

Analysis at the Sequence Level of Mutations Induced by the Ultimate Carcinogen *N*-Acetoxy-*N*-2-Acetylaminofluorene

by Robert P. P. Fuchs,* Nicole Schwartz* and Michel P. Daune*

The covalent binding of an ultimate carcinogen to the DNA bases or phosphate groups creates a premutational lesion that *in vivo* is processed by the repair, replication and recombination enzymes, and eventually may be converted into a mutation. Being interested in the way that an initial premutational event is converted into a stable heritable mutation, we have sequenced stable mutations in a gene that has formed covalent adducts *in vitro* with *N*-acetoxy-*N*-2-acetylaminofluorene (*N*-AcO-AAF), a model for the ultimate metabolite of the rat liver carcinogen 2-acetylaminofluorene, AAF). *In vivo* studies have shown the mutagenicity of AAF and its derivatives in both bacterial and eukaryotic systems. *N*-AcO-AAF reacts *in vitro* with DNA leading mainly to the formation of a guanine adduct, *N*-2-(deoxyguanosin-8-yl)-acetylaminofluorene (80%) and to at least three minor adducts. Studies by our group showed that binding of *N*-AcO-AAF to DNA resulted in a local distortion of the DNA helix around the C-8 adduct (the insertion-denaturation model).

We describe here the analysis of forward mutations induced in the tetracycline-resistance gene of pBR322 by directing the chemical reaction of the carcinogen to a small restriction fragment (*Bam*HI-*Sal*I) inside the antibiotic-resistance gene. Mutants are selected for ampicillin (Ap) resistance and tetracycline (Tc) sensitivity. The plasmid DNA of such mutants was analyzed for sequence changes in the fragment where the AAF binding had been directed.

We show here that the mutations are mainly frameshifts involving GC base pairs and that certain base pairs (hotspots) are affected at high frequencies.

Introduction

An important step in the carcinogenic process is thought to be the initial attack of the DNA molecule by a so-called ultimate carcinogen. In fact, more than 90% of the carcinogens tested are mutagens in bacterial systems (1). The premutational event is the covalent binding of the ultimate carcinogen to the DNA bases or phosphate groups. The chemical structure of the adducts formed, and to a lesser extent the structural changes induced in the DNA double helix in the neighborhood of the adducts, has been extensively studied during the last ten years. However the crucial question is "How will the different repair, replication and recombination enzymes handle these chemically modified bases?" In other words, since the end point of this initial step is a mutation, "How is this initial premutational event converted into a stable and heritable

mutation?" *N*-Acetoxy-*N*-2-acetylaminofluorene (*N*-AcO-AAF) is a model ultimate metabolite of the strong rat liver carcinogen 2-acetylaminofluorene (AAF). *In vivo* studies have shown the mutagenicity of AAF and its derivatives in both bacterial (2, 3) and eukaryotic systems (4). *N*-AcO-AAF reacts *in vitro* with DNA leading mainly to the formation of a guanine adduct (5), *N*-2-(deoxyguanosin-8-yl)-acetylaminofluorene (80%) and also to at least three minor adducts (N. Schwartz, R. P. P. Fuchs and M. P. Daune, unpublished results), one of which is characterized as 3-(deoxyguanosin-*N*-2-yl)-acetylaminofluorene (6).

Studies from our group led to the general conclusion that binding of *N*-AcO-AAF to DNA resulted in a local distortion of the DNA helix around the C-8 adduct (7-9). We have called this structural alteration the insertion-denaturation model (10). A similar model has been proposed by other investigators (11).

In this paper we describe the analysis of forward mutations induced in the tetracycline-resistance

*Institut de Biologie Moléculaire et Cellulaire du CNRS, 15 rue Rene Descartes, 67084 Strasbourg Cédex, France

gene of pBR322 by directing the chemical reaction of the carcinogen to a small restriction fragment (*Bam*HI, *Sal*I) inside the antibiotic-resistance gene. A preliminary report of this work has recently been published (12).

