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.

12.9 REFERENCES

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