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# Sites of termination of *in vitro* DNA synthesis on ultraviolet- and *N*-acetylaminofluorene-treated φX174 templates by prokaryotic and eukaryotic DNA polymerases

(exonuclease/mutagenesis/SOS repair)

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**ABSTRACT** *In vitro* DNA synthesis on a φX174 template primed with a restriction fragment and catalyzed by the *Escherichia coli* DNA polymerase I large (Klenow) fragment (pol I) terminates at the nucleotide preceding a site that has been altered by ultraviolet irradiation or treatment with *N*-acetylaminofluorene. Termination on ultraviolet-irradiated templates is similar when synthesis is catalyzed by *E. coli* DNA polymerase III holoenzyme (pol III), phage T4 DNA polymerase, a polymerase α from human lymphoma cells, or avian myeloblastosis virus reverse transcriptase. 3'→5' exonuclease activity cannot be detected in the reverse transcriptase and DNA polymerase α preparations. On *N*-acetylaminofluorene templates, pol I, pol III, and T4 polymerase reactions terminate immediately preceding the lesion, whereas reverse transcriptase-catalyzed reactions and, at some positions in the sequence, polymerase α-catalyzed reactions terminate at the site of the lesion. Substitution of $Mn^{2+}$ for $Mg^{2+}$ changes the pattern of pol I-catalyzed termination sites. The data suggest that termination is a complicated process that does not depend exclusively on the 3'→5' exonuclease activity associated with many polymerases.

Induced mutagenesis is a process that occurs when the DNA synthetic machinery of a cell approaches a damaged nucleotide. In *Escherichia coli*, an inducible mechanism, the SOS repair pathway, plays a crucial role in mutagenesis (1, 2). In some way, the process of bypassing a lesion results in mutation. Because *in vivo* experiments are difficult to interpret mechanistically, we have adapted an *in vitro* system—φX174 DNA primed by a single restriction fragment—as a useful model system for DNA synthesis. Using this model, others (3, 4) have carried out a transfectional analysis of the production of mutation, and we have determined the biochemical nature of the molecules synthesized on damaged templates (5).

DNA synthesis by either the *E. coli* DNA polymerase I large (Klenow) fragment (pol I) or the *E. coli* DNA polymerase III holoenzyme (pol III) is blocked by the presence of UV-induced pyrimidine dimers (6–8) or of adducts of *N*-acetylaminofluorene (AAF) (9) in the DNA template. pol I terminates synthesis one nucleotide before the site of a pyrimidine dimer or guanine that has reacted with AAF (5). There are two distinct mechanisms that might be responsible for termination; (*i*) either the enzyme is unable to insert a nucleotide opposite a damaged base or (*ii*) an inserted nucleotide is rapidly removed by the 3'→5' exonuclease editing function associated with the polymerase. The second of these explanations is favored by Villani *et al.* (8), who have suggested that inhibition of exonuclease activity could permit a polymerase to bypass a lesion in the DNA. This would provide a simple mechanism for the inducible error-prone repair believed to be responsible for the bulk of mutagenesis following UV irradiation (1, 2). A test of this hypothesis would be to distinguish the contributions of polymerase and nuclease functions in the process of termination. We have set out to do this in two ways: (*i*) by studying a number of DNA polymerases, including some that have and some that lack associated nuclease activity, and (*ii*) by using conditions that affect the polymerase and nuclease functions of a single enzyme in different ways.

We have examined termination of synthesis in an *in vitro* DNA synthesizing system by using as a template φX174 DNA primed with purified restriction fragments. The templates were either irradiated with UV light or treated with *N*-acetoxy AAF, and the products of synthesis were analyzed on DNA sequencing gels (5).

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## MATERIALS AND METHODS

**DNA.** The preparation of DNA and the construction of templates have been described (5, 9). Analysis on agarose gels showed the templates to be intact, suggesting little or no breakage at the sites of either AAF adducts or pyrimidine dimers in the DNA.