Material and Methods

The *E. coli* strains used were AB 1157 or AB 1886 (13). N-AcO-AAF (^3H -ring) was synthesized as described previously (14) (specific activity: 196 mC/mole). N-AcO-AAF (^3H -ring) reaction with supercoiled plasmid DNA was performed in 10mM Tris, 1mM EDTA, pH 8, buffer (TE buffer) containing 5% of ethanol (DNA concentration: 50 $\mu\text{g}/\text{mL}$). Removal of unbound fluorene derivatives was achieved by four successive ethanol precipitations. The number of AAF residues bound per plasmid molecule was determined as previously described.

Samples of pBR322 reacted with N-AcO-AAF to various extents (ranging from 0 to 2.5% of modified bases) were digested with *Bam*HI, and *Sal*I restriction enzymes (Boehringer, Mannheim). The large fragment (16S fragment) and the small fragment (6S fragment) were separated and purified either by velocity sedimentation on sucrose

gradients (5% to 20%) or by electrophoresis on 0.8% agarose or on 8% polyacrylamide gels followed by electroelution. T4 DNA ligase (Biolabs) was used to ligate the unmodified 16S fragment with either the unmodified 6S fragment of the 6S fragments obtained from the various AAF-modified pBR322 samples (6S-AAF). The ligation was performed under the conditions specified by the T4 DNA ligase manufacturer. The DNA fragment concentrations were 16.5 $\mu\text{g}/\text{mL}$ for the 16S fragment and 7.5 $\mu\text{g}/\text{mL}$ for the 6S fragment.

Ultraviolet Irradiation of the Cells prior to Transformation

In some cases, the *E. coli* cells were ultraviolet-irradiated prior to the transformation procedure. This treatment was used to induce the cellular SOS response. The cells were ultraviolet-irradiated as a suspension in 0.01M MgSO_4 with a germicidal lamp (15 W, Phillips) at a dose giving about 50% survival (i.e., 60 J/m^2 for the wild type strain, AB 1157; 6 J/m^2 for the *uvrA* strain, AB 1886). The cells were then incubated in LB medium for 30 min at 37°C to allow expression of the SOS function.

E. coli Transformation and Selection of the Ampicillin-Resistant (Ap^{R}) and Tetracycline-Sensitive (Tc^{S}) Clones

The *E. coli* was made competent for transformation by the classical CaCl_2 treatment procedure (16). The different ligation mixtures were diluted by a factor of 100 in 10mM Tris, 10mM CaCl_2 and 10mM MgCl_2 (pH 7) and used to transform the competent cell suspension by mixing one volume of the DNA solution with two volumes of the concentrated *E. coli* suspension. Following the transformation procedure, the cells were spread on LB plates containing Ampicillin (50 $\mu\text{g}/\text{mL}$) and incubated at 37°C overnight. The clones were then replated on LB plates containing tetracycline (20 $\mu\text{g}/\text{mL}$). Clones which grew on Ap but not on Tc were scored as Ap^{R} , Tc^{S} mutants. Such individual mutant clones were then grown further in LB medium plus Ampicillin for preparation of the plasmid DNA contained in these clones. Plasmid DNA was purified either on a small scale (10 mL of culture) by an adaptation of the method of Clewell and Helinski (17) or on a larger scale (1l culture) by a NaCl/SDS lysis procedure followed by a CsCl/Ethidium bromide centrifugation step (18).

DNA Sequence Analysis of the Mutants

Plasmids were digested with *Bam*HI and *Sal*I restriction enzymes and ^{32}P end-labeled at their 5'

