

Site-Specific Pausing of Deoxyribonucleic Acid Synthesis Catalyzed by Four Forms of *Escherichia coli* DNA Polymerase III[†]

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ABSTRACT: Sites on an fd DNA template which terminate synthesis catalyzed by each of four forms of *Escherichia coli* DNA polymerase III have been identified at single nucleotide resolution. Results were obtained by comparing the products made by forms of DNA polymerase III with products generated from the same 3'-terminus by using the dideoxynucleotide sequencing method, on high-resolution polyacrylamide gel electrophoresis. Each form of DNA polymerase III generates products of distinct lengths ending at a limited number of preferred sites of synthesis termination. The addition of auxiliary subunits to the DNA polymerase III core form of the enzyme has a distinct functional effect on primer

elongation and specificity of polymerase pausing. Most sites (65%) can be correlated to positions of potential secondary structure in the template arising via local hydrogen-bonding interactions. The proximity of polymerase pausing to sites adjacent to hairpin stems was related to the size of the enzyme since the smaller core form of DNA polymerase III generally paused at sites which were closer to the base of these structures than the larger holoenzyme. The occurrence of termination sites is markedly affected by the inclusion of spermidine or *Escherichia coli* single-stranded DNA binding protein in the reaction mixtures. Additionally, a nucleotide composition specificity of pause sites has been observed.

Termination of DNA synthesis in vitro has been the subject of a number of previous studies. Work on the mechanism of elongation catalyzed by *Escherichia coli* DNA polymerase II demonstrated that the progress of this enzyme is impeded at specific sites of secondary structure in the DNA template (Sherman & Geftter, 1976). However, when the template was complexed with *E. coli* single-stranded DNA binding protein (SSB),¹ the barriers to synthesis were eliminated and the enzyme remained associated with the same primer-template molecule for extensive intervals of synthesis. Similar barriers to extensive synthesis have been observed with the DNA polymerase isolated from vaccinia virus (Challberg & Englund, 1979), bacteriophage T4 (Huang et al., 1981; Roth et al., 1982), and DNA polymerase α from *Drosophila melanogaster* (Villani et al., 1981). In these experiments, the addition of the T4 helix-destabilizing protein (gene 32 protein) or *E. coli* SSB removed many of the barriers to extensive synthesis catalyzed by the T4 polymerase and DNA polymerase α holoenzyme, respectively. DNA polymerase α pause sites have also been mapped in template regions which lack the potential for formation of stable hairpin structures (Kaguni & Clayton, 1982; Weaver & DePamphilis, 1982). Analysis of these sites at the resolution of a single nucleotide revealed that pausing occurs at specific DNA sites. These results indicate that the pausing observed with DNA polymerases may be attributed to a combination of the effects of template secondary structure and sequence.

The DNA polymerase III holoenzyme is a complex multisubunit enzyme that is responsible for most of the replicative synthesis in *Escherichia coli* [for a review, see McHenry & Kornberg, (1981)]. Minimally, the holoenzyme contains seven subunits: α , ϵ , θ , β , γ , δ , and τ (McHenry & Kornberg, 1977; McHenry & Crow, 1979; McHenry, 1982). DNA polymerase III (α , ϵ , and θ), the catalytic core of holoenzyme, is capable of limited synthesis primarily in short gaps created by nuclease treatment of duplex DNA. This core DNA polymerase III is inactive on natural chromosomes such as G4 DNA (McHenry & Kornberg, 1977; McHenry & Crow, 1979). Two additional DNA polymerase III forms have been isolated: DNA polymerase III' (α , ϵ , θ , and τ) and DNA polymerase III* (α , ϵ , θ , τ , γ , and δ). These forms display functional properties intermediate between DNA polymerase III and the holoenzyme (Fay et al., 1981, 1982; McHenry, 1982).

The four catalytically active forms of *E. coli* DNA polymerase III have demonstrated marked differences in the rate of DNA synthesis, the extents of processive DNA synthesis, and the lengths of the template utilized for synthesis for each enzyme form. When tested on a randomly primed fd DNA template, DNA polymerase III holoenzyme synthesizes a product many times larger than that made by the DNA polymerase III core enzyme (Fay et al., 1981). When products were measured at enzyme excess, such that enzymes would repeatedly interact with each 3'-terminus, the disparity in product sizes suggested that the progression of DNA polymerase III may be blocked by sequences or structures in the template which are less effective at blocking the holoenzyme. The ability to pass these sites must result from the association of the holoenzyme auxiliary proteins with the core enzyme. Similar analyses with the other two holoenzyme subassemblies, DNA polymerase III' and DNA polymerase III*, indicated

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¹ Abbreviations: dNTP, deoxynucleoside triphosphate; ddNTP, di-deoxynucleoside triphosphate; RF, replicative form; SSB, *Escherichia coli* single-stranded DNA binding protein; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; bp, base pair(s); BSA, bovine serum albumin; AMV, avian myeloblastosis virus; holoenzyme, DNA polymerase III holoenzyme; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

that the association of only some of the full complement of auxiliary proteins significantly changes the sizes of products synthesized by these forms compared to products of the core enzyme form (Fay et al., 1982). Additionally, the activities of each of these polymerases are markedly affected by spermidine and single-stranded DNA binding protein, factors that influence the DNA template structure.

The present study was undertaken to (a) identify structures or sequences in the template which promote termination of synthesis, (b) to determine if changes in template secondary structure mediated by spermidine or SSB would modify the progression of each enzyme form, and (c) to determine whether the presence of particular subunits associated with the DNA polymerase III core alters the pattern of pause sites.

Materials and Methods

Proteins and Enzymes. *E. coli* DNA polymerase III, DNA polymerase III', DNA polymerase III*, and DNA polymerase III holoenzyme were prepared by the methods of McHenry & Crow (1979), McHenry (1982), Fay et al. (1982), and McHenry & Kornberg (1977), respectively. AMV reverse transcriptase was obtained through the Office of Program Resources and Logistics, Viral Cancer Program, National Cancer Institute, from Dr. Joseph Beard. *E. coli* single-stranded DNA binding protein (SSB) was prepared by the method of Chase et al. (1980). Restriction endonuclease *Sau*3A1 was purchased from New England Biolabs. *E. coli* DNA polymerase I large fragment was purchased from New England Nuclear Corp. Spermidine trihydrochloride was purchased from Sigma Corp.

Nucleotides and Polynucleotides. Unlabeled dNTPs were purchased from ICN. The dideoxynucleoside triphosphates (ddNTPs) were purchased from P-L Biochemicals. [^3H]dTTP (78.7 Ci/mmol) and [α - ^{32}P]dATP (3200 and 800 Ci/mmol) were purchased from New England Nuclear Corp. Poly(dA) ($S_{20,w}$ = 6.8 S) and oligo(dT)₁₀ were purchased from Miles Laboratories and Sigma Corp., respectively. Poly(dA)-oligo(dT)₁₀ 20:1 complexes (containing adenine and thymine in a molar ratio of 20:1) were prepared by annealing poly(dA) and oligo(dT)₁₀ at 37 °C for 5 min in 0.1 mM EDTA and 10 mM Tris-HCl (pH 7.5).

