Volume 8 Number 19 1980 Nucleic Acids Research

Termination of in vitro DNA synthesis at AAF adducts in the DNA

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Received 23 June 1980

ABSTRACT

DNA synthesis catalyzed by E. coli polymerases I or III is inhibited on templates containing N-acetoxy-acetylaminofluorene-reacted adducts. Termination of synthesis occurs just before the site of the adduct. Synthesis on ØX174 templates primed with restriction fragments and treated with AAAF can be visualized on DNA sequencing gels. Comparison of the amounts of the different newly synthesized fragments with those calculated from the probability of termination as determined from the average number of adducts per molecule shows that synthesis terminates, rather than stutters, at each adduct. This method may be useful for detecting the bypass of lesions.

INTRODUCTION

The production of induced mutations requires the active involvement of cellular processes to convert the initial insult into a change in the base sequence of DNA (1). Since loss of excision repair capacity leads to enhanced induced mutation rates, it is mostly likely that the mutagenic process occurs when a cell replicates damaged DNA. Some DNA damage may lead to base change without interruption of DNA synthesis, for example, the deamination of adenine or cytosine by nitrous acid. Other lesions such as UV-induced pyrimidine dimers and bulky aromatic hydrocarbon adducts first block DNA replication, and mutations may then arise as the cell attempts to overcome or bypass the block (2).

In E. coli this latter process appears to be carried out by an inducible repair mechanism, termed SOS repair (3). DNA replication in UV-irradiated bacterial cells results in gaps in the newly synthesized DNA, opposite pyrimidine dimers. Most of these are processed without mutation by a recombination mechanism (4); however, some of them are filled by error-prone de novo DNA synthesis directed by the SOS system,the enzymology of which has still to be elucidated. We have, therefore, started to search for in vitro systems which will permit a mechanistic analysis of termination and/or bypass by DNA polymerases at lesions in the DNA template.

A start in the development of such a system has been made by a number of

investigators (5,6,7) using ØX174 DNA as a template. The presence of pyrimidine dimers or benzo(a)pyrene diolepoxide (BPDE) residues in this template produces an absolute block to synthesis catalyzed by DNA polymerases I and III from E. coli (5,7). On ØX174 DNA templates that have been UV-irradiated or treated with either BPDE or N-acetoxy-acetylaminofluorene (N-acetoxy-AAF) synthesis catalyzed by E. coli DNA polymerase I produces DNA fragments terminating one nucleotide before an altered base (8). Since production of fragments on a sequencing gel is not alone sufficient to determine whether synthesis is permanently halted or simply delayed temporarily, we report here experiments that examine in detail termination of DNA synthesis at AAF adducts.

MATERIALS AND METHODS

(a) Enzymes

E. coli pol I, large fragment (Klenow) lacking 5'→ 3' exonuclease was purchased from Boehringer-Mannheim. E. coli pol III holoenzyme, 3.5×10^5 units/mg protein (9), was a gift from Dr. U. Hübscher.

Restriction endonuclease Hae II was purchased from Bethesda Research Laboratories, Inc.

(b) Preparation of primed ØX174 DNA

Single stranded ØX174 DNA was extracted from purified phage with phenol, alcohol precipitated and resuspended in 10 mM Tris HCl pH 7.5, 0.25 mM EDTA. Contaminating oligonucleotides annealed to the DNA were removed by adding formamide to 50% and passing the DNA through a Sephacryl S-200 column. Double stranded replicative form ØX174 DNA was prepared by a modification of the method of Eisenberg et al. (10) including an ethidium bromide/CsCl equilibrium density gradient step.

Restriction fragments were prepared by digesting 200 µg replicative form of ØX174 DNA with 100 units of Hae II restriction enzyme and separating the fragments by electrophoresis on a preparative 1% agarose gel. Individual bands, identified by ethidium bromide staining, were cut out, the agarose dissolved in 5 M $NaClO_4$ and then loaded onto a small hydroxyapatite column in 0.04 M sodium phosphate buffer (pH 6.8). The DNA was eluted in 1 ml of 0.5 M phosphate buffer, ethidium bromide was extracted with isoamyl alcohol, and then the fragments were dialyzed against 2.5 mM Tris HCl pH 7.5, 0.1 mM EDTA, precipitated with ethanol and redissolved in 0.25 ml of 10 mM Tris HCl pH 7.5, 0.25 mM EDTA.

