Frequency and Fidelity of Translesion Synthesis of Site-specific N-2-Acetylaminofluorene Adducts during DNA Replication in a Human Cell Extract*

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We have previously analyzed the effects of site-specific N-2-acetylaminofluorene (AAF) adducts on the efficiency and frameshift fidelity of SV40-based DNA replication in a human cell extract (Thomas, D. C., Veaute, X., Kunkel, T. A., and Fuchs, R. P. P. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 7752–7756). Here we use two sets of substrates to examine the probability of replication termination and error-free and error-prone bypass of AAF adducts. The substrates contained site-specific adducts at one of three guanines in a NAr1 sequence (5'-GGCGCC-3') placed within the lacZα reporter gene and located on the template for either leading or lagging strand replication. The presence of the adduct at any position strongly reduces the efficiency of a single round of replication in a HeLa cell extract. Product analysis reveals preferential replication of the undamaged strand and termination of replication of the damaged strand occurring one nucleotide before incorporation opposite either a leading or lagging strand adduct. Products resistant to restriction endonuclease cleavage at the adducted site were generated in amounts consistent with 16–48% lesion bypass during replication. Most of this bypass was error-free. However, two-nucleotide deletion errors were detected in the replication products of DNA containing an AAF adduct in either the leading or lagging strand but only when present at the third guanine position. Collectively, the data suggest that the replication apparatus in a HeLa cell extract generates a template-primer slippage error at an AAF adduct once for every 30–100 bypass events.

DNA replication in eukaryotic cells requires multiple proteins that function in an asymmetric manner to coordinately synthesize the leading and lagging strands (Kornberg and Baker, 1992). Given the asymmetry of replication of duplex DNA, unrepaired DNA adducts may have different potentials for blocking replication or promoting replication infidelity depending on whether the adduct is located on the leading or lagging strand. To examine this, we have been using the SV40 origin-dependent replication system (for review, see Stillman (1994), and references therein) as a model for human chromosomal replication. With this system, replication of undamaged DNA in mammalian cell extracts is highly accurate (Roberts and Kunkel, 1988; Hauser et al., 1988; Thomas et al., 1991). However, replication of DNA containing randomly placed cyclobutane pyrimidine dimers (CPDs) is highly mutagenic (Thomas and Kunkel, 1993; Carty et al., 1993). The assay system was adapted (Roberts et al., 1991) to determine whether, as a result of the asymmetry of replication, CPD-induced errors arise at different rates during leading and lagging strand synthesis. Overall average error rates were equal for the leading and lagging strand replication machinery, with strand-specific differences in fidelity observed at some nucleotide positions (Thomas et al., 1993).

For several years, we have studied the effects of an encounter between the replication fork and the major adduct of a known carcinogen, N-2-acetylamino fluorene (AAF) located at one template position. Studies in Escherichia coli have revealed that AAF, which binds primarily at the C-8 position of guanine, is a strong mutagen for -1 and -2 frameshift mutations (Fuchs et al., 1981; Koffel-Schwartz et al., 1984; Schaeper et al., 1990), particularly within repetitive sequences that permit slippage to form misaligned intermediates (Burnouf et al., 1989; Lambert et al., 1992). Mutagenesis within these sequences is strongly dependent on adduct location. Template modification at the 3’ most base in a run of guanines or at the third guanine in the Nar1 recognition sequence 5’-GGCGCC-3’ is much more mutagenic than at the other guanine residues because stable intermediates can form during replication (Lambert et al., 1992; Garcia et al., 1993; Milhe et al., 1994). More recently, we have extended these studies in human cell extracts using the SV40-based replication system. We examined the efficiency and frameshift fidelity of replication of DNA containing a sitespecific AAF adduct present at one of the three guanine positions in the Nar1 sequence, each on the leading strand template relative to the first fork to encounter the adduct (Thomas et al., 1994). The primary effect of the adduct was to inhibit replication. When replication of the damaged template did occur, evidence was obtained for error-free lesion bypass with all three substrates, and for error-prone lesion bypass with the substrate that carries the AAF adduct at the third guanine.

