Mutations Induced by 1-Nitrosopyrene and Related Compounds During DNA Replication in Human Cells and Induction of Homologous Recombination by These Compounds

Veronica M. Maher, Nitai P. Bhattacharyya, M. Chia-Miao Mah, Janet Boldt, Jia-Ling Yang, and J. Justin McCormick

_Michigan State University, East Lansing, MI_

Includes the Commentary of the Institute's Health Review Committee

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SYNOPSIS OF RESEARCH REPORT NUMBER 55

RELATIONSHIP OF NITROPYRANE-DERIVED DNA ADDUCTS TO CARCINOGENESIS

BACKGROUND

Diesel engine exhaust contains hundreds of chemicals, both in its vapor phase and also adsorbed onto small, respirable, carbon particles. Nitropyrenes, which form during diesel fuel combustion, are a major class of these chemicals. Nitropyrenes cause mutations (structural changes in DNA, the cell's genetic material) and are carcinogenic in some animals. Mutations may occur when inhaled nitropyrenes are metabolized to active derivatives that combine with DNA. Such combinations of chemicals with DNA are called DNA adducts. Because adducts interfere with normal DNA replication, their presence can result in mutations that may ultimately affect a gene's function and initiate carcinogenesis.

Carcinogenesis is a multistep process involving a number of changes in normal cellular activities. Because cancer cells proliferate faster than normal cells, scientists have proposed that normal restraints on proliferation may be lost during carcinogenesis. Protooncogenes and tumor suppressor genes are normal genes that help regulate cell proliferation. Mutations can convert protooncogenes to oncogenes (cancer-causing genes), which stimulate cell proliferation above normal levels. Furthermore, because tumor suppressor genes inhibit cell proliferation, losing their function removes a control on proliferation. This loss of function may occur by a combination of mutation and a process called homologous recombination. In this latter process, two DNA segments combine with each other. Such recombination can cause a loss of tumor suppressor gene function.

This study used sophisticated molecular biology techniques to examine whether nitropyrene-DNA adducts cause changes, such as mutation and homologous recombination, that can alter the function of genes that confer normal restraints on cell proliferation.

APPROACH

To study the effect of nitropyrene-DNA adducts on mutation, Dr. Veronica Maher and colleagues exposed a specific gene, in culture, to each of two nitropyrene derivatives. They then (1) measured the number of adducts formed by each derivative, (2) analyzed the chemical structure of the adducts, and (3) determined the region of the DNA the adducts formed. When the investigators allowed the genes containing adducts to replicate, mutations occurred. They compared the kinds of mutations in the untreated gene with the mutants of treated genes, one that had replicated, and one that had not been exposed to nitropyrene derivatives. To determine the relationship between the location of DNA adducts and mutation, the investigators compared the regions in DNA where adducts formed to those regions where mutations occurred. In another series of experiments, the researchers exposed genes to nitropyrene derivatives to determine if nitropyrene-DNA adducts stimulated homologous recombination.

RESULTS AND IMPLICATIONS

Exposing a specific gene, in culture, to activated derivatives of 1-nitropyrene and 1,6-dinitropyrene produced DNA adducts. The investigators found that the principal binding site on the DNA was the same for the two nitropyrene derivatives tested, and the kinds of mutations that resulted from these adducts were similar. Mutations occurred at several similar regions in DNA; however, each nitropyrene derivative also caused mutations at distinctive regions of the DNA, producing a specific mutation "fingerprint" for each chemical. Such fingerprints have the potential for providing useful biomarkers in cases of suspected human exposure to mutagens by furnishing cellular evidence of exposure and identifying the type of mutagen involved. The investigators established that nitropyrene-induced mutations occurred primarily at regions of DNA where adducts preferentially formed. In addition, exposing a specific gene to activated 1-nitropyrene increased homologous recombination; the extent of recombination increased with increasing levels of DNA adducts.

In summary, Dr. Maher and colleagues used two model cell culture systems to demonstrate that DNA adducts produced by nitropyrene derivatives cause mutations and stimulate homologous recombination. Gene mutations can convert protooncogenes to oncogenes, or can begin the process leading to loss of tumor-suppressor gene function. The latter process can be completed by homologous recombination. Thus, these findings may have important implications for understanding the mechanism of diesel exhaust-induced carcinogenesis, and support the hypothesis that exhaust constituents, such as nitropyrenes, can cause two types of genetic changes that are implicated in carcinogenesis.

This Statement is a summary prepared by the Health Effects Institute (HEI) and approved by the Board of Directors, of a research project sponsored by HEI from 1997 to 1999. This study was conducted by Dr. Veronica M. Maher and associates at Michigan State University. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.
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**HEI Research Report Number 55**

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The Statement is a nontechnical summary, prepared by the HEI and approved by the Board of Directors, of the Investigators' Report and the Health Review Committee's Commentary.

## II. INVESTIGATORS' REPORT  Veronica M. Maher et al. ........................................ 1

When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three outside technical reviewers and a biostatistician. The Report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in the selection or management of the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee, and, if necessary, revise their report.

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## III. COMMENTARY  Health Review Committee .................................................. 41

The Commentary on the Investigators' Report is prepared by the HEI Health Review Committee and staff. Its purpose is to place the study into a broader scientific context, to point out its strengths and limitations, and to discuss the remaining uncertainties and the implications of the findings for public health.

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ABSTRACT

The transformation of normal human cells into cancer cells is a multistep process. Evidence suggests that a minimum of five independent steps (changes) are required for the development of certain kinds of human cancer, as well as for malignant transformation of human cells in culture. Mutations are one of the mechanisms involved in bringing about such changes. A single DNA base substitution mutation can activate an oncogene or inactivate a tumor suppressor gene. Because the action of tumor suppressor genes is to prevent cells from becoming malignant, the activity of both copies of such genes must be eliminated before suppression is lifted. Homologous mitotic recombination between a mutant tumor suppressor gene allele and its non-mutant allele is one mechanism for accomplishing this.

The present study was designed to investigate the mechanisms by which certain carcinogenic compounds found in diesel exhaust particles and structurally-related N-substituted aryl carcinogens induce such base substitution mutations and homologous recombination events in mammalian cells in culture, including human cells. The system we employed to determine rapidly the kinds of mutations induced by these compounds, as well as the location of the point mutations in the target gene, involved a circular DNA molecule (plasmid) carrying a small target gene, supF. The target gene was exposed in vitro to radiolabeled compounds and then was allowed to replicate in human cells where the mutations were formed. The sites of mutation induction were compared with the sites of stable binding of the carcinogens to the DNA (adducts).

The system used to determine whether these agents could induce homologous recombination consisted of a thymidine kinase-deficient mouse L cell line with a recombination substrate stably integrated into the genome. To determine whether or not excision repair was involved in the mechanism by which carcinogens induced recombination, the recombination substrate was introduced into an excision repair-proficient human cell line and two repair-deficient human cell lines. These cell lines were then compared for the frequency of recombination induced by the agents.

All four N-substituted aryl compounds tested in the supF mutagenesis assay produced mainly base substitutions involving guanosine-cytosine (G+C)* base pairs, primarily G+G → thymidine-adenine (T+A) transversions. However, 1,6-dinitropyrene adducts, formed by exposing the plasmids to 1-nitro-6-nitrosopyrene in the presence of a reducing agent, also induced a significant proportion (17%) of single G+C base-pair deletions. Although there were common sites in the supF genes at which mutations frequently were seen in mutants derived from plasmids exposed to one or the other compound, i.e., “hot spots” in common among some of the other mutation spectra, each agent induced its own unique set of hot spots and “cold spots.” There was a good correlation between hot spots for initial adduct formation and for subsequent mutation induction within the human host cell.

1-Nitrosopyrene, 1-nitro-8-nitrosopyrene, and N-acetoxy-2-acetylaminofluorene were tested in the mouse cell recombination assay and compared with what was found for two structurally related carcinogens, the 7,8-diol-9,10-epoxide of benzo[a]pyrene and 4-nitroquinoline-1-oxide. 1-nitro-6-nitrosopyrene was inactive in these mouse cells, but each of the other agents caused a concentration-dependent increase in the frequency of recombination.

The frequency of recombination induced by 1-nitrosopyrene was compared in the three human cell strains constructed for this purpose. The dose required to cause a specific increase in frequency of recombination in the extremely repair-deficient strain was significantly lower than that required for the normally repairing strain. The dose required for the strain with low, residual repair capacity was slightly higher. These data strongly suggest that the presence of un-
excised DNA adducts, rather than the excision repair process itself, stimulates intrachromosomal homologous recombination in human cells.

INTRODUCTION

The growing concern over the availability of oil as a source of fuel for automobiles has prompted consideration of increased reliance on diesel engines. This, in turn, has led to increased interest in the human health effects of exposure to diesel engine emissions and diesel exhaust particles. Long-term exposure to high concentrations of diesel exhaust emissions increases lung tumor incidence in rats and mice (Ishinishi et al. 1986); and the activity is associated with the particulate fraction of the diesel exhaust (Heinrich et al. 1988). The predominant nitrated polycyclic aromatic hydrocarbon in diesel exhaust is 1-nitropyrene (1-NP), but 1.3-, 1.6-, and 1.8-dinitropyrenes (1.3-, 1.6-, and 1.8-DNP), which are tumorigenic in laboratory animals (Nesnow et al. 1984; Ohtake et al. 1984; Tokiwa et al. 1986; Wislocki et al. 1988; King 1987; Iwagawa et al. 1989), also have been detected. 1-Nitropyrene also has been shown to cause tumors in some experiments (El-Bayoumy et al. 1982, 1984; Hirose et al. 1984; Ohtake et al. 1984; Wislocki et al. 1988), as has its partially reduced metabolite, 1-nitrosopyrene (1-NOP) (Wislocki et al. 1986).

Our interest in these carcinogens and structurally related N-aryl compounds stems from our ongoing efforts to understand the mechanisms by which normal human cells are transformed into malignant cells (McCormick and Maher 1988). It is now recognized that malignant transformation both in vivo and in vitro is a multistep process and that mutations can bring about one or more of the steps or changes involved. In fact, a single DNA base substitution mutation can activate a cellular proto-oncogene, such as H-ras, into an oncogene (Tabin et al. 1982), and the kinds of base changes in the oncogenes of the tumor-derived cells are specific to the carcinogen used to induce the tumors (Barbacid 1987). A single base substitution also can inactivate tumor suppressor genes (Malkin et al. 1990; Stratton et al. 1990). Because the action of tumor suppressor genes is to prevent the cell from becoming malignant, the function of both alleles of such genes within the cell must be eliminated before a malignant tumor can form (Cavenee et al. 1983, 1985). Evidence suggests that one mechanism by which this can occur is homologous mitotic recombination between a mutant allele and the nonmutant allele. Such recombination renders the cell homozygous for the mutant form of the gene and eliminates the corresponding suppressor function (Cavenee et al. 1983, 1985; Scrable et al. 1990).

There are, however, very few assay systems available for detecting such rare mitotic recombination events or for determining whether the frequency of such events can be increased by exposure to carcinogens. Therefore, we turned to a model system initially developed by Liskay and Stachel (1983), involving extrachromosomal homologous recombination, to test the hypothesis that carcinogens can induce recombination.

With the support of the Health Effects Institute, we showed previously that 1-NP and its metabolite, 1-NOP, can induce mutations in an endogenous gene of diploid human fibroblasts in culture (Patton et al. 1986). Other investigators showed that 1.6-DNP is mutagenic in bacteria (Beland et al. 1985; Rosenkranz and Meredith 1985), and in mammalian cell assays (Li and Dutcher 1983; Takayama et al. 1983). When the present study was proposed in 1986, there were several unanswered questions: How do these compounds cause mutations? Do the human cellular processes distinguish between DNA adducts formed by structurally related carcinogens or do they treat them all in a similar fashion? What impact does nucleotide excision repair have on such mutagenesis? Can such agents induce homologous recombination in mammalian cells, and if so, what mechanisms are involved? The present study was designed to answer these questions.

To examine the mechanisms by which 1-NP and 1.6-DNP induce mutations at the sequence level, we proposed to determine whether there are specific sites in a small target gene at which the DNA adducts formed by partially reduced derivatives of these two carcinogens, i.e., 1-NOP and 1-nitro-6-nitrosopyrene (1-N-6-NOP), preferentially cause mutations. If such specific sites exist, we wanted to explore whether these mutations correspond to sites of preferential binding of the compound to the DNA. We also wanted to see whether two structurally-related carcinogens, N-acetoxy-2-acetylaminofluorene (N-AcO-AAF), and N-acetoxy-N-trifluoroacetyl-2-aminofluorene (N-AcO-TFA-AF), which bind covalently to DNA at the same position as 1-NP and 1.6-DNP (i.e., form adducts at the C8 position of guanine), would induce the same kinds of mutations as 1-NOP or 1-N-6-NOP and at the same sites in the target gene.

When we proposed this study in 1986, we had extensive experience inducing mutations in the hypoxanthine(guanine)phosphoribosyltransferase (HPRT) gene, an endogenous gene of diploid human cells. However, there was no practical way to analyze mutations in this gene at the sequence level. Such studies are now quite feasible, in part because of the technique developed in this laboratory by Yang and associates (1989) to copy mRNA directly from a lysate of a small clone of mutant cells into cDNA and amplify the cDNA 1011-fold and then sequence the product directly.
This technique eliminates the need to prepare very large populations of cells derived from a mutant clone, a difficult achievement if diploid finite life span human cells are the target cells. We have applied this technique extensively (Chen et al. 1993, 1991; Lukash et al. 1991; McGregor et al. 1991; Yang et al. 1991). However, this technique was not available in 1986, and, therefore, the mutagenesis assay system we proposed to use consisted of a plasmid that carries a defined target gene and is capable of replicating in mammalian cells as well as in bacteria (a shuttle vector). We exposed the plasmid DNA to the N-substituted aryl compound to be tested, analyzed the number and kinds of adducts formed (the residues covalently bound to the DNA), and then transfected the plasmids into human cells to be replicated. The progeny plasmids were recovered from the human cells, and those containing a mutated target gene were identified in indicator bacteria. The specific mutational changes and their locations in the target sequence were determined by sequencing the target gene in those mutants. Our target gene, the supF gene, which codes for a tyrosine suppressor tRNA, is ideal for such a study because its small size makes it relatively easy to analyze at the sequence level. This gene also is sensitive to single base-pair changes at almost any position in the 85-base-point region of the tRNA (Kraemer and Seidman 1988) and, therefore, is ideal for determining hot spots and cold spots, i.e., sites where there is a nonrandom distribution of mutations induced by carcinogens. The human host cell for plasmid replication was the kidney cell line 293, which gives a very low spontaneous background frequency of supF mutants (Yang et al. 1988).

To examine the ability of 1-NP and related carcinogens to induce homologous recombination, we proposed at the outset of this research to expose a mouse L cell line, 338M, containing two defective copies of a herpes simplex 1 virus thymidine kinase (tk) gene, to either various concentrations of 1-NOP or to the structurally related carcinogens, and then select for cells that had acquired a functional gene by homologous recombination. We and our colleagues (Wang et al. 1988) were the first to show that several other chemical carcinogens, including the 7,8-diol-9,10-epoxide of benzo[a]pyrene, \( \frac{\pm}{\pm} \)-7\( \beta \)-8\( \alpha \)-dihydroxy-9\( \alpha \),10a-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), induced dose-dependent increases in the frequency of intrachromosomal homologous recombination in this cell line. However, we could not determine whether the mechanism or mechanisms involved DNA repair processes. In the present study, we proposed to use structurally related radiolabeled carcinogens and compare their ability to induce recombination as a function of the number of DNA adducts formed, and thus see whether the cellular processes involved in recombination dealt with the carcinogens in a common manner. We further proposed to transfer the homologous recombination substrate into the genome of a series of human cell strains that differed in their capacity to remove such adducts from their DNA. If successful, in generating a suitably matched set of cell strains, we planned to use the strains to determine whether these N-substituted aryl carcinogens induce recombination by stimulating excision repair processes, which might give rise to single-stranded intermediates that are subject to recombination, or whether homologous recombination was stimulated by the presence of such adducts remaining unexcised in DNA.

**SPECIFIC AIMS**

The long-range purpose of the research was to gain insight into the mechanisms by which N-substituted aryl carcinogens, such as 1-NP and 1,6-DNP, cause the genetic changes involved in the malignant transformation of mammalian cells. We particularly wanted to shed light on the mechanisms by which such agents induce mutations in human cells in culture. To this end, we wanted to see whether human cells distinguish between structurally related DNA damage, i.e., whether each agent would cause a unique spectrum of mutations. A related purpose was to find out whether these carcinogens could induce homologous recombination in mammalian cells, and if so, whether this would be achieved by mechanisms involving DNA excision repair processes.

**STUDIES OF MUTAGENESIS**

The specific goals of the mutagenesis experiments were:

1. to determine the frequency of mutations induced in a target gene (supF) when a plasmid containing structurally related adducts formed by 1-NP, 1,6-DNP, N-AcO-TFA-AF, or N-AcO-AAF replicated in human cells, and compare these on the basis of equal numbers of adducts formed per plasmid to determine which agents were more effective mutagens;
2. to compare the ability of these adducts to interfere with bacterial transformation, as one measure of their biological activity;
3. to compare the spectrum of mutations induced by each agent, i.e., the kinds of mutations and their locations in the target gene;
4. to determine the nature of the adducts formed and to determine indirectly their distribution within the target gene and to compare the frequency of adduct formation at particular sites with the frequency of mutations induced at these same sites; and
5. to compare the results obtained with these four N-substituted aryl carcinogens, which form adducts primarily or exclusively at the C8 position of guanine, with our previous findings with a related carcinogen, BPDE, which binds to the N\(^2\) position of guanine.

STUDIES OF HOMOLOGOUS RECOMBINATION

The specific goals of the homologous recombination experiments were:

1. to determine whether 1-NOP, 1-N-6-NOP, or N-AcO-AAF can induce intrachromosomal homologous recombination in the mouse L cell line;
2. to compare the frequency of recombination induced by these structurally related agents with the frequency observed previously with BPDE on the basis of equal concentration applied, equal cell killing, and equal numbers of DNA adducts formed;
3. to determine the molecular nature of the recombinational events induced by these agents and compare the results with what is obtained spontaneously; and
4. to attempt to construct suitably matched strains of excision repair–proficient and excision repair–deficient human cells containing a low number of integrated copies of the recombination substrate, and then use them to determine whether the mechanism of 1-NOP–induced recombination involves nucleotide excision repair processes.

MATERIALS AND METHODS

STUDIES OF MUTAGENESIS

Chemical Carcinogens

All of the radiolabeled carcinogens were generally tritiated. 4-Nitroquinoline-1-oxide (4-NQO) (204 mCi/mmol), N-AcO-AAF [4.7 Ci/mmol], and BPDE [692 nCi/mmol] were purchased from Chemsyn Science Laboratories (Lenexa, KS). N-Acetoxy-N-trifluoracetyl-2-aminofluorene (63 mCi/mm mol) was generously supplied by Dr. Charles M. King of the Michigan Cancer Foundation (Detroit, MI). 1-Nitropyrene (217 mCi/mm mol at a purity of 99.9%) and 1-N-6-NOP (764 mCi/mm mol) were generously supplied by Dr. Frederick A. Beland of the National Center for Toxicological Research (Jefferson, AK). Unlabeled 4-NQO and N-AcO-AAF were obtained from the National Cancer Institute Carcinogenesis Research Program. Unlabeled 1-NOP and 1-N-6-NOP were supplied by Dr. Beland. All four agents were supplied as solids and stored at –20°C in a desiccator.

Plasmid Shuttle Vector

When this investigation was begun in 1987, it was not feasible to isolate endogenous genes, such as the HPRT gene, from mammalian cells to analyze them at the DNA sequence level for changes in structure. Therefore, we used the shuttle vectors pZ189 and pS189, which were exposed to the test compound in vitro and then allowed to replicate in human cells in which the mutations occurred. The shuttle vector pZ189, generously supplied by Dr. Michael Seidman (Osaka Pharmaceutical, Rockville, MD), is made up of 5,504 base pairs and contains the gene for tyrosine suppressor transfer RNA, supF, which is flanked by two genes essential for survival of the plasmid in its bacterial host under selective conditions: the genes for ampicillin resistance and for the bacterial origin of replication. It also contains the origin of replication gene and the large tumor antigen gene from simian virus 40, which allow the plasmid to replicate in human cells (Seidman et al. 1985). This plasmid was employed in our mutagenesis studies of BPDE, 1-NOP, N-AcO-TFA-AF, and N-AcO-AAF, which were carried out or begun before pS189 had been constructed. For the last compound tested, 1-N-6-NOP, we used shuttle vector pS189. This vector is identical to pZ189 except that Dr. Seidman deleted 170 nonessential base pairs between the supF gene and the gene for ampicillin resistance. This lowered the background mutations by decreasing the chance of detecting mutants containing large rearrangements involving the supF gene (Seidman 1989).

The supF gene is an exquisite target for this kind of study for many reasons: because it codes for a tRNA, rather than coding for a protein, one does not have the problem of virtually every third base being unimportant for coding; there are no stretches of genetic material that do not code (introns); and there is no large fraction of the coding region that is "silent," i.e., not responsive to base pair alterations because the corresponding amino acids do not play an important role in the function of the protein so that such mutations are not found when selection is applied. A base substitution, or deletion, or insertion at almost any of the sites making up the structure of the tRNA gene eliminates the suppressor function of the tRNA and results in an identifiable phenotypic change (Kraemer and Seidman 1989). This makes the gene exceptionally useful because one does not have to analyze thousands of transfectants to find one that exhibits a nonfunctional target gene. Another advantage is that there is no selection applied with the supF gene. The replicated plasmids are merely rescued from the human host cells, transferred into indicator bacteria, and those that happen to have a mutation inactivating the target supF gene are identified by the color of the bacterial colony they formed.
When this research began, no one had determined the spectrum of mutations induced by carcinogens in the HPRT gene. However, recently, Maher and associates (Chen et al. 1990, 1991; Yang et al. 1991) determined the spectrum for BPDE in the coding region of the HPRT gene, and found mutations that were very similar to those that they (Yang et al. 1988) found with BPDE by using the shuttle vector pZ189 and allowing it to replicate in human 293 cells.

**Bacterial Cells**

The bacterial host used for identifying plasmids with a mutant supF gene was *Escherichia coli* SY204 (Sarkar et al. 1984), which is sensitive to ampicillin, and cannot produce β-galactosidase unless the nonsense mutation in that gene is suppressed by a suppressor tRNA, such as supF. Bacteria that contain a functioning supF gene are able to produce β-galactosidase, and thus form blue colonies on agar containing an indicator dye and an inducer of the galactosidase gene (Yang et al. 1988). Mutants that have lost a functioning supF gene form white or very light blue colonies.

**Human Host Cell Line and Culture Conditions**

The human host used was an embryonic kidney cell line, designated 293 (Graham et al. 1977) that was obtained from Dr. Michele Calos of Stanford University (Stanford, CA). It was chosen because it allows efficient replication of shuttle vectors with a low frequency of background mutations (Lekowski et al. 1984). The cells were grown in Eagle's minimal essential medium supplemented with 0.2 mM L-serine, 0.2 mM L-aspartic acid, 1 mM sodium pyruvate, and 10% fetal calf serum (culture medium) in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C.