Fragments were denatured by heating to 100°C for 5 min and then annealed to single stranded ØX174 DNA at 65°C for 90 min in 100 mM KCl. The concentration of single stranded DNA was 50 µg/ml and the ratio of fragment to single stranded DNA

was 1.2:1 (mole:mole) which should result in priming of approximately 55% of the template molecules.

(c) N-acetoxy-AAF treatment of DNA

Single stranded DNA at a concentration of 400-500 µg/ml was incubated at 37°C with [^{14}C]N-acetoxy-AAF (52.5 mCi/mmole, Midwest Research Institute) at 1-50 µg/ml in 10 mM Tris pH 7.5 for 60-180 min. Unreacted N-acetoxy-AAF was extracted with isoamyl alcohol and the DNA was dialyzed against 10 mM Tris HCl pH 7.5, 0.25 mM EDTA. The number of adducts per molecule was determined from the ratio of radioactivity to OD_{260}.

(d) DNA polymerase reactions

The reaction mixture for incorporation assays with pol I contained 50 mM Tris HCl pH 8.0, 8 mM $MgCl_2$, 5 mM dithiothreitol, 50 µM each of the four deoxynucleoside triphosphates, 0.006-0.12 pmoles of template DNA and 0.19 or 0.74 units of DNA polymerase I. The TTP was [^{3}H]-labeled at a specific activity of 1 µCi/nmole. Incubation was at 37°C and incorporated radioactivity was measured by precipitation of the DNA onto GF/C glass fiber or Whatman 3 MM paper filters which were washed with 5% trichloroacetic acid/1% sodium pyrophosphate, ethanol, and then dried for liquid scintillation counting.

(e) Agarose gels

The DNA samples to be analyzed were loaded onto 10 cm, 1.4% agarose tube gels and electrophoresed for 2 hrs at 50 volts in 40 mM Tris HCl pH 7.8, 20 mM sodium acetate, 1 mM EDTA. Single stranded ØX174 DNA or RF DNA, with or without Hae II digestion, was either run on parallel gels or mixed with the radioactive samples before loading onto the gel to serve as markers. The gels were stained in 0.5 µg/ml ethidium bromide to locate the DNA and cut into 1.25 or 2.5 mm thick slices. To count [^{3}H]-labeled DNA, 1 ml of water was added to each slice and the agarose dissolved by heating to 100°C before liquid scintillation counting in Aquasol.

(f) Sequencing gels

The procedures for the polymerase reactions, preparation of samples and running of sequencing gels were based on the method of Sanger et al. (11) and have been described previously (8).

A theoretical curve for the amount of termination at successive guanines can be calculated from the probabilities, q, of terminating at any guanine, and p, of continuing past , where p = (1-q). Thus the proportion of newly synthesized chains terminating at the n^{th} guanine in a sequence is $p^{n-1}q$ and the cumulative amount of termination occurring up to quanine n is $1-p^n$. For a sequence containing N guanines, the proportion of chains terminating up to position n as a function of the total termination in the sequence is $\frac{1-p^n}{1-p^N}$.

Autoradiographs of sequence gels were scanned on a Gilford 250 spectrophotometer and the intensities of the bands determined by cutting out and weighing the peaks. The DNA template used for the sequence experiments contained 113 AAF adducts, that is, 0.09 adducts per guanine residue. Thus, if a single adduct were an absolute block to DNA synthesis and assuming that reaction of N-acetoxy-AAF with guanine is random with regard to position in the sequence, the probability of a synthesized strand terminating at any guanine, q, would be 0.09. The labeling procedure (refs 8 and 11) consists of a pulse of high specific activity [α-^{32}P]TTP followed by a chase of cold TTP and there are relatively few sites for incorporation of TTP interspersed between the guanines being examined. We therefore assume that most of the incorporation of [^{32}P]occurs in the region at the 5' end of the newly synthesized chains so that each chain is equally labeled and the amount of radioactivity is a fair measure of the number of chains.