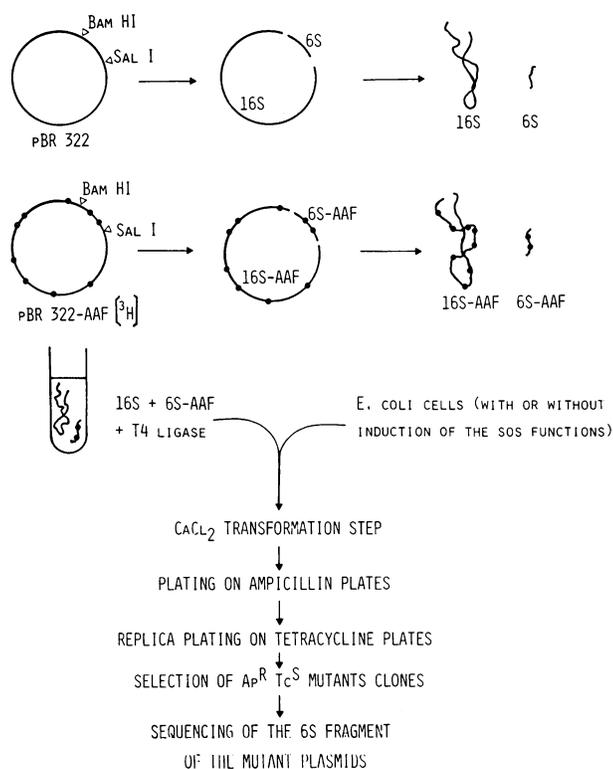


FIGURE 1. Strategy for the site-directed mutagenesis experiment.

extremities with T4 DNA kinase (Boehringer, Mannheim). Strand separation and sequencing were performed according to the method of Maxam and Gilbert (19).

Results

The strategy that was used to obtain mutants in the tetracycline resistance gene, within the small restriction fragment (*Bam*HI, *Sal*I), is outlined in Figure 1. This restriction fragment (275 base pairs long: 6S fragment) modified to various extents with N-AcO-AAF [³H-ring] was reinserted by *in vitro* ligation into the nonreacted large (*Bam*HI, *Sal*I) restriction fragment (16S fragment). This large fragment contains both the gene coding for the β -lactamase (Ap resistance gene) and the origin of replication. The ligation mixture was used to transform CaCl₂-treated *E. coli* recipient cells. Mutants are selected for Ampicillin (Ap) resistance and tetracycline (Tc) sensitivity.

Frequency of Obtention and Restriction Enzyme Analysis of the Ap^R Tc^S Mutants

Mutation frequencies were calculated as the ratio of Ap^R Tc^S clones/Ap^R clones. The mutation frequency in the control experiment in which the 16S fragment was ligated to a nonmodified 6S fragment was 0.4% when no ultraviolet treatment was applied to the bacteria prior to the transformation step. This frequency was similar (0.6%) when the ultraviolet treatment was applied. When analyzed by gel electrophoresis, the plasmid DNAs isolated from such clones were always shorter in length than the original pBR322. In general, the size reduction ranged from 0.2 to 0.8 kb. The restriction analysis pattern showed that these mutant DNAs had retained the unique *Eco* RI site but that in general they had lost both the *Bam*HI *Sal*I restriction sites. We call these mutants class I mutants and suggest that they mainly arise from the dimerization of the 16S fragment. Such dimers, which have the Ap^R Tc^S phenotype, are then converted to smaller plasmids (monomers) through *in vivo* recombination. Work is in progress to lower this mutation background by using an alkaline phosphatase-treated 16S fragment. Class I mutants were easily recognized and excluded from the pool of mutants to be sequenced.

When 6S-AAF fragments ligated to the nonmodified 16S fragment are used to transform *E. coli* one finds a decrease in the transformation efficiency with increasing levels of bound AAF residues. (Fig. 2). The extent of this AAF-dependent inactivation of transformation is strongly related to the general repair genotype of the recipient cell (R. P. P. Fuchs and E. Seeberg, manuscript in preparation). One

also finds a corresponding increase in the mutation frequency, provided the cells are exposed to UV prior to the transformation step. The mutant DNAs isolated from such experiments fall into two classes when analyzed by gel electrophoresis: class I mutants defined as in the control experiment, and class II mutants, exhibiting the original size of pBR322 and retaining both *Bam*HI and *Sal*I restriction sites.