Preparation of Specifically Primed fd Templates. Preparation of single-stranded fd bacteriophage DNA and the duplex replicative form (RF) DNA were previously described (Matson et al., 1980). *Sau*3A1 restriction fragments of fd RF DNA were prepared in a reaction (1 mL) containing 50 mM NaCl, 6 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 100 μg of BSA, 250–500 μg of fd RF DNA, and *Sau*3A1 (50–100 units).² Reactions were incubated at 37 °C for at least 3 h. The reactions were terminated by the addition of EDTA (pH 7.5) and SDS to 10 mM and 0.1%, respectively, and heated to 65 °C for 5 min. The resulting four *Sau*3A1-generated restriction fragments (*Sau*3A1-A, 3425 bp; *Sau*3A1-B, 2144 bp; *Sau*3A1-C, 507 bp; and *Sau*3A1-D, 332 bp) were separated by electrophoresis through a 1.0% low melting temperature agarose gel (12.5 \times 25 \times 0.3 cm). Electrophoresis was conducted at constant voltage (2.5 V/cm) for 8–10 h in a Tris-acetate (40 mM Tris base and 20 mM acetic acid, pH 8.0) buffer system at 4 °C. Following electrophoresis, the gel was stained for 15 min in gel buffer containing ethidium bromide (1 $\mu\text{g}/\text{mL}$) and visualized under long-wave ultraviolet illumination. Individual DNA fragments were cut from the gel

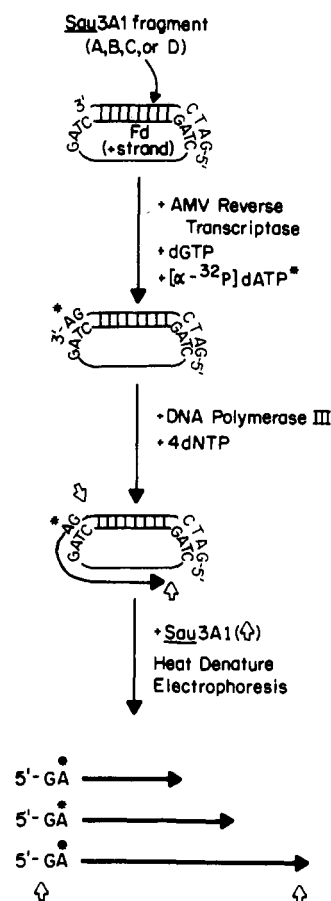


FIGURE 1: Procedure used to determine nucleotide position and frequency of pausing by DNA polymerase III enzyme forms at specific sites on *Sau*3A1 restriction fragment primed fd DNA templates. ^{32}P -Labeled nascent DNA products are digested with *Sau*3A1 at regenerated recognition sites (5'-↓GATC-3', some of which are designated by the open arrow), heat denatured, and fractionated by electrophoresis through an 8% polyacrylamide gel. Locations of pause sites are determined by comparing the length of 5'- ^{32}P -labeled nascent DNA chains with the DNA sequence of the primer-template. Since each nascent DNA chain contains the same amount of radioactivity, regardless of its length, the frequency of enzyme pausing at specific template sites is proportional to the intensity of [^{32}P]DNA bands observed on polyacrylamide gels.

and purified from the gel matrix by the method of Smith (1980).

Each of the purified *Sau*3A1 restriction fragments was separately used to specifically prime single-stranded fd phage DNA. Individual *Sau*3A1 DNA fragments were denatured by heating at 100 °C for 3 min followed by rapid cooling in an ice-water bath. Single-stranded fd DNA was added until the restriction fragments were in a 3–6-fold molar (polymer molecule) excess. The annealing reaction was adjusted to 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1 mM EDTA and incubated at 65 °C for 4–6 h followed by slow cooling. Primer-templates constructed in this way were concentrated by precipitation of the DNA with 2 volumes of ethanol.

Primer-templates were specifically labeled at the 3'-OH terminus of the *Sau*3A1 restriction site (5'-↓GATC-3') of each primer fragment with one residue of [α - ^{32}P]dATP. A similar approach has been successfully employed to detect synthesis pause sites of mammalian DNA polymerase α (Weaver & DePamphilis, 1982). Details of the priming and labeling process are shown diagrammatically in Figure 1. Two distinct advantages are derived from this approach: (i) The progress of each DNA polymerase can be monitored at the resolution of a single nucleotide when ^{32}P -labeled nascent chains are

² One unit of *Sau*3A1 is the amount of enzyme required for the complete digestion of 1 μg of λ DNA in 1 h at 37 °C.

fractionated by gel electrophoresis in parallel with template sequence information. (ii) Since each nascent DNA product is labeled at only one residue, the DNA band intensities observed following gel electrophoresis represent the relative number of ^{32}P -labeled DNA chains of various lengths. End-labeling reactions (20 μL) contained 3 μg of unique primer-template DNA, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 10 mM MgCl_2 , 10 mM dithiothreitol, 0.04 mg/mL BSA, 10 μM dGTP, and 3 μM [α - ^{32}P]dATP (3200 Ci/mmol). The reaction was incubated with 12 units (total) of AMV reverse transcriptase for 2 h at 42 $^\circ\text{C}$. The reaction mixture was terminated with the addition of EDTA to 25 mM, adjusted to 100- μL total volume with 10 mM Tris-HCl (pH 8.0) and 10 mM NaCl, and deproteinized by two phenol extractions and one chloroform extraction.

Further purification of ^{32}P end-labeled primer-templates was achieved through the use of a minicolumn centrifugation procedure described by Penefsky (1979) and adapted in this laboratory for the purification of DNA. The minicolumn consists of a 1-mL disposable tuberculin syringe (Pharmaseal Inc.) fitted with a porous polyethylene frit (70- μm pore size; Bolabs, Inc.) and filled with 1 mL (bed volume) of Sephadex G-50 fine resin (Pharmacia) previously equilibrated in deionized, distilled water at room temperature. Before the end-labeled primer-template mixture was loaded, the resin was partially dehydrated by spinning in a swinging bucket rotor for 2 min at 700 rpm (100g) in a benchtop centrifuge (Sorvall, Model GLC-1). The end-labeled primer-template mixture (100 μL) was applied to the top of the column and recentrifuged as described above. Material excluded from the column had the following characteristics: (i) The recovery of input DNA was 80–95%. (ii) The introduction of single- or double-stranded nicks in the DNA was negligible. (iii) Primer-templates purified in this way generated the expected DNA sequence (Beck et al., 1978) in Sanger dideoxynucleotide sequencing reactions. (iv) Less than 0.0003% of [α - ^{32}P]dATP (free triphosphate) was excluded from the resin. (v) Contaminating solvents are completely included within the resin. (vi) Use of resin equilibrated in water desalts the DNA. The excluded DNA was desiccated in a Speed Vac concentrator (Savant Instruments) and resuspended in 20 μL of buffer (20 mM Tris-HCl, pH 7.5, 5 mM NaCl, and 0.1 mM EDTA) at a concentration of end-labeled primer-template of approximately 0.15 mg/mL.

DNA Sequencing. DNA sequencing starting with the four *Sau3A1* unlabeled restriction fragment primed fd templates was performed essentially as described by Sanger et al. (1977) with the following alteration. Sufficient *Sau3A1* nuclease was added during the dATP chase reaction to allow for complete cleavage of the regenerated restriction sites.