RESULTS

The templates used for DNA synthesis in these experiments consist of single stranded ØX174 DNA molecules to which are annealed individual purified restriction fragments to act as unique primers. On such a template, pol I is capable of completely replicating the available region of single stranded DNA (12). With an untreated template, incorporation of nucleotides reaches a maximum level (Fig. 1A) which is proportional to the amount of template added (Fig. 1B & D). The product formed consists mainly of fully replicated molecules and migrates on agarose gels at the position expected for nicked double stranded circular DNA (Fig. 2). Incorporation on unprimed DNA was 5-10% of that on primed templates and analysis of the product on agarose gels indicated that all the synthesis was taking place on linear (nicked) molecules as a result of loop back priming of the free 3' end (data not shown). Treatment of the template DNA with N-acetoxy-AAF results in a reduction in the incorporation of nucleotides into the DNA by pol I (Fig. 1). Synthesis reaches a maximum level which is less than that for the untreated DNA, and the extent of incorporation is proportional to the amount of template added, indicating that a smaller region of the template DNA is available for synthesis. Similar results are obtained by synthesis by pol III holoenzyme (Fig. 1C).

The extent of DNA synthesis has been measured on a number of templates constructed from DNA containing various numbers of adducts and primed with different restriction fragments. In each case the amount of synthesis was compared to that on an untreated template primed with the same restriction fragment. The amount of synthesis decreases as the number of AAF adducts in the single stranded region of the template is increased (Fig. 3). There is only one 3' hydroxyl primer on these templates, and if termination occurred at the site of an AAF adduct, synthesis could not be

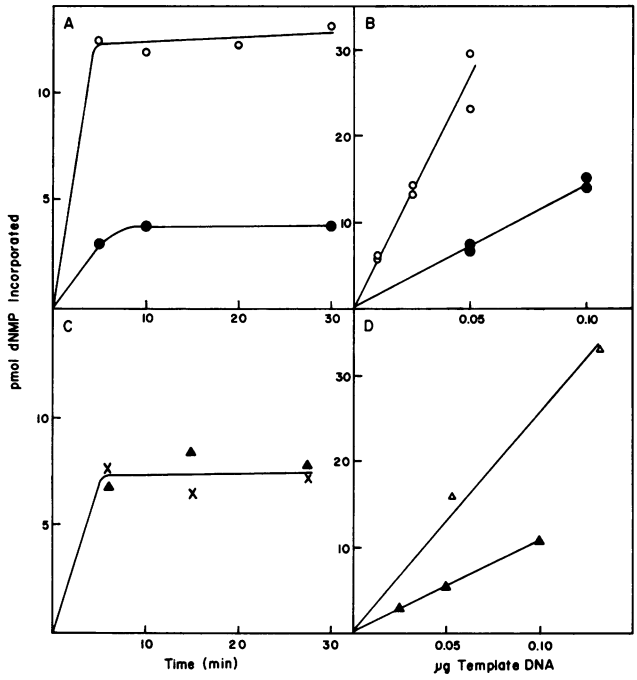


Figure 1. Extent of synthesis by Pol I on intact and AAF-reacted, primed ØX174 DNA templates. A, Time course of synthesis by pol I with 0.025 µg DNA template primed with Hae II fragment 4. o–o, unreacted template;●–●, template containing 5.5 AAF adducts per molecule. B, Dependence of incorporation by pol I on amount of template added. Incubation was for 30 min. Symbols as in A. C, Time course of synthesis with 0.05 µg DNA template primed with Hae II fragment 1 and containing 5.5 AAF adducts per molecule. ▲–▲, pol I; x--x, pol III holoenzyme. D, Dependence of incorporation by pol I on amount of template added. Templates were primed with Hae II fragment 1. Incubation was for 30 min. Δ–Δ, unreacted template; ▲–▲, template containing 5.5 AAF adducts per molecule.

reinitiated. This would result in an amount of residual synthesis, R, for a given number of adducts, N, given by $R = (N + 1)^{-1}$ as shown by the dashed line in Fig. 3. The calculated best fit to the data (solid line) indicates that while AAF adducts are acting as blocks to DNA synthesis, there is a divergence between the measured number of adducts in the template and the observed number of blocks to synthesis. As discussed later, this may in part be due to the effective length of DNA available for synthesis in the untreated templates being less than expected.

