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# Replication of acetylaminofluorene-adducted plasmids in human cells: Spectrum of base substitutions and evidence of excision repair

(*supF* gene/DNA sequencing/aromatic amine/DNA repair/mutational spectrum)

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**ABSTRACT** **In rats fed the liver carcinogen 2-acetylaminofluorene (AAF), the two most abundant types of DNA adduct are *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene and its deacetylated derivative. When plasmids carrying AAF adducts replicate in bacteria, the predominant mutations are frameshifts, whereas with deacetylated (AF) adducts, they are mainly base substitutions, just as we found when plasmids carrying AF adducts replicated in human cells. We have investigated the frequency and spectrum of mutations induced when a shuttle vector carrying AAF adducts (85% bound to the C8 position of guanine, 15% to the $N^2$ position) replicated in human cells. The frequency induced per initial AAF adduct was higher than with AF adducts, but the kinds of mutations were similar—i.e., 85% base substitutions, principally G·C → T·A transversions. There was good correlation between the "hot spots" for mutations and hot spots for AAF adduct formation, suggesting that mutational hot spots reflect preferential binding of the carcinogen to DNA. $^{32}$P-postlabeling analysis of the adducts before and after the DNA was transfected into the human cells showed that there was no deacetylation of AAF adducts and that 85% of both types of adducts were removed within 3.5 hr, most probably by excision repair.**

2-Acetylaminofluorene (AAF) forms two major DNA adducts *in vivo*, *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF) and its deacetylated derivative, dG-C8-AF. Physicochemical studies suggest that dG-C8-AAF and dG-C8-AF differ significantly in the way they affect the conformation of DNA. Models have been proposed in which dG-C8-AAF causes a major local distortion of the DNA helix by inducing the modified guanine to assume the *syn* conformation (1, 2), whereas dG-C8-AF retains a normal orientation without causing a major change in the B-DNA structure (3). *In vitro* DNA polymerization studies on single-stranded bacteriophage DNA show that dG-C8-AAF causes T4 DNA polymerase to terminate polymerization one nucleotide prior to the site of the adduct, whereas dG-C8-AF induces termination after incorporation of a nucleotide opposite the modified guanine (4). Such termination also occurs with modified T7 DNA polymerase, but the latter enzyme bypasses dG-C8-AF lesions frequently (5). These results predict a significant difference in the kinds of mutations induced by AAF and AF adducts and, perhaps, in their location in a target gene.

Studies of mutations induced in the tetracycline-resistance gene of pBR322 when plasmids carrying AAF adducts replicate in *Escherichia coli* show that AAF adducts induce frameshifts predominately (6), whereas AF adducts give mainly base substitutions, predominantly G·C → T·A transversions (7). To see if this were true with human cells as host, we exposed a shuttle vector, pZ189, to *N*-acetoxy-2-acetylaminofluorene (N-AcO-AAF) or *N*-acetoxy-*N*-trifluoroacetyl-2-aminofluorene (N-AcO-TFA-AF) to obtain AAF or AF adducts, respectively, introduced the DNA into human cell line 293 to be replicated by the human cell polymerases, and examined the progeny plasmids for the frequency and spectra of mutations induced in the small target gene *supF*. An advantage of the shuttle-vector assay is that one can determine the nature of the adducts initially formed and their location in the target gene before transferring the plasmids into the human cells. Progeny plasmids carrying mutations in *supF* are identified using indicator bacteria, and the nature and sites of the mutations are determined by sequencing. We have used this assay to study a series of structurally related carcinogens that bind predominantly to guanine (8–11), in order to get insight into the mechanisms by which they cause mutations.

