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Genetics

2-(*N*-acetoxy-*N*-acetylamino)fluorene mutagenesis in mammalian cells: Sequence-specific hot spot

(simian virus 40/chemical carcinogen/DNA secondary structure/DNA repair)

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ABSTRACT **Mutations induced by 2-(*N*-acetoxy-*N*-acetylamino)fluorene were studied using temperature-sensitive simian virus 40 (SV40) mutants as probe in monkey kidney cells. *In vitro* treatment of the SV40 virions with 2-(*N*-acetoxy-*N*-acetylamino)fluorene increased mutagenesis and decreased survival in the viral progeny. A lethal hit of approximately 85 acetylaminofluorene adducts per SV40 genome was calculated. UV irradiation of cells prior to infection did not modify the results. Molecular analysis of independent SV40 revertants showed that 2-(*N*-acetoxy-*N*-acetylamino)fluorene induces base substitutions that are located not opposite putative acetylaminofluorene adducts but next to them. Moreover, a hot spot of mutation restoring a true wild-type genotype was observed in 10 of the 16 revertants analyzed. This hot spot, not targeted opposite a major DNA lesion, was not observed using UV light as damaging agent in the same genetic assay. Two models involving the stabilization, by acetylaminofluorene adducts, of the secondary structure of a specific quasipalindromic SV40 sequence are proposed to explain this sequence-specific hot spot.**

The recent discovery that some human cancers are associated with single point mutation in the *ras* gene family (1–3) enhanced the interest in the molecular mechanisms by which mutations arise. The most convenient method to approach mutagenesis in mammalian cells is to use exogenous probes (4–8). These probes must be well-defined and entirely dependent on the host cell for replication and for repair. Simian virus 40 (SV40) is the virus that best fits these requirements. Indeed, the SV40 system allowed Bourre and Sarasin (8) to show that UV light gives rise to base substitution opposite putative UV-induced lesions.

2-(*N*-Acetoxy-*N*-acetylamino)fluorene (AAAF) has been widely used to induce tumors in animals. This carcinogen binds both to proteins and to guanine in nucleic acids. Among the DNA lesions (dG-AAF), the major one, 2-[*N*-(deoxyguanosin-8-yl)-*N*-acetylamino]fluorene, gives rise to local modifications in the DNA structure (9). Such adducts are mutagenic in bacterial cells, leading essentially to targeted frameshift mutations (10, 11). Since very little is known about AAAF mutagenesis in mammalian cells, we have determined the mutational specificity of this carcinogen on the SV40 large tumor (T) antigen gene and compared it with the UV-induced mutations determined in the same genetic system—composed of a reversion assay from a temperature-sensitive tsA58 SV40 mutant to wild-type growth at the restrictive temperature.

We report here that *in vitro* treatment of SV40 by AAAF induces both a decrease in progeny survival and an increase in viral mutagenesis. The molecular analysis of AAAF-induced revertants reveals a hot spot of mutation not localized opposite a major DNA lesion in more than 60% of independently isolated mutants. We hypothesize that a specific DNA structure stabilized by an AAF adduct is responsible for the hot spot.