The products of synthesis by pol I were analyzed by agarose gel electrophoresis. The product synthesized on unreacted primed ØX174 DNA consists mainly of double stranded circular molecules (Fig. 2), and when this is digested with Hae II before

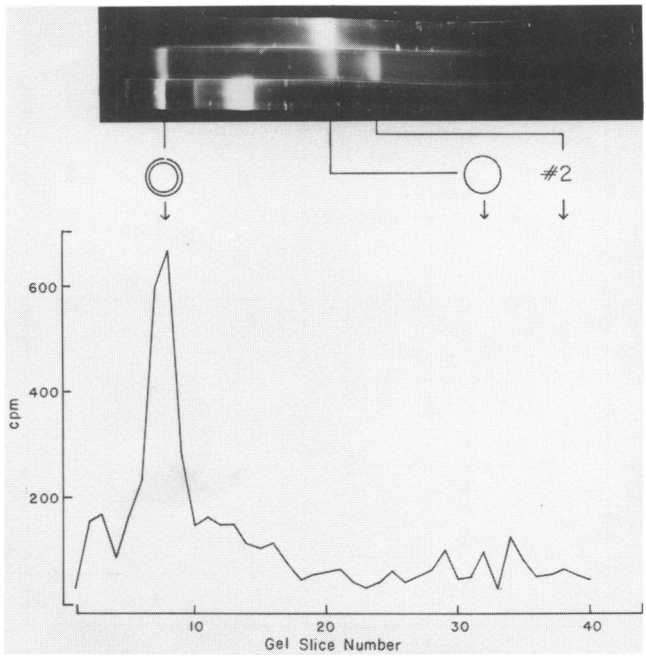


Figure 2. The product synthesized by pol I on ØX174 DNA primed with Hae II fragment 2, analyzed by electrophoresis on a 1.4% agarose gel. Top and bottom gels were run with unlabeled ØX174 DNA, single stranded or double stranded RF, respectively, as markers. The products of synthesis by pol I in the presence of [^{3}H]-TTP on unreacted ØX174 DNA primed with Hae II fragment 2 are shown on the center gel, which was sliced to determine the position of incorporated [^{3}H] (chart). The bands on this gel at the positions of single stranded DNA and of Hae II fragment 2 are unannealed components of the template.

electrophoresis (Fig. 4A), restriction fragments 1, 3, 4, 5, and 6 are all found in amounts approximately corresponding to the size of the fragments (fragments 7 and 8 are too small to be resolved on these gels). On the template containing 3.3 adducts (Fig. 4B) synthesis of fragments 1 and 3 is greatly reduced while fragments 4, 5 and 6 are present in almost normal amounts . From the product synthesized on the template with 15.6 adducts, only fragment 4 can be identified. Thus, as the number of AAF adducts on the template is increased there is a progressive loss of synthesized restriction fragments, with those furthest from the priming fragment being lost first. The small, and constant, amount of fragment 2 synthesized is presumed to be due to loop back self priming by some of the unannealed primer in the reaction mix.

When templates with sufficiently large numbers of adducts are used, the synthesized products are small enough to be characterized by sequence analysis. Using

