Abasic sites from cytosine as termination signals for DNA synthesis

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ABSTRACT

DNA with abasic sites has been prepared by deamination of cytosine followed by treatment of the product with uracil N-glycosylase. Termination in vitro on such templates does not occur until treatment with uracil N-glycosylase. DNA terminated one base before abasic sites created from C's has been used as a template in "second stage" reactions. With enzymes devoid or deficient in 3'>5' exonuclease activity purines, particularly adenine, are preferentially added opposite the putative abasic site. 2-Aminopurine behaves more like adenine than like guanine in these experiments. Polymerase β preferentially incorporates A opposite abasic sites produced from T, and G opposite abasic sites produced from C. We have eliminated an obvious artefact (e.g. strand switching) which might account for this observation.

INTRODUCTION

In previous work from this laboratory (1) it has been suggested that apurinic/apyrimidinic (AP, abasic) sites are blocks to DNA synthesis in vitro. These blocks need not be absolute since as we (1) and others (2,3) have reported, particular DNA polymerases are able to insert nucleotides opposite the AP position and even to continue synthesis past this site. We observed that Escherichia coli DNA polymerase I and other polymerases had a strong preference for the insertion of adenine opposite the AP site(4). This observation with an in vitro system is consistent with the observed specificity of mutation induced at AP sites (5) and by compounds whose mutagenicity might be mediated by abasic sites(6). Our conclusion that polymerases prefer A depends on the non-informational nature of the abasic site produced by the removal of T. However, it has been suggested (7) that through "a combination of van-der Waals interactions and stacking interactions" such sites might still retain some coding information. For this reason we have carried out an investigation in which an AP site is generated from a C, in order to determine the generality of our previous observation.

For this study we used the system previously described (1) in which

a (+) M13 viral strand is multiply insulted by the introduction of abasic (apurinic/apyrimidinic, AP) sites and is then used as a template for DNA synthesis. The net result of the experimental system is a gel similar to the sequencing gels obtained in Sanger's dideoxynucleotide technique (8) but with template lesions serving as chain terminators. In this investigation we show that polymerases retain their preference for A when an abasic site is generated from a C. However, there appears some specificity relating to the source of the abasic site when polymerase β is used. Finally, we are able to use the system to assay both 3'>5' exonuclease activity and polymerase action to illustrate the nucleotide specificity of the base analog, 2-aminopurine.

MATERIALS AND METHODS

Enzymes and chemicals: E.coli DNA polymerase Klenow fragment was obtained from Boehringer Mannheim, and is referred to as polI (Kf) throughout this paper. Bacteriophage T4 DNA polymerase was obtained from BRL. AMV reverse transcriptase was from Life Sciences. DNA polymerase α was prepared by Dr. K. Bose from the human lymphoma line Daudi (9). Polymerase β from Novikoff hepatoma, was purified by Mosbaugh and Meyer and obtained from Dr. T. Kunkel. Uracil N-glycosylase was prepared and assayed as described (1). Phage M13 and E. coli strains BW313 and HfrC71 were obtained and used as detailed (1). Deoxynucleoside triphosphates (HPLC grade) were purchased from Pharmacia PL-Biochemicals. Dr. Myron Goodman kindly provided the 2-aminopurine deoxyriboside triphosphate (2-APdTP) used in some of the experiments. Buffers were as used before (1), except that Hepes was substituted for Tris in all solutions. Growth of cells and preparation of phage was as described before (1). Preparation of DNA, restriction fragments, details of DNA polymerase reactions and first and second stage reactions were as previously detailed (1).