When a ColE1-derived plasmid is replicated in E. coli, site-specific AAF adducts induced both -1 and -2 frameshift mutations at a 20-fold higher frequency when the adduct is located on the lagging strand as compared to the leading strand (Veaute and Fuchs, 1993). We hypothesized that the asymmetry of leading and lagging strand replication enzymology in

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1 The abbreviations used are: CPD, cyclobutane pyrimidine dimer; AAF, N-2-acetylamino fluorene; AAF adduct, the major adduct N-(2'-deoxyguanosin-8-yl)-2-acetylamino fluorene; SV40, simian virus 40; T antigen, SV40 large tumor antigen; RF I DNA, covalently closed, circular, double-stranded DNA; RF II DNA, nicked, circular, double-stranded DNA; pol, polymerase.
human cells might also affect the frequency of mutations induced by AAF adducts. For this study we constructed two sets of four DNA substrates, each containing the SV40 replication origin and either no damage or a single, site-specific AAF adduct at one of the three guanines in a NarI sequence. In both sets of substrates, the adducts are located asymmetrically relative to the origin of replication, such that they will be encountered first by either the leading or lagging strand replication apparatus. After replication in a human cell extract, we performed product analyses to quantitatively compare the probabilities of replication termination, error-free bypass and sequence context-dependent, error-prone bypass.

**EXPERIMENTAL PROCEDURES**

Materials—E. coli strains and construction of substrates containing site-specific AAF adducts have been described previously (Burnouf et al., 1988; Veauve and Fuchs, 1993). The substrates (Fig. 1A) are described below. Unmodified substrate (G0) was prepared by the same procedure as for AAF-containing DNAs, starting with mock treatment of the oligonucleotide used to insert the adduct. Radionucleotides were from Amersham Corp.; T antigen was from Molecular Biology Resources. Enzymes were from New England Biolabs or U. S. Biochemical Corp. Extracts were prepared as described (Li and Kelly, 1985).

Replication Reactions and Product Analysis—Reactions (25 μl) contain[3H]dCTP, 100 μM dNTPs, 10–20 ng of DNA, and other components as described (Roberts and Kunkel, 1993) were incubated for 1 h (or for times indicated in Fig. 2) at 37°C. One-tenth of each reaction was collected on filters following precipitation with trichloroacetic acid to determine total incorporation. Following purification of the DNA (Roberts and Kunkel, 1993), an aliquot of each replication reaction was analyzed by electrophoresis (55 V) on a 1.1% agarose gel containing 0.2 μg/ml ethidium bromide. The dried gel was exposed to a phosphor screen for scanning and products quantitated using a Molecular Dynamics Phosphorimager.

Measurement of Repair in Extracts—Repair of adducts was measured by incubating [3H]dCTP in the absence of T antigen for 1 h. Following purification of the DNA by the same procedure used for replication products, the samples were treated with 1 unit of restriction endonuclease BsaHI (an isoschizomer of NarI) for 1 h at 60°C. The samples were resolved by agarose gel electrophoresis, and the dried gel was quantitated as described above. Repair (expressed in percent) was calculated by dividing the sum of the two cleavage products (cut) by the sum of the intensities of the bands resistant to cleavage (uncut) plus the two cleavage products, after subtracting background values for a blank region of the gel.

Analysis of Lesion Bypass—Purified DNA samples were digested with 1 unit of AvaiI for 1 h at 37°C to linearize the DNA and then with 1 unit of BsaHI for 1 h at 60°C. The samples were resolved by agarose gel electrophoresis, and the dried gel was quantitated as described above. Lesion bypass was calculated as described in the legend to Table 1.

Determination of Reversion Frequencies—Following purification, replicated DNA samples were digested with DpnI. The lacZ α-complementation reversion frequencies for control and replicated DNAs were determined by electroporation of E. coli strain JM103 to score blue and white colonies on LB indicator plates containing ampicillin, as described (Burnouf et al., 1988). The assay scores errors that restore the correct reading frame (lacZ') from the +2 reading frame (lacZ) of the starting DNA as blue colonies. White colonies represent the remaining total of replicated products.