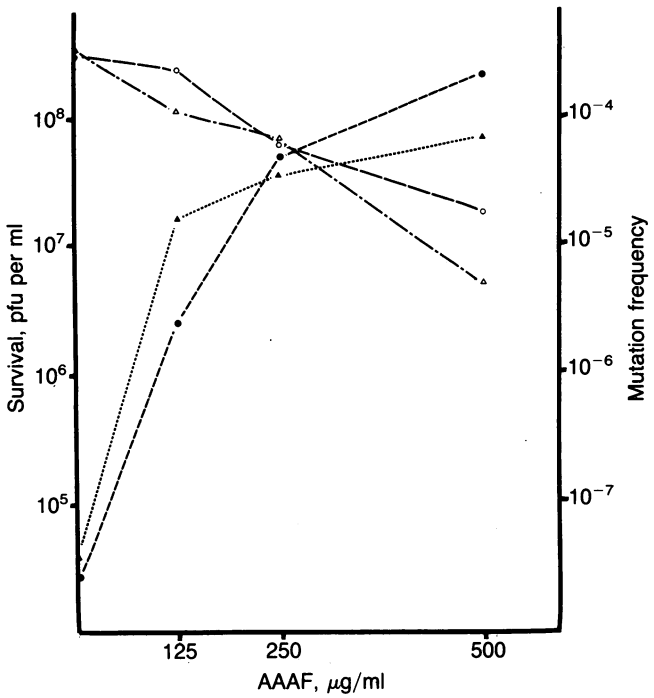

FIG. 1. Survival [plaque-forming units (pfu) per ml; open symbols] and mutation frequency (closed symbols) of the viral progeny obtained from tsA58 SV40 virion treated *in vitro* with various concentrations of AAAF. CV-1P cells were infected with tsA58 SV40 virus that had been treated for 2 hr *in vitro* with AAAF at 0–500 μg/ml. Cells were either untreated (○, ●) or irradiated with 10 J/m² of UV light (254 nm) 24 hr before infection (△, ▲). Each point is the average of duplicate plates of cells infected with serial dilutions of lysates. The errors involved in these measurements are estimated to be between 10% and 20%.

MATERIALS AND METHODS

Cells and Virus. African green monkey kidney cells (CV-1P and MA134 cells) were cultured in Dulbecco's modified Eagle's medium (GIBCO) containing 7% fetal bovine serum. The temperature-sensitive tsA58 early SV40 mutant was grown in MA134 cells and purified by the polyethylene glycol procedure (12). Supercoiled form I DNA was purified by CsCl/ethidium bromide equilibrium centrifugation. Virus infections were carried out at 37°C for 2 hr on confluent

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Abbreviations: AAAF, 2-(*N*-acetoxy-*N*-acetylamino)fluorene; AAF, acetylaminofluorene; SV40, simian virus 40; T antigen, large tumor antigen.