Abasic DNA from which cytosines were removed was prepared as follows: 1 ml M13 DNA (280 μ g/ml in 1xSSC) was mixed with 3 ml of 4M NaHSO₃, pH 6.0, as described by Shortle and Nathans (10), and the mixture was layered with mineral oil and incubated in the dark in the presence of 0.5mM hydroquinone, for 50 min at 37°C. Extensive dialysis against degassed buffers was as detailed (10), first at pH 6.8 and 0°C, and then at 37°C and pH 9.2 to hydrolyze the bisulfite adduct, followed by final dialysis at 4°C against 2 mM Tris-Cl pH 8.0, 2mM NaCl, 0.25mM EDTA. The resulting deaminated DNA was precipitated with ethanol and resuspended in 250 μ l of 20mM Hepes pH 8.0-1 mM EDTA. To obtain abasic DNA, this

preparation was treated with Uracil N-glycosylase as described before for DNA where T's were substituted with U's (1). The resulting abasic sites were stabilized by NaBH_4 reduction as previously described (1).

RESULTS

When deaminated DNA prepared by bisulfite reduction as described in Materials and Methods was used as a template for primer extension with either *Escherichia coli* DNA pol I (Kf) or T4 DNA polymerase (T4 pol) no blocks to synthesis were observed within 40 nucleotides of the primer and only slight termination was observed beyond that, indicating that the bisulfite treatment did not result in an inefficient template. Incubation of the bisulfite treated molecule with uracil N-glycosylase converts it into a template on which termination occurs. As in our previous experiments (1, 11, 12, 13 14), termination on the newly synthesized strand with T4 DNA polymerase occurs one nucleotide 3' to the site of the putative lesion on the template strand. With Pol I (Kf) termination occurs before and/of opposite the lesion depending on the site. In the portion of the gel shown in Fig. 1, termination synthesis catalyzed by T4 DNA polymerase occurs one nucleotide before C's in the template at positions 24, 28, 39, 46, 47, 50, 58, and 63. The difference in the catalytic behavior of DNA polymerases can be seen in the pol I (Kf) lanes. At position 25 a light band is seen opposite the putative abasic site. At positions 29 and 40 the main band with pol I occurs opposite the lesion. Termination occurs mainly before the lesion at positions 50, 58, and 63. The difference between *E. coli* pol I and T4 DNA polymerase has previously been interpreted as due to the different ratios of 3'→5' exonuclease/polymerase in the two enzymes (14).

We next used the template with abasic sites derived from C's for a second stage reaction (1, 12). DNA terminated just before lesions is isolated from a reaction mixture similar to that described above and is then used as a substrate for a second polymerase reaction in which individual dNTP's are added one at a time (Fig. 2). In this experiment we tested the ability of different polymerases to catalyze the addition of single dNTP's to a primer terminated before a C-derived abasic site. The overall results fit a pattern in which polymerases α and AMV reverse transcriptase (which lack nuclease activity) elongate by the preferential addition of purines, particularly A. Polymerase β is either inert or elongates slightly only with dGTP. With *E. coli* pol I (Kf) the

FIGURE 1: Termination pattern of polI(Kf) and T4 DNA polymerase on a template with abasic sites derived from C. Single stranded M13 DNA treated with bisulfite as described under Materials and Methods was incubated with uracil N-glycosylase before priming with Taq I fragment D. Extension of primer and pattern of termination by polI(Kf) - lane 1; by T4 polymerase - lane 2; lanes 3 and 4 are respective controls with deaminated, but not glycosylase treated, template. T,C,A,G - dideoxynucleotides in Sanger's sequencing lanes, corresponding to the newly synthesized complementary strand.

exonucleolytic activity dominates in this experiment and for both types of polymerase, those with and without nuclease activity, the behavior of the analog 2-amino purine mimics that of dATP. It is simplest to begin the detailed analysis by considering the behavior of polymerases without associated nucleases (lanes 3, 4 and 5; Fig. 2). Polymerase β (lane 3) stops at position 17 just before a template C at position 18. Elongation occurs with pol α and AMV reverse transcriptase with dATP, dGTP, and with 2-APdTP. At this position TTP also seems to produce elongation. A faint band indicating elongation is seen with dCTP and polymerase α . At position

FIGURE 2: Second-stage single nucleotide addition by DNA polymerases on an abasic DNA template produced by removal of C's.