The present results with AAF adducts show that, as we found for AF adducts (8), 85% of the mutations consisted of base substitutions, mainly G·C → T·A transversions. But none of the "hot spots" for mutations induced by AAF adducts were identical to those found with AF adducts; each agent induced its own unique spectrum. The correlation between sites of mutation induction by AAF adducts and sites of adduct formation, as inferred from polymerase termination, was high, but not every site that exhibited a high frequency of initial adduct formation was the site of a high frequency of mutations, suggesting that DNA repair eliminated adducts prior to induction of mutations. This was confirmed by $^{32}$P-postlabeling studies of transfected pZ189.

## MATERIALS AND METHODS

**Cells and Plasmid.** The human cell line 293, *E. coli* strain SY204, and shuttle vector pZ189 have been described (9).

**Formation of AAF Adducts on the Plasmid.** Purified plasmid DNA dissolved in 2 mM sodium citrate buffer (pH 7.0) at 1 mg/ml was added to freshly prepared ethanol solutions of generally tritiated N-AcO-AAF (63 mCi/mmol; 1 Ci = 37 GBq) at the designated concentrations and incubated at 37°C for 30 min. The compound was supplied by Charles M. King, Michigan Cancer Foundation, Detroit. Unbound carcinogen was removed by repeated ether extractions, followed by extraction with phenol buffered to pH 7.0 with sodium

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Abbreviations: AAF, 2-acetylaminofluorene; dG-C8-AAF, *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene; dG-C8-AF, *N*-(deoxyguanosin-8-yl)-2-aminofluorene; dG-$N^2$-AAF, 3-(deoxyguanosin-$N^2$-yl)-2-acetylaminofluorene; N-AcO-AAF, *N*-acetoxy-2-acetylaminofluorene; N-AcO-TFA-AF, *N*-acetoxy-*N*-trifluoroacetyl-2-aminofluorene.

citrate. The DNA was precipitated with ethanol, redissolved in sodium citrate buffer (pH 7.0), and stored at −20°C.

**Transfection and Rescue of Replicated Plasmids.** The human cells were transfected as described (9). Progeny plasmids derived from individual transfections were assayed separately for mutant *supF* genes to distinguish mutations that occurred frequently from putative siblings.

**Bacterial Transformation and Mutant Characterization.** As described previously in more detail (9), *E. coli* SY204 cells were transfected with progeny plasmids and selected for ampicillin resistance. Those that received a plasmid with a nonfunctional (mutant) *supF* gene formed white or light blue colonies, instead of the dark blue colonies formed with an intact gene. The plasmids with a mutant *supF* gene were analyzed for gross rearrangements by gel electrophoresis. The *supF* gene of those with normal patterns was sequenced using Sequenase 2.0 (United States Biochemical), dideoxynucleotides, and [α-[$^{35}$S]thio]dATP.

**Determination of Sites of AAF-DNA Adducts.** The frequency of adduct formation at various positions in *supF* was estimated from the degree of inhibition of polymerization by the Klenow fragment of *E. coli* DNA polymerase I (10), using primer end-labeling with [γ-$^{32}$P]ATP, which eliminates the need to take into account the number of adenine bases incorporated during polymerization, and polymerization with [α-[$^{35}$S]thio]dATP, which gives more distinct bands than $^{32}$P. A rightward primer, complementary to the transcribed strand near the 5′ end of the gene, and a leftward primer, complementary to the opposite strand at positions 211–230, were used. DNA from the four dideoxy sequencing reactions, carried out on an untreated template, served as size markers. The relative intensities of the bands on the autoradiogram of the gel containing end-labeled oligonucleotide fragments were determined using a digital image analyzer (8).

**Characterization of AAF-DNA Adduct Profile.** The profile of adducts before and after DNA was transfected into human cells was obtained by $^{32}$P-postlabeling using the 1-butanol enrichment procedure, followed by thin-layer chromatography (12). The adducts were identified and quantified by comparison to a dG-C8-AF DNA standard, derived from the reaction of calf thymus DNA with [*ring*-$^{3}$H]*N*-hydroxy-2-aminofluorene, or to a 3-(deoxyguanosin-$N^2$-yl)-AFF (dG-$N^2$-AFF) and dG-C8-AAF DNA standard, derived from the reaction of [*ring*-$^{3}$H]*N*-acetoxy-AAF with salmon testes DNA. To eliminate extracellular plasmids, transfected cells were exposed to DNase I (50 μg/ml, Sigma) at 37°C for 30 min and washed twice with phosphate-buffered saline, pH 7.2.