**Enzymes.** pol I lacking 5'→3' exonuclease was purchased from Boehringer-Mannheim. T4 DNA polymerase was a gift from Nicholas Cozzarelli, pol III was a gift from U. Hübscher, and avian myeloblastosis virus (AMV) reverse transcriptase was provided by J. W. Beard. DNA polymerase α from Daudi human lymphoma cells was prepared as described (10). One unit of enzyme directed the incorporation of the following amounts of nucleotide into DNA at 37°C: pol I and T4 DNA polymerase, 10 nmol/30 min; pol III, 1 pmol/min; AMV reverse transcriptase, 1 nmol/10 min; and DNA polymerase α, 1 nmol/60 min.

Restriction endonuclease Hae II was purchased from Bethesda Research Laboratories (Rockville, MD).

**Enzyme Assays.** DNA polymerase activity was determined by using activated salmon testis DNA and [$^3$H]TTP (35 cpm/pmol) as described (10). Endonuclease activity was monitored by using φX174 replicative form [$^3$H]DNA as described (10) and 19 units of AMV reverse transcriptase. 3'→5' exonuclease activity was determined by two separate assays: (*i*) 3'-$^3$H-end-labeled DNA (27,000–40,000 cpm/μg of DNA) was prepared by treating activated salmon testis DNA with $^3$H-labeled deoxynucleoside triphosphates (250 cpm/pmol each) and DNA polymerase α. Exonuclease activity was measured as described (10). (*ii*) 5'-$^{32}$P-labeled oligodeoxythymidylic acid hexamer ($d[T]_6$) was prepared by treating $d[T]_6$ with T4 polynucleotide kinase and [γ-$^{32}$P]ATP, followed by purification of the product through two Sephadex G-50 columns. Thin layer chromatography indi-

Abbreviations: AAF, *N*-acetylaminofluorene; pol I, *Escherichia coli* DNA polymerase I large (Klenow) fragment; pol III, *Escherichia coli* DNA polymerase III holoenzyme; AMV, avian myeloblastosis virus; $d[T]_6$, oligodeoxythymidylic acid hexamer.

cated no contaminating [$\gamma$-$^{32}$P]ATP in the preparation. This labeled d[T]$_6$ was used for nuclease assay in the following way: 0.25 nmol of labeled d[T]$_6$ diluted with unlabeled d[T]$_6$ or 0.025 nmol of labeled d[T]$_6$ was incubated at 37°C with either 0.036 or 0.36 unit of T4 polymerase, 5.2 or 52 units of pol III, 0.32 or 0.96 unit of pol I, 19 units of AMV reverse transcriptase, or 0.6 unit of DNA polymerase $\alpha$ from Daudi cells in a total volume of 10 $\mu$l of 50 mM Tris·HCl, pH 7.5/8 mM $MgCl_2$/5 mM 2-mercaptoethanol. Samples were taken at intervals and chromatographed with 2 M pyridine formate buffer (pH 3.5) on polyethyleneimine thin layer plates (Brinkmann). The radioactive spots of d[T]$_6$ or its degradation products were detected by overnight exposure of the plates to x-ray film.

**Polyacrylamide DNA Sequencing Gels.** Standard polymerase reactions for sequencing gels were carried out as described (5) in 10-$\mu$l reaction mixtures containing [$^{32}$P]TTP (1–2 $\mu$Ci; ≈300 Ci/mmol; 1 Ci = 3.7 × $10^{10}$ becquerels), 0.06 pmol of template DNA, and one of the following enzymes: pol I (0.2 unit), AMV reverse transcriptase (19 units), T4 DNA polymerase (0.36 unit), or DNA polymerase $\alpha$ (2.4 units). For pol III (52 units), the reaction mixture also contained 5 mM ATP and 5 mM spermidine·HCl. The amounts of polymerases used were chosen to give maximum incorporation of label. When expressed in the same units, the polymerizing activities were comparable for all enzymes, except AMV reverse transcriptase for which the amount used was ≈30 times that used for pol I (however, there were no changes in the respective band patterns when the concentration of pol I was increased 10-fold or when the AMV reverse transcriptase concentration was reduced by four-fifths).

Sequence standards were produced by using pol I with chain-terminating nucleotides (11). One microgram of unlabeled sonicated carrier DNA was added to the samples, which were diluted 1:1 with 8 M urea/0.2 M NaOH/0.005% bromphenol blue/0.005% xylene cyanol FF and analyzed on a 20% polyacrylamide gel (12). The sequence is numbered from the site of the *Hae* II restriction cut and is two less than the numbering used previously (5), which was taken from the center of the *Hae* II recognition site.

