymes in mammalian cells (i.e., the condensed structure of DNA in chromatin) could influence both the induction and processing of DNA damage within different parts of the genome. For most somatic cells, the expression of a large part of the genome is not needed for specialized function. Thus, DNA repair can operate with a strong preference for processing damage in functionally important genomic domains.

A large body of evidence suggests that regions of chromatin engaged in replication and transcription are in a more open conformation than the inactive bulk chromatin. From the observed hypersensitivity of transcriptionally active chromatin to certain nucleases, differences in chromatin conformation may play a role in determining the accessibility of genomic sequences to repair enzymes. Early studies aimed at elucidating the role of chromatin structure in DNA repair compared properties of bulk chromatin to those of chromatin that had been enriched for transcriptionally active sequences by selective digestion with enzymes that degrade DNA. Although such studies provided evidence that the initial distribution and removal of several bulky chemical adducts were influenced by chromatin structure (Bohr *et al.*, 1987), this approach has been limited by the digestion technique. Analysis of repair in the special case of non-transcribed α-DNA of

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monkey cells (Smith, 1987) revealed a complex pattern of repair heterogeneity, probably mediated by chromatin structure.

Recently, analysis of DNA damage and repair has become possible in specific sequences of the genome, allowing study of well-characterized loci in different cells and under different regulatory conditions. Although, most such studies have focused on damage induced by UV-light in cultured mammalian cells, limited data are now also available for *E. coli*, *Saccharomyces cerevisiae*, and *Drosophila melanogaster*.

In this chapter, evidence is summarized on the heterogeneity of repair of different types of damage in various cellular systems. Not an exhaustive description of various aspects and agents, this chapter is more of a discussion of the demonstration of "preferential repair" as the most notable result obtained from studying specific sequences. A more detailed discussion may be found in Smith and Mellon (1990).

10.2 REPAIR OF UV-INDUCED LESIONS

10.2.1 CYCLOBUTANE PYRIMIDINE DIMERS

The method to measure repair of cyclobutane pyrimidine dimers (CPD) in defined DNA sequences was described initially for CHO cells containing amplified dihydrofolate reductase (DHFR) gene sequences (Bohr *et al.*, 1985). The CPD content of DNA fragments obtained by restriction of genomic DNA can be determined by using the enzyme T4 endonuclease V, which makes single strand incisions specifically at CPD sites. DNA is denatured and fractionated by size in alkaline agarose gels, transferred to a supporting membrane and hybridized with an appropriate probe. Comparison of intensities of bands in DNA samples treated and untreated with T4 endonuclease V allows calculation of the actual frequency of CPD in a DNA fragment and as a measure of its repair.

10.2.1.1 Rodent cells

Removal of pyrimidine dimers from the *DHFR* gene in CHO cells has been studied most intensively. The demonstration that the removal of CPD was much faster and more efficient from the *DHFR* gene itself than from inactive flanking sequences or the genome overall (Bohr *et al.*, 1985; Table 10.1) suggests that the high UV-survival of cultured rodent cells—despite their levels of overall repair being lower than those of human cells—results from proficient repair of essential genes. However, this consequence may occur only in concert with the efficient removal of other photolesions such as (6-4) photoproducts (Smith and Mellon, 1990). The results also demonstrated that overall repair capacity is not necessarily a valid parameter to predict biological endpoints such as cytotoxicity and mutagenesis.

Table 10.1 Preferential removal of cyclobutane pyrimidine dimers from the DHFR gene in cultured mammalian cells

Cell	Time (h)	% T4 endonuclease-sensitive sites removed			Genome overall
		A	B	C	
Human 6A3	4	52	69	32	
	24	85	100	69	70
CHO B-11	4	51	82	12	
	24	62	89	10	15

Cells irradiated with 10 J/m^2
Data from Mellon *et al.* (1987)
A = both strands analyzed simultaneously; B = transcribed strand; C = non-transcribed strand.

Fine-structure analysis of repair in the *DHFR* region has been facilitated by its extensive characterization, and has made available probes for study of different regions of the *DHFR* gene and flanking sequences. The 3′ half of the *DHFR* gene appears to be less well repaired than the 5′ half (Bohr *et al.*, 1986). This feature is not generic to active genes in rodent or human cells, since efficient repair at 3′ ends of other genes has been described (Mayne *et al.*, 1988). A fragment located several kb 3′ to the gene and proximal to a putative replication origin region and nuclear matrix attachment region (MAR) exhibited poor repair. Whether this situation is common to this type of MAR and to what extent this might relate to association of DNA with nuclear matrix proteins has yet to be determined.

The repair levels of different regions of the *DHFR* gene locus can be influenced by altering DNA methylation levels (Ho *et al.*, 1989). In CHO cells containing only about 40% of the normal level of DNA methylation, repair in the 3′ end of the gene increased to resemble repair in the 5′ end. Although the overall repair increased two-fold, repair of the fragment containing the MAR was not increased.

Additional evidence for proficient repair of transcriptionally active sequences in rodent cells has come from studies of several other systems. The HMG CoA reductase gene in CHO cells and the HPRT gene in V79 cells have exhibited efficient repair in backgrounds of low overall repair. In the HPRT gene, the domain of preferential repair appears to extend out in the 3′ direction for several kb. Mouse 3T3 cells have been shown to remove CPD from the active c-*abl* protooncogene, but not from the inactive c-*mos* gene (Madhani *et al.*, 1986). Repair in genes of the major histocompatibility locus of the mouse has been shown to be inefficient in cells not expressing them (e.g., fibroblasts), but efficient in expressing them (e.g., B cells). In B cells, poor repair was observed in a fragment containing an unexpressed pseudogene that lies between two expressed (and repaired) genes (Haqq and Smith, 1987).

Further insight into the role of transcription in preferential repair was provided

by measurements of repair in two complementary strands (Table 10.1). Mellon *et al.* (1987) discovered that the preferential repair of the *DHFR* gene in CHO cells is confined to the transcribed strand, both in cell lines containing amplified *DHFR* loci and in those unamplified for *DHFR* loci. The removal of CPD from the non-transcribed strand was as poor as from the genome overall. This effect suggests that chromatin accessibility alone cannot account for preferential repair in these cases, and implies some role for the transcription process itself in the removal of CPD in these rodent cells in culture.

10.2.1.2 Human cells

Human cultured cells differ from rodent cells by an efficient and nearly complete removal of CPD from their genomes at biologically relevant UV doses. Therefore, what has been called "preferential repair" in human cells reflects primarily differences in rates of repair. A rapid repair of transcriptionally active DNA was postulated some years ago from the kinetics of recovery of UV-inhibited RNA synthesis in normal and UV-sensitive human cells. Resumption of RNA synthesis following UV-irradiation was much faster than the overall removal of CPD. Mellon *et al.* (1986) demonstrated that the *DHFR* gene in a human cell line is repaired two- to three-fold faster than the genome overall; further studies revealed that this preferential repair could be attributed solely to more rapid repair of the transcribed strand. The non-transcribed strand was repaired in a manner resembling the genome overall: more slowly and less completely than the transcribed strand (Table 10.1; Mellon *et al.*, 1987). In both human (Mellon *et al.*, 1987) and CHO cells, preferential repair has also been shown in the flanking region 5′ to the gene that occurs on the DNA strand opposite to the one in *DHFR*, a feature consistent with the presence of divergent transcription units in these regions.

In primary fibroblasts, the adenosine deaminase (ADA), β-actin, and *DHFR* genes have also been shown to exhibit preferential repair (Mayne *et al.*, 1988; Kantor *et al.*, 1990). For the ADA gene, the repaired domain includes some sequences directly 3′ to the transcription unit. However, DNA fragments at the 3′ end of the ADA gene exhibited no preference of repair of the transcribed strand, due perhaps to the presence of a convergent transcription unit overlapping the gene. An ADA antisense transcript has been reported recently, suggesting that both strands of this part of the gene are transcribed. Few data on transcriptionally inactive sequences are available for human cells. The transcriptionally inactive 754 locus has been found to be repaired much more slowly than active regions, perhaps even slower than the genome overall (Venema *et al.*, 1990; Kantor *et al.*, 1990), suggesting the possibility of different levels of repair among inactive sequences in human cells.

Heterogeneity in removal of CPD has been reported in UV-sensitive human cells. Analysis of CPD removal in active genes in xeroderma pigmentosum group C (XP-C) cells revealed a complex picture. Repair of the 3′ end of the ADA gene in XP-C was indistinguishable from that in normal cells, whereas the 5′ end of the ADA

gene (the *DHFR* and β-actin genes) were repaired less than in normal cells, but still much more efficiently than the genome overall (Venema *et al.*, 1990; Kantor *et al.*, 1990). Strand-specific analysis indicates that removal of CPD from active genes in XP-C cells is restricted to the transcribed strand only; the high repair in the 3′ end of the ADA gene may thus be the result of a convergent transcription unit. The inactive 754 locus exhibits the deficient repair characteristic of the genome overall in these cells. Together, these results suggest that the residual repair (15% of normal) in XP-C is confined largely to domains containing transcriptionally active DNA, perhaps explaining the relatively high UV-resistance of non-dividing XP-C cells compared to cells of other XP groups (Kantor and Elking, 1988).

Analysis of repair in Cockayne's syndrome (CS) cells has provided valuable insight into the nature and importance of preferential repair. In these UV-sensitive cells exhibiting normal overall repair but poor recovery of RNA synthesis following UV, the active ADA and *DHFR* genes (Mayne *et al.*, 1988) as well as the c-*abl* gene were repaired at the same rate and to the same extent as the inactive 754 gene or genome overall, respectively. Thus they appear to lack the rapid repair observed in normal cells. The repair of active genes in CS may be even less efficient than the overall repair.

10.2.1.3 *D. melanogaster*

Preliminary studies on removal of CPD in the *Drosophila* cell line K^c has revealed no consistent differences between active and inactive genes. The rate of repair of the active gene coding for RNA pol II was exceptionally fast compared to the overall repair of the genome. However, repair of the active Gart and the inactive Notch and White loci resembled the removal of CPD from the genome overall. Further analysis of preferential repair in specific sequences in these cells may provide important clues to the determinants of preferential repair in those cell types exhibiting it.

10.2.1.4 Yeast

Repair of CPD in functionally different genomic regions in *S. cerevisiae* was reported by Terleth *et al.* (1989). These investigators took advantage of the mating type determinant system of yeast, comparing removal of CPD in two identical α-mating type loci differing only in their expression. The active MAT α-locus was found to be repaired significantly faster than the inactive HML α-locus in a wild-type strain. In a mutant, expressing both loci repair of the HML locus was similar to the active MAT locus in wild-type cells.

Recent results obtained from screening of RAD mutants for effects on preferential repair have revealed an interesting similarity to mammalian cell mutants. RAD 16 mutants, which exhibit moderate UV-sensitivity, are able to remove CPD from the

active MAT locus, but not from the inactive HML locus (Terleth *et al.*, 1989). In this respect RAD 16 cells resemble most cultured rodent cells and XP-C cells.

Smerdon and Thoma (1990) have examined repair of UV damage in exquisite detail in the yeast TRURAP mini-chromosome, actually a 2.6 kb plasmid whose chromatin structure has been mapped. Plasmid DNA prepared from irradiated cells was examined in a modification of the technique described above, in which the probe hybridizes to one end of the restriction fragment. This process allows mapping of the CPD sites to within about 40 bases, and formation and removal of dimers at individual sites can be monitored. The transcribed strand of the active *URA3* gene was repaired much more rapidly than the other strand, while slow repair occurred on both strands of a region containing the *ARS1* sequence and "stable nucleosomes." Still another region of the plasmid exhibited rapid repair of both strands; both strands of this region may be transcription templates. Any individual plasmid molecule contained only about one CPD.

10.2.1.5 *E. coli*

Recently, strand-specific preferential repair has been demonstrated for the genes of the *lac* operon in *E. coli* (Mellon and Hanawalt, 1989). In the uninduced state (after 40 J/m^2), repair in each of the two strands of a restriction fragment in the locus displayed roughly linear kinetics, with about 50% of the CPD removed in 20 minutes. Under inducing conditions, repair of the transcribed strand was very rapid, being more than 60% complete in only 5 minutes, whereas repair of the other strand was similar to that found in the uninduced cells. This clearly demonstrates that strand-specific preferential repair of active genes is not a unique feature of eukaryotes, and that chromatin structure *per se* is not a required factor for selective removal of CPD from active genes.

10.2.2 (6-4) PHOTOPRODUCTS

Although the rate of induction of (6-4) photoproducts in DNA by UV was initially considered to be about 10% of CPD (Franklin and Haseltine, 1986), recent data indicated that their induction may be significantly higher, and that the biological relevance of this lesion is greater than previously understood. Only limited information is available concerning the induction and repair of (6-4) photoproducts in different parts of the genome, because no convenient and quantitative assay is currently available. Very recent work suggests that these lesions, unlike CPD, may be highly favored to form in the inter-nucleosome linker regions of chromatin, like many chemical adducts (Gale and Smerdon, 1990; Mitchell *et al.*, 1990). This effect may be relevant to the more rapid repair of (6-4) photoproducts compared to CPD, and to the existence of several mutants that are altered in their repair of only one of these two major products.

Nairn *et al.* (1985) investigated the removal of (6-4) photoproducts in CHO cells using a radioimmunoassay and observed no differences in either their induction or removal between chromatin enriched in transcriptionally active sequences and bulk chromatin. About 40% was removed from both chromatin fractions in 2 hours following 20 J/m^2. In these experiments, the fractionation procedure relied on the selective precipitation of transcriptionally active chromatin after limited digestion with micrococcal nuclease. Whether the enrichment was high enough to detect preferential repair is unclear.

An indication of preferential repair of (6-4) photoproducts in active genes was derived from a study of the *DHFR* region in UV-irradiated CHO cells. Thomas *et al.* (1989) measured (6-4) photoproducts in specific sequences by using two enzymes from *E. coli*: DNA photolyase to remove specifically the CPD, and UVR-ABC nuclease to incise the DNA at sites of remaining damage thought to be almost exclusively (6-4) photoproducts. No differences were found in the frequencies of (6-4) photoproducts in the *DHFR* gene or the transcriptionally inactive 3′ flanking sequence over doses up to 60 J/m^2. The (6-4) photoproducts appeared to be removed more efficiently from the *DHFR* gene than from the non-coding sequence, i.e., 55% and 35% removed, respectively, during 8 hours repair following 40 J/m^2 UV. However, the total extent and rate of removal were much less than previously reported for (6-4) photoproducts in the genome overall. The authors suggested that this might arise from the greater UV doses they used. In addition, whether repair of the 3′ non-coding sequence truly reflects repair of the bulk chromatin is questionable. More information on repair of genes and inactive sequences, including data about strand specificity, is needed to draw conclusions about preferential repair of these lesions. However, this study does document heterogeneity in repair of (6-4) photoproducts, at least for rodent cells.

10.2.3 REPAIR HETEROGENEITY DETERMINED FROM REPAIR SYNTHESIS

Preferential repair of DNA damage has also been analyzed by labelling the newly synthesized DNA resulting from excision-repair synthesis, either with radioactive thymidine or bromodeoxyuridine, and then fractionating the DNA according to some property and examining the distribution of repair label. For example, Cohn and Lieberman (1984) labelled repair patches with BrdUrd, isolated the DNA fragments containing BrUra with a specific antibody, and analyzed their molecular weight distributions. They concluded that for certain doses and times after irradiation, the distribution of repaired sites in human fibroblasts was not random.

This type of analysis may often be less specific with regard to the lesions under study than direct adduct measurements. For example, in the case of UV-induced damage, repair incorporation reflects the removal not only of CPD, but also of (6-4) photoproducts and other lesions. The rates of removal and associated patch sizes need not be the same for all lesions. Indeed, recent experiments suggest that the

repair synthesis observed immediately after UV irradiation originates predominantly from removal of (6-4) photoproducts. A class of human trichothiodystrophy cells and a mutant V-79 cell (VH-1) both exhibit alterations in repair synthesis at early times after UV that correlate well with deficiencies in the removal of (6-4) photoproducts (Zdzienicka *et al.*, 1988; Broughton *et al.*, 1990). Photoreactivation (mediated by microinjected photolyase) in human fibroblasts has little effect on repair synthesis (measured autoradiographically) early after UV, but produced significant effects later (Roza *et al.*, 1990). Cohn and Lieberman (1984) found an apparent random distribution of repaired sites in the first 2 hours after irradiation with moderate doses of UV, but a non-random distribution for many hours thereafter. This effect might argue against preferential repair of (6-4) photoproducts; but at the irradiation doses used, the interlesion distance of the (6-4) photoproducts may have been too large compared with the DNA fragment size to allow detection of a non-random distribution in this case.

In Leiden, much attention has been paid to the analysis of the distribution of UV-induced repair sites with respect to their association with the nuclear matrix, a structure known to be associated with transcriptionally active DNA. Under certain conditions, repair occurred preferentially at the nuclear matrix in confluent human fibroblasts (Mullenders *et al.*, 1988). This preferential repair was only apparent during a short (15 minute) period directly following irradiation and only at low UV-doses (5 to 10 J/m^2), and was not observed in primary fibroblasts after high doses (30 J/m^2; Mullenders *et al.*, 1988) or in transformed human cells (Harless and Hewitt, 1987). The association of those sites repaired immediately after UV with the matrix was stable during continued incubation. This matrix-associated repair may reflect primarily the removal of (6-4) photoproducts from transcriptionally active DNA, since it was observed only very early after irradiation. Removal of CPD seems to be too slow to account for this observation, even from the transcribed strands of active genes.

The preferential localization of repaired sites in DNA associated with the nuclear matrix had been observed previously in XP-C cells, even under conditions (high UV doses and longer times) where it is not observed in normal cells (Mullenders *et al.*, 1984, 1988). From these results and the hypersensitivity of repaired DNA to endogenous nucleases (Player and Kantor, 1987) XP-C cells can be inferred to be proficient in repair of at least some fraction of transcriptionally active DNA but deficient in repair of inactive DNA. A heterogeneity in the location of repair sites in XP-C cells was also demonstrated when XP-C DNA was treated with the CPD specific enzyme T4 endonuclease, and analyzed in alkaline sucrose gradients (Mansbridge and Hanawalt, 1983). On average, the molecules containing repair sites were considerably longer than the bulk of the DNA molecules suggesting that some domains in the chromatin of XP-C cells were repaired much more efficiently than the bulk chromatin. This phenomenon, also referred to as "domain-limited repair," was not observed in normal human fibroblasts, even in the first 2 hours following irradiation (Mullenders *et al.*, 1986). Direct analysis of the removal of CPD from active and inactive genes has confirmed that XP-C cells have the

capacity to repair domains containing active genes, but are unable to repair inactive DNA (Mayne *et al.*, 1988; Kantor *et al.*, 1990).

When repair synthesis was analyzed in confluent CS cells (Mullenders *et al.*, 1988), results opposite to those observed for XP-C cells were found. In these cells, DNA associated with the nuclear matrix appeared to be reduced in repair synthesis when compared with the remainder of the loop DNA. These results suggest that CS cells may not only be deficient in the preferential repair of active sequences, but that these sequences may actually be repaired less efficiently than the bulk of the DNA. Whether these observations in CS cells reflect only the repair of (6-4) photoproducts is unknown. Direct analysis of removal of these lesions from specific sequences in CS cells has not been reported. For CPD, thorough analysis of the repair of the separate complementary strands will be needed to determine whether the repair deficiency extends beyond a lack of preferential repair. Presently, factors involved in preferential repair may govern not only the rate but also the extent of repair of transcriptionally active genes.

Leadon and Snowden (1988) studied UV-induced repair synthesis in defined fragments of genes by labelling the repair sites with BrdUrd, separating repaired from non-repaired restriction fragments with an antibody specific for BrUrd-containing DNA, and determining the distribution of specific sequences between the two classes of DNA by hybridization. This method does not measure actual lesion frequencies, and does not distinguish the effects of repair patches produced during removal of different adducts. Transcribed members of the metallothionein (MT) family in human cells were initially repaired more rapidly than non-transcribed MT genes or the genome overall. This effect was observable at shortly (2 hours) following irradiation, and thus suggests that (6-4) photoproducts are preferentially repaired in the active MT genes compared with the genome overall.

For rodent cells, domain-limited repair and the association of repair with the nuclear matrix have been studied in confluent primary Syrian hamster cells (Mullenders, 1986), that exhibit the poor repair of CPD characteristic of established rodent cell lines. Repair patches labelled with ^{3}HdThd during a 2-hour period following UV-exposure were found to be uniformly distributed within the genome when the DNA was analyzed for domain-limited repair or for the preferential association with the nuclear matrix. Also when repair was measured over longer (24 hours) periods, no heterogeneity in distribution of repaired sites was observed. Given the very selective removal of CPD from active genes in rodent cells, this lack of domain-limited repair and matrix-associated repair is unexpected. The most likely explanation seems to be that in these rodent cells, unlike XP-C, that are deficient in repair of (6-4) photoproducts, the repair of these lesions accounts for a large fraction of the repair synthesis observed. The distribution pattern of repaired sites, therefore, most likely represents primarily the repair of (6-4) photoproducts, that are repaired proficiently in the entire genome, thus masking the observation of domain-limited and matrix-associated repair. These experiments were carried out before the effect of dose on the matrix association of repaired DNA was observed with normal human cells, and used relatively high doses; at lower doses these kinds

of measurements will likely reflect the repair heterogeneity in rodent cells.

10.3 ALTERABLE REPAIR OF UV DAMAGE IN MAMMALIAN GENES

With two exceptions, the studies discussed above compared repair in sequences of different transcriptional activity in the same cells. In a few cases, attempts have been made to study the relation between repair and transcription in mammalian cells by modulating gene activity in a given cell system by exogenous factors, and examine effects on repair. What is considered rapid repair in mammalian cells (i.e., a considerable fraction of lesions removed in several hours) could be facilitated by rates of transcription that would be considered low. Interpretation of such experiments can also be complicated by the fact that inducing treatments can also bring about changes in chromatin structure of the genes under study, and may influence overall repair efficiency. MT genes, whose transcription can be strongly enhanced by metals and steroids, have been studied in rodent and human cells. The possibility that UV itself may induce MT gene transcription complicates the interpretation of such experiments. Okumoto and Bohr (1987) measured removal of CPD in a CHO cell line containing amplified MT I and MT II genes. Repair measurements were made in DNA fragments which, in addition to the small MT genes, consisted largely of flanking sequences of unknown organization. The outcome of this study was that a 1000-fold increase in transcription of MT I gene by induction with zinc (assumed from results of others) led to variable increases of repair up to a maximum of two-fold. No enhancement of repair was observed in the *DHFR* gene. Although the results suggest that gene activity may determine the efficiency of repair of CPD in active genes in CHO cells; a precise description of its role in this system cannot be made mainly due to the lack of characterization of the DNA fragments used. The same problem was faced in the study of Leadon and Snowden (1988) who used an immunological technique to investigate repair in defined DNA fragments in human cells. This method allows simultaneous determination of repair in various size classes of DNA fragments. At early times after UV the MT genes that are constitutively expressed at a basal level exhibited about twice the amount of repair as the inactive MT pseudogenes or the average for the genome overall. Induction by either cadmium or steroids increased the repair observed by another factor of two, suggesting that the rate of repair in these genes was related to the rate of transcription. However, the nuclease hypersensitivity of the mouse MT genes increases after stimulation with cadmium, indicating changes in chromatin structure as well.

Another method to modulate gene activity is to study genes in cells at different stages of differentiation in culture. Studies with rat cells have revealed interesting, but unexpected, results. Kessler and Ben-Ishai (1988) studied repair of CPD in the creatine kinase (CK) gene in rat myogenic cells, that differentiate to form myotubes upon serum deprivation, and express the CK gene. They were unable to observe any significant removal of CPD in 24 hours in the CK gene in growing or

differentiated cells.

At Stanford University, repair has also been studied using two rat cell cultured cell systems which can be induced to differentiate, L8 muscle cells and PC12 nerve cells. In both types of undifferentiated cells, inactive sequences were poorly repaired, as expected. However, CPD were removed from active genes much more slowly than anticipated from our observations with genes in CHO, mouse, and human cells. In the differentiating L8 cells, repair of both constitutively active (pyruvate kinase) and induced (creatine kinase) genes remained slow, requiring several days to reach appreciable repair levels. This situation might explain the results of Kessler and Ben-Ishai (1988). However, even unexpressed genes (e.g., serum albumin, GAP43) exhibited slow, but proficient, repair. In PC12 cells, growth associated protein (GAP-43) was induced from a basal level in undifferentiated cells to a very high level by supplementing the culture medium with nerve growth factor. However, this treatment increased only moderately (30 to 50%) the repair of the GAP-43 gene.

10.4 REPAIR OF OTHER TYPES OF DAMAGE

Although the excision-repair system in mammalian cells recognizes a large variety of lesions, the rate and extent of processing of various adducts can be different, and can depend on the cell type. Both rodent and human cells in culture remove (6-4) photoproducts rapidly and efficiently; whereas human cells exhibit extensive removal of CPD from the genome overall, whereas rodent cells do not. Information about repair of other types of adducts in defined regions of the genome is vital to basic mechanisms of preferential repair and its biological importance.

Studies of the influence of chromatin structure on the induction and repair of DNA damage other than UV-induced lesions have been mainly concerned with damage induced by reactive chemicals. The scarce data available for ionizing radiation are contradictory. Nose and Nikaido (1984) observed no differences in the induction of DNA strand breaks and alkaline sensitive lesions in defined DNA fragments of active and inactive genes, whereas Chiu *et al.* (1986) reported that DNA regions containing transcriptionally active DNA were more susceptible to damage by ionizing radiation. The reasons for this discrepancy are unclear. Several chemical agents have been characterized with respect to the distribution and repair of their adducts within chromatin. Heterogeneity in distribution of DNA adducts has been found at the levels of the nucleosome, in DNAase I hypersensitive regions of chromatin, in nuclear matrix associated DNA and loop DNA, and, in a few cases, in specific DNA sequences. With chemical agents, the possibility of heterogeneity in the initial distribution of adducts, which may in itself influence rates or extent of repair must be considered. The study of preferential repair of CPD in eukaryotic cells has been greatly facilitated by the uniform distribution of CPD within the genome. With the exception of ribosomal DNA, all genes investigated so far have exhibited CPD frequencies comparable with the average

frequency in the genome. The low frequency of CPD in ribosomal DNA can be attributed to its high guanine and cytosine content (Rajagopalan *et al.*, 1984).