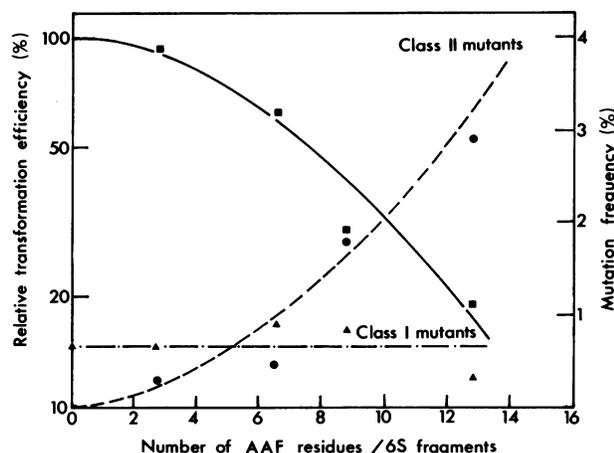


FIGURE 2. Relative transformation efficiency and mutation frequencies as a function of the number of AAF residues bound to the 6S fragment: (■) relative transformation efficiency (log scale); (▲) class I mutants; (●) class II mutants. The transformation of *E. coli* AB 1157 strain has been performed after ultraviolet irradiation of the cells (60 J/m²) as described in the legend to Figure 1. Selection of the transformants was done on LB plates containing ampicillin (50 μ g/mL). Mutants were selected by replica plating on LB plates containing tetracycline (20 μ g/mL).

The frequency of class II mutants increases with the level of AAF modification and reaches about 3% at the highest level tested (Fig. 2). It should be stressed that only the class II mutation frequency is a function of -AAF modification and dependent on ultraviolet irradiation of the host cell (for the conditions, see legend to Fig. 1).

Sequence Analysis of Nine pBR322 Ap^R Tc^S Mutants

Nine class II mutant plasmids were isolated from either the wild type *E. coli* strain, AB 1157, or the corresponding *uvrA* mutant strain, AB 1886. The double *Bam*HI/*Sal*I digested DNA was ³²P-end-labeled at the 5' extremities and sequenced according to the Maxam and Gilbert technique (19). The sequence of the wild type 6S fragment of pBR322 was found to be identical to the sequence published by Sutcliffe (20). In all of the nine class II mutants we found a mutation located within the 6S fragment. All of the mutants showed a deletion of

guanine-AAF adduct that might favor the hairpin structure shown in Figure 4. Due to the multicopy state and to the recessivity of the mutations that are scored in our system, the conversion of the premutagenic lesion into a stable mutation most likely occurs simultaneously in both strands prior to replication.

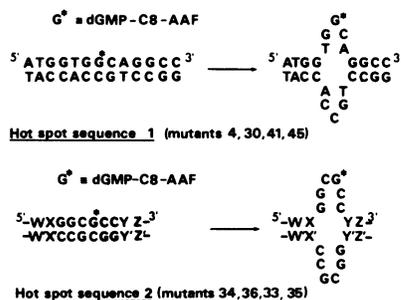


FIGURE 4. Hypothetical hairpin structure at hot spot sequences 1 and 2. According to the insertion-denaturation model proposed by Fuchs and co-workers (7, 10), there is a local denaturation of the helix around the guanine-AAF adduct that might favor the hairpin structure.

The molecular mechanism by which the mutation is being fixed remains to be elucidated.

This work has been supported by Grant No. 79.7.0664 from the D.G.R.S.T. (Délégation Générale a la Recherche Scientifique et Technique).