**Exonuclease Activity of Polymerases.** The DNA polymerase preparations were tested for the presence of 3'→5' exonuclease activity by using salmon testis DNA labeled at the 3' ends with $^3$H-labeled nucleotides as substrates. Pol I assayed on denatured DNA released 7.2 (pmol/min)/unit of polymerizing activity, and T4 polymerase assayed on native DNA released 35.4 (pmol/min)/unit of polymerizing activity. No activity against either native or denatured substrate was detected for AMV reverse transcriptase or the Daudi $\alpha$ polymerase at minimum levels of detection of <0.02 and <0.1 (pmol/min)/pol I polymerizing unit, respectively. Because some exonucleases are more active on short DNA chains (13), the enzymes were tested by using 5'-$^{32}$P-labeled d[T]$_6$ as substrate and monitoring the hydrolysis to penta- and tetranucleotides by thin layer chromatography. Although both pol III and T4 DNA polymerase were very active on this substrate, no activity was detected with pol I, AMV reverse transcriptase, or the Daudi $\alpha$ polymerase at concentrations of polymerizing activity that were, respectively, 60, 360, and 2 times greater than that used for pol III. AMV reverse transcriptase and the $\alpha$ polymerase were also tested on substrates of ColE1 or $\phi$X174 replicative form superhelical DNA and found to be free from any endonucleolytic activity.

**Termination on UV-Irradiated DNA.** When the products of synthesis on an untreated template were analyzed (Fig. 1), a number of bands were found on the sequence gel, most of which

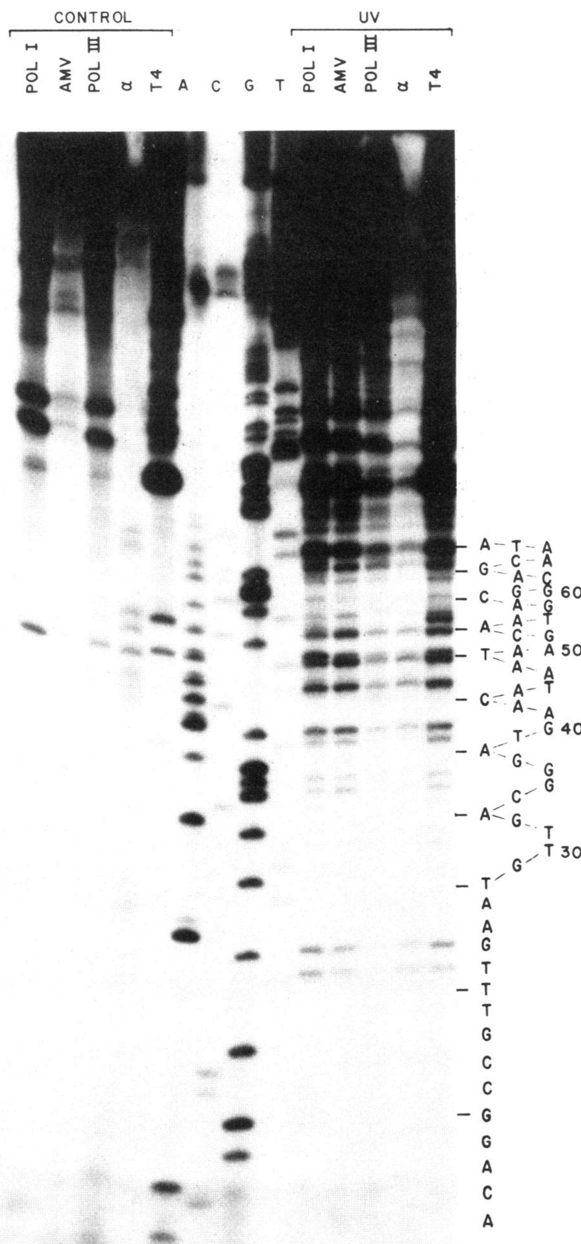


FIG. 1. Polyacrylamide gel analysis of products synthesized on either untreated or UV-irradiated DNA templates by pol I, AMV reverse transcriptase, pol III, DNA polymerase $\alpha$, and T4 DNA polymerase. The DNA templates were primed with *Hae* II restriction fragment 5; UV irradiation was 1500 J/m$^2$. Lanes A, C, G, and T are sequence standards synthesized by pol I with dideoxy chain-terminating nucleotides on an unirradiated template. The sequence given is of the synthesized strand.

could be identified as *Hae* II restriction fragments generated as the enzymes replicated the template. Some additional bands found with T4 polymerase were probably due to termination at sites of secondary structure in the template to which the T4 polymerase was particularly susceptible (ref. 14; unpublished observations). A series of termination bands found with the Daudi $\alpha$ polymerase reflected the limited ability of this enzyme to copy long regions of single-stranded DNA.