Gel Electrophoresis. Electrophoresis was conducted through 8% polyacrylamide gels (40 \times 34 \times 0.04 cm) with a 19:1 ratio of acrylamide to *N,N'*-methylenebis(acrylamide) in 7 M urea, 100 mM Tris-borate (pH 8.3), and 2 mM EDTA. Electrophoresis was carried out at room temperature and 1500 V for 2–3 h. Bromphenol Blue and Xylene Cyanol FF dyes were used to monitor the progress of DNA fragments. Following electrophoresis, the polyacrylamide gels were covered with plastic wrap, overlaid with Kodak X-Omat RP film, and exposed at -70°C . A Dupont Cronex Lightening Plus intensifying screen was utilized. To ensure a linear relationship between the amount of radioactivity and film response, multiple exposures of each gel were made.

Reactions Using DNA Polymerase III Core-Containing Enzymes. Reaction mixtures (10 μL) contained 20 mM

Tris-HCl, pH 7.5, 15 mM DTT, 200 $\mu\text{g}/\text{mL}$ BSA, 20% glycerol, 100 μM each of dATP, dCTP, dGTP, and dTTP, and approximately 300–600 ng of end-labeled *Sau3A1* restriction fragment primed fd DNA and DNA polymerase III.³ Synthetic reactions using DNA polymerase III core-containing enzymes were catalyzed under conditions of enzyme excess with respect to available 3'-OH termini. Approximately 40 units of the appropriate enzyme was added to a reaction such that the enzymes could repeatedly interact with available primer termini. Addition of higher enzyme levels at the beginning of the reaction did not result in higher levels of total synthesis (data not shown). Reactions were incubated at 30 $^\circ\text{C}$ for the times indicated in the figure legends. Synthesis was terminated at the indicated times by heating the sample at 65 $^\circ\text{C}$ for 5 min, at which time *Sau3A1* (1.0 unit) was added. The reaction was allowed to proceed until the cleavage at the regenerated sites was complete (about 30 min). Two volumes of 95% formamide, containing 10 mM EDTA and 0.1% each of Bromphenol Blue and Xylene Cyanol FF, was added, and the reaction mixture was boiled for 3–5 min. The samples were subjected to electrophoresis as described above.

Computer Analysis. Since the entire sequence of the fd DNA is known (Beck et al., 1978), computer-directed search programs of nucleotide sequences capable of forming secondary structure via local intrastrand interactions, or interstrand interactions between the template and the plus or minus strands of the primer fragment, were formulated. The identification of potential secondary structures (hairpins) in a 300-nucleotide region adjacent to the primer termini was accomplished by comparing the hydrogen-bonding capacity of 12 nucleotide long template segments with adjacent sequences which were chosen at single nucleotide intervals. Potential hairpin structures which contained at least 22 hydrogen bonds (within complementary base pairs) in a 12-nucleotide section of the stem of each structure were compiled.⁴

The search for long-range interactions was conducted in the following way. Template sequences which were 35 nucleotides long were chosen such that the first nucleotide at the 3'-end of each sequence would correspond to positions of polymerase pausing. These sequences were matched with other possible complementary 35 nucleotide long sequences (chosen at 5-nucleotide intervals) on the fd template strand and the plus and minus strands of the restriction fragment used to prime synthesis. Complementarity was based upon potential hydrogen-bonding interactions (within complementary base pairs) rather than the formation of base pairs. This approach was taken so that dA-dT base pair formation, a weaker interaction, would be weighted less heavily than dG-dC base pair formation, an interaction that would have a greater effect in stabilizing template secondary structure. The above analysis was repeated by substituting a randomly generated 35 nucleotide long sequence for the pause site region on the fd DNA template. These random sequences were generated by assuming a nucleotide composition consistent with that of fd DNA, such that they would appropriately model the actual

³ Units for the DNA polymerase III forms are as follows: One unit of DNA polymerase III core or DNA polymerase III' is 1 pmol of total nucleotide incorporated per min on an activated salmon sperm DNA template. One unit of either DNA polymerase III*, when supplied with saturating levels of β , or DNA polymerase III holoenzyme is 1 pmol of total nucleotide incorporated per min on a bacteriophage G4 DNA template using priming in situ with *dnaG* primase.

⁴ When secondary structures were predicted by using other similar criteria (e.g., a larger stem section and correspondingly more potential hydrogen bonds), essentially the same pattern of potential secondary structures was obtained.

tested sequence. Potential long-range hydrogen-bonding interactions which exceeded interactions seen with randomly generated sequences were compiled. Additionally, a program was developed to characterize individual nucleotide concentrations within a region surrounding specified positions. These programs were written in Fortran and implemented on a DEC system 10 computer. Programs are available upon request.

Results

Synthetic Pausing of DNA Polymerase III Core and Holoenzyme on Specifically Primed fd DNA. Initial determination of the positions of pausing of DNA synthesis focused on the core and holoenzyme forms of DNA polymerase III because of the disparate average size ranges of products synthesized on randomly primed fd DNA templates. When in excess over template DNA molecules, the core enzyme synthesized products which averaged 50 nucleotides in length after repeated interaction of the polymerase with the template, while the holoenzyme generated products which averaged several hundred nucleotides in length (Fay et al., 1981). To examine the influence of template sequence and structure on polymerization by DNA polymerase III enzyme forms, four unique primer-template systems were constructed for DNA synthesis in vitro.

When products of DNA polymerase III made on *Sau3A1* restriction fragment primed fd templates are subjected to high-resolution polyacrylamide gel electrophoresis, a number of distinct pause sites⁵ of synthesis are observed which are located nonrandomly along the templates. In reactions using either 3'-³²P-labeled *Sau3A1*-A or *Sau3A1*-B fragment primed fd DNA templates (Figure 2; lanes 1 and 2, respectively), more than 30 synthetic pause sites were observed after 60 min of DNA polymerase III catalyzed activity. The nucleotide at the 3'-terminus of the nascent 5'-³²P-labeled DNA chains was determined by direct comparison of the fragment length with the DNA sequence of the primer-template. Both major and minor pause sites were observed as indicated by the varying band intensities of ³²P-labeled nascent chains. Major template-specific barriers to DNA polymerase III occurred between positions 2149 and 2137⁶ on the *Sau3A1*-A primed template, and between positions 5566 and 5545 on the *Sau3A1*-B primed templates.

The pause sites did not represent an absolute kinetic barrier to the progression of DNA polymerase III on any of the primed fd templates. On *Sau3A1*-D fragment primed fd templates, the combination of additional enzyme and longer incubation times resulted in an increase in the fraction of primers (332 nucleotide long *Sau3A1*-D fragment) utilized for synthesis, while nascent 5'-³²P-labeled DNA chains which had previously accumulated at sites close to the primer terminus were extended (Figure 3A,B). Nascent 5'-³²P-labeled DNA fragments eventually accumulated at template sites which were more than 300 nucleotides from the primer terminus after 120 min of core enzyme activity.