**Transfection and Rescue of Replicated Plasmid**

Human 293 cells (0.5 to 1 × 10⁶), plated into a series of 150-mm diameter dishes, were transfected with 6 µg of plasmid, using calcium phosphate coprecipitation (Graham and Van der Eb 1973), but without the glycerol shock. After 40 to 48 hours, low molecular weight DNA (plasmid) was extracted and separated from cellular DNA (Hirt 1967), purified with buffered phenol, treated with RNase A (50 µg/mL) at 37°C for one hour and proteinase K (100 µg/mL) at 50°C for two hours, extracted with buffered phenol/chloroform, precipitated with ethanol, and further purified by drop dialysis (Sillhavy et al. 1984). The DNA was treated with DpnI to digest any DNA that still had the bacterial methylation pattern of input plasmid. (Plasmids that have replicated in human cells no longer have such a pattern and are immune from digestion with this enzyme.) DNA extracted from the target cells in each individual dish was kept separate from other DNA so that after the plasmid DNA had been sequenced we could distinguish mutations occurring frequently (hot spots) from putative siblings arising from within the same population.

**Bacterial Transformation and Mutant Identification and Characterization**

Plasmids to be assayed for an inactive (mutated) supF gene were transferred into SY204 using the method described by Hanahan (1983), and the bacterial transformants that took up a plasmid were selected for resistance to ampicillin on plates containing indicator dye, an inducer of β-galactosidase, and ampicillin (Yang et al. 1987). Bacteria that contained a functioning supF gene formed blue colonies on indicator agar; bacteria with a mutant supF formed white or very light blue colonies. The frequency of supF mutants was determined from the number of these latter colonies expressed as a fraction of the total colonies. Each candidate mutant colony was streaked on fresh plates to confirm the phenotype. If they proved to be white or light blue, the bacterial cells in the colony were grown to very large numbers overnight in broth, and the plasmid DNA was extracted from the bacteria, dissolved in buffer, and analyzed for altered DNA mobility (gross alterations) by electrophoresis on 0.8% agarose gels (Maniatis et al. 1982). Plasmids that did not show any evidence of gross alterations, i.e., no size change greater than 150 base pairs, were analyzed further by a secondary bacterial transformation to ensure that the cells' inability to produce β-galactosidase actually reflected inactivation of the supF gene. The mutated supF genes were then sequenced using a modification of the dideoxyribonucleotide method of Sanger and associates (1977) and ³⁵S-labeled adenosine 5'-triphosphate (Yang et al. 1987). Plasmids were denatured with alkali to generate single-strand templates (Zagursky et al. 1985), and polymerization was carried out using modified T7 DNA polymerase (Sequenase, United States Biochemical Corp., Cleveland, OH).

**Treatment of Plasmids with Radiolabeled Carcinogen**

A stock supply of pZ189 or pS189 plasmids was prepared by amplifying a colony of bacteria overnight by the method described; an alkaline lysis procedure was used to obtain the plasmids, which were purified by ethidium bromide and cesium chloride density gradient centrifugation (Maniatis et al. 1982). A small volume of a solution containing a generally tritiated carcinogen of high specific activity was added to a small volume of plasmid DNA solution. The nature of the solvent used depended upon the particular carcinogen being tested (see below). For the formation of 1-NP
or 1,6-DNP adducts, DNA was dissolved (at 300 to 500 μg/mL in 10 mM helium-purged sodium citrate buffer, pH 5.0) and added to a freshly prepared ethanol solution of 1-NOP or 1-N-6-NOP. This was incubated at 37°C for two hours in the presence of 20 mM ascorbic acid, which served as a reducing agent to generate the hydroxyamine. For the formation of AAF adducts, or their deacetylated form, 2-aminofluorene (AF), DNA was dissolved (at 1 mg/mL in 2 mM sodium citrate buffer, pH 7.0) and added to a freshly prepared ethanol solution of N-AcO-AAF or N-AcO-TFA-AF and incubated at 37°C for 30 minutes. The reaction mixtures were protected from light. At the end of the incubation, the unbound carcinogen was removed by repeated phenol-chloroform extractions, ether extraction, or both methods; this was followed by three successive precipitations with ethanol (Maniatis et al. 1982). The number of moles of carcinogen residue bound per mole of DNA plasmid was calculated from the absorbance profile of the DNA and the specific activity of the carcinogen, using one optical density unit (O.D.) = 50 μg DNA/mL.

Characterization of the DNA Adducts Formed

To analyze for 1,6-DNP adducts, 32P-postlabeling was used with 0.2 μg plasmid DNA diluted with 2 μg carrier DNA, and slight modifications of the n-butanol enhancement procedure employed by Gupta (1985). DNA adducts were then separated by the contact-transfer method of Lu and associates (1986). Details of the process, which was carried out under the direction of Dr. Beland, are described by Boldt and colleagues (1991). To identify the types of adducts formed by 1-N-6-NOP, we ran analyses of N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene (dG-C8-1-A-6-NP) as a standard. For deacetylated adducts formed by N-AcO-TFA-AF, high-pressure liquid chromatography (HPLC) was employed under the direction of Dr. Charles M. King. Adducts were characterized following hydrolysis with anhydrous trifluoroacetic acid for one hour at 70°C. The trifluoroacetic acid was evaporated, and 150 μL of 80% methanol was added. The recovery rate was approximately 80%. The products of the hydrolysis were analyzed by reverse-phase HPLC using a Waters (New Bedford, MA) C18 Bondapak column in the method described by Mail and coworkers (1989). The profile of adducts initially formed on the plasmids by N-AcO-AAF, and of those remaining after the plasmids had been transfected into the human host cells was obtained by 32P-postlabeling using the n-butanol enrichment procedure; this was followed by thin-layer chromatography (Gupta 1985). The adducts were identified and quantified by comparing them with an N-(deoxyguanosin-8-yl)-1-amino-6-nitropyrene (dG-C8-AAF) DNA standard, derived from the reaction of calf thymus DNA with [ring-3H]N-hydroxy-2-amino-5-fluorene, or with a 3-(deoxyguanosin-N2-yl)-acetylaminofluorene (dG-N2-AAF) and dG-C8-AAF DNA standard, derived from the reaction of [ring-3H]N-AcO-AAF with salmon testes DNA. To eliminate extracellular plasmids, transfected cells were exposed to DNase I (50 μg/mL) (Sigma, St. Louis, MO) at 37°C for 30 minutes, and washed twice with phosphate-buffered saline (pH 7.2).

Determination of Sites of Carcinogen-Induced Adducts

To estimate the positions of adducts in the supF gene, we used the in vitro DNA polymerase-stop assay of Moore and Strauss (1979) and the Klenow fragment of E. coli polymerase I (Yang et al. 1988). The reactions were run not only with 35S-labeled adenosine 5'-[α-thio]triphosphate, but also with the primer end-labeled with γ-32P-labeled adenosine 5'-triphosphate. Plasmids with adduct levels of 25 to 70 adducts per plasmid (i.e., 0.5 to 1.5 adducts per supF gene per strand) were denatured and annealed with the appropriate primer. In our experiments with the location of adducts formed by 1-NOP and N-AcO-TFA-AF, which were the first of such experiments to be carried out, we used a 20mer leftward primer complementary to the nontranscribed strand and located 130 base pairs 5' to the beginning of the structural portion of the tRNA in the supF gene (Yang et al. 1988; Mah et al. 1989). For assaying the location of adducts formed by 1-N-6-NOP and N-AcO-AAF in our later experiments, two 20mer primers were used, a rightward primer, complementary to the transcribed strand at the beginning of the 200-base-pair supF gene, and the leftward primer. Polymerization was performed by the method used for the sequencing reaction, except that the deoxynucleotides were omitted, and the adducts were allowed to cause the termination (Yang et al. 1988; Boldt et al. 1991; Mah et al. 1989, 1991).

DNA from the four dideoxy sequencing reactions, carried out on an untreated template, was electrophoresed on the same gel to serve as DNA size markers. The relative intensities of the bands on the autoradiograph of the gel using γ-32P-labeled adenosine 5'-triphosphate end-labeled primer were determined by a digital image analyzer (Visage 110, Bio Image, Eastman Kodak Co., Rochester, NY). We used whole-band analysis software, which provides band quantification by defining boundaries of bands based on inflection points and integrating the optical density of the whole band, and presenting these data as percentages of the total density of the material being analyzed in each lane of the gel.

STUDIES OF HOMOLOGOUS RECOMBINATION

Plasmid for Recombination

Plasmid pJS-3 contains duplicated copies of an Htk gene and a selectable marker gene, aminoglycoside phospho-
transferase (neo), which confers resistance to an analog of neomycin, Geneticin (Grand Island Biological Co., Grand Island, NY); the plasmid was constructed and kindly provided by Dr. R. Michael Liskay (Yale University, School of Medicine, New Haven, CT). The plasmid (Figure 1) carries a 2.5-kilobase pair (kb) BamHI fragment containing an Htk mutant gene number 8 (tk8) and a 2.0-kb HindIII fragment containing Htk mutant gene number 26 (tk26). Both Htk genes are full length and contain all the necessary regulatory sequences required for their expression, but each contains an 8-base pair Xhol linker inserted at a unique site (Liskay et al. 1984). Because the HindIII site was created at the original PvuI site of the Htk gene, the tk26 gene lacks 500 base pairs from the 5’ end of the promoter region; however, these are not necessary for expression. The Xhol linker insertion mutations map in the coding region of the Htk gene at positions 735 (mutant 26) and 1,220 (mutant 8). The neo gene is located between the two Htk genes. Plasmid pJK2, a pBR322 derivative containing an intact Htk gene, was provided by Dr. Susan Conrad of the Department of Microbiology at Michigan State University for use in reconstruction experiments.

Mouse and Human Cell Strains and Culture Conditions

The mouse tk L cell line 333M, which was provided by our colleague, Dr. Liskay, contains a single stably integrated copy of plasmid pJS-3 (Liskay et al. 1984). A second mouse L cell line, used as a negative control, contains only one copy of the Htk gene (mutant 26). The cloning efficiency of the 333M mouse cells ranged from 60% to 90%. An osteosarcoma-derived human cell strain, 143 tk-1, was purchased from the NIGMS Human Genetic Mutant Cell Bank (Camden, NJ); RD tk-, a rhabdomyosarcoma-derived human cell strain, was obtained from Dr. Conrad; and XP12ROSV40 tk-, a Simian virus 40-transformed xeroderma pigmentosum (XP) cell strain was obtained from Dr. Peter Herrlich of Körersforschung, Karlsruhe, Germany. The cloning efficiencies ranged from 40% to 70% for the 143 tk- cells, 20% to 50% for the XP strain, and 5% to 25% for the RD cells. These mammalian cells were cultured in the same manner as the 293 cells, except that 15% fetal calf serum was used for the 333M mouse L cells. Selection of tk+ mouse L 333M recombinants was carried out using the same culture medium, but it was supplemented with deoxyadenosine (2 x 10^-5 M), hypoxanthine (1 x 10^-4 M), amethopterin (2 x 10^-6 M), and thymidine (3 x 10^-5 M) (CHL). Because of the amethopterin, cells require an exogenous source of thymidine and a functional thymidine kinase protein for DNA synthesis.

Treatment of Cells with Carcinogens

Cells in exponential growth were dislodged with trypsin and plated at 10^6 cells/cm². Six hours later, the culture medium was replaced with serum-free medium, and the carcinogen to be tested was delivered from stock solutions into the dishes by micropipette. For 3-NQO, a 317 mM stock solution of labeled or unlabeled compound was prepared in ethanol and stored at -20°C for repeated use. The other carcinogens were dissolved just before each use. The solvent for N-acetylaminofluorene (N-acetyl-aminofluorene, N-acetylacetone, N-acetylaminofluorene) was anhydrous ethanol; anhydrous dimethylsulfoxide was used for 1-nitroso-2-nitrosoaniline; and anhydrous acetone was used for BPDE. When labeled N-acetyl-aminofluorene was used, the specific activity was lowered to 562 mCi/mmole with the unlabeled compound. The final concentration of solvent in the medium did not exceed 0.5%. At the end of the one-hour treatment period, the medium was removed. The cells either were rinsed with phosphate-buffered saline, given fresh culture medium, and incubated at 37°C, or they were used to assay the number of sister chromatid exchanges equal to bound to DNA or cell survival (Bhattacharya et al. 1989).

Assay for Recombination

Exponentially growing target cells were plated into 10-
cm diameter dishes at a density of 10^4 cells/cm^2. For each
determination, the total number of dishes used was ad-
justed to have at least 2 x 10^6 surviving cells after treat-
ment, plus one dish for assaying cell survival. After six
hours, the medium was replaced with medium that lacked
serum, and the cells were treated with either the carcino-
gen or the solvent control. One set of cells for each dose was
plated immediately at cloning density to determine sur-
vival. The rest of the cells were incubated at 37°C in a fresh
culture medium. Selection of the tk+ recombinants with
CHAT was begun 18 hours later, and the selection medium
was renewed every 2 to 3 days, until macroscopic colonies
were formed (12 to 14 days). Representative colonies were
isolated for further analysis, and the other colonies were
stained and counted. The number of colonies observed for
the treated population was corrected for the number of vi-
able cells, as determined from the accompanying cytotoxic-
ity studies. The frequency of induced recombination was
determined by subtracting the background frequency ob-
erved in the control dishes, which were treated with sol-
ten only (Bhattacharyya et al. 1989).

Assay of Cell Survival (Cytotoxicity)

The cytotoxic effect of the carcinogens was determined
from the decrease in colony-forming ability of the treated
populations compared with that of the solvent-treated con-
trol cells. After exposure, cells in one dish per dose were
dislodged with trypsin and plated at cloning densities,
which were adjusted to give rise to 35 to 60 macroscopic
colonies per 100-mm diameter dish, using four to six dishes
per determination. After 12 to 14 days with one refedding
with culture medium, the colonies were stained and coun-
ted, and the survival was calculated from the relative
cloning efficiency.

Assay for Retention of the neo Gene

Colonies resistant to CHAT were isolated and transferred
to small dishes containing CHAT. The medium was
replaced with fresh CHAT the following day. When the cells
had almost reached confluence, they were trypsinized, and
a small volume of the cell suspension was transferred to
duplicate 23-mm diameter wells to be tested for Geneticin
resistance. The rest of the cells were transferred to flasks to
be propagated for DNA isolation and blot hybridization or
to be stored in liquid nitrogen. The next day, the medium
in one set of the duplicate wells was exchanged for medium
containing 200 µg/mL of active Geneticin to determine
whether the recombinant cells retained the neo gene. The
medium was renewed every two to three days, and the
growth of the cells was monitored microscopically after 8
to 12 days (Bhattacharyya et al. 1989, 1990a).

DNA Blotting and Hybridization

Individual CHAT-resistant colonies, isolated by the
method described above, were grown and expanded to
25 x 10^6, and their DNA was extracted (Bhattacharyya et
al. 1989). Restriction enzyme digestion using BamHI, HindIII,
and XhoI was carried out under the conditions recom-
manded by the supplier (New England BioLabs, Beverly,
MA). Digested DNA (12 µg) was electrophoresed on 0.8% agarose gels, transferred to Gene Screen Plus (E.I. du Pont
de Nemours and Co., Wilmington, DE) and hybridized with a
32P-labeled probe consisting of the 25-kbp BamHI frag-
ment containing the Htk gene. As shown in Figure 1, pJS-3
was constructed in such a way that one Htk gene is released
on a 2.5-kbp BamHI restriction fragment and the other on
a 2.0-kbp HindIII fragment. If the XhoI linker is still present
in the Htk genes, digestion with XhoI releases a 1.5-kbp fragment and a 1.0-kbp fragment from the larger gene, and a
1.5-kbp fragment and a 0.5-kbp fragment from the small
Htk gene.

Determination of the Number of Residues Covalently
Bound to Cellular DNA

To determine the percentage of tritium-labeled carcino-
gen residues remaining on parental DNA after a 24-hour
period of growth, we prelabeled cellular DNA with methyl-
labeled 14C-RIR (51.3 mCi/mmol) (Amersham Interna-
tional, Arlington Heights, IL), and measured the 3H:14C ratio at
time zero and time 24 hours. Because parental 333M cells
are deficient in thymidine kinase enzyme (tk), they could
not be used for prelabeling. Therefore, tk+ recombinant
cells were used for these experiments. Cells were grown ex-
ponentially for 48 hours in a culture medium containing
14C-thymidine (TR) (0.005 to 0.01 µCi/mL), then trypsi-
nized, pooled, and plated at a density of 10^4 cells/cm^2 for
treatment with 4-NQO or 1-NOP, or at 3 x 10^4 cells/cm^2 for
BPDE or N-AcO-AAF, using 10 x 10^6 to 30 x 10^6 cells for
each treatment. After six hours, the cells were treated with
labeled carcinogens for one hour. One set of cells was har-
vested immediately and analyzed for cell survival and for
the number of covalently bound residues per 10^6 DNA nu-
cleotides, using the method described above for pZ189. Af-
after 24 hours, the second set of treated cells was harvested
and similarly analyzed. The percentage of residues remain-
ing was calculated from the 3H:14C ratios at time 24 hours
compared with the percentage remaining at time zero (Bha-
tacharyya et al. 1989).

DNA Transfection and Selection of Transfectants
Resistant to Geneticin

The three human cell strains were transfected with pJS-3
(0.1 µg to 1.5 µg, using dimethylsulfoxide and Polybrene
[Sigma] at a concentration of 5 μg/mL (Bhattacharyya et al. 1990a). The transfected population was selected for Geneticin resistance 24 hours later by adding 200 μg active Geneticin per mL of medium. Resistant cells were allowed to form colonies for 15 to 20 days, with one refeding with selective medium before they were isolated and propagated for further study (Bhattacharyya et al. 1990a).

**Reconstruction Studies to Determine Optimal Selection Conditions**

To determine the optimal concentration of the compounds used to select tk+ human cells, the pTk2 plasmid with an intact tk gene was introduced into tk- parental 143 cells, and the population was selected with medium containing 2 x 10^-5 M deoxycytidine, 1 x 10^-4 M hypoxanthine, 3 x 10^-5 M thymidine, and various concentrations of the amethopterin. The results indicated that with the other three compounds used at the concentrations specified, the optimal concentration of amethopterin was 4 x 10^-7 M.

To determine the optimal cell density for selection, tk+ cells (50 to 100) were plated in 100-mm diameter dishes, with a lawn of 10^5 to 10^6 tk- parental cells. Selection with CHAT was started after approximately 24 hours. The results indicated that at a cell density of 3 x 10^5 to 10 x 10^5 cells plated per dish, recovery of the 143-derived and XP-derived tk+ cells was 100%, and that of RD-derived tk+ cells was 60% (Bhattacharyya et al. 1990a).

**Determination of Rate of Recombination**

The rate of recombination in the series of newly constructed transfected human cell strains was determined from fluctuation analysis tests developed by Luria and Delbruck (1943). For each assay, a group of 10 to 20 parallel subcultures of an individual transfected cell strain was seeded with approximately 300 clonable cells, and the number of cells in each parallel subculture was expanded to approximately 10^7 cells (approximately 2^15 doublings). The frequency of CHAT-resistant recombinants in 4 x 10^6 cells from each of the 10 to 20 parallel subcultures was determined in the method described above, and the rate of spontaneous recombination was calculated utilizing the method of Capizzi and Jameson (1973).

**Determination of Unscheduled DNA Synthesis as a Measure of Excision Repair Capacity**

Cells in culture medium were plated into two sets of 35-mm diameter dishes (10^5/dish). The next day, the medium was replaced with culture medium containing hydroxyurea (10-mm). Thirty minutes later, the medium was removed, and the cells were rinsed and irradiated with UV 254nm light from a germicidal lamp (15 J/m^2) or sham-irradiated in the method described by Patton and colleagues (1984). The cells were refed with culture medium supplemented with hydroxyurea and 3H-thymine (20 μCi/mL, 30 Ci/mmole) and incubated at 37°C for three hours. The extent of incorporation of 3H-thymine was determined by the method described by Grossmann and associates (1985), and the incorporation induced by ultraviolet (UV) radiation was calculated. A second method measured the number of silver grains per cell nucleus. Cells (10^5) in culture medium were seeded into 60-mm diameter dishes containing coverslips. The next day the cells were given hydroxyurea, irradiated with UV (15 J/m^2), and allowed three hours to incorporate 3H-thymine in the method described. After three hours, the coverslips were processed for autoradiography in the method described by Maher and colleagues (1979).

**RESULTS**

**STUDIES OF MUTAGENESIS**

**Comparison of the Mutagenic Effect of 1-Nitropyrene and 1,6-Dinitropyrene Residues in the Shuttle Vector Assay**

To compare the frequency of mutants induced by 1-NP adducts and 1,6-DNP adducts and determine the nature of the mutations in the supF gene, plasmids were treated with various concentrations of tritiated 1-NP or 1-N-6-NOP in the presence of ascorbic acid, which served as a reducing agent to generate N-hydroxy-1-aminopyrene and N-hydroxy-1-amino-6-nitropyrene, respectively. In vitro exposure of DNA to either of these hydroxylamines under slightly acidic conditions results in the formation of one major adduct, N-(deoxyguanosin-8-yl)-1-aminopyrene (dG-C8-1AP) or dG-C8-1A-6-NP, respectively (Howard et al. 1963; Djuric et al. 1988). An earlier study (Patton et al. 1986) used HPLC analysis to show that human cells exposed to 1-NP or 1-NOP also form only one major adduct, which cochromatographed with dG-C8-1AP. In the present study, 1-NOP was the first N-substituted aryl carcinogen to be assayed for its mutagenicity in the shuttle vector system. The best plasmid available for our use at that time was pZ189. The results of our study of 1-NOP included a comparison with the results we had obtained previously using BPDE (Yang et al. 1988). The HPLC analysis carried out under the direction of Dr. Beland indicated that 1-NOP formed only one major adduct, which cochromatographed with the authentic standard N-(deoxyguanosin-8-yl)-1-aminopyrene (dG-C8-1AP) (data not shown).

The fourth N-substituted aryl compound to be assayed in this study was 1-N-6-NOP. By that time, pS168, a derivative of pZ189 (Seidman 1988) had become available. It was employed because its design reduced the frequency of back-
ground mutants from $1.3 \times 10^{-4}$ to $0.8 \times 10^{-4}$. After we had exposed pS189 to various concentrations of 1-N-6-NOP, Dr. Beland and his colleagues employed $^{32}$P-postlabeling to analyze a portion of the DNA sample that had been exposed to the highest concentration of 1-N-6-NOP for the kinds of adducts formed. The results indicated the formation of a single type of adduct (see Figure 2) that migrated to the same position on polyethyleneimine cellulose plates as the authentic standard, dG-C8–1-A-6-NP. The results of the study with the 1-N-6-NOP-treated shuttle vector in human cells have recently been published (Boldt et al. 1991).