## RESULTS

**Characterization of *N*-AcO-AAF-Treated Plasmids.** There was a linear relationship between the number of AAF residues per plasmid and the concentration of *N*-AcO-AAF used (Fig. 1*A*). The frequency of *supF* mutants as a function of AAF residues per plasmid, after replication in cells, was nonlinear (Fig. 1*B*), whereas with AF residues, the response was linear throughout the range of adducts tested (up to 40 residues per plasmid) (8). The mutant frequency per $10^4$ plasmids for 20 AAF or AF residues per plasmid was ≈12.5; for 40 residues, it was ≈50 for AAF and ≈25 for AF; and for 70 residues, it was 128 for AAF, while extrapolation predicts only 44 for AF.

A mutation frequency of $128 \times 10^{-4}$ is 98 times higher than the background frequency, $1.3 \times 10^{-4}$ (Table 1). Of the 42 background mutants analyzed, 8 showed gross alterations [i.e., deletions or insertions of >150 base pairs (bp)]. None of the 114 mutants analyzed that were derived from *N*-AcO-AAF-treated plasmids showed such changes. A representative fraction of the mutant plasmids that did not show altered gel mobility were further analyzed by DNA sequencing of *supF*. Of the control mutants sequenced, 67% (18/27) contained deletions or insertions of ≥4 bp. In contrast, only 5% (4/77) of the *N*-AcO-AAF-induced mutants sequenced showed such alterations.

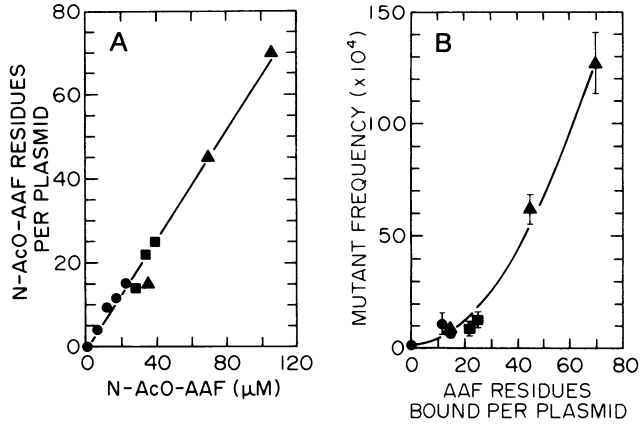


FIG. 1. (*A*) AAF residues formed per plasmid as a function of concentration. (*B*) Frequency of *supF* mutants as a function of residues per plasmid. Error bars refer to the standard error of the frequencies obtained from a series of individual transfection experiments made with each set of treated plasmids. The various symbols indicate data from plasmids exposed to N-AcO-AAF in three separate experiments.

**Kinds of Mutations Induced by *N*-AcO-AAF and Locations in *supF*.** Table 2 shows the kinds of sequence alterations observed in unequivocally independent mutants, along with data for plasmids carrying AF residues. Of the mutants induced by AAF residues, 85% exhibited 1- or 2-bp substitutions, a result identical to that found with AF residues. With control mutants, 51% contained deletions. The types of base substitutions induced by AAF and AF residues also were very similar (Table 3).

The specific locations of 78 point mutations induced by AAF residues are shown in Fig. 2. For purposes of comparison, the spectra of 50 mutations induced by AF residues (8) and 17 spontaneous mutations (8–11) are included. With AAF mutants, prominent hot spots, defined as a site in a spectrum of 50 or more mutations where at least 8% of the mutations are located, were found at positions 122, 127, and 155. With AF mutants, hot spots were found at positions 123, 133, 159, and 169. None of the hot spots were common to both AAF and AF residues. A unique −1 frameshift in the run of five G·C base pairs (positions 172–176) occurred frequently with AAF residues (6% of the total); this was not true with AF residues.