Barriers to the progression of holoenzyme-catalyzed synthesis were also observed on the specifically primed fd DNA templates. In reactions using 3'-³²P-labeled *Sau3A1*-B fragment primed fd DNA, holoenzyme progression was arrested at a discrete number of sites located within the limits measurable by sequence analysis, approximately 200 nucleotides from the primer terminus (Figure 4, lanes 2-5). Approxi-

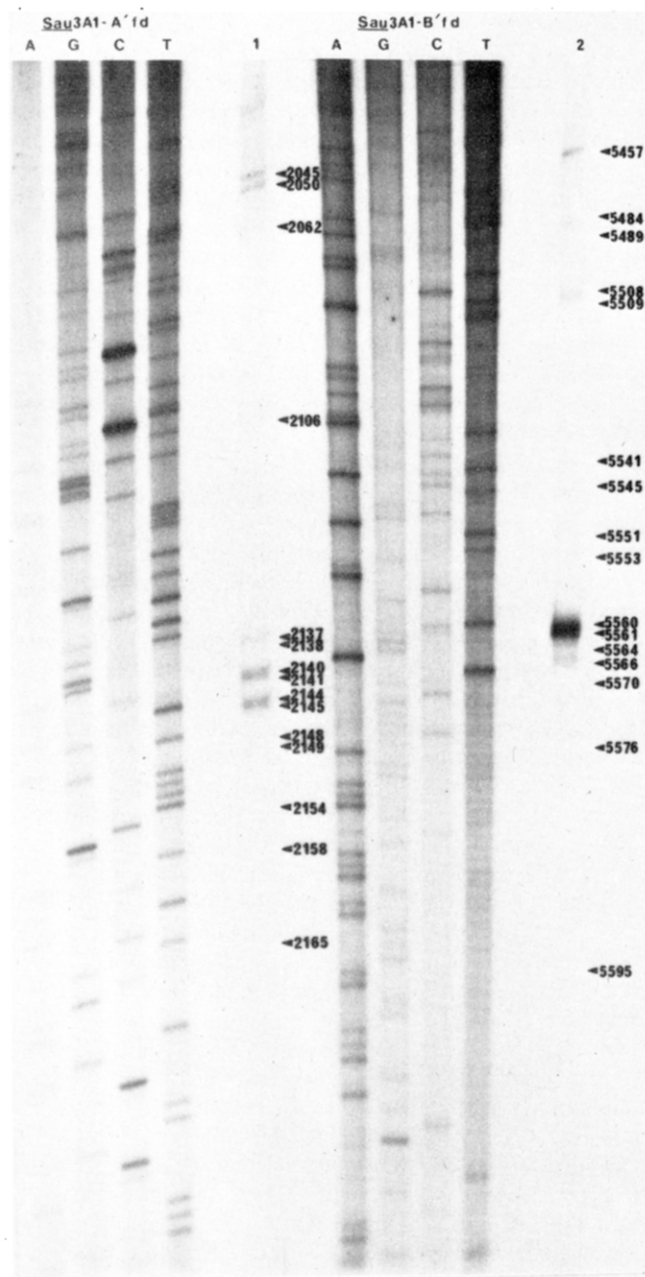


FIGURE 2: Autoradiogram of products made by DNA polymerase III core enzyme on *Sau3A1*-A and -B fragment primed fd DNA. Reactions were performed as described under Materials and Methods. Lanes 1 and 2 correspond to the 5'-³²P-labeled nascent DNA products of synthesis catalyzed by DNA polymerase III (40 units) after 1 h of synthesis on 3'-³²P-labeled *Sau3A1*-A and -B fragment primed templates, respectively. Reaction mixtures contained 0.75 μ g of primer-template DNA. The dideoxynucleotide sequence determination using ddATP, ddGTP, ddCTP, or ddTTP (listed as A, G, C, and T, respectively) is shown for both primer-template combinations. The numbers to the right of lanes 1 and 2 represent the nucleotide position of core polymerase pausing on *Sau3A1*-A and -B fragment primed fd templates, respectively. The primer termini for *Sau3A1*-A and -B primed templates are located at positions 2225 and 5650, respectively.

mately 50% of the nascent 5'-³²P-labeled DNA chains synthesized by the holoenzyme were too long to be resolved (>200 nucleotides) and, in fact, remained at the top of the gel and are not shown in the autoradiogram presented. In contrast to core enzyme catalyzed reactions, the holoenzyme was capable of utilizing and extending all of the primer termini present in the reaction within 30 s as evidenced by the loss of the 2144 nucleotide long band in lanes 1 and 18 which rep-

⁵ The term pause site refers to the template position directly following the last nucleotide incorporated by one of the DNA polymerases.

⁶ Numbering of nucleotides starts at the unique *HindII* (*HpaI*) cleavage site (Beck et al., 1978).

