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Alternative Explanation for Excision Repair Deficiency Caused by the *polA*ex1 Mutation

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An investigation of the mechanism of the *polA*ex1 mutation in vitro suggested that the excision repair deficiency observed in vivo does not result from an inability of the enzyme to nick translate. The defect appears to reside in the inability of the enzyme to effectively generate a nick structure to serve as a substrate for DNA ligase.

Traditional models of excision repair in *Escherichia coli* have suggested that an incision step introduces a nick into the DNA 5′ to a thymidine dimer. DNA polymerase I binds at this site and synthesizes from the 3′-hydroxyl terminus, while simultaneously degrading with 5′ → 3′ exonuclease activity (nick translation) through the dimer, removing it, and then dissociating before ligation (1). Therefore, it was not surprising that *E. coli* containing the *polA*ex1 mutation, a temperature-sensitive defect in 5′ → 3′ exonuclease activity, was found to be excision repair deficient (3).

However, some recent work of Sancar and Rupp (7a) has demonstrated that the *E. coli* incision enzyme complex (*uvrABC*) makes two cleavages, one at the eighth phosphodiester 5′ to the pyrimidine dimer and one at the fourth or fifth phosphodiester 3′ to the dimer. The resultant short DNA segment containing the dimer is incompletely base paired and is easily dissociable from the larger DNA, leaving a 12- to 13-nucleotide gap. Therefore, nick translation should not be necessary for repair; the polymerase need only fill in this short gap to form a ligatable substrate. Why then does a defect in the 5′ → 3′ exonuclease activity of DNA polymerase I impair excision repair? One explanation is that the polymerase section of the molecule alone is not sufficient for gap closure and the presence of the 5′ → 3′ exonuclease in its native, active conformation is somehow required to generate a nick that can be a substrate for DNA ligase. To test this hypothesis, we constructed a tritiated ColE1 plasmid DNA template containing an average of 0.6 gap 20 nucleotides long per DNA molecule to examine the repair process. The number of 3′-hydroxyl termini and the amount of single-strand template per 3′-hydroxyl terminus were determined by the method of Hockensmith and Bambara (2). The plasmid was first nicked with pancreatic DNase (1 ng/300 nmol of DNA) for 8 min at 23°C, and the number of available 3′ termini was determined by treating the preparation with excess *E. coli* DNA polymerase I, 10 μM dCTP, 10 μM dATP, and 10 μM [^{3}H] dTTP (77 Ci/mmol) to add an average of three nucleotides per 3′ end. These nicks were then widened to gaps by the action of *E. coli* exonuclease III (a gift from L. Loeb), which sequentially degrades from the 3′ end. The size of these gaps was determined by incorporating radiolabeled nucleotides with T4 DNA polymerase, which lacks a 5′ → 3′ exonuclease and is able to fill gaps exactly to ligatable ends without causing strand displacement (5). The gap length which we calculated varied from 18 to 22 nucleotides depending on the method of determination, and we used an average value of 20 nucleotides. We tested the abilities of purified *E. coli* DNA polymerase I, mutant *polA*ex1 DNA polymerase I, and the DNA polymerase I large fragment (in which the 5′ → 3′ exonuclease was proteolytically removed) to function with *E. coli* DNA ligase to convert the DNA template to a closed circular duplex. Conversion was detected by subsequent sedimentation on alkaline sucrose density gradients to determine the fraction of DNA converted to a closed circular form.

Figure 1 shows the results obtained with reaction mixtures containing DNA polymerase and DNA ligase with the gap-containing DNA template. The relative areas under the closed circular DNA and open circular DNA peaks could be used to assess the number of breaks in the molecules (6). The polymerases were all adjusted to equivalent activities at both 30 and 43°C on a ColE1 DNA template with 200 nucleotide gaps

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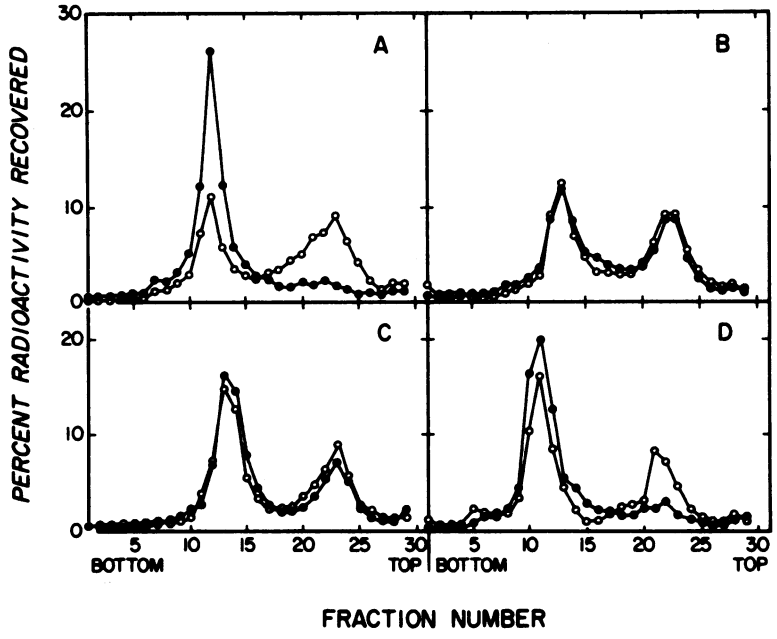