In describing the results of these two studies, we will indicate the compound used to treat the DNA as 1-NOP or 1-N-6-NOP. However, when referring to the adducts formed (covalently bound carcinogen residues) on the DNA, we will specify 1-NP adducts (residues), or 1,6-DNP adducts (residues), respectively, because the adducts formed are those that would be formed by the parent carcinogens.

After the plasmids were treated with various concentrations of radiolabeled 1-NOP or 1-N-6-NOP in the presence of ascorbic acid, we determined the frequency of adducts per plasmid in the method previously described. We also assayed an aliquot of each sample of treated plasmid for its ability to make bacteria resistant to ampicillin. This assay was not essential to our purposes, but it represented a simple rapid test to obtain comparative information about which type of adduct was more effective in interfering with bacterial transformation. The carcinogen-treated plasmids and solvent-treated control plasmids then were introduced into human 293 cells by transfection in the method previously described and were allowed approximately 44 hours for replication. The progeny plasmids were harvested, and the frequency of those plasmids with an inactivating mutation in the supF gene was determined by transferring them into indicator bacteria, in the method described earlier.

In summarizing the data on mutagenicity in this section and the data on intrachromosomal homologous recombination induced by carcinogen exposure later in the report, we will use figures as well as tables. In the figures showing linear or quadratic relationships, the lines of curves were determined by least squares regression. For linear relationships, the slopes of the line $\pm$ SE are reported in the text describing the results. When slopes are compared, statistically significant values are noted (defined as values with $p < 0.05$). For the linear relationship and log scale for the dependent variable, as with the survival of DNA transforming efficiency or cell cloning ability, the $D_0$ (the dose required to reduce the survival to 37% of any value along the exponential portion of the curve) is reported.

As shown in Figure 3a, there was a linear increase in the number of 1-NP residues per plasmid as a function of the concentration of 1-NOP (slope: $2.4 \pm 0.13$). This was also true for 1,6-DNP residues (Figure 4a), except for the plas-

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**Figure 2.** Autoradiogram obtained from $^{32}$P-postlabeling pS189 DNA that had been reacted with A-hydroxy-1-amino-6-nitropyrene, generated from 1-N-6-NOP. (Reprinted from Boldt et al. 1991.)

**Figure 3.** Number of 1-NOP adducts per plasmid pS189 as a function of concentration of 1-NOP in the presence of ascorbic acid (A). Relative frequency of transformation of bacteria to ampicillin resistance as a function of the number of 1-NOP adducts per plasmid (B). (Error bars indicate the standard errors of the mean.) Also shown is the frequency of supF mutants as a function of the number of 1-NOP adducts per plasmid (C). The error bars refer to the standard errors of the supF mutant frequencies obtained from a series of individual human cell transfection experiments made with each set of treated plasmids. Data from plasmids treated with 1-NOP in three separate experiments are indicated with $\Delta$ and $\bullet$. (Reprinted from Yang et al. 1988.)

**Figure 4.** Adduct formation (A); bacterial transformation efficiency (B); and frequency of mutants (C) obtained with 1-N-6-NOP-treated pS189, carried out as in Figure 3. (Reprinted from Boldt et al. 1991.)
mids exposed to 35.5 μM 1-N-6-NOP, which exhibited an adduct level of only 38.5 (slope: 1.3 ± 0.9). Linear extrapolation of the least squares line using only the values for 15, 25, 26, and 28 μM predicts that 35.5 μM should yield 53 adducts per plasmid. A value of 53 adducts per plasmid is also consistent with the biological data obtained using this particular batch of 1-N-6-NOP-treated plasmids (see below). Additional assays of the adduct level in this particular batch of DNA were not possible because by the time the need for such reassay was recognized, the sample had essentially been used up in testing its mutagenicity.

There was a 1.85-fold difference in the slopes of the lines shown in Figures 3a and 4a, indicating that more adducts per plasmid were formed per μM 1-NOP than per μM 1-N-6-NOP. This difference may reflect only a difference in the degrees of reduction by ascorbic acid. Once formed, 1-NP adducts caused three times as much interference with bacterial transformation as did 1,6-DNP adducts (Figure 3b, slope: −0.075 ± 0.005, D₀ = 5.8). For Figure 4b, the slope of the line, calculated using the values for 23, 31, 34, and 44 1,6-DNP residues per plasmid, was −0.025 ± 0.002, D₀ = 17. The relative bacterial transformation seen in a series of experiments using the plasmids that had been treated with 35.5 μM 1-N-6-NOP gave a mean value of 5.5%. Extrapolation of the line shown in Figure 4b indicates that a survival value of 5.5% corresponds to approximately 54 1,6-DNP residues per plasmid.

Comparison of the mutation frequency data shown in Figures 3c and 4c indicates that 1,6-DNP residues are more effective than 1-NP residues in causing mutations in the supF gene. Figure 3c shows a linear increase in mutants as a function of 1-NP residues per plasmid (slope: 0.56 ± 0.02). Twenty residues per plasmid calculates to 11.2 x 10⁻⁴ supF mutants. In contrast, the curve for 1,6-DNP residues is quadratic. The solid line in Figure 4c shows the best fit using the value for 38.5 residues per plasmid for the DNA exposed to the 35.5 μM concentration of 1-N-6-NOP; the broken line shows the best fit curve for the data, using the 53 residues per plasmid for that DNA sample and the value predicted from Figure 4a and supported by the data in Figure 4b. The curve that contains the broken line is defined by 1.77 − 0.041y + 0.21y², SE ± 0.0075. At 20 residues per plasmid, the mutant frequency is 9.4 x 10⁻⁴. At 40 residues per plasmid, it is 41 x 10⁻⁴; at 60 residues, it is 75 x 10⁻⁴.

What is not obvious from Figure 3c and 4c is that the background frequency of defective supF genes obtained using solvent-treated pZ189 was 1.3 x 10⁻⁴, but with pS189, it was only 0.8 x 10⁻⁴. This lower background is an advantage in such analysis. What is more, the fraction of background mutants that are not worth sequencing because they exhibit gross alterations (altered gel mobility) was 17% for pZ189 (5 out of 30), but only 9% for pS189 (3 out of 32) (see Tables 1 and 2). These results were expected because the deletion of 170 unnecessary base pairs between the supF gene and the essential gene for ampicillin resistance in pS189 makes mutants containing such gross rearrangements less likely to be recovered.

A total of 58 mutants from 1-NOP-treated pZ189 plasmids and 30 from untreated pZ189 plasmids were assayed in

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**Table 1. Analysis of Mutants Obtained by Transformation of Escherichia Coli with Progeny of 1-Nitrosopyrene-Treated pZ189 Generated During Replication in 293 Cells**

<table>
<thead>
<tr>
<th>Adducts per Plasmid</th>
<th>Number of Human Cell Transformation Experiments</th>
<th>Number of supF Mutants/Number of Transformants</th>
<th>Frequency of supF Mutants (x 10⁴)</th>
<th>Plasmids with Altered Gel Mobility</th>
<th>Number of supF Genes Sequenced</th>
<th>Number with Deletions</th>
<th>Number with Insertions</th>
<th>Number with Point Mutations</th>
<th>Characterization of Sequenced Mutants</th>
<th>Frequency of Mutants with Point Mutations (x 10⁴)</th>
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<tr>
<td>0</td>
<td>14</td>
<td>31/218,750</td>
<td>1.4</td>
<td>5/30</td>
<td>25</td>
<td>13</td>
<td>3</td>
<td>9</td>
<td>0.4</td>
<td>2.3</td>
</tr>
<tr>
<td>5.7</td>
<td>2</td>
<td>4/12,905</td>
<td>3.1</td>
<td>0/4</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>16.2</td>
<td>4</td>
<td>18/20,200</td>
<td>8.9</td>
<td>3/17</td>
<td>10</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>6.2</td>
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<tr>
<td>29.1</td>
<td>4</td>
<td>27/16,705</td>
<td>16.2</td>
<td>1/26</td>
<td>20</td>
<td>2</td>
<td>0</td>
<td>18</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>62.5</td>
<td>4</td>
<td>42/11,720</td>
<td>35.8</td>
<td>2/41</td>
<td>30</td>
<td>1</td>
<td>2</td>
<td>27</td>
<td>30.2</td>
<td></td>
</tr>
</tbody>
</table>

* Table taken from Yang et al. 1988.
* Alteration visible on agarose gel (greater than 150 base pairs).
* Deletion of 0 to 150 base pairs.
* Insertion of 0 to 20 base pairs.
* Substitution, deletion, or insertion of 1 to 2 base pairs.
* Calculated by multiplying the fraction of mutants with point mutations by the observed frequency (column 4). The fraction of mutants with point mutations is the number of mutants with point mutations (column 9) divided by the sum of the total plasmids sequenced (column 8) and the number of plasmids that showed altered gel mobility, and therefore, were not sequenced (numerator, column 5).
* These data for the controls do not include those obtained later and shown in Tables 5 and 6.
Mutations and Homologous Recombination Induced by Nitropyrenes and Related Compounds

Table 2. Analysis of Mutants Obtained by Transformation of Escherichia Coli with Progeny of 1-Nitro-6-nitropyrene-Treated pS189 Generated During Replication in 293 Cells

<table>
<thead>
<tr>
<th>Adducts</th>
<th>Number of Human Cell Transfection Experiments</th>
<th>Number of supF Mutants/Number of Transformants</th>
<th>Frequency of supF Mutants (× 10^4)</th>
<th>Plasmids with Altered Gel Mobility/Number Examined</th>
<th>Total Plasmid supF Genes Sequenced</th>
<th>Characterization of Sequenced Mutants</th>
<th>Frequency of Mutants with Point Mutations (× 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13</td>
<td>32/390,781</td>
<td>0.8</td>
<td>3/32</td>
<td>15^h</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>6</td>
<td>42/22,500</td>
<td>18.6</td>
<td>1/39</td>
<td>38</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>31</td>
<td>5</td>
<td>24/13,330</td>
<td>17.4</td>
<td>0/24</td>
<td>24</td>
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<td>0</td>
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<td>34</td>
<td>1</td>
<td>26/13,426</td>
<td>19.4</td>
<td>1/26</td>
<td>26</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>38</td>
<td>1</td>
<td>17/2,493</td>
<td>58.8</td>
<td>0/17</td>
<td>17^i</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>44</td>
<td>5</td>
<td>18/4,163</td>
<td>43.2</td>
<td>1/10</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

^a Table taken from Boldt et al. 1991.

^b Alteration visible on agarose gel (greater than 150 base pairs).

^c Deletion of 9 to 150 base pairs.

^d Insertion of 10 to 30 base pairs.

^e Substitution, deletion, or insertion of 1, 2, or 3 base pairs.

^f Calculated by multiplying the fraction of mutants with point mutations by the observed frequency (column 4). The fraction of mutants with point mutations is the number of mutants with point mutations (column 5) divided by the sum of the total plasmids sequenced (column 6) and the number of plasmids that showed altered gel mobility, and therefore, were not sequenced (numerator, column 5).

^g These data are derived from this study with pS189 and do not include control data from pZ184 that appear in Tables 1, 6, and 7.

^h Five of these had rearrangements that did not alter their gel mobility.

^i One mutant contained a gross rearrangement that did not alter its gel mobility.

agarose for altered electrophoretic mobility to detect gross alterations (deletions or insertions greater than 150 base pairs) (Table 1, column 5). The majority of those mutants that did not show such alterations (64 1-NOP mutants and 25 control mutants) then were analyzed further by DNA sequencing. The results show that in addition to the 5 out of 30 control mutants with gross alterations, 13 more contained deletions ranging from 9 to 150 base pairs, and 3 more exhibited insertions ranging from 10 to 20 base pairs, giving a total of 21 out of 30 (70%) exhibiting such changes.

Only 9 of the 30 mutants examined (30%) contained point mutations in the supF gene, defined as base pair substitutions, deletions, or insertions of one to three base pairs. Thus, the frequency of point mutations in solvent-treated control plasmids that had been transsected into human cells and allowed to replicate was only 0.4 × 10^-4, compared with an overall mutant frequency of 1.4 × 10^-4 (29%). In contrast, the frequency of point mutations observed in the mutants obtained from the progeny of plasmid containing 29.1 or 62.5 1-NP adducts per plasmid, following their replication in the human host cells, reached 85% (13.9 out of 16.2 and 30.2 out of 35.8, respectively). This difference is statistically significant.

Similarly, 124 1-NOP-treated pS189 plasmids and 32 untreated pS189 plasmids were analyzed (Table 2). The results with pS189 show that the background frequency of supF mutants containing point mutations was 0.3 × 10^-4, which is very similar to what was found with pZ189. Again, the increase in frequency of supF genes with point mutations in the progeny of 1-NOP-treated plasmids was statistically significant (Table 2, column 10).

The kinds of mutations induced by 1-NOP and 1-NOP differed significantly from those found in untreated pZ189 plasmids (Table 3). In contrast to the 27% base substitution for untreated plasmids, the majority of the mutations in the progeny of the treated plasmids were base substitutions, with 74% for 1,6-DNP residues and 85% for 1-NP residues. This is highly significant (p<0.0001). Table 3 and Figure 5 also point out that the frequency of single base-pair deletions (minus one frameshift mutation) induced by 1,6-DNP residues (16 out of 98) is significantly higher than that seen with 1-NP residues (1 out of 60) (p<0.01). The kinds of base substitutions induced by these two carcinogens were very similar, both being mainly G+C → T+A (Table 4).

Figure 5 diagrams the 65-base-pair section of the supF gene, which corresponds to the structural part of the tRNA gene. Figure 5 also shows not only the location of the mutations induced by 1,6-DNP and 1-NP adducts and the spontaneous mutations, but also how frequently a particular change occurred. In analyzing the data, we defined a prominent hot spot as any site at which at least 8% of the point mutations, in a sample size of at least 50 observed point mutations, were located. With 1,6-DNP adducts, three prominent hot spots were found (positions 123, 144, and 159). In addition, the region from 172 to 176 represented a hot spot for deletions of a single G+C base pair (minus) one frameshift mu-
Table 3. Comparison of Independent Sequence Alterations Generated in the supF Gene by Replication of 1-Nitrosopyrene- or 1-Nitro-6-nitrosopyrene-Treated Plasmids in Human Cells

<table>
<thead>
<tr>
<th>Sequence Alterations</th>
<th>Untreated Plasmids (^b)</th>
<th>1-N-6-NOP-Treated Plasmids</th>
<th>1-NOP-Treated Plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single base substitution</td>
<td>7</td>
<td>65 (^c)</td>
<td>48</td>
</tr>
<tr>
<td>Two base substitutions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tandem</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 20 bases apart</td>
<td>1</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>&gt; 20 bases apart</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Deletions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single G•C pair</td>
<td>3</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>Single A•T pair</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tandem base pairs</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4-20 base pairs</td>
<td>7</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>21-100 bases</td>
<td>8</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Insertions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single G•C pair</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Single A•T pair</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&lt; 20 bases</td>
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<td>2</td>
<td>0</td>
</tr>
<tr>
<td>21-100 bases</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tandem deletion/substitutions</td>
<td>0</td>
<td>4 (^c)</td>
<td>4 (^c)</td>
</tr>
<tr>
<td>Gross rearrangement</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>98</td>
<td>60</td>
</tr>
</tbody>
</table>

\(^a\) Table taken from Beldt et al. 1991.

\(^b\) These data for the control mutants include our published results with the supF gene obtained with untreated pZ189 and pS189 plasmids (Yang et al. 1987, 1989; Mih et al. 1989; Beldt et al. 1991).

\(^c\) One mutant has both one base substitution and one tandem deletion/substitution.

Figure 5. Location of independent point mutations induced by 1,6-DNP and 1-NP adducts in the structural region of the supF RNA gene. The DNA strand shown is the 5' to 3' strand synthesized from the rightward primer. The point mutations observed in the progeny of the 1-N-6-NOP- and 1-NOP-treated plasmid are placed below the sequence. The mutations found in mutants from untreated plasmids are placed above the line. The rectangles containing \(7T\)'s represent the deletion of G•C nucleotides and the introduction of a \(T\) into the indicated sequence. The open rectangle represents the deletion of three nucleotides. The caret shows the location of an inserted thymidine. The asterisk and the bracket indicate that it is not possible to determine which nucleotide within a run is involved in the mutation. The mutations underlined represent tandem mutations. Every tenth base and the anticodon is underlined. (Reprinted from Beldt et al. 1991.)
Table 4. Comparison of the Kinds of Base Substitutions Generated in the supF Gene during Replication of 1-Nitrosopyrene- or 1-Nitro-6-nitrosopyrene-Treated Plasmids or Untreated Plasmids in Human Cells

<table>
<thead>
<tr>
<th>Base Substitution</th>
<th>Untreated Plasmids</th>
<th>1-N-6-NOP-Treated Plasmids</th>
<th>1-NOP-Treated Plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transversions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G:C→T:A</td>
<td>9 (69%)</td>
<td>52 (64%)</td>
<td>33 (61%)</td>
</tr>
<tr>
<td>G:C→G:C</td>
<td>1 (8%)</td>
<td>10 (15%)</td>
<td>8 (15%)</td>
</tr>
<tr>
<td>A:T→T:A</td>
<td>0</td>
<td>1 (1%)</td>
<td>3 (5.5%)</td>
</tr>
<tr>
<td>A:T→G:G</td>
<td>2 (15%)</td>
<td>0</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Transitions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G:C→A:T</td>
<td>1 (8%)</td>
<td>15 (18%)</td>
<td>6 (11%)</td>
</tr>
<tr>
<td>A:T→G:C</td>
<td>0</td>
<td>2 (2%)</td>
<td>3 (5.5%)</td>
</tr>
<tr>
<td>Total</td>
<td>13 c</td>
<td>81 d</td>
<td>54 e</td>
</tr>
</tbody>
</table>

* Table taken from Boldt et al. (1991).
* These data for the control mutants include our previously published results with the supF gene (Yang et al. 1987, 1988; Mah et al. 1989).
* Mutants with base substitutions made up 27% of the total independent mutants sequenced (see Table 3).
* Mutants with base substitutions made up 74% of the total independent mutants sequenced.
* Mutants with base substitutions made up 85% of the total independent mutants sequenced.

the run of G·Cs; for 1-NP adducts, the unique hot spots were positions 109 and 127.

Correlation Between Sites of Binding by 1,6-Dinitropyrene and Sites of Mutations

To determine whether these mutational hot spots corresponded to hot spots for carcinogen binding in the supF gene, we carried out the DNA synthesis-stop assay, in which the presence of bulky adducts interferes with DNA replication. Figure 6 shows a characteristic gel. As previously noted in the Materials and Methods section, for the polymerase-stop assay, 35S-labeled adenosine-5′-triphosphate and 32P-end-labeled primer were used. Both assays gave similar results. For qualitative analysis (photographs), we used the 35S-labeled samples because these gave sharper bands. For quantitative analysis, we used the 32P-end-labeled samples. In order to obtain samples of good quality for band density analysis, replicate autoradiographs of the polymerization termination gels were developed after various lengths of exposure to ensure that bands were neither underexposed nor overexposed. With 1-N-6-NOP-treated samples, the bands indicative of chain termination corresponded to positions one nucleotide 5′ to virtually every cytosine in the DNA sequencing standard lane, which indicated that DNA synthesis was terminated one base before each guanine in the template. In one run of three guanines, the polymerization also terminated directly opposite the last guanine. No bands corresponding to positions one nu-

Figure 6. Polyacrylamide gel showing the sites of termination in the supF gene of the Klenow fragment of E. coli polymerase I on a template containing 0.6 1,6-DNP adducts per 230 bases by using the in vitro DNA polymerase-stop assay. The lanes labeled GATC are dideoxy sequence standards obtained by incubating untreated DNA templates with Sequenase and dideoxyribonucleotides. Lane 37 shows the product of the stop assay carried out on a template prepared for 1-N-6-NOP-treated pSI89 containing 37 1,6-DNP adducts per plasmid; lane 0 represents a template from untreated plasmid. The stop assay reactions for the templates shown here were run using 35S-labeled adenosine 5′-triphosphate. The reaction was chased with unlabeled dATP for 15 minutes. The numbers on the left identify the location of bases in the supF gene. (Reprinted from Boldt et al. 1991.)
cleotide away from any base other than guanine were seen, and there was little or no interference with polymerization on the untreated control template.

Figure 7 diagrams the relative frequency of the polymerase terminations (shown by the length of the solid vertical lines) in both strands of the supF gene carrying an average of 0.6 1,6-DNP residues per strand. Figure 7 also shows the location and frequency of the mutations induced by 1,6-DNP residues. In this study, which was carried out more recently than the study with 1-NP-modified plasmids, interference with polymerization by adducts on either strand was determined using two primers (Boldt et al. 1991); in the earlier study with 1-NP-modified plasmids, only one strand was assayed (Yang et al. 1988). Displaying the relationship in this manner, with the structure of the supF gene included in the diagram, shows which sites are most prominent for binding or mutation formation, but it also shows the surrounding (neighboring) sequences that may well influence such formation. Therefore, such a diagram is more informative than a two-dimensional (scatterplot) graph. The very prominent sites for mutation induction at position 144 and in the region 172 through 176, as well as the less prominent sites at positions 109 and 127, exhibited a high frequency of chain termination (adduct formation). However, there was relatively little interference with polymerization at position 159, even though this was a very prominent mutational hot spot. Some of the lack of correlation between adduct formation and mutation frequency can be explained by so-called “silent mutations.” For example, positions 142 and 146 exhibited a high frequency of chain termination, but there were no mutations at 142, and only one at 146. Cumulative data from studies with the supF gene (Knaemel and Seidman 1989) demonstrate that out of the more than 480 supF mutants analyzed, no mutations have ever been found at position 142, and mutations at position 146 cause a phenotypic change only if there is a corresponding mutation at another site. Thus, these regions are silent.

The results of a similar study conducted with 1-NP-modified plasmid DH109 carrying between 0.2 and 1.5 residues per strand of the supF gene is shown in Figure 8 (Yang et al. 1988). Only the rightward primer was used. Again, the gel pattern of the DNA bands (data not shown) corresponded to positions one nucleotide before virtually every cytosine in the DNA-sequencing standard lane. No bands corresponding to positions one nucleotide away from any base other than guanine were seen, and there was no evidence of any interference with polymerization when an untreated template was used. The pattern of bands did not vary significantly with the number of 1-NP adducts per molecule of plasmid. The frequency of DNA adducts, as estimated with this assay, ranged from 0% (position 131) to 6.6% (position 143) for 1-NP adducts. The chain terminations by 1-NP adducts were most frequently located at position 143, but no mutations were observed at that position. This probably was because mutations at this site do not affect the function of the trNA. (Position 143 is unpaired in the spur of the cloverleaf structure of the trNA.) The frequency of terminations at positions 109, 114, 142, 149, 163, and 169 was approximately the same (approximately 3%), but mutation only occurred at position 109. In fact, position 109 represents a prominent

Figure 7. Relative frequency of 1,6-DNP adducts in the 5' to 3' and 3' to 5' strand of the structural region of the supF gene, as judged by the polymerase-stop assay, and the location of carcinogen-induced base substitutions. The Klenow fragment of DNA polymerase I was used with either the rightward or the leftward sequencing primer to determine polymerase-stop sites (panel shown above the coding sequence of the supF gene). The relative intensities of the bands on the autoradiograph were determined by digital image analyzers. The data, expressed as percentages of the total band densities, are represented by the length of the lines. The base substitutions shown below the supF cDNA gene are the guanine base changes found in the corresponding strand, as taken from Figure 5. (Reprinted from Boldt et al. 1991.)