10.4.1 AFLATOXIN B_1 ADDUCTS

Leadon and Snowden (1988) used their immunological technique to examine excision-repair in DNA fragments containing MT genes and pseudogenes in human cells treated with aflatoxin B_1. Shortly after treatment, results resembled those for UV irradiation. The fragments encompassing active genes contained more repaired sites than did fragments with inactive genes or the bulk of the DNA. However, at later times, unlike the case for UV, the repair in the fragments containing silent sequences remained low. Interpretation of results with aflatoxin are complicated by the fact that the rate of spontaneous release of the adducts is considerable, leaving apurinic sites in the DNA subject to repair by a different pathway.

10.4.2 ACETYLAMINOFLUORENE ADDUCTS

Differential repair of lesions induced by *N*-acetoxy-acetylaminofluorene (NA-AAF) has been reported for CV-1 monkey cells infected with simian SV40 virus (Brown *et al.*, 1987). The initial adduct frequency in cellular DNA was about twice that in viral DNA. Viral DNA repair was on average twofold faster than that of cellular DNA, but a viral DNA region containing the major nuclease hypersensitive sites was repaired at twice the rate of the rest of the viral genome. The more open structure of SV40 chromatin could result in greater accessibility to repair systems (Shelton *et al.*, 1980), but does not explain the lower adduct frequency in viral DNA. The formation of NA-AAF adducts in the non-transcribed highly condensed α-DNA of monkey cells was found to be slightly higher; their removal was slightly slower than in the bulk DNA. Removal of NA-AAF adducts from α-DNA was enhanced following UV-irradiation, suggesting that the heterochromatic α-chromatin has to be converted into a less condensed configuration to facilitate access to repair enzymes (Smith, 1987).

The induction and repair of NA-AAF adducts in specific sequences has been measured in the CHO cell line originally used by Bohr *et al.* (1985) to measure gene-specific repair of CPD (Tang *et al.*, 1989). In this case, the *E. coli* UVR-ABC nuclease was used to cleave DNA at the site of adducts. Similar frequencies of adducts were formed in 14 kb fragments of the *DHFR* gene and the 3′ non-transcribed sequence. Removal of adducts from the two specific fragments and from the genome overall followed the same kinetics, with 60 to 70% removal after 24 hours. This level of repair is surprisingly high, since less efficient repair has been reported in monkey cells (10% in 6 hours), CHO cells (30% in 24 hours, exclusively C^8-AAF), and human cells (50% in 24 hours, mainly C^8-AAF). Thus, removal of NA-AAF adducts appears to be clearly different from the selective

removal of CPD observed in the same CHO cell line, and resembles more closely the repair of CPD in human cells. However, the results of other experiments may indirectly suggest the existence of selective repair of NA-AAF adducts in active genes. Repair in the CHO line AT3-2 and in a UV-hypersensitive derivative have shown cross sensitivity to NA-AAF. Only C^8-AAF adducts were formed, and repair of the genome overall was slow but identical in both cell lines. Although repair of bulk DNA was similar, unobserved differences in extent of repair of active genes may account for different cytotoxic responses. In addition, assuming that NA-AAF adducts were formed exclusively with guanine, the observation that mutations in the *DHFR* gene were selectively recovered from the non-transcribed strand of the gene (Carothers *et al.*, 1989) may indicate preferential repair. However, this effect might also reflect a biased distribution of potential sites for the type of mutation selected in these experiments.

10.4.3 PSORALEN ADDUCTS

One of the first examples of intragenomic repair heterogeneity in mammalian cells was the extremely inefficient removal of psoralen photo-induced monoadducts and cross-links from the heterochromatic, non-transcribed α-DNA of quiescent cultured monkey cells. Since this effect was not observed in actively growing cells and since UV damage was efficiently repaired in α-DNA, the poor removal of psoralen adducts seemed likely to arise from the unusual chromatin structure of α-DNA, rather than from its inactivity (Smith, 1987). Interestingly, total adduct frequencies in α-DNA resembled those in bulk DNA; a lower cross-link to monoadduct ratio was observed, however, and might be the result of lower flexibility of DNA in α-chromatin.

Removal of psoralen DNA cross-linking and cross-linkable monoadducts from specific sequences has been studied by two methods. The first is an adaptation of that used for CPD, in which rapidly renaturing (cross-linked) restriction fragments are separated from fragments containing no cross-links by gel electrophoresis, and cross-link frequencies in specific sequences are determined by hybridization to specific probes. Cross-linking is measured directly by this method, and cross-linkable monoadducts are measured by determining the increase in cross-linking brought about by further irradiation of the purified DNA. These monoadducts comprise about 65% of the initial monoadducts formed by the irradiation of cells. Using this method, Vos and Hanawalt (1987) showed that the removal of DNA cross-linking from the *DHFR* genes of human and CHO cells proceeds more rapidly than removal of cross-linkable monoadducts. Comparison of repair in active genes with repair in silent sequences has not been reported using this method. Whether the observed repair is more rapid than that in the overall genome is difficult to assess due to the wide variation reported in the literature for rates of repair of these adducts. The rates of removal of monoadducts observed certainly appear slower than corresponding rates for CPD removal, but the possible effects

of the cross-links also present are difficult to assess.

A second method to measure repair of psoralen adducts involves shearing isolated genomic DNA to an appropriate size and separating cross-linked and noncross-linked molecules on special density gradients. Fractions from gradients can be assayed for DNA radioactivity to analyze the overall genome, and also slot-blotted to filters for hybridization to various specific probes. Using this method, cross-links were reported to be formed to a greater extent in the human *DHFR* gene than in the bulk of the genome, but to a much lesser extent in the inactive c-*fms* protooncogene. Removal of cross-links was efficient in *DHFR* (90% in 24 hours), barely detectable in c-*fms* and at an intermediate level in the overall genome (31%). More investigation, especially comparisons between neighboring active and inactive sequences and genes in different states of activity, is clearly needed.

10.4.4 ADDUCTS OF SIMPLE ALKYLATING AGENTS

Several investigators have utilized fractionation of chromatin to investigate selective alkylation or repair in different regions of chromatin. Such studies have revealed remarkable heterogeneity in the distribution of lesions within chromatin, perhaps most strikingly demonstrated by distinct hotspots of O^6-ethylguanine at the bases of DNA loops in rat brain cells exposed to *N*-ethyl-*N*-nitrosourea (Nehls *et al.*, 1984). Ryan *et al.* (1986) fractionated chromatin into "active" chromatin, nuclear matrix associated DNA, and bulk chromatin; they measured the methylated purine content of these fractions at various times after exposing rats to dimethyl-nitrosamine. Regions of active chromatin and nuclear matrix DNA tended to be methylated more readily than bulk chromatin. Removal of *N*-methylated purines was rather uniform in the various fractions, although repair in active chromatin was slightly faster than in the other fractions. In contrast to N-methylpurines, wide variations in repair of O^6-methyl guanine were observed in the various chromatin fractions, repair being most rapid in active chromatin. Nuclear matrix DNA, representing about 2.5% of the total DNA and being depleted of active DNA by the isolation method employed, was nearly unrepaired for up to 20 hours. The latter may be due to the presence of "bent" DNA at the bases of loops (Sykes *et al.*, 1988), since non-bent DNA has been shown to be refractory to repair of O^6-methyl-guanine *in vitro* (Boiteux *et al.*, 1985).

Nose and Nikaido (1984) measured induction and repair of methylnitroso-guanidine-induced DNA strand breaks, alkaline labile sites in the active collagen and of the inactive β-globin gene of human fibroblasts. The rate and extent of repair in both loci was similar. This finding is in agreement with the study of Ryan *et al.* (1986), provided that lesions under study are assumed to be apurinic sites formed from *N*-methylpurines.

Similar conclusions can also be drawn from studies with CHO cells. Scicchitano and Hanawalt (1989) measured the repair of *N*-methylpurines in the *DHFR* gene and flanking sequences in CHO cells exposed to dimethylsulphate. To assay these

adducts, their heat lability was exploited to introduce apurinic sites in the DNA, which were then converted into single strand breaks. Formation of *N*-methylpurines was found to be the same in the *DHFR* gene and in the 3′ inactive sequence. Repair reached a level of 70% after 12 hours following treatment, and was virtually complete after 24 hours. No differences were observed between the gene and its flanking sequence, and analysis with strand specific probes indicated no differences in repair rates of the transcribed and non-transcribed strands of the *DHFR* gene. Overall repair in the genome measured by alkaline sucrose gradient centrifugation was somewhat slower and less complete (i.e., 70% repair in 24 hours).

Together, these results suggest that repair of O^6-methylguanine may occur preferentially in transcriptionally active DNA. Repair of *N*-methylpurines is uniform within chromatin, and in the case of active genes not dependent on transcription itself. The absence of preferential repair of *N*-methylpurine may be related to the repair system involved in processing these adducts. Preferential repair of 3-methyladenine would probably have gone unobserved in these experiments due to the preponderance of 7-methylguanine adducts. Chromatin structure may not restrict access of DNA to small glycosylases and AP endonucleases, and the absence of strand-specific repair may be due to lack of blockage of transcription, assuming that such blockage accounts for strand-specific repair of CPD. However the same arguments could apply to O^6-methylguanine, that is repaired by a small, specific methyltransferase. In this case, a preferential localization of the enzyme in transcriptionally active chromatin could underlie preferential repair, but further verification of this possibility awaits both data on gene-specific repair of O^6-methylguanine as well as availability of purified enzyme.

10.5 CONCLUSIONS

Current understanding of repair heterogeneity is blurred considerably. In areas such as removal of CPDs, enough data existed from different sources to begin to discern some detail. The strand-specific repair of active genes in *E. coli*, yeast, and cultured mammalian cells suggested that this process is probably not mediated by simple accessibility (diffusion controlled), but is somehow coupled to transcription itself. A model for the mechanism of this preferential repair is that transcription blockage at these lesions somehow targets the repair system. This model can be tested using *E. coli*, in which advanced knowledge of the repair process exists. Recently, findings have indicated that CPDs block transcription *in vitro* at the site of the lesion and that a relatively stable RNA polymerase–DNA complex is formed. In the system so far developed, purified UVR-ABC nuclease proteins have no increased affinity for this substrate.

How strand-specific repair fits into the overall picture of repair heterogeneity is unclear. Results with CS cells raise the possibility that a specific factor not only hastens repair of active genes, but is also required for their efficient repair, at least for CPD. Clearly, some intragenomic repair heterogeneity is not mediated directly

by the transcription process. In several cases, inactive DNA or regions flanking genes are repaired better than the genome overall. XP-C cells appear to restrict repair of both CPDs and (6-4) photoproducts—perhaps all bulky adducts—to certain chromatin domains; these domains may be larger than transcription units. These observations suggest that accessibility factors influence repair as well. The difference in the rate of removal of (6-4) photoproducts and CPD from human and rodent genomes may reflect different requirements for some accessibility factor due to their differing distributions between core and linker DNA. However, the simple idea that rodent cells carry out primarily repair of active genes (because they lack some factor necessary to make inactive chromatin accessible) seems inconsistent with their proficient removal of AF adducts from inactive sequences.

The complex systems developed by eukaryotic cells for managing their large amounts of DNA, containing sequences with differing functions, undoubtedly result in multiple hierarchies for DNA repair; that a simple picture is not emerging should not be surprising. An attempt to reconcile the available data with models of chromatin scanning and processing by repair systems may be found in the article by Smith and Mellon (1990), but the monograph serves primarily to emphasize our ignorance of such processes. The usual statement that more information is needed to understand the phenomenon under study certainly applies in this case. However, besides the need for more data about repair of various lesions in different genes, cell types, tissues, and organisms, knowledge of the details of chromatin architecture must advance considerably before understanding of repair heterogeneity increases in clarity.

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11 Molecular Analysis of Mutations in Endogenous Genes

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11.1 INTRODUCTION

The mutagenic potential of chemical agents and radiation is determined primarily by the nature and quantity of the lesions introduced in DNA. Most mutagens induce multiple types of lesions, each with different mutagenic properties resulting in a variety of mutations. To obtain a clear picture of the various types of mutations caused by a mutagen, target genes capable of detecting the majority of induced mutations are needed. Detecting mutations has been limited to those genes in which a mutation causes a phenotypic change such that mutants can be isolated through selective killing of cells or organisms carrying the non-mutated gene, or where the phenotypic change is recognizable by visual inspection. However, not all mutations in a gene give rise to a mutant phenotype, because some mutations either do not alter (e.g., "wobble bases") or only partially reduce the activity of the gene product.

11.2 FACTORS INFLUENCING MUTATION SPECTRA

The distribution of mutagen-induced DNA sequence alterations in a given gene of a particular cell or organism is known as a mutation spectrum. Factors likely to influence the shape of such a mutation spectrum are described below.

11.2.1 DNA LESIONS

Although a mutagenic agent often gives rise to a variety of DNA lesions, only a fraction of such lesions have mutagenic potential. For instance, some lesions might directly miscode or distort the DNA helix, inserting incorrect nucleotides during replication of DNA; whereas strand breaks in the DNA probably give rise to deletions or to chromosomal rearrangements. Furthermore, each lesion has its own

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intrinsic mutagenic potential; some lesions can be present in excess without being mutagenic, whereas other minor lesions are highly effective mutagens. The best studied DNA lesions are summarized in Table 11.1, together with examples of some DNA-damaging agents causing them.

Table 11.1 Mutation spectra caused by genotoxic agents

Major type of DNA lesions	DNA damaging agents
Single and double strand breaks	Ionizing radiation, bleomycin
Pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts	UV light (254 nm)
Alkylated bases, alkyl-phosphotriesters	Alkylating agents
Inter- and intra-strand DNA cross-links	Psoralen + 340 nm UV light, mitomycin C, *cis*-platinum-compounds
Bulky adducts	*N*-acetoxy-2-acetylamino-fluorene, benzo[*a*]pyrene, aflatoxin B_1

A lesion is not necessarily directly mutagenic, but may be unstable and form secondary lesions with mutagenic properties (e.g., AP-sites; Loeb and Preston, 1986). Many mutagens have to be metabolically activated before they become effective. The presence or absence of mutagen activating or deactivating processes in various cell types may lead to different types of DNA lesions and, consequently, to different mutation spectra generated in cells of different organs.

11.2.2 DNA STRUCTURE AND SEQUENCE

The way DNA is organized in the cell influences the shape of a mutation spectrum. In mammalian cells for example, the introduction of lesions over the genome by some mutagens is non-random. Ethylation of the O^6-position of guanine by *N*-ethyl-*N*-nitrosourea (ENU) varies by a factor up to 800 between different regions of the genome (Nehls *et al.*, 1984). These lesions are introduced in larger amounts in areas of chromosomal DNA that have a more "open" structure, possibly representing the promoter regions of actively transcribed genes. The shielding of DNA against free radical reactions by chromosomal proteins is probably reduced at these sites.

The DNA nucleotide sequence can also influence the mutation spectrum. The

frequency of formation of a particular lesion at a certain base often depends not only on the DNA sequence of adjacent nucleotides but also on the secondary structure of the DNA. This effect may result in nucleotide sites with a high incidence ("hot spots") and a low incidence ("cold spots") of adduct formation.

11.2.3 DNA REPAIR

A mutation spectrum can be influenced by the extent of DNA repair. In both prokaryotic and eukaryotic cells, several DNA repair mechanisms capable of removing lesions from DNA exist (Friedberg, 1985). Some types of DNA damage can be repaired directly. In various organisms, alkyl transferases have been detected that are capable of removing an alkyl group from alkylated bases. Bulky lesions that are blocks for DNA replication may be repaired through nucleotide excision-repair, which removes a short segment of DNA containing the adduct. Other damaged bases can be removed through a process called base-excision-repair. Both types of excision-repair overlap partly, since they sometimes act on the same DNA adducts. The process of excision-repair is probably error-free, because the information concerning the correct nucleotide sequence is available from the complementary DNA strand. However, some types of damage are not removed from the DNA, and persist even through replication. So called tolerance mechanisms allow the replication machinery to bypass some of the lesions and repair the gaps that may be generated in daughter strands when the replication fork encounters bulky adducts, causing reinitiation of DNA synthesis downstream of the lesion. Indications exist that DNA polymerases preferentially insert adenine opposite non-coding lesions such as apurinic sites (Kunkel, 1984), a process that can give rise to mutations. In mammalian cells, polymerase δ and polymerase α are implicated to act as the leading and lagging strand polymerase, respectively (Prelich and Stillman, 1988). The ability to bypass potential mutagenic lesions in the DNA during replication are likely to be different for various DNA polymerases. This situation and the fact that the leading and lagging strand are synthesized in a different manner (i.e., continuous versus discontinuous) may result in a strand bias in the distribution of mutations.

For several DNA adducts, removal from the mammalian DNA occurs in a non-random fashion (Bohr *et al.*, 1985; Gupta *et al.*, 1985; Leadon, 1986; Ryan *et al.*, 1986). Removal of UV-induced pyrimidine dimers, for instance, is more rapid in those parts of the genome transcribed actively; and a strand preference exists in favor of the transcribed DNA strand (Mellon *et al.*, 1987). Repair-deficient strains or cell lines are available for most organisms used in mutation studies. Mutation spectra determined in cells with repair-proficient and repair-deficient genetic background are likely to enhance understanding of the molecular mechanisms of DNA repair.

11.3 TARGET GENES FOR ANALYSIS OF MUTATION SPECTRA

11.3.1 INTRODUCTION

The analysis of mutation spectra may also be useful for purposes other than obtaining information on DNA structural features and on cellular processes involved in mutation induction. In carcinogenesis, oncogene activation is sometimes accompanied by the appearance of specific mutations in one of the cellular proto-oncogenes (Barbacid, 1986). Activation of the three *ras* oncogenes has been shown to occur through specific point mutations, which have been localized at codons 12, 13, and 61. Specific deletions and/or somatic loss of heterozygosity for regions of the genome have been observed for other rare human tumors including:

1. osteosarcoma (chromosome 13, band q14),
2. Wilms' tumor (chromosome 11), and
3. acoustic neuroma (chromosome 22).

Recently, similar deletion events and specific loss of heterozygosity have been observed in more frequent human tumors including:

1. carcinoma of the lung (chromosome 3p),
2. breast (chromosome 13), and
3. colon (chromosome 5, band q21-22).

Depending on the type of mutation needed for activation of a particular oncogene, large differences are expected to exist between various mutagenic carcinogens in their ability to induce particular types of tumors. Analysis of mutations in a relatively large DNA target sequence should provide insight into the capability of mutagenic carcinogens to introduce the type of mutations necessary for activation of specific oncogenes.

11.3.2 CHROMOSOMAL TARGET GENES FOR MUTATION STUDIES

The most commonly used systems for mutation studies are cultured mammalian cells, *Escherichia coli*, yeast, and *Drosophila melanogaster*. Mutation studies in mammalian cells using endogenous genes as target for mutation induction are usually performed with cultured rodent or human cell lines. The choice of target genes for mutagenesis studies is rather limited, because a well defined selection procedure must exist for cells with a mutated gene. Genes on integrated shuttle vectors are also being used as targets for mutagenesis. The genes that serve as targets for mutation induction in the different systems are listed in Table 11.2; however, mutation spectra have not been determined for each.

Table 11.2 Target genes for mutagenesis

Organism	Target gene
E. coli	*lacI*, *gpt*
Yeast	*SUP4*, *URA3*
D. melanogaster	*white*, *vermilion*, *adh*
Mammalian cells	*hprt*, *aprt*, *dhfr*, *tk*, *ouabainr*, *gpt*

In contrast to most bacterial and yeast genes, *Drosophila* and mammalian genes have a complex structure of intron and exon sequences and a relatively large size. These features have delayed large scale molecular analysis of mutations in mammalian and *Drosophila* genes. The development of the polymerase chain reaction (PCR) procedure (Saiki *et al.*, 1985), that allows *in vitro* amplification of specific DNA sequences, has made possible the rapid determination of the nature of mutations in specific DNA fragments. Two oligonucleotide primers, that flank the DNA segment to be amplified and that can hybridize to opposite strands, are used in repeated cycles of denaturation, primer annealing, and extension of the primers with a DNA polymerase. This procedure results in specific amplification of the target DNA sequence more than one billion-fold. The PCR-based technique has recently been used to analyze mutations in the mammalian *hprt*, *aprt*, *dhfr*, and chromosomally integrated *gpt* genes and the *Drosophila white* and *vermilion* loci. Some spectra determined at these loci are discussed below.

11.3.3 SPONTANEOUSLY OCCURRING MUTATIONS

The actual change in DNA sequence at the autosomal adenine phosphoribosyltransferase (*aprt*) locus in cultured mammalian cells has been determined in a series of spontaneous mutants (De Jong *et al.*, 1988; Phear *et al.*, 1989). In both cases, Chinese hamster ovary (CHO) cells that are hemizygous for the *aprt* gene were used. The spectra obtained by the two laboratories show large differences. Ninety % of the mutations determined by De Jong *et al.* (1988) were base-pair substitutions, of which 80% were GC→AT transitions and a pronounced "hot spot" was found. The mutation spectrum determined by Phear *et al.* (1989), however, consists for 35% of deletions, duplications, and base insertions. Furthermore, in their spectrum only 40% of the detected base-pair changes were GC→AT transitions and the "hot spot" detected by De Jong was not the site of any of their mutations. The fact that with the same target gene and in the same cell line different spontaneous spectra are generated in different laboratories may result from the use of different culture conditions. For instance, some batches of serum used for cell culture media contain variable amounts of nucleosides, that might influence nucleotide pools; imbalances in nucleotide pools are known to induce mutations

(Phear and Meuth, 1989a, 1989b), and can greatly influence the spectra of spontaneous mutations.

The mutation spectrum of spontaneous mutations at the *gpt* gene in mouse cells was determined by Ashman and Davidson (1987). This prokaryotic gene is located on a retroviral shuttle vector, that is integrated into chromosomal DNA. Deletion was the most frequent event (67%) among these mutants, more than half of those being due to a 3 bp deletion at a single site. At this "hot spot" a trinucleotide was repeated. The same 3 bp deletion "hot spot" was also found in the spontaneous spectrum at the *gpt* gene (Tindall and Stankowski, 1989); although in that study, a different integrated shuttle vector and CHO cells were used. No preference for a specific change was found among the mutants having a base substitution, but somewhat more transversions than transitions were detected.

Spontaneous mutations at the X-linked *hprt* locus as determined by Southern blot analysis consisted of about 10 to 20% of large deletions in cultured chinese hamster cells (Fuscoe *et al.*, 1983; Stankowski *et al.*, 1986) and 40 to 50% in human lymphoblastoid cells (Genett and Thilly, 1988; Monatt, 1989). Sequence analysis of 16 spontaneous *hprt* mutants from V79 CHO showed that seven of them lacked one or more exons from PCR amplified *hprt* cDNA. These mutants were probably caused by a mutation in a splice acceptor site, that was confirmed for three of them by sequence analysis of the involved splice junction.

Introduction of the methodology for the isolation of *hprt* T lymphocytes from human blood provides an opportunity to determine *in vivo* mutation spectra (Albertini *et al.*, 1982a; Morley *et al.*, 1983). Therefore, epidemiological studies now have the potential to correlate mutation spectra with occupational and environmental exposures to genotoxicants. Analysis of 31 mutant T lymphocytes from healthy male donors, between 30 to 40 years of age, showed that in 17, a single nucleotide change, including five frameshifts, was present. Like in V79 Chinese hamster cells, a high proportion (12 out of 31 mutants) of splice mutants was also observed.

Molecular analysis of human germ line *hprt* mutations in Lesch–Nyhan patients showed that more than 80% of these mutations are point mutations (Yang *et al.*, 1984). Sequence analysis of 29 of these mutants showed that base pair substitutions were the major type of changes (Gibbs *et al.*, 1989, Davidson *et al.*, 1989). Comparison of the somatic and germinal *hprt* mutation spectra showed that both spectra are in close agreement, apart from a discrepancy in the fraction of splice mutants that is much lower (6%) in Lesch–Nyhan and gouty arthritis patients.

11.3.4 ALKYLATING AGENTS

Alkylating agents react with N- and O-atoms in DNA. The most prominent types of adducts caused by these mutagens are phosphotriesters and N^7-alkyl-guanine (N^7-alkyl-G), N^3-alkyl-adenine (N^3-alkyl-A), O^6-alkyl-guanine (O^6-alkyl-G), O^4-alkyl-thymine (O^4-alkyl-T), and O^2-alkyl-thymine adducts (O^2-alkyl-T). The relative

proportion of these adducts differs tremendously among alkylating agents. Some agents (e.g. dimethylsulphate (DMS), methylmethanesulphonate (MMS), and ethylmethane-sulphonate (EMS)) react predominantly with DNA through a bimolecular substitution (S_N2) reaction forming a transition complex with strong nucleophiles, particularly base nitrogens. Other agents like *N*-methyl-N-nitrosourea (MNU), *N*-ethyl-*N*-nitrosourea (ENU), *N*-nitroso-*N*,*N*-dimethylamine (DMN), *N*-nitroso-*N*,*N*-diethylamine (DEN), *N*-nitroso-*N*-methyl-*N*-α-acetoxymethylamine (NMAM), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG), 1-(2-hydroxyethyl)-1-nitrosourea (HENU) almost exclusively alkylate DNA through stable carbonium ions, the kinetics of which are unimolecular (S_N1). Hence, these compounds tend to react relatively more extensively with weakly nucleophilic oxygen positions. The GC→AT transition has been found to be the predominant kind of change (>95%) after treatment with MNU (Richardson *et al.*, 1987a; Burns *et al.*, 1988a), MNNG (Richardson *et al.*, 1987b; Reed and Hutchinson, 1987; Burns *et al.*, 1987; Gordon *et al.*, 1988; Kohalmi and Kunz, 1988; Kunz *et al.*, 1989), ENNG (Richardson *et al.*, 1988; Kunz *et al.*, 1989), DMN (Horsfall *et al.*, 1989), NMAM (Horsfall and Glickman, 1989), HENU (Richardson *et al.*, 1988), EMS (Burns *et al.*, 1986; Kohalmi and Kunz, 1988), and DMS (Zielenska *et al.*, 1989) in *E. coli*, yeast, and mammalian cells. This base substitution is probably the result of mispairing of O^6-alkyl-G with thymidine during replication. For the S_N1 compounds, the GC→AT transition shows a strong sequence context, namely the predominance of mutation at guanine residues preceded (5′) by a purine (Richardson *et al.*, 1987a; Burns *et al.*, 1988b). However, chemicals with S_N2 characteristics (DMS and EMS) do not seem to have this 5′ flanking base effect (Burns *et al.*, 1986; Zielenska *et al.*, 1989). AT→GC transitions detected in ENU spectra probably result from O^4-ethyl-T, which can mispair with guanine. In the spectrum of ENU in *Drosophila* (Pastink *et al.*, 1989) and mammalian cells, however, a substantial number (30 to 40%) of AT→TA and AT→CG transversions is also induced possibly by O^2-ethyl-T or N^3-ethyl-A.