FIG. 1. Alkaline sucrose sedimentation of 20-nucleotide, gap-containing, ^{3}H-labeled ColE1 DNA that was not treated (○) or was treated with DNA polymerase I and DNA ligase (●) at 30°C (A); treated with DNA polymerase I large fragment and DNA ligase at 30°C (●) or 43°C (○) (B); treated with *polA*ex1 polymerase and DNA ligase at 30°C (●) and 43°C (○) (C); and treated with *polA*ex1 DNA polymerase at 43°C (●) or DNA polymerase large fragment at 43°C (○), each followed by heat inactivation and subsequent incubation with T4 polymerase and DNA ligase (D). Each standard reaction mixture (0.045 ml) contained 50 mM Tris-hydrochloride (pH 8.0), 8 mM $MgCl_2$, deoxynucleoside triphosphates (30 μM each), 25 μM NAD, 5 mM β-mercaptoethanol, 150 mM NH_4Cl, 15 μM ^{3}H-labeled ColE1 DNA, and 0.15 μg of DNA ligase (a gift from I. R. Lehman). Each form of DNA polymerase I used was present at a concentration of 10 U/reaction. DNA polymerase I was purchased from New England Biolabs, Inc. The DNA polymerase I large fragment (Klenow fragment) was purchased from New England Nuclear Corp. *polA*ex1 mutant polymerase was prepared by the method of Uyemura et al. (8). T4 DNA polymerase (Miles Laboratories, Inc.) was present at a concentration of 0.4 U/reaction. The reaction time was 30 min, and reactions were terminated by centrifugation through Sephadex G-50 (Pharmacia) to remove unreacted deoxyribonucleoside triphosphates (7).

per molecule, so that polymerase activity differences could not affect the results. Figure 1A shows the results with untreated DNA and DNA reacted with DNA polymerase I in the presence of DNA ligase. DNA reacted with DNA polymerase I in the absence of DNA ligase resulted in a sucrose gradient sedimentation profile that waas indistinguishable from the profile of untreated DNA. When both enzymes were present at 30°C, 85% of the original gaps were repaired. Similar results were obtained at 43°C (data not shown). The polymerase large fragment and the *polA*ex1 polymerase were also tested with DNA ligase for the ability to repair at 30 and 43°C (Fig. 1B and C). The mutant polymerase was less efficient than the wild type at generating a ligatable substrate, repairing 34% of the original gaps at 30°C. When the temperature was increased to 43°C, only 22% repair was observed. The proteolyzed polymerase I large fragment was ineffective at either temperature, repairing only 21 and 22% of the gaps at 30 and 43°C, respectively. With respect to the polymerase large fragment our results are consistent with those of Lundquist and Olivera (4), who showed that this polymerase fragment displaces nucleotides at the 5′ terminus of a nick to produce single-stranded tails that resist ligation.

There are two reasons why the ligase substrate may not be generated by the *polA*ex1 polymerase: (i) the defective polymerase cannot close the gap down to a nick; and (ii) the defective polymerase immediately begins to displace the strand ahead of it once the gap has been closed, providing no ligatable intermediate. If the first alternative were predominant, subsequent synthesis at a 3′ terminus would generate a DNA ligase substrate.

We designed a series of experiments to test for these mechanisms. Under identical conditions T4 DNA polymerase, wild-type DNA polymerase I, *polA*ex1 mutant DNA polymerase I, and the DNA polymerase I large fragment were allowed to synthesize for 30 min at 43°C in the

absence of DNA ligase on the 20-nucleotide gap-containing template by using [α-^{32}P]dATP (New England Nuclear Corp.) and three unlabeled deoxyribonucleoside triphosphates. Nucleotide incorporation was determined from the ^{32}P incorporation corrected for counting efficiency. Maximum synthesis by T4 DNA polymerase yielded 21 nucleotides per 3′ terminus, DNA polymerase I added an average of 67 nucleotides per 3′ terminus, the polymerase large fragment added an average of 55 nucleotides per 3′ terminues, and *polA*ex1 polymerase added an average of 36 nucleotides per 3′ terminus. Therefore, each form of DNA polymerase I added more than enough nucleotides to completely fill the gap. The addition of DNA ligase to the reaction mixtures reduced the average number of nucleotides added by the wild-type polymerase, the *polA*ex1 mutant polymerase, and the polymerase large fragment by 30, 1, and 4%, respectively. However, synthesis remained well in excess of gap length.