Figure 8. Relative frequency of 1-NP adducts in the 3' to 5' strand of the coding region of the supF gene, as judged by the polymerase-stop assay, and the location of 1-NP-induced base substitutions in the corresponding 5' to 3' strand. The methods employed were the same as those described in Figure 7, except that only the rightward primer was used. (Reprinted from Yang et al. 1988.)
Mutational hot spot for 1-NOP, and 169 represents a prominent hot spot for a related carcinogen. Thus, the intensity of the bands in the stop assay gel shows that some of the apparent lack of correlation between the hot spots for mutation induction and hot spots for adduct formation can be explained by silent mutations. However, as presented later in the Discussion Section, this does not fully explain the lack of correlation.

Comparison of the Mutagenic Effect of 2-Acetylaminofluorene and 2-Aminofluorene Residues in the Shuttle Vector Assay

The well-studied liver carcinogen AAF forms two major adducts in vivo, dG-C8-AAF and its deacetylated form dG-C8-AF. The latter is very similar to the dG-C6-1AP formed by 1-NP and 1-NOP. Models and biological studies in bacteria suggest that dG-C8-AAF produces very different kinds of mutations from those found with dG-C8-AF. Therefore, for our second and third series of experiments with N-substituted aryl compounds, we exposed pZ189 to N-AcO-AAF to produce dG-C8-AAF, and to N-AcO-TFA to induce deacetylated dG-C8-AF adducts. The results of these studies have been published (Mah et al. 1990, 1991). Aliquots from samples of N-AcO-TFA-treated DNA were analyzed by reverse-phase HPLC to determine the nature of the adducts formed. Two peaks of radioactivity were observed: a major peak of at least 90%, which eluted after 28 minutes of exposure, and variable quantities of a minor peak, which eluted after 19 minutes of exposure. Consistent with previous studies (Tang and Lieberman 1963; Johnson et al. 1966), the major peak corresponded to intact dG-C8-AF, while the minor peak represents a ring opened form of this adduct (data not shown). No other adducts were seen.

To characterize the kinds of DNA adducts formed in vitro by N-AcO-AAF, we collaborated with Drs. Beland and S. J. Culp, who carried out 32P-postlabeling analysis. The adduct pattern obtained is shown in Figure 9, along with that of a sample containing the three authentic standards used; those are dG-C8-AF, dG-C8-AAF, and dG-N2-AAF. The most intense areas correspond to dG-N2-AAF and dG-C8-AF. The radioactivity located between these two major areas is artifactual. There was almost no radioactivity (less than 0.3%) in the area corresponding to dG-C8-AF. (The 32p-postlabeling intensities for dG-C8-AAF and dG-N2-AAF are not representative of the amounts of these two adducts present in a sample, because reconstruction studies indicate that dG-N2-AAF postlabels much more efficiently than dG-C8-AAF.) Comparing the data from the N-AcO-AAF-treated DNA with that from the standards showed that 85% of the adducts were dG-C8-AAF, and 15% were dG-N2-AAF. These results are in agreement with the findings of Kriek and associates (1967) and Yamasaki and colleagues (1977).

As indicated in Figures 10a and 11a, both agents showed a linear relationship between the concentration of carcinogen and the number of adducts formed (slope for AF adducts: 1.36 ± 0.03; slope for AAF adducts: 0.64 ± 0.02), with N-AcO-TFA being twice as active as N-AcO-AAF in forming adducts. However, AAF adducts interfered with bacterial transformation more than AF adducts did (Figure 11 slope: -0.024 ± 0.002, D₀ = 18; Figure 10 slope: -0.011 ± 0.001, D₀ = 39).

The AAF adducts induced more mutations per adduct than did AF adducts. The mutant response for AF adducts was linear (Figure 10c, slope: 0.40 ± 0.08). The mutant response for AAF adducts showed considerable curvature with a significant quadratic coefficient (Figure 11c, slope: 0.05y ± 0.025y² ± 0.005). At lower levels of adducts, i.e., up to 20 AAF or 4F residues per plasmid, the frequencies of mutants as functions of adduct levels were similar. How-

Figure 9. Autoradiogram of 32P-postlabeling polyethylenimine-cellulose plates showing the adduct patterns of N-AcO-AAF-treated pZ189 containing 70 adducts per plasmid (A), and dG-C8-AF, dG-C8-AAF, and dG-N2-AAF standards (B). (Reprinted from Mah et al. 1991.)

Figure 10. Number of AF adducts formed per pZ189 as a function of concentration of N-AcO-TFA-AF (A), frequency of transformation (B), and frequency of supF mutants (C). Error bars indicate the standard errors of three determinations. (Reprinted from Mah et al. 1989.)
Figure 11. Number of AAF adducts formed per pZ189 (A); decrease in transforming ability (the error bars indicate the standard errors of four determinations) (B); and frequency of supF mutants induced (the error bars refer to the standard errors of the supF mutant frequencies obtained from a series of individual human cell transfection experiments made with each set of N-AcO-AAF-treated plasmid) (C). Data from plasmids treated with N-AcO-AAF in three separate experiments are indicated with •, △, and ■. (Reprinted from Mah et al. 1991.)

However, at higher levels of adducts, the mutant frequencies with AAF residues increased more than those with AF residues. Thus, at 40 AAF residues per plasmid, the mutant frequency ($52.5 \times 10^{-4}$) was more than two times higher than with 40 AF residues per plasmid ($17.6 \times 10^{-4}$).

Tables 5 and 6 give the data on the number of mutants analyzed from these various treated plasmids and the frequency and kinds of mutations observed. A total of 118 mutants from N-AcO-TFA-AF-treated plasmids and 114 mutants from N-AcO-AAF-treated plasmids were examined, with 50 and 77 mutants sequenced, respectively. The data in Tables 7 and 8 summarize the kinds of mutations and the types of base substitutions induced by AAF and AF adducts. As discussed above in connection with Tables 3 and 4, the kinds of mutations differed significantly from those obtained with untreated pZ189. The data also show that there was no significant difference in the kinds of mutations or base substitutions induced by AF and AAF adducts.

2-Aminofluorene adducts cause mainly base substitutions in bacteria, although AAF adducts cause mainly minus one frameshift mutations (Koefel-Schwartz et al. 1984; Bichara and Fuchs 1985). Therefore, the question of deacetylation of AAF adducts in the human cells following transfection was considered, even though such a process would not be likely. To test this hypothesis, cultures of 293 cells were transfected with 18 µg of pZ189 carrying 70 AAF adducts per plasmid. After three hours, extracellular DNA was eliminated using DNase I. Plasmid DNA was extracted from the human cells immediately or after an additional 8.5 or 175 hours. The DNA was analyzed by $32^P$-postlabeling for the number and kinds of adducts, and the data were compared to those obtained with nontreated plasmids. The plasmid DNA extracted from human cells 3.5 hours after transfection showed the same adduct profile as nontreated plasmid. No deacetylation of AAF adducts was observed. However, the level of adducts in the plasmid had been decreased to 75% of the original level (data not shown). Control studies indicated that replication of transfected plasmids had not occurred yet (Mah et al. 1991).

Comparison of the data in Tables 7 and 8 with the data for 1,6-DNP and 1-NP adducts, summarized in Tables 3 and

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Table 5. Analysis of Mutants Obtained by Transformation of Escherichia Coli with Progeny of N-Acetox-N-trifluoracetyl-2-aminofluorene-Treated pZ189 Generated During Replication in 293 Cells

<table>
<thead>
<tr>
<th>Adducts per Plasmid</th>
<th>Number of Human Cell Transfection Experiments</th>
<th>Number of supF Mutants/Number of Transforms</th>
<th>Frequency of supF Mutants ($\times 10^{10}$)</th>
<th>Plasmids with Altered Gel Mobility/Number Examined</th>
<th>Plasmid $supF$ Genes Sequenced</th>
<th>Total Characterization of Sequenced Mutants</th>
<th>Frequency of Mutants with Point Mutations ($\times 10^{10}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td>42/313,753</td>
<td>1.3</td>
<td>8/40</td>
<td>26</td>
<td>14 3 9</td>
<td>0.3</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>6/23,053</td>
<td>2.6</td>
<td>0/4</td>
<td>1</td>
<td>0 8 1</td>
<td>1</td>
</tr>
<tr>
<td>8.6</td>
<td>2</td>
<td>30/51,610</td>
<td>5.8</td>
<td>5/24</td>
<td>2</td>
<td>0 0 2</td>
<td>2</td>
</tr>
<tr>
<td>22.6</td>
<td>3</td>
<td>38/27,740</td>
<td>13.7</td>
<td>2/9</td>
<td>19</td>
<td>2 0 17</td>
<td>11.1</td>
</tr>
<tr>
<td>33.2</td>
<td>3</td>
<td>25/22,640</td>
<td>11.0</td>
<td>1/20</td>
<td>10</td>
<td>0 1 9</td>
<td>9.0</td>
</tr>
<tr>
<td>40.7</td>
<td>3</td>
<td>44/22,696</td>
<td>19.4</td>
<td>1/41</td>
<td>18</td>
<td>1 0 17</td>
<td>17.4</td>
</tr>
</tbody>
</table>

* Table taken from Mah et al. (1990).
* Alteration visible on agarose gel (more than 150 base pairs).
* Deletion of 9 to 150 base pairs.
* Insertion of 10 to 30 base pairs.
* Substitution, deletion, or insertion of 1, 2, or 3 base pairs.
* Calculated by multiplying the fraction of mutants with point mutations by the observed frequency (column 4). The fraction of mutants with point mutations is the number of mutants with point mutations (column 9) divided by the sum of the total plasmids sequenced (column 6) and the number of plasmids that showed altered gel mobility, and therefore, were not sequenced (numerator, column 5).
* These data for the control mutants include pZ189 (Yang et al. 1987, 1988; Mah et al. 1989).
Table 6. Analysis of Mutants Obtained with Progeny of N-Acetoxy-2-acetylaminofluorene-Treated pZ189 Generated during Replication in 293 Cells

<table>
<thead>
<tr>
<th>Adducts per Plasmid</th>
<th>Number of Human Cell Transfection Experiments</th>
<th>Number of supF Mutants/Number of Transfomers</th>
<th>Frequency of supF Mutants (x 10^5)</th>
<th>Plasmids with Altered Gel Mobility/Number Examined</th>
<th>Total supF Genes Sequenced</th>
<th>Characterization of Sequenced Mutants</th>
<th>Frequency of Mutants with Point Mutations (x 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40</td>
<td>44/326,744</td>
<td>1.3</td>
<td>8/42</td>
<td>27</td>
<td>15/3/9</td>
<td>0.3</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>6/5,552</td>
<td>10.8</td>
<td>0/6</td>
<td>4</td>
<td>0/0/4</td>
<td>10.8</td>
</tr>
<tr>
<td>15</td>
<td>13</td>
<td>33/48,281</td>
<td>6.8</td>
<td>0/32</td>
<td>25</td>
<td>1/0/23</td>
<td>6.3</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>9/10,143</td>
<td>8.9</td>
<td>0/6</td>
<td>2</td>
<td>0/0/2</td>
<td>8.9</td>
</tr>
<tr>
<td>22</td>
<td>8</td>
<td>23/26,487</td>
<td>8.7</td>
<td>0/22</td>
<td>17</td>
<td>1/0/16</td>
<td>8.2</td>
</tr>
<tr>
<td>25</td>
<td>8</td>
<td>16/12,610</td>
<td>12.7</td>
<td>0/14</td>
<td>11</td>
<td>1/0/10</td>
<td>11.5</td>
</tr>
<tr>
<td>45</td>
<td>4</td>
<td>100/16,068</td>
<td>62.2</td>
<td>0/28</td>
<td>16</td>
<td>0/0/16</td>
<td>62.2</td>
</tr>
<tr>
<td>70</td>
<td>4</td>
<td>89/6,972</td>
<td>127.7</td>
<td>0/6</td>
<td>2</td>
<td>0/0/2</td>
<td>127.7</td>
</tr>
</tbody>
</table>

* Table taken from Mah et al. (1991).
* Alteration visible on agarose gel (greater than 150 base pairs).
* Deletion of 9 to 150 base pairs.
* Insertion of 10 to 30 base pairs.
* Substitution, deletion, or insertion of 1 or 2 base pairs.
* Calculated by multiplying the fraction of mutants with point mutations by the observed frequency (column 4). The fraction of mutants with point mutations is the number of mutants with point mutations (column 9) divided by the sum of the total plasmids sequenced (column 6) and the number of plasmids that showed altered gel mobility, and therefore, were not sequenced (numerator, column 3).
* These data for the control mutants include previously published results with the supF gene obtained with untreated pZ189 plasmids (Yang et al. 1987; 1986; Mah et al. 1989).
* One mutant with gross rearrangement.

4. indicates that these structurally related carcinogens, which form adducts primarily or exclusively at the C8 position of guanine, are very similar in the kinds of point mutations they induce. The notable exception is that 1,6-DNP residues caused a significantly greater frequency of minus one frameshift mutations in the run of 5 G-C base pairs discussed above. The AAF residues also induced minus one deletions in that region, but the difference between AAF and AF adducts in inducing such frameshift mutations was not statistically significant.

The spectra of unequivocally independent supF mutants obtained with AAF and AF residues are compared in Figure 12. There were no hot spots in common between these two structurally related carcinogens. Prominent hot spots for N-AcO-AAF-induced base pair substitutions were found at positions 122, 127, and 155, whereas N-AcO-TFA-AF induced prominent hot spots at positions 123, 133, 159, and 169. With AAF residues, only 6.3% (5 out of 79) of the mutations observed were minus one frameshift mutations in the run of 5 G-C base pairs found in region 172 through 176, the same region where the minus one frameshift mutations caused by 1,6-DNP residues occurred at a frequency of 13% (13 out of 98). With AF residues, no such mutations were observed.

Table 9 compares the hot spots found in the spectrum of each of the four N-substituted aryl carcinogens. Each carcinogen induced at least one unique hot spot: 109 for 1-NP residues; 144 and the region 172 through 176 for 1,6-DNP; 122 and 155 for AAF; and 189 for AF. The spectrum induced by BPDE residues (Yang et al. 1987) showed two hot spots, 109 and 123, which are similar to those induced by 1-NP residues. However, unlike the spectrum for 1-NP residues, the spectrum for BPDE residues showed a cold spot at 127 and 159. Thus, each aryl compound produced a unique set of hot spots and cold spots, i.e., a unique spectrum of mutations.

Comparison Between Sites of Adducts and the Location of Mutations Induced by N-Acetoxy-2-acetylaminofluorene and N-Acetoxy-N trifluoroacetyl-2-aminofluorene

The results of the DNA polymerase termination assay, carried out to determine the sites and frequencies of carcinogen-DNA adduct formation in the supF gene, are shown in Figures 13 and 14. With N-AcO-AAF-treated plasmids, which were studied later than the N-AcO-TFA-AF-treated plasmids, both a leftward and a rightward primer were employed. Figure 15 shows a representative gel for the N-AcO-AAF-treated plasmids. In this particular assay, the leftward sequencing primer was used to determine the location of adducts on the 5’-3’ strand of the supF gene, beginning at position 210. The arrow points to a band, at position 123, that is notably darker than the others, indicating a hot spot.
Table 7. Comparison of Independent Alterations Generated in the \textit{supF} Gene by Replication of Carcinogen-Treated or Untreated Plasmids in Human Cells\textsuperscript{a}

<table>
<thead>
<tr>
<th>Sequence Alterations</th>
<th>Number of Independent Mutants with This Change</th>
</tr>
</thead>
</table>
|                      | Untreated Plasmids\textsuperscript{b}  | \(N\text{-AcO-AAF-}
|                      | Treated Plasmids | Treated Plasmids |
| Single base substitution | 7 | 62 | 42 |
| Two base substitutions | 27% | 85% | 86% |
| Tandem | 1 | 0 | 1 |
| \(\leq 20\) bases apart | 2 | 3 | 0 |
| > 20 bases apart | 0 | 2 | 1 |
| Three base substitutions | 0 | 0 | 1 |
| > 20 bases apart | 2% | 8% | 2% |
| Deletions | 3 | 7 | 8 |
| Single G+C pair | 1 | 0 | 0 |
| Single A+T pair | 0 | 2 | 1 |
| Tandem base pairs | 51% | 13% | 8% |
| 4-20 base pairs | 7 | 3 | 2 |
| > 20 bases | 8 | 1 | 1 |
| Insertions | 1 | 1 | 2% |
| Single G+C pair | 1 | 0 | 0 |
| Single A+T pair | 0 | 1 | 1 |
| < 20 bases | 8% | 1% | 2% |
| Gross rearrangements | 5 | 1 | 2% |
| Total | 37 | 79 | 51 |

\textsuperscript{a} Table taken from Mah et al. 1991.

\textsuperscript{b} These data are for the control mutants include our published results with the \textit{supF} gene obtained with untreated pZ189 and pS189 plasmids (Yang et al. 1986, 1988; Mah et al. 1988; Boldt et al. 1991).

Table 8. Comparison of the Kinds of Base Substitutions Generated in the \textit{supF} Gene During Replication of Carcinogen-Treated or Untreated Plasmids in Human Cells\textsuperscript{a}

<table>
<thead>
<tr>
<th>Base Substitutions</th>
<th>Number of Independent Mutants with This Change</th>
</tr>
</thead>
</table>
|                    | Untreated Plasmids\textsuperscript{b}  | \(N\text{-AcO-AAF-}
|                    | Treated Plasmids | Treated Plasmids |
| Transversions | 9 (69%) | 47 (61%) | 32 (65%) |
| G+C→T+A | 1 (8%) | 11 (17.5%) | 8 (17%) |
| G+C→C+G | 0 | 0 | 0 |
| A+T→T+A | 2 (15%) | 0 | 0 |
| A+T→C+G | 2 (15%) | 0 | 0 |
| Transitions | 1 (8%) | 12 (17%) | 9 (18%) |
| G+C→A+T | 0 | 2 (3%) | 0 |
| A+T→G+C | 0 | 2 (3%) | 0 |
| Total | 13\textsuperscript{c} | 72\textsuperscript{d} | 49\textsuperscript{e} |

\textsuperscript{a} Table taken from Mah et al. 1989.

\textsuperscript{b} These data include published results with untreated plasmids (Yang et al. 1987, 1988; Mah et al. 1989; Boldt et al. 1991).

\textsuperscript{c} Mutants with base substitutions made up 27% of the total independent mutants sequenced.

\textsuperscript{d} Mutants with base substitutions made up 85% of the total independent mutants sequenced.

\textsuperscript{e} Mutants with base substitutions made up 86% of the total independent mutants sequenced.
Mutations and Homologous Recombination Induced by Nitropyrenes and Related Compounds

Figure 12. Location of independent point mutations induced in the structural region of the supF RNA gene of N-AcO-AAF- and N-AcO-TFA-AF-treated pZ89 plasmid. The point mutations observed in the progeny of the treated plasmid are placed below the sequence; those from untreated plasmids above the line. Each square represents a deleted nucleotide. The caret shows the location of an inserted cytosine. The bracket indicates that it is not possible to determine which nucleotide within a run is involved in the mutation. The mutations underlined represent tandem mutations. Every tenth base and the anticodon is underlined. (Reprinted from Mah et al. 1991.)

for termination of polymerization. This corresponds to a termination site (adduct) at position 122 on the template, which is a hot spot for AAF-induced mutations.

The Klenow fragment of DNA polymerase does not bypass dG-C8-AAF adducts (Sehm et al. 1989). The pattern of the DNA bands obtained with DNA containing AAF adducts corresponded to positions one nucleotide before virtually every cytosine in the DNA-sequencing standard line, indicating that, as with the other compounds, DNA synthesis was terminated one base before each guanine in the template. No bands corresponding to positions one nucleotide away from any base other than guanine were seen.

Each of the hot spots for mutation induction (positions 122, 127, and 155) also showed a relatively high frequency of carcinogen binding, although some sites that showed high carcinogen-adduct formation were cold for mutation induction (for example, positions 99, 102 through 105, and 111). A similar correlation was found for AF adducts (Figure 14). The fact that some sites showed high carcinogen-adduct formation but very low mutation induction (for example, positions 99, 102 through 105 and 111) can be explained by the silent mutations discussed earlier. Even though mutations at these particular sites have been found because they produced phenotypic changes (Kraemer and

Figure 13. Relative frequency of AAF adducts in the 5' to 3' and 3' to 5' strands of the structural region of the supF gene, as judged by the polymerase-stop assay, and the location of the mutations observed. The methods used were the same as those described in Figure 7. (Reprinted from Mah et al. 1991.)

Table 9. Sites of Hot Spots\textsuperscript{a} in the Structural Region of the supF Gene Induced by Covalently Bound Residues of the Four N-Substituted Aryl Carcinogens Studied and by the 7,8-Diols-9,10-epoxide of Benzo[a]pyrene

<table>
<thead>
<tr>
<th>1-Nitropyrene</th>
<th>1,6-Dinitropyrene</th>
<th>2-Acetaminofluorene</th>
<th>2-Aminoanthracene</th>
<th>BPDE\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>109</td>
<td></td>
<td></td>
<td></td>
<td>109</td>
</tr>
<tr>
<td>123</td>
<td>123</td>
<td></td>
<td></td>
<td>123</td>
</tr>
<tr>
<td>127</td>
<td></td>
<td></td>
<td></td>
<td>133</td>
</tr>
<tr>
<td>+</td>
<td>144</td>
<td></td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>159</td>
<td>159</td>
<td></td>
<td></td>
<td>169</td>
</tr>
<tr>
<td>172–176</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} In the spectrum we obtained for 1-NP, 1,6-DNP, AAF, AF, and BPDE residues, a hot spot, defined as a site in a spectrum of at least 50 mutations, where at least 6% of the mutations located, consisted of at least 5, 8, 6, 5, or 7 independent mutations, respectively.

\textsuperscript{b} The data for BPDE are taken from Yang et al. (1987) for purposes of comparison.

\textsuperscript{c} The asterisk indicates a cold spot, that is, a site where 2% or less of the observed mutations were located.
Seidman (1989), the kinds of mutations that have been scored at these sites do not include G+C → T+A transversion, the major kind of mutation induced by AAF residues. For example, the only kind of mutation ever found at position 99 was a G+C → A+T transition; however, this type of mutation represents only 17% of the total base substitutions induced by AAF residues in the supF gene. Both G+C → A+T transitions and G+C → T+A transversions at position 111 have caused a phenotype change in the supF gene. However, the latter transversion, which is the predominant kind of base substitution induced by dG-C8-AAF in this gene, produced a phenotypic change only if it occurred as a tandem mutation involving this site. Similarly, even though G+C → T+A transversions were detected at positions 102 through 105 with other agents, dG-C8-AAF probably would not induce this kind of mutation at this sequence (Fuchs and colleagues [Koffel-Schwarz et al. 1984]). These investigators showed that dG-C8-AAF mainly induces minus one frameshift mutations in a sequence constituted of repeated bases. No minus one base pair deletions in positions 102 through 105 in the supF gene have been detected in more than 480 mutants studied (Kraemer and Seidman 1980).