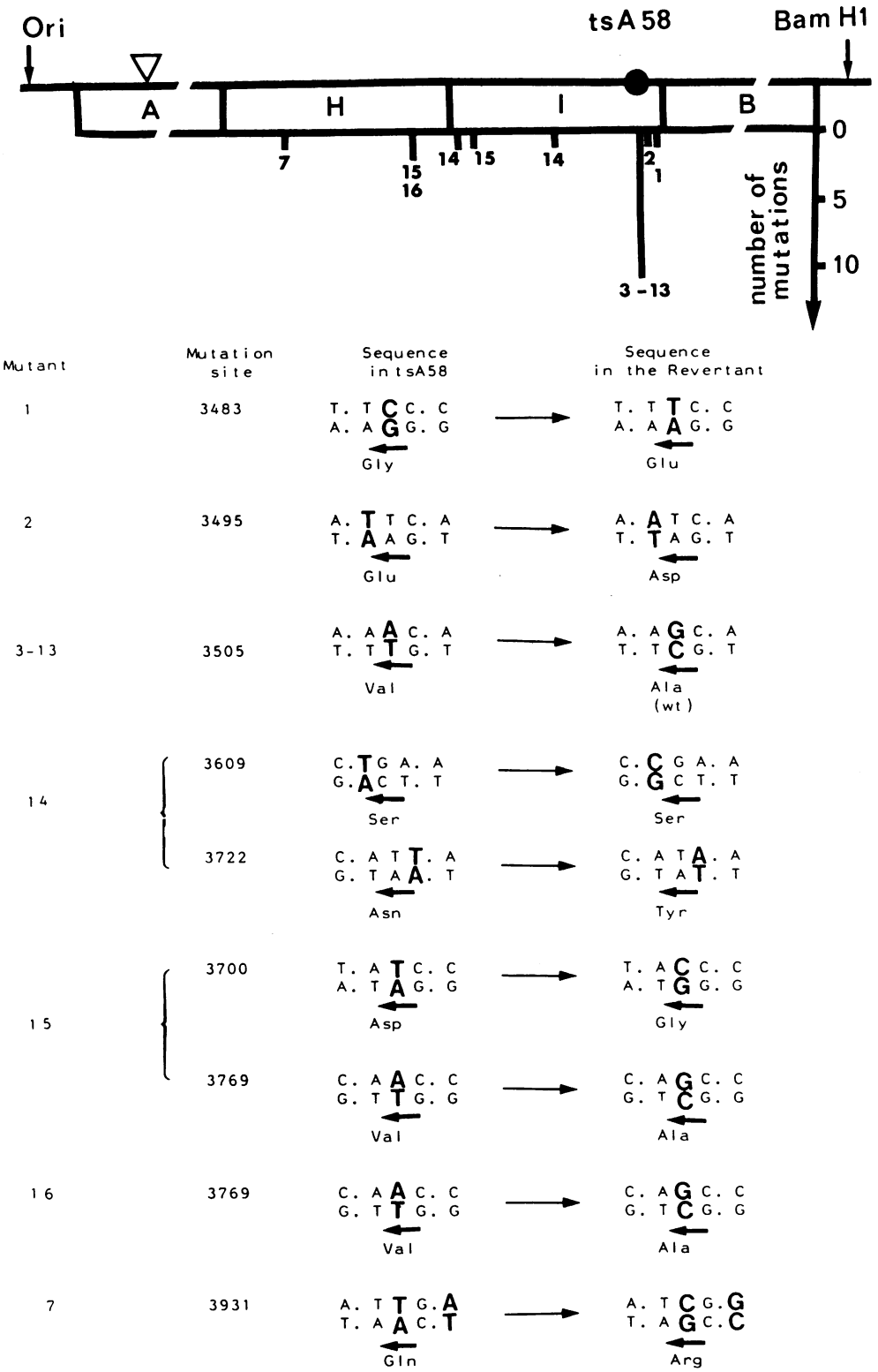

FIG. 2. Location and DNA sequence of mutation sites of AAAF-induced mutants. Various concentrations of AAAF were used as described for Fig. 1. Revertants were isolated from AAAF-treated virus particles. (*Upper*) Location of sites. Vertical bars below the map indicate the distribution of the mutations in revertants 1–16; lengths of the bars are proportional to the number of independently isolated revertants. The origin of replication (Ori), the mutation tsA58, the *Bam*HI restriction site, and the splicing region (▽) of the T-antigen gene are shown. A, H, I, and B refer to the fragments resulting from digestion with *Hin*cII plus *Hin*dIII. (*Lower*) Sequences at mutation sites. The tsA58 mutation is mapped at position 3505 and results in substitution of valine for the wild-type (wt) alanine. Mutation sites are given by nucleotide numbers of the published SV40 nucleotide sequence (16). For mutants 14 and 15, brackets show two independent reversion sites found in the same revertant genome. Codon frames of the amino acid residues are indicated by arrows (5′→3′) below the nucleotide sequences.

CV-1P cells previously washed with phosphate-buffered saline (PBS: 0.14 M NaCl/2.7 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH7.6).

AAAF-Treatment and Quantification of AAF Adducts. Virus suspensions in PBS were treated for 2 hr at 37°C in the dark with various concentrations of AAAF (a gift from R. P.

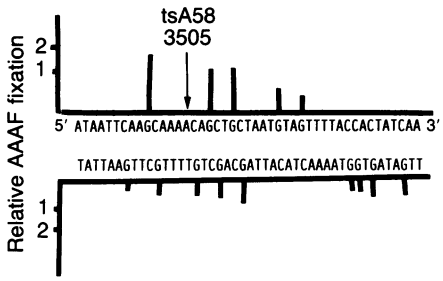

FIG. 3. Relative fixation of AAAF on deoxyguanosine residues around the tsA58 mutation. The relative index of AAAF fixation, determined from nucleotide 3491 to nucleotide 3535, including the mutation hot spot at position 3505 (tsA58), was calculated as the percentage of arrest of the 3'→5' exonuclease activity of bacteriophage T4 polymerase (11). Purified ^{32}P-labeled I fragment was treated *in vitro* with AAAF (0.5 μg/ml), as described in *Materials and Methods*, in order to obtain ≈0.3 adduct per I fragment.