Analysis of Replication of the Two Strands—Replicated samples were processed as usual and digested with PvuII and EcoRI in a 30-μl reaction, using conditions optimal for PvuII. The samples were de-salted, lyophilized, dissolved in 10 μl of formamide loading dye, and resolved on an 8% polyacrylamide sequencing gel. Bands on the dried gel were quantitated as described above.

**RESULTS**

Description of Substrates—Two sets of DNA substrates were prepared (Fig. 1, A and B). Each set consists of a control lacking an AAF adduct and substrates with an AAF adduct on one of

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2 We refer to repair of AAF adducts as any process leading to loss of the adduct such that the NarI sequence is rendered sensitive to BsaHI restriction endonuclease.
stances that the closest fork must travel from the SV40 origin to the first nucleotide of the NarI site are 520 and 585 nucleotides for the leading and lagging strand vectors, respectively, distances representing 19 and 21% of the total size of the plasmid. For simplicity, the substrates of each series are referred to as leading or lagging strand substrates and identified by the position of the adduct (G0, G1, G2, or G3). The lacZα gene coding sequence of all substrates is in the +2 reading frame, yielding white colonies on indicator plates. Mutations restoring the correct reading frame (e.g. two-nucleotide deletions) are detected as blue colonies.

Replication Time Course—Previous replication reactions with substrates containing site-specific AAF adducts were performed for 4 h (Thomas et al., 1994), potentially allowing enough time for more than one round of replication. This could significantly alter the product distribution obtained with substrates in which only one of the two strands contains a bulky adduct. Therefore, we began the present study by analyzing the replication products obtained at different incubation times to measure the fraction of products representing more than one round of replication. Both strands of the input DNA are methylated at adenosines in 5′-GATC-3′ sequences. A single round of replication generates hemimethylated DNA, whereas multiple rounds yield unmethylated DNA. The proportion of unmethylated, replicated DNA can be quantitated by digesting the radiolabeled reaction products with restriction endonuclease MboI. This enzyme cleaves only unmethylated DNA, leaving hemimethylated products intact.

The results of a time course for T antigen-dependent replication of undamaged and damaged DNA are shown in Fig. 2. Radiolabeled replication products are detected after a 30-min reaction, and product yield significantly increased in incubations of 1 h or longer. No MboI cleavage products were detected in 30 min. As the incubation time is increased, substantially more MboI cleavage products were observed. In 1-h reactions with G0 DNA, these products comprise approximately 7% of the total. This increases to 29% at 2 h and 38% by 3 h. For G3 DNA, these values were about 5% at 1 h, 29% at 2 h, and 51% at 3 h. Based on these data, we chose to perform subsequent replication reactions for 1 h, because the products at this time point predominantly reflect one round of replication and the yield of replicated DNA was adequate for further analysis.

Measurement of Adduct Removal in the Extract—To more accurately quantitate lesion bypass efficiency and replication fidelity, we next examined the extent of repair of AAF adducts by the same HeLa cell extract used for replication. We took advantage of an earlier observation that the AAF-modified NarI site is insensitive to cleavage by restriction endonuclease BsaHI (Thomas et al., 1994). Radiolabeled control and modified DNAs were incubated in the extract in the absence of T antigen. The products were purified, treated with BsaHI, and resolved on an agarose gel (Fig. 3). The undamaged G0 sample (lagging strand substrate) was completely cleaved. In contrast, while most of the G1 and G3 DNA products were insensitive to cleavage, some digestion was observed. Quantitative analysis of the results in Fig. 3 suggests that after a 1-h incubation in the extract, 14% of the G1 and G3 modified substrates were rendered adduct-free.