STUDIES OF HOMOLOGOUS RECOMBINATION

Ability of Structurally Related Polycyclic Aromatic Carcinogens to Induce Homologous Recombination

Wang and associates (1988) used a tk-deficient mouse L cell line, 333M, which contains a single integrated copy of the plasmid construct shown in Figure 1, to show that ultraviolet (UV) radiation, mitomycin C (a carcinogen that forms crosslinks between strands in DNA), N-methyl-N-nitro-N-nitrosoguanidine (a simple alkylating agent), and BPDE (a polycyclic aromatic carcinogen) can cause homologous recombination in a dose-dependent manner. To gain insight into the mechanism by which such agents cause recombination between homologous genes in mammalian cells, we compared the abilities of four structurally related, N-substituted aryl carcinogens to induce recombination as a function of applied concentration, level of cytotoxicity induced, and number of DNA adducts initially formed and remaining in the target cells 24 hours after treatment (Bhattacharyya et al. 1989). The carcinogens tested were 1-NOP, 1-N-6-NOP, N-acetyl-2-AAF, and 4-NQO, a nitrocompound that, like 1-NOP and 1-N-6-NOP, requires cellular activation before it can bind covalently to DNA (Tada and Tada 1976; Kawazoe 1981). For the sake of comparison, the frequency of recombination induced by the structurally related carcinogen BPDE as a function of the initial number of DNA adducts formed also was determined.

Before assaying for recombination, we determined the extent of cell killing expected from each concentration used in order to estimate the number of target cells to use per assay. We wanted to adjust the number of dishes so as to have
a minimum of $2 \times 10^6$ surviving cells per determination. The number of target cells per dish for each determination was kept uniform ($10^4$ cells/cm$^2$).

The frequency of recombinants is calculated from the observed number of CHAT-resistant colonies divided by the number of surviving target cells, and the latter information is determined in each recombination experiment by subculturing one set of cells immediately after carcinogen treatment and plating them at low (cloning) density. Therefore, we carried out reconstruction studies to be sure that the survival of cells plated at low density was the same as that of cells left undisturbed for 18 hours and then selected in CHAT medium.

Figure 16 shows the results obtained with three $N$-substituted arylnitrosamines, as a function of the concentration applied. Each gave a dose-dependent increase in frequency of $tk^+$ recombinants, cells able to survive selection with CHAT medium. The results of preliminary cytotoxicity experiments are included in the top panels of Figure 16, along with the cytotoxicity results that accompanied the recombination assays. In a series of cytotoxicity experiments, with 1-N-6-NOP, using doses comparable to 1-NOP doses, no cell killing was observed in this mouse L cell line. The lack of cytotoxicity indicates that in these cells the chemical was not activated into a form capable of binding to cellular macromolecules. Therefore, we increased the concentrations from 1.0 $\mu$M to as high as 10 $\mu$M. At that concentration, a fine precipitation of the compound could be seen in the dishes, but cell survival decreased only slightly below that of the untreated control. Therefore, we did not test this carcinogen further in the 333M cell line.

One way to compare agents for their ability to induce recombination is to use the slope of the least squares line defining the frequency of recombinants induced as a function of applied concentration. The slopes of the lines in the bottom half of Figure 16 are: 1-NOP, 66.6 $\pm$ 14.1; $N$-AcO-AAF, 21.7 $\pm$ 6.4; 4-NQO, 66.7 $\pm$ 27.4. The data in Figure 16 indicate that this value per $10^6$ viable cells per $\mu$M concentration of carcinogen is 66 for 4-NQO, 69 for 1-NOP, and 22 for $N$-AcO-AAF. Another way to compare agents is to determine the frequency of recombinants induced as a function of the cytotoxic effect (recombinogenic efficiency). Figure 17a gives this information for the three carcinogens (slopes: 1-NOP, 57.8 $\pm$ 7.1; $N$-AcO-AAF, 50 $\pm$ 6.2; 4-NQO, 36.7 $\pm$ 4.8).

Figure 16. Cell killing and induction of homologous recombination by 1-NOP, $N$-AcO-AAF, and 4-NQO as a function of their concentration in the cell culture medium. Cells were treated for one hour at a density of $10^6$ cells/cm$^2$. Immediately after treatment, one set of cells was assayed for survival. The rest were allowed to begin expression. Selection for $tk^+$ recombinants with CHAT medium was begun after 18 hours. The background frequencies observed in the solvent-treated controls accompanying each individual experiment have been subtracted. For the four independent experiments with 1-NOP, these ranged from $13 \times 10^{-4}$ to $18 \times 10^{-4}$; for the five independent experiments with $N$-AcO-AAF, these ranged from $9 \times 10^{-4}$ to $35 \times 10^{-4}$, with an average of $19 \times 10^{-4}$; for the five independent experiments with 4-NQO, these ranged from $12 \times 10^{-4}$ to $26 \times 10^{-4}$, with an average of $23 \times 10^{-4}$. Individual data points are shown as $\Phi$. (Reprinted from Bhattacharyya et al. 1989.)

Figure 17. A: Induction of recombination as a function of the extent of cell killing. The data are taken from the experiments shown in Figure 16. Data points for $N$-AcO-AAF have been omitted for the sake of clarity and the results are indicated by a dashed line. B: Cell killing as a function of the number of radiolabeled residues initially bound to DNA. C: Frequency of recombination as a function of the initial number of DNA adducts formed. The slopes of the lines in Figure 17 were not obtained from additional experiments. Instead, they were derived from the slopes of the lines shown in Figure 16 in order to display the relationship between induction of recombination and adduct formation. The relationships for 1-NOP, 4-NQO, and $N$-AcO-AAF that are shown in Figure 17 were derived from the experimental data shown in Figures 16a and 16b. The data for BPDE were derived from the binding data of Figure 17a, and the recombination and survival data were taken previously from Figure 1 of Wang and associates (1988) (see text). The following symbols are used in this figure: $\bullet$ = 1-NOP; $\text{O}$ = $N$-AcO-AAF; $\text{O}$ = 4-NQO; and $\Delta$ - BPDE. (Reprinted from Mahar et al. 1990.)
Although the data for BPDE are not shown in Figure 1a, they are available from the study by Wang and associates (1968) (slope: 56.4 ± 3.4). The symbols in Figure 1a indicate data from the experiments shown in Figure 1b. The individual data points for N-AcO-AAF, which were very close to those shown for 1-NOP, have been omitted for the sake of clarity, and the relationship is shown by a broken line.

A third way to compare agents is to express the induced frequency as a function of the number of residues covalently bound to DNA. To obtain these relationships, cells were treated with various concentrations of radiolabeled carcinogen, and the number of DNA adducts formed per 10^6 nucleotides was determined, along with the corresponding degree of cytotoxicity. These results are shown in Figure 1b. For the sake of comparison, we also included BPDE in this study (slopes: BPDE, 0.33 ± 0.007; 4-NQO, 0.017 ± 0.002; 1-NOP, 0.012 ± 0.0003, N-AcO-AAF, 0.006 ± 0.0008). Once we had determined the cytotoxicity of each agent as a function of the number of adducts formed, we used these data to estimate the frequency of recombination per adduct from the relationship between recombination and cytotoxicity (i.e., from the data shown in Figure 1a). These indirectly derived relationships between the frequency of induced recombinants and the initial number of DNA adducts (recombinogenic effectiveness) are shown in Figure 1c. For each agent, the frequency increased linearly with the number of adducts (slopes: BPDE, 1.86 ± 0.42; 1-NOP, 0.63 ± 0.09; 4-NQO, 0.63 ± 0.12; N-AcO-AAF, 0.30 ± 0.05). The BPDE was significantly more effective at inducing recombination than the other carcinogens; it was followed by 1-NOP, 4-NQO, and N-AcO-AAF (the ratios of the slopes of the lines were 6.2:2.3:2.1:1.0).

Relative Rate of Adduct Removal by 333M Cells

The observed differences in recombinogenic effectiveness could reflect intrinsic differences in the mechanism of action of the carcinogens. But those observations might instead be the result of differences in the cells' rate of removal of adducts from their DNA. To see if the latter explanation was correct, we compared the rate of loss of the various adducts by measuring the initial number of adducts per 10^6 nucleotides and the number remaining after 24 hours, adjusting the doses to obtain approximately equal levels of survival (20% to 50% of the untreated control), rather than equal numbers of adducts. The results (Figure 1d) showed that the 333M mouse cell line has a relatively slow rate of excision of such damage (no more than 30% lost in 24 hours), but there was no significant difference in the rates of removal of NOP-induced adducts and N-AcO-AAF-induced adducts, or between those rates and the rate of removal of 4-NQO-induced adducts.

Characterization of the Nature of the Recombination Events from Analysis of the Products Obtained

The recombination substrate in the pJS-3 plasmid was designed to provide information about the product of recombination (Liskay et al. 1984). Therefore, if the recombinational event involves a single reciprocal exchange within a chromatid or a single unequal exchange between chromatids, only a single wild-type copy of the Htk gene will be present, and the neo gene, coding for resistance to Geneticin, will be lost. If the event consists of a nonreciprocal transfer of wild-type information, i.e., gene conversion, the Htk gene duplication and the neo gene will be retained (Figure 1). To determine the type of recombination event induced by these carcinogens, representative CHAT-resistant colonies were isolated from independent experiments and tested for resistance to Geneticin. The results indicated that 82% of the events induced by all three agents were consistent with gene conversions. Molecular analysis by Southern blot hybridization, using the Htk gene as the probe, confirmed these results. Each of the Geneticin-resistant recombinants retained the Htk gene duplication, with one Htk gene being wild type, i.e., lacking an Xhol restriction site. The Geneticin-sensitive recombinants, on the other hand, contained only a single (wild-type, Xhol-resistant) Htk gene (data not shown).
Preparation of a Suitable Series of Human Cell Strains Containing the Substrate for Recombination

To determine whether the recombination was stimulated by the presence of unrepaird lesions remaining in DNA or by the process of nucleotide excision repair of such damage, we introduced the Htk recombination substrate system into a series of established (infinite life span) cell lines that differed in their ability to carry out excision repair of DNA damage. Extracts from human cells were known to catalyze strand transfer between a linear duplex and homologous, circular single-stranded DNA (Hsieh et al. 1986; Cane et al. 1987; Kenne and Ljunquist 1987; Lopez and Coppey 1987; Fishel et al. 1988), suggesting that human cells would be capable of carrying out homologous recombination. To use the Htk assay, these human cell lines had to be tk-deficient, i.e., both copies of the endogenous tk allele had to be inactivated. We located three tk-deficient, infinite-life-span human cell lines, designated 143 tk-, RD tk-, and XP12RSV40 tk-, and tested them for their sensitivity to 254 nm UV radiation to estimate their excision repair capacity. The 143 cells showed a survival similar to that of normal diploid human fibroblasts (D0, approximately 5 J/m²); the RD cells showed an intermediate sensitivity (D0, approximately 2 J/m²); and, as expected, the XP cells showed an extreme sensitivity (D0, approximately 0.3 J/m²). Because the parental cell lines are tk-, it was not possible to use UV radiation-induced incorporation of 3H-TDR as a measure of their nucleotide excision repair capacities, but such measurements subsequently were carried out with Htk recombining derived from each of the parental lines.

Before introducing the pJS-3 plasmid into these cell lines, we also assayed 10 x 10⁶ to 20 x 10⁶ cells of each for spontaneous revereration of the endogenous tk genes and found no revertants. In the course of the work described below, an additional 5 x 10⁷ to 1 x 10⁸ cells from each of the cell lines were assayed for tk+ cells with negative results, indicating that the frequency of spontaneous revereration of the endogenous tk genes in these three parental tk- cell lines was less than 1 x 10⁻⁸ or 2 x 10⁻⁸. Therefore, these three strains proved suitable for our purpose. The plasmid was linearized at its unique ClaI site (Figure 1) to increase the chance that the integrated plasmids would contain the 2 Htk genes intact with the neo gene located between them. The plasmid was then introduced into populations of parental cells. Transfectants were selected for Geneticin resistance. Representative transfectants from each cell line were isolated, propagated in the absence of Geneticin, and assayed for the ability to undergo spontaneous recombination to yield CHAT-resistant cells. Approximately 25% of the transfectants were capable of producing CHAT-resistant recombinants, indicating that the Htk substrate was integrated and intact (Bhattacharyya et al. 1990a). We used Southern blotting analysis to confirm this, to see what kinds of spontaneous recombination events had occurred, and also to determine the number of copies of pJS-3 integrated into the transfectants. Representative Southern blotting analysis data for CHAT-resistant recombinants are shown in Figure 19.

As shown in Figure 1, double digestion of DNA from transfectants with BamHI and HindIII should release both Htk genes as individual bands of 2.5-kbp and 2.0-kbp. Both bands should still contain the XhoI, producing 1.5-, 1.0-, and 0.5-kbp fragments. The odd-numbered lanes in Figure 19 indicate DNA digested with BamHI and HindIII. As expected, they show bands at 2.5 and 2.0-kbp (except for lanes 5, 7, and 9, which are from recombinants of cell strain RD-12, which lost one of its BamHI sites during transfection). The even-numbered lanes indicate DNA digested with BamHI, HindIII, and XhoI. We expected that if one of the Htk genes became wild type, i.e., lost the XhoI site, either the 2.5-kbp or 2.0-kbp band would be resistant to XhoI digestion. This is what was found for every CHAT-resistant cell tested, indicating that each of those cells arose from recombination. Because of its small size, the 0.5-kbp fragment is visible only in lanes 4, 16, and 20 of this photograph (Figure 19). However, the fragment can be seen in all appropriate lanes in the original autoradiograph (Bhattacharyya et al. 1990a). Table 10 summarizes the kinds of events seen. Similar results were obtained by determining the loss of Geneticin resistance (data not shown) (Bhattacharyya et al. 1990a), which indicated that the kinds of spontaneous

![Figure 19. Southern blot analysis of the Htk gene in genomic DNA from various CHAT-sensitive XP cell strains. DNA in each of the odd-numbered lanes was digested with HindIII and BamHI to release the Htk gene as individual bands (2.5 or 2.0 kbp). The DNA in the even-numbered lanes was digested with HindIII, BamHI, and XhoI to identify XhoI-resistant wild-type Htk genes. Lanes 1 through 4 contain DNA from RD-3-derived clones (RD-3 contains two copies of the substrate, which accounts for the appearance of a 1.0-kbp band). Lanes 5 through 8 contain DNA from RD-12-derived clones. (In strain RD-12, one of the BamHI sites of 2.5 kbp fragments of Htk gene was modified during transfection and integration giving a higher position of the band released by BamHI digestion.) Lanes 11 through 14 contain DNA from XP1-derived clones; lanes 15 and 16 contain DNA from tk transfectant XP-8; and lanes 17 through 20 contain DNA from XP8-derived clones. (Reprinted from Bhattacharyya et al. 1990a.)](image-url)
Table 10. Molecular Characterization of Htk⁺ Recombinants\(^a\)

<table>
<thead>
<tr>
<th>pJS-3-Transfected Cell Strain</th>
<th>Number of Htk⁺ Recombinants Tested</th>
<th>Type of Recombination Event</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0 kbp Wild Type (Tk26)</td>
<td>2.5 kbp Wild Type (Tk8)</td>
</tr>
<tr>
<td>143-7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>143-15</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>XP-6</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>XP-7</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>RD-3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>RD-12</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^a\) Table taken from Bhattacharyya et al. (1990a).

\(^b\) The number of recombinants that contain a wild-type copy of the indicated gene and also retain a gene containing the 8-base-pair frameshift mutation.

recombinants resembled those seen in the 333M mouse L cell line. Most of those recombinants (67%) retained the duplicated Htk gene, indicating that they arose from non-reciprocal transfer of information.

The construction of pJS-3 also facilitates the determination of the number of integrated copies of the recombination substrate. If the substrate is present in a single copy, digestion with BamHI or HindIII produces a hybridization pattern containing only two bands, i.e., a 2.5-kbp band or a 2.0-kbp band, respectively, and a much larger junction fragment, a band in a higher position on the gel. Similarly, if there are two copies of the substrate (nontandem), there are also three bands, one at position 2.5-kbp or 2.0-kbp (with BamHI or HindIII, respectively) and two larger junction fragments. The results of such analysis of our candidate transfectant strains are shown in Table 11.

The Htk⁺ recombinants derived from each of the three parental cell lines were assayed for UV-radiation-induced incorporation of \(^3\)H-dR (unscheduled DNA synthesis). As predicted from their survival curves after the UV radiation, the 143 cells exhibited a large number of cells lightly labeled with silver grains; the RD cells showed an intermediate number of such cells (three to four times fewer than did the 143 cells); and the XP12ROSV40 cells showed no evidence of lightly labeled cells (data not shown). Ultraviolet radiation–induced incorporation of \(^3\)H-dR in the presence of hydroxyurea produced results consistent with these findings. There was no increase above the background level with the XP12ROSV40 cells; with RD cells, the level was \(5 \times 10^4\) cpm greater than the background level; and the level was \(380 \times 10^4\) cpm higher than the background level with 143 cells (Bhattacharyya et al. 1990a).

Table 11. Number of Copies of Integrated Plasmids in Independent pJS-3 Transfectants\(^a,b\)

<table>
<thead>
<tr>
<th>Parental Cell Line</th>
<th>pJS-3-Transfected Geneticin-Resistant Cell Strain (Subclone)</th>
<th>Number of Integrated Copies of Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>143 (tk^-)</td>
<td>143-15</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>143-7</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>143-9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>143-14</td>
<td>2 (with rearrangements)</td>
</tr>
<tr>
<td>RD (tk^-)</td>
<td>RD-12</td>
<td>1 (lacking a Bam site)</td>
</tr>
<tr>
<td></td>
<td>RD-3</td>
<td>2</td>
</tr>
<tr>
<td>XP12ROSV40 (tk^-)</td>
<td>XP-6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>XP-7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>XP-8</td>
<td>Multiple</td>
</tr>
</tbody>
</table>

\(^a\) Only the transfectants that yielded productive recombinational events were tested for plasmid copy number.

\(^b\) Table taken from Bhattacharyya et al. (1990a).
Table 12. Rate of Spontaneous Recombination in Various Transfectants

<table>
<thead>
<tr>
<th>pJS-3-Transfected Cell Strain</th>
<th>Experiment Number</th>
<th>Number of Parallel Subcultures</th>
<th>Mean Number of Cells per Subculture ($\times 10^{-6}$)</th>
<th>Mean Number of CHAT-Resistant Clones per Subculture</th>
<th>Recombination per $10^6$ Cells per Cell per Generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>143-7</td>
<td>1</td>
<td>19</td>
<td>10.2</td>
<td>44.9</td>
<td>3.703</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8</td>
<td>8.3</td>
<td>43.5</td>
<td>2.092</td>
</tr>
<tr>
<td>143-9</td>
<td>1</td>
<td>14</td>
<td>6.7</td>
<td>49.8</td>
<td>17.985</td>
</tr>
<tr>
<td>143-14</td>
<td>2</td>
<td>12</td>
<td>10.5</td>
<td>12.9</td>
<td>883</td>
</tr>
<tr>
<td>143-15</td>
<td></td>
<td>15</td>
<td>6.8</td>
<td>6.1</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10</td>
<td>9.2</td>
<td>7.3</td>
<td>145</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10</td>
<td>8.3</td>
<td>3.2</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>8.4</td>
<td>81.2</td>
<td>36.533</td>
</tr>
<tr>
<td>RD-3</td>
<td>1</td>
<td>15</td>
<td>6.4</td>
<td>23.3</td>
<td>484</td>
</tr>
<tr>
<td>RD-9</td>
<td></td>
<td>15</td>
<td>7.9</td>
<td>20.4</td>
<td>5.705</td>
</tr>
<tr>
<td>RD-12</td>
<td>1</td>
<td>15</td>
<td>9.4</td>
<td>52.9</td>
<td>1.744</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15</td>
<td>12.5</td>
<td>52.9</td>
<td>11.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>6.2</td>
<td>159.2</td>
<td>21.569</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>10.9</td>
<td>274.5</td>
<td>783.461</td>
</tr>
</tbody>
</table>

*Table taken from Bhattacharyya et al. (1990a).

Number of cells in the subcultures at the time of assaying for the frequency of tk− cells.

Determination of the Rate of Spontaneous Recombination Among the Various Transfectant Cell Strains

The frequency of recombination, i.e., the number of CHAT-resistant cells in a population at the time of selection, does not give information on the rate of spontaneous recombination in a particular cell strain containing the recombination substrate. For this information, fluctuation analysis tests were carried out. As shown in Table 12, the rate per $10^6$ cells per cell generation for the 143-derived cell strains ranged from 0.15 to 1.9, with a mean of 0.91. The RD-derived strains ranged from 0.6 to 1.2, with a mean of 0.83; and the XP12ROSV40-derived strains ranged from 3.0 to 4.1, with a mean of 3.6. The variance was always greater than the mean number of recombinants, validating the fluctuation test (Bhattacharyya et al. 1990a).