On the UV-irradiated template, many more bands were produced by all the enzymes and, as previously shown for pol I (5), these bands corresponded to the sites of pyrimidine dimers in the template. All the bands were found one nucleotide before

the first of two adjacent pyrimidines in the template. It is particularly striking that not only do all five enzymes show termination at dimers but that each enzyme terminates in the same position.

**Termination on AAF-Treated DNA.** Adducts of AAF are also blocks to DNA synthesis (9) and, as previously reported (5), the principal termination bands for pol I are found (Fig. 2) one nucleotide before the positions of guanines (the known sites of reaction of AAF) in the template. A similar pattern was also seen for T4 DNA polymerase and pol III. For AMV reverse transcriptase and Daudi $\alpha$ polymerase, however, the termination bands were at different positions from those for the prokaryotic enzymes. In the case of AMV reverse transcriptase, termination occurred at the level of the guanines in the template—i.e., as if a nucleotide were inserted opposite an AAF adduct. In other experiments (not shown), we have observed that, on an AAF-containing template that has been treated with pol III, the termination pattern can be converted from "pol III-like" to "AMV-like" by subsequent incubation with reverse transcriptase if deoxyribonucleoside triphosphates are present during the second incubation. In the absence of evidence for activity associated with these enzymes that might result in other than a 3′ hydroxyl terminus, we conclude that the two types of termination pattern we have observed are due to the addition of an extra nucleotide opposite the guanyl–AAF adduct when synthesis is catalyzed by AMV reverse transcriptase. The position of the termination bands relative to the AAF adducts obtained with the $\alpha$ polymerase depended on the position of the adduct in the template (see below).

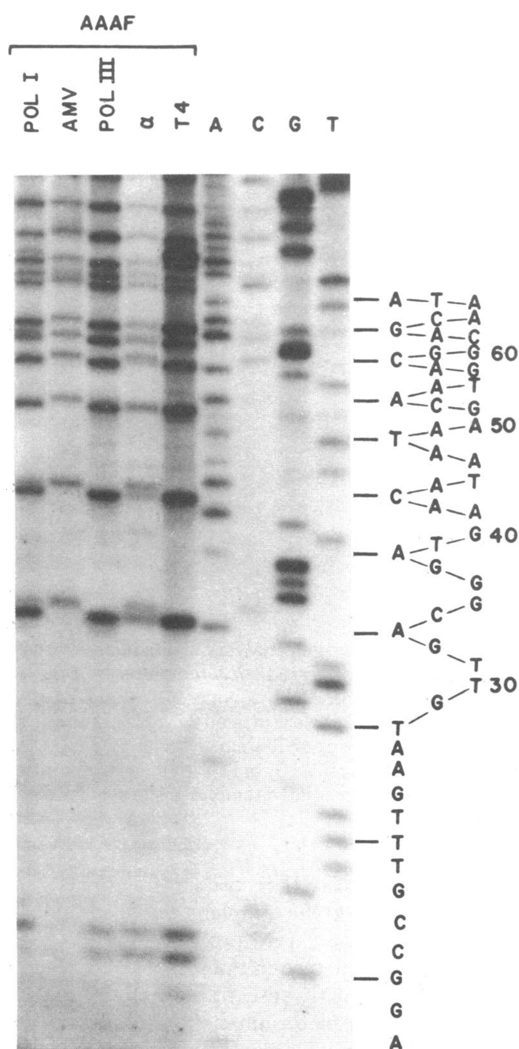


FIG. 2. Polyacrylamide gel analysis of products synthesized on an AAF-treated template by pol I, AMV reverse transcriptase, pol III, DNA polymerase $\alpha$, and T4 DNA polymerase. The DNA template was primed with *Hae* II restriction fragment 5 and contained 113 AAF residues per $\phi$X174 molecule.