**Correlation Between Sites of AAF Adducts and Location of Mutations.** To see whether the hot spots for N-AcO-AAF-induced mutations reflected hot spots for DNA binding, we

Table 1. Analysis of mutants obtained with N-AcO-AAF

| No. of adducts per plasmid | Mutants/ transformants, no./no. | Mutant frequency × $10^4$ | No. of *supF* genes sequenced | No. with point mutations |
|---|---|---|---|---|
| 0 | 44/326,744* | 1.3 | 27 | 9 |
| 12 | 6/5,552 | 10.8 | 4 | 4 |
| 15 | 33/48,261 | 6.8 | 25 | 23 |
| 15 | 9/10,143 | 8.9 | 2 | 2 |
| 22 | 23/26,487 | 8.7 | 17 | 16 |
| 25 | 16/12,610 | 12.7 | 11 | 10 |
| 45 | 100/16,068 | 62.2 | 16 | 16 |
| 70 | 89/6,972 | 127.7 | 2 | 2 |

*These data with untreated pZ189 include published results (8–10); the frequency in the present study was 13/107,994.

Table 2. Kinds of sequence alterations

| Mutation(s) | No. of occurrences: Untreated* | | N-AcO-AAF-treated | | N-AcO-TFA-AF-treated† | |
|---|---|---|---|---|---|---|
| Substitution | | | | | | |
| Single base | 7 | | 62 | | 42 | |
| Two-bases | | | | | | |
| Tandem | 1 | (27%) | 0 | (85%) | 1 | (86%) |
| Nontandem | 2 | | 5 | | 1 | |
| Three-bases | 0 | | 0 | | 1 | (2%) |
| Deletions | | | | | | |
| Single G·C pair | 3 | | 6 | | 1 | |
| Single A·T pair | 1 | (51%) | 0 | (13%) | 0 | (8%) |
| ≥4 bases | 15 | | 4 | | 3 | |
| Insertions | | | | | | |
| Single G·C pair | 1 | | 1 | | 0 | |
| ≤20 bases | 2 | (8%) | 0 | (1%) | 1 | (2%) |
| Rearrangements | 5 | (14%) | 1 | (1%) | 1 | (2%) |
| (Total) | 37 | | 79 | | 51 | |

*These data include published results with untreated plasmids (8–11).
†These data are taken from ref. 8.

carried out the DNA polymerase stop assay (13) to estimate the sites and frequencies of adduct formation in the gene. *E. coli* DNA polymerase I Klenow fragment was used, since ordinarily it does not bypass dG-C8-AAF adducts (5). Fig. 3 shows the relative intensities of the termination sites as judged from the autoradiographic intensities of the bands on the gel using pZ189 carrying 25 AAF adducts—i.e., an average of ≤0.5 adduct per strand of *supF*. Fig. 4 shows an example. There was no evidence of interference with polymerization with untreated plasmids, supporting the assumption that, under our experimental conditions, the density of the bands at various positions on the gel reflects the chance of adduct-induced premature termination of polymerization. Care was taken to ensure that the length of exposure of the gels was such that differences in the density of the bands on the autoradiograms were clearly visible.

The pattern with AAF adducts corresponded to positions 1 nucleotide prior to virtually every cytosine in the DNA-sequencing standard lane, indicating that DNA synthesis was terminated 1 base prior to each guanine in the template. No bands corresponding to positions 1 nucleotide away from any base other than guanine were seen. Hot spots 122, 127, and 155 showed a relatively high frequency of chain termination (adducts), but some sites that showed high adduct formation did not exhibit mutations (e.g., positions 99, 102–105, and 111).