Comparison of Sensitivity of the Cell Strains to Killing and Recombination Induced by 1-Nitrosopyrene and Ultraviolet Radiation

We showed previously (Patton et al. 1986) that XP12BE fibroblasts from complementation group A were significantly more sensitive than normal fibroblasts to the killing

from Bhattacharyya et al. (1990b). The following symbols are used in this figure: • = 143-7 cells; □ = RD-12 cells; and △ = XP7 cells.
Table 13. Representative Example of Data Used to Determine Frequency of Ultraviolet Radiation- and 1-Nitrosopyrene-Induced Recombination in the Three Cell Strains

<table>
<thead>
<tr>
<th>Agent and Dose</th>
<th>Cell Strain</th>
<th>Survival of Control (%)</th>
<th>Target Cells (× 10^6)</th>
<th>Viable Cells (× 10^6)</th>
<th>CHAT-Resistant Colonies</th>
<th>CHAT-Resistant Cells per 10^6 Viable Cells</th>
<th>Induced Frequency (× 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultraviolet (J/m^2)</td>
<td>XP-7</td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>14</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>0</td>
<td>XP-7</td>
<td>42</td>
<td>4</td>
<td>1.7</td>
<td>35</td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td>0.2</td>
<td>XP-7</td>
<td>31</td>
<td>6</td>
<td>1.9</td>
<td>51</td>
<td>27</td>
<td>20</td>
</tr>
<tr>
<td>0.4</td>
<td>XP-7</td>
<td>14</td>
<td>10</td>
<td>1.4</td>
<td>54</td>
<td>42</td>
<td>35</td>
</tr>
<tr>
<td>0.6</td>
<td>RD-12</td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>35</td>
<td>17 (28)</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>RD-12</td>
<td>60</td>
<td>4</td>
<td>2.4</td>
<td>100</td>
<td>42 (69)</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>RD-12</td>
<td>55</td>
<td>6</td>
<td>3.3</td>
<td>127</td>
<td>39 (64)</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>RD-12</td>
<td>16</td>
<td>10</td>
<td>1.6</td>
<td>106</td>
<td>86 (110)</td>
<td>82</td>
</tr>
<tr>
<td>0</td>
<td>143-7</td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>13</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>143-7</td>
<td>84</td>
<td>2</td>
<td>1.7</td>
<td>22</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>143-7</td>
<td>66</td>
<td>4</td>
<td>2.7</td>
<td>54</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>143-7</td>
<td>32</td>
<td>6</td>
<td>1.9</td>
<td>41</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>1-Nitrosopyrene</td>
<td>XP-7</td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>78</td>
<td>39</td>
<td>—</td>
</tr>
<tr>
<td>0</td>
<td>XP-7</td>
<td>77</td>
<td>3</td>
<td>2.3</td>
<td>143</td>
<td>62</td>
<td>23</td>
</tr>
<tr>
<td>0.05</td>
<td>XP-7</td>
<td>42</td>
<td>6.5</td>
<td>2.7</td>
<td>350</td>
<td>128</td>
<td>89</td>
</tr>
<tr>
<td>0.10</td>
<td>XP-7</td>
<td>20</td>
<td>8.5</td>
<td>1.7</td>
<td>319</td>
<td>188</td>
<td>149</td>
</tr>
<tr>
<td>0.15</td>
<td>RD-12</td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>66</td>
<td>33 (55)</td>
<td>—</td>
</tr>
<tr>
<td>0.05</td>
<td>RD-12</td>
<td>60</td>
<td>3</td>
<td>1.8</td>
<td>97</td>
<td>54 (90)</td>
<td>35</td>
</tr>
<tr>
<td>0.10</td>
<td>RD-12</td>
<td>34</td>
<td>4</td>
<td>1.36</td>
<td>97</td>
<td>71 (118)</td>
<td>63</td>
</tr>
<tr>
<td>0.15</td>
<td>RD-12</td>
<td>22</td>
<td>9</td>
<td>2</td>
<td>197</td>
<td>98 (164)</td>
<td>109</td>
</tr>
<tr>
<td>0</td>
<td>143-7</td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>13</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>0.5</td>
<td>143-7</td>
<td>50</td>
<td>6</td>
<td>3</td>
<td>67</td>
<td>23</td>
<td>16</td>
</tr>
<tr>
<td>0.8</td>
<td>143-7</td>
<td>13</td>
<td>8</td>
<td>1</td>
<td>47</td>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td>1.2</td>
<td>143-7</td>
<td>4.8</td>
<td>10</td>
<td>0.48</td>
<td>29</td>
<td>60</td>
<td>54</td>
</tr>
</tbody>
</table>

*Table taken from Bhattacharyya et al. (1996b).*

and mutagenic effects of 1-NOP. XP12BE cells cannot remove covalently-bound 1-NOP residues from their DNA (unpublished data). In contrast, normally repairing human fibroblasts readily excised such adducts, with more than 75% removed in 12 hours (Maher et al. 1988; Yang et al. 1988). Therefore, 1-NOP represents a multiringed chemical carcinogen that forms bulky DNA adducts that are repaired by an excision process defective in XP cells, i.e., nucleotide excision repair. Because of this, we compared the sensitivity of these human cell strains to the killing effect of 1-NOP and 1-N-6-NOP, and of UV radiation. The results for 1-NOP and UV radiation are included in the top panels of Figure 20. As expected, the 143-7 cells were relatively resistant, compared with the other two strains. The XP-7 cells were the most sensitive. The 1-N-6-NOP was tested in the 143-7 and XP-7 cells only. There was little or no cell killing in either strain by doses that were within the solubility of the compound. When we exposed the XP-7 cells to concentrations as high as 10 μM, a precipitate formed, and the cells lifted up from the dishes. In spite of this, we assayed one series of these XP-7 cells for induction of recombination by 1-N-6-NOP, using 5-μM and 10-μM doses. There was no evidence of induction.

We compared the frequency of recombination induced in the three cell strains by 1-NOP as well as by UV radiation (Bhattacharyya et al. 1996b). The data are shown in the bottom panels of Figure 20 and in Table 13. All three strains exhibited a concentration-dependent increase in the frequency of recombinants. The slope (899.2 ± 78) of the line shows the relationship between 1-NOP-induced recombinants and applied concentration for the XP-7 cells, which are totally devoid of excision repair capability. For the RD-12 cells, which have an intermediate level of excision repair capacity, the slope was 444.9 ± 113.4; and for the repair-proficient 143-7 cells, it was 38.2 ± 3.5. Southern blot analysis of CHAT-resistant recombinants induced by UV radiation or 1-NOP (Figure 21) and determination of the loss of Geneticin resistance showed that very few recombinants...
(approximately 7%) resulted from single reciprocal exchanges. This result is similar to the fraction observed with spontaneous recombinants from these human cell strains.

**DISCUSSION**

**STUDIES OF MUTAGENESIS**

One important advantage of the shuttle vector plasmid mutagenesis system we used is that it allowed us to treat plasmids in vitro, determine the frequency of adduct formation, and analyze the kinds of adducts formed. We then were able to introduce the DNA into the human cell line, where the mutations were generated with the human cell DNA replication apparatus. This system allowed us to compare the mutagenic activity of structurally related carcinogens on the basis of equal numbers of adducts formed per plasmid. A second very important advantage is the small size of the target gene supF. Because only 85 base pairs make up the structural portion of the tRNA, this particular gene has been used in a large number of mutagenesis studies (Kraemer and Seidman 1989). The small size allows a reasonable time period to sequence a sufficient number of independent supF mutants to saturate the gene, i.e., to determine at which sites particular genetic changes will result in a phenotypic change. The investigator then can detect a mutation and identify hot spots and cold spots induced in the gene by various mutants. A much greater length of time spent sequencing would be required to saturate an endogenous mammalian cell gene, such as the HPRT gene, and obtain similar information on unique spectra. (The HPRT gene is composed of 44,000 base pairs, with 654 base pairs making up the coding region of the DNA.)

We used this shuttle vector system to gain insight into the mechanisms by which 1-NP, 1,6-DNP, and two other structurally related carcinogens induced mutations when DNA, carrying adducts formed by these carcinogens, replicates in human cells. In particular, we wanted to see whether the four chemicals were approximately equal in their ability to induce mutations. We wanted to compare the chemicals, not on the basis of equal concentration administered, but on the basis of equal numbers of DNA adducts initially formed on the DNA. We also wanted to determine whether the four chemicals produced virtually the same kinds of mutations and whether or not they did so with approximately equal frequency at various sites in the target gene. In other words, would each agent produce its own distinctive pattern of mutation hot spots in the supF gene? Could one get a “signature” to indicate that a particular mutagenic agent had been at work?

Our results showing the spectra of mutations induced by these adducts (Figures 5 and 12), as well as our data estimating the frequency of adduct formation at the various sites in the supF gene (Figures 7, 8, 13, and 14), strongly support our conclusion that the observed mutagenesis was targeted to sites where adducts occurred. This is because all four agents bind predominantly, if not exclusively, to guanine, and the majority of the mutations observed with the carcinogen-treated plasmids involved G:C base pairs (90% for AF adducts, 97% for 1,6-DNP adducts, and 87% for 1-NP adducts). The 32P-postlabeling analysis of plasmids exposed to N-acetyl-a-flavin or 1-N-6-NOP did not reveal any evidence of adduct formation with any base other than guanine. The same results were found with the HPLC analysis of plasmids exposed to N-acetyl-2-flavin or 1-NOP. However, it has been shown that 1-NP can bind to poly A:poly T oligonucleotides (Kinosuchi and Ohnishi 1986). Thus, 1-NP residues bound to adenine may have formed at a low frequency in p2189 in our study. If this did occur, it would help to explain why 13% of the base substitutions we observed with 1-NP-treated plasmids involved A:T base pairs.

The kind of base substitution mutations induced by the four agents were very similar to each other (Tables 4 and 5), with 61% to 66% being G:C → T:A transversions. Several mechanisms could explain the predominance of G:C → T:A transversions. Because all four agents bind principally or exclusively to guanine, these transversions may have resulted from apurinic sites in DNA caused by loss of guanine carrying carcinogen residues. If this latter reaction occurred, and if human cell DNA polymers inserted adenosine triphosphate opposite an apurinic site during replication, as observed by Kunkel (1984) and by Loeb and Preston (1986) in their in vitro polynucleotide studies, this combination of events could have yielded such transversions. However, the adducts we studied are not highly labile, making depurination an unlikely mechanism for the mutagen-
sis they produced. Furthermore, Bichara and Fuchs (1985) studied mutations induced in an N-hydroxy-aminofluorene-treated plasmid during replication in E. coli and evaluated the mutagenic potency of the apurinic lesions in their assay. They found that simple depurination events at added dG-C6-AF residues resulting in G-C → T-A transversion mutations accounted for less than 10% of the induced mutations in their experiments. Another possible explanation for the predominance of G-C → T-A transversions is that the bulky adducts formed in DNA so distort its structure that the added guanine is no longer recognized as instructive for replication, and the polymerases in the human cells preferentially insert an adenine nucleotide opposite them because of this lack of instruction (Strauss et al. 1982). However, not all the base substitutions were G-C → T-A. G-C → C-G transversions and G-C → A-T transitions occurred, both at about the same frequency of 15%.

Another possible explanation for the base substitutions we observed is that changes in the conformation of guanine caused by these bulky carcinogen residues allowed stable purine-purine mispairing (or purine-pyrimidine mispairing, e.g., guanine-thymine) that went undetected and resulted in mutations. Brown and colleagues (1986) showed that in a synthetic decyododecamer, unmodified guanine could pair stably with adenine in the syn position without distorting the helix. Using a different decyododecamer, crystal structures of G(syn)A(anti) mismatches were observed at near-neutral pH (Brown et al. 1989). Furthermore, Norman and associates (1986) found evidence that in a double-stranded oligonucleotide containing a single dG-C6-AF adduct, adenine opposite the modified guanine produced a stable structure that placed the AF in the B-DNA minor groove, with the guanine syn. The fact that the AAF adducted guanine assumes the syn conformation in AAF-modified DNA helix (Grunberger et al. 1970; Fuchs 1975; Santell et al. 1980) may allow the replication machinery to insert an adenosine triphosphate opposite the modified guanine to form a stable mispairing. This could explain the high proportion of G-C → T-A transversions that we observed. The G-C → C-G and G-C → A-T base changes we observed reflect less stable mispairing with guanine or thymine nucleotides opposite the modified guanine. Whether such stable mispairing with adenine with guanine is modified by 1-NP, 1,6-DNP, or AAF adducts has not yet been determined in such model experiments.

The relationship between the frequency of mutants obtained with plasmids carrying AF adducts or 1-NP adducts and the initial number of adducts formed per plasmid was linear (Yang et al. 1988; Mah et al. 1989). This type of linear response was also found with BPDE adducts (Yang et al. 1987). However, with 1,6-DNP adducts and AAF adducts, the curve defining this relationship had a significant quadratic coefficient, so that the frequency of mutants induced per adduct increased as the initial number of residues increased. The reason for this nonlinear increase is not yet known. The adducts formed by these two chemicals are larger than 1-NP or AF adducts. Unlike these adducts, 1,6-DNP and AAF adducts produce minus one frameshift mutations in the supF gene at detectable frequencies of 16% (16 out of 98) and 7% (6 out of 72), respectively. These adducts may cause greater distortion of the supercoiled plasmids when the number of adducts per plasmid increases, and this distortion may allow greater slippage, resulting in frameshift mutations. However, in the case of AAF adducts, we did not find that the plasmids carrying the greatest number of adducts were the ones that produced more frameshift mutations (data not shown). Adducts formed by BPDE, which we expected to be just as distorting, did not cause deletions.

The 1,6-DNP adducts induced a high frequency (13%) of minus-one frameshift mutations, specifically in a run of 5 guanine residues at a specific site on the gene (position 172 through 176). The AAF adducts showed similar results, but at a lower frequency (7%). The AF adducts and 1-NP adducts did not produce any such frameshifts (Figures 5 and 12). Examining the frequency of terminations of polymerization in vitro (Figures 7, 8, 13, and 14) shows that approximately 29% of the covalent binding to that strand of the plasmid by 1-N-6-NOP occurred in the region of 172 through 176. Approximately 20% of the binding by 1-NOP, approximately 11% of the binding by N-AcO-AAF, and approximately 7% of the binding by N-AcO-TFA-AF occurred in that region. Thus, DNA initial binding alone cannot explain the difference in the frequency of minus one frameshift mutations induced in that run of 6 G-C base pairs. The mechanism for this particular type of mutation may be the strand slippage model of Streisinger and colleagues (1966). The reason why such mutations were not found in the run of 4 guanines in the opposite strand of the plasmid (positions 102 through 105), even though binding occurred there, may be that the longer run of G-C base pairs allows more slippage than the shorter run.

As with the induction of minus one frameshift mutations, one possible explanation for the fact that each compound exhibited a unique hot spot for mutations is that the binding of each chemical to specific sites is not uniform, i.e., more adducts are formed at particular unique sites. Figures 7, 8, 13, and 14 show, for example, that N-AcO-AAF bound to site 155 of the 3' strand of the supF gene at a frequency of approximately 8%, whereas the other agents bound at a frequency no greater than 2% at that site. Only AAF adducts showed position 155 as a hot spot for mutations. Similarly, for the 5' strand of the gene, 1-N-6-NOP bound to site 144 at a very high frequency (15%), whereas N-AcO-AAF
formed adducts at a frequency of only 4%. Only 1,6-DNP adducts showed a hot spot at site 144. Again, the clear difference between N-AcO-TFA-AF and N-AcO-AAF, i.e., the hot spot at AAF-induced mutations at site 127, can be explained by the relatively high binding of N-AcO-AAF to that position (13%) compared to that of N-AcO-TFA-AF (only 2%).

Nevertheless, binding cannot be the only factor because our study of the rate of loss of AAF adducts from pZ189 following transfer of the treated plasmids into the excision repair-proficient human cell line indicated that a substantial proportion of the initial adducts are removed during the period before the plasmids can be replicated. If excision repair is influenced by the DNA sequences surrounding the adduct, the spectrum of mutations produced is affected. The surrounding (neighboring) sequence at the site of the adduct is also expected to influence the chance of the polymerases incorporating an incorrect nucleotide across from the adduct. This represents another factor that can affect the spectrum.

Although all the factors that play a role in the mutagenic process cannot yet be accounted for, it is clear from our study that carcinogen adducts induce mutations in DNA, most probably targeted to adducts, and that each agent studied produced its own characteristic spectrum of hot spots in the supF gene when plasmids carrying such adducts were allowed to replicate in human cells. These results support the hypothesis that specific carcinogens cause mutations in specific sites in a target gene, such as the endogenous HPRT gene or specific cellular protooncogenes. Thus, it may be possible to use mutational spectra as biomarkers for human exposure to particular chemical agents. We have recently examined the spectrum of mutations induced in the coding region of the endogenous HPRT gene of diploid human fibroblast cells by BPDE (Chen et al. 1990, 1991; Yang et al. 1991). The kinds of mutations found in those studies were very similar to those found in the supF gene using the shuttle vector assay (Yang et al. 1989). With the support of the Health Effects Institute, we currently are extending such experiments to include mutations induced in human T-lymphocytes by 1-NOP.

STUDIES OF HOMOLOGOUS RECOMBINATION

Mitotic recombination, which results in the loss of one allele of the two alleles of a gene, has been observed in numerous malignant cells, either transformed in culture or derived from tumors arising in vivo. It is considered to play a causal role in certain human cancers. As reviewed by Caveness and colleagues (1983, 1985) and by Scrable and associates (1990), the loss of heterozygosity, detected prin-

cipally by Southern blotting analysis (DNA-DNA hybridization), has been shown to occur in such forms of cancer as retinoblastoma, Wilms tumor, astrocytoma, rhabdomyosarcoma, and, more recently, colon cancer (Fearon and Vogelstein 1990). Evidence suggests that both alleles of certain dominant genes responsible for normal control of cell proliferation and suppression of malignant growth must be eliminated in order for a cell to become cancerous. If only one allele of the gene is mutated, the loss of heterozygosity, i.e., the loss of the other allele, allows the mutant allele carrying a recessive mutation to be expressed in the cell. Perhaps the clearest example of such a suppressor gene being eliminated by mitotic homologous recombination comes from a series of studies with cells from retinoblastoma tumors (reviewed by Scrable et al. 1990). The protein coded for by the retinoblastoma gene blocks the progression of cells from the resting state into the DNA replication phase of the cell cycle. When both copies of this gene are lost, the cell can replicate continually. When a functional gene is returned to a malignant retinoblastoma-derived cell by transfection or electroporation, cell replication ceases.

Of course, it is possible to eliminate both alleles in a single cell by inducing mutations in both of them, but the chances of doing this are very low. The inheritance of an inactivating mutation in one allele of such a tumor suppressor gene predisposes the cell to cancer because only one other inactivating event must occur to eliminate the suppressor function. However, in cells containing two copies (alleles) of a particular tumor suppressor gene, the sequence of events that appears to occur most commonly is the acquisition of a point mutation or deletion in one allele, followed by mitotic recombination (for example, gene conversion) between the mutant allele and the wild-type allele, which eliminates the latter allele. To be certain that mitotic recombination has occurred, rather than the loss of a whole chromosome followed by reduplication of the other chromosome, it is necessary to demonstrate that marker genes on either side of the putative tumor suppressor gene have not been lost. At the present time, such studies are limited; however, evidence of allelic loss by recombination is increasing.

Our working hypothesis was that repeated exposure to carcinogens can move normal cells through the multistep process of malignant transformation, not only by inducing mutations in critical dominant-acting protooncogenes, but also by inactivating one of the alleles of a dominant suppressor gene and then stimulating mitotic recombination, which eliminates the second wild-type allele of such a suppressor gene. To test the hypothesis that carcinogen-induced DNA damage increases the frequency of such homologous recombination between genes that are within the
human genome and to study the effect of DNA repair on this induction in order to understand the mechanisms involved, we used the model system described in this report.

Our experiments involving the mouse L cell line 333M of Liskay and associates (1984) were the first to demonstrate a dose-dependent increase in the frequency of intrachromosomal homologous recombination by a series of chemical carcinogens (Wang et al. 1988; Bhattacharyya et al. 1989). 1-Nitrosopyrene, 4-NQO, and N-AcO-AAF induced such recombination in the mouse L cell line. However, 1-N-6-NOP did not produce this recombination, probably because it was not metabolized into a reactive intermediate. 1-Nitrosopyrene also induced recombination in the human cell strains, but, again, 1-N-6-NOP did not, probably for the same reason. Preliminary experiments suggest that N-AcO-AAF also induces such recombination. The fact that exposing cells in culture to such carcinogens induces recombination suggests that, in addition to inducing point mutations in relevant oncogenes in human cells in the body, carcinogens also may induce homologous recombination, thus contributing in two ways toward induction of the malignant state.

The types of recombinational events we observed to be induced by 1-NOP, N-AcO-AAF, and 4-NQO in the mouse L cell line were similar to those types reported by Wang and coworkers (1988). Of these events, 82% were consistent with gene conversion, and 18% involved single reciprocal exchanges. These results are very similar to the figures of 85% and 15% found for spontaneous recombinational events in this 333M cell line (Liskay et al. 1984; Wang et al. 1988), suggesting that the mechanisms involved in carcinogen-induced recombination are similar to those operating in untreated cells. On the basis of the number of adducts formed, BPDE was the most efficient in inducing recombination, followed by 1-NOP and 4-NQO; N-AcO-AAF was the least efficient. The data suggest that the DNA damage caused by exposing the mouse L cells to these carcinogens stimulates recombination, causing it to occur at a significantly higher frequency than when it occurs spontaneously. However, our finding that the relationship between the frequency of recombination and the initial number of DNA adducts was not the same for the four polycyclic aromatic carcinogens (Figure 1Aa) suggests that the mechanism of recombination recognizes intrinsic differences in the nature of the adducts. For example, BPDE binds principally to the N2 position of guanine (Weinstein et al. 1976); 1-NOP and N-AcO-AAF bind principally to the C8 position of guanine (Heflich et al. 1986, 1988); and 4-NQO binds to both guanine and adenine (Ikenaga et al. 1977). It probably is not fortuitous that the mutagenic effectiveness of this same series of four carcinogens in diploid human fibroblasts, using resistance to 6-thioguanine as the genetic marker, follows the same order as that shown in Figure 16 (Heflich et al. 1980; Aust et al. 1984; Patton et al. 1986; Sato et al. 1986).

If recombination were stimulated by the repair processes involved in removing adducts induced by these multiranged carcinogens, then the difference in the effectiveness of a particular agent, i.e., the frequency of recombination induced per initial frequency of DNA adducts formed, might reflect the rate of removal of adducts from DNA by excision repair. However, with the mouse L cell line, it was not possible to determine the role of excision repair in this induction of recombination. This is because rodent cell lines in culture do not carry out extensive excision repair of damage from their overall genome. Instead, they preferentially remove damage from specific active genes (Bohr et al. 1983). It was for this reason that we transfected the recombination substrate into three human cell lines that differed in their excision repair abilities (Bhattacharyya et al. 1990a). The availability of such cell strains allowed us to determine the role that nucleotide excision repair plays in the induction of homologous recombination between endogenous genes in mammalian cells.

Before trying to use comparative studies to examine this role of nucleotide excision repair, we had to see if these strains had comparable spontaneous rates of recombination. Excision repair-deficient mutants of E. coli K12 bacteria exhibit an enhanced background frequency of spontaneous recombination (Zieg et al. 1978), and excision repair-defective rad3 mutants and other mutants of Saccharomyces cerevisiae sensitive to UV radiation exhibit higher background frequencies of spontaneous mitotic recombination than do strains that repair normally (Boram and Roman 1976; Kern and Zimmerman 1978; Aguilera and Klein 1988; Montelone et al. 1988). However, excision-defective S. cerevisiae mutants carrying rad1 show decreased frequencies of spontaneous mitotic recombination (Schiastl and Prakash 1988).

We found no significant difference between the repair-deficient RD-derived cell strains and the repair-proficient 143-derived strains with regard to their mean rates of spontaneous intrachromosomal homologous recombination (0.8 and 0.9 events per 10^6 cells per generation, respectively), even though the former strain is less capable of carrying out nucleotide excision repair than the latter. The mean rate of spontaneous recombination in the cell strains derived from the XP cell line from complementation group A, which is very deficient in such repair, was 3.8 events per 10^6 cells per cell generation. Whether this four-fold difference is significant is being investigated.