The spectra of the methylating agents MNU and MMS (Nivard *et al.*, 1989) in germ cells of *Drosophila* are completely different from all other investigated species. For both agents, very few GC→AT transitions were recovered among the point mutations, but transversions (especially AT→TA) predominated. The explanation might be that the amount of time available between initial damage and the fixation of the mutation during replication is much longer in the case of postmeiotic germ cells than for all other investigated systems. No removal of DNA adducts occurs during this time period, but a higher proportion of secondary lesions such as at AP-sites, caused by chemical instability of alkylated purines, may be formed. After fertilization, O^6-metG may be rapidly removed from the DNA by methyltransferase(s) in the female oocyte. Taken together, these processes dramatically change the initial adduct spectrum, and, consequently, the mutation spectrum. This large difference in spectra in *Drosophila* was not observed for the ethylating agent ENU, probably because, analogous to *E. coli* alkyltransferases, removal of an ethyl group from the O^6-position of guanine by alkyltransferase is

much less efficient than removal of a methyl group. The generation time of the cells of the organisms used for the generation of mutation spectra varies greatly. For some mutagens, the adduct spectrum, therefore, changes with time due to the action of DNA repair enzymes and the formation of secondary lesions. The adduct spectrum at the time of replication determines the shape of the mutation spectrum, and differences are to be expected between different organisms for a mutation spectrum by the same mutagen.

In addition to inducing point mutations, alkylating agents are well known to be clastogenic (Preston *et al.*, 1981; Moore *et al.*, 1989). In mouse lymphoma cells and also in human lymphoblastoid TK6 cells, mutants can show structural loss of the TK locus (Little *et al.*, 1987; Applegate *et al.*, 1990).

11.3.5 CROSS-LINKING AGENTS

The nature of mutations induced by the anti-tumor drug *cis*-diaminedichloroplatinum(II) (cisplatin) was determined for 28 mutants in the CHO *aprt* gene (De Boer and Glickman, 1989). Base substitutions, frameshifts, short deletions, and duplications were found. Many mutations occur at or close to the sequences 5′-AGG-3′ and 5′-GAG-3′, that are thought to be the sequences where the majority of cisplatin-induced intra-strand cross-links are formed. Apparently, inter-strand cross-links between guanines or adenines in opposing strands do not contribute significantly to the induction of *aprt* mutations in the case of cisplatin, although they are highly cytotoxic. When the distribution of the 5′-AGG-3′ and 5′-GAG-3′ sequences on the two DNA strands is compared with the sites were mutations occurred, most mutations seemed to be caused by cisplatin adducts in the non-transcribed strand. This strand preference may be a reflection of more efficient repair of the transcribed strand or be due to differences in the damage distribution.

Bulky adducts

Metabolic activation of the polycyclic hydrocarbon benzo[*a*]pyrene, a well-studied environmental mutagen and carcinogen, yields the major reactive metabolite (±)-*trans*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE), which predominantly binds to the exocyclic N^2 amino group of guanine. Molecular analysis of 59 BPDE-induced mutations at the *aprt* gene of CHO cells showed that in all cases gene inactivation was the result of a point mutation (Mazur and Glickman, 1988). The predominant mutation was the GC→TA transversion, which comprised 62% of the spectrum, but also other base substitutions (almost exclusively transversions) and frameshifts were recovered. The majority of BPDE-induced mutations were targeted within runs of G:C base pairs, half of them being runs of guanines flanked by adenine residues. The observation that in mammalian cells BPDE predominantly induces point mutations has also been made by other

investigators. Southern blot analysis of *hprt* mutants from V79 Chinese hamster cells (King and Brookes, 1984) and dihydrofolate reductase (*dhfr*) mutants from CHO cells (Carothers *et al.*, 1988) showed that all of the *hprt* mutants and all but one of the *dhfr* mutants, had retained their normal restriction patterns. Sequence analysis of 14 *dhfr* mutants showed that 10 mutants were caused by GC→AT transversions and three mutants by a −1 frameshift (Carothers and Grunberger, 1990).

The nature of mutations induced by the aromatic amine *N*-acetoxy-2-acetylaminofluorene (AAAF), has been investigated at the *dhfr* locus in CHO cells. AAAF reacts predominantly at the C^8-position of deoxyguanosine residues, yielding two types of adducts, 2-[*N*-deoxyguanosin-8-yl)-*N*-acetyl]-aminofluorene (dG-AAF) and 2-[*N*-deoxyguanosin-8-yl)aminofluorene (dG-AF). AAF adducts caused a larger distortion of the DNA conformation than AF adducts. Of 29 investigated AAAF-induced *dhfr* mutations about 30% were large gene alterations (Carothers *et al.*, 1986). Sequence analysis of 20 mutants with point mutations showed that 75% of these were of the GC→TA transversion type (Carothers *et al.*, 1989). About 40% of the mapped mutants displayed greatly reduced steady-state levels of *dhfr* mRNA. In all cases, these mutants contained premature translation stop codons. From the remaining 10 base substitution mutants with normal levels of *dhfr* mRNA, five contained a nonsense codon in the sixth exon. In contrast to stop codons in exon VI, nonsense codons in exons II to V probably caused the *dhfr* mRNA to become unstable. The observation that quite often mRNA transcribed from a mutant gene has a decreased stability, has also been found for the *hprt* gene by other investigators (Vrieling *et al.*, 1988; Thacker and Ganesh, 1989; Liber *et al.*, 1989).

Base analogues

Mutagenesis by the thymidine analogue 5-bromodeoxyuridine (BrdUrd) has been investigated in mouse cells using the *E. coli gpt* gene on an integrated retroviral shuttle vector as target for mutations (Davidson *et al.*, 1988). BrdUrd mutagenesis in mammalian cells has been suggested as being dependent upon the perturbation of endogenous deoxycytidine metabolism; furthermore, mutations could arise from misincorporation of BrdUrd into DNA, driven by unbalanced deoxynucleotide pools available for DNA synthesis. The great majority of BrdUrd-induced mutations were found to be GC→AT transitions, probably caused by misincorporation of BrdUrd opposite guanine in DNA. A high degree of sequence specificity was observed, since 95% of the induced GC→AT transitions occurred in a sequence of two adjacent guanine residues. In about 90% of the cases, the guanine residue involved in mutation was the one in the more 3′ position.

Southern analysis of *hprt* CHO mutants induced by the purine analogs 9-ß-D-arabinosyl-2-fluoroadenine (F-ara-A) and 9-β-arabinofuranosyladenine (ara-A) (Huang *et al.*, 1989), which have antiviral and antitumor activities, showed that the

great majority had lost all or part of the *hprt* gene. Incorporation of *F-ara-A* into DNA is thought to cause termination of replicating segments, resulting in localized deletions of genetic material. Ara-A, however, has been found to be incorporated in DNA suggesting that a mechanism other than chain termination is involved in the induction of gene deletions by this analogue.

Ionizing radiation

Ionizing radiation was the first genotoxic agent identified (Muller, 1927), and is one of the most common carcinogenic agents to which humans are exposed, because of background radiation and exposure during medical treatment. Ionizing radiation generates oxygen radicals, that can react with the genetic material. DNA damage induced by ionizing irradiation consists of DNA strand breaks (both double- and single-stranded), base-free (AP) sites, and modified DNA bases. Analysis of mutations at different loci (*hprt, tk, dhfr, gpt, white*) after irradiation with X-rays, γ-rays, neutrons, accelerated argon ions, and α-particles showed that in the majority of the investigated genes large deletions or other types of chromosomal rearrangements including mitotic recombination or gene conversion were the predominant type of mutation (Vrieling *et al.*, 1985; Thacker, 1986; Stankowski and Hsie, 1986; Urlaub *et al.*, 1986; Fuscoe *et al.*, 1986; Little *et al.*, 1987; Gibbs *et al.*, 1987; Pastink *et al.*, 1987; Batzer *et al.*, 1988; Kronenberg and Little, 1989; Applegate *et al.*, 1990). However, 80% of the mutations induced by γ-rays at the *aprt* gene of CHO cells, where the *aprt* gene is in a hemizygous state (Breimer *et al.*, 1986; Miles and Meuth, 1989), were point mutations, including base changes, frameshifts, multiple changes, and small deletions. Compared to the spontaneous spectrum determined by the same group, the target sequences for both frameshifts and small deletions appeared to be different, and γ-rays induced more multiple base changes. A similar spectrum of *aprt* mutations induced by γ-rays in the same CHO cell line was found. That the fraction of large deletions among the *aprt* mutations induced by ionizing radiation in this cell line is much lower than found in other chromosomal loci may be due to the specific cell line used in these studies, which carry only one copy of the *aprt* gene (and possible adjacent genes). If an essential gene is located adjacent to the *aprt* gene, large deletions are lethal under hemizygous conditions. Analysis of deletion mutants from *hprt* deficient T lymphocytes has shown that deletions can extend for as far as 5 cM into the flanking region of the gene. In addition, genes in hemizygous conditions may be unable to undergo homologous mitotic recombination or gene conversion.

Examination of termini of ionizing radiation-induced small deletions in the *white* gene of *Drosophila* (Pastink *et al.*, 1988) and the *aprt* gene in Chinese hamster cells (Miles and Meuth, 1989) showed that the majority of those occurred between short direct repeats, one copy of which is retained in the mutant gene. The formation of these small deletions can be explained by recombination or by "slipped mispair" during DNA replication (Albertini *et al.*, 1982b).

The variability in the proportion of deletion mutants by a certain mutagenic agent at different loci may be much more a reflection of the differences in the genetic organization of these genes, than a result from the mutagenic character of the agent.

UV light

Short wavelength (i.e., 254 nm) UV irradiation results in the formation of various adducts in DNA. The major UV-induced lesion is the cyclobutane pyrimidine dimer that is formed between two adjacent pyrimidines. Dimers have been demonstrated to play a significant role in cell killing and mutagenesis. Pyrimidine dimers in genomic DNA of mammalian cells have been shown to occur randomly (Bohr *et al.*, 1985) indicating no significant bias between large domains of chromatin. Recently, however, dimer distribution in core DNA of nucleosomes was reported to not be uniform (Gale *et al.*, 1987), but that a 10.3-base periodicity exists that may be related to the inherent structure of the nucleosome, and reflects histone–DNA interactions. The order of preference for dimer formation at possible dipyrimidine sites was shown to be TT > TC = CT > CC (Setlow and Carrier, 1966).

Another UV-induced lesion that may play an important role in the mutagenic effects of UV light is the pyrimidine-pyrimidone (6-4) photoproduct (Lippke *et al.*, 1981; Brash and Haseltine, 1982). Like pyrimidine dimers, this lesion is also formed at dipyrimidine sites. The alkali lability of at least some (6-4) photoproducts was used to determine that these photoproducts are almost exclusively formed at TC and CC sequences (Brash *et al.*, 1987; Bourre *et al.*, 1987). Some discrepancy exists about the relative frequency of cyclobutane pyrimidine dimers and (6-4) photoproducts. Recent measurements using an antibody-assay specific for (6-4) photoproducts showed that the ratio of dimers versus (6-4) photoproducts is about 3:1, which is now generally accepted (Mitchell 1988).

Analysis of UV-induced mutations at the CHO *aprt* locus (Drobetsky *et al.*, 1987) showed that mutations consisted of single, tandem double, and non-tandem double-base substitutions. Ninety-four percent of the UV-induced mutations appeared to be targeted to dipyrimidine sites. Like in *E. coli*, about 70% of the base substitutions were GC→AT transitions (Schaaper *et al.*, 1987).

UV-induced single and tandem base changes at a chromosomally integrated *gpt* gene in CHO cells were also predominantly GC→AT transitions, half of the mutations being at four "hot spots" (Romac *et al.*, 1989). Nonsense mutations and mutations eliminating the ATG initiation codon were about 50% of the mutations, suggesting that most amino acid changes in the *gpt* protein do not decrease phosphoribosyltransferase activity enough to let the cell survive under the selective conditions used.

The molecular nature of mutations induced by UV light has been investigated in *hprt* mutants from V79 Chinese hamster cells isolated after exposure to 12 J/m^2

(Vrieling *et al.*, 1989) or 2 J/m^2 and from CHO cells (strain AA8) exposed to 2 J/m^2. Among the mutants analyzed, all possible base-pair changes were present, 60% being transversions. Of the mutations, 95% occurred at dipyrimidine sites, and, therefore, are probably caused by UV-induced photoproducts at these sites. At 2 J/m^2, both in V79 and in CHO cells, more than 86% of the mutations were caused by photoproducts in the non-transcribed strand of the *hprt* gene. This effect was 65% at 12 J/m^2. This strand bias for mutation induction towards the non-transcribed strand was probably caused by preferential repair of photoproducts from the transcribed strand of the *hprt* gene. The less pronounced strand bias at 12 J/m^2 might be caused by saturation of preferential DNA repair processes at high doses.

UV-induced mutations were also analyzed in the repair-deficient cell lines V-H1 and UV-5, which are UV sensitive derivatives of V79 and CHO, respectively. Both belong to complementation group 2 of UV-sensitive Chinese hamster cell lines. Removal of pyrimidine dimers from an 18 kb EcoRI fragment of the *hprt* gene was completely deficient in V-H1 cells. UV-induced mutation induction in V-H1 and in UV-5 was about seven times higher per unit dose than in normal V79 and CHO cells (Zdzienicka *et al.*, 1988). Most UV-induced (2 J/m^2) single base-pair changes and tandem double mutations in V-H1 and UV-5 were GC to AT transitions. Furthermore, in contrast with normal V79 cells and CHO cells, 95% of the base pair changes were caused by photoproducts in the transcribed strand of the *hprt* gene (Table 11.3). In the absence of DNA repair, mutational events were expected to be equally distributed over both DNA strands. Although more dipyrimidine sequences are present in the exon sequences of the transcribed strand, this difference alone is insufficient to explain the strand bias in V-H1 and UV-5 (Vrieling *et al.*, 1989). The same observation has been made in repair-deficient human fibroblasts (XP-A).

Table 11.3 Distribution of mutagenic photoproducts over the DNA strands

Cell→	V79	V79	CHO	V-H1	UV-5
	12 J/m^2	2 J/m^2	2 J/m^2	2 J/m^2	2 J/m^2
DNA strand↓					
Transcribed	6	3	2	10	21
Non-transcribed	11	17	17	1	1

To explain this unexpected finding, some other mechanism apart from DNA repair may be acting with different error efficiencies for the two DNA strands decreasing mutation induction in the non-transcribed strand. For instance, this mechanism could be the fidelity of DNA replication. The fidelity of the DNA polymerases involved in replication may be different when synthesizing the leading or the lagging strand. The strong processivity of polymerase δ might force it to incorporate a nucleotide opposite photoproducts in the template of the leading DNA

strand (translesion synthesis). However, when synthesizing the lagging DNA strand polymerase α might be able to start from a new initiation site downstream from a photoproduct, thereby generating a gap that can be filled by recombination at a later time. This last process is likely to cause less errors than translesion synthesis. The consequence of this model is that, in the case of the *hprt* gene, the transcribed strand corresponds to the template of the leading strand.

UV-induced mutation spectra were also determined in yeast, but only under repair proficient conditions. Like in the *hprt* gene, no predominance of GC→AT transitions was found at the URA3 locus (Lee *et al.*, 1988). Half of the base substitutions were transversions, whereas the majority of the transitions were AT→GC base changes.

11.4 CONCLUSIONS

The rate and efficiency of the different DNA repair processes on the various potentially mutagenic lesions in the different target genes are of vital importance for the ultimate shape of the mutation spectra. The phenomena of non-random introduction and removal of DNA lesions is very small, given the observation that, for some genes, the target sequence for mutations which will give rise to a mutant phenotype. Furthermore the fact that for some target genes multi-locus deletions may be lethal makes it necessary to determine the spectra of genetic events in mammalian cells with several different target genes in order to determine the generality of a mutation spectrum observed in a specific gene.

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12 Molecular Analysis of Mutations in Shuttle Vectors and Transgenic Animals

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12.1 INTRODUCTION

Research focusing on the pathways by which chemicals induce mutagenic changes and neoplastic transformation has been an area of intense study for the past 40 years (Miller and Miller, 1978). Overwhelming evidence indicates that mutations can result from replication errors induced by the products formed from the reaction between nucleophilic centers in DNA and the ultimately genotoxic species (Miller, 1981). The mechanisms governing the mutagenic processing of DNA adducts within prokaryotic cells are relatively well understood (Miller, 1983). For example, genetic analysis using the *lacI* gene of *Escherichia coli* (Miller, 1983) or similar systems (LeClerc *et al.*, 1984; Wood *et al.*, 1984; Koffel-Schwartz *et al.*, 1984) has revealed that individual chemicals or types of radiation produce distinctive mutational spectra. Examination of the mutational fingerprint left behind after the processing of a damaged gene has helped workers identify the putative DNA lesions responsible for the genetic changes observed in bacterial systems. Furthermore, the evaluation of mutational spectra generated in *E. coli* strains that are altered in DNA replication or repair functions has defined the precise genetic requirements for mutagenesis induced by these agents.

The premise is tempting to accept the validity of extrapolation of data on the mutational spectra of DNA damaging agents in bacteria to more complex systems, such as humans, that are less amenable to facile genetic analysis. However, risks exist in accepting this line of reasoning too readily, because comparatively little is known about how mammalian cells cope with DNA damage. Hence, a number of workers have focused their efforts on the development of systems to establish

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mutational spectra in mammalian systems. Three approaches have been taken toward this goal. The first is the use of endogenous genes of mammalian cells in culture as targets for genotoxicants. Two approaches to be reviewed are (a) the use of genes carried on shuttle vectors as the targets for mutation, and (b) the use of transgenes in intact animals to study mutagenesis *in vivo*. This paper on shuttle vectors and transgenic animals will examine critically how well these systems for mutation analysis provide relevant genetic data. For more detailed reviews specifically on shuttle vectors, the reader is referred to DuBridge and Calos (1988) and Sarasin (1989a, b).

12.2 OPERATIONAL FEATURES OF SHUTTLE VECTOR AND TRANSGENIC ANIMAL ASSAYS FOR MUTAGENESIS

Shuttle vectors share two basic design features. First, the vector must be capable of being replicated within both mammalian and bacterial, usually *E. coli*, hosts. Second, provisions must be made for mutant screening or selection, usually after transfer of the vector or a portion of it into *E. coli*.

12.2.1. TRANSIENTLY REPLICATING, EPISOMAL SHUTTLE VECTORS

Recombinant shuttle vectors that transiently replicate within mammalian cells were the first type to be established. These vectors contain the genes necessary for replication of SV40 viral sequences in permissive cells (e.g., the SV40 origin of replication and the gene encoding the SV40 large T antigen) and achieve a very high copy number (10^4 to 10^5/cell) within the host. Typically, the vector is replicated within the nucleus, and assumes a normal nucleosomal structure. DNA damage is introduced into the target gene of the vector either *in vitro* before the vector is transferred into the host or *in vivo* by treatment of cells stably carrying the vector as an episome. The vectors replicate in mammalian hosts for a short time, usually 2 to 3 days, before the host dies; consequently, the duration of experiments using this system is limited to this relatively short time period.

The early experiments of several laboratories indicated that the passage of shuttle vectors through mammalian cells led to an unacceptably high level of spontaneous mutation (~1%) (Calos *et al.*, 1983; Razzaque *et al.*, 1983; Lebkowski *et al.*, 1984; Ashman and Davidson, 1984). Most spontaneous mutations were deletions. Although the exact reason for the spontaneous deletions was never established unequivocally (Razzaque *et al.*, 1984; Lebkowski *et al.*, 1984), second generation systems were developed in which the background was reduced by one to two orders of magnitude. One strategy that proved effective in attaining a manageably low background involved the insertion into the shuttle vector of essential DNA sequences at positions flanking the marker gene in which mutations were evaluated (Seidman *et al.*, 1985). A loss of the mutation target by deletion would lead to the

loss of an essential gene for plasmid viability. A second approach was the use of human 293 cells (Lebkowski *et al.*, 1985), a cell line that, for reasons still not understood, is less destructive to shuttle vectors passed through them. Other human cell lines also appear to generate a relatively low spontaneous mutation frequency.

12.2.2 STABLY REPLICATING EPISOMAL VECTORS

A disadvantage of the SV40-based vectors described above is the aforementioned high spontaneous mutation frequency and the temporal limitation of 2 to 3 days before the transfected host cell dies. The development of stable episomal vectors largely circumvented both of these problems. Several research laboratories established vectors based either on the Epstein–Barr virus (EBV) (Drinkwater and Klinedinst, 1986; DuBridge *et al.*, 1987) or the bovine papilloma virus (BPV; Ashman and Davidson, 1985; MacGregor and Burke, 1987). These shuttle vectors are maintained at a low copy number (10 to 100/cell), repeatedly cycle with the host under host control, and do not kill the host. The latter feature makes it possible to treat cells harbouring the vector with a mutagen; alternatively, the approach of modifying the vector *in vitro* and then transferring it into the host for fixation of mutation is also feasible (Ingle and Drinkwater, 1989). Although in one early study a BPV-based vector suffered a high spontaneous mutation frequency (~1%) (Ashman and Davidson, 1985), much more reasonable values were obtained with the EBV systems (Drinkwater and Klinedinst, 1986; DuBridge *et al.*, 1987), that displayed spontaneous mutation frequencies of 10^{-5} to 10^{-6}. Despite this impressive characteristic, disadvantages to most of the systems have developed to date. The first stems from the low copy number of the stably maintained vector in the mammalian cell, which limits the sensitivity of the assay and necessitates the use of a large number of cells in each experiment; consequently, experiments with EBV vectors are very labour intensive. The second limitation is the high toxicity to host cells treated with many DNA damaging agents. By reducing the yield of mutants, the overall sensitivity of the assay is diminished. Treating the shuttle vector with the mutagen prior to transfection circumvents this constraint. Recent studies have aimed at combining the high copy number of the SV40 vectors with the genetic stability of the EBV and BPV systems (DuBridge *et al.*, 1987; Heinzel *et al.*, 1988). The results of these investigations have been promising, but have not yielded an optimal system.

12.2.3 CHROMOSOMALLY INTEGRATED SHUTTLE VECTORS

A third class of shuttle vector has the target gene integrated within the host chromosome for mutation fixation, and then the genetic target is recovered for mutation enumeration and qualitative analysis. A type of integrated shuttle vector contains the SV40 origin of replication (Breitman *et al.*, 1982; Ashman and

Davidson, 1984; Ellison *et al.*, 1989a). Integration occurs after transfection into cells that are not permissive for replication of SV40 (e.g., Chinese hamster cells). The presence of the integrated DNA within the cell is usually selected for by antibiotic resistance, typically against G418 analogs. In the specialized case of vectors containing the *E. coli gpt* gene, this gene serves both as the transfection marker as well as the target gene for mutagenesis (Ashman and Davidson, 1984). Early results in this area indicated that recovery of the integrated vector sequences from the host genome was accompanied by frequent artefactual mutations. A more optimistic future for this general approach is possible, however, with the advent of strategies to detect mutations in the mammalian cell (Ashman *et al.*, 1986; Drobetsky *et al.*, 1989b) or to efficiently recover the target sequences by the polymerase chain reaction (Ellison *et al.*, 1989b).

An alternative to the SV40-based vector system utilizes the *in vitro* packaging of bacteriophage λ to excise the integrated mutational target DNA from the high molecular weight DNA of mammalian cells (Glazer *et al.*, 1986). Mutations within the target gene are scored after infection of *E. coli* with the recovered phage. Recently, this λ packaging system has been adopted for the analysis of mutations induced in transgenic animals (Gossen *et al.*, 1989). The vector, microinjected into the fertilized egg of a mouse, integrates into the host genome, and becomes part of the genetic complement of the resulting transgenic animal. Gossen *et al.* (1989) initially demonstrated the feasibility of this approach, and established that the spontaneous mutation frequency of the recovered *lacZ* gene is attractively low ($<10^{-5}$). Although more technically demanding than the cell culture systems described above, this system offers exciting possibilities to examine the organotropic effects of mutagens and carcinogens in intact animal models.

12.3 MUTATIONAL SPECIFICITY OF ULTRAVIOLET LIGHT

Ultraviolet light (UV) is one of the most common DNA-damaging agents in the human environment. Its DNA-damage spectrum includes predominantly cyclobutane pyrimidine-pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts.

The mutation spectrum of UV light has been determined by using the shuttle vector systems detailed above. After discovering methods to control the high spontaneous background of mutations in a transiently replicating SV40-based episomal vector, Calos and Miller (Lebkowski *et al.*, 1985) and Seidman and coworkers (Seidman *et al.*, 1985; Hauser *et al.*, 1986) successfully achieved dose-dependent increases in induced mutant fractions over background in UV-irradiated plasmids replicated in mammalian cells. The system developed by Calos and Miller uses the 1 kb *lacI* gene of *E. coli*, which encodes the repressor of the β-galactosidase gene, as the mutation target in an SV40-derived vector harboured episomally within human 293 cells. The forward assay scores for clones that have inactive *Lac* repressor, owing to the *lacI*$^+$ to *lacI*$^-$ mutation. These

colonies produce β-galactosidase, and thus are blue in the presence of the β-galactosidase indicator dye, X-gal; the parental *lacI*$^+$ colonies are colourless. A fourfold increase in mutant fraction was observed over a range of UV fluences from 0 to 70 J/m^2. At the highest dose of UV used, the induced mutation frequency was 1.5×10^{-3} over a background of 3.5×10^{-4}. The Seidman laboratory took a complementary approach that involved UV-irradiating a vector prior to its introduction into the mammalian host cell. Their vector, pZ189, had the 0.15 kb *supF* tRNA gene as the mutation target; it was replicated within monkey kidney CV1 cells. Mutant progeny derived from irradiated plasmids, after transfer into *E. coli*, failed to suppress an amber mutation in the *lacZ* gene of the host, and hence displayed colorless or light-blue colonies on β-galactosidase indicator plates. The induced mutation frequency, 6×10^{-3}, at a UV dose of 500 J/m^2 to the plasmid, was a 20-fold increase above the background for this system. This assay achieved an acceptably low background mutation frequency by having essential genetic elements flanking the mutation target in the shuttle vector, whereas that of the Calos laboratory achieved the same goal by using 293 cells as the host.