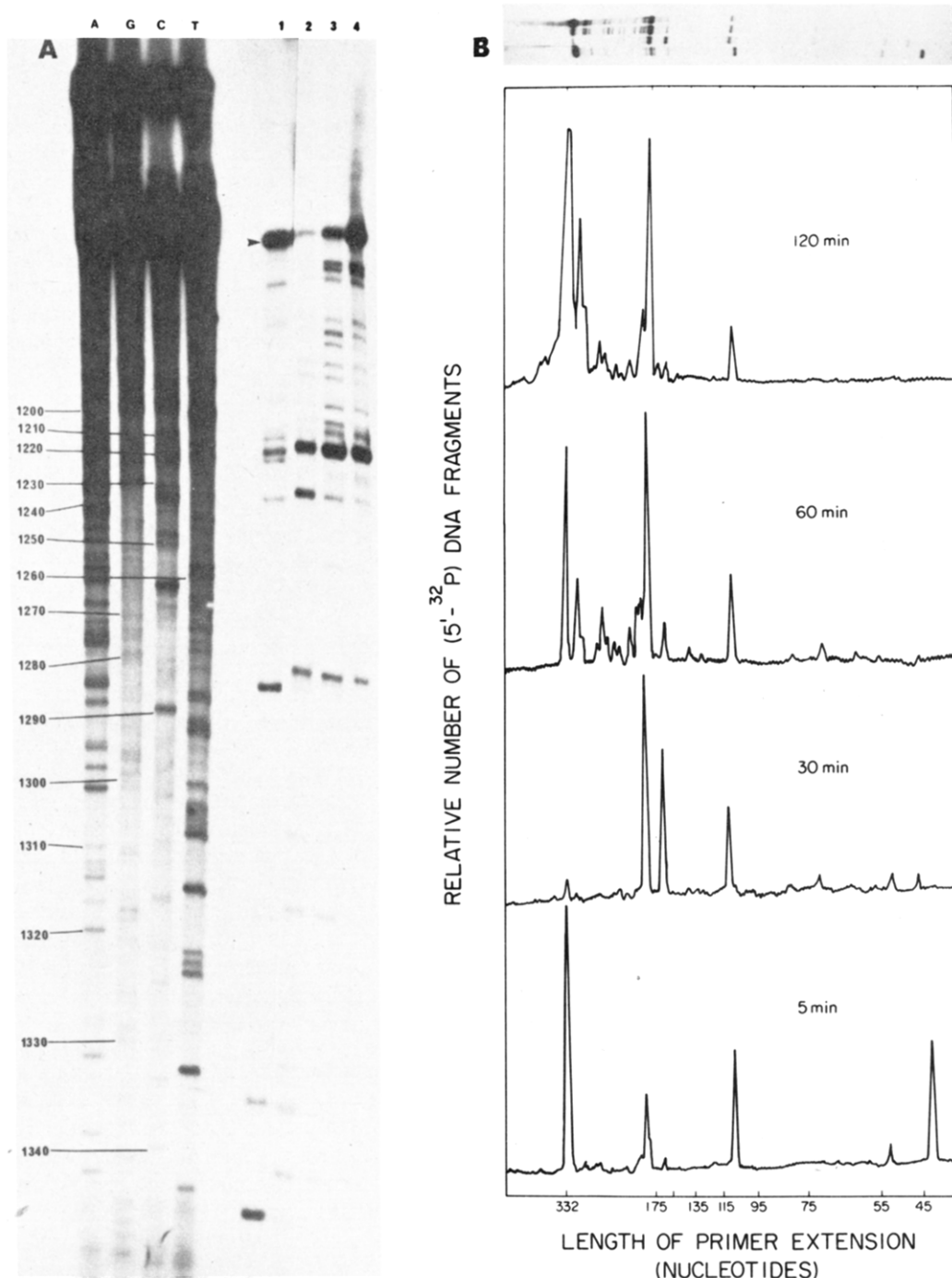


FIGURE 3: (A) Autoradiogram of time-dependent progression of DNA polymerase III core enzyme on 3'-³²P-labeled *Sau*3A1-D fragment primed fd DNA templates. The reaction mixture (10 μL) was incubated at 30 °C with 23 units of DNA polymerase III and 1.0 μg of primer-template DNA. Aliquots (2.0 μL) were removed after 5, 30, 60, and 120 min of core enzyme catalyzed DNA synthesis (lanes 1, 2, 3, and 4, respectively). Additional fresh enzyme (23 units) was added to the reaction mixture at the following times: 15, 30, and 60 min. ³²P-labeled DNA chains were treated as described under Materials and Methods prior to electrophoresis. *Sau*3A1-D primer fragments not extended by DNA polymerase III are shown by the arrow. The DNA sequence of this template region as determined in dideoxynucleotide sequencing reactions using ddATP, ddGTP, ddCTP, or ddTTP (listed as A, G, C, and T, respectively) is shown. The numbers on the left indicate nucleotide positions in the fd DNA template. The primer terminus is located at position 1386. (B) Densitometer tracing of lanes 1-4 in (A). Core enzyme progression is from right to left.

resents the unextended 3'-³²P-labeled *Sau*3A1-B fragment.

Most of the 5'-³²P-labeled nascent DNA chains synthesized by the holoenzyme on the *Sau*3A1-B primed fd template accumulated at position 5565 within the first 3 min of the reaction. This holoenzyme pause site occurs in a region of the fd template adjacent to the origin of fd replication which has been shown to have a high degree of template secondary structure (Gray et al., 1978; Huang & Hearst, 1980). Significant progression through this local region of secondary

structure occurred only after 10 min. On other *Sau*3A1 fragment primed fd DNA templates, a portion of the holoenzyme molecules present in each reaction failed to traverse several of the potential template barriers even after 60 min of incubation, resulting in the irreversible termination of primer extension at these sites.

The pattern of template-directed pausing in holoenzyme-catalyzed reactions changed when the polyamine spermidine (4 mM) was added to the reaction mixture (Figure 4, lanes

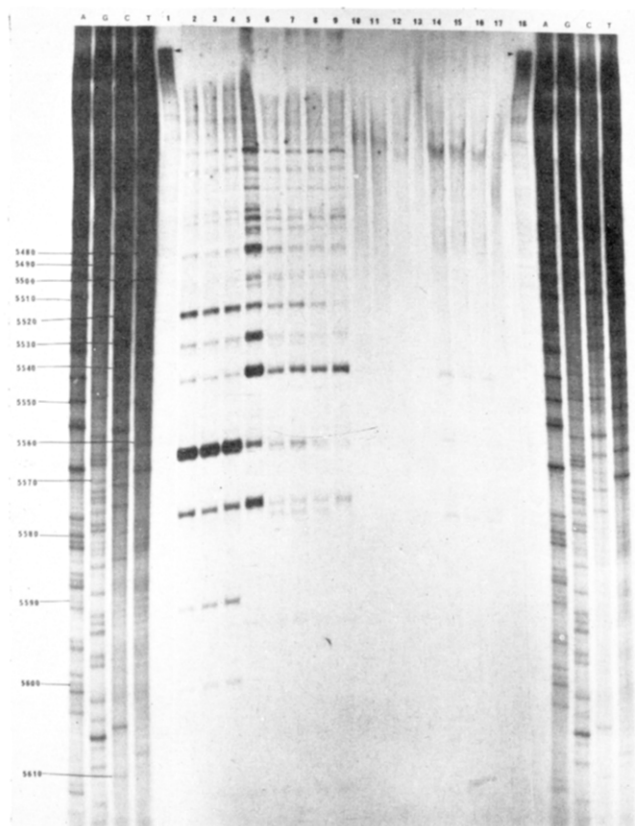


FIGURE 4: Autoradiogram of nascent 5'- ^{32}P -labeled DNA chains synthesized by DNA polymerase III holoenzyme on 3'- ^{32}P -labeled *Sau3A1*-B fragment primed fd DNA templates. Reaction mixtures (10 μL) were incubated at 30 $^{\circ}\text{C}$ with 40 units of DNA polymerase III holoenzyme and 0.5 μg of primer-template DNA. Aliquots (2.0 μL) were removed at intervals and treated as described under Materials and Methods prior to electrophoresis. The holoenzyme reactions shown are the following: holoenzyme alone (lanes 2–5); holoenzyme supplemented with 4 mM spermidine (lanes 6–9); holoenzyme supplemented with 1.0 μg of SSB (lanes 10–13); holoenzyme supplemented with both 4 mM spermidine and 1.0 μg of SSB (lanes 14–17). The 5'- ^{32}P -labeled nascent DNA products of synthesis after 0.5, 1, 3, and 10 min of incubation are shown in the first four lanes of each reaction set. Synthetic activity, expressed as picomoles of nucleotide incorporated per microgram of *Sau3A1*-B primed DNA, after 10 min of incubation was as follows: holoenzyme alone, 80.8 pmol; holoenzyme plus spermidine, 152.8 pmol; holoenzyme plus SSB, 195.2 pmol; holoenzyme plus both spermidine and SSB, 188 pmol. Lanes 1 and 18 contain unreacted, heat-denatured 3'- ^{32}P -labeled *Sau3A1*-B fragment primed fd DNA (0.04 μg). The *Sau3A1*-B fragment is shown by the arrow. The DNA sequence of this template region as determined in the dideoxynucleotide sequencing reactions using ddATP, ddGTP, ddCTP, or ddTTP (listed as A, G, C, and T, respectively) is shown. The numbers on the left indicate nucleotide positions in the fd DNA template. The primer terminus is located at position 5650.