In the majority of the spontaneous recombinational events observed in the human cell strains (86%, 38 out of
43), the neo gene was retained, which is consistent with nonreciprocal transfer of wild-type genetic information (gene conversion). In the other 12%, only a single copy of the Htk gene remained, reflecting a single reciprocal exchange within a chromatid or a single unequal exchange between sister chromatids. This 88:12 ratio of nonreciprocal to reciprocal spontaneous recombinational events is similar to the 85:15 ratio of spontaneous events observed with these genes in the 333M mouse L cell line (Liskay et al. 1984). We found no evidence of double reciprocal exchanges within a chromatid, an event which would generate two Xhol-sensitive sites in one of the two Htk genes. Letsou and Liskay (1986), who studied spontaneous recombination between the Htk genes integrated into the 333M mouse L cell line, also found no incidence of this. Our assay system (Figure 1) cannot distinguish between gene conversion and double unequal reciprocal exchanges between sister chromatids because cells that do not contain a wild-type Htk gene are not recovered after selection for growth in CHAT medium. But double reciprocal exchanges probably did not account for our results because single reciprocal exchanges occurred only 12% of the time. Analogous studies in S. cerevisiae, in which one can distinguish between the two types of events, also show that gene conversion is a very frequent event in recombination (Klein 1984; Jackson and Fink 1985).

Some of the Can in resistant transfectant human cell strains, obtained after transfer of plasmid pJS-3 into our parental cell lines, did not give rise to CHAT-resistant cells. Otherwise several possible explanations for such results. During the transfections, one of the Htk genes may have been inactivated by a gross rearrangement (insertion, deletion, etc.). Such damage to incoming plasmids during the transfection process is well-documented (Calos et al. 1983; Razaque et al. 1983). A second possibility is that the site of integration of the substrate in the genome affected its ability to recombine or to express the wild-type Htk gene (position effect). For example, integration near the centromere may result in some physical constraint for recombination between the two Htk genes. This possibility is under investigation. However, the important point is that we succeeded in generating three human cell strains that contained a single intact copy of a stably integrated substrate for assaying homologous recombination. These strains exhibited low rates of similar spontaneous recombination, yet differed in their excision repair capacities.

These three cell strains were used to determine whether excision repair stimulates recombination. Their sensitivity to killing by UV radiation or 1-NOP was correlated with their lack of nucleotide excision repair capacity (top panels of Figure 20). The XP-7 cells, which are completely lacking in excision repair, were the most sensitive. Their UV radiation and 1-NOP survival curves are virtually identical to what was found previously with the diploid XP fibroblast cell line XP12BE, which also lacks the capacity to repair UV radiation or 1-NOP-induced DNA damage (Patton et al. 1984, 1986). The 143-7 cells, which are normally proficient in excision repair, were the most resistant. Their survival curves were equal to those found previously with excision repair-proficient diploid human fibroblasts derived from normal neonates (Patton et al. 1986; Maher et al. 1988). The RD-12 cells showed intermediate survival curves, which was consistent with the low, but detectable level of excision repair capability that was observed in these cells (Bhattacharyya et al. 1990a).

The sensitivity of these three cell strains to homologous recombination induced by UV radiation or 1-NOP was correlated inversely with their capacity to excise DNA damage caused by these agents (bottom panels of Figure 20). The most repair-deficient cell strain, XP-7, showed the highest frequency of recombination; the RD-12 cell strain, which has an intermediate repair capacity, was more resistant, and the 143-7 cell strain, which is repair-proficient, had the lowest frequency of recombination induced by these carcinogens. The 143-7 cells can rapidly remove lesions in DNA caused by UV radiation or 1-NOP, whereas the XP-7 cells cannot carry out such repair and the RD-12 cells do so only at a low rate (Bhattacharyya et al. 1990b). Thus, the observed recombination cannot be the result of intermediates generated by nucleotide excision repair. The differences in induced frequency also cannot be caused by differences in the rates of spontaneous recombination in these cell strains because their rates are so similar (Bhattacharyya et al. 1990a). Similar results using UV radiation as the DNA damaging agent and an independent series of human cell strains containing a related recombination substrate, were obtained in this laboratory by Tsujimura and associates (1990).

These differences in recombination frequencies that we observed cannot simply reflect different amounts of damage induced because, for a given dose of UV radiation, all three strains received equal amounts of UV radiation-induced damage. The results indicate that recombination between the duplicated Htk genes was stimulated by the presence of lesions remaining unexcised in the DNA some time after treatment, after the 143-7 cells have had time to remove most of the lesions and the RD-12 cells have been able to remove some of them. The unexcised lesions remaining in the cellular DNA may have stimulated recombination directly, for example, by blocking DNA synthesis, leading to the generation of discontinuities (single-stranded regions) that initiate recombination. We favor this hypothesis (Bhat-
tacharyya et al. 1990b; Maher et al. 1990). Of course, it is possible that the presence of the unexcised lesions interfered with DNA synthesis, RNA synthesis, or other cellular processes, and such interference indirectly resulted in homologous recombination by inducing an as yet unidentified process. Whatever the mechanism, the presence of unexcised adducts or photoproducts in these human cells increased the frequency of homologous recombination without drastically altering the ratio of the kinds of recombination events. The vast majority, 91% (51 out of 56), of the products analyzed represented gene conversions; this is similar to the findings for spontaneously arising intrachromosomal homologous recombination in these cell strains. Whether this difference in ratio, 91:9 versus 88:12, is significant requires additional studies.

Our data showing that UV radiation-induced recombination frequencies, including single reciprocal exchanges, are higher in cells that cannot remove such lesions or cells that excise them only slowly, are consistent with the hypothesis that a pathway for transferring lesions from parental strands to daughter strands (by recombination) functions in these human cells. To understand how DNA replication can proceed on a damaged template, Forance (1983) assayed UV-irradiated human cells for the presence of photoproducts (as revealed by endonuclease-sensitive sites) in thedaughter strands of DNA. He found that for a given dose of UV radiation, the number of endonuclease-sensitive sites in the daughter strands was higher for XP cells than for normal human cells. Evidence for the presence of pyrimidine dimers in the daughter strand of Simian virus-40 DNA has also been obtained in UV-irradiated monkey cells (Das-Gupta and Summers 1980).

In summary, our recombination data shown in Figure 20 suggest that excision repair lowers the frequency of homologous recombination by removing prerecombinogenic lesions that would otherwise induce such recombination. The recent recognition that mitotic recombination can play a role in carcinogenesis by eliminating tumor suppressor genes (Cavenoe 1983, 1985) strongly suggests that carcinogens not only cause activating mutations in dominant oncogenes and inactivating mutations in tumor suppressor genes, but also can function to allow the latter to exert their effect in the cell and bring it ultimately to the malignant state.

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Mutations and Homologous Recombination Induced by Nitropyrenes and Related Compounds


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Mutations and Homologous Recombination Induced by Nitropyrenes and Related Compounds


ABOUT THE AUTHORS

Veronica M. Maher obtained a Ph.D. in molecular biology at the University of Wisconsin, Madison, in the McArthur Laboratory for Cancer Research, and carried out postdoctoral studies at Yale University School of Medicine. Dr. Maher is a Professor in the Department of Biochemistry and the Department of Microbiology at Michigan State University, as well as Co-Director of the Carcinogenesis Laboratory. She also serves as the Associate Dean for Graduate Studies in the College of Osteopathic Medicine. Her current research interests include molecular mechanisms of mutagenesis and recombination in human cells; chemical- and radiation-induced carcinogenesis; and the effect of DNA repair on these processes.

J. Justin McCormick received a Ph.D. in cell physiology at
Catholic University, Washington, D.C., and carried out postdoctoral studies at the McArdle Laboratory for Cancer Research at the University of Wisconsin. He is a Professor in the Department of Biochemistry and Department of Microbiology at Michigan State University, as well as Co-Director of the Carcinogenesis Laboratory. He is also the Associate Director of the Cancer Etiology Program at the Cancer Center at Michigan State University, and serves as the Associate Dean for Research in the College of Osteopathic Medicine. His current research interests include the role of oncogenes in the transformation of human cells and chemical- and radiation-induced multistep carcinogenesis in human cells.

Nitai P. Bhattacharyya received a Ph.D. in physics from Calcutta University, Calcutta, India, in 1985. He carried out postdoctoral studies in the Carcinogenesis Laboratory at Michigan State University from 1986 through 1989. He is now a scientist on the staff of the Saha Institute of Nuclear Physics in Calcutta. His research interests include the molecular mechanisms of carcinogenesis, recombinant, and carcinogenesis, as well as DNA repair.

Janet Boldt received an M.D. and a Ph.D. in toxicology from the University of Hamburg, Germany, in 1989, and carried out postdoctoral studies in molecular biology in the Carcinogenesis Laboratory at Michigan State University from 1989 to 1991. She is currently pursuing a medical residency in Germany. Her research interests include the molecular mechanisms of carcinogenesis and mutagenesis.

Michael Chin-Miao Mah received an M.D. from the Federal University of Minas Gerais, Brazil, in 1986, and a Ph.D. in microbiology from Michigan State University in 1991, under the direction of Professor Maher. She pursued a year of postdoctoral studies in the Carcinogenesis Laboratory at Michigan State University and is currently a research associate at the Institute of Molecular and Cellular Biology, Strasbourg, France, with Dr. Robert Fuchs. Her research interests include the molecular and cellular biology of carcinogenesis and mutagenesis.

Jia-Ling Yang received a Ph.D. in biochemistry from Michigan State University in 1986 under the direction of Professor Maher. She pursued postdoctoral studies for one year in the Carcinogenesis Laboratory at Michigan State University and then at the Academia Seneca in Taipei, Taiwan. In 1991, she accepted a position as Associate Professor in the Department of Life Sciences at Ching-Hwa University in Taiwan. Her research interests include the molecular and cellular biology of environmental mutagenesis and DNA repair.

**PUBLICATIONS RESULTING FROM THIS RESEARCH**


Maher VM, Bhattacharyya NP, Wang Y, Tsujimura T, Liskay


**ABBREVIATIONS**

| AAF       | 2-acetylaminofluorene |
| AF        | 2-aminofluorene       |
| A-T       | adenine-thymine       |
| BPDE      | (±)-7β,8α-dihydroxy-9α,10α-epoxyp-78,9,10-tetrahydrobenzo[a]pyrene |
| C-G       | cytosine-guanine      |
| CHAT      | deoxycytidine, hypoxanthine, aminopterin, thymidine |
| dG-C8-AF  | N-(deoxyguanosin-8-yl)-1-aminofluorene |
| dG-C8-1-A-6-NP | N-(deoxyguanosin-8-yl)-1-amin-6-dinitropyrene |
| dG-C8-1-AF | N-(deoxyguanosin-8-yl)-1-amino-6-dinitropyrene |
| dG-N2-AAF | 3-(deoxyguanosin-N2-yl)-acetylaminofluorene |
| 1,3-DNP   | 1,3-dinitropyrene      |
| 1,6-DNP   | 1,6-dinitropyrene      |
| 1,8-DNP   | 1,8-dinitropyrene      |
| G-C       | guanine-cytosine      |
| HPLC      | high-pressure liquid chromatography |
| HPRT      | hypoxanthine(guanine)-phosphoribosyl-transferase |
| Htk       | Herpes simplex 1 virus thymidine kinase gene |
| kbp       | kilobase pair         |
| N-AcO-AAF | N-acetoxy-2-acetylaminofluorene |
| N-AcO-TFA-AF | N-acetoxy-N-trifluoroacetyl-2-aminofluorene |
| 1-N-6-NOP | 1-nitro-6-nitrosopyrene |
| 1-NOP     | 1-nitrosopyrene       |
| 1-NP      | 1-nitropyrene         |
| 4-NQO     | 4-nitroquinoline-1-oxide |
| O.D.      | optical density unit  |
| T+A       | thymine-adenine       |
| ThR       | thymidine             |
| tk        | thymidine kinase enzyme |
| UV        | ultraviolet           |
| XP        | xeroderma pigmentosum |
INTRODUCTION

A Request for Applications (RFA 86-2) that solicited proposals for "Health Effects of Diesel Emissions" was issued by the Health Effects Institute (HEI) in the summer of 1986. In response to the RFA, Dr. Veronica Maher, of Michigan State University, submitted a proposal entitled "Kinds of Mutations Induced in Human Cells by 1-Nitrosopyrene, 1-Nitro-8-Nitrosopyrene, and Related Aromatic Amine Derivatives and Their Induction of Homologous Recombination." The HEI approved the three-year project, which began in July 1986. Total expenditures were $395,517. The Investigators' Report was received at HEI for review in March 1991. A revised report was received in December 1991 and was accepted by the Health Review Committee in April 1992. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. The Health Review Committee's Commentary is intended to place the Investigators' Report in perspective as an aid to the sponsors of HEI and to the public.

REGULATORY BACKGROUND

The U.S. Environmental Protection Agency (EPA) sets emissions standards for diesel engines and vehicles under Section 202 of the Clean Air Act, as amended in 1990. Section 202(a)(1)(A) of the Act directs the Administrator of the EPA to "prescribe (and from time to time revise) . . . standards applicable to the emissions of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Section 202(a)(3)(A)(i) of the Act, as amended by Section 201 of the 1990 Amendments, specifically directs the Administrator to set standards for the "emissions of hydrocarbons, carbon monoxide, oxides of nitrogen, and particulate matter from classes . . . of heavy-duty vehicles or engines . . . ."

The EPA has taken a variety of regulatory actions with respect to diesel engines and vehicles under the authority given it by Sections 202(a)(1) and (a)(3)(A)(i). The EPA has set emissions standards initially made applicable to all engines and vehicles produced in a given model year. Engines and vehicles of the same class that are produced in succeeding years must also comply with these existing standards, unless the EPA establishes a new set of standards.

Section 202(a)(4)(A) states that, to comply with emission requirements of the Clean Air Act Amendments, emission control technologies used in new motor vehicles or engines after model year 1978 should not "cause or contribute to an unreasonable risk to public health, welfare, or safety in its operation or function." To assess the extent to which unreasonable risk may occur, Section 202(a)(4)(B) requires, in part, that the use of such control technologies be considered in terms of the extent it has on concentrations of unregulated pollutants and whether alternative technologies or methods can be used to produce less risk to public health.

With respect to heavy-duty diesel engines and vehicles, the EPA issued emissions standards in 1980 that specified limits for hydrocarbons, carbon monoxide, and oxides of nitrogen applicable to engines and vehicles produced in the 1985 model year. New standards later were promulgated for the 1988 and 1989 model years, with the 1988 model year standards adding limits to particulate matter emissions. Emission standards for the 1991 and 1994 model years have also been set by the EPA and were revised most recently in 1989 and 1990.

The 1990 Amendments to the Clean Air Act include several provisions that deal with diesel engines and vehicles. Section 202(a)(3)(B)(ii) of the Act, as amended by Section 201 of the 1990 Amendments, sets out new emissions standards for oxides of nitrogen produced from diesel-powered heavy-duty trucks. This Section requires that, beginning in model year 1998, all diesel-fueled heavy-duty trucks will emit not more than 4.0 grams of nitrogen oxides per brakehorsepower-hour.

Emissions from buses are a focus of several provisions in the 1990 Amendments. Section 202(f) of the Act, added by Section 207(b) of the 1990 Amendments, requires increasingly demanding particulate matter emission standards for buses not covered by Section 219 of the Act. Sections 219(a) and (b) of the Act, as added by Section 227 of the 1990 Amendments, requires the EPA to promulgate regulations "applicable to urban buses for the model year 1994 and thereafter" that will reduce particulate matter emissions by as much as 50%.

SCIENTIFIC BACKGROUND

The health effects of exposure to diesel engine exhaust became a focus of intensive research as use of these engines in light-duty passenger automobiles and vans increased during the early 1970s. Today, although diesel-powered au-
tomobiles do not represent a large segment of the light-duty fleet in the United States, heavy-duty diesel engines are widely used in trucks, buses, trains, farm equipment, machinery, and ships. Over the last decade, the findings of many researchers have raised the question of whether exposure to diesel exhaust presents possible health risks. Diesel engine exhaust has been categorized as a "potential occupational carcinogen" by the United States National Institute for Occupational Safety and Health (1988) and as "probably carcinogenic to humans" by the International Agency for Research on Cancer (1989). This is the result of epidemiologic data and diesel engine exhaust's mutagenic and carcinogenic properties (both discussed below).

**DIESEL CARCINOGENICITY**

Whole diesel engine exhaust is composed of two phases: a vapor phase containing organic chemicals, and a particulate phase containing particles with adsorbed organic chemicals. The importance of the particulate phase of diesel soot in lung tumor development has been demonstrated by the lack of tumors in rats exposed to exhaust from which particles had been removed by filtration (Heinrich et al. 1986; Iwai et al. 1986; Brightwell et al. 1989).

Diesel exhaust particles are composed of a dense carbonaceous core containing adsorbed, combustion-derived organic compounds that account for 15% to 45% of the total particulate mass (International Agency for Research on Cancer 1989). Among these organic compounds are polycyclic aromatic hydrocarbons (PAHs)* and their nitrated derivatives (nitro-PAHs). Because of their small size (0.1 to 0.5 μm), diesel particles are readily respirable and deposit in the deepest regions of the respiratory tract (Chan et al. 1981; reviewed by McClellan 1987). Interest in the potential carcinogenicity of the organic compounds adsorbed to diesel particles was derived from reports that organic solvent extracts of these particles are carcinogenic in mouse skin bioassays (Kotin et al. 1955; Nesnow et al. 1983), and are mutagenic in the Ames Salmonella typhimurium assay (Huizingh et al. 1978).

Epidemiological investigations of the relationship between occupational exposure to diesel exhaust and cancer have produced conflicting results (reviewed by McClellan 1987; reviewed by Mauderly 1992). However, recent studies provide suggestive evidence that chronic exposure to high concentrations of diesel engine exhaust produces a slightly elevated incidence of bladder and lung cancer (Silverman et al. 1988; Garshick et al. 1987, 1988; International Agency for Research on Cancer 1989).

Animal studies provide evidence that diesel exhaust is a pulmonary carcinogen in rats (Heinrich et al. 1986; Iwai et al. 1986; Mauderly et al. 1987; Brightwell et al. 1989). Exposure of rats to whole diesel exhaust induced a dose-dependent increase in both benign and malignant forms of adenomatous tumors and squamous tumors at soot concentrations of 3.5 mg/m³ or higher. No such response occurred in rats exposed to a lower level of diesel soot (2.3 mg/m³) (Ishinishi et al. 1988). In contrast, exposure of hamsters to soot concentrations as high as 8.6 mg/m³ did not produce tumors (Heinrich et al. 1986; Brightwell et al. 1989).

Two theories have been advanced to account for the carcinogenicity of diesel exhaust in rats. The first theory proposes that compounds adsorbed to diesel particles may induce carcinogenesis by interacting with DNA in target cells and producing mutations. Replication of mutated DNA can produce abnormal progeny that develop tumorigenic properties. Alternatively, because rat lung tumors developed after exposure to high concentrations of diesel soot, Vostal (1986) proposed that overloading normal lung clearance mechanisms could induce tumors by a nonspecific effect of the foreign material on the replication of sensitive lung epithelial cells. Spontaneous, or chance, mutations can take place during normal cell replication. In response to the presence of foreign material, lung cells can increase their replication rate, enhancing their probability of undergoing spontaneous mutational events leading to tumorigenesis.

This research report focuses on the genetic changes caused by exposing cells to two nitro-PAHs found adsorbed to diesel particles. Dr. Maher and colleagues proposed that these chemicals can induce carcinogenesis by genetic changes that activate naturally occurring protooncogenes and inactivate naturally occurring tumor suppressor genes. The remainder of this section will discuss the genotoxicity of these nitro-PAHs and their impact on carcinogenesis.

**NITROPYRENES IN DIESEL EXHAUST**

Nitropyrenes are a class of nitro-PAHs containing one or more nitro groups covalently bound to carbon atoms. Nitro-PAHs form when the parent PAHs, present in fuel or produced by the incomplete combustion of fuel, react with nitrogen oxides in the presence of trace amounts of nitric acid (Pitts et al. 1978). This reaction takes place under a variety of circumstances: during fuel combustion; when PAHs in combustion products react with nitrogen oxides in engine emissions; or when gas-phase or particle-adsorbed PAHs are oxidized and nitrated in the atmosphere by hydroxyl radicals and nitrogen oxides (Pitts 1987). By reacting with DNA, several nitro-PAHs display both mutagenic and carcinogenic activities in relation to mammalian cells (Hefflich et al. 1986a; Rosenkranz and Howard 1986). Thus,*

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*A list of abbreviations appears at the end of the investigators' Report for your reference.
this class of compounds represents a potential risk to human health. Studies with organic solvent extracts of diesel soot particles established that the nitropyrene-containing fraction accounted for approximately half of the total mutagenic activity with regard to bacteria (Huisingsh et al. 1979). 1-Nitropyrene (1-NP) is mutagenic in hamster and human cells, as well as in bacteria (Takayama et al. 1983; Heffrich et al. 1985, 1986a,b; Patton et al. 1986; Eddy et al. 1987). However, most of the mutagenic activity of the nitropyrenes in bacteria is accounted for by 1,6-dinitropyrene (1,6-DNP) and 1,8-dinitropyrene (1,8-DNP), which are found in much lower concentrations in diesel particle extracts than 1-NP (Mermelstein et al. 1981; Rosenkrantz 1982; Salmeen et al. 1984). The tumorigenic properties of 1-NP and the dinitropyrenes display a similar relationship: 1,6- and 1,8-DNP are substantially more tumorigenic than 1-NP (Heffrich et al. 1986b; Tokiwa and Ohnishi 1986), which exhibits weak tumorigenicity in weanling female rats (Ishida et al. 1991).

Nitropyrenes differ from many other PAHs because they do not require the addition of a mixture of exogenous oxidizing enzymes to produce mutations in bacteria. Instead, after nitropyrenes enter target cells, they undergo enzymatic reduction to arylhydroxylamino derivatives, and, in the case of dinitropyrenes, possibly conversion to their O-esters (Djuric et al. 1985). These derivatives spontaneously form reactive arylhydroxylaminium ions that bind covalently to DNA, forming DNA adducts (Rosenkrantz and Howard 1986).

The formation of DNA adducts is believed to be the mechanism by which many chemicals exert the mutagenic effects that may be an early step in tumor induction (reviewed by Harris et al. 1987). The results from two studies support, but do not prove, a relationship between DNA adduct formation and lung tumor development after exposure to diesel engine exhaust. Maunderly and coworkers (1987) demonstrated that benign and malignant tumors formed exclusively in peripheral lung tissue after chronic exposure of rats to diesel exhaust. Bond and colleagues (1988) showed that peripheral lung tissue was the site of the highest levels of DNA adducts in rats exposed to a similar level of diesel exhaust. The similarity between DNA adducts formed in cultures of human tissues exposed to a variety of chemicals and those in laboratory animals in which these chemicals induce cancer, provides additional support for a link between adduct formation and tumor induction (Harris et al. 1987).