For pol I on AAF-treated templates, secondary bands have previously been seen two or even three nucleotides before the site of the lesion but bands opposite the lesion were never observed (5). For this preparation of pol I, no bands two or more nucleotides before the lesion were found, but less intense bands opposite the lesion could be seen (see Fig. 2). This difference between the two enzyme preparations was reproducible. For the particular AMV reverse transcriptase experiment shown, although bands corresponding to the guanines at positions 19 and 20 were absent, such bands have been observed in other experiments. Bands corresponding to a guanine at position 15 in the template were occasionally missing from several of the enzymes.

**Changes in Termination Pattern of pol I.** Replacing $Mg^{2+}$ by $Mn^{2+}$ significantly decreases the fidelity of synthesis by pol I and other DNA polymerases, which results in misincorporation of nucleotides into the DNA (3, 15). In our system, replacing the 8 mM $Mg^{2+}$ by 0.5 or 1.0 mM $Mn^{2+}$ produced a number of changes in the position of termination observed for pol I (Fig. 3). At most positions in the sequence, the use of $Mn^{2+}$ resulted in a much more frequent termination with a nucleotide opposite the AAF adduct. In addition, at certain guanines (e.g., those at positions 61 and 76), bands were either missing or greatly diminished in intensity, possibly due to failure to terminate. These modifications of the termination pattern were brought about by the effect of $Mn^{2+}$ on pol I; that they were not due to electrophoresis artifacts was shown by experiments in which the $Mg^{2+}$-$Mn^{2+}$ difference was present only during the synthesis step of the reaction.

To test the suggestion by Villani *et al.* (8) that termination by pol I is effected by the 3′→5′ exonuclease activity of this enzyme, we examined the relative activities of polymerase and exonuclease in the presence of $Mn^{2+}$ (Table 1). At the $Mn^{2+}$ concentrations at which we observed a change in termination pattern, the ratio of exonuclease to polymerase activity was actually higher than that with $Mg^{2+}$. For T4 polymerase, the opposite result was observed; addition of up to 0.5 mM $Mn^{2+}$ preferentially inhibited the exonuclease activity, although it had no effect on the termination pattern on AAF-treated DNA (data not shown); at all concentrations of $Mn^{2+}$ tested, the band pattern was identical to that obtained with $Mg^{2+}$.

## DISCUSSION

Termination of *in vitro* DNA synthesis by pol I occurs at the sites of the known principal lesions produced by AAF, benzo[*a*]pyrene diol epoxide, and UV light (5). It is important to quantitate the sensitivity of our method to determine whether any synthesis "bypasses" lesions in the DNA. A maximum estimate for the frequency of bypass can be obtained from a determination of the relative amounts of termination at successive lesions. When termination is at a guanyl–AAF adduct, for example, the proportion of chains terminating up to position $n$ in a sequence containing $N$ guanines is $(1 - P^n)/(1 - P^N)$, where $P$ is the probability of proceeding past a guanine, as determined from the number of AAF residues per guanine in the molecule (9). The best fit of the data from five of our experiments (9) is to a curve having 100% termination at each lesion, but the standard deviation (11–14% of the mean for the first six