Replication of Modified Substrates in the Extract—T antigen-dependent replication reactions were performed for 1 h in the same HeLa cell extract. In three separate experiments, all modified substrates were replicated ~30% less efficiently than was the unmodified control substrate when total T antigen-dependent nucleotide incorporation was considered. Following precipitation and extraction of the products, a portion of each sample was subjected to electrophoresis on an agarose gel (Fig. 4). The alternate lanes marked (−) for BsaHI + AvaI treatment represent the undigested products of reactions with G0, G1, and G3 DNAs. This product distribution is typical of the
Estimation of Bypass Efficiency—To determine if the replicated, damaged DNA retained the AAF adduct, replication products were digested first by Mbol to cleave products having undergone more than one round of replication, and then by Avall and BsaHI. Avall was included to achieve better resolution of fragments of interest, while BsaHI-resistant products are inferred to have retained the AAF adduct (or to have a sequence change at the NarI site; see below).

The results are shown in Fig. 4, in the alternate lanes marked (+) for BsaHI + Avall treatment. Digestion of the products of replication of the undamaged G0 substrates with Avall and BsaHI yielded several bands as predicted from complete digestion and from the location of the known sites (Fig. 1 and legend to Fig. 4). The largest of these bands is shown in the autoradiogram (indicated as cut), while higher mobility bands are not shown. Bands at these same positions were observed with the G1 and G3 substrates in both orientations. However, a new band (indicated as uncut) was observed with the G1 and G3 substrates. For the lagging strand G1 and G3 substrates, the mobility of this band is as predicted by lack of incision at the NarI site containing the AAF adduct. The same is true for the leading strand vector, but here the mobility of the “uncut” band is slightly lower, as predicted by the different location of the added NarI site relative to its neighbors. The data are consistent with the interpretation that this band represents replicated DNA containing the AAF adduct.
cytosine content and DNA repair (see above). For the products of replication of all six modified substrates, the sums of the pixel values for the termination bands and the 120-nucleotide product are similar to the values for the complementary 124-nucleotide fragment (Table II). The relative values are not exactly 100%, perhaps reflecting low levels of second-round synthesis and/or some replication by the fork arriving from the other direction.

Values for the ratio of termination band intensity to the 124-nucleotide fragment intensity ranged from 69 to 95% (Table II). We suggest that these represent the probability that replication terminates upon encountering an adduct. Correspondingly, the ratio of the 120-nucleotide fragment intensity to the 124-nucleotide fragment intensity may approximate the probability of lesion bypass, which is needed for synthesis of full-length restriction fragment. These probabilities ranged from 16 to 27%.

AAF Adduct-induced Error Rates with Opposite-orientation Substrates—We next compared frameshift replication error rates when the AAF adduct was present on the leading or lagging strand relative to the closest fork emanating from the SV40 origin. Reactions were performed as above, and replicated samples were digested with DpnI to remove unreplicated or incompletely replicated molecules. Controls included untreated substrates and those incubated in the extract but with-
out T antigen. These controls were not subsequently treated with DpnI, as this would destroy the unreplicated (fully methylated) molecules. DNA samples were introduced by electroporation into a E. coli α-complementation host, and cells were plated to score white colonies as well as blue colonies resulting from restoration of the correct reading frame in the lacZ reporter gene.

As noted previously, transfection of unreplicated G1 or G2 DNAs, or the same DNAs incubated with the extract in the absence of T antigen, yielded reversion frequencies only slightly above the background established with undamaged G0 DNA (Thomas et al., 1994). We have noted similar results with the G1 and G2 substrates in either orientation (Table III). Control values obtained for the G3 DNAs were higher than the other DNAs, ranging from 4.0 to 6.6 × 10^{-4} (Table III). These higher values are consistent with earlier studies demonstrating that the G3 adduct is mutagenic in E. coli (Burnouf et al., 1990; Veauve and Fuchs, 1993).

Only the products of replication of the G3 substrates yielded reversion frequencies above background levels (Table III). For the leading strand vector the frequencies were about 6-fold above the minus-T antigen control, indicating that most of the blue colonies reflected errors generated during replication in the HeLa cell extract. Reversion frequency values for the lagging strand vector were roughly 3-fold higher than with the leading strand vector and ~20-fold above background reversion frequencies. Sequence analysis of revertants from the G3 reaction products demonstrated the deletion of a GC dinucleotide from the NarI site.