Control T4 lane - products of first-stage by T4 DNA polymerase. Incubation with polI(Kf), polymerase α and AMV reverse transcriptase was as described (1). Polymerase β incubations (0.03 units/lane) were carried out for 30 min at 35°C at the presence of 70 mM NaCl. Nucleotide triphosphates were used in all reactions at 200 μ M. Polymerase reactions were terminated by heating at 65°C for 5 min, then digested with Taq I for one hour at 65°C. Numbering is from the 3'OH end of the primer (Taq I fragment E).

* * * * *

-T-T-C-A-T-A-T-C-G-G-C-C-T-T-A-T-C-C-A-C-A-T-A-G-T-G-G-C-5' (template)

20 25 30 35 40

lane 1 - pol I(Kf); lane 2 - polI(Kf) heated at 42°C for 20 min in 50 mM Hepes pH 8.0, then immediately chilled and added to the incubation mix; lane 3 - polymerase β ; lane 4 - polymerase α ; lane 5 - AMV reverse transcriptase. * - putative abasic sites.

22, polymerase α and AMV reverse transcriptase catalyze elongation with dATP. AMV reverse transcriptase also elongates with dGTP. At positions 26 and 27, polymerase α elongates with dATP and with dGTP; polymerase β elongates only with dGTP. At position 35 both polymerase α and AMV reverse

transcriptase elongate with dATP or with 2-APdTP. Elongation with dGTP is appreciable with AMV reverse transcriptase, less so with pol α and least (but detectable) with polymerase β . No change occurs with dCTP and there is slight elongation with TTP. As observed before (11), elongation is not uniform at all positions in the sequence.

The position of the bands with pol I (Kf) can be accounted for by supposing that instead of elongation, there has been exonucleolytic degradation until the first base complementary to the dNTP added is reached where the polymerase "idles". For example one sees bands with dATP at position 30, presumably the result of degradation from position 32 whereas with dGTP a heavy band appears at position 28. With dCTP, the band is at position 26. At this position 2-APdTP behaves more like dATP than like dGTP. We suppose that the difference between these results and those obtained earlier in this laboratory (e.g., 14) are due to a higher 3'>5' exonuclease/polymerase ratio in these preparations. Heating the polymerase preparation as suggested by Lecomte and Doubleday (15) to selectively inactivate the 3'>5' exonucleolytic activity, does not change the results (Fig. 2, lanes 2).

During the course of our work we modified the original protocol (12) so that the first stage, 32 P-labeled product was digested with the restriction endonuclease prior to the second stage polymerase incubation in order to make stopping the reaction and loading on the sequencing gel more convenient. Experiments on the specificity of incorporation opposite abasic sites made from either T or C using this revised protocol with pol I (Kf) gave results as expected from our previous experiments (Fig. 3). Purines but not pyrimidines were added opposite abasic sites. We added dAMP (lane b) or dGMP (lane c) to inhibit the 3'>5' pol I exonuclease (Fig. 3 lanes a; 11). As can be seen (Fig. 3, left) pol I (Kf) adds A opposite abasic sites derived from T at positions 31 and 34. G is added at position 31 only in the presence of dAMP. Similar results were obtained on a template with abasic sites derived from C's. Only purines promote elongation. At sites 27 and 38, A and G are about equally effective although we have not quantitated these gels as in our previous studies (1). Using the revised protocol, we observed that polymerase β inserted G opposite abasic sites produced from C (see Fig. 3 right, positions 23, 27 and 38) and A opposite sites generated by removal of T's (Fig. 3 left, positions 22, 25 and 31) as though it remembered the correct base. Only polymerase β displays this selectivity.