A different strategy used to probe UV mutagenesis was used originally by Summers and colleagues and involved irradiating mouse LTK$^-$ cells that had the *supF* mutation target integrated within the host genome (Glazer *et al.*, 1986). Mouse cells carrying multiple copies of the vector were irradiated, and the integrated vectors were recovered by taking advantage of bacteriophage λ sequences that provided the ability for *in vitro* DNA packaging. Analysis of mutations was by plaque color, and was similar to that described above for the *supF* sequence on the pZ189 plasmid. The induced mutation frequency by UV at a single dose of 12 J/m^2 was 1×10^{-4}, representing a fivefold increase over the background obtained with unirradiated cells. Recently, a similar approach was reported in which the target for UV mutagenesis was the *aprt* gene carried on a vector integrated in the genome of a Chinese hamster ovary cell line (Drobetsky *et al.*, 1989b). The results of this study correlated with the mutations observed after similar treatment of an endogenous *aprt* locus within Chinese hamster ovary cells (Drobetsky *et al.*, 1989a).

The types of detectable UV-induced mutations are similar in each of the aforementioned systems, and the data are indeed strikingly consistent with the mutational specificity of UV light in bacteria (Todd and Glickman, 1982). Moreover, the spectrum of base substitution mutations in the *lacI* gene replicated in human cells is very similar to that using the same target sequence in *E. coli*, although frameshifts, that are significant features of the bacterial spectrum (~30% of all mutations), occur infrequently in the mammalian system (Hsia *et al.*, 1989). The G:C→A:T transition constitutes 80 to 90% of all induced mutations in mammalian cells. This situation occurs when the *supF* or *lacI* target genes are on a plasmid irradiated *in vitro* (Hauser *et al.*, 1986; Hsia *et al.*, 1989), when cells harboring the *supF* gene integrated in the host genome are irradiated *in vivo* (Glazer *et al.*, 1986), and when the *lacI* target is maintained episomally within cells and is irradiated *in vivo* (Lebkowski *et al.*, 1985). The spontaneous spectrum in these

systems consisted of large deletion mutations, which constitute up to half of all mutations, with the balance being either small deletions or point mutations (Hauser *et al.*, 1986; Bredberg *et al.*, 1986). The local changes are primarily G:C→A:T and G:C→T:A at roughly equal frequency. This latter spontaneous mutation is also a minor feature of the UV-induced spectrum, although to rule out aspects of experimental design, such as artefactual abasic site formation that might have contributed to these changes, is difficult (Lebkowski *et al.*, 1985).

The G:C→A:T transition that dominates the UV mutation spectrum occurs mainly at the 3′-nucleotide of a 5′-pyrimidine-cytosine-3′-dimer (Brash *et al.*, 1987). Speculatively, the observed mutation may arise from the insertion of adenine opposite the modified cytosine residue of the dimer. The exact chemical nature of the pyrimidine-cytosine (6-4) photoproduct that causes this mutation is unknown at present, but available data suggest that both the pyrimidine-pyrimidone (6-4) and cytosine-containing cyclobutane dimers are premutagenic lesions (Brash *et al.*, 1987). However, photoreactivation of a UV-irradiated plasmid prior to transfection reduced the induced mutation frequency substantially (Protic-Sabljic *et al.*, 1986). Cytosine-containing cyclobutane dimers thus appear to constitute the major proportion of the mutagenic lesions; and other lesions, presumably (6-4) photoproducts, are responsible for a significant fraction of the remaining mutations. Hot spots of mutagenesis appear in UV-irradiated plasmids, but these do not correlate with significantly elevated levels of photoproduct formation in the same DNA sequences (Hauser *et al.*, 1986; Brash *et al.*, 1987). In excision-repair-defective human xeroderma pigmentosum complementation group A cells, enhanced mutagenesis was observed compared to normal human cells (Bredberg *et al.*, 1986). The mutation spectrum of UV-irradiated DNA in the repair-deficient cells was similar to that in normal cells, with the exception that there were relatively more G:C→A:T transitions and fewer G:C→T:A transversions. In ataxia telangiectasia and Cokayne's syndrome cells, the mutation spectrum of UV-irradiated DNA was similar to that in normal cells.

12.4 MUTATIONAL SPECIFICITY OF DNA ALKYLATING AGENTS

Human exposure to DNA alkylating agents occurs from several environmental and endogenous sources (Bartsch *et al.*, 1987). Overwhelming evidence has shown that the principal mutation induced by alkylation of DNA in bacterial systems is the G:C→A:T transition (Coulondre and Miller, 1977), that likely occurs due to the misreplication of O^6-alkylG residues in DNA (Basu and Essigmann, 1988). Also this mutation is the chief genetic change observed in oncogene sequences activated by alkylating agents in animals (Zarbl *et al.*, 1985). The second most frequent mutation in bacterial systems is the A:T→G:C transition, that is thought to be caused by alkylation of the O^4-atom of thymine residues in DNA (Singer, 1986). Several laboratories have investigated the mutational specificity of alkylating agents in either shuttle vector or transgenic animal models.

12.4.1 CONVENTIONAL SHUTTLE VECTOR SYSTEMS

Calos and coworkers treated human 293 cells harbouring the *lacI* SV40-based shuttle vector with ethyl methanesulphonate (EMS) at doses ranging from 0 to 1 mg/ml (Lebkowski *et al.*, 1986). At the highest dose an induced mutation frequency of 4×10^{-3} was obtained, which was 10-fold above background. Ninety-eight % of the induced point mutants that gave rise to a nonsense codon had the G:C→A:T transition; the spontaneous spectrum was essentially as described above for UV. Similar findings were made by Ashman *et al.* (1986) and Ashman and Davidson (1987) using an integrated retroviral vector in mouse A9 cells. The target for mutation in the integrated vector was the bacterial *gpt* gene, and the vector was recovered from the murine host cell by COS cell fusion. Also using EMS, Drinkwater and coworkers (Ingle and Drinkwater, 1989) observed the same mutation using an EBV-based vector treated *in vitro* and then transferred into human lymphoblastoid cells for mutation fixation.

Studies on two alkylnitrosoureas, MNU and ENU, have been carried out with EBV-based shuttle vectors maintained episomally in either human 293 cells (DuBridge *et al.*, 1987) or human lymphoblastoid cells (Eckert *et al.*, 1988). Analysis of the MNU-induced mutants in the *lacI* gene showed again the predominance of the G:C→A:T transition. ENU also induced the G:C→A:T change as the major mutational event (~50%), although many mutations were also at A:T base pairs. Specifically, the A:T→T:A mutation represented 20% of all mutations and the A:T→G:C and A:T→C:G changes accounted for 17% and 9%, respectively. The authors reasoned on the basis of its known mutational specificity (Singer, 1986) that O^4metT could be responsible for the A:T→G:C changes. They also speculated that the remaining mutations may be due to alkylation at the O^2 of thymine, a reaction that occurs more frequently with ENU than with EMS or MNU.

12.4.2 TRANSGENIC ANIMAL MODELS

The mutation spectrum of ENU has recently been investigated in transgenic animals (Gossen *et al.*, 1989). The system utilizes the *E. coli lacZ* gene integrated in each cell of the intact organism as the target for mutation. After 7 days of treatment with various doses of ENU, DNA from brain and liver were isolated. These tissues were studied, because in the newborn animal they are target and non-target organs for carcinogenesis, respectively. Brain is susceptible to carcinogenesis owing to its relative lack of the methyltransferase enzyme that repairs O^6MetG. The *lacZ* sequences were recovered from the host DNA by *in vitro* λ packaging, and transfected into *E. coli* for mutant enumeration and characterization. Mutants were detected at comparable frequencies from both liver and brain DNA (~3 to 6×10^{-5}). Four mutants were sequenced from brain DNA, three of which showed the expected G:C→A:T transition, with the remaining mutant showing a G:C→T:A transversion.

No mutant retrieved from the liver DNA was sequenced; hence, no conclusions on the utility of the technique for analysis of organotropic effects of DNA damaging agents can be reached at this time. Nevertheless, the great potential of this approach for the evaluation of the mutational specificity of DNA damaging agents has been established, although a final evaluation of the general utility of this technique must await the results of more detailed studies.

The transgenic model circumvents the key deficiency of the single cell models described elsewhere in this review. Specifically, none of the DNA damaging agents evaluated in single cell models has required enzymatic activation by, for example, cytochrome P_{450} or flavin containing monooxygenases. Such activation systems (or enzymatic detoxification systems) could, in principle, be provided either in the single cell systems exogenously or endogenously by way of the molecularly cloned genes. This area has received little attention in the literature.

12.4.3 SITE-SPECIFIC MUTAGENESIS BY INDIVIDUAL ALKYL-DNA ADDUCTS

The mutational spectrum of a DNA damaging agent often suggests the chemical identity of a DNA adduct that might have caused a specific feature of the spectrum. Hypotheses thus generated can be tested by synthesizing an oligonucleotide containing that adduct, inserting it by recombinant DNA techniques into a shuttle vector, and then replicating the singly adducted genome *in vivo*. This general approach has been useful in bacterial systems (Basu and Essigmann, 1988); recently, it has been extended to evaluate the possible mutagenic effects of O^6-substituted guanines in mammalian cells. Ellison *et al.* (1989a; 1989b) constructed an SV40-based shuttle vector containing either O^6MetG or O^6EtG within a restriction endonuclease recognition site. The vector was transfected in parallel into two isogenic Chinese hamster ovary cell lines, where one member of the pair was deficient and the other was proficient in mammalian O^6-alkylG DNA–alkyltransferase activity. The vector integrated into the genome of the host, which was non-permissive for replication of SV40 viral sequences. Following fixation of the mutation, the area of the genome in the vicinity of the originally adducted site was amplified by the polymerase chain reaction and the mutation frequency was evaluated by determining the fraction of DNA that was refractory to cleavage by the restriction enzyme. In the repair-proficient cells, the mutation frequency of both adducts was low and usually indistinguishable from the background of the assay (~2%). In repair-deficient cells, by contrast, the mutation frequency of O^6MetG was approximately 20%, and that of O^6EtG was half this value. The true mutation frequencies of the lesions are twice these values, because only one strand of the shuttle vector was modified, and the unadducted strand should have engendered only wild type progeny. The mutations of both adducts were almost exclusively G→A transitions, which is in accord with the predominant mutation induced by alkylating agents in the shuttle vector systems described above.

The same general approach has been taken by Mitra *et al.* (1989), who prepared a retrovirus-based vector containing O^6MetG or O^6-benzylguanine (O^6BzG) in either the first or second base of the twelfth codon of the *Ha-ras* gene. Rat cells were the host for replication; the vector was integrated into the host genome; mutants were screened among progeny that contained activated *Ha-ras* sequences using hybridization probes. Both DNA adducts are only weakly mutagenic in this system, possibly because of the repair-proficient phototype of the host. The mutation frequencies of O^6MetG and O^6BzG were ~1% and 0.5%, respectively. The adducts induced the same amount and types of mutation regardless of their presence at the first or second base of the codon. Not all mutations could be scored in this assay, but both adducts induce G→A transitions; in addition, O^6BzG causes G→C and G→T transversions. The general conclusions of this study are in accord with those of Ellison *et al.* (1989b).

12.5 MUTATIONAL SPECIFICITY OF AROMATIC AMINES, AMIDES AND NITRO COMPOUNDS

Genotoxic aromatic amines occur widely in nature and are commonly used in industry. Generally these compounds require metabolic activation for mutagenicity. Although abundant data are available on the mutational specificity of these compounds in *E. coli* (Koffel-Schwartz *et al.*, 1984; Bichara and Fuchs, 1985; Gupta *et al.*, 1988), and in endogenous mammalian genes *in vivo* (Carothers *et al.*, 1989), a limited number of studies has been done to address the same issue by using shuttle vectors replicated in mammalian systems.

12.5.1 2-AMINOFLUORENE DERIVATIVES

Maher and her colleagues have used the *supF* system developed by Seidman *et al.* (1985) to investigate the mutagenic effects of chemically reactive forms of *N*-acetyl-2-aminofluorene (AAF) or 2-aminofluorene (AF) (Maher *et al.*, 1989; Mah *et al.*, 1989). The vector was modified with either *N*-acetoxy-2-acetylaminofluorene (AAAF), that forms *N*-acetylated adducts at the C^8 of guanine (i.e., AAF adducts), or *N*-acetoxy-*N*-trifluoroacetyl-2-aminofluorene, which forms the same DNA adduct without the *N*-acetyl group (i.e., AF adducts). At equal levels of DNA modification (~20 adducts per plasmid), the mutation frequencies of both plasmids are approximately the same ($\sim 1 \times 10^{-3}$), which is a 10-fold increase above background. With the AF-modified plasmid, in excess of 90% of the mutants are point mutations and, of these, 98% are base substitutions (Mah *et al.*, 1989). Two-thirds of these are G:C→T:A transversions, which outcome is largely in accord with the bacterial mutational spectrum of AF (Bichara and Fuchs, 1985). The remaining mutations are also targeted at G:C pairs, and are equally divided between the two remaining possible base substitutions.

AAAF has been shown to cause mainly frameshift mutations in bacteria (Koffel-Schwartz *et al.*, 1984). The mutational specificity of this compound in a globally modified vector has been investigated in mammalian cells (Gentil *et al.*, 1986). The results show a predominance of mutations at A:T base pairs adjacent to putative AAF-guanine adduct formation sites. These data are seemingly inconsistent with the spectrum detected in bacteria (Koffel-Schwartz *et al.*, 1984), but the system used by Gentil *et al.* (1986) would be blind to the detection of most frameshifts. Site-specific mutagenic analysis of the C^8-guanine adduct of AAF has been reported in COS cells, where the adduct was found to induce both G:C→C:G and G:C→T:A transversions at equal frequencies (Moriya *et al.*, 1988). These data are inconsistent with the bacterial mutation spectrum of AAAF, but they are in accord with data from endogenous mammalian assays where a similar mutational specificity has been observed after treatment of these cells with the same compound (Carothers *et al.*, 1989). Furthermore, the genetic change detected in the activated c-*Ha-ras* protooncogene of mice treated with *N*-hydroxy-AAF is the G:C→T:A transversion (Wiseman *et al.*, 1986). A possibility suggested for the disparity between the bacterial and mammalian mutational specificity of AAAF is that mammalian cells may possess an adduct deacetylase that converts the AAF adduct to the corresponding AF adduct *in vivo* (Moriya *et al.*, 1988).

12.5.2 NITROPYRENE DERIVATIVES

Nitropyrenes are produced during the combustion of diesel fuels. Several members of this group, notably 1-nitropyrene and 1-nitrosopyrene (1-NOP), have been shown to be potent mutagens (Heflich *et al.*, 1985; Stanton *et al.*, 1988). Maher and coworkers have studied the mutagenic specificity of 1-NOP in a mammalian shuttle vector system (Yang *et al.*, 1988). In these studies, pZ189 was treated *in vitro* with 1-NOP in the presence of ascorbic acid, a mixture that putatively yields the predominant 1-aminopyrene adduct at the C^8 of guanine, which is also the major DNA adduct of 1-NOP (Heflich *et al.*, 1985). Transfection of modified plasmids into human 293 cells produced a dose-dependent increase in mutation frequency. At the highest level of modification (63 adducts per plasmid), the mutation frequency was 4×10^{-3}, which exceeds by 25-fold the background observed in this system. An analysis of the sequence alterations induced by 1-NOP showed that 85% are single base substitutions and that 10% are deletions. Sixty % of the base substitutions are G:C→T:A transversions. By using DNA sequencing techniques to map the positions of adducts in the modified shuttle vector, no correspondence was demonstrated between the positions of DNA damage and the sites of mutations. These genetic results are markedly different from those observed in bacteria where, using the λ *cI* gene as the target, 80% of the mutations involve the addition or deletion of a single G:C base pair (Stanton *et al.*, 1988).

12.5.3 *N*-METHYL-4-AMINOAZOBENZENE DERIVATIVES

The mutagenic activity of a chemically reactive form of *N*-methyl-4-aminoazobenzene (MAB), an aminoazo dye and hepatocarcinogen, has been investigated in a shuttle vector replicated in mammalian cells (Ingle and Drinkwater, 1989). MAB reacts with DNA to form adducts primarily at either the C^8 (60%), the N^2 (20%), or the N^7 (20%) atoms of guanine (Beland *et al.*, 1980; Tarpley *et al.*, 1982). An EBV-based shuttle vector was modified *in vitro* and transfected into a human lymphoblastoid cell line, where mutations were fixed. DNA adducts were not quantified but the mutation frequency at the highest dose (0.4 mM) evaluated was 1.5×10^{-2}, that was a 40-fold increase above background. MAB was found to be a versatile mutagen, inducing mutations at both G:C and A:T base pairs. Forty percent of all mutations are large deletions, while 60% are point mutations. The most abundant mutation in this latter class is G:C→A:T, comprising 33% of the base substitutions. The second-most frequently detected mutation is the (-1) frameshift at G:C pairs (22% of all point mutations). In *Salmonella typhimurium*, MAB derivatives caused a pattern of mutation that is quite consistent with that observed in the mammalian shuttle vector system (Mori *et al.*, 1980). The genetic change detected in activated oncogenes of MAB-induced tumors is the G:C→T:A transversion (Ingle and Drinkwater, 1989). This mutation represents only 10% of all mutations in the mammalian spectrum.

12.6 MUTATIONAL SPECIFICITY OF POLYCYCLIC AROMATIC HYDROCARBONS

12.6.1 BENZO[*a*]PYRENE (BP)

BP is an environmental contaminant that arises primarily from the combustion of organic matter. Much evidence suggests that BP reacts mainly with DNA by way of a diol-epoxide (BPDE) intermediate and forms DNA adducts predominantly at the N^2 atom of guanine (Conney, 1982). The mutagenic specificity of BPDE has been investigated in the *supF* gene in SV40-based shuttle vectors.

Yang *et al.* (1987b) transfected the shuttle vector pZ189 that had been modified with up to 15 BP residues per plasmid into human 293 cells. The results showed a dose-dependent increase in mutation frequency; at the highest dose the mutation frequency was 4×10^{-3}, which was a 25-fold enhancement over background. Almost all BPDE-induced mutants are the result of local genetic changes, and, of these, 80% are single or double base substitution mutations. Eighty-two % of the base substitutions are transversions targeted at G:C pairs; G:C→T:A mutations were three- to fourfold more frequent than G:C→C:G. Hot spots for mutation occur in runs of guanine residues. Two other studies were conducted using the same target gene replicated in monkey cells (Yang *et al.*, 1987a; Roilides *et al.*, 1988). These studies showed essentially the same results as those of Yang *et al.* (1987b). Taken

together, all of these data on BPDE are in accord with the mutational spectrum observed in the *lacI* gene in bacteria (Eisentstadt *et al.*, 1982). These results are also consistent with the type of mutations observed in the endogenous *aprt* gene of Chinese hamster ovary cells (Mazur and Glickman, 1988; Carothers and Grunberger, 1990).

12.6.2 BENZO(*c*)PHENANTHRENE

One of the configurational isomers of the diol epoxide of this hydrocarbon (BPhDE) is the most carcinogenic polycyclic hydrocarbon derivative yet synthesized (Levin *et al.*, 1986). Its reaction products with DNA have been characterized as adducts at the exocyclic amino groups of guanine and adenine (Agarwal *et al.*, 1987). Bigger *et al.* (1989) modified a derivative of pZ189, containing the *supF* gene as the mutation target, to adduction levels from 0 to 25 adducts per plasmid. Replication in 293 cells caused a dose-dependent increase in mutation frequency corresponding at the highest dose to a 60-fold increase over background. As with BP, most of the induced mutations were base substitutions, predominantly transversions at both A:T and G:C pairs. The A:T→T:A change accounted for 35% of all mutations, followed by G:C→T:A and G:C→C:G transversions, which represented 29% and 15% of the mutations, respectively.

12.7 MUTATIONAL SPECIFICITY OF OTHER DNA DAMAGING AGENTS

12.7.1 IRON(III)/HYDROGEN PEROXIDE/EDTA

Endogenously and exogenously mediated oxidation of DNA or treatment of DNA with ionizing radiation yields a wide variety of products, including base modifications, apurinic sites and strand breaks (Von Sonntag, 1987). In view of this multiplicity of DNA lesions, that the mutation spectrum observed following DNA oxidation or irradiation displays a wide variety of genetic changes. The predominant changes observed in both bacterial and mammalian systems, however, are base substitutions, mainly the G:C→A:T transition (Glickman *et al.*, 1980; Ayaki *et al.*, 1986; Grosovsky *et al.*, 1988; Tindall *et al.*, 1988). Base substitutions at A:T pairs also contributed significantly to the observed mutation spectrum (Storz *et al.*, 1987; Loeb *et al.*, 1988).

The spectrum of oxygen radical mutations induced in the *supF* gene of pZ189 has been assessed in monkey cells (Moraes *et al.*, 1989). Mutagenesis is dose-dependent; at a mutation frequency of 10^{-2}, a 60-fold increase over the spontaneous level of mutagenesis has been achieved. Particularly noteworthy is that the induced mutation spectrum is a quantitatively amplified version of the spontaneous spectrum, suggesting that oxidative damage is a major contributor to

the background of mutations occurring by replicating the vector in simian cells. However, subtle differences exist between the oxidation and spontaneous spectra. Specifically, whereas the predominant base substitution mutation in both cases is the G:C→A:T transition, base substitutions of all types at A:T pairs are increased from a total of ~2% in the spontaneous spectrum to about 15% in the mutation spectrum of the oxidized vector. Furthermore, 75% of all oxidation-induced deletions of less than 100 bp occur at two A:T base pairs in the gene.

12.7.2 SAFROLE

The hepatocarcinogen safrole is a naturally occurring compound in some plants and spices. It reacts with DNA after metabolic activation to form adducts principally with guanine at the N^2 position. Adducts also form to lesser extents at the N^7 and C^8 positions of guanine and at the exocyclic amino group of adenine (Phillips *et al.*, 1981; Wiseman *et al.*, 1985). The mutational spectrum by the direct-acting 1′-acetoxy derivative of safrole was assessed at the *tk* locus of an EBV-derived shuttle vector modified *in vitro* and transfected into human lymphoblastoid cells (Ingle and Drinkwater, 1989). At a level of mutagen giving a mutation frequency of 4×10^{-3} (a 10-fold increase over background) 70% of the induced mutants were point mutations. Most classes of base substitutions and single base deletions were represented in the spectrum and, of these, two-thirds were positioned at G:C pairs.

Safrole induced activating base substitution mutations at codons 12 and 61 in the c-*Ha-ras* protooncogene treated with activated safrole *in vitro* and then transfected into NIH 3T3 cells (Ireland *et al.*, 1988). The same proto-oncogene was activated in liver tumors obtained from mice treated with safrole (Wiseman *et al.*, 1987). Base substitution mutations in the activated proto-oncogenes were observed at both G:C and A:T base pairs, which is in accord with the mutational specificity of safrole in the shuttle vector system described above.

12.7.3 8-METHOXYPSORALEN (8-MOP)

Psoralens are bifunctional alkylating agents that, in a photochemical reaction, bind DNA primarily at the 5,6-double bond of thymine residues. Both monoadducts and inter-strand T-T cross-links can be formed, depending upon the irradiation dosing regimen used (Cimino *et al.*, 1985). Bredberg and Nachmansson (1987) investigated the mutational effects of 8-MOP plus UV in the *supF* gene of pZ189 in monkey cells. A 16-fold increase in mutation frequency above background (7×10^{-4}) was obtained only under conditions favoring cross-link formation. Although only a small number of mutants was sequenced, 85% were single base substitutions, with one-third positioned at either of the two central bases of a 5′-ATAT-3′ site. This sequence is an expected hot spot for cross-link formation (Sage and Moustacchi, 1987). Overall, however, mutations were more abundant

at G:C sites, which does not seem to correlate with the known DNA adducts formed by this drug. A possible explanation for the mutations at G:C sites comes from an examination of the mutation spectrum, that reveals that half of the G:C-targeted mutations occur at one hot spot in which the mutation site is flanked by multiple A:T pairs. Hence, the G:C-targeted mutations may occur at sites adjacent to the position of 8-MOP adduct formation.

The mutations observed at A:T base pairs in the shuttle vector are consistent with the mutational spectrum of 8-MOP in the *lacZ* gene inserted into bacteriophage M13 (Piette *et al.*, 1985). However, the predominant mutation in *E. coli*, the A:T→C:G transversion, was not observed in the shuttle vector replicated in the mammalian cells. Furthermore, no G:C-targeted mutations were observed in the bacterial system.

12.8 CONCLUSIONS

Careful examination of studies in which shuttle vectors were used to evaluate the mutational specificity of DNA damaging agents revealed that the field developed well after a slow start, but that its future prospects are not as bright as its accomplishments in the recent past. First, several approaches have been developed that have successfully overcome the unacceptably high background mutation frequency that hampered the early development of the field. Moreover, in the past few years, many mutational spectra of different DNA damaging agents have been evaluated, especially in the *lacI* and *supF* systems. Equally noteworthy is that the mutational specificity of most genotoxicants corresponds well to expectations based on the results of similar analyses of endogenous genes in mammalian cells or in prokaryotic systems. The shuttle vector systems also permit the mapping of adduct formation sites in the same DNA sequence in which mutations are fixed; presently, this goal has not been achieved for endogenous genes of intact cells or organisms treated with a genotoxicant. Lastly, the advances made in shuttle vector design have made it possible to conduct site-specific mutagenesis studies using single DNA adducts in mammalian cells (Ellison *et al.*, 1989a, b).