Fuchs, IBMC, Strasbourg, France) dissolved in dimethyl sulfoxide. At the end of the incubation, 2% fetal bovine serum was added and viruses were dialyzed overnight against PBS. ^{32}P-labeled SV40 DNA (3×10^6 cpm/μg) was treated with [^{3}H]AAAF (from R. P. Fuchs; specific activity, 200 Ci/mol; 1 Ci = 37 GBq) under the same conditions, except that the DNA was purified by two ether extractions, two chloroform extractions, and two ethanol precipitations. The ^{3}H/^{32}P ratio was measured after trichloroacetic acid precipitation on Millipore filters and allowed us to quantify the number of DNA adducts per SV40 genome.

UV Irradiation. After being washed with PBS, cell monolayers were irradiated at 254 nm (10 J/m^2) with a germicidal lamp at a fluence rate of 0.12 J·M^{-2}·sec^{-1}, 24 hr before infection.

Mutation Assay. The mutation assay is based upon the reversion from a temperature-sensitive growth phenotype to a wild-type growth phenotype at 41°C. The temperature sensitivity of the tsA58 mutant is due to a mutation in the early T-antigen gene (8, 13). CVP-1 cells, either untreated or UV-irradiated, were infected with SV40 virus and kept for 3 days at the permissive temperature of 33°C. Cells were then scraped off the plates and frozen and thawed three times. Titers of the viral progeny were determined at both 33°C and 41°C by a standard plaque assay procedure on CVP-1 cells (5). The mutation frequency is defined as the ratio of plaques growing at 41°C to the total number of virus growing at 33°C in the progeny. Wild-type virus has no selective advantage during the lytic cycle at 33°C when compared to the temperature-sensitive mutants (5, 14).

Purification of Revertants. Plaques growing at 41°C were isolated independently, each mutant being picked from a different Petri dish after the viral cycle. Moreover, infecting viruses were treated with various concentrations of AAAF (125–500 μg/ml). We thus ensured that all the revertants studied were derived from individual events during virus growth. Stock viruses were grown on MA134 cells and their titers were measured at both 41°C and 33°C to ensure that they really corresponded to phenotypic revertants. Purified form I DNA was prepared from each individual revertant.

Marker Rescue and DNA Sequencing. DNA from the tsA58 revertants was digested with *Hin*cII and *Hin*dIII restriction enzymes, and the resulting fragments were purified by electrophoresis in 6% polyacrylamide gels. Fragments A, H, I, and B, spanning the early SV40 region, were individually hybridized *in vitro* with the tsA58 form II DNA, as described by Lai and Nathans (15). After transfection of the different hybrid molecules into CV-1P cells, the production of temperature-independent virus allowed us to determine which fragment coded for the reversion site. The fragments able to complement the tsA58 mutation were labeled at the 5' end with polynucleotide kinase and [γ-^{32}P]ATP and then sequenced by the method of Maxam and Gilbert (17).

Direct Detection of True Wild-Type Revertants. The wild-type genome has two overlapping *Fnu*4H1 restriction enzyme recognition sites (at nucleotide positions 3505 and 3508), whereas the tsA58 genome has only one, at position 3508, due to the tsA58 point mutation at 3505. (Nucleotide numbering is according to ref. 16.) Revertant DNA were digested with *Hin*cII and *Hin*dIII restriction enzymes, labeled at the 3' end with the Klenow fragment of DNA polymerase I, and then digested with *Fnu*4H1. Among the fragments obtained with *Fnu*4H1 there is a doublet of 30 and 33 nucleotides in the wild type, whereas the tsA58 gives only a 33-nucleotide-long fragment that can be separated in a 16% polyacrylamide DNA sequencing gel and indicates directly whether the original tsA58 sequence is still present.

Location and Quantification of Lesions on SV40 DNA. The ^{32}P-labeled fragment resulting from the digestion of SV40 with *Hin*cII plus *Hin*dIII and including the tsA region (fragment I) was treated *in vitro* with AAAF as described above. It was then digested with the associated 3'→5' exonuclease activity of bacteriophage T4 polymerase, in a buffer containing 33 mM Tris acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, and 0.1 mg of bovine serum albumin per ml. The lesions, which blocked the enzyme, gave rise to discrete bands, which were then visualized on a 20% DNA sequencing gel. Taking into account the radioactivity present in each band, we calculated a fixation index corresponding to the amount of adduct linked to a given nucleotide (11).

