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

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-acetylaminoanthrionine (N-AcO-AAF, a model for the ultimate metabolite of the rat liver carcinogen 2-acetylaminoanthrionine, 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 (BamHI-SalI) 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-acetylaminoanthrionine (N-AcO-AAF) is a model ultimate metabolite of the strong rat liver carcinogen 2-acetylaminoanthrionine (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-N2-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
gene of pBR322 by directing the chemical reaction of the carcinogen to a small restriction fragment (BamHI, SalI) 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 (H-ring) was synthesized as described previously (14) (specific activity: 196 mC/mmole). N-AcO-AAF (H-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 μg/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 extends (ranging from 0 to 2.5% of modified bases) were digested with BamHI, and SalI 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 μg/mL for the 16S fragment and 7.5 μg/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 MgSO4, with a germicidal lamp (15 W, Phillips) at a dose giving about 50% survival (i.e., 60 J/m2 for the wild type strain, AB 1157; 6 J/m2 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 (ApR) and Tetracycline-Sensitive (TcS) Clones

The E. coli was made competent for transformation by the classical CaCl2 treatment procedure (16). The different ligation mixtures were diluted by a factor of 100 in 10mM Tris, 10mM CaCl2, and 10mM MgCl2 (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 μg/mL) and incubated at 37°C overnight. The clones were then replated on LB plates containing tetracycline (20 μg/mL). Clones which grew on Ap but not on Tc were scored as ApR, TcS 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 BamHI and SalI restriction enzymes and 32P end-labeled at their 5'
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 (BamHI, SalI), is outlined in Figure 1. This restriction fragment (275 base pairs long; 6S fragment) modified to various extents with N-AcO-AAF[H-ring] was reinserted by in vitro ligation into the nonreacted large (BamHI, SalI) 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 ApR TcS Mutants

Mutation frequencies were calculated as the ratio of ApR TcS clones/ApR 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 BamHI SalI 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 ApR TcS 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 BamHI and SalI restriction sites.

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 ApR TcS 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 BamHI/SalI 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
either a single GC base pair or a doublet of adjacent GC-CG base pairs. Two of these mutants (numbers 35 and 36) also had a second mutation (Table 1).

Two hot spot sequences for mutagenesis were found within the collection of the nine mutants.

**Hot Spot Sequence 1.** As shown in Table 1, four out of the nine mutants (mutants number 4, 30, 41 and 45) show a deletion of a single G residue at position 520 or 521. (Numbering starts clockwise from the unique Eco RI site). It should be noted that the four mutants have arisen under quite different conditions (i.e., in two different strains, with or without ultraviolet induction of the SOS functions, with very different AAF modification levels).

**Hot Spot Sequence 2.** Mutants 34, as shown in Figure 3, and 36 exhibit a -2 deletion within the alternating GGC sequence at positions 435-438 (deletion of a GC sequence at position 435-436 or 437-438, or of a CG sequence at position 436-437). Mutant 33 also exhibits a -2 deletion within a GGC sequence at position 548-551. The mutation in mutant 35, although being different (-1 deletion of G 416 and double transition at 414-415) also takes place within the same 6-nucleotide long sequence, GGC-GCC, sequence that is in common to all four mutants 34, 36, 33, 35. This given sequence, that is found three times within the 6S fragment, can therefore be considered as a mutational hot spot. Such a GC deletion in an alternating GC sequence was shown in vivo to be a hot spot for reversion of the mutation, his D 3052, in Salmonella by the mutagen 2-nitrosfluorene (21). It is striking to find that the same type of mutation occurs in both a reversion and a forward mutation assay.

**Conclusion**

The reason why such particular sequences are hot spots for mutagenesis is not clear. Whether or not such sequences are hot spots for the AAF binding reaction itself is presently under investigation. Alternatively, it is more likely that the processing of the premutational lesions is strongly sequence-dependent. It is noteworthy that at both hot spots the sequence is such that one can draw a short “hairpin-type” secondary structure (Fig. 4). Such hairpins are too short to be stable by itself but might be highly stabilized by the conformational change that -AAF introduces when bound to C-8 of guanine. As stated by the insertion-denaturation model proposed by Fuchs and co-workers (7, 10), there is a local denaturation of the helix around the

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**Table 1**

| Description of the mutation | Mutant number | Strain | Induction of SOS functions | Average number of bound AAF residues/6S fragment | Sequence in the neighborhood of the mutation |
|----------------------------|---------------|-------|---------------------------|-----------------------------------------------|---------------------------------------------|
| - 1 deletion of G 520 or 521 | 4             | AB 1886 | +                         | 2.0                                           | G G G T A T G G C G C A G G C C G           |
|                             | 30            | AB 1886 | -                         | 2.8                                           |                                             |
|                             | 41            | AB 1157 | +                         | 13.8                                          | T C T A C G C C G G A C G A T C G T         |
|                             | 45            | AB 1157 | +                         | 2.8                                           |                                             |
| - 1 deletion of G 399 or 390 | 32            | AB 1157 | +                         | 8.8                                           | G T G C T G G C C G C T A T A T C G         |
| - 2 deletion of GC 435-436 or 437-438 or CG 436-437 | 34 | AB 1157 | +                         | 6.6                                           |                                             |
|                             | 36            | AB 1157 | +                         | 8.8                                           |                                             |
| Addition of a C within sequence with 526-528 | 36 |          |                            | 8.8                                           | G T G C A G G C C G T G G C C G           |
| - 2 deletion of GC 548-549 or 550-551 or CG 549-550 | 33 | AB 1157 | +                         | 13.8                                          | A C T G T T G G C C C C A T C T C C         |
| - 1 deletion of G 416 and double transition at 414-415 | | 35 | AB 1157 | +                         | 8.8                                           | C A T C A C C G G C C C A C A G G T double transition [A T] |

*The sequences appearing in this table are the wild type sequences with the numbering defined by Sutcliffe (20). The bases involved in the deletion mutations are boxed with dotted lines. The different possibilities to obtain a given mutated sequence are shown. Mutant 36 exhibits two mutations: a - 2 deletion as in mutant 34 and a + 1 addition of a C residue within the sequence CCC at positions 526-528. Mutant 35 has got a - 1 deletion of a G at position 416 and a double transition, GC → AT, at position 414-415.*
FIGURE 3. Part of the sequence of mutant 34 and of the wild type DNA showing the –2 deletion of a CG doublet within the hot spot sequence GGCGCC.
guanine-AAF adduct that might favor the hairpin structure shown in Figure 4. Due to the multiplicity state and to the recessivity of the mutations that are scored in our system, the conversion of the premutagenic state and likely occurs simultaneously in both strands prior to replication.

\[ \text{Hairpin structure:} \]

\[ \text{DNA adduct:} \]

\[ \text{Hot spot sequence 1:} \]

\[ \text{Hot spot sequence 2:} \]

**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.

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