6–9). The effect of spermidine in the reactions was 2-fold. First, the fraction of ^{32}P -labeled nascent DNA chains which accumulated within 200 nucleotides of the primer terminus of the *Sau3A1*-B primed fd template was reduced with respect to holoenzyme reactions catalyzed in the absence of spermidine. This can be attributed to the stimulation in activity normally observed in spermidine-supplemented holoenzyme reactions on both synthetic homopolymer and natural DNA templates (Tabor & Tabor, 1976; Fay et al., 1981). Second, spermidine has the apparent capacity to strengthen preexisting barriers in the template strand in addition to creating new ones. The most intense pause sites observed in spermidine-supplemented holoenzyme reactions occurred in regions of computer-predicted local secondary structure in the template strand (Figure 5).⁴ This result implies that spermidine-enhanced

holoenzyme activity on natural DNA templates can partially be attributed to the capacity of this factor to discourage polymerase pausing at sites which do not correspond to hairpin structures.

Template-specific barriers to holoenzyme progression were removed when the *Sau3A1* primer-template was coated with *E. coli* SSB (approximately 3 μg of SSB/ μg of fd DNA; Figure 4, lanes 10–13). In these reactions, most of the radioactivity associated with nascent DNA chains remained at the top of the gel and is not shown. However, in reactions containing an SSB-coated primer-template in addition to spermidine (4 mM), the progression of the holoenzyme was arrested at the same population of pause sites seen in the spermidine-supplemented holoenzyme reactions, albeit to a lesser extent (Figure 4, lanes 14–17). The loss of pause sites when SSB is present, and the partial reemergence of secondary structure related pause sites when SSB and spermidine are present, suggests that secondary structure is a significant determinant of pause sites.

Loss of Holoenzyme Activity as a Result of Pausing. The ability of the holoenzyme to synthesize through pause sites on the specifically primed fd templates was demonstrably different in the presence or absence of SSB. In the absence of SSB, the capacity of the holoenzyme to progress through the majority of the observable pause sites (within 200 nucleotides of the primer terminus) on the *Sau3A1*-A fragment primed fd templates was not significantly improved by the combination of additional enzyme and longer incubation times (Figure 6, lanes 1–3). Those sites which halted the progress of the holoenzyme correlated with template regions that have a high degree of secondary structure as indicated by reactions catalyzed in the presence of spermidine (Figure 6, lane 4).

In holoenzyme-catalyzed reactions supplemented with ATP (500 μM), a 2-fold stimulation in enzymatic activity has been observed on synthetic homopolymer templates (Burgers & Kornberg, 1982). This elevated level of holoenzyme activity can be attributed to the ability of ATP to facilitate the formation of stable initiation complexes at 3'-OH termini (Wickner, 1976; Johanson & McHenry, 1982; Burgers & Kornberg, 1982). However, in holoenzyme reactions on *Sau3A1*-A fragment primed fd DNA which were catalyzed in the presence of ATP (500 μM), enhanced holoenzyme movement through pause sites was not observed (Figure 6, lane 6). The results suggest that the observed irreversible termination of primer extension at some pause sites is not related to a necessity of the holoenzyme to undergo ATP-related complex formation with 3'-termini.

In contrast, primed templates coated with SSB allowed progression of the holoenzyme past all pause sites (Figure 6, lane 5). Clearly, the fate of holoenzyme molecules which are arrested at pause sites on templates which are not coated with SSB differs greatly from that of holoenzyme molecules on SSB-coated templates.

The apparent inability of the holoenzyme to progress through a portion of the template barriers found on *Sau3A1* fragment primed fd templates could be attributed to a structural alteration or inactivation of the polymerase which occurs as a consequence of pausing. An alternative explanation is that the holoenzyme remains active at the pause site but is incapable of synthetic activity through the site. To distinguish between these two alternatives, measurements were made of the maximum levels of holoenzyme-catalyzed synthesis on *Sau3A1* primer-templates in the presence or absence of SSB. The maximum level of holoenzyme synthesis on *Sau3A1*-D primed templates coated with SSB was approximately 2-fold