The data showing preferential replication of the undamaged strand (Fig. 5C) led us to next examine whether multiple rounds of replication of the undamaged strand lead to an underestimate of AAF-dependent replication infidelity. To test this, we treated the G3 product DNA with MboI to digest second-round replication products. The effect on reversion frequencies was negligible with both G3-modified substrates (Table III, values in parentheses).

The appearance of BsaHI-resistant replication products consistent with lesion bypass (Fig. 4) suggests that AAF adduct blockage of replication (Fig. 5C) is not absolute. Therefore, we next examined whether longer incubation times influenced reversion frequencies. The effect on reversion frequencies for the substrate containing the lagging strand adduct were negligible (values of 94, 120, and 110 × 10^{-4}, respectively, for incubations of 60, 90, and 120 min). However, the reversion frequencies for the substrate containing the leading strand adduct were increased (values of 33, 51, and 77 × 10^{-4}, respectively, for incubations of 60, 90, and 120 min). Thus, the ~3-fold difference between leading and lagging strand values for a 1-h incubation was reduced to only a 1.4-fold difference after 2 h.

Influence of Proofreading on AAF-induced Replication Errors—We next attempted to determine whether the rate of AAF-induced deletion errors is affected by replication conditions that modulate proofreading. The contribution of proofreading to the fidelity of replication of undamaged DNA (reviewed by Roberts and Kunkel (1995)) has been assessed by increasing the dNTP concentration in the reaction. This selectively stimulates the rate of polymerization from a mispaired or misaligned intermediate, allowing less opportunity to edit the mistake. Alternatively, proofreading exonuclease activity can be inhibited by adding to the reaction a dNMP, the end product of exonuclease action. Both approaches were used in the present study for replication of the two G3-modified substrates (Table IV). With the leading strand substrate, the reversion frequency of replication products from reactions containing 1000 μM dNTPs was 12-fold higher than from reactions containing 10 μM dNTPs, while an intermediate reversion frequency was obtained for the 100 μM reaction. A similar effect was seen for the lagging strand substrate, but the maximum difference was 4.4-fold. Note that in a 1-h reaction containing 1000 μM dNTPs, the two substrates are copied with about equal fidelity (Table IV, compare 470 × 10^{-4} to 440 × 10^{-4}). The addition of 2 mM dGMP to reactions containing 100 μM dNTPs had little effect on reversion frequencies with either G3-modified substrate.

**Discussion**

The present investigation is an extension of our earlier study (Thomas et al., 1994) of the efficiency and fidelity of replication
in human cell extracts of DNA containing unique AAF adducts located at defined template positions. The two main objectives here were to be as quantitative as possible and to compare the effects of adducts on replication by leading and lagging strand replication proteins. Thus, we first established conditions to obtain primarily one round of replication in vitro (1-h incubation, Fig. 2) and then determined the level of adduct repair activity in the extract (14%, Fig. 3) under these conditions. Subsequent analyses of the replication products (Figs. 4 and 5, Tables II-IV) then permit a quantitative estimate of the probability of replication termination and error-free and error-prone translesion synthesis.

The primary effect of an AAF adduct is termination of replication. The present study precisely maps the site of termination as occurring after incorporation opposite the template nucleotide immediately preceding the adduct, in all six site-specifically modified substrates examined (Fig. 5C). We did not observe detectable termination at any upstream template positions or after incorporation opposite the adduct itself. Thus, any incorporation that does occur opposite the adduct likely has one of three fates: (i) correct extension, (ii) misalignment followed by extension (see below), or (iii) excision by a nuclease. The termination pattern observed during synthesis by the human replication apparatus is much simpler than that observed with the replicative DNA polymerase III holoenzyme of E. coli (Belgine-Valladier et al., 1994). That multisubunit enzyme complex terminates synthesis at several upstream locations preceding site-specifically AAF-adducted guanines. The pattern observed in Fig. 5C is also simpler than that observed with exoendonuclease-deficient DNA pol ω and pol β, which terminate synthesis both before and opposite AAF adducts (Rabkin and Strauss, 1984, and references therein). Incorporation studies with AAF-adducted substrates using eukaryotic DNA polymerases having intrinsic S′ → S′′ exonuclease activities have not been reported.