FIGURE 3: Incorporation of dNTP's opposite abasic sites formed from T's (left) or C's (right). Arrows indicate positions in the gel opposite putative abasic sites. The sequence shows the newly synthesized strand with dots indicating abasic sites from T and stars abasic sites from C. Substrates were prepared separately, primed with the same primer (Taq I fragment D), and incubated with polymerase at the same time. dA, dG, dideoxy sequence standards; T4, first stage product; a, incubated with polI(Kf); b, plus dAMP (5mM); c, plus dGMP; d, rat Novikoff hepatoma pol β . Position numbering in these gels is from the first A in the newly synthesized extension of the primer, so that for comparison with other gels in this paper C(20) will be C(22), A(30) is A(32) etc. [Reproduced from (12)]

Our modified protocol permitted an interpretation other than base recollection (M. Casadaban, personal communication). If in the process of isolating the first stage product, there were strand switching so that a newly synthesized strand, blocked before a lesion, switched to a template with a non reacted base at this position, then the normal complementary base would be added. Although this explanation does not account for the variations observed with different enzymes or for the observed site specificity (Fig. 3), we wanted to see whether strand switching could be demonstrated, especially since digestion with Taq I (used in these experiments) includes an incubation at 65°C that might have resulted in denaturation of the newly synthesized strand and a switching of template strands before the addition of polymerase. We therefore compared the patterns observed when first stage product was mixed with an excess of (+) strand and either boiled (Fig. 4 A) or not (Fig. 4 B). The template was prepared so that abasic sites were derived from C's. It is possible to adjust conditions so that strand switching occurs but we conclude that it

FIGURE 4: Single nucleotide addition opposite an abasic site: Second stage reaction with forced strand-switching
Abasic DNA formed by removal of C's, was primed with Taq I fragment D and used in first stage synthesis with T4 DNA polymerase. The 32 P labelled first stage products were either:
A (left part of gel) - boiled and reannealed with excess of untreated wild type single stranded M13 DNA in the presence of 0.1M KCl to facilitate strand switching, or,
B (right part of gel) - only mixed on ice with the DNA and the salt.
lane 1, T4 synthesis control of first stage; lanes 2-4, with $200\text{ }\mu\text{M}$ dATP; lanes 5-7, with $200\text{ }\mu\text{M}$ dGTP. Lanes 3 and 6 contain also 5mM dGMP. Pol I (Kf) was used in 2,3,5, and 6. Pol β , in lanes 4 and 7. ddT,C,A,G are dideoxy sequencing lanes.

is not a serious problem in our second stage experiments. The effect of forced strand switching can be seen in part A of Fig. 4. At positions 29, 40, 48, and 51 with both pol I and pol β there is an overwhelming elongation produced with dGTP. Only in the presence of dGMP is there appreciable elongation produced by pol I (Kf) with the addition of dATP. We suppose this to be the result of misincorporation due to the suppression

FIGURE 5: Single nucleotide addition opposite abasic sites in DNA by pol I (Kf) and pol β . (a) sites derived from T's. (b) sites derived from C's. Substrates used for first stage with T4 polymerase were primed with Taq I fragment C. The first stage products for each of the reactions appear in the T4 lane. For the pol I (Kf) reactions, 50 and 200 μ M were used for each of the nucleotides. For pol β , only 200 μ M was used. A, G, 2AP, C, T - deoxynucleoside triphosphates of adenine, guanine, 2-amino purine, cytosine and thymine.

The sequence appearing in this gel is:

-G-C-C-T-T-C-G-T-T-T-G-A-G-G-T-T-G-T-C-C-A-G-T-C-C-T-A-A-5' (template strand)

25 30 35 40 45

of editing exonuclease activity. The results are quite different when the mixture including excess unreacted (+) strand is not boiled. Under these conditions, and in the presence of 5 mM dGMP, the specificity of pol I (Kf) for A is clear. At position 28, elongation with A is predominant, at position 40 only A produces elongation and the same seems true at position 48. In this experiment pol β was inert under conditions where forced strand switching did not occur but this is not always the case (see Fig. 5).