Table 3. Kinds of base substitutions observed

| Base change | No. of mutants observed: Untreated* | | N-AcO-AAF-treated | | N-AcO-TFA-AF-treated† | |
|---|---|---|---|---|---|---|
| Transversions | | | | | | |
| G·C → T·A | 9 | (69%) | 47 | (65%) | 32 | (65%) |
| G·C → C·G | 1 | (8%) | 11 | (15%) | 8 | (17%) |
| A·T → T·A | 0 | | 0 | | 0 | |
| A·T → C·G | 2 | (15%) | 0 | | 0 | |
| Transitions | | | | | | |
| G·C → A·T | 1 | (8%) | 12 | (17%) | 9 | (18%) |
| A·T → G·C | 0 | | 2 | (3%) | 0 | |
| (Total) | 13 | | 72 | | 49 | |

*These data include published results with untreated plasmids (8–11).
†These data are taken from ref. 8.

**Analysis of Initial AAF Adduct Profile and the Kinds and Amount of Adducts Remaining After Transfection.** Since AF adducts give mainly base substitutions in *E. coli*, but AAF adducts do not (6, 7), it was important to determine whether, by some chance, deacetylation of AAF adducts occurred in the human cells following transfection. Therefore, cultures of 293 cells were transfected with 18 μg of pZ189 carrying 70 AAF adducts per plasmid. After 3 hr, extracellular DNA was eliminated using DNase I. Plasmid DNA was extracted immediately after DNase I treatment or after an additional 8.5 or 17.5 hr. The DNA was analyzed by $^{32}$P-postlabeling for the number and the kinds of adducts, and the data were compared to those obtained with nontransfected plasmids.

The adduct pattern is shown in Fig. 5*A*. The most intense areas corresponded to 3-(deoxyguanosin-$N^2$-yl)-2-acetylaminofluorene (dG-$N^2$-AAF) and dG-C8-AAF (Fig. 5*B*). The radioactivity located between these two major areas is artifactual. There was almost no radioactivity (<0.3%) in the area corresponding to dG-C8-AF. (The $^{32}$P-postlabeling intensities for dG-C8-AAF and dG-$N^2$-AAF are not representative of the amounts of these two adducts present in a sample, since reconstruction studies indicate that the latter postlabels much more efficiently than the former.) Comparison of the data from the N-AcO-AAF-treated DNA with the standards showed that 85% of the adducts were dG-C8-AAF and 15% were dG-$N^2$-AAF (Fig. 5). Analysis of plasmids extracted from human cells 3.5 hr after transfection—i.e., immediately after DNase I treatment—gave a similar adduct profile (data not shown). No deacetylation of AAF adducts was observed.

Scintillation counts of material eluted from the areas corresponding to dG-C8-AAF, dG-$N^2$-AAF, and dG-C8-AF obtained from polyethyleneimine-cellulose plates of N-AcO-AAF-treated DNA before transfection into human cells and 3.5 hr, 12 hr, and 21 hr after transfection showed that by 3.5 hr, the initial adduct level per plasmid had been decreased to 15% (data not shown). By 12 hr, the number of adducts remaining per plasmid was only 5% that of the nontransfected plasmids. Bacterial transformation experiments with DNA exposed to restriction enzymes that distinguish DNA that has replicated in human cells from input DNA showed that no plasmid replication had occurred up to that time. After an additional 9 hr (i.e., 21 hr since transfection of the human cells), the level of adducts in the plasmid was still ≈5%. Transformation studies indicated that by that time, ≈30% of the input plasmids had replicated.