To detect the products evolved as a result of 5′ → 3′ exonuclease activity, the polymerase assay was repeated, this time in the presence of unlabeled deoxynucleoside triphosphates. The reactions were terminated, and the product sizes were determined by column chromatography on Bio-Gel A5m (Bio-Rad Laboratories). The elution profiles of the template reacted with the wild-type polymerase showed a peak of radioactivity corresponding to the single nucleotides evolved as a result of 5′ → 3′ exonuclease activity (nick translation). The relative size of the single nucleotide peak represented 0.3% of the total DNA. The elution profiles of the template reacted with either the polymerase large fragment or the *polA*ex1 mutant polymerase failed to show any products less than 100 nucleotides long (i.e., smaller than the exclusion limit of the column [data not shown]), implying that polymerization in excess of gap length by either of these enzymes occurs solely by strand displacement.

To examine the extent of strand displacement, the 5′ termini of the gap-containing templates were labeled with ^{32}P. Each enzyme was allowed to polymerize on this template, and the reactions were terminated by heating the mixtures to 70°C for 5 min. A sample of each reaction preparation was then treated with S1 nuclease (Sigma Chemical Co.) to degrade the single-stranded 5′ ends displaced by polymerization. Under these conditions, the maximum release of acid-soluble ^{32}P at 30 or 43°C occurred after reaction with wild-type polymerase I followed by S1 nuclease; we defined this release as the 100% value for this experiment. At both 30 and 43°C, the amount of ^{32}P released after reaction with the *polA*ex1 polymerase or the polymerase large fragment without S1 nuclease was not significantly different from the amount released by S1 nuclease alone (considered the 0% value for this experiment). However, after reaction with the polymerase large fragment at 43°C, subsequent treatment with S1 nuclease released 82% of the ^{32}P. At 30 and 43°C reaction with the *polA*Ex1 polymerase followed by S1 nuclease released 53 and 44% of the ^{32}P, respectively. These results indicate that somewhat less than one-half of the 5′ ends were strand displaced by the action of the *polA*ex1 polymerase at the restrictive temperature.

To detect the presence of residual gap structures after polymerization, the polymerase assay was repeated at 43°C in the absence of DNA ligase. After 30 min, the reactions were terminated by heating the mixtures for 10 min at 70°C. Bacteriophage T4 DNA polymerase and DNA ligase were then added, and the reaction was continued for 30 min at 37°C. When the wild-type polymerase I reaction was terminated and reconstituted with T4 DNA polymerase and DNA ligase, 76% of the original gaps were repaired (data not shown). When the polymerase I large fragment was reacted, inactivated, and reconstituted with T4 DNA polymerase and DNA ligase, only 15% of the original gaps were repaired (Fig. 1D). In contrast, when the *polA*ex1 polymerase was used in this manner, 62% of the original gaps were repaired (Fig. 1D). This result indicates that in most gaps synthesis by the *polA*ex1 polymerase leaves a residual gap structure which is not a substrate for ligation.

To determine the sizes of these residual gaps, the *polA*ex1 DNA polymerase was reacted with the template and four deoxynucleoside triphosphates for 30 min at 30 and 43°C. The reaction mixtures were heat inactivated and then centrifuged through Sephadex G-50 (Pharmacia Fine Chemicals, Inc.) to remove unincorporated nucleotides. The reaction was reconstituted with T4 DNA polymerase and α-^{32}P-labeled deoxynucleoside triphosphates and further reacted for 30 min at 37°C. Approximately one additional nucleotide was added by the T4 polymerase after initial incorporation by the *polA*ex1 polymerase at either temperature.

In conclusion, the *polA*ex1 DNA polymerase is expected to function poorly in excision repair at elevated temperatures based on the properties of the pure enzyme in vitro. We examined the process of gap filling by this enzyme and found that the 5′ → 3′ exonuclease dysfunction prevents the formation of a ligatable substrate after a gap is filled. At restrictive temperatures only nominal repair can be obtained with *polA*ex1 polymerase and DNA ligase, although an amount of nucleotides well in excess of gap size has been added. In direct contrast to results

obtained with the polymerase I large fragment, heat inactivation of *polA*ex1 reactions followed by treatment with T4 polymerase and DNA ligase did result in significant gap repair. An analysis of products on sizing columns showed no mono- or oligonucleotide products evolved from the *polA*ex1 polymerase reaction. Strand displacement of 5′ termini was observed on a fraction of the gaps. We conclude that with the *polA*ex1 polymerase, synthesis on a large population of 3′ termini stops short of the 5′ terminus, leaving a residual template that can be extended by T4 polymerase and DNA ligase for complete repair. This limited synthesis is masked by a smaller population of 3′ ends which have sustained significant synthesis via strand displacement, resulting in a total incorporation per gap that is much greater than the gap size.

Our results suggest that at restrictive temperatures in vivo the *polA*ex1 DNA polymerase does not efficiently close most gaps to nicks and that, with those which are closed, begins to strand displace before ligation can occur.

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