2-Amino purine is widely used as an adenine analog. The structural similarity between these two purines can be demonstrated in two ways in our experiments. As has been pointed out recently (16) the 3'→5' exonuclease sites and the polymerization sites are separated on the *E. coli* pol I molecule. Structural relationships between the different purines can

therefore be observed at both the exonuclease and the polymerization levels in the same type of experiment (Fig. 5). As pointed out above, the preparations of pol I (Kf) recently used in our experiments appear to have more exonuclease activity than those used previously (compare Fig. 1 and Fig. 2). Incubation of an abasic template, prepared either by the removal of T's (Fig. 5 left) or by the removal of C's (Fig. 5 right), with a single dNTP and pol I (Kf) leads to the "simplification" of the gel pattern: fewer bands are visible compared to the T4 first stage product and these bands are more intense. As discussed earlier in regard to a different sequence (above, Fig. 2), the result of incubation of a single base with pol I (Kf) can be interpreted as a digestion in the 3'→5' direction to a position of idling opposite a base complementary to the nucleotide added. Examination of Fig. 5 shows that with both templates addition of the 2- amino purine nucleotide leads to an accumulation of radioactivity at sites at which an A would normally be added. There are differences from the result obtained with dATP. For example, both reactions with 2-AP and A lead to an accumulation at positions 40 and 45. However, there are stops with 2-AP at position 30 whereas with A, there is no stop visible at this position but rather opposite the T at position 27 (with 2-AP there are stops at this position as well). The pattern of bands with dGTP is almost identical on either template (Fig. 5) indicating that degradation is the major reaction.

Polymerase β behaves in this experiment (Fig. 5) as though it can distinguish abasic sites derived from C from those derived from T. This is the same result as seen in Fig. 3 but with a protocol which reduces the possibility of a strand exchange. The protocol for Fig. 3 involved heat treating (65°C, 60 min.) a Taq I reaction mixture to create a primer of 20 to 40 nucleotides. In the revised protocol, the primer is the size of the restriction fragment (600 to 800 nucleotides) and the mixture is kept on ice until incubation with the polymerase at 35°C. Since polymerase action is terminated before treatment of the second stage reaction product with restriction enzyme, any "strand switching" (Fig. 4) should be minimized. Both dATP and 2-APdTP permit strand elongation by polymerase β on templates with T-derived abasic sites (Fig. 5 left). Elongation at some, but not all sites is seen only with dGTP on sites produced by the removal of C's (Fig. 5 right).

DISCUSSION

Our results confirm the report that polymerases add purines opposite abasic sites with a preference for the addition of A (1) and this

preference holds even when the abasic site originates from a C. Four aspects of this work require additional comment: a) the role of the 3'→5' exonuclease in determining the result of termination experiments; b) the possible role of "strand switching"; c) the illustration of the specificity of 2-amino purine; and d) the curious behavior of polymerase β preparations.

The interaction between polymerization and 3'→5' exonuclease activity in proofreading by pol I has been studied in a number of laboratories (e.g. 17). We have already commented (1, 11, 13, 14) on the role of this interaction in determining the site of termination of DNA synthesis. Our view (14) is that termination makes editing possible. Support for this view is the observation that polymerases without exonuclease still terminate and that inhibition of exonuclease activity with nucleoside monophosphates may alter the ratio of termination at or before a lesion but does not itself permit bypass. Relevant to this point is a seeming inconsistency in the results presented herein. In Fig. 1, we show that pol I (Kf), at least at certain sites, is able in a first stage reaction to permit elongation to directly opposite the site of an abasic site. In contrast, (Fig. 2, 4, 5) in a second stage reaction, the preparations of polymerase we are now using result only in exonucleolytic degradation. It is not only the presence of all four dNTP's that permits elongation since in other recent experiments (data not shown), the presence of all four dNTP's prevented degradation in a second stage reaction but did not lead to elongation. The difference between our current and past enzyme preparations is seen by the almost complete degradation seen in these experiments on incubation with water (data not shown) as compared to the relative stability on incubation seen in our previous studies (1). We suppose that there is a difference in the action of polymerase when it is moving processively on a template as in a stage one reaction as compared to the distributive mode of action in stage two reactions (18). This presumed difference during processive synthesis fits the model of polymerase structure proposed by Ollis et al. (16) in which the enzyme molecule after combination with its substrate folds around the double helix. These workers (16) also point out the spatial separation between exonuclease and polymerase binding sites with the mononucleotides binding only at the exonuclease site.