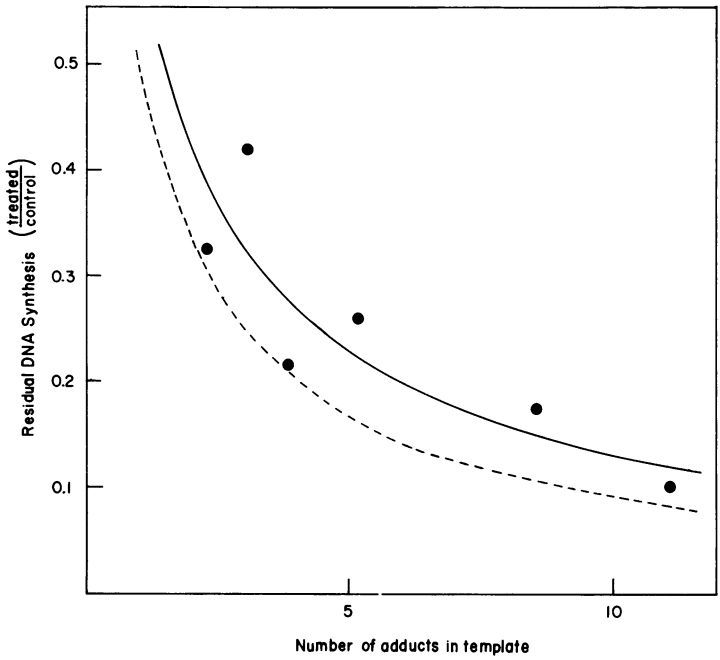
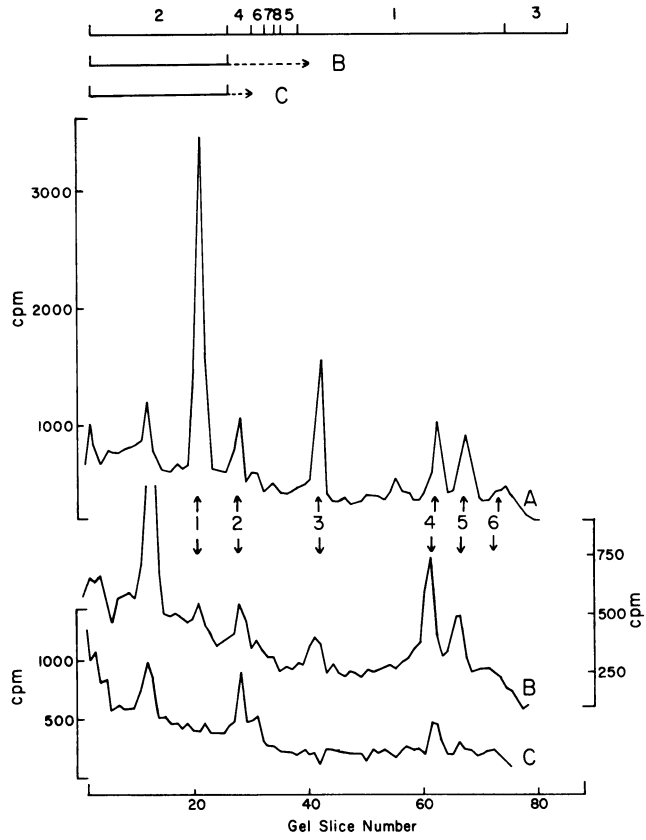


Figure 3. The reduction in synthesis by pol I as a function of the number of AAF adducts in the single stranded region of the template. The amount of residual synthesis was determined by comparing incorporation on paired untreated and AAF-reacted templates in experiments similar to those shown in Fig. 1.

these techniques, we have previously demonstrated (8) that the principal sites for termination by pol I occur one nucleotide before guanines in the template, guanine being the known site for addition of AAF. The autoradiograph in Fig. 5A shows the product of synthesis by pol I on a template containing 113 AAF adducts separated on a 20% polyacrylamide gel. Each band corresponds to the position of a guanine in the template. From a scan of the autoradiograph (Fig. 5B) the sizes of the peaks can be used to make an estimate of the relative frequency of termination at each guanine. The region scanned contains 20 guanines, and where there is difficulty in resolving individual bands, the peaks for several bands have been grouped together for quantitation. The cumulative frequency of termination has been plotted versus the position of the guanine in the sequence. The data shown in Fig. 5C is the mean, with standard deviations of five separate experiments. The dashed line in Fig. 5C is the relationship between termination and guanine position, calculated from the assumption that each AAF adduct is an absolute block to elongation by pol I (see Methods): less than 100% termination by



	Relative incorporation					
Fragment:	1	2	3	4	5	6
A	0.68	0.18	0.65	1.0	1.46	0.82
B	0.08	0.12	0.14	1.0	0.81	0.52
C	0.05	0.22	0	1.0	0.32	0.45