REFERENCES

- McCann, J., Choi, E., Yamasaki, E., and Ames, B. N. Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 carcinogens. *Proc. Natl. Acad. Sci. (U.S.)* 72: 5135-5139 (1975).
- Ames, B. N., Gurney, E. G., Miller, J. A., and Bartsch, H. Carcinogens as frameshift mutagens: metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. *Proc. Natl. Acad. Sci. (U.S.)* 70: 782-786 (1973).
- Santella, R. M., Fuchs, R. P. P., and Grunberger, D. Mutagenicity of 7-iodo and 7-fluoro derivatives of *N*-hydroxy and *N*-acetoxy-*N*-2-acetylaminofluorene in the *Salmonella typhimurium* assay. *Mutat. Res.* 67: 85-87 (1979).
- Landolph, J. R., and Heidelberger, C. Chemical carcinogens produce mutations to ouabain resistance in transformable C 3H/10 T^{1/2} Cl 8 mouse fibroblasts. *Proc. Natl. Acad. Sci. (U.S.)* 76: 930-934 (1979).
- Kriek, E., Miller, J. A., Juhl, V., and Miller, E. C. 8-(*N*-Fluorenylacetyl)guanosine and arylamidation reaction product of guanosine and the carcinogen *N*-acetoxy-*N*-2-fluorenylacetylamine in neutral solution. *Biochemistry* 6: 177-182 (1967).
- Westra, J. G., Kriek, E., and Hittenhausen, H. Identification of the persistently bound form of the carcinogen *N*-acetyl-2-aminofluorene to rat liver DNA *in vivo*. *Chem.-Biol. Interact.* 15: 149-164 (1976).
- Fuchs, R., and Daune, M. Physical studies on deoxyribonucleic acid after covalent binding of a carcinogen. *Biochemistry* 11: 2659-2666 (1972).
- Fuchs, R. P. P., and Daune, M. P. Dynamic structure of DNA modified with the carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene. *Biochemistry* 13: 4435-4440 (1974).
- Fuchs, R. P. P. *In vitro* recognition of carcinogen-induced local denaturation sites in native DNA by S1 endonuclease from *Aspergillus oryzae*. *Nature*, 257: 151-152 (1975).
- Fuchs, R. P. P., Lefèvre, J. F., Pouyet, J., and Daune, M. P. Comparative orientation of the fluorene residues in native DNA modified by *N*-acetoxy-*N*-2-acetylaminofluorene and two 7-halogen derivatives. *Biochemistry* 15: 3347-3351 (1976).
- Grunberger, D., and Weinstein, I. B. Conformational changes in nucleic acids modified by chemical carcinogens. In: *Chemical Carcinogens and DNA* (P. Grover, Ed.), CRC Press, Vol. 2, Boca Raton, FL, pp. 59-94.
- Fuchs, R. P. P., Schwartz, N., and Daune, M. P. Hot spots of frameshift mutations induced by the ultimate carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene. *Nature* 294: 657-659 (1981).
- Howard-Flanders, P., Boyce, R. P., and Theriot, L. Three loci in *E. coli* K-12 that control the excision of pyrimidine dimers and certain other mutagen products from DNA. *Genetics* 53: 1119-1136 (1966).
- Lefevre, J. F., Fuchs, R. P. P., and Daune, M. P. Comparative studies on the 7-iodo and 7-fluoro derivatives of *N*-acetoxy-*N*-2-acetylaminofluorene: binding sites on DNA and conformational change of modified deoxytrinucleotides. *Biochemistry* 17: 2561-2567 (1978).
- de Murcia, G., Lang, M. C., Freund, A. M., Fuchs, R. P. P., Daune, M. P., Sage, E., and Leng, M. Electron microscopic visualization of *N*-acetoxy-*N*-2-acetylaminofluorene binding sites in Col E1 DNA by means of specific antibodies. *Proc. Natl. Acad. Sci. (U.S.)* 76: 6076-6080 (1979).
- Cohen, S. N., Chang, A. C. Y., and Hsu, L. Non-chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. (U.S.)* 69: 2110-2114 (1972).
- Clewell, D. B., and Helinski, D. R. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. *Proc. Natl. Acad. Sci. (U.S.)* 62: 1159-1166 (1969).
- Katz, L., Kingsbury, D. K., and Helinski, D. R. Stimulation by cyclic adenosine monophosphate of plasmid deoxyribonucleic acid-protein relaxation complex. *J. Bacteriol.* 114: 557-591 (1973).
- Maxam, A. M., and Gilbert, W. A new method for sequencing DNA. *Proc. Natl. Acad. Sci. (U.S.)* 74: 560-564 (1977).
- Sutcliffe, J. G. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR 322. *Cold Spring Harbor Symp. Quant. Biol.* 43: 77-90 (1979).
- Isono, K., and Yourno, J. Chemical carcinogens as frameshift mutagens: Salmonella DNA sequence sensitive to mutagenesis by polycyclic carcinogens. *Proc. Natl. Acad. Sci. (U.S.)* 71: 1612-1617 (1974).