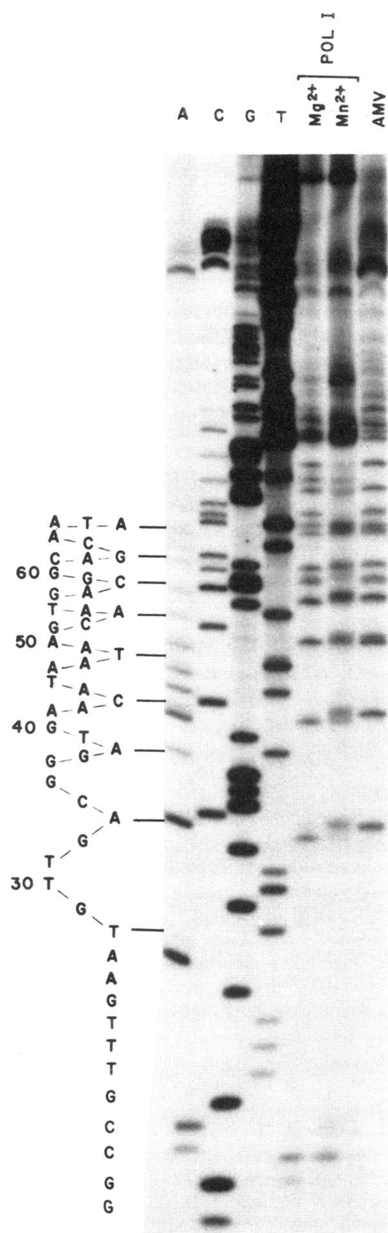


FIG. 3. Effect of divalent cation on sites of termination by pol I on AAF-treated DNA. Reactions were identical to those in Fig. 2 except for replacement of 8 mM $MgCl_2$ by 0.5 mM $MnCl_2$ where indicated.

residues) makes it impossible for us to exclude the hypothesis of as much as 10% readthrough. Although similar quantitation data are not available for all the enzymes, we have identified sites of termination for pol III, a mammalian $\alpha$ polymerase, T4 DNA polymerase, and AMV reverse transcriptase, as well as for pol I.

With UV-irradiated DNA, all five enzymes terminate at positions one nucleotide preceding the site of a pyrimidine dimer in the template. Similar results have been obtained (ref. 5; unpublished data) with regard to the adducts formed by benzo[*a*]pyrene diol epoxide. The sites of termination on AAF-treated DNA, however, show interesting variations. The principal site of reaction of AAF is at the guanines in the DNA (16), and all the positions of termination occur at or near these sites. Two guanyl–AAF adducts have been reported in double-stranded DNA (16), but the distribution of these in single-stranded $\phi$X174 DNA is unknown. The only sites of termination for pol III and T4 polymerase and the principal site of termination for pol I are at nucleotides immediately preceding guanyl–AAF adducts. Our data suggest that the termination sites for AMV reverse transcriptase are one nucleotide further—i.e., with a base inserted opposite the lesion.

With the mammalian $\alpha$ polymerase, there is some variation in termination site. Where a nucleotide is inserted opposite a lesion, as at positions 34, 43, and 58, the nucleotide preceding the guanyl–AAF adduct is thymidine, but at positions 19, 20, 52, 61, and 64, where termination occurs almost exclusively before the adduct, the preceding nucleotide is cytidine or, in one case, guanosine (see Fig. 2). In experiments in which other priming fragments were used, doublets were more ambiguous than those in positions 34, 43, and 58. Nonetheless, we considered 5 out of 9 bands at the site of the treated nucleotide to be significant when preceded by thymidine as compared with 3 out of 14 when preceded by cytidine. Such a specificity could be related to differences in stacking interactions between AAF and neighboring bases (17), where a base-stacking interaction with AAF was observed for adenine but not for uracil (18).

Villani *et al.* (8) have suggested that termination of DNA synthesis by pol I and pol III at pyrimidine dimers may be due to the 3'→5' exonuclease activities of these enzymes and thus that mammalian enzymes, which lack such activities, are capable of continuing past the lesions. It has further been suggested that "SOS induced" UV mutagenesis in *E. coli* could result from inhibition of the exonuclease function of pol III, possibly by an accumulation of deoxynucleotide monophosphates (8, 19). All the enzymes we tested terminated at lesions and, in the case of pyrimidine dimers, the position of termination was identical for all five enzymes. For two of the enzymes, AMV reverse transcriptase and the mammalian $\alpha$ polymerase, we detected no exonuclease activity (exonuclease/polymerase ratios of these enzymes were less than 1/1750 and 1/350, respectively, of the ratio of T4 DNA polymerase). The difference between the bacterial enzymes and AMV reverse transcriptase in the position of termination at AAF adducts could be interpreted to mean that the exonuclease activities of the bacterial enzymes play a role in termination at AAF adducts; however, exonuclease activity cannot account for all the AAF results. Even though pol I possesses exonuclease activity, it can, under certain conditions, insert a nucleotide opposite the adduct, and it is also clear