Figure 4. The products of synthesis by pol I on ØX174 DNA primed with Hae II fragment 2, analyzed on 1.4% agarose gels following Hae II digestion. The insert shows the Hae II restriction fragment map and the calculated average extent of synthesis from the primer at which the polymerase would encounter an AAF adduct. A, Unreacted template; B, Template containing 3.3 AAF adducts per molecule; C, Template containing 15.6 AAF adducts per molecule. Relative incorporation = cpm in fragment/size of fragment; normalized in relation to fragment 4.

the polymerase at any adduct would give results falling below this line. The closeness of the actual results to the prediction suggests that the fragments found on these sequence gels are indeed the result of irreversible termination of synthesis at the sites of AAF adducts.

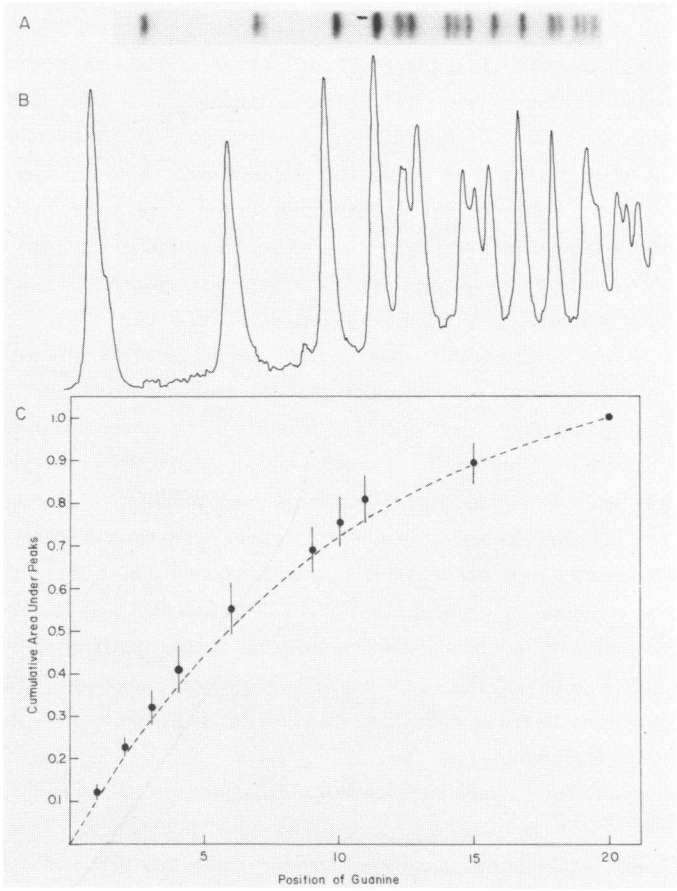


Figure 5. Quantitation of termination by pol I using sequence gels. A, Channel from 20% polyacrylamide sequence gel on which have been run the products of synthesis by pol I on a template containing 113 AAF adducts. Each fragment terminates one base before the position of a guanine in the template (ref 8). The smallest fragments are on the left, so that synthesis is proceeding from left to right, and the smallest and largest termination fragments in the section of the gel shown are 33 and 118 nucleotides long respectively. B, Densitometric scan of gel shown in A; C, Cumulative summation of peak sizes from scan through 20 successive guanines (total area under peaks set as 1.0). Data shows mean and standard error of five separate experiments.

DISCUSSION

In developing this in vitro system to measure termination of DNA synthesis at lesions in the DNA, we had two major objectives: to determine whether a particular lesion is a block to synthesis by DNA polymerases, and to be able to detect

circumstances in which synthesis might bypass such lesions. Previous reports (5,7,13) have concluded that UV-induced pyrimidine dimers and adducts of BPDE in DNA are blocks to synthesis by pol I and pol III of E. coli. However, none of these systems have been able to eliminate the problem of illegitimate priming; that is, if a DNA template contains multiple 3'-OH termini that can act as primers for DNA polymerase, synthesis may reinitiate downstream from a site of termination. In our experiments these problems have been overcome by constructing a template with a single purified restriction fragment as primer and by the use of gel filtration to separate the template DNA from contaminating oligonucleotides. This system therefore provides a method for quantitation of termination and/or bypass at lesions in the DNA.