## DISCUSSION

Seventy out of 72 base substitutions involved G·C base pairs, $^{32}$P-postlabeling analysis of the N-AcO-AAF-treated plasmids showed that all adducts involved guanine, with ≈85% dG-C8-AAF and ≈15% dG-$N^2$-AAF, and no deacetylation of adducts occurred *in vivo*. These observations strongly suggest that the mutations were targeted to AAF adducts. The two base substitutions involving A·T pairs were found in mutants derived with plasmids treated with low doses of N-AcO-AAF and might represent background mutations. Moriya *et al.* (14), studying mutations induced by a site-specific dG-C8-AAF in plasmids replicating in a monkey cell line, also found that the mutations were targeted. Additional support for targeted mutagenesis comes from our polymerase stop-assay data. All three mutation hot spots (sites 122, 127, and 155) showed a high frequency of chain termination, suggesting that the spectrum of mutations reflects, at least in part, preferential binding of the carcinogen to specific sites. A striking example of this was found at positions 155 and 156 (Fig. 3). The guanine at position 155 in the transcribed strand (shown 3′ to 5′ in that figure) is a hot spot for mutations and binding. The guanine at position 156 in the opposite strand

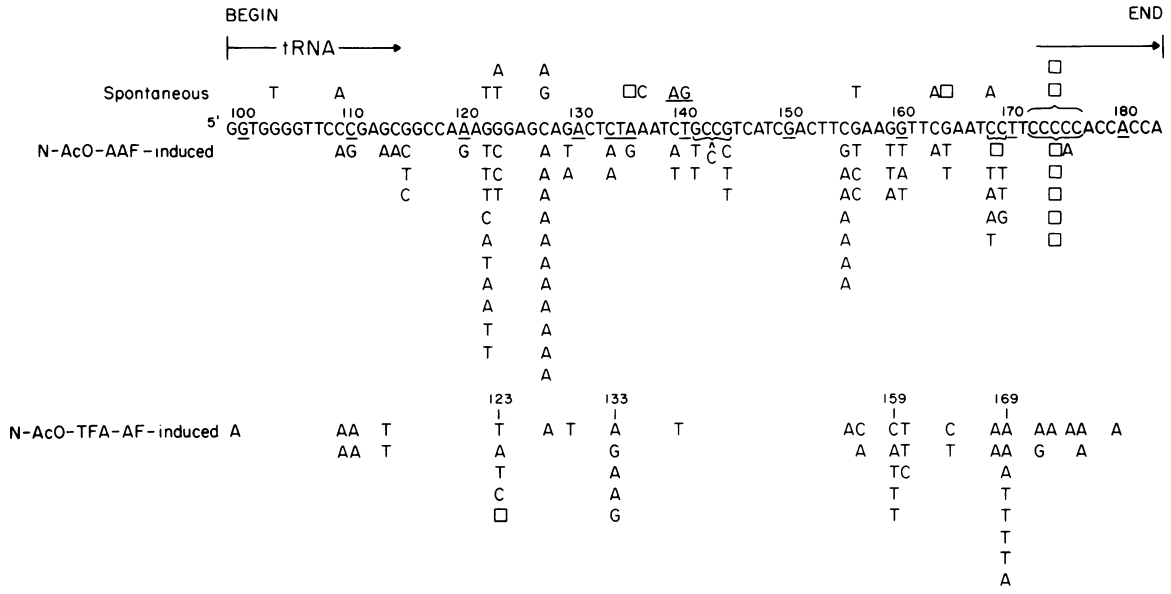


FIG. 2. Location of independent point mutations in the *supF* gene. The strand shown is the 5′-to-3′ strand synthesized from the rightward primer. Point mutations observed in the progeny of the carcinogen-treated plasmids are placed below the sequence; those from untreated plasmids are placed above the line. Squares represent deleted nucleotides; caret shows the location of an inserted cytosine (somewhere between 141 and 144). Brackets indicate where it is not possible to determine which nucleotide within a run is involved in the mutation. Every tenth base and the anticodon are underlined.

has a much lower frequency of mutations and a correspondingly lower level of binding. Yet these guanines are located in identical surrounding sequences (5′-CTTC*G*AAG-3′).