RESULTS

Survival and Mutagenesis. Fig. 1 shows survival and mutation frequency after treatment of tsA58 virions with AAAF. A large increase in mutation frequency was observed even at a low toxicity level. Neither enhanced mutation nor enhanced virus survival were observed in the viral progeny from UV-irradiated cells. Similar results were obtained by treating SV40 tsB201 DNA with AAAF *in vitro*, from which we calculated a lethal hit of roughly 85 adducts per SV40 genome (data not shown).

Molecular Analysis of SV40 Revertants. We isolated revertants after AAAF treatment of viral particles so as to characterize them at the molecular level. To localize the reversion site on the revertant genomes, we purified DNA fragments resulting from digestion with *Hin*cII and *Hin*dIII and we tested their ability to complement the tsA58 mutation by the marker-rescue technique (15). Among 16 independent revertants, 85% of the reversion sites were localized around the tsA mutation in the I fragment, and 15% of reversion sites were in the H fragment (Fig. 2). Digestion of revertant DNA with the restriction enzyme *Fnu*4H1 showed that roughly 65% of the AAAF revertants were true genotypic revertants—i.e., that the original sequence of the wild-type had been restored (Fig. 2). For three of these true revertants, DNA sequence analysis was performed to confirm the sequence deduced from the *Fnu*4H1 site. In the other revertants, the tsA58 mutation was still present, and the restoration of the wild-type growth phenotype at 41°C was due to a mutation at a second site. For one of these revertants (mutant 15), two mutation sites were detected, one in the I fragment (position 3700) and one in the H fragment (position 3769), either of which is able to independently restitute an active T antigen. For one true genotypic revertant (mutant 7), a second mutation, localized at position 3931 in the H fragment, is also able to complement the tsA mutation. One silent mutation was also detected in revertant 14 (position