The presence of BsaHI-resistant radiolabeled products (Fig. 4) suggests that AAF adducts are present in the replicated DNA and, thus, are not absolute blocks to replication. Quantitative analysis shows that approximately 17 to 24% of the products are BsaHI-resistant with all substrates examined. (Note that the ~1% mutagenesis for two-base deletions at the Nal site observed with the G3 vectors (Table III) should not contribute significantly to the population of BsaHI-resistant products). Since these products are derived from replication of both the damaged and undamaged strand, the efficiency of bypass per encounter with an AAF adduct in the damaged strand is 2-fold higher than this value, i.e. 34–48%. Lesion bypass efficiencies estimated from quantitative analysis of full-length restriction fragments versus termination bands (Fig. 5C) are also high, ranging from 16 to 27% (Table II). The data suggest that the human replication apparatus in a HeLa cell extract can bypass one-sixth to one-half of all AAF adducts encountered. This is remarkable given the evidence for high fidelity replication with undamaged substrates (Izuta et al., 1995), and references therein). It is also remarkable from a structural perspective. Although the structures of the major eukaryotic cellular replicative DNA polymerases are not yet known, some information does exist for Klenow DNA polymerase (Joyce and Steitz, 1994), human immunodeficiency virus type 1 reverse transcriptase (Kohlsteadt et al., 1992; acoboma-Molina et al., 1993), T7 RNA polymerase (Sousa et al., 1993), and pol β (Pelletier et al., 1994). These studies suggest numerous contacts between DNA polymerases and template-primers that might be severely perturbed by the presence of an adduct as bulky as the AAF adduct studied here. A bulky adduct might be expected to strongly reduce polymerase binding and/or translocation, as evidenced by the AAF-induced termination of synthesis observed with numerous DNA polymerases (e.g. see above). Thus, frequent bypass by the multiprotein replication complex implies a major role for replication accessory proteins in assisting lesion bypass. Logical candidates for modulating lesion bypass during eukaryotic chromosomal replication of damaged DNA are proteins that influence polymerase template-primer binding and/or translocation, such as proliferating cell nuclear antigen, which confers high processivity to pol δ (for review see Stillman (1994), and references therein).

The data in Table III demonstrate that replication of the G3-modified substrate is mutagenic, whereas replication of the other two modified substrates is not. This is consistent with a model wherein the replication apparatus first incorporates cytosine opposite the modified guanine (Lambert et al., 1992). The template-primer then slips such that the two terminal bases in the primer (3′-CpG-5′) hydrogen-bond with a repeated downstream complementary 5′-GpC-3′ dinucleotide in the template, thus forming a misaligned intermediate (Schaaper et al., 1990). Continued synthesis from this intermediate leads to a two-base deletion error. This model predicts that mutagenesis should result from adducts in the G3 but not the G1 or G2 positions, because, given the sequence at the NarI site, only the former allows formation of a misaligned intermediate stabilized by two terminal base pairs. This prediction was realized in E. coli (Burnouf et al., 1989; Veau and Fuchs, 1993), in our previous study of T antigen-dependent replication in a HeLa cell extract (Thomas et al., 1994) and in this study.

The revertant frequencies in Table III are for G3 adduct-dependent replication errors among total replication products generated in a single round of replication and can therefore be used to estimate quantitatively the probability of error-free versus error-prone bypass. Given the average ~3-fold bias in replication of the undamaged strand (Fig. 5C), a revertant frequency of 1% for the lagging strand G3-modified substrate (Table III) suggests that 3% of the products of replication of the damaged strand are mutagenic with this substrate in a 1-h incubation. A similar approach suggests that ~1% of bypass events is mutagenic in a 1-h reaction with the leading strand G3-modified substrate. These estimates are limited to errors that restore the correct reading frame, which is in this and our previous study are two-base deletions.