That some sites showed high adduct formation, yet little or no mutation induction, suggests that in addition to binding, other factors such as DNA repair and neighboring sequences also determine the spectrum of mutagenesis. Therefore, we also analyzed the plasmids for the number of adducts remaining after transfection into the host cells, using $^{32}$P-postlabeling analysis. The results showed that prior to DNA replication, the frequency of adducts was decreased to ≈5% of the initial level.

The information obtained from $^{32}$P-postlabeling analysis of plasmids carrying 70 adducts (i.e., 1.1 per 85-bp *supF* gene) allowed us to estimate the mutagenic effectiveness of AAF adducts (i.e., the frequency at which they induce an error during DNA replication). Transformation studies with plasmids rescued from human cells at various times after transfection indicated that plasmid replication did not occur within the first 12 hr, but by 21 hr, 30% of the plasmids had replicated. If none of the 70 adducts per plasmid had been lost, and if during the first round of replication, every adduct gave rise to a mutation, the mutant frequency would have been $\approx 50 \times 10^{-2}$. But, if only ≈5% of the initial AAF adducts remained at the time of replication, and if the mutations were generated during replication of template DNA containing adducts, the yield of mutants should have been only 5% as frequent, $\approx 2.5 \times 10^{-2}$. The observed frequency was $1.28 \times 10^{-2}$, which is very close to the theoretical yield. Since not every base substitution in the 85 bp of the structural portion of *supF* inactivates the function of the tRNA, our data indicate that

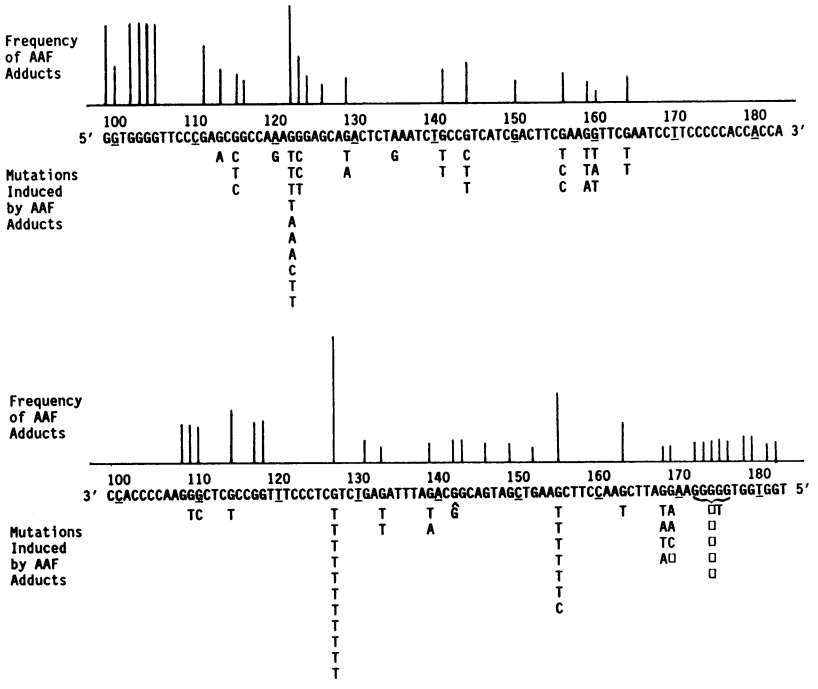


FIG. 3. Relative frequency of AAF adducts in the 5′-to-3′ and 3′-to-5′ strand of *supF*, as judged by the polymerase stop assay, and location of the corresponding point mutations. Relative frequency of termination (density of bands) is represented by the length of the lines shown above the gene. The base substitutions shown below the gene are the guanine base changes found in the corresponding strand.