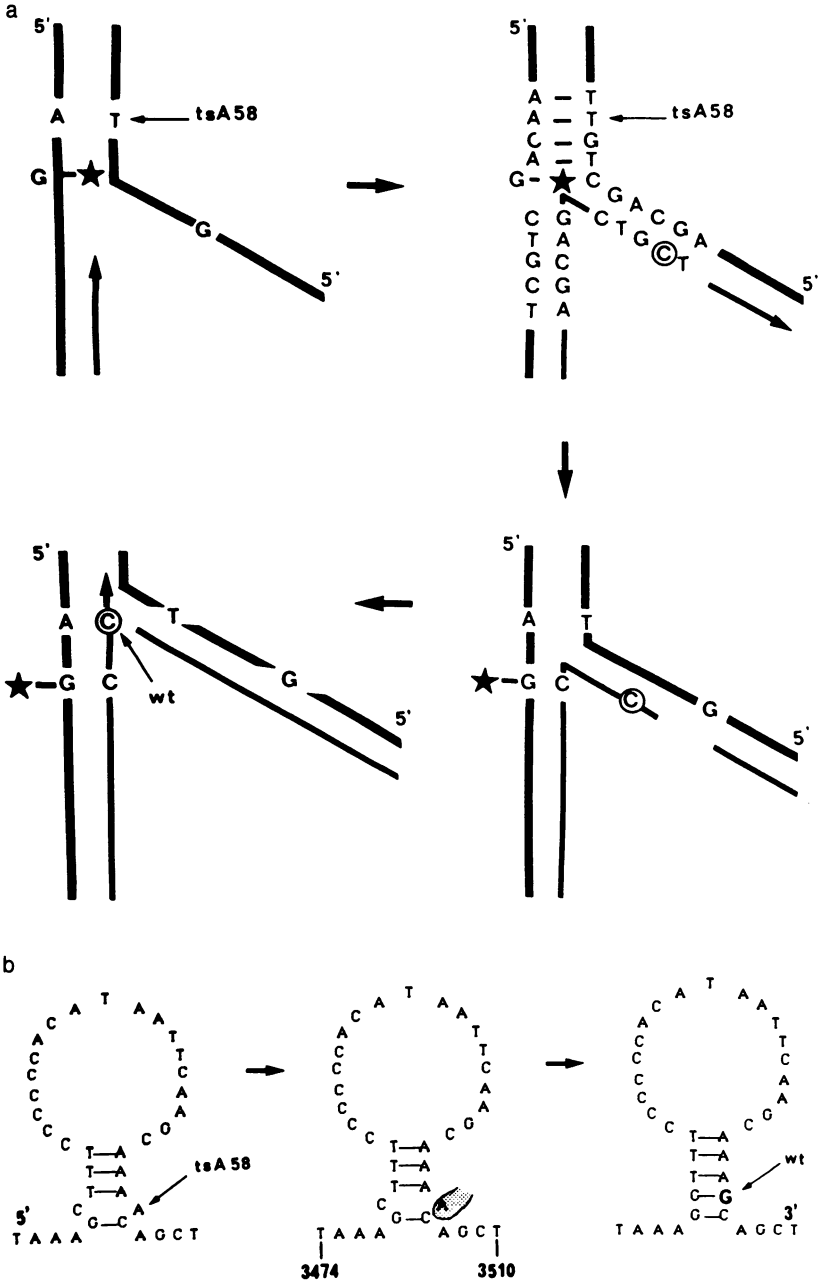

FIG. 4. (*a*) Strand-switch model for AAAF mutagenesis. The strand-switch model for semiconservative DNA replication at a dG-AAF adduct is based on the fact that the sequence from nucleotide 3504 to nucleotide 3511 (tsA58 mutation is at nucleotide 3505) of the leading strand is identical to the sequence of the lagging strand from nucleotide 3506 to 3513, except for one base (G at position 3511 and A at position 3505). The dG-AAF lesion (star) represents a block to the DNA polymerase, which can therefore use the opposite strand as a template (upper schemes). The regular DNA synthesis on this strand will displace the abnormal structure by a branch-migration process giving rise to the usual replication fork. In such a case, the dG-AAF lesion has been bypassed but an A–C mismatch appears at the tsA58 mutation site (lower schemes). In the absence of repair, the second round of replication will produce tsA58 molecules and true revertants in a 1:1 ratio. (*b*) Role of a quasipalindromic sequence during AAAF mutagenesis on SV40 DNA. The quasipalindromic sequence found around the tsA58 sequence allows the formation of a secondary structure that could be stabilized by a dG-AAF lesion. The C–A mismatch present in the stem corresponding to the tsA58 sequence may be repaired, leading to a fully base-paired structure. The normal base-pairing of a G opposite the C during this repair process will give rise to the wild-type (wt) SV40 sequence.

3609). There is no significant correlation between the number of adducts per genome and these double mutations.

Distribution of AAAF Lesions Along SV40 DNA. The location and the frequency of AAF adducts on a SV40 DNA fragment around the tsA58 mutation were determined by using a T4 DNA polymerase assay (11). DNA was treated *in vitro* with AAAF to induce approximately 0.3 lesion per I fragment, corresponding roughly to 6 adducts per SV40 genome, in order to avoid too many early stops of the T4 DNA polymerase. It can be seen (Fig. 3) that all deoxyguanosine residues react with AAAF. The frequency of AAF adducts on deoxyguanosine residues varies no more than by a factor of 4 as a function of the flanking sequences. No evident hot spot of AAF addition is observed in the SV40 DNA spanning the tsA58 mutation site.

DISCUSSION

AAAF treatment is mutagenic in mammalian cells as measured with SV40 as a molecular probe. Results are identical

when one uses two different genetic loci on SV40, one involved in the early T antigen gene and the other involved in the late viral protein 1 (VP1) gene (data not shown). The lethal hit calculated from the radioactivity linked to the DNA after treatment with [^{3}H]AAAF corresponds approximately to 85 adducts per SV40 genome. The exact chemistry of the lethal event is not known. The lethality could therefore be due to either major or minor AAF-adduct species, as well as to a secondary modification of these adducts, such as a depurination event (6). When cells were UV-irradiated prior to infection in order to induce the SOS response, neither increased survival nor enhanced mutagenesis was observed, in contrast to what was previously found using UV-irradiated SV40 virus (5, 18).