Two asymmetries are observed during T antigen-dependent replication of AAF adduct-modified DNAs in the extract. These are a 3-fold difference in AAF adduct-induced replication fidelity with the two opposite-orientation G3-modified substrates (Table III) and preferential replication of the undamaged strand (Fig. 5C). The latter could reflect uncoupling of the first replication fork to encounter the adduct, such that repli-
cation of the undamaged strand continues while replication of the damaged strand is completely or transiently blocked. Evidence exists that uncoupling can occur under some circumstances in E. coli (Koffel-Schwartz et al., 1987)\(^3\) and during SV40 replication in vivo (Burhans et al., 1991). Alternatively, the first fork may not replicate either strand beyond the lesion, with preferential replication of the undamaged strand accomplished by the fork arising from the other direction in the circular substrate. By this same logic, the 3-fold difference in revertant frequencies can be explained in more than one way. If bypass is catalyzed by the first fork to encounter the adduct, then the difference suggests that the leading strand replication apparatus is 3-fold more accurate than the lagging strand apparatus. Alternatively, error-prone lesion bypass replication may be accomplished only by the fork arising from the other direction. For example, it is formally possible that much or all of the error-prone lesion bypass synthesis with both G3-modified substrates is catalyzed by the lagging strand replication apparatus. Consistent with this idea, mutagenesis with the leading strand G3 vector increases with increasing incubation time, while that with the lagging strand substrate remains about the same. Given two replication forks and use of a circular substrate, several explanations are consistent with the existing data. It is also possible that the lesion bypass synthesis in the extract is catalyzed by proteins other than those normally comprising a replication fork. It may be possible to resolve these issues in the future using reactions reconstituted from purified proteins or using much larger replicons or substrates containing a replication termination signal.

Several observations suggest that the misaligned intermediate believed to be responsible for the AAF-dependent deletion errors might be edited by proofreading during replication. First, the evidence presented here for substantial bypass implies that incorporation opposite an AAF adduct is possible, but the bands in Fig. 5C indicate termination of incorporation one base before the adduct but not opposite the adduct itself. This may be interpreted in light of the observation that proofreading-proficient Klenow polymerase generated a termination pattern similar to that seen here, while the exonuclease-deficient polymerase terminated synthesis after incorporation opposite the adduct (Belguise-Valladier et al., 1994). This indicates that a proofreading exonuclease can remove incorporations opposite the adduct. Moreover, the presumed pretranslational intermediate in this study contains two terminal base pairs and an unpaired modified GpC dinucleotide in the template strand. Several studies suggest that undamaged misaligned intermediates formed during synthesis by proofreading-proficient DNA polymerases (Bebenek et al., 1990; Bebenek and Kunkel, 1990; Thomas et al., 1991; Kunkel et al., 1994) and during T antigen-dependent replication in extracts (Roberts et al., 1993) are subject to editing by exonucleases. In this study, the rate of AAF-induced deletion errors was substantially increased by increasing the dNTP concentration in the reaction (Table IV). This observation is consistent with selective stimulation of polymerization from a misaligned intermediate, allowing less opportunity to proofread the error. The fact that the dNTP-dependent increase was greater with the substrate containing the adduct in the leading strand relative to the first fork to encounter the adduct is consistent with more active proofreading by the leading strand replication apparatus. As discussed above, other explanations are also possible. The second approach to modulate deletion error rates by modulating proofreading, inhibition of exonuclease activity by adding dGMP to the reaction, yielded very small effects (Table IV). Lack of a monophosphate effect on frameshift fidelity during T antigen-dependent replication was also observed with undamaged substrates in an earlier study (Roberts et al., 1993). The reason for this is unclear, especially since addition of dGMP to a replication reaction clearly reduces base substitution fidelity with normal substrates (Roberts et al., 1991; Izuta et al., 1995) and with a dNTP analog (Minnick et al., 1995).

The estimates of termination and error-free and error-prone bypass presented here are for replication in a HeLa cell extract. It will be interesting to obtain similar estimates using extracts of normal human fibroblasts (Boyer et al., 1993) or cell lines having defects in mismatch repair or nucleotide excision repair.

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\(^3\) R. P. P. Fuchs, unpublished observations.