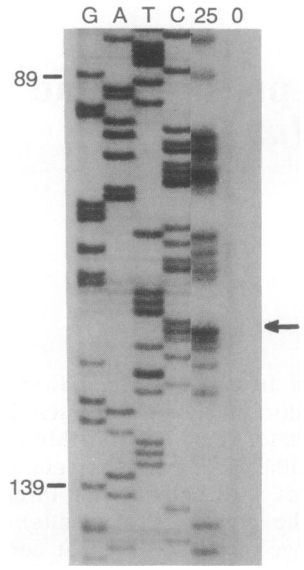


FIG. 4. Autoradiogram of polyacrylamide gel showing the sites of polymerization termination. The leftward sequencing primer was used to determine the location of adducts on the 5'-to-3' strand. Lanes G, A, T, and C are dideoxy sequence standards obtained by incubating untreated DNA templates with Sequenase and dideoxyribonucleotides. Lane 25 shows the products from a template containing 25 AAF adducts per plasmid; lane 0 represents a template from untreated plasmid. The reactions shown here were run using [$\alpha$-[$^{35}$S]thio]dATP followed by unlabeled dATP for 15 min. Numbers at left identify the location of bases. Arrow points to position 123, a hot spot for termination of polymerization. It corresponds to a termination site (adduct) at position 122 on the template, a hot spot for AAF-induced mutations. [$\gamma$-$^{32}$P]ATP gave similar results.

the mutagenic effectiveness of AAF adducts is very high indeed, >50%.

The majority of the base substitutions were G·C → T·A transversions. Various mechanisms could explain this observation. As discussed previously (8–11), if DNA polymerases in the host cell preferentially insert an adenine nucleotide opposite a depurination or noninstructional adduct (15–17), this would explain the predominance of these transversions. Alternatively, AAF residues may have changed the conformation of guanine so that stable G·A mispairing occurred and went undetected. Evidence indicates that such mispairing

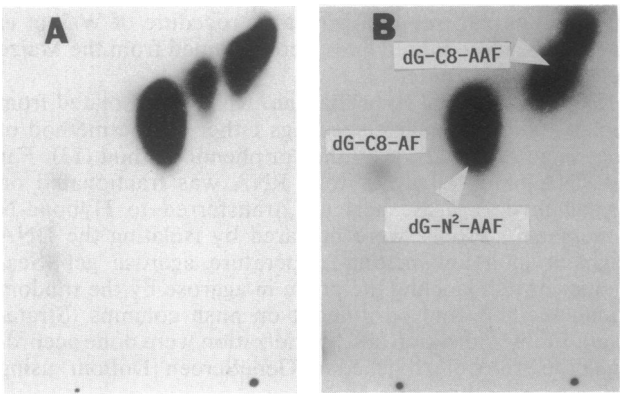


FIG. 5. Autoradiogram of $^{32}$P-postlabeling polyethyleneimine-cellulose plates showing the adduct patterns of N-AcO-AAF-treated pZ189 containing 70 adducts per plasmid (*A*) and dG-C8-AF, dG-C8-AAF, and dG-N$^2$-AAF standards (*B*).

can occur in synthetic oligonucleotides, with dG-C8-AAF assuming the *syn* conformation (18).

Our results with the *supF* gene, showing that in human cells AAF adducts primarily induce base substitutions, differ significantly from the frameshift results found in the tetracycline-resistance gene when plasmids containing AAF adducts replicated in *E. coli* (6). This difference might reflect the difference in the target gene sequences or a difference between prokaryotes and eukaryotes in the mechanism of mutagenesis.

In our assay, AAF residues were more efficient in causing mutations than AF residues, even though the kinds of mutations were very similar. Since AAF adducts are more bulky than AF adducts, they might cause more interference with DNA replication. If so, this interference could also explain why AAF residues induced −1 frameshifts more frequently than did AF residues (8% vs. 2%). Suggestion that more bulky adducts can cause more interference with replication comes from our finding that 1,6-dinitropyrene adducts are twice as mutagenic as less bulky 1-nitropyrene adducts (10, 11) and induce many more −1 frameshifts (16% vs. 1.5%). (More than 80% of these occurred in that same run of five guanines at positions 172–176, but none occurred in the run of three guanines or four guanines.) These deletions might be the result of strand slippage (19).

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