Table 1. Effect of divalent cation on DNA polymerase and 3'→5' exonuclease activity

| | $Mg^{2+}$, mM | $Mn^{2+}$, mM | Polymerase activity* | Exonuclease activity† | Polymerase activity/exonuclease activity |
|---|---|---|---|---|---|
| pol I | | | | | |
| | 8 | — | 29.1 | 7.2 | 0.25 |
| | — | 0.2 | 2.4 | 5.6 | 2.33 |
| | — | 0.5 | 22.3 | 6.1 | 0.27 |
| | — | 0.75 | 8.3 | 5.0 | 0.60 |
| | — | 1.0 | 7.2 | 3.3 | 0.46 |
| T4‡ | | | | | |
| | 5 | — | 53.4 | 35.4 | 0.66 |
| | 5 | 0.2 | 57.1 | 17.1 | 0.30 |
| | 5 | 0.5 | 49.5 | 12.1 | 0.24 |
| | 5 | 0.75 | 16.1 | 11.0 | 0.68 |
| | 5 | 1.0 | 11.1 | 7.6 | 0.68 |

* Expressed as pmol incorporated per min per unit.

† Measured by using native (for T4) or heat-denatured (for pol I) 3'-$^3$H-labeled salmon testis DNA and expressed as pmol released per min per unit.

‡ The use of 0.5 mM $Mn^{2+}$ in the absence of $Mg^{2+}$ completely abolished the polymerase activity of T4.

that the mammalian $\alpha$ polymerase, for which we can detect no exonuclease, is capable of terminating at the same position, for some of the AAF adducts, as the bacterial enzymes.

Further evidence that the exonuclease activity is not the sole determinant of the position of termination comes from the results with $Mn^{2+}$. Substituting $Mn^{2+}$ for $Mg^{2+}$ increases the likelihood that pol I-catalyzed reactions will terminate at a nucleotide opposite an AAF adduct. This change occurs despite the fact that the use of $Mn^{2+}$ does not reduce the exonuclease activity/polymerase activity ratio of the enzyme. In contrast, addition of $Mn^{2+}$ selectively inhibits the exonuclease activity of T4 polymerase but does not affect the pattern of termination. Because $Mn^{2+}$ did not compleely inhibit the exonuclease activity of T4 polymerase, we cannot assume that complete loss of this activity would not affect termination.

At least two aspects of our studies appear to parallel the results of "misincorporation" studies. AMV reverse transcriptase has been reported to synthesize DNA with decreased "fidelity" and, in contrast to the prokaryotic polymerases, appears to insert a nucleotide at the level of the AAF-reacted base. Substitution of $Mn^{2+}$ for $Mg^{2+}$ results in a decreased "fidelity" of synthesis by pol I (15), although such substitution in our pol I system leads to the insertion of nucleotides at the level of the reacted base and alters the band pattern in as yet unexplained ways. Notwithstanding such parallel behavior, the phenomenon of misincorporation is fundamentally different from that observed here. Misincorporation involves complex kinetic behavior at each nucleotide, and the outcome is determined by the interrelationships among the rates of polymerization and proofreading at the site of the potential misincorporation and of elongation past the potentially misincorporated base (2). The $3' \rightarrow 5'$ exonuclease may have a significant effect on misincorporation only at low nucleotide concentrations such that the rate of addition of the nucleotide immediately following the site of the misincorporation is reduced, allowing more time for the exonuclease to excise the terminal base (4, 20, 21). The situation is quite different when an adduct that effectively prevents elongation is present on a nucleotide. It is even possible that, during replication of lesions that seriously impair the capacity of a sequence to act as a template for primer elongation, it is termination of synthesis that permits action of the exonuclease rather than the reverse.

The substitution of $Mn^{2+}$ for $Mg^{2+}$ with pol I does not produce the same result as with AMV transcriptase. At those sites at which termination bands appear to be absent when $Mn^{2+}$ is used with pol I on an AAF-reacted template, bands are always found with AMV reverse transcriptase. We have also observed (unpublished result) that $Mn^{2+}$ affects the pattern of termination by pol I on UV-irradiated templates similarly, leading to bands opposite the sites of the pyrimidine dimers, rather than at the preceding nucleotide.

These differences suggest that the process of termination is a complicated one and may involve a number of different factors, including the nature of the lesion, the choice of cation, and the characteristics of the particular DNA polymerase involved. Although our experiments do not completely define the precise properties of a polymerase that govern its ability to either terminate or bypass a lesion, it is clear that the evidence does not suggest an exclusive role for $3' \rightarrow 5'$ exonuclease activity in these processes.

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