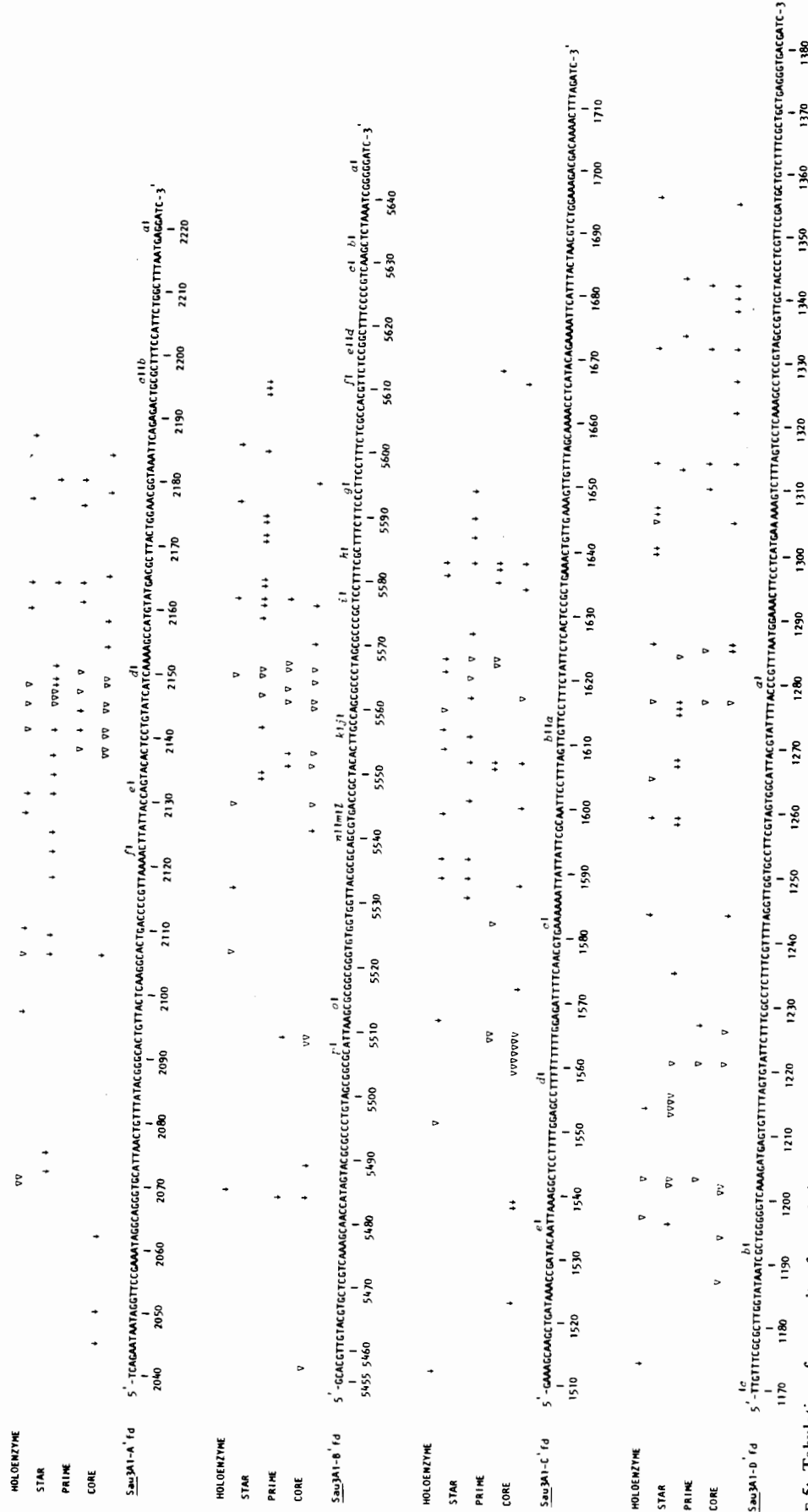


FIGURE 5. Tabulation of pause sites for each form of DNA polymerase III observed on the four *Sau3A1* fragment primed fd DNA templates. The location of each pause site after 1 h of synthesis by each form of the polymerase in the absence of SSB and spermidine is shown above. DNA polymerase III, DNA polymerase III', DNA polymerase III*, and DNA polymerase III holoenzyme incorporated an average of 12.75, 32.4, 102, and 122.5 pmol of nucleotide per μ g of reacted *Sau3A1* restriction fragment primed fd DNA, respectively, after 30 min of incubation. Levels of nucleotide incorporation varied on the four primer-template combinations; however, the ratios of synthesis by the four enzyme forms remained relatively constant. The nucleotide sequence of the fd template strand is written in the 5' to 3' direction. Synthesis is from right to left. The primer termini on the *Sau3A1*-A, -B, -C, and -D fragment primed templates are at positions 2225, 5650, 1718, and 1386, respectively. Major (V) and minor (+) polymerase pause sites were determined on the basis of band intensities of 32 P-labeled nascent DNA chains in 8% polyacrylamide gels. Vertical lines on the sequence represent the first hydrogen-bonded base pair encountered by the polymerase of potential hairpin structures arising from local hydrogen-bonding interactions. Criteria for hairpin structures required the formation of at least 22 hydrogen bonds in a 12-nucleotide section of the

stem of each structure.⁴ The double-stranded regions of potential hairpin structures encountered on each of the primer-template combinations are as follows: *Sau3A1*-A' fd, (a) 2221-2210 and 2123-2112, (b) 2198-2187 and 2019-2008, (c), 2197-2184 and 2028-2015, (d) 2151-2140 and 2090-2079, (e), 2133-2123 and 1977-1967, and (f) 2123-2113 and 2090-2080; *Sau3A1*-B' fd, (a) 5646-5635 and 5428-5417, (b) 5634-5624 and 5522-5512, (c) 5630-5618 and 5456-5444, (d) 5618-5607 and 5493-5482, (e) 5617-5606 and 5527-5516, (f) 5612-5597 and 5527-5512, (g) 5595-5584 and 5456-5445, (h) 5585-5574 and 5515-5504, (i) 5578-5567 and 5551-5540, (j) 5559-5540 and 5536-5517, (k) 5557-5546 and 5521-5510, (l) 5544-5533 and 5467-5456, (m) 5542-5533 and 5523-5514, (n) 5541-5531 and 5511-5501, (o) 5515-5501 and 5420-5406, and (p) 5508-5496 and 5396-5384; *Sau3A1*-C' fd, (a) 1613-1603 and 1542-1532, (b) 1612-1600 and 1485-1473, (c) 1583-1572 and 1513-1502, (d) 1559-1551 and 1546-1538, and (e) 1536-1525 and 1439-1428; *Sau3A1*-D' fd, (a) 1281-1270 and 1208-1197, (b) 1193-1177 and 1160-1144, and (c) 1170-1158 and 1113-1101. The nucleotide positions are as numbered in the fd sequence of Beck et al. (1978).

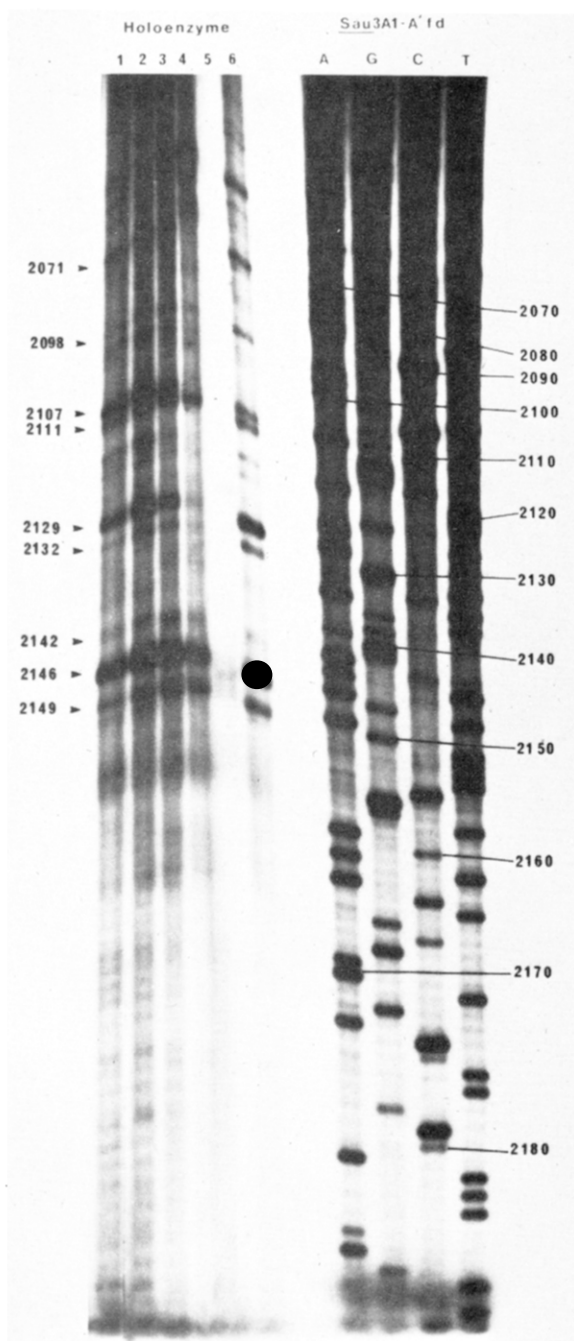


FIGURE 6: Autoradiogram of time-dependent progression of DNA polymerase III holoenzyme on 3'- ^{32}P -labeled *Sau3A1-A* fragment primed fd DNA templates. The reaction mixture (20 μL) was incubated at 30 $^{\circ}\text{C}$ with 40 units of the holoenzyme and 1.2 μg of the primer-template DNA. Aliquots (3.0 μL) were removed after 1 and 10 min of holoenzyme-catalyzed DNA synthesis (lanes 1 and 2). Portions (3.0 μL) of the remaining reaction mixture were transferred to reaction tubes containing one of the following, and synthesis was continued for a total of 60 min: no additions (lane 3), spermidine (2 mM final concentration) (lane 4), SSB (0.6 μg) (lane 5), or ATP (500 μM) (lane 6). Additional fresh holoenzyme (40 units) was added to each reaction after 30 min of synthesis. ^{32}P -labeled DNA chains were treated as described under Materials and Methods prior to electrophoresis. The DNA sequence of the template region as determined in dideoxynucleotide sequencing reactions using ddATP, ddGTP, ddCTP, or ddTTP (listed as A, G, C, and T, respectively) is shown. The numbers to the left represent nucleotide positions on the fd template of holoenzyme pausing. The numbers to the right represent nucleotide positions in the fd DNA template as determined in the sequencing reactions. The primer terminus is located at position 2225.