The limited synthesis obtained with pol β to opposite lesions (see Fig. 2) is different from what might be expected based on the results of

Kunkel et al. (19). These workers concluded that both pol α and β had significant ability to bypass abasic sites in DNA and that pol β produced a higher amber reversion frequency than did α . The results in the present paper are similar to those reported by Rabkin and Strauss (11) who observed a limited ability of polymerase β to add nucleotides opposite an acetyl aminofluorene adduct using a protocol identical to that used in these experiments. All three investigations used polymerase β from the same source. The most obvious difference is in the protocols, since the Kunkel et al (19) experiment involves copying relatively long stretches of template whereas our "second stage" protocol involves only the addition of single nucleotides to a primer. This distinction should make an important difference for processive enzymes since as shown by Grosse et al. (18), the incorporation of the first nucleotide following a 3' end obeys more stringent specificity rules than does the addition of nucleotides further along. However, pol β is reported to be "entirely nonprocessive" (20).

We started this investigation in an attempt to demonstrate that the specificity observed with abasic sites derived ultimately from T's did not depend on that derivation. The observed preference for purines and particularly A's has been confirmed, both for polymerase α and AMV reverse transcriptase (Fig. 2) and for E. coli pol I (Kf) in the presence of dNMP (Fig. 4). Some additional comment needs to be made on what is perhaps the most surprising aspect of these experiments, the preferential incorporation by polymerase β of guanine opposite certain sites derived from cytosine and of adenine opposite sites derived from thymine. We have considered one obvious artefact, a strand switching which could make an unreacted site available as a template. Such switching can be forced (Fig. 4) but does not seem likely in the absence of heating even in the presence of an artificially large amount of unreacted substrate. The explanation must, at least in part, depend on the polymerase used because the phenomenon is not observed with polymerase α (Fig. 2) or with E. coli pol I (Kf) in the presence of nucleotide monophosphates (Fig. 4). Any explanation must also take into account the protocol of a "second state" reaction which requires separation of enzyme and DNA at a point at which synthesis has stopped just prior to the lesion and then mixing fresh enzyme and DNA in a new reaction mixture. We assume that the addition of this first nucleotide must have the characteristics of a distributive reaction regardless of the enzyme. It has been suggested that either residual strand switching or a "slippage" of the newly synthesized strand at sites where the 3' nucleotide in the

template is identical to the base removed (as in Fig. 5) could account for what appears to be a "memory" of the missing base at that position. This hypothesis avoids speculating on enzyme behavior for which there seems to be no obvious physical explanation. Although stacking interactions are considered to play as large a role in the stabilization of the double helix as hydrogen bonding (21), examination of the base preceding the insertion at the positions we studied indicates that the base selection (e.g. G over A opposite former C's) could not be predicted on the basis of the most favored base stacking interaction. Notwithstanding the attractiveness of the idea of residual strand switching or slippage, we still need to account for the different behavior of the polymerases on these templates. Attempts to prepare templates with abasic sites derived from purines by heat and acid treatments in order to further study the phenomenon have not so far been successful due to the breaks which accompany the heating reaction.

A variety of factors affect the site of termination. Since termination as it appears in this technique is an equilibrium process, these factors include the nature of the polymerase, the substrate and the sequence surrounding the site at which events occur. These experiments also illustrate the biochemical relationship between 2-amino purine and adenine. 2-Amino purine is recognized as adenine at the exonucleolytic site and incorporated like adenine at the polymerization site although with some differences in detail, as might be expected from the complexity of factors governing its incorporation in different sites (22).

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