Despite the attributes indicated above, the field is likely to struggle during the next few years for several reasons. A key limitation of the shuttle vector approach is evident upon examination of the types of DNA damaging agents that have (or have not) been studied. Specifically, these are chemical mutagens that require enzymatic activation for their genotoxic effects to be manifested. Most chemical mutagens of relevance to human health fall in this category, and the mammalian cells conventionally used as hosts cannot metabolically activate mutagens. Chemists have often been able to provide electrophilic intermediates; the synthetic work usually requires a long lead time, and workers in the shuttle vector field have nearly exhausted the supply of such intermediates prepared over the years for other purposes. Of course, circumventing synthetic work may be not only possible but also conceptually desirable, by using microsomes or purified monooxygenase

preparations to achieve a more natural activation of chemical mutagens. In practice, however, such preparations are usually contaminated with nucleases that damage the plasmid and compromise its viability once inside the mammalian cell. Another approach would be to express molecularly cloned genes encoding the xenobiotic activation systems in the host cells for replication of shuttle vectors. With a few exceptions (Crespi *et al.*, 1989; Davies *et al.*, 1989; Hansen *et al.*, 1989), little work has been done in this area. Maintaining consistently high levels of expression of the cytochrome P_{450} genes has proven to be a problem that needs to be addressed.

Finally, a decade ago, shuttle vectors were widely hailed as the anticipated means by which mutational specificity data could be generated on DNA damage in mammalian cells. Nevertheless, in this day of sophisticated chemical and molecular biological techniques, one should ask whether the shuttle vector is the best approach toward the goal of efficiently generating high quality data. For example, the ability to analyze mutations in endogenous DNA sequences of mammalian cells by the polymerase chain reaction has provided an attractive alternative. Moreover, as indicated above, transgenic animals offer much promise as an improved vehicle by which genetic data on mutagens are likely to be generated in future studies. This model is still largely on the intellectual drawing board, although one published paper has given rise to optimism (Gossen *et al.*, 1989). The attractive features of the model are that it provides biochemically relevant metabolic activation and detoxification of carcinogens in the cells of intact organisms, and it also permits organotropic and even inter- and intraspecies comparisons to be made. The eventual coupling of transgenic technology with established animal models for carcinogenesis will enable the most direct conclusions to be made on the relevance of specific patterns of mutagenesis to carcinogenesis. Sufficiently sensitive methodology for achieving these goals in animals treated with low levels of DNA-damaging agents is not yet available, but this technology is within reach.

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13 Structure–Activity Relationships: Computerized Systems

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13.1 INTRODUCTION

Applications of computational methods to study relationships between chemical structure and biological activity have contributed substantially to our current understanding of the interactions of chemicals with biological systems. The number and sophistication of such applications have increased, and will continue to increase, with advances in computer hardware and software, with the availability of larger biological databases, and with increased understanding of molecular-level interactions in biological systems. Such advances have enhanced both the scope and detail of the biological problems that can be successfully treated by computational structure–activity relationships (SAR) techniques.

This paper presents important issues relative to the application of computational

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SAR techniques to problems in environmental chemical hazard evaluation. Such problems are most often characterized by not only a lack of biological activity data for the chemicals of concern but also a lack of understanding concerning molecular-level mechanisms of activity. The goal is to develop SAR models that can rely on existing biological activity data on chemical analogs to estimate the toxicity of the chemicals of interest. SAR models such as these may play important roles in preliminary assessments, and may help direct future experimental and theoretical investigations.

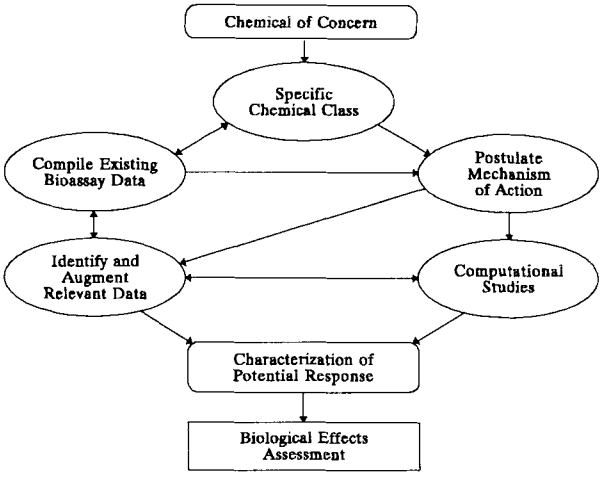

Figure 13.1 The process of developing information for the structure–activity assessment of the potential biological effects of a chemical or chemical class

This paper presents important generalizations relative to the application of computational SAR techniques, problems particular to the SAR modelling of genotoxicity and cancer endpoints, and an illustrative analysis of a chemical class—the organic halides. Additional issues relating to database development, biologically based comparative assessment approaches, and a weight-of-evidence evaluation scheme are also discussed.

13.2 SAR AS A PROCESS

The relationship among various components of an SAR study is complex, as illustrated in Figure 13.1. The initial stages involve specification of the chemical class and compilation of bioassay data for the biological activity endpoint of interest. Specification of these components limits and defines the scope of the SAR modelling study. This information together with knowledge of chemical properties

and potential interactions with biomolecular targets contributes to the derivation of possible mechanisms of toxicity. Mechanistic hypotheses can suggest the type of computational study that would be most useful, and the results of such a study can provide a quantitative basis for the organization and differentiation of chemicals within a class. Continuing feedback may occur between computational studies, knowledge concerning the mechanisms of action, and the assembly of a relevant bioassay database for the chemical class. Postulating mechanisms of action can be the basis of experimental bioassay strategies, and may suggest a means to extrapolate the results of one type of bioassay to another in order to estimate toxic endpoints of interest. Ultimately, SAR modelling of potential biological responses based on the current state of understanding of molecular-level mechanisms should provide a rational basis to extrapolate existing information to untested chemicals.

The feedback loops indicated in Figure 13.1 can greatly enhance the relative contributions of each component in the SAR study. This situation, in turn, can lead to a improved understanding of the fundamental processes involved in a biological process, and can improve the ability to characterize potential responses of untested members of the chemical class. Generally, such feedback requires closer interactions between theoretical chemists, synthetic organic chemists, and experimental biologists. For example, an underutilized yet potentially very powerful approach involves the identification of one or more particular compounds that may have no environmental significance, but which, if tested, could be extremely informative in refining mechanistic hypotheses.

The SAR model derived from Figure 13.1 pertains to the underlying processes specific to the particular bioassay and chemical class chosen. Frequently, however, a chemical class is specified based on little or no knowledge concerning the mechanisms of toxic action of its members, but rather by consideration of common chemical structural and reactivity characteristics. Incomplete class specification or the inclusion of chemicals that act through alternate mechanisms can confuse and limit the utility of the resulting SAR model.

As studies progress and additional information becomes available, efforts should continue towards revising and updating chemical class specifications to incorporate expanding knowledge. In addition, alternative means to specify chemical classes from a biological perspective should be investigated. Furthermore, an SAR study requires a consensus concerning the activity assignment of a specific chemical in a given bioassay. Response criteria required to distinguish a biologically active molecule from an inactive one may be subject to controversy. This problem is particularly acute for complex endpoints such as carcinogenicity. Hence, weight-of-evidence schemes, that attempt to formulate a consensus outcome from such data, can provide a useful starting point for an SAR study. Other types of SAR approaches could seek to incorporate an array of bioassay data into the modelling of a complex biological endpoint. For example, a select group of genetic toxicity bioassays might provide a useful basis for SAR modelling of carcinogenicity. These characteristics are examples of only a few of the preliminary considerations that impact on the selection of any SAR modelling method and its determination

of predictive value.

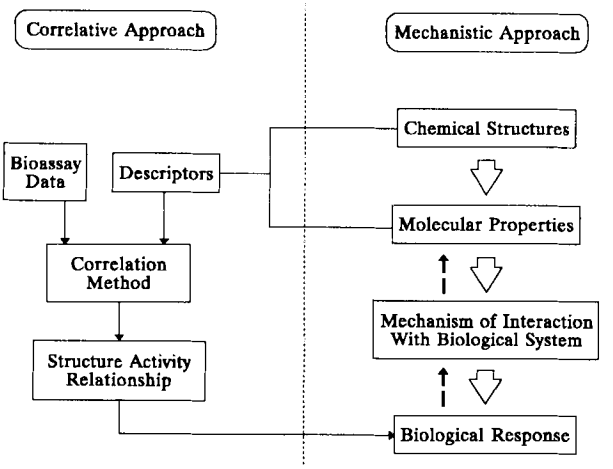

Figure 13.2 Generic components of a structure-activity study

13.3 CORRELATIVE AND MECHANISTIC APPROACHES

Two extreme but complementary SAR approaches exist which attempt to model the relationship between chemical structure and biological response: one from a correlative and the other from a causal perspective (Figure 13.2). In each case, the chemical structure is responsible for a molecule's physical properties (such as the molecular electrostatic potential, electrophilicity or nucleophilicity, solubility, and partition coefficient). These properties determine the biological response through specific biomolecular interactions. In the causal approach, understanding the mechanism of action and the underlying molecular-level interactions that determine activity is the key step to estimating possible biological responses of untested class members. The tools of computational chemistry, such as quantum mechanical and semi-empirical programs to compute minimum energy structural configurations, atomic charges, electronic properties, and relative molecule energies, are used to probe and model the molecular-level basis for biological activity in these approaches. Since studies used to validate the mechanisms of action are critical and may require additional investigation of related chemicals, interactions among experimentalists and theoreticians are extremely valuable in this approach (Figure 13.1).

The left half of Figure 13.2 represents the strictly correlative approach, in which little or no knowledge concerning the causal basis for activity is presumed. A large number of descriptors based on chemical structure and molecular properties is computed for each member of a class of chemicals. This descriptor set is then

merged with biological activity information, and a statistical or pattern recognition technique is used to identify relevant descriptors that are significantly correlated with a particular biological response. These descriptors, and in some cases the quantitative weights associated with their contributions, are the basis of the SAR model for the prediction of biological response.

In practice, these two approaches (Figure 13.2) are rarely separate, and SAR model development ideally should incorporate elements of each. Mechanistic considerations can and should influence the types of molecular descriptors that are considered in a correlative SAR study. Likewise, the results of a purely correlative study have limited utility if considered independent from mechanistic understanding. By considering the descriptors that account for activity correlations and developing insight concerning their relationship to possible mechanisms of activity, the validity of the SAR model and confidence in its predictions are enhanced. Computational SAR models incorporating elements of both the causal and correlative approaches likely will play an increasingly important role in identifying the features of chemical structure conditionally required for the biological activity or inactivity within a series of compounds.

13.4 CORRELATIVE SAR TECHNIQUES

The allure of the correlative SAR approach is that little prior knowledge of mechanisms is required. Several such approaches have been applied to problems in genotoxicity and carcinogenicity, that are most often characterized by large amounts of data but very little molecular-level understanding of mechanisms of activity. Since the application of such approaches is likely to increase in conjunction with extensive data collection efforts currently under way, a sampling of available approaches is considered and contrasted. General reviews on correlative methods are contained in Jurs *et al.* (1985), McKinney (1985), Enslein *et al.* (1983, 1987), Golberg (1983), and Frierson *et al.* (1986). The reader is referred to the literature for a more detailed discussion of each method and its specific applications: ADAPT (Jurs *et al.*, 1983; Stouch and Jurs, 1985), SIMCA (Wold and Sjostrom, 1977; Dunn and Wold, 1980, 1981), CASE (Klopman, 1985; Klopman *et al.*, 1985a, 1987a), and TOPKAT (Enslein and Craig, 1982; Enslein *et al.*, 1983, 1987). For illustrative purposes, one technique, the CASE program, will be considered in more detail, and applied to a sample analysis of the organic halides as a class.

Input to a correlative SAR program usually consists of graphical or coded molecular structures of the chemical of interest, and qualitative or quantitative measures of biological activity corresponding to a common biological endpoint. This information constitutes the database for study. Molecular descriptors are then either input from external sources or calculated internally. The term "molecular descriptor" refers to any physico-chemical or substructural parameter, either experimentally measured or calculated, that attempts to encode an aspect of molecular structure. Descriptors fall into four major categories—topological,

geometrical, physicochemical, and electronic—and pertain either to the entire molecule or a local region of a molecule. Specific examples include: connectivity indices (Randic, 1975); fragment substructures; moments of inertia; molecular volume; partition coefficients; and HOMO or LUMO (highest occupied or lowest unoccupied molecular orbital) energies. Descriptors are discarded that have either linear dependencies on other descriptors already included in the analysis, or that account for little activity variation within the database. A correlation step employs a statistical or pattern recognition method to determine associations between remaining descriptors and activity variation within the database. The final set of molecular descriptors, and in some cases the statistical weights associated with these descriptors are the basis of the SAR model used for subsequent activity predictions.

Correlative SAR programs vary with regard to the number and type of molecular descriptors considered, the degree of user input in the selection of descriptors, the analysis used to determine which descriptors are relevant to activity, and the final form of the SAR prediction model. In addition, many programs operate in more than a single mode (SAR or QSAR) and with more than one type of correlation or pattern recognition method. A wide variety of molecular descriptors can be supplied for a TOPKAT, SIMCA, or ADAPT analysis—some are calculated internally and others must be supplied from external sources. A CASE analysis, by contrast, is based on a single type of descriptor—molecular skeletal fragments. Two problems are associated with the use of a large number of descriptors in correlative SAR approaches. When any type of regression statistics is used, strict limits exist on the number of initial descriptors that can be considered in relation to the number of molecules in the database to avoid chance correlations and meaningless results (Topliss and Edwards, 1979). Second, while numerous diverse descriptors relate to molecular structure in a general sense, their relation to each other or to specific physico-chemical processes is often unclear. Hence, to assess their causal significance, which ultimately limits the utility of the model, may be difficult (Frierson *et al.*, 1986; Rosenkranz *et al.*, 1990). Hence, SAR models that attempt to restrict the range of possible descriptors based on general mechanistic relevance have some advantages over unrestricted methods. The descriptor generation and selection processes in TOPKAT are semi-automated, and involves calculation and consideration of several descriptors. An ADAPT or SIMCA analysis, by contrast, requires greater user expertise and user involvement in generating and selecting descriptors. The CASE descriptor selection process is fully automated, and involves an exhaustive calculation of all possible descriptors. However, in this method, the descriptors are all the same type. Although the number or size of fragments needed to model a relevant molecular parameter could be exceedingly large, an advantage of the CASE approach is that molecular fragments may provide more insight into a possible mechanistic interpretation than a combination of dissimilar descriptors.

The specific correlative SAR methods also differ with regard to the type of correlation processes used to evaluate and select relevant descriptors. TOPKAT

uses a multivariate regression method to determine the smallest set of descriptors that best reproduces the activity variation within the database. The resulting quantitative SAR (QSAR) equation is based on the premise that a discrete increment of activity can be associated with any type of molecular descriptor. The most common application of CASE makes qualitative activity predictions based on the presence or absence of one or more significant fragments. Significance is determined by deviation of the fragment incidence distribution within the database from a random binomial distribution among activity classes. SIMCA and ADAPT are classified as pattern recognition approaches, and use linear discriminate or principle components clustering analysis methods to derive an SAR model. Molecules represented by a string of descriptor values are positioned in a multi-dimensional space, where each descriptor is represented by a separate axis in this space. Molecules that have similar descriptor values (presumably chemically and biologically similar) tend to cluster in the same regions of this descriptor space. If the position of a molecule within this space meaningfully correlates with activity, then a qualitative activity prediction is based on the position of a new compound within the delineated active or inactive regions of this space. The linear discriminant analysis used most commonly in ADAPT attempts to determine the descriptor space that gives the best planar separation of the entire data set into an inactive and active molecular region. The principle components cluster approach used by SIMCA searches instead for significant clustering of similar molecules associated with either activity or inactivity. In this approach, one or more significant clustering patterns can be associated with each activity category, where the category can include different measures of activity (such as active versus inactive) or different mechanisms of activity (Dunn and Wold, 1981).

13.4.1 THE CASE SYSTEM

Since the CASE method will be applied to a sample analysis for illustrative purposes, it is considered in greater detail. The CASE methodology has been described on several occasions (Klopman 1984; Klopman and Macina, 1987; Klopman *et al.*, 1985b, 1987a; Rosenkranz and Klopman, 1989). CASE is fully automated and selects its own molecular fragment descriptors from a user selected "training" set composed of active and inactive molecules and their associated activities. The descriptors are easily recognizable single, continuous structural fragments, of length two to ten non-hydrogen atom units embedded in the complete molecule. The descriptors are classified as either activating (biophore) or inactivating (biophobe) fragments. The ability of CASE to select biophores readily recognized as being part of a molecule is a major advantage of the method. Chemical "intuition" traditionally has been used to associate the presence or absence of particular functional groups with biological activity. Indeed, this approach was taken recently by Ashby and Tennant (1988) and Ashby *et al.* (1989) in identifying "structural alerts" for carcinogenesis. Enzymes and other biological

receptors recognize moieties much larger than the groups usually considered. While CASE is limited in its ability to predict important steric or 3-dimensional conformational requirements for activity, it can generate substructures of intermediate size that may be associated with biological activity. Since a major aim is to elucidate the basis of the action of toxicologically active molecules, the identification by CASE of structural components embedded in the molecule offers a tool that may be exploited to investigate structural sites of metabolism or receptor binding or to test other hypotheses.

Once a "training set" of chemicals has been assimilated, CASE can be queried regarding the expected activity of molecules of unknown activity. On the basis of the presence or absence of the previously identified biophores and bioprobes, CASE estimates activity or lack thereof. In addition, CASE can use the descriptors to perform an *ad hoc* multivariate regression analysis (QSAR) that results in a projected potency (Frierson *et al.*, 1986; Klopman *et al.*, 1987a, b; Rosenkranz and Klopman, 1989). Experience with CASE indicates that databases consisting of at least 30 to 50 congeneric (structurally similar) chemicals distributed among inactive, marginally active, and active chemicals are required for a correlative analysis.

13.4.2 CHEMICAL CLASSIFICATION AND REPRESENTATION

An important aspect of SAR modelling that has particular relevance to a correlative SAR study is the initial database compilation. The statistical nature of such methods imposes numerical and representation requirements on the database under consideration. The composition of the database is generally restricted to a chemical class of structurally, and presumably mechanistically related compounds. This chemical classification usually imposes limits on the structural diversity of the compounds under consideration, with structural features common to all members of a chemical class considered necessary but not sufficient requirements for activity. Hence, even in a strictly correlative study, some structure-mediated aspects of an assumed common mechanism of action are usually implicitly incorporated before analysis.

An alternative means to assess chemical class membership from a strictly biological perspective has been suggested by Garrett *et al.* (1984, 1986) and Waters *et al.* (1988c), using pattern recognition techniques. This approach classifies chemicals according to a similar overall pattern of genetic or other biological activity based upon biologically effective dose. Thus, groups of chemicals may be identified that display qualitatively and quantitatively similar biological responses across species without knowledge of their chemical structures. Such classifications may result in novel or unusual groupings of chemicals that appear to be structurally unrelated, but that may exert their biological activity through similar mechanisms. Using computerized profile matching techniques, rapid identification of subsets of chemicals is possible from large databases with similar profiles for such

classification purposes. Correlative SAR techniques, such as CASE, may then be used to ascertain those structural components that may be responsible for biological activity or inactivity.

Some correlative SAR approaches have a limited capacity to evaluate structurally diverse databases. This capability strongly depends, however, on whether each mechanistically distinct chemical class is adequately represented within the database. Careful analysis of such databases could facilitate the identification of mechanistically distinct chemical classes that cross traditional class boundaries. However, any further analysis of such data should proceed on the smaller circumscribed data classes. A danger is that large, diverse databases are being analyzed indiscriminately, and used to satisfy numerical representation requirements for SAR without being examined subsequently to see if they have satisfied important class representation requirements. A CASE or TOPKAT analysis, in principle, is capable of yielding independent sets of descriptors when analyzing a combined database of mechanistically distinct classes subject, however, to the above precautions. Also, a principle-components-clustering approach, such as used in SIMCA, is particularly well suited to distinguishing separate patterns of activity within a database.

In addition to limits imposed on the nature of chemicals that can be included in a particular database, data representation requirements dictate the type and number of compounds that should be included for a correlative SAR study. Confident estimates can be made only for chemicals whose descriptor values lie within the bounds of the model. In addition, sufficient numbers of compounds in each activity category and descriptor class should be included to provide adequate statistics and confidence levels for reliable estimations. In general, the more diverse the database, the more descriptors are needed to span the variation within the database. In practice, few of these conditions are adequately met for most classes of compounds of interest. Data gaps and deficiencies are a common and sometimes serious problem associated with correlative SAR studies. Such studies are dependent ultimately on the availability of high quality biological databases, and, as a result, have been limited to a few particular bioassays and endpoints.

13.4.3 SAR MODELLING OF GENOTOXICITY AND CARCINOGENESIS

Several features and important unresolved issues pertaining to the modelling of genotoxicity and carcinogenicity endpoints that impact on SAR studies remain. On first inspection, an abundance of data available for use in SAR modelling studies appears to exist. On closer examination, however, several serious deficiencies and problems become apparent. The range of bioassays that have been used to evaluate thousands of chemicals for genotoxicity is wide. However, the diversity of chemical structures represented is tremendous, and very few chemicals have been tested in the full range of bioassays. Only a few select bioassays, such as the *Salmonella* test, exist for which sufficient data representation requirements are met within a wide variety of chemical classes. Such bioassays were designed as

experimental surrogates of specific processes thought to be important components in mechanisms of carcinogenicity. However, the structural determinates for activity within a particular bioassay, the relationships among bioassays, and the relationships among bioassays and the processes being modelled are often unclear and subject to controversy.

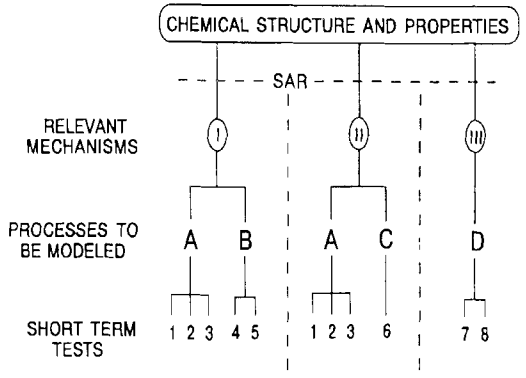

Figure 13.3 A paradigm using the relationships between chemical structure and carcinogenesis to select a short-term test battery for a specific class of chemicals

The complex nature of the carcinogenicity endpoint in relation to the underlying multi-step processes and surrogate short-term tests is illustrated schematically in Figure 13.3. Ideally, a battery of short-term tests (1 to 8) for the evaluation of a given chemical should address the relevant processes (A, B, C, and D) within the array of possible mechanisms (I, II, and III). Several postulated mechanisms for carcinogenesis have been reported (Huberman and Barr, 1985), and more than one may apply depending on the chemical class under consideration. These mechanisms often involve multi-step processes, with DNA damage and repair being important components. Short-term tests attempt directly or indirectly to model the processes of interest. In practice, however, available short-term tests are imperfect predictors of the processes being modelled, and the processes themselves are often neither known nor clearly understood. Hence, major uncertainties are present at every level in Figure 13.3. SAR modelling studies can also be involved at every level (Figure 13.3) in trying to reduce these uncertainties and to establish meaningful correlations and mechanistic hypotheses. Correlative SAR modelling studies that attempt to identify relevant structural features can be performed at the first level using carcinogenicity as the endpoint, and at the last level using the particular bioassay data as the endpoint. At the second level, specific processes thought to be relevant to carcinogenicity, such as DNA-adduct formation, can be modelled at the molecular level for specific chemicals and chemical classes using the tools of theoretical chemistry. At the last level, SAR modelling can also help

elucidate the underlying basis for activity in a particular short-term bioassay. Also at this level, profitable interactions between theoreticians and experimentalists to generate new data for mechanistic hypothesis refinement are feasible. This knowledge, in turn, can be used to define the combination of short-term tests likely to estimate most accurately the chemical processes of concern.

In the absence of information concerning the optimal selection of short-term tests to model the carcinogenicity endpoint, the current strategy must incorporate redundant assays for processes that are imperfectly modelled and independent tests to adequately model each unique process. Judgment concerning which tests are independent or redundant can be made only after a careful evaluation of all data. Finally, the selection of tests should depend on a proven capability of each test to model structural analogues within a chemical class of interest.

The remainder of this monograph will consider in greater detail the composition of available databases, methods to organize and represent such data in terms of profiles, and the sample evaluation of the class of organic halides.

13.5 GENETIC TOXICOLOGY DATABASES: THE GENE-TOX AND NTP DATABASES

Currently, more than 180 test systems or bioassays exist to evaluate agents that induce genetic or related effects, and these test systems may be conveniently organized into a series of phylogenetic endpoint categories (IARC, 1987a; Waters *et al.* 1988a). The number of chemicals for which data exist is greater than 18200, with more than 60000 references in the database at the Environmental Mutagen Information Centre (EMIC) at Oak Ridge National Laboratory (Wassom, 1980).

Three major peer-reviewed genetic toxicology databases are currently under development in the US One is the database on short-term *in vitro* and *in vivo* genetic tests being developed by the National Toxicology Program (NTP). The other two genetic toxicology databases, being assembled by the US Environmental Protection Agency (EPA), are the Gene-Tox database and the Genetic Activity Profile (GAP) described under "Genetic Activity Profiles."

The NTP database uses standardized laboratory protocols and blind testing procedures in selected laboratories. While relatively few short-term tests have been employed by the NTP, the number of chemicals evaluated to date, particularly in the Ames test, is substantial. The NTP database offers advantages of internal consistency and comparability; however, the representation of chemical analogs in the NTP database at its current size is sparse, except for Ames test data. Although the requirements of SAR analysis are not considered by the NTP in chemical selection at the present time, the NTP's databases for Salmonella mutagenicity and the rodent carcinogenicity have been utilized extensively for SAR studies. An advantage of the NTP databases is that each chemical is jointly tested for carcinogenicity and mutagenicity in *Salmonella* and for the presence of "structural alerts" for genotoxicity (Ashby and Tennant, 1988; Ashby *et al.*, 1989). Thus, the

determination of whether the chemicals associated with specific CASE biophores are also mutagens is relatively straightforward.