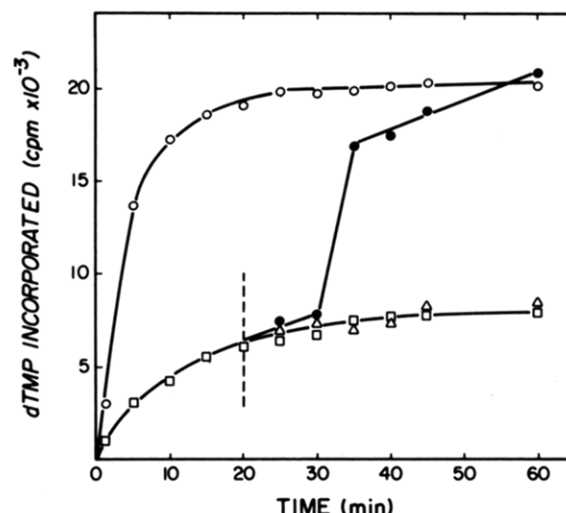


FIGURE 7: Time dependence of synthesis by DNA polymerase III holoenzyme on *Sau3A1-D* fragment primed fd DNA templates. Reaction mixtures (50 μL) contained 1.5 μg of unlabeled primer-template DNA, 50 μM [^3H]dTTP (10 Ci/mmol), and approximately 126 units of DNA polymerase III holoenzyme. Other additions are as described under Materials and Methods. Aliquots (2.0 μL) were removed at regular intervals, and acid-insoluble radioactivity was determined. [^3H]dTMP incorporation is plotted vs. time of incubation at 30 $^{\circ}\text{C}$. The open circles represent holoenzyme activity on templates supplemented with SSB (4.8 μg). The open squares represent the activity of the holoenzyme alone. Both reactions were supplemented with additional holoenzyme (63 units) after 15 and 30 min of holoenzyme-catalyzed activity. Portions (13 μL) of the reaction with holoenzyme alone were transferred to two tubes containing SSB (1.2 μg) at $t = 20$ min and were reacted at 30 $^{\circ}\text{C}$ in the presence (O) or absence (Δ) of additional holoenzyme (63 units) added at $t = 30$ min.

greater than on templates which were not coated (Figure 7). When additional holoenzyme was added to templates which were not coated with SSB, no further synthesis was observed. Apparently, the presence of SSB at the start of the reaction removes hairpin structures, thereby facilitating the continued progression of the polymerase.

In the absence of SSB, progression of the holoenzyme through the majority of pause sites does not occur. In an effort to reconstitute holoenzyme molecules which may have lost auxiliary subunits as a result of pausing at template barriers, excess levels of purified β subunit were added to holoenzyme reactions after 20 min of synthetic activity. This polypeptide had no effect on the level of synthetic activity and was incapable of restarting stalled holoenzyme molecules at pause sites (data not shown). In contrast, if SSB is added after the holoenzyme has already completed synthesis up to pause sites on the *Sau3A1-D* primed template (20 min), further synthesis is observed only if additional fresh holoenzyme is added.⁷

In a similar experiment, SSB and a second higher primer density synthetic template [poly(dA)-oligo(dT)₁₀ 20:1] were added to a reaction in which maximum levels of holoenzyme-catalyzed activity on the *Sau3A1-D* primed template had been achieved (20 min). If the stalled holoenzyme at pause sites was still catalytically functional, the presence of saturating levels of substrate in the form of a high primer

⁷ In a control experiment, the holoenzyme was incubated with reaction components, template, and three dNTPs such that synthesis would pause not at secondary structures but at sites requiring the incorporation of the missing nucleotide. Addition of the fourth dNTP at 30 min resulted in a level of DNA synthesis equivalent to 50% of that which occurs when all four dNTPs are added initially. This result implies that the holoenzyme paused at template barriers is not losing all of its activity simply as a result of incubation in the reaction.

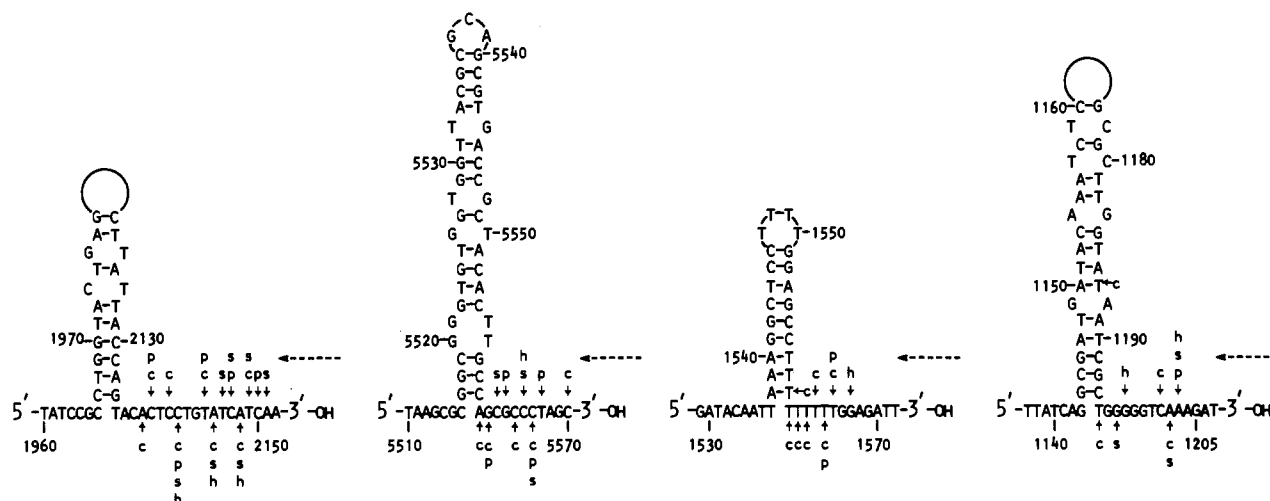


FIGURE 8: Positions of polymerase pausing at major barriers to enzyme progression. The nucleotide sequence of each hairpin structure is illustrated to emphasize potential base pairing. Pause sites (†) are presented for DNA polymerase III core (c), DNA polymerase III' (p), DNA polymerase III* (s), and DNA polymerase III holoenzyme (h). The numbers refer to the position of the indicated nucleotide in the fd DNA sequence. The direction of DNA synthesis is indicated by the horizontal arrows.

density template should result in renewed holoenzyme-catalyzed DNA synthesis. However, no additional synthesis was observed on either template unless fresh holoenzyme was added to the reaction (data not shown). Apparently, once the holoenzyme stalls at pause sites, it is changed in such a way that it is no longer catalytically functional. Furthermore, new enzymes cannot initiate synthesis at pause sites unless they are structurally altered by SSB.

Comparison of Site-Specific Pausing of DNA Polymerase III Core-Containing Enzymes on Specifically Primed fd DNA. A similar analysis to that presented above using DNA polymerase III' and DNA polymerase III* was undertaken to establish the effects of the particular auxiliary subunits present in each of the DNA polymerase III enzyme forms on the frequency and specificity of pausing. Since these two subassemblies of the holoenzyme have been shown to generate products at enzyme excess that are intermediate in size between those made by the core and holoenzyme (McHenry, 1982; Fay et al