DNA ADDUCTS AND MUTATIONS

Carcinogenesis develops as a result of changes in normal cellular processes that control growth, differentiation, and development. These changes are driven by inherited, spontaneous, and carcinogen-induced genetic and nongenetic (epigenetic) events (Shields and Harris 1991). Initiation, the first step in carcinogenesis, can be induced by a chemical carcinogen reacting with DNA to form DNA adducts. Adducts can interfere with DNA's normal replication process, and when DNA replication proceeds on DNA templates containing such adducts, mistakes (mutations) are introduced into the new strands. When new strands containing mutations replicate, the changes are made permanent and are found in new cell generations (Wogan and Gorelick 1985; Cohen and Ellwein 1990). Heffrich and colleagues (1985) described both DNA adduct formation and gene mutations in Chinese hamster ovary cells exposed to 1-nitropyrene (1-NOP) (a partially reduced derivative of 1-NP that forms before the arylhydroxylamino derivative and the active arylhydroxylaminium ion). Similar studies were carried out with diploid human fibroblasts (Patton et al. 1986). Mutated cells, which persist in tissue long after the initiating agent has disappeared, can be converted to a tumor in later, epigenetic stages of promotion and progression (Wigley and Balmain 1991; Strauss 1992).

Because most mammalian and bacterial cells have at least some capacity to correct lesions in DNA, not all DNA adducts remain in DNA long enough to cause mutations (Griffin 1991). In a process called excision repair, specific enzymes excise DNA sequences containing adducts, while other enzymes repair the damage by synthesizing a new DNA segment and adding it to the original molecule. Humans show interindividual differences in their ability to carry out DNA repair (Harris 1989), with some individuals being markedly deficient. The latter, who suffer from syndromes characterized by deficiencies in DNA repair are prone to the development of several forms of cancer. For example, individuals with xeroderma pigmentosum have an increased risk of sunlight ( ultraviolet)-induced skin cancer (Wogan and Gorelick 1985). Because their skin cells cannot excise ultraviolet-induced DNA lesions, these individuals are hypersensitive to mutations induced in the cells in those parts of their body exposed to sunlight (Maher et al. 1976, 1979). Even in individuals with normal repair capacities, mutations can be introduced if replication takes place before excision repair enzymes remove DNA adducts and add new DNA (Konze-Thomas et al. 1982).

Dr. Maher and her colleagues studied two possible changes in genes that contained unexcised adducts after exposure to compounds present on diesel exhaust particles: (1) production of gene mutations, which can activate protooncogenes to oncogenes, and (2) homologous recombination, which can play a role in the inactivation of tumor suppressor genes. Because these two processes are at the core of this study, they are described in more detail in the following section.
ROLE OF MUTATION AND HOMOLOGOUS RECOMBINATION IN ACTIVATION OF PROTOONCOCGENES AND INACTIVATION OF TUMOR SUPPRESSOR GENES

Because cancer cells have a proliferative rate higher than that of normal cells, one mechanism of cancer development is likely to involve a disruption of normal restraints on cell proliferation (Alberts et al. 1989). There are two genetic routes that might cause uncontrolled cell proliferation. The first route is by making a normal stimulatory gene, a protooncogene, hyperactive. The second route is by inactivating a tumor suppressor gene.

Protooncogenes are normal genes expressed during embryogenesis and normal post-natal growth. They code for proteins that appear to play crucial roles in normal cellular growth and differentiation (Stowers et al. 1987). Protooncogenes can be activated to oncogenes by mutation in only one of a diploid cell's two gene copies (alleles) (Alberts et al. 1989). Reddy and coworkers (1982) established that a mutational change at even a single nucleotide in a DNA molecule can activate a protooncogene to an oncogene. If the mutation occurs in the region of a gene coding for a specific protein, the protein may be produced in normal amounts, but may be more active than its normal counterpart. If, instead, mutation occurs in a control region, the gene may be overexpressed, producing a protein with normal activity, but in greater than normal amounts (Alberts et al. 1989). Changes in at least 17 protooncogenes (either in gene expression or in the gene product) have been implicated in human malignancies (Bishop 1991). The eventual dysregulation of growth and differentiation enhances the probability of neoplastic transformation (Harris 1991).

In contrast to the hyperactivity associated with oncogenes, tumor suppressor genes have been recognized by the oncogenic consequences of their loss of expression. Tumor suppressor gene products are believed to counterbalance normal proliferation-driving protooncogene products (Sager 1992). Therefore, inactivation or loss of a normal tumor suppressor gene frees a cell of the gene's inhibitory effect on cell proliferation (Alberts et al. 1989). Changes in the activity of at least 12 tumor suppressor genes have been linked indirectly to the development of a variety of human tumors (Bishop 1991).

A link between the normal activity of a tumor suppressor gene and inhibition of cell proliferation was demonstrated by Kasten and colleagues (1991). The investigators exposed human cells to carcinogens and noted that as the protein product of the p53 tumor suppressor gene accumulated, the cell cycle slowed down just before DNA synthesis. Slowing the cell cycle caused a transient "switching off" of cell proliferation. This was interpreted as allowing extra time for the cells to repair carcinogen-damaged DNA, thereby decreasing the chance of mutational events producing potentially tumorigenic cells (Kasten et al. 1991; Lane 1992). The importance of normal p53 tumor suppressor gene activity is illustrated by the observation that its loss is the most common genetic alteration found in human cancers (Levine et al. 1991; Wafik et al. 1992).

In order for the activity of most tumor suppressor genes to be lost, inactivating changes must occur in both gene alleles (Alberts et al. 1989). This contrasts with the change in only one allele that is sufficient to convert a protooncogene to an oncogene. Inactivation of a tumor suppressor gene can begin with a mutation, eliminating the activity of one allele (Stratton et al. 1990). The activity of the second allele can be lost by a process called homologous mitotic recombination (Fearon and Vogelstein 1990; Scoble et al. 1990). During cell replication, DNA can undergo a wide variety of recombinations (rearrangements) that alter gene expression (Rubnitz and Subramani 1985). Recombinations are initiated by enzymatic cleavage of specific sequences in double-stranded DNA, producing complementary single-strand DNA sequences that pair, or recombine, at regions with the homologous sequences (Chakrabarti et al. 1985; Chakrabarti and Seidman 1986; Lin et al. 1987). Recombination between DNA sequences of a mutated tumor suppressor gene allele and homologous sequences of the active allele inactivates the second allele, thus causing the loss of gene activity.

In summary, increasing evidence suggests that transformation of a normal cell to one with a tumorigenic capacity involves both activation of protooncogenes to oncogenes, and inactivation of tumor suppressor genes (Fearon and Vogelstein 1990). Because mutation and homologous recombination play critical roles in these genetic changes, Dr. Meher and colleagues examined the ability of the activated products of two nitropyrenes found in diesel exhaust, and related compounds, to produce changes in DNA that drive these processes.

JUSTIFICATION FOR THE STUDY

Through RFA 86–2, entitled "Health Effects of Diesel Emissions," HEI solicited applications for studies aimed at providing an understanding of the relative importance of exposure to the various components of diesel exhaust, including carbonaceous particles, their associated organic compounds, and gases, on tumorogenesis. An additional area of interest was the further development and applica-
tion of techniques to detect and quantify markers of exposure and dose of diesel emission products, such as DNA and protein adducts. Seven studies were funded under RFA 86-2. Three of these studies investigated the relationship between particle-associated organic compounds and carcinogenicity in rats. Four of the studies dealt with various aspects of nitropyrenes, including their metabolic activation, biomonitoring via adduct formation, and mutagenicity.

This report presents the results of Dr. Maher's second HEI-funded study. In her first study, Maher and associates (1988) compared the in vitro cytotoxic and mutagenic effects of 1-NP and 1-NOP on fibroblast cells from normal human subjects, an individual with the excision-repair-deficient disease Xeroderma pigmentosum, and a person with an inherited predisposition to malignant melanoma.

In the present study, Maher and colleagues hoped to gain insight into the mechanisms by which N-substituted aryl carcinogens cause genetic changes involved in carcinogenesis. The genetic changes they proposed to study (DNA mutation and homologous recombination) have been implicated in two critical steps that can convert a normal cell to one with a tumorigenic capacity. These steps are the activation of protooncogenes to oncogenes and the inactivation of tumor suppressor genes. Because these experiments were responsive to the RFA and were expected to provide valuable information on the carcinogenic potential of some of the organic compounds that are adsorbed to diesel particles, the HEI Research Committee recommended funding for the study, which began in July 1987.

OBJECTIVES AND STUDY DESIGN

The long-range objective of the study was to determine the mechanisms by which N-substituted aryl carcinogens, such as 1-NP or 1,6-DNP, cause genetic changes, and how these changes might be involved in the malignant transformation of mammalian cells.

The investigators’ specific aims were:
1. To determine whether the adducts formed by each compound cause a unique spectrum of mutations (changes in the sequence of the organic bases that make up the DNA molecule) during replication of a target gene, and whether these mutations occur primarily at sites of carcinogen binding (sites of adduct formation); and
2. To determine whether these carcinogens induce homologous recombination in mammalian cells, and, if so, whether recombination occurs by mechanisms involving DNA excision-repair processes.

MUTAGENESIS

The key component of the mutation assay system was a plasmid (a small, circular DNA molecule) carrying a defined target gene that served as a marker for mutation. In addition to the target gene, the investigators added other genetic components that allowed the plasmid to replicate, first in mammalian cells, and then in bacteria. This was termed a “shuttle vector.”

In place of 1-NP and 1,6-DNP, the investigators used partially reduced nitropyrene derivatives: 1-NOP, the partially reduced derivative of 1-NP, and 1-nitro-6-nitrosopyrene (1-N-6-NOP), the partially reduced derivative of 1,6-DNP. Using partially reduced derivatives was necessary because plasmids were exposed to the nitropyrenes in vitro, in the absence of enzymes required for reduction in the pathway converting nitropyrene to their activated forms. Each partially reduced nitropyrene derivative was incubated with plasmids in the presence of ascorbic acid. This further reduced each derivative to the corresponding arylhydroxylamine, a metabolite that forms the aryltintrinemium ion that reacts with plasmid DNA to form a covalently bound adduct.

After separating the plasmids from ascorbic acid and unreacted nitrosopyrenes, the investigators measured the number of bound adducts. They then added the plasmids containing adducts to a human embryonic kidney cell line, where mutations in the target gene occurred during plasmid replication. The replicated plasmids were recovered from the human cells and separated from cellular DNA by taking advantage of the plasmids’ lower molecular weight. Those plasmids containing a mutated target gene were identified by adding the plasmids to a specific bacterial strain. The researchers identified those bacteria containing a plasmid with a mutated target gene by observing the color of the colonies formed in the presence of a dye. Bacteria with a plasmid containing a functional (nonmutated) target gene synthesized an enzyme that reacted with the dye, producing blue colonies. Bacteria containing a plasmid with a nonfunctional (mutated) target gene do not produce this enzyme, and thus form white colonies. Dr. Maher and colleagues then isolated plasmids from bacteria containing a mutated target gene. By comparing the sequence of DNA bases (adenine, thymine, guanine, cytosine) in the target gene with the sequence of bases in the gene from untreated plasmids (which had also replicated in cell culture), the investigators identified the specific mutational changes that had been induced by each nitropyrene and the location of these mutations within the gene.

The investigators then determined whether there were “hot spots,” sites where a large fraction of the mutations
were found in the target gene, and whether these mutational hot spots corresponded to hot spots for carcinogen binding (sites of frequent adduct formation). Because the DNA polymerase enzymes that synthesize new DNA use an existing DNA molecule as a template, bulky adducts on DNA interfere with in vitro replication by stopping the polymerase just before the site of an adduct. The investigators used this interference with in vitro replication to determine the location on the target gene where the polymerase reaction was terminated and the frequency of polymerase terminations at a particular site (hot spots for carcinogen binding). These data were compared with the location and frequency of mutations in the gene induced by exposing plasmids to 1-N-6-NOP and 1-NOP (hot spots for mutations). (For details of this assay, please refer to the Investigators' Report.)

Two structurally related carcinogens, N-acetoxy-2-acetylaminofluorene (N-AcO-AAF) and N-acetoxy-N-trifluoracet-32l-aminofluorene (N-AcO-TFA-AF), react with the same atom in DNA (the C8 position of guanine) as 1-NOP and 1-N-6-NOP. Therefore, the investigators also determined whether these compounds induced the same kinds of mutations as nitropyrenes and whether they occurred at the same sites in the target gene.

The target gene for mutation was the supF gene, which coded for the synthesis of a tyrosine suppressor transfer RNA. The small size of the target gene permitted easier analysis at the DNA sequence level and determination of hot spots for mutation induction by DNA adducts. The investigators proposed that an additional advantage was that mutations at almost any site in the gene's structural portion eliminate gene function. An advantage of the human host cell line used for plasmid replication was that it produces few spontaneous supF mutants.

**HOMOLOGOUS RECOMBINATION**

The investigators next compared the ability of 1-NOP, 1-N-6-NOP, N-AcO-AAF, and 4-nitroquinoline-1-oxide to induce homologous recombination as a function of both their concentration and the number of DNA adducts formed. 4-Nitroquinoline-1-oxide was tested because, like 1-NOP and 1-N-6-NOP, it is an N-substituted polycyclic aromatic carcinogen that must be activated by reduction before it can bind covalently to DNA.

First, the investigators added increasing amounts of each compound to cultures of a mouse cell line engineered to contain two inactive alleles (copies) of a gene that normally codes for the synthesis of the enzyme thymidine kinase. They then placed the carcinogen-exposed cells in a selective culture medium, designed so that the cells needed an active thymidine kinase enzyme in order to survive and grow. Obtaining an active thymidine kinase required a productive recombination event between the two inactive, mutant alleles that yielded a nonmutant allele. The frequency of cells acquiring a functional gene by recombination was then identified by measuring the frequency of colony formation in the selective medium. These data were compared with the frequency of colonies formed by cells that had not been exposed to the test compounds.

To compare the frequency of homologous recombination on the basis of the number of DNA adducts formed, the investigators exposed the cells containing the plasmid to radioactively labeled carcinogens (whose relative abilities to induce recombination were known from earlier experiments) and determined the frequency of recombination and the number of DNA adducts formed.

In earlier studies, Dr. Maher and her coworkers established that several chemical carcinogens induced dose-dependent increases in the frequency of homologous recombination in the same mouse cell line (Wang et al. 1988). However, they could not determine whether the final steps in recombination involved retention of DNA adducts, or their removal by repair mechanisms. To gain insight into the role of DNA adducts and repair in recombination, Dr. Maher and coworkers inserted DNA carrying the two inactive gene alleles into the genome of three human cell lines with different capacities for removing adducts from DNA. By exposing each cell line to 1-NOP and comparing the frequencies of recombination, the investigators were able to determine whether recombination was favored in those cells with active DNA repair systems, or whether recombination was stimulated by unexcised adducts remaining in DNA.

**TECHNICAL EVALUATION**

**ATTAINMENT OF STUDY OBJECTIVES**

The investigators successfully investigated the mechanistic bases of two phenomena associated with the induction of cancer: mutations and recombinational events. They demonstrated that nitropyrenes, chemicals present in diesel and other emissions, induced specific mutational events and affected the frequency of recombinational events. They also established that two structurally related compounds that bind to the same area of DNA as the nitropyrenes had similar effects. Thus, the study sheds light on the potential of these ubiquitous compounds to participate in two rate-limiting processes that have been implicated in cancer initiation and progression.
ASSESSMENT OF METHODS AND STUDY DESIGN

The experiments were well designed, clearly constructed, and provided much useful data.

The methods were state of the art at the time the study began. The shuttle vector system, used in the mutation assay, was a valuable first step in analyzing the types of mutations induced in human cells by this group of model mutagens and carcinogens. In 1992, this was the only feasible method for comparing mutational spectra caused by exposure to different carcinogens. Shuttle vector systems offered convenience, the ability to do site-specific studies, and to saturate targets with specific adducts. However, recent advances, such as polymerase chain reaction technology, make it possible to analyze mutational events in more relevant genetic targets, such as normal chromosomal genes, rather than in artificially constructed shuttle vectors.

The homologous recombination studies provide additional evidence for the potential genetic toxicity of these chemicals.

STATISTICAL METHODS

The data are presented thoroughly and in a manner allowing interpretation by the reader. The investigators provided suitable regression slopes, standard errors, and other requisite statistical information. The statistical analyses showed how precisely the experiments differentiated the behavior of the different carcinogens.

In figures showing linear or quadratic relationships, the investigators determined the curves by least squares regression analysis. For linear relationships, slopes and standard errors were presented. Statistically significant differences in slopes were defined as values with p < 0.05. One method used by the investigators to compare the ability of the different nitropyrenes to induce homologous recombination was to compare the slopes of the least squares line defining the frequency of recombinants induced as a function of applied concentration. Because the frequency of recombination does not provide information on the rate of spontaneous recombination in a cell line containing the recombination substrate (plasmid), the investigators used fluctuation analysis to obtain these values.

Figures 7 and 8 in the Investigators' Report correlate the sites of mutation, identified by DNA sequencing, with the sites of adduction, as located by interrupted DNA polymerization. These diagrams consist of two one-dimensional scales laid side by side. To examine the correlation of the scales, it is necessary to "walk through" the diagram point by point, which appears to be an inefficient procedure. The authors did not make use of any of the common two-dimensional devices for depicting a bivariate relationship, such as the standard statistical scatterplot, nor did they provide a quantitative basis for measuring correlation and detecting hot and cold spots.

RESULTS AND INTERPRETATIONS

This section presents a selection of those findings that may most aid in assessing the relationship of DNA adduct formation with mutations and homologous recombination. For a full discussion of Dr. Maher's findings, please refer to the Investigators' Report.

Mutagenesis

The number of adducts formed per plasmid, at similar nitropyrene concentrations, was greater after plasmids were exposed to 1-NOP than when they were exposed to 1-N-6-NOP. Because reduction by ascorbic acid produces intermediates that are subsequently converted to the active derivatives binding to DNA, this result may have been due to a greater reduction of 1-NOP to its arylhydroxylation derivative, producing an increased level of its active arylnitrenium ion. Alternatively, the absence of O-esterification of 1-N-6-NOP (Djuric et al. 1985), under the in vitro conditions used, may have resulted in the 1-N-6-NOP's slower conversion to its active arylnitrenium ion.

The mutation frequency data demonstrated that adducts formed by the dinitropyrene derivative caused mutations in the supF gene more effectively than those formed by 1-NOP. An interesting finding is that, after exposure to 1-N-6-NOP, the frequency of mutations induced per adduct increased as the number of adducts increased. The investigators provided no explanation for this non-linear increase.

Exposing plasmids to 1N-6-NOP and 1-NOP, as well as N-AcO-AAF and N-AcO-TFA-AAF, produced a spectrum of mutations in the target gene that was characteristic for each chemical. Although there were areas in the gene that showed significant numbers of mutations (hot spots) after individual exposures to several of the chemicals, there were also specific areas where mutations appeared only after exposure to one of the chemicals.

After plasmids were exposed to each chemical, the kinds of mutations induced in the target gene by the DNA adducts were generally similar. These were predominantly substitutions of a guanine-adenine base pair by a thymine-adenine base pair. However, 1-N-6-NOP adducts induced another type of mutation, deletion of a single guanosine-cytosine base pair, at a rate nine times that of 1-NOP adducts.
The investigators established that adduct formation occurred predominantly at guanine residues in the target gene. This would agree with the majority of the nitrosopyrene-induced mutations involving substitutions of guanine-cytosine base pairs.

A high frequency of DNA polymerase termination (sites of adduct formation) occurred at prominent sites of mutation. Therefore, the investigators found a positive correlation between sites of carcinogen binding and sites of mutation.

**Homologous Recombination**

Most of the chemicals studied increased the frequency of recombination, compared with untreated control cells. Because recombination can occur by more than one mechanism, the investigators studied the nature of the recombinational events. They found that, although the chemicals increased the rate of recombination, the nature of the recombinational events was the same as that occurring spontaneously in control cells. The proportion of recombination via gene conversion (the majority of events) versus single reciprocal exchanges, was the same in both control and treated cells. Those readers who are interested in the molecular nature of the two recombination mechanisms are referred to Dr. Maher's lucid description in the Investigators' Report.

By using three cell lines that differed in their ability to carry out excision-repair processes, Dr. Maher and her colleagues demonstrated a positive correlation between the frequency of recombinational events and the number of adducts remaining unexciised in the DNA molecule, that is, the presence of unexciised adducts on DNA stimulated the frequency of homologous recombination.

An interesting finding is that, although 1-NOP, 4-nitroquinoline-1-oxide, and N-AcO-AAF induced intrachromosomal homologous recombination, 1-N-6-NOP did not. This was in spite of the fact that numerous reports indicate that the parent 1,6-DNP causes mutations and breaks in DNA in several other mammalian cell lines studied. The investigators suggested that a possible explanation for this result is a lack of DNA adduct formation in these cells. However, because intrachromosomal homologous recombination has been suggested as playing a significant role in the inactivation of tumor suppressor genes (although its requirement is not absolute), and in view of the established carcinogenicity of 1,6-DNP, the 1-N-6-NOP results raise some questions concerning the adequacy of this experimental system.

**IMPLICATIONS FOR FUTURE RESEARCH**

The use of the shuttle vector was appropriate at the time this study began. However, advances such as polymerase chain reaction technology now make it possible to study mutational changes in more relevant genetic targets. The shuttle vector experiments described in this study demonstrate that each compound produced subtly different mutational spectra. In principle, this suggests that it may be possible to link mutations in the somatic cells of potentially exposed individuals with specific chemical agents (Thilly et al. 1989).

As part of a new study supported by HEI, Dr. Maher is applying newer technology to study the mutational spectra induced in the coding region of an endogenous human gene exposed in culture to 1-NP and/or to diesel emission particles.

**CONCLUSIONS**

This report provides evidence that nitropyrenes increase the frequency of mutations and homologous recombination. Both 1-NOP and 1-N-6-NOP produced DNA adducts and induced gene mutations. (Because DNA adducts were formed in vitro in the absence of activating enzymes, these partially reduced intermediates were used as surrogates for the parent compounds, 1-NP and 1,6-DNP, which are present on diesel exhaust particles.)

The kinds of base substitution mutations induced by the nitropyrene derivatives were similar, regardless of which chemical was used for exposure. However, each chemical produced a unique spectrum of mutations, that is, each one caused large numbers of mutations (hot spots) in different areas in the DNA molecule, producing a specific "fingerprint." Such fingerprints have the potential to provide useful biomarkers in cases of suspected human exposure to mutagens, by furnishing cellular evidence of exposure and identifying the type of mutagen involved. Comparing the frequency of adduct formation at various sites in the DNA molecule with the location of mutational hot spots provided evidence that mutations occurred at prominent sites of adduct formation.

Exposing cells to 1-NOP (as well as to several related compounds) increased the frequency of homologous recombination. A cell line that could not repair DNA lesions by excision-repair processes was more sensitive to 1-NOP induced homologous recombination than were cells that pos-
sessed this capacity. Therefore, the presence of DNA adducts stimulated homologous recombination.

Because both types of genetic changes studied by Dr. Maher and her colleagues have the potential to remove normal restraints on cell proliferation, this study provides insight into possible mechanisms by which exhaust constituents, such as nitropyrenes, can cause genetic changes that are implicated in carcinogenesis.

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