The largest peer-reviewed and publicly available database has been developed through the EPA Gene-Tox Program (Waters and Auletta, 1981). Gene-Tox data are available for more than 4000 chemicals evaluated in 73 different short-term bioassays. About 330 chemicals have been tested in five or more different kinds of bioassays. The Gene-Tox Program was designed to assess the current status of genetic bioassays rather than to evaluate individual chemicals. However, all chemicals tested in the bioassays under evaluation are represented by overall positive or negative classification for the given chemical bioassay. These classifications are rendered by peer review committees in the process of periodically considering the accumulated data for an individual test system. The qualitative Gene-Tox data have recently been combined with the mutagenicity database of the Registry of Toxic Effects of Chemical Substances (RTECS) that includes information on dose. RTECS is searchable on-line through the US National Library of Medicine's Toxicology Data Network (TOXNET). The mutagenicity data in RTECS have not been peer reviewed, and do not correspond to those data reviewed by Gene-Tox committees.

The Gene-Tox Program also has reviewed the animal cancer bioassays for 506 compounds, and has classified the results qualitatively as follows: 252 as Sufficient Positive; 99 as Limited Positive; 40 as Sufficient Negative; 21 as Limited Negative; one as Equivocal; and 93 as Inadequate (Nesnow *et al.*, 1986). The preponderance of positive data in Gene-Tox for both mutagenicity and carcinogenicity has restricted assessments of test and battery performance largely to issues of sensitivity and positive estimation (Nesnow and Bergman, 1988). Despite these limitations, data from the Gene-Tox Program have been used in conjunction with ADAPT, CASE, and TOPKAT in the SAR assessment of genetic toxicity and potential carcinogenicity (Klopman *et al.*, 1985a; Enslein *et al.*, 1987). These correlative SAR programs have relied heavily on the results of the Ames test that represents by far the largest number of chemicals in the Gene-Tox database.

From the point of view of test battery selection, analyses of the Gene-Tox database have been performed by Rosenkranz *et al.* (1984) and Ray *et al.* (1987). The Gene-Tox report of Ray *et al.* (1987) deals specifically with the identification of specialized batteries of bioassays applicable to specific chemical classes. The report concludes that the small number of chemicals within any chemical class for which more than two or three assays have been performed severely limits definitive conclusions regarding chemical class-specific test batteries. Ideally, if data from short-term test batteries were available for a large number of compounds, the data sets could be used to investigate common or different mechanisms of chemical action among various tests in a battery, and would be more useful for SAR investigations.

13.5.1 GENETIC ACTIVITY PROFILES

To enhance the utility of genetic bioassay data in the evaluation of individual chemicals, a quantitative presentation format has been developed which incorporates Gene-Tox and NTP as well as other published data (Garrett *et al.*, 1984; IARC, 1987a; Waters *et al.*, 1988b). The GAP is a bar graph (Figure 13.4; symbols used: (-) = no exogenous activation; (^) with exogenous activation; majority response (+/-) = a solid vertical line to mean LDU value; conflicting data = dashed vertical line from the origin through all conflicting data points) that displays information on the various genetic and related tests that have been performed using a given chemical, including: (a) the phylogenetic levels of the test systems; (b) the genetic endpoints that the tests represent; and (c) the dose of the chemical that has yielded a positive or negative response in each test. Thus, a profile permits direct visual assessment of the responses of an array of short-term tests applied to a chemical and facilitates a computer-based comparison of genetic activity for chemical selection and SAR model development (Waters *et al.*, 1988c). GAP matching techniques can indicate biological similarities across chemical structural classes or differences within classes which could have mechanistic significance (Garrett *et al.*, 1984, 1986; Waters *et al.*, 1988c). They can also assist in pinpointing differences in genotoxic responses among species.

The current GAP database includes profiles and data listings for approximately 350 agents tested in nearly 200 different genetic bioassays. Many agents represented in the GAP database (IARC, 1987a) have been evaluated in animal cancer tests and in epidemiological studies (ATSDR, 1986; IARC, 1987b). Because of its quantitative component, the GAP database should be useful in quantitative SAR studies. Limitations of the GAP database include size, chemical class representation, and a preponderance of positive data. Fortunately, for purposes of the sample SAR investigation, both the GAP and Gene-Tox databases contain a large proportion of negative results for the organic halides.

13.5.2 PROBLEMS WITH SOME EXISTING DATABASES

Two significant shortcomings of available databases are the lack of both standardization of test protocols and consensus interpretation of the data. Thus, several analyses have been performed comparing the Gene-Tox and US National Toxicology Program data compilations (Klopman *et al.*, 1990; Rosenkranz and Klopman, 1990a; Rosenkranz *et al.*, 1990). In a detailed analysis of the *Salmonella* mutagenicity results, a significant overlap was found between the structural determinants identified in each data set, even though the specific chemicals in these databases did not overlap greatly (Klopman *et al.*, 1990; Rosenkranz and Klopman, 1990a). This finding implies a common mechanistic basis for the activity of the two sets of compounds. However, a comparison of the CASE results derived from the NTP and Gene-Tox databases for sister chromatid exchange as well as chromosomal aberrations revealed very little commonality between the CASE substructures identified in the NTP versus the Gene-Tox database for each assay

(Rosenkranz *et al.*, 1990). Since the chemical class representation within the two databases differs, this result could be a reflection of class-specific mechanisms of activity for these assays. The two databases were developed differently: The NTP data were generated using coded chemicals of known purity, following a rigid experimental protocol and statistical criteria for the consistent interpretation of the results, whereas the Gene-Tox results were abstracted from the published literature and submitted to individual peer panels for their expert judgment and overall assessment.

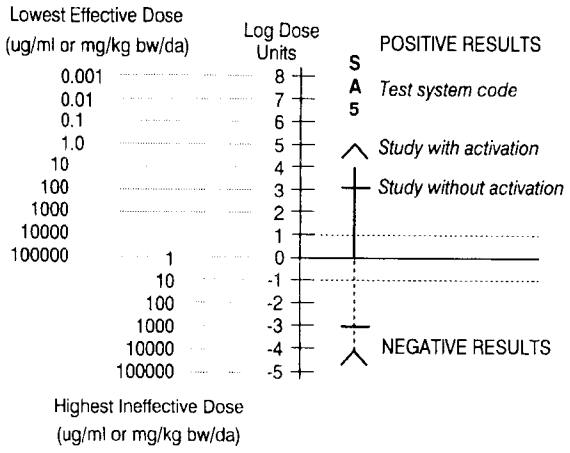

Figure 13.4 Genetic activity profile: LED and HID converted to logarithmic dose units (LDU).

Another problem with these databases relates to the standardization of carcinogenicity results. Thus, the compilation of Gold *et al.* (1984, 1986, 1987) theoretically is well suited for the study of the structural basis of carcinogenicity, since it also provides a measure of carcinogenic potency in the most sensitive species. Many chemicals studied under the aegis of the NTP were included in the compilation of Gold *et al.* (1984, 1986, 1987, 1989). On several occasions, a chemical listed as non-carcinogenic by the NTP was given a potency (TD_{50}) value by Gold *et al.*, and hence was interpreted by CASE as being carcinogenic in the analysis of the latter data. Thus, when the two databases were used independently as training sets for CASE, different predictions resulted. When, however, the Gold *et al.* TD_{50} values were used for the chemicals designated as positive carcinogens by NTP, excellent quantitative correlations with carcinogenic potency were obtained (Nesnow, 1990).

The NTP database is used for most of the present CASE analyses, because both carcinogenicity and short-term test data are available for the class of organic halides under consideration. Generally, however, given the paucity of data available,

agreement on the interpretation of multiple species/sex carcinogenicity data are needed to facilitate not only SAR studies but also the validation of short-term test data performance.

13.6 INTEGRATION OF STRUCTURAL CONCEPTS WITH DATABASE EVALUATION

A plethora of information exists about the genotoxic properties of molecules and, to some extent, their carcinogenicity. Furthermore, some SAR methodologies have limited ability to investigate non-carcinogenicity data sets with respect to the structural basis of mutagenicity, genotoxicity, and carcinogenicity subject to data representation requirements (Klopman *et al.*, 1990; Rosenkranz and Klopman, 1990a, b). The feasibility of integrating some of these techniques is discussed below. To accomplish this, the CASE methodology is used, because it has proven especially effective for comparing the structural basis of various biological endpoints (Klopman *et al.*, 1990; Rosenkranz and Klopman, 1990b). The advantages of the CASE program are augmented by the availability of GAPs from the GAP database and the use of profile-matching techniques.

The first step in the process of integrating these methodologies is database compilation. A qualitative genetic toxicology database on the organic halides, derived from the larger Gene-Tox and GAP databases is used as an illustration. Each organic halide has been evaluated in 10 or more short-term tests; the class has been divided into aryl halides, saturated alkyl halides, and unsaturated alkyl halides. Some compounds span more than one chemical class. Organic halides, that represent a general class of chemicals for which the evaluation of the potential carcinogenicity in terms of short-term test data is particularly difficult, provide a major challenge for the application of various computerized SAR and comparative biological assessment tools. Approximately one-third of these organic halides produce primarily positive responses in short-term tests; about one-half are essentially negative in the Ames *Salmonella* tester strains; and about one-third are negative in most of the other short-term tests. Approximately one-half of these halides are positive in one or more rodent cancer bioassays. Of the remaining one-half, a majority are equivocal for carcinogenicity or have been inadequately evaluated for carcinogenicity. Only a few of these chemicals are considered to be negative for carcinogenicity in animal studies.

13.7 SAR ESTIMATIONS

Using a training set consisting of 254 diverse chemicals derived from the NTP carcinogenicity test results, CASE identified major biophores found in organic halides that are associated significantly with carcinogenicity in rodents (Rosenkranz and Klopman, 1990c). These biophores were used by CASE to estimate the

carcinogenicity of a group of halogenated chemicals that were not included in the original training set (Table 13.1). When the Gold *et al.* (1984, 1986, 1987, 1989) database was used to derive the CASE fragments, the resulting predictions were reasonably different, i.e., the CASE predictions were in agreement for 75% of the chemicals (Table 13.1). Based upon the results of the *Salmonella* mutagenicity assays for the same chemicals, the mutagenicity of these chemicals was also estimated (Table 13.2).

Table 13.1 Some significant biophores associated with the probability of carcinogenicity of halogenated chemicals in rodents

Fragment size = *** 1 2 3 4 5 6 7 8 9 10 SUB		Number	Inactives	Marginals	Actives	Probability
Cl -CH =		4	0	0	4	0.063
Cl -CH_2-		19	5	2	12	0.058
Br -CH-		3	0	0	3	0.125
Br -CH_2-		3	0	0	3	0.125
O -CH_2-CH-		6	0	1	5	0.031
Cl -C*-Cl		5	1	0	4	0.109
Cl -CH -Cl		3	0	0	3	0.125
Cl -C -C	<2-Cl>	9	2	1	6	0.090
C =CH -C =C -		49	11	6	32	0.001
CH =C -C =CH -	54	14	7	33		0.005
CH =CH -C.=C.-	3	0	0	3		0.125
O -PO -O -CH_3	<3-O>	4	0	1	3	0.125
CH =CH -C =C -	<3-O>	7	0	0	7	0.008
Cl -C =C -C =CH -	<4-Cl>	4	0	0	4	0.063

C. = a carbon atom shared by two rings.
<2-Cl> = a chlorine atom at position 2 from the left.

During these analyses, chemicals were identified (Table 13.3) that are estimated to be carcinogenic in only one species but not in the other. This conclusion follows from the CASE identification of species-specific biophores. Furthermore, for halothane, a chemical may possess a biophore for one species yet may have a biophore for the other.

The accuracy of SAR estimates is very much a function of the quality and composition of the databases employed. Of the 60 organic halides evaluated in one

or more rodent carcinogenicity bioassays, 54 are reported in the NTP, IARC, and Gene-Tox databases, and a "consensus judgment" has been assigned. The 54 organic halides for which carcinogenicity data exist have been listed in order from negative to positive according to the consensus judgment. Although this group is incomplete with respect to the carcinogenicity of the 60 halides, substantial agreement exists for data generated by the NTP and the evaluations of IARC and Gene-Tox. Where adequate positive data exist (i.e., NTP, IARC, or Gene-Tox display classifications of LP, CE, S, or SP consensus judgment), the CASE program accurately estimates the carcinogenicity of organic halides. For those halides where inadequate or limited carcinogenicity data exist and for halides negative for carcinogenicity, CASE predictions are much worse. In fact, when the NTP carcinogenicity data are used to train CASE, several possible carcinogens not adequately represented in the training set (i.e., those labelled LP, CE, S, or SP consensus judgment) are not predicted by CASE. These agents include acridine mustard, benzyl chloride, vinylidene chloride, and pentachlorophenol. The correlation of carcinogenicity based on the consensus judgment with *Salmonella* mutagenicity is very poor for the halides studied, with only 51% concordance.

13.7.1 PROFILE MATCHING

To determine patterns or similarities in genetic activity profiles for a group of chemicals, the data for each possible pair of chemicals was examined by computer over the entire series of tests to find common test results (Figure13.5; j_a (abbreviated a) and j_b (abbreviated b); bioassays 2 and 4 are common to both chemicals; however, results are positive in test 4 for chemical b and negative for chemical a). The comparative evaluation of chemicals used the binomial distribution to determine the probability (p) that concordant results would occur by chance among the common tests in two different profiles. The p value was the primary function for evaluating the significance of a qualitative match between a pair of chemicals. The agreement in the relative magnitudes of common profile lines was determined by calculating the dose-related function (DRF). A DRF was computed for common concordant test results only, and is termed the DRF_c. The p and the DRF_c value may be used as screening tools to obtain matched profiles at preselected levels of significance and quantitative agreement.

Using a p value cutoff of $p \leq 0.05$, two highly statistically significant matches were obtained for the aryl halides. The matches were between the structural analogue pairs 2,4-D and 2,4,5-T and between p,p′-DDD and p,p′-DDE. For the saturated alkyl halides, four matches of the previously stated quality were found. Matches were found for carbon tetrachloride with halothane and with 1,1,2,2-tetrachloroethane, and ethylene dibromide was matched with dibromochloropropane and with epichlorohydrin.

Table 13.2 Estimation of carcinogenicity in rodents and comparison of estimations for two *Salmonella* mutagenicity databases for halogenated compounds

CAS no.	Chemical	C/R	Mutagenicity Overall	Mutagenicity %
00071-55-6	1,1,1-Trichloroethane	+	+	67.0
00079-34-5	1,1,2,2-Tetrachloroethane	+	-	20.0
00096-12-8	1,2-Dibromo-3-chloropropane	+	+	82.9
00106-93-4	1,2-Dibromoethane	+	+	80.0
00107-06-2	1,2-Dichloroethane	+	+	66.0
00078-87-5	1,2-Dichloropropaned	+	+	74.0
01746-01-6	2,3,7,8-TCDD	+	-	7.0
00093-76-5	2,4,5-Trichlorophenoxyacetic	+	-	7.0
00088-06-2	2,4,6-Trichlorophenol	+	-	38.0
00094-75-7	2,4-Dichlorophenoxyacetic acid	+	+	38.0
00151-67-7	2-Bromo-2-chloro-1,1,1-trifluor\	m	+	63.0
00101-14-4	4,4′-Methylenebis(2-chloroben\	+	-	86.7
00051-21-8	5-Fluorouracil	+	-	0
16238-56-5	7-Bromomethyl-12-methylBA	+	+	63.0
24961-39-5	7-BromomethylBA	+	+	63.0
00309-00-2	Aldrin	+	-	7.0
01912-24-9	Atrazine	+	-	0
00100-44-7	Benzyl chloride	-	+	63.0
00314-40-9	Bromacil	+	+	67.0
00075-25-2	Bromoform	+	+	63.0
00133-06-2	Captan	+	m	57.0
00056-23-5	Carbon tetrachloride	+	-	0
00056-75-7	Chloramphenicol	m	m	55.6
00108-90-7	Chlorobenzene	-	-	25.0
00075-45-6	Chlorodifluoromethane	-	-	0
00067-66-4	Chloroform	+	-	36.0
00439-14-5	Diazepam	-	-	36.2
00072-55-9	Dichlorodiphenyldichloroethylene	+	-	38.0
00075-09-2	Dichloromethane	+	+	66.0
00062-73-7	Dichlorvos	+	m	57.0

GAPs showed that the compounds named above are negative in the Ames test, but positive in *Saccharomyces cerevisiae* for gene mutation, mitotic recombination and gene conversion. No highly significant matches were obtained for the unsaturated alkyl halides. However, at lower levels of significance ($p \leq 0.05$), some structurally related compounds (e.g., aldrin, dieldrin, heptachlor) displayed matches. If the stringency of matching criteria are relaxed ($p \leq 0.05$) and the focus is placed on matches based on positive test results, matches from some of the saturated alkyl halides become apparent. According to this criterion, ethylene dibromide matches with dibromochloropropane, epichlorohydrin, benzyl chloride, tris (2,3-dibromo-

Table 13.2 (Continued)

CAS No.	Chemical	C/R	Mutagenicity Overall	Mutagenicity Per Cent
00060-57-1	Dieldrin	m	-	7.0
00072-20-8	Endrin	m	-	7.0
00106-98-8	Epichlorohydrin	+	+	84.7
00074-96-4	Ethyl bromide	+	+	80.0
00133-07-3	Folpet	m	m	57.0
00076-44-8	Heptachlor	+	-	7.0
00146-59-8	ICR 170	-	m	43.5
17070-44-9	ICR 191	-	+	63.6
00054-42-2	Iododeoxyuridine	m	-	0
00058-89-9	Lindane	-	-	0
00094-74-6	MCPA	+	-	38.0
00072-43-5	Methoxychlor	+	-	0
00074-83-9	Methyl bromide	NT	NT	0
00150-66-6	Monuron	+	-	33.3
00303-47-9	Ochratoxin A	+	-	0
00072-55-9	p,p′-DDE	+	-	38.0
00082-68-8	Pentachloronitrobenzene	-	-	13.3
00087-86-5	Pentachlorophenol	-	-	7.0
67774-32-1	Polybrominated biphenyl	+	-	0
00709-98-8	Propanil	+	-	10.1
00072-54-8	Tetrachlorodiphenylethane	+	-	25.6
00127-18-4	Tetrachloroethylene	+	-	30.0
00052-68-6	Trichlorfon	+	-	0
00079-01-6	Trichloroethylene	+	-	0
01582-09-8	Trifuralin	+	+	70.0
00126-72-7	Tris(2,3-dibromopropyl)phosph\	+	+	74.4
00075-01-4	Vinyl chloride	+	-	0
00075-35-4	Vinylidene chloride	-	-	0

C/R = carcinogenicity in rodents

propyl)-phosphate, and dichloromethane. These pair-wise matches can be displayed graphically. Clearly the more structurally complex compounds such as tris(2,3-dibromopropyl)phosphate, that have greater structural diversity than most of the saturated alkyl halides identified as matches, will likely belong in more than one chemical class. The results of profile matching are consistent with the presence in each of these compounds of the CASE *Salmonella* biophore [(*X*)-CH_2-] where *X* = Cl, Br, or I. Where marginal matches (0.05) were included, all compounds with the (*X*)-CH2- biophore were identified. Structural alerts [(*X*)-CH_2- and/or $C(X)_4$ where *X* = H, F, Cl, Br, or I in any combination] have also been reported by Ashby *et al.* (1989). A computerized SAR method, a biological comparative assessment approach, and expert opinion lead to similar conclusions in this example.

Table 13.3 Identification of chemicals estimated to be carcinogenic in one species but not in another

CAS no.	Chemical	Carcinogenicity Rat	Mouse
00079-34-5	1,1,2,2-Tetrachloroethane	-	m
00078-87-5	1,2-Dichloropropane	-	+
00151-67-7	2-Bromo-2-chloro-1,1,1-trifluoroethane	-	+
00309-00-2	Aldrin	-	m
00075-25-2	Bromoform	-	+
00133-06-2	Captan	-	m
00056-23-5	Carbon tetrachloride	-	m
00067-66-4	Chloroform	-	+
00072-55-9	Dichlorodiphenyldichloroethylene	-	+
00133-07-3	Folpet	-	m
00076-44-8	Heptachlor	-	m
00094-74-6	MCPA	-	+
00072-55-9	p,p′-DDE	-	+
00127-18-4	Tetrachloroethylene	-	+
00079-01-6	Trichloroethylene	-	+
01582-09-8	Trifuralin	-	m
00075-01-4	Vinyl chloride	-	+
00101-14-4	4,4′-Methylenebis(2-chlorobenzene)	+	-
00051-21-8	5-Fluorouracil	+	-
00106-89-8	Epichlorohydrin	+	-
00150-66-6	Monuron	+	-
00709-98-8	Propanil	+	-

- = negative; + = positive; m = marginal

13.8 WEIGHT-OF-EVIDENCE SCORING SYSTEM

Committee 1 of the International Commission for Protection Against Environmental Mutagens and Carcinogens (ICPEMC) has been involved for several years in the development of a computer-based methodology to assess the evidence from short-term genetic tests that a chemical is a mutagen (Lohman *et al.*, 1990). The evaluative approach selected by ICPEMC Committee 1 is based on a "weighted test" scoring system. Input data for this methodology have been obtained from the GAP database described above. Data are first combined into a test score. The test score is the product of several factors representing dose, the sign of the test result (+ or -), and the use of metabolic activation. This process is repeated for each type of test included in the battery, and the results are combined and weighted over a sequence of data reduction steps. The results from each test (and replicate) are used in generating the overall assessment of the chemical being evaluated. The analysis is continued through the hierarchy of information, agglomerating data from individual tests into results for classes representing similar kinds of tests into

overall *in vitro* or *in vivo* values and then combining these into a final score for the agent.

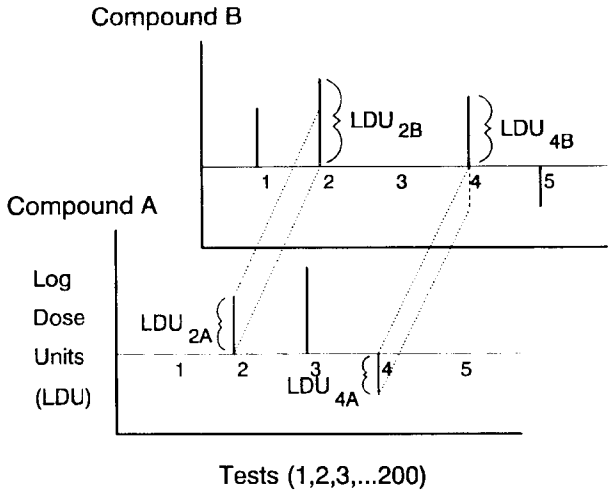

Figure 13.5 Genetic activity spectra for five bioassays of two chemicals

Table 13.4 illustrates the final score from the Committee 1 evaluation compared to the present "consensus judgment" for carcinogenicity. The three compounds found negative in carcinogenicity testing (pentachloronitrobenzene, halothane, and methoxychlor) are at the negative end of the ICPEMC ranking. The ranking clearly differentiates chemicals that display quantitative differences in genotoxicity. The last eight compounds (on the positive end) of the ICPEMC weight-of-evidence ranking (Table 13.4) are detected, and grouped by profile matching techniques. Five compounds (1,2-dichloroethane, dibromo-chloropropane, ethylene dibromide, tris(2,3-dibromopropyl)PO_4, and epichlorohydrin) are considered to have sufficient evidence of carcinogenicity. Also a number of the sufficient positive carcinogens are not detected in genotoxicity assays, and, therefore, display an overall negative score.

ICPEMC Committee 1 is investigating a consensus view with carcinogenicity as the endpoint of choice (Nesnow, 1990). For practical use, the test weights may be subject to adjustment following internal and/or external standardization. For such a standardization, data on long-term carcinogenicity studies in rodents, epidemiological data, or SAR may be used.

In addition to its utility in weight-of-evidence evaluations, the ICPEMC Committee 1 scoring method is intended to serve as a mechanism to evaluate test system consensus for diverse kinds of tests. As illustrated by Figure 13.6, the combined agent score for 34 halogenated organic compounds may be plotted versus the score for the test class. Figure 13.6 shows results for sister-chromatid

exchange, chromosomal aberrations, and cell transformation in mammalian cells *in vitro*. These results indicate that test class scores for sister-chromatid exchange and chromosomal aberrations are most consistent with consensus. Although cell transformation assays are inconsistent with consensus, these assays display a positive class score for so-called non-genotoxic carcinogens such as chloroform and carbon tetrachloride. The *in vivo* test classes that are most consistent with consensus are (a) heritable damage in insects; and (b) chromosomal aberration in mammals (data not shown). With respect to carcinogenicity, *in vivo* mammalian test classes (especially micronuclei and dominant lethal) have yielded negative scores for limited and sufficient carcinogens more frequently than did *in vitro* test classes (data not shown).

An expert system such as CASE can be based as easily on weight-of-evidence or consensus evaluations as any other biological endpoint. In fact, in the present instance such consensus evaluation was found to improve the CASE prediction accuracy. As mentioned earlier, while CASE was only partially successful in performing the QSAR portion of the program on the TD_{50}-based carcinogenicity database assembled by Gold *et al.* (1984, 1986, 1987, 1989), it was very successful in doing so when the TD_{50} values were modified by a weight-of-evidence carcinogen ranking scheme (Nesnow, 1990). Furthermore, CASE was also readily able to learn the human intelligence-based rules for identifying "structural alerts" of genotoxicity. Thus, the databases compiled using human intelligence/intuition weight-of-evidence appeared to provide a valuable resource and adjunct to purely computer-driven algorithms.

13.9 SUMMARY AND DISCUSSION

This chapter has discussed several general issues relating to the application of computational SAR methods, contrasted a few particular correlative SAR methods, considered general problems relating to indirect or direct SAR modelling of the carcinogenicity endpoint, and discussed database considerations. The role of SAR and biologically based techniques in chemical classification and test selection, and a weight-of-evidence scoring scheme have also been discussed.