N-acetoxy-AAF reacts with guanine in DNA, the major product being N-acetyl-N-(guan-8-yl)-2-aminofluorene, with a minor product reacting at the N^2 position (14). Other adducts may possibly occur; chemical loss of the N-acetyl group could result in formation of guanyl-2-aminofluorene (15) and reaction with adenine has been suggested (16). The base-displacement model of Grunberger and Weinstein (17) suggests that in native DNA the glycosyl linkage of 8-substituted deoxyguanosine is rotated so that the AAF residue is inserted into the DNA coplanar with adjacent bases, while the N^2 adduct does not appear to cause major distoration of the DNA helix (14). It is unclear what the structural configuration of guanyl-AAF would be at the junction between double stranded and single stranded DNA in a partially replicated molecule. AAF adducts in DNA have been shown to result in an absolute block for synthesis by RNA polymerase at or near the site of the adduct (16).

The results presented here indicate that DNA synthesis catalyzed by either pol I or pol III holoenzyme from E. coli is blocked by AAF adducts. The kinetics of incorporation on AAF-reacted templates, together with the proportionality between extent of incorporation and amount of added template, show that only a limited amount of the template may be copied before termination occurs. Analysis of the products of synthesis locates the sites for termination at the position of guanyl-AAF in the template (8).

We have used two methods to quantitate the amount of termination occurring. The first method, using the reduction in the amount of template available for synthesis (Fig. 3) shows a discrepancy between the number of AAF adducts and the number of termination sites. Comparing these results with the theoretical prediction appears to indicate the number of termination sites to be approximately 70% of the number of adducts. Since with both pol I and pol III there is a complete halt to incorporation on AAF-reacted templates that does not change with either prolonged incubation or use of higher concentrations of enzyme it is unlikely that termination is leaky. Another possibility is that only one of the guanyl-AAF adducts is a block to synthesis, for

instance, the major C-8 adduct that comprises about 80% of the total. Since our experiments cannot identify the nature of the adducts blocking synthesis, this possibility cannot be ruled out; however, for the reasons given below we think this unlikely.

Our second method of quantitation measures the amount of termination at successive guanines in a sequence by estimating the amount of radioactivity in the bands on a DNA sequencing gel by scanning the resulting autoradiograph (Fig. 5). These results are consistent with every AAF adduct being a site for termination of synthesis. If this is correct, the discrepancy in the incorporation experiments would indicate a non-random error in our residual synthesis determinations. One such error can be identified. Several of our preparations of ØX174 DNA contain significant numbers of linear (i.e., nicked) molecules. The presence of a single break will have little effect on incorporation on AAF-reacted templates where termination occurs after a limited amount of synthesis, but on unreacted templates complete replication would be impossible and the amount of incorporation would be less than expected. This would result in an overestimation of the amount of residual synthesis on the equivalent AAF-reacted template. Taking all the data, we therefore conclude that most, and probably all of the AAF adducts formed at guanines in the DNA act as complete blocks to DNA synthesis by pol I. The limited data we have suggests that the same is also true for pol III holoenzyme.

One disadvantage to the system is that many enzymes, such as T4 DNA polymerase and mammalian α polymerases, synthesize very poorly on templates containing large regions of single stranded DNA and thus provide problems in quantitating termination. On the other hand, for those enzymes for which we can detect termination of synthesis, use of this system should enable us to identify additional factors or conditions that may allow bypass of lesions, and begin to unravel the mechanisms that are involved in the process of error-prone repair.

ACKNOWLEDGEMENTS

We would like to thank the Carcinogenesis Program, NCI, for supplying [^{14}C]-labeled N-acetoxy-AAF. This research was supported in part by grants from the National Institutes of Health (GM 07816, CA 14599, CP 85669) and the Department of Energy (EV 765-02-2040). We would also like to thank Professor Tom Nagylaki for helpful discussions.

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