All revertant genomes are characterized by at least one base-pair substitution that is responsible for the phenotypic reversion. Ten of the 16 independent revertants we obtained were found to possess the true wild-type genotype, due to the direct transition of the T·A base pair of the tsA58 mutation (Fig. 2). The other revertants are due to mutation at another site—in most cases, a substitution at a T·A base pair. Since AAAF binds covalently to the C-8 or the N^2 position of deoxyguanosine residues, we were expecting that AAAF-induced substitutions be localized opposite deoxyguanosine residues. However, if the mutagenic lesion were not a major dG-AAF adduct, mutations could, of course, be found opposite other bases. Indeed the AAAF-induced SV40 revertants do not show targeted mutagenesis opposite putative deoxyguanosine lesions. All but one of the base substitutions are at A·T sites and 90% are adjacent to G·C sites. These data may represent a targeted process one base before or after the AAAF lesion. This result is not due to a bias of our genetic assay, since we know it is able to detect targeted mutagenesis. Experiments using the same protocol carried out with UV-irradiated SV40 showed targeted mutagenesis opposite putative pyrimidine dimers or pyrimidine-pyrimidone(6-4) products (8). This assay is also able to detect base pair substitutions at G·C sites (unpublished data).

In the prokaryotic forward-mutation assay described by Koffel-Schwartz *et al.* (10), 90% of the mutations were frameshift mutations, and only 10% were base-pair substitutions. The screening assay we used cannot detect frameshift mutations. In these conditions, any frameshift mutation leads to the production of an inactive T antigen, which is lethal for the virus. The high percentage of true genotypic revertants recovered after AAAF treatment does not correlate with a strong stop of the 3′→5′ exonuclease activity of the T4 polymerase, as shown in Fig. 3, where a relative fixation index has been calculated for the deoxyguanosine residues. This calculation was possible only after treating SV40 DNA *in vitro*. Indeed, when the intact virus was treated, the number of DNA fragments with AAF adducts able to stop the exonuclease was too small to allow accurate quantification of the AAF-adduct frequency. However, no strong stop for the T4 DNA polymerase was detected in the tsA58 area. Two hypotheses may explain the AAAF induction of a high percentage of true genotypic revertants. First, according to the strand-switch model described by Ripley (19), a strand switch may occur during the normal semiconservative DNA synthesis at an AAF adduct, as described in Fig. 4*a*. The replicated DNA sequence will therefore be conserved, except for the position 3505, which will present a mismatch. In the absence of repair, the second round of replication will then give rise to tsA58 and wild-type DNA molecules. The second model is based on the presence of a quasipalindromic structure that may permit the formation of a secondary structure, at the tsA58 site, with a mispairing at position 3505 (Fig. 4*b*). Such a hairpin is short and probably unstable; however, according to the insertion/denaturation model proposed by Fuchs *et al.* (9), an AAF adduct might stabilize the hairpin (20). As shown by De Boer and Ripley (21) with the T4 *rII* gene, the repair of noncomplementary bases to improve the pairing in the secondary structure predicts that base substitutions will occur at sites of mismatches. At site 3505 (Fig. 4*b*), the noncomplementary C–A could be repaired to form T·A or C·G. In the latter case, the wild-type genotype would be restored. Interestingly, the reversion sites 3495 and 3483 are localized in the same quasipalindromic structure (Fig. 4*b*). The latter mutation could arise via a similar mechanism by pairing positions 3478–3484 with positions 3500–3506 and correcting the C–C mismatch.

In these two models of sequence-directed mutagenesis, the various DNA-structure intermediates should be favored by AAF lesions, since no true revertant has been found in the same genetic system after UV irradiation. Indeed, the UV-induced lesions may well destabilize the secondary structures. No enzymatic process is known in mammalian cells to produce such sequence-directed mutagenesis, but somewhat similar models have been detailed in prokaryotes (19, 21, 22) and proposed for the genetic variation of the interferon genes (23).

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