A database on 60 organic halides, a general class considered difficult to test and evaluate for carcinogenicity, served as a vehicle for discussion of specific methods. Organic halides have frequently proven to be carcinogenic in animal studies. However, no existing mechanistic model adequately accounts for this activity, and several compounds are considered to be non-genotoxic carcinogens.

The so-called "genotoxic" and "non-genotoxic" carcinogens appear to fall into two groups: "genotoxic" carcinogens generally cause cancers in multiple species, in both sexes and at multiple sites, while "non-genotoxic" carcinogens appear to be greatly restricted in their specificity (Ashby and Tennant, 1988; Gold *et al.*, 1989). Additionally, "genotoxic" carcinogens generally display an enhanced carcinogenic potency (Rosenkranz and Ennever, 1990) and a decreased lipophilicity. The vast

majority of recognized human carcinogens are genotoxicants (Ennever *et al.*, 1987; Shelby, 1988; Bartsch and Malaveille, 1989), while presumed human non-carcinogens appear to be non-genotoxic (Ennever *et al.*, 1987).

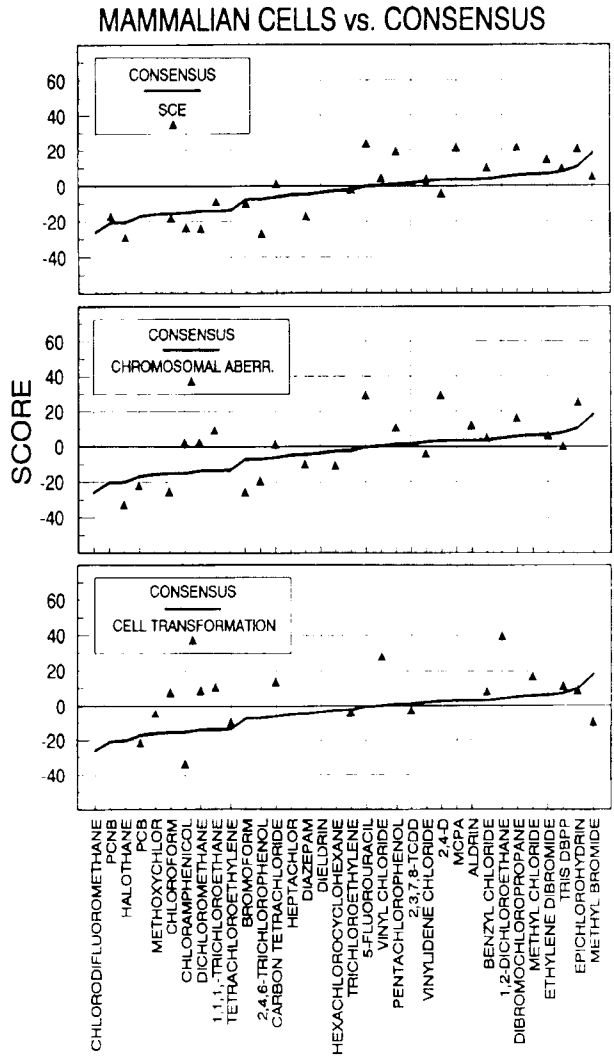

Figure 13.6 Scores for primary DNA damage and overall consensus judgment using ICPEMC Committee 1 approach

The genotoxicity of many of the halogenated organic compounds considered is undetected in the Ames test. Furthermore, poor correspondence exists between the

results of the Ames test when compared with the "consensus judgment" for carcinogenicity. The CASE programme was able to estimate accurately the carcinogenicity of many organic halides. Where only inadequate or limited data were available and for halides the data were negative for carcinogenicity, CASE estimates were less accurate. Even when NTP carcinogenicity data were used to train CASE, several agents labelled by consensus judgment as "limited positive," "clear evidence," "sufficient positive" were not estimated by CASE to be carcinogenic. Perhaps part of the explanation for the dichotomy in the predicted versus the actual carcinogenicity response lies in the dose necessary to cause a response in a short-term test versus that required to produce cancer in a rodent: The dose required for carcinogenicity is frequently 100 times that necessary to produce a response in a short-term test. Data presented in the GAP format for ethylene dibromide (Figure 13.7) illustrate that even among short-term tests, the doses needed to produce various responses range over several orders of magnitude. Thus, the mere presence of a chemical fragment or substituent may be insufficient to model chemical reactivity in a particular reaction mechanism that is important for carcinogenicity. Additional investigation is needed to determine the quantitative relationships between chemical structure, reactivity, and carcinogenicity for this chemical class.

The ICPEMC Committee 1 weight-of-evidence scheme may be useful to assess quantitative genotoxicity data and their relationship to carcinogenicity. In the enclosed illustration, the scheme was successful at separating many of the positive genotoxic carcinogens from known putative non-carcinogens. The scheme could not, however, deal effectively with the non-genotoxic carcinogens. Evidence presented elsewhere (McCoy *et al.*, 1990; Rosenkranz and Klopman, 1990b) suggests that CASE may be able to recognize structural fragments associated with some non-genotoxic carcinogens.

Prospectively, a useful approach may involve the application of biologically based comparative techniques (profile matching) and weight-of-evidence schemes for the subsetting of chemicals within a large quantitative genetic toxicology database. In this way, correlative SAR methods may be more effectively applied to identify the substructural elements responsible for particular biological responses and to suggest biologically plausible mechanisms of action. Clearly, additional investigation along the lines discussed in this chapter is necessary to further the utility of the various computerized techniques that may be applicable in the study of SAR across species.

Table 13.4 Carcinogenicity of 34 halides ranked using the ICPEMC Committee 1 approach

Compound	Carcinogenicity evaluation	ICPEMC score
Chlorodifluoromethane	L	-26.05
Pentachloronitrobenzene	LN	-20.45
Halothane	LN	-20.21
Polychlorinated biphenyls	S	-16.88
Methoxychlor	SN	-15.71
Chloroform	S	-15.28
Chloramphenicol	L	-15.11
Dichloromethane	S	-13.85
1,1,1-Trichloroethane	I	-13.81
Tetrachloroethylene	S	-13.48
Bromoform	CE	-7.29
2,4,6-Trichlorophenol	S	-7.22
Carbon tetrachloride	S	-6.18
Heptachlor	LP	-5.06
Diazepam	I	-4.68
Dieldrin	L	-3.67
Hexachlorocyclohexane	LP	-2.70
Trichloroethylene	LP	-2.34
5-Fluorouracil	I	-0.26
Vinyl chloride	S	0.20
Pentachlorophenol	CE	1.09
2,3,7,8-TCDD	S	1.38
Vinylidene chloride	LP	2.59
2,4-D	L	3.14
MCPA	L	3.29
Aldrin	LP	3.34
Benzyl chloride	LP	3.58
1,2-Dichloroethane	S	4.58
Dibromochloropropane	S	5.71
Methyl chloride	I	6.37
Ethylene dibromide	S	6.60
Tris(2,3-dibromopropyl)PO_4	S	7.77
Epichlorophydrin	S	10.60
Methyl bromide	L	18.33

CE = clear evidence of carcinogenicity; I = inadequate evidence of carcinogenicity; L = limited evidence of carcinogenicity; LN = limited negative;

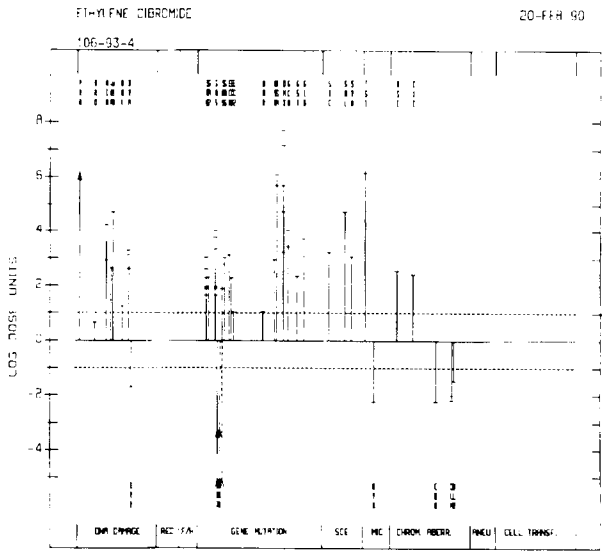

Figure 13.7 GAP of ethylene dibromide

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14 Structure–Activity Relationships: Experimental Approaches

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14.1 INTRODUCTION

Effects of genotoxic agents can vary by species, sex, and individual, as well as by cell type and stage in the same individual. Among the variables causing diversity in response are the types of adducts formed with DNA, their persistence, mutagen dose, and the time interval between the formation of premutagenic lesions and DNA replication. In spite of these variables, the question remains whether cause–effect relationships can be established for action spectra of genotoxicants that have significance beyond the experimental or environmental conditions under which the correlations are established. This problem is of foremost importance to assess relative risks due to exposure to genotoxic agents and to formulate conclusions from data obtained from different assays systems. Therefore, improvement of the understanding of underlying mechanisms by which genetic alterations are mediated is imperative. Therefore, all of the foregoing factors must be addressed before assessments of genetic hazards produced by synthetic and naturally occurring mutagenic chemicals can be made.

14.2 EARLY APPROACHES

Early attempts at classifying biological effects of mutagens occurred in the 1960s when fundamental chemistry principles were applied to catalogue the substitution reactions of alkylating agents (AAs) with nucleophiles in nucleic acids and proteins (Ross, 1962). The relative reactivity of various alkylating agents towards different nucleophilic centres in biomacromolecules was considered in terms of the rates of these substitution reactions, and according to the principle of Swain and Scott (1953). Swain and Scott developed a two-parameter equation to correlate the

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relative rates of reactions of various nucleophilic reagents (e.g., water, acetate, pyridine, thiosulphate) with a series of substrates (alkyl halides, esters, epoxides) in water solution. This linear relationship is described by

$$\log \left(\frac{k_y}{k_{H_2O}}\right) = s \times n \qquad (14.1)$$

where

k_{H2O} is a rate constant for reaction with water,

k_y is the corresponding rate constant for reaction with any other nucleophilic reagent,

s is a constant characteristic of the substrate, and

n is a constant characteristic of the nucleophilic reagent.

Usually, methyl bromide is chosen as a standard substrate, for which $s = 1.00$ to determine values of n; from n values, s values can be obtained for direct acting agents of unknown nucleophilic selectivity.

The Swain–Scott empirical linear relationship has proven to be highly valuable to understand the great diversity in genetic activity profiles (GAP) shown by the large group of alkylating agents. This diversity is primarily the consequence of varying alkylation patterns in DNA and proteins. These patterns have been reviewed in detail on several occasions (Hemminki, 1983; Singer and Grunberger, 1983; Coles, 1984–85; Saffhill *et al.*, 1985). For instance, AAs with high s values, typified by methyl bromide (MBr) or methyl methanesulphonate (MMS), show high selectivity in their reactions with nucleophiles, and are particularly efficient towards centers of high nucleophilicity, such as N^7 of G and N^3 of A in DNA, and of -RS$^-$ and -RSH in proteins. The opposite type of AA has a strong affinity for exocyclic oxygen, and is represented by *N*-ethyl-*N*-nitrosourea (ENU) and *N*-ethyl-*N'*-nitro-N-nitrosoguanidine (ENNG). As a consequence of variation in intrinsic adduct distribution, GAPs of even closely related AAs vary considerably, as was first shown by Ehrenberg and coworkers (1966) in studies of plants. By systematically analyzing the biological effects of several monofunctional alkyl methanesulphonates in barley (Osterman-Golkar *et al.*, 1970), successful attempts have been made to obtain for AAs semi-quantitative correlations of mutagenic, clastogenic, and cytotoxic activities with chemical structure and physico-chemical characters. Similar studies on other higher plants (Gichner and Veleminsky, 1967), mammalian cells in culture (Couch and Hsie, 1978; Couch *et al.*, 1978), and *Drosophila* (Vogel and Natarajan, 1979a, b) further supported the feasibility of the concept.

To illustrate the value of physico-chemical properties in predicting general GAPs, the relative reactivities of AAs with different nucleophiles are compared in Table 14.1. The relative reaction rates with thiosulphate to acetate increase much faster for epichlorohydrin (ECH; factor = 2382) and methyl bromide (MBr; factor = 4380)

than for isopropyl methanesulphonate (iPMS; factor = 10), reflecting the higher nucleophilic selectivity of agents with a high s value. Consequently, most modifications by ECH and MBr are on proteins but not on DNA, that may cause cell lethality, and thus demonstrating mutagenic properties of high s value chemicals may often be difficult experimentally. Aside from reactivity, absorption and stability have a major impact on mutagenic or carcinogenic responses in a living organism (Figure 14.1).

Table 14.1 Relative reactivity of alkylating agents with nucleophiles compared with water

		$k_y \div k_{H2O}$			
		1	2	3	3/1
Substrate	s	Acetate (n=2.72)	Pyridine (n=4.43)	Thiosulfate (n=6.36)	
Isopropylmethane-sulfonate (iPMS)	0.28	6	12	60	10
Ethyl methane-sulfonate (EMS)	0.64	55	200	11300	205
β-Propiolactone (PL)	0.77	125	590	80000	640
Epichlorohydrin (ECH)	0.93	340	2250	810000	2382
Methyl bromide (MBr)	1.00	525	4000	2300000	4380

From Swain and Scott (1953) and Ross (1962)

This whole concept (i.e., analysis of multiple genetic endpoints in relation to specific chemical properties of the genotoxic agent) has not been applied. Progress in understanding how genotoxic chemicals exert their biological effects across species has been impeded by the lack of a concept considering both physico-chemical properties and GAPs of chemicals. The single end-point approach, relating induced genetic damage to exposure dose is of limited value for quantitative structure–activity considerations despite of its wide application in more than 150 tests for mutagenicity. For example, attempts to correlate the carcinogenic potency of chemicals with qualitative mutagenicity data obtained from a single

mutagenicity test turned out to be an oversimplification of a complex matter, as demonstrated for a set of AAs tested in the *Salmonella* assay (Bartsch *et al.*, 1983).

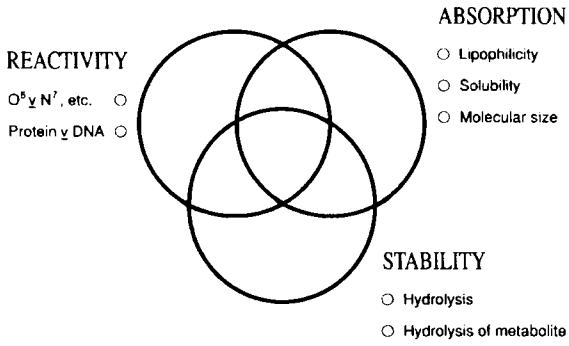

Figure 14.1 The three major physico-chemical factors influencing the extent to which a (DNA-reactive) parent chemical or metabolite may elicit a mutagenic or carcinogenic response in a living organism

14.3 CRITERIA AND METHODS FOR INTERSPECIES COMPARISONS

A most critical point in comparative mutagenesis studies with cellular systems or multicellular organisms is measurement of dose, i.e., the actual dose at the genetic material of the target cell under analysis. The external dose is not an adequate parameter to compare the genotoxic effects of chemicals, because of the large variation in their reactivity, stability, and absorption (Figure 14.1). Exact dose measurements at the genetically significant target are possible only when labelled mutagens or monoclonal antibodies are used (Drake, 1975); except for a few model compounds (mostly monofunctional AAs) such information is scanty. Another problem is that the types of DNA adducts and their relative distribution in DNA have been determined only for a minority of genotoxic chemicals.

To enable comparisons for a wide range of genotoxic agents, information is needed on the spectrum of genetic alterations produced in an assay system and the consistency of this spectrum over a range of organisms. Thus, if a genotoxic agent showed marked specificity for the production of a particular type of genetic damage, the consistency of this specificity must be defined over a wide range of organisms, and, if not, the variables causing heterogeneity in genotoxic effects must

be elucidated. The analytical tools available for such a comprehensive analysis comprise both traditional biological endpoints and recent molecular genetic techniques, that may be complemented by DNA dosimetry (Table 14.2). Some of the perspectives and limitations of the experimental possibilities are discussed below.

14.3.1 GENOTOXIC EFFECTIVENESS

Determinations of genotoxic effectiveness are based on the amount of chemical exposure (exposure concentration or dose) needed for the production of a specified frequency of mutations (Hussein and Ehrenberg, 1975). Factors that affect responses to a mutagen include uptake, penetration of and migration to the genetically significant target cells, rate of hydrolysis, and metabolic fate.
Consequently, even closely related genotoxicants may differ vastly in their mutagenic effectiveness. Typical examples include the small AAs: in *Salmonella typhimurium* strains TA98 and TA100 *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is more than 1000-fold more effective than dimethylnitrosamine (DMN) (Sugimura, 1982), despite their similar nucleophilic selectivity. Therefore, mutagenic effectiveness (i.e., expressing mutation induction per unit external dose) has little value to compare the response to mutagens among species.

14.3.2 GENOTOXIC EFFICIENCY

Ideally, the extent and the distribution of DNA adducts that result from interaction with genotoxic agents should be used for interspecies comparisons. Presently, such data exist mainly for the small group of methylating and ethylating carcinogens.

14.3.3 RELATIVE GENOTOXIC EFFICIENCY

14.3.3.1 Relative carcinogenic potency

Analysis of reactions of small AAs with DNA in animals showed that neither the absolute amount of reaction with DNA nor the reaction at the N^7 atom of guanine could be correlated with carcinogenic potency (Schoental, 1967: Swann and Magee, 1968; 1971). By contrast, the relative ability to react with the O^6 atom of guanine (O^6-G) correlated well with carcinogenic action (O'Connor *et al.*, 1979), confirming the prediction of Loveless (1969) that the induction of mutations and cancer is correlated with O^6-G alkylation.

In studying the complex relationships between types of DNA damage and biological endpoints such as tumor formation, Bartsch and coworkers (Bartsch *et al.*, 1983; Barbin and Bartsch, 1986, 1989) used median TD_{50} estimates for rodents,

and compared these to the nucleophilic selectivity of mono- and bifunctional direct-acting alkylating agents. Nucleophilic selectivity of AAs was expressed by Swain–Scott constants s and/or the ratios of O^7-/O^6-alkyl guanine. A positive linear relationship between the log of TD_{50} estimates and s values was established for 17, mostly monofunctional, AAs (Barbin & Bartsch, 1989; Vogel *et al.*, 1990). In contrast, seven bifunctional alkylating agents (all antineoplastic drugs); chloroethylene oxide (CEO) and two methyl halides were more potent carcinogens than the above 17 AAs. These agents showed a higher carcinogenic potency than would have been predicted on the basis of their nucleophilic selectivity. With the seven cross-linking agents, no simple correlation was apparent between the TD_{50} of each AA and its s value. Strikingly, their TD_{50} values remained within narrow limits for six of the seven bifunctional antitumor drugs (Table 14.3), despite a range of s values between 0.53 (BCNU) and 1.39 (melphalan; MEL). This observation suggests that their ability for cross-linking DNA, rather than a definite nucleophilic selectivity, is the predominant cause of their high carcinogenic potency (Vogel *et al.*, 1990).

Both in terms of risk assessment models and mechanisms of action, a crucial question raised by these studies is what causes the remarkable differences in tumorigenic potency existing even between closely related carcinogens such as the direct-acting methylating agents (Table 14.4)? Answers may be forthcoming from a comprehensive analysis of genetic action spectra of carcinogens including characterization of induced mutational spectra at the molecular level, the role of unstable and persistent DNA lesions in relation to DNA repair, and the dose-dependency of each process.

Multiple genetic endpoints in relation to nucleophilic selectivity

Mutational spectra produced in various repair backgrounds can provide information on the role of different premutagenic lesions and on the significance of repair for mutation fixation. Isolation and subsequent characterization of carcinogen-induced base-sequence changes is of particular relevance when attempting to relate specific DNA adducts to the product (i.e., induced genetic damage). However, analysis of mutational spectra should not be restricted to sequence determination of newly induced mutations, but should involve a broader elaboration of the genetic action spectrum of the genotoxic agent under study. This outcome may be achieved by a combined application of molecular and general genetic techniques. The eukaryotic systems available for such a combined approach are lower eukaryotes (*Neurospora*), mammalian cells in culture, *Drosophila*, and the mouse (Table 14.5).

Extensive work on small AAs revealed the feasibility to link, under repair-proficient conditions, certain types of DNA alkylation products with various genetic endpoints in mammalian cells (Natarajan *et al.*, 1984) and in *Drosophila* (Vogel and Natarajan, 1979a, b). In V79 cells, AAs of low s values were less clastogenic than those with high s values, as indicated by the ratio of *hprt* mutations/breaks per

Table 14.2 Strategy and experimental tools for comparative analysis

Analysis of	System(s)	Information
1. **Genotoxic effectiveness**		qualitative
Single genetic endpoint in relation to exposure dose	prokaryotic + eukaryotic	(less suited for analysis of SAR)
2. **Mutagenic/carcinogenic efficiency**		quantitative
Single or multiple biological endpoints in relation to DNA dose (DNA dosimetry)	eukaryotic + mammalian	
3. **Relative genotoxic efficiency**		semi-quantitative
3.1 TD_{50} in rodents versus nucleophilic selectivity	mouse, rat, + hamster	
3.2 Measurement of multiple genetic endpoints compared with nucleophilic selectivity of mutagen	*Neurospora*, *Drosophila*, + mammalian cells in culture	
3.3 Genetic damage in relation relation to altered DNA repair condition	*Neurospora*, *Drosophila*, + mammalian cells	
3.4 Biological dosimetry (= relating genetic endpoints to each other) mammalian cells,+ mouse (?)	*Neurospora*, *Drosophila*,	
3.5 Cross comparisons		
4. **Molecular spectra of procarcinogen-induced mutations**		
DNA base alterations induced by *in vivo* systems (?) carcinogens requiring metabolic activation		types of ultimate electrophiles
5. **SAR considerations**		
Estimation and analysis of genotoxic properties from structural and physico-chemical properties		priority setting for in-depth analysis

100 cells (Table 14.5). A more recent analysis (Table 14.6), in which the ratios (CA/SLRL) of chromosome breakage events (CA, measured as ring-X loss) to recessive lethal mutation (SLRL) induction was compared with the nucleophilic

selectivity of monofunctional AAs, confirmed earlier conclusions (Zijlstra and Vogel, 1988). All these data suggest a correlation between the ability of AAs for base nitrogen alkylation in DNA and their chromosome-breaking efficiency. Conversely, AAs acting more extensively at the DNA base oxygen atoms, appeared to have a higher potential for mutation induction, as measured by traditional genetic techniques, such as the *Drosophila* sex-linked recessive lethal assay.

Table 14.3 Comparison of median TD_{50} (mg/kg bw) in rodents and s values for seven antitumour drugs with cross-linking properties

Compound	s	TD_{50}
Bis(chloroethyl)nitrosourea (BCNU)	0.53	34
Mitomycin C (MitC)	0.81	0.68
Busulfan (BUS)	0.90	<140
Thio-tepa (t-TEPA)	1.10	131
Mechlorethamine (MEC)	1.18	17
Chlorambucil (CA)	1.26	92
Melphalan (MEL)	1.39	67

The molecular techniques now available enable one to verify or modify earlier hypotheses and conclusions. In fact, direct miscoding mutagenesis due to oxygen modification seems the predominant mechanism leading to mutations with ENU-type agents for which O^6-EtG and O^4-EtT mediated transition mutations are the most common sequence changes in human cells (Eckert *et al.*, 1988), Drosophila (Pastink *et al.*, 1989), and in *Escherichia coli* (Richardson *et al.*, 1987b). Deletion mutations were either not found (Pastink *et al.*, 1989) or found very rarely (Kelley *et al.*, 1985). Compared with the effects of ENU, in *Drosophila*, the molecular mutational spectra changed for agents of higher nucleophilic selectivity, such as DES, EMS, MNU, and MMS (Nivard, 1991).

With MMS, next to intra-locus deletions (18%) and A:T→G:C transitions (20%), an increasing proportion of transversion mutations (56%) was found (Nivard *et al.*, 1992). By contrast, 95 to 100% of single base changes induced by DMS, EMS, MNU, and MNNG at the *lacI* gene of *E. coli* were transitions, mostly of the G:C→A:T type (Burns *et al.*, 1986, 1987, 1988; Richardson *et al.*, 1987a; Zielenska and Glickman, 1988). By analogy, all single base changes induced by EMS in mouse cells (Ashman and Davidson, 1987) and 53 of 54 mutations induced in human cells (Lebkowski *et al.*, 1986) were found to be G:C→A:T transitions. This discrepancy between *Drosophila* and cellular systems could be due to the shorter time for which the latter are at risk for mutagenesis. Alkylation at the N^7 and N^3 positions of purine bases, if not removed by repair enzymes, labilizes the glycosylic bond, leading to depurination (Loeb, 1985). This effect is time-dependent (Singer and Grunberger,1983), which could be one reason why in prokaryotic or other cellular systems, where the time interval between interaction with DNA and mutation fixation is short, mutation induction correlates best with O-alkylation.

Thus, altered spectra might be produced in more complex *in vivo* system, as recently shown for MMS for which 52% of mutations (mosaic mutations isolated from the F_2) fixed only after several rounds of replication represented A:T→T:A transversions. This value is significantly higher than the 25% A:T→T:A transversions found among *vermilion* mutations fixed immediately after fertilization (Nivard *et al.*, 1992).

Table 14.4 Tumorigenic potency of methylating AAs in relation to their nucleophilic selectivity

Chemical	*s*	O^6-/7-Alk-guanine[a]	TD_{50} (mg/kg bw)[b]
N-Methyl-*N*-nitrosourea (MNU)	0.42	0.11	28
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)	0.42	0.11	103
Dimethylnitrosamine (DMN)	—	0.10/0.13	70.2[c]
Methyl methanesulfonate (MMS)	0.86	0.004	12200
Trimethyl phosphate (TMP)	0.91	—	167000

[a] Vogel and Natarajan (1982)
[b] Barbin and Bartsch (1989)
[c] procarcinogen

14.3.3.2 Altered DNA repair

Classification of genotoxic carcinogens with regard to the magnitude of altered mutation induction (hypermutability) in repair-defective condition revealed a positive linear relationship between enhanced mutagenic effectiveness in excision-repair defective (*exr*$^-$) cells in *Drosophila* and the nucleophilic selectivity for a series of 18 carcinogens, representing either monofunctional and cyclic alkylating agents:

where,

$r = 0.79$ ($p<0.01$)

f_{exr-}/f_{exr+} denotes the enhancement ratio for mutation induction, and

$$\frac{f_{exr^-}}{f_{exr+}} = 12.4s - 1.9 \qquad (14.2)$$

s is the Swain–Scott constant (Vogel, 1989).

Table 14.5 Enhancement ratios for mutation induction in three repair-deficient systems in comparison with TD_{50} values for mono-functional AAs

Chemical	s	TD_{50}[a]	*Drosophila* *mei-9*	*Neurospora* *uvs-2*	CHO[b] *27-1*
MMS	0.86	12200	8.2	7.7	2.2
β-Propiolactone (PL)	0.77	562	6.3	4.0	NT
EMS	0.67	[110][c]	2.7	2.0	3.0
MNU	0.42	28	5.8	5.0	NT
MNNG	0.42	103	2.6	2.0	NT
ENU	0.26	11	1.3	NT	1.0

[a] TD_{50} in mg/kg per bw
[b] HPRT locus
[c] TD_{50} for diethyl sulfate
s = 0.64
NT = no test

For some agents, similar studies were conducted with *Neurospora* and Chinese hamster ovary cell lines (Zdzienicka and Simons, 1986). Table 14.7 compares mutagenicity data from three different systems with TD_{50} estimates for rodents (Barbin and Bartsch, 1989). Median TD_{50} values decreased with decreasing s, and the relative ranking for hypermutability response in *Drosophila mei-9* paralleled that in *Neurospora uvs-2*. However, the similarity in response of the two genetic systems to monofunctional AAs was not found for cross-linking agents: both *mei-9* and *mus-201* mutants of *Drosophila* (both are *exr*$^-$) were insensitive to cross-linking agents (Vogel, 1989), while the nucleotide excision-repair defective *uvs-2* mutant is not (deSerres and Brockman, 1986).

The striking parallelism in response to AAs between the two repair mutants of *Drosophila* and *Neurospora* and the positive correlations of TD_{50} values and hypermutability indices with nucleophilic selectivity suggest efficient error-free repair of ring nitrogen alkylation products. Further support for this conclusion comes from analysis of base sequence changes of *Drosophila vermilion* mutations produced in *exr*$^-$ genotypes after treatment with MMS: 63% of all changes (vs. 25% in wild-type) resulted from a modified adenine (Nivard *et al.*, 1992).

Table 14.6 Endpoints for measuring relative genotoxic efficiency (RGE)

System	Measurement of RGE	Specific loci[a]
Neurospora	Point (or intracistronic mutations) versus multi-locus deletions	*ad-3A*, *ad-3B*
Mammalian cells in culture	Chromosomal aberrations, SCEs and point mutations in relation to cell killing	*hprt*[a]
Drosophila	Chromosome breakage effects (chromosome loss, translocations) relative to X-linked recessive lethals (700 loci)	*white*[a], *adh*[a], *vermilion*[a]
Mouse	Specific-locus mutations, dominant lethals, and cataracts, translocations	7 loci

[a]Used for analysis of DNA base sequence changes

Suggestive evidence also exists that other cellular factors also play a role in the hypermutability response (Vogel, 1989). These factors are (a) the faster rate of removal of methyl adducts compared with ethyl derivatives (also well-established from repair studies on mammalian cells; Brent *et al.*, 1988), and (b) the length of the time period between DNA modification and onset of replication. Despite the many variables, a positive correlation was found between relative efficiency ranking of carcinogens and enhancement ratios for hypermutability. This finding suggests a strong function of DNA repair in the initial process of tumorigenesis.

Active repair also seems to be the reason that a large series of genotoxic carcinogens, mostly of high nucleophilic selectivity, failed to produce significant numbers of specific-locus mutations in early germ cells in the mouse (Table 14.8).

14.3.3.3 Biological dosimetry

The most desirable dosimetry procedure would be that providing information on the extent and the distribution of DNA adducts resulting from interaction with genotoxic agents. Where such data are unavailable, as is often the case, two or more biological endpoints may be compared with one another. For instance, a perusal of Table 14.9 reveals that the results of studies with AAs in CHO cells or

Table 14.7 Nucleophilic selectivity (s value) and relative efficiency for chromosome aberrations versus mutation in *Drosophila* (CA/SLRL)[a] and V79 cells[b]

Chemical	s	CA/SLRL[a]	Mutation($\times 10^{-5}$) /breaks per 100 cells[b]
Ethylnitrosourea (ENU)	0.26	0.04	13.7/31.1[c]
N-Ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG)	0.26	0.10	no test
Isopropyl methanesulphonate (iPMS)	0.29	0.17	no test
Methylnitrosourea	0.42	0.23	2.9
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)	0.42	0.19	no test
Diethylsulfate (DES)	0.64	0.13	no test
Ethyl methanesulfonate (EMS)	0.67	0.32	0.7
Dimethylsulfate (DMS)	0.86	0.44	1.0/1.1
Methyl methanesulfonate (MMS)	0.86	0.55	0.4
Trimethylphosphate (TMP)	0.91	0.35	no test

[a]Ratio of ring-X chromosome loss (CA) to recessive lethals (SLRL) in *Drosophila*; data from Zijlstra and Vogel (1988).
[b]Ratio of *hprt*-locus mutations to the number of breaks in V79 cells; data from Natarajan *et al.* (1984).
[c]Two experiments.

Drosophila are in remarkable agreement, showing a relationship with s values in both systems, if mutation induction is related to cytotoxicity (efficiency measurement). For both *Drosophila* and the CHO system, however, no relationship exists between s values and genotoxic effect, when mutation induction is compared per unit of dose. This situation is not surprising, because analysis on an equal-molar basis (mutagenic effectiveness) takes no account of the differences among AAs in rate constants, rates of decomposition, and other characteristics (Osterman-Golkar *et al.*, 1970; Vogel and Natarajan, 1982). Biological dosimetry may also be used for genotoxicant classification, even when information on nucleophilic

selectivity is unavailable (Table 14.10). For example, in *Drosophila*, the ratio of ring-X loss (CA) to recessive lethals (SLRL) has prognostic value in terms of functionality of genotoxic agents (Zijlstra and Vogel, 1988). Thus, the 14 genotoxic agents listed in Table 14.10, based on their CA/SLRL ratios, clearly fall into two distinctly different groups: cross-linking versus monofunctional agents.

Table 14.8 Induction of specific locus mutations in (102/E1 x C3H/E1) F_1 male mice

Compound	Germ cell stage		Ref.
	Postspermatogonia[a]	Spermatogonia	
Chlormethine	+	-	[1]
Cyclophosphamide	+	-	[2]
Diethylsulfate	+	-	[3]
Ethyl methanesulfonate	+	-	[4]
Ethylnitrosourea	-/+[b]	+	[5,6]
Methyl methanesulfonate	+	-	[7,8]
Mitomycin C	-	+	[8]
Procarbazine, HCl	+	+	[9,10]
Vincristine	-	-	[11]

[a]For specification of different postspermatogonial germ cell stages.

[b]Among 15542 offspring, only one complete specific locus mutation, but nine mosaic mutations were obtained.

References:
[1] Ehling and Neuhäuser-Klaus (1989a)
[2] Ehling and Neuhäuser-Klaus (1988a)
[3] Ehling and Neuhäuser-Klaus (1988b)
[4] Ehling and Neuhäuser-Klaus (1989b)
[5] Favor *et al.* 1990)
[6] Ehling and Neuhäuser-Klaus (1984)
[7] Ehling and Neuhäuser-Klaus (1990)
[8] Ehling (1978)
[9] Kratochvilova *et al.* (1988)
[10] Ehling and Neuhäuser (1979)
[11] Ehling *et al.* (1988).

Comparisons of various endpoints have also been applied to the study of germline mutagenesis in the mouse, where tests for heritable translocations, dominant lethals, and specific-locus mutations can be combined with screening for dominant-cataract mutations, protein-charge mutations, and enzyme-activity mutations (Ehling *et al.*, 1985). This work is still in its inventory phase, since, apart from X-irradiation, only few model carcinogens (cyclophosphamide, ENU, MMS, mitomycin C, and procarbazine) have been analyzed by this multiple-endpoint approach (Ehling *et al.*, 1985; Ehling, 1988).

Table 14.9 Mutagenicity of alkylating agents in CHO cells and in *Drosophila*

Activity per unit exposure dose (mutagenic effectiveness):

CHO cells: EMS < ENU < DMS < MMS < MNU < ENNG < MNNG

Drosophila: EMS = iPMS < MNU < MMS+DMS < ENU

Activity relative to cytotoxicity (mutagenic efficiency):

CHO cells[a]: DMS < MMS < MNU < MNNG < iPMS < ENNG < ENU < DES < EMS

Drosophila[b]: DMS = MMS < MNU, iPMS < EMS < ENU

[a]HPRT mutants at 10% survival (Couch and Hsie, 1978; Couch *et al.* 1978; Natarajan *et al.*, 1982).

[b]Dose that produced 4% SLRL at 50% survival (Vogel and Natarajan, 1979a).

Cross-comparisons of TD_{50} and genotoxic properties

Two chemicals provide examples of the relationships between TD_{50} and genotoxic properties: Vinyl chloride (VC) and 7,12-dimethylbenz[*a*]anthracene (DMBA).

Apart from the well-studied class of small monofunctional AAs, few attempts have been made to relate carcinogenic potency estimates to formation and persistence of specific DNA adducts. An example is that of 2-chloroacetaldehyde (CAA) and of chloroethylene oxide (CEO) in VC carcinogenesis. Proteins and RNA, not DNA, are the major targets for CAA, and CEO is the metabolite responsible for VC carcinogenicity (Singer and Bartsch, 1986). Analysis of the genotoxic properties of both CAA and CEO provide strong support for such a conclusion (Table 14.11). CEO was shown to cause G:C→A:T transition mutations in *E. coli*, that could arise from the hypothetical N^4-(2-chlorovinyl)-deoxycytidine (Barbin and Bartsch, 1986). Interstrand cross-links have been demonstrated *in vitro* in salmon sperm DNA treated with CAA; its cross-linking property is also apparent from the CA/SLRL ratio of 19.0 in *Drosophila* (Table 14.11). In the *Drosophila* repair test with the excision-repair defective strain *mei-9*, CAA (a highly toxic agent) acts as a weak mutagen (a doubling of the spontaneous mutation rate was found) under exr^+ condition. In the absence of intact excision-repair, no effect was found. Its low genotoxic potential is in keeping with its *s* value of 1.3. Consequently, CAA could not be characterized by the *Drosophila* repair assay. The *s* value of CEO (0.71) is similar to that of glycidaldehyde (GA, 0.83), 1,3-propane sulphone (PS, 0.71), and ß-propiolactone (PL, 0.77); thus hypermutability effects comparable with these three agents are anticipated. By contrast, no effect at all was

found for CEO (Table 14.10). This observation is in remarkable agreement with the mechanism suggested for CEO-mutagenesis in *E. coli*. This agent mutated *recA*$^-$ strains more efficiently than a *recA*$^+$ strain. The absence of any elevated response in the *exr*$^-$ genotype in *Drosophila* (Vogel, 1989) suggests that direct miscoding may also be the mutagenic pathway of CEO in *Drosophila*.

7,12-Dimethylbenz[*a*]anthracene (DMBA) is another agent that gives no enhanced response in the *Drosophila* repair assay (Table 14.11). Thus, for three agents for which direct miscoding mutagenesis is likely to be a significant pathway (i.e., ENU, CEO, and DMBA), TD_{50} values indicated high tumorigenic potential (Table 14.11). Analysis is needed of DNA sequence changes of mutations induced in both repair-intact and repair-deficient cells, utilizing human and other mammalian cells, and *Drosophila*.

14.4 MOLECULAR GENETIC SPECTRA OF PROCARCINOGENS

Determination of molecular mutational spectra (i.e., "fingerprint" analysis) induced by both procarcinogens and their presumptive or established genotoxic metabolites is expected to provide information on the kind of DNA modifications involved. Yet unknown genotoxic action pathways may become apparent from such an analysis. For instance, for initial reaction products that they produced with DNA, methylating and ethylating AAs were among the best characterized genotoxic agents. Both *s* values and O^6-/O^7-alkylG ratios have been determined for most members of this group. However, in spite of close similarities of the O^6-/O^7-EtG ratio of DEN and ENU, the spectra of mutations found in the *Drosophila vermilion* gene are not the same (Pastink *et al.*, 1989; Sierra *et al.*, 1989). A considerable part of DEN-induced base sequence changes is transversions (46%), whereas 79% of those induced by ENU were transition mutations. By analogy, substantial numbers of transversions (C:G→A:T or A:T→T:A) were also found in activated *H-ras* genes from DEN-induced mouse liver tumor DNA (Stowers *et al.*, 1988). Whether a high constitutive activity of O^6-alkylG DNA-transferase, that is present in the liver (Pegg, 1983), or whether other factors are responsible for the unexpected mutational pattern remains unclear.

Another example of an unexpected finding is the significantly lower hypermutability indices of MNNG and DMN in *Drosophila* compared with MNU (Vogel, 1989). For DMN, bioactivation products other than the methylating species (e.g. formaldehyde and/or HO-dependent activation) could be the reason that characterization of this group by their O^6-/O^7-metG ratios does not fully describe their genotoxic properties (Malaveille *et al.*, 1987). Thus substantial heterogeneity in DNA base change spectra can be expected even for "closely related" carcinogens.

Table 14.10 Cross-comparisons between genotoxic properties of carcinogens and their tumorigenic potency (TD_{50} estimates)

AAs	*s*	TD_{50}[a]	*exr*⁻/*exr*⁺ CA/SLRL *Drosophila*	27-1 CHO[c]	Suggested mechanism[b]
ENU	0.26	11	1.3	0.04	1.0	direct
DMBA	—	61	1.4	<0.04[c]	nt	direct?
CEO	0.71	72-0.43[d]	0.74	nt	nt	direct?
PS	0.71	1760	4.9	0.08	nt	indirect?
PL	0.77	562	6.3	0.39	nt	indirect?
GA	0.83	5960	12.3	2.56	nt	indirect?
MMS	0.86	12200	8.2	0.55	2.0	indirect/ mispairing
CAA	1.3	10000/ 15000[d]	inconcl	19	nt	crosslinks (weak)

[a]TD_{50} in mg/kg bw; from Barbin and Bartsch (1989).
[b]Predominant mechanism suggested to be either direct miscoding or indirect miscoding/misrepair mutagenesis.
[c]DMBA, a well established mutagen in *Drosophila*, did not measurably increase the frequency of ring-X loss (CA)
[d]The carcinogenic potency of CEO ranged from 0.43 to 72 mg/kg bw, and the estimate for CAA is 10 to 15 g/kg bw (Barbin and Bartsch, 1989).
[e]Zdzienicka and Simons (1986); *HPRT* locus.

14.5 SAR AMONG CARCINOGENS/MUTAGENS IN GENERAL

In the foregoing discussion, emphasis was placed on simple alkylating agents (AA); the fact that AAs are not a single uncomplicated series is perhaps the most important point to emerge from that discussion. Thus, the production of those mutations critical to carcinogenicity and the nature and extent of the carcinogenicity induced by an AA were shown to be dependent upon a matrix of variables, the major ones being either chemical (hydrolytic half-life, intrinsic reactivity, type of reactivity) or biological (extent and sites of reaction, response to adducts in terms of timing and fidelity of repair, or replication prior to repair). Within this context, no general and simple correlation should be expected—rather, subtle correlations between two parameters are to be expected once all other variables have been normalized. One could actually turn the matter around, and suggest that to expect simple axioms such as "gene mutagens will also always be clastogens" would be misleading. Once simple correlations are not sought or contrived, meaningful correlations are likely to emerge.

Moving away from relatively simple AAs into the broad middle ground of chemical carcinogenicity, the problems faced become immense, due mainly to the requirement for metabolic activation to produce a DNA-reactive (electrophilic)

Table 14.11 Ratios of ring-X loss (CA) to recessive lethals (SLRL) for a series of 14 genotoxic agents (Zijlstra and Vogel, 1988)

Chemical	CA/SLRL	Classification[a]
1,2-Dibromoethane (DBE)	0.37	monofunctional
Diethylnitrosamine (DEN)	< 0.04	monofunctional
Dimethylnitrosamine (DMN)	0.16	monofunctional
Dacarbazine (DTIC)	0.09	monofunctional
1-Phenyl-3,3-dimethyltriazene (PDMT)	0.22	monofunctional
Procarbazine (natulan, PC)	0.14	monofunctional
1-(2,4,6-Trichlorophenyl)-3,3-dimethyl-triazene (Cl_3PDMT)	0.29	monofunctional
Busulfan (BUS)	5.40	cross-linking
2-Chloroacetaldehyde (CAA)	19.0	cross-linking
2-Chloroethyl methanesulfonate (CEMS)	2.77	cross-linking
Cisplatin (DDP)	6.79	cross-linking
Cyclophosphamide (CPA)	8.54	cross-linking
Hexamethylphosphoramide (HMPA)	11.98	cross-linking
TEPA	14.60	cross-linking

[a]CA/SLRL values > 2 property for cross-linking

intermediate. Thus, once one includes the concept of metabolic transformation of a chemical, the possibility of forming several different electrophiles must be entertained, along with the likelihood of encountering metabolic pathways that will be directed towards elimination of the molecule from the target area, DNA. Adding to the complexity of attempting to simulate *in vitro* mammalian metabolism *in vivo* by an induced rodent liver subfraction (S9 mix), the situation approaches insolubility. This complexity must be accepted if progress is to be made. For example, the reference carcinogen benzo[*a*]pyrene is known to be mutagenic in the *Salmonella* assay (+S9 mix) via formation of the 4,5-epoxide, yet carcinogenic via formation of the bay region diol epoxide (Rosenkranz *et al.*, 1985). Likewise, the *N*-acetyl group on the 2-aminofluorene/DNA adduct drastically affects the response of DNA to the adduct—yet a complex of metabolic factors determines the presence or absence of this acetyl group, factors that vary among species and strains of rodents, and hence among the several S9 mixes used in routine *in vitro* testing. That reaction with isolated DNA of the model ultimate carcinogen for 2-acetylaminofluorene (2AAF), the *N*-acetoxy derivative 2-AAAF, failed to mimic the true interaction of 2AAF with cellular DNA emphasizes the complication of studying procarcinogens. Therefore, general SARs must be used as an adjunct to a scientific approach of estimating and understanding chemical carcinogenesis—not

as a means to bypass thought and experimentation. The following general considerations exist:

1. Chemical structure. The major chemical groupings associated with chemically induced cancer are shown in Figure 14.2 (Ashby *et al.*, 1989). These groups are usually associated with activity in short-term genotoxicity assays and with genotoxic carcinogenesis (Ashby and Tennant, 1988; Shelby, 1988; Ashby *et al.*, 1989; Bartsch and Malaveille, 1989; Tomatis *et al.*, 1989). An obvious hierarchy exists, and more agents of suspect structure are known than have been shown to be genotoxic *in vitro*, genotoxic *in vivo*, and/or carcinogenic.
2. Sites of carcinogenesis. Tomatis *et al.* (1989) recently warned against requiring coincidence between sites of carcinogenesis in humans and animals before animal data are regarded as an adequate model of these human effects. Nonetheless, certain patterns of carcinogenesis in rodents are emerging that can influence trans-species extrapolations, including those to humans. Thus, agents that are genotoxic (i.e., reactive to DNA) are more likely to be active across species and in several tissues, while non-genotoxic agents have a higher chance of being species- or sex- or site-specific in their rodent carcinogenicity. Furthermore, a sub-group of rodent tissues seems to be sensitive only to genotoxicants; this knowledge may contribute to trans-species extrapolations (tissues such as Zymbal's gland, skin, and circulatory system; Ashby *et al.*, 1989; Tomatis *et al.*, 1989). Thus, qualification of the term "carcinogen" is probably a necessary precursor to discern SARs of value useful in understanding trans-species extrapolation of cancer data.
3. Chemical studies. That small changes in chemical structure can produce large and predictable changes in biological activity, and that the synthesis and biological evaluation of key chemical entities are areas of this science that have been neglected, and which could be usefully reinstituted. The fact that replacement of the two protons on MNU with alkyl groups can extend the hydrolytic half-life of this agent 1000-fold (Figure 14.3), while retaining alkylating potential, is an illustration of this principle.
4. Metabolic Studies. Although not routinely practical, detailed metabolic and toxicokinetic studies are usually the surest way to rationalize the apparent species-specificity of a procarcinogen; such studies usually enable confident estimates of human carcinogenic potential for a rodent carcinogen.

14.6 CONCLUSIONS AND PERSPECTIVES

Much of the information in this review has been derived from work with alkylating carcinogens. This monograph illustrated how a combination of both classical and molecular genetic methods may be used to acquire information across species on cause–effect relationships in the action of genotoxicants. The analytical tools available for such a comprehensive analysis include determination of multiple

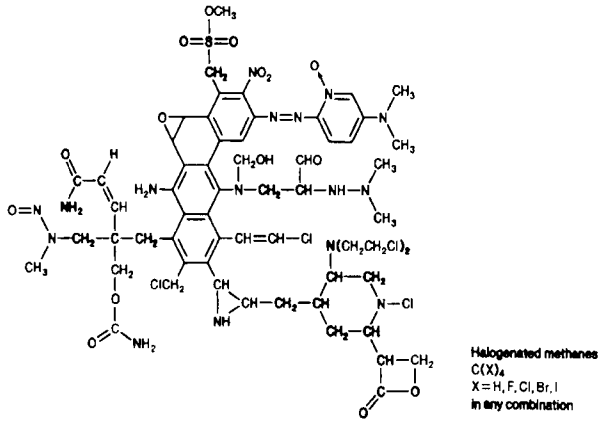

Figure 14.2 Main structural features of model chemical: DNA-reactivity, mutagenicity, and genotoxic carcinogenicity (structurally alerting fragments provided in Ashby *et al.*, 1989)

genetic endpoints related to fundamental physico-chemical properties of genotoxicants (such as Swain–Scott's *s* value), and modification of DNA repair condition and tumorigenic potency (TD_{50} compared with initial DNA interaction patterns). Although a systematic analysis of carcinogen-induced molecular mutational spectra has just begun, this approach promises to be particularly relevant to our understanding of the actions of genotoxic carcinogens in the following ways:

1. By comparing genetic action spectra of procarcinogens with those of their established or presumptive metabolites; such an analysis may help identify reactive intermediates relevant for the formation of premutagenic lesions.
2. By determining DNA base sequence changes of carcinogen-induced mutations produced in repair-intact versus repair-deficient condition; appropriate test systems (e.g., transgenic mice) have yet to be developed, before the likely link between DNA repair and carcinogenesis can be properly tested for mammals.

The correlations for relative potency ranking already established between tumorigenic potency in rodents, hypermutability response in *exr*$^-$ strains of *Drosophila* and *Neurospora*, and nucleophilic selectivity of AAs make it very likely that such a correlation is likely to be found for mammals.

To select proper model compounds, priority may be given to

1. Genotoxic carcinogens characterized as having low TD_{50} values (e.g., DEN,

DMBA, ENU, CEO, MitC), which is probably due to their ability for direct-miscoding (monofunctional agents) and/or cross-linking potential.

2. The mechanisms underlying the generally low tumorigenic potency of small AAs with a high *s* value; for human exposure, agents known to form cyclic adducts with nucleic acids are particularly relevant. However, one problem inherent with their generally high nucleophilic selectivity is their low mutagenic efficiency in relation to cytotoxicity, that makes the production of mutations at a single locus a cumbersome task. Some scientists hesitate to include this type of genotoxic agent into molecular mutational spectral analysis.

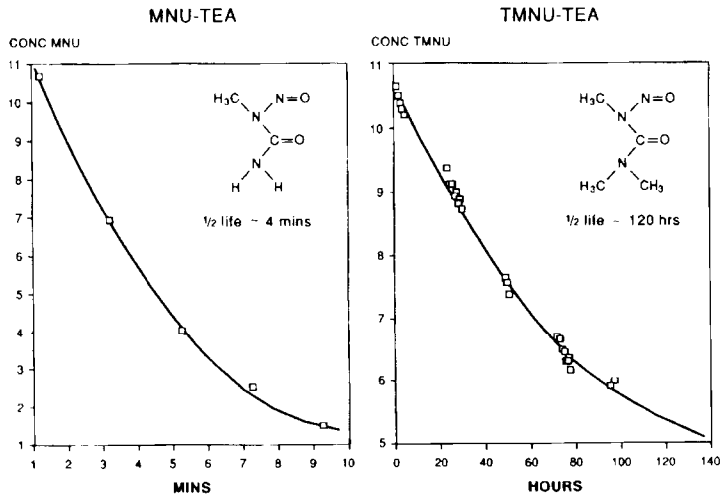

Figure 14.3 Extending the hydrolytic half-life 1000-fold results from replacing two protons on methylnitrosourea (MNU) to produce trimethylnitrosourea

14.7 REFERENCES

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IPCS Joint Activity 19
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METHODS TO ASSESS DNA DAMAGE AND REPAIR

Interspecies Comparisons

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Numerous chemical and physical agents in the environment are capable of reacting with DNA. These potentially DNA-damaging substances include not only man-made compounds, but also those of natural origin (e.g. food constituents and ultraviolet radiation). Fortunately, mammalian species possess powerful defence systems to overcome the potentially harmful effects of such agents.

This monograph, and the workshop from which it is derived, evaluates the current state of knowledge of defence mechanisms and applies this knowledge to estimate the risk to health of humans exposed to substances that alter genetic material. The analysis is meant to be an overview rather than an exhaustive treatment of new and promising approaches. To limit possible bias introduced by a "windowed" view from the use of particular species, a specific endpoint, or a limited class of DNA-damaging agents, emphasis is placed on integrating information obtained from various disciplines.

This evaluation deals predominantly with mechanisms of activation/detoxification of DNA-damaging agents, organotropic and cell-structure effects, the induction and repair of DNA damage, and the molecular and phenotypical analysis of mutation induction.

Carcinogens can cause injury to humans and their environment. *Methods to Assess DNA Damage and Repair* provides guidance for the structured acquisition of key information to reduce cancer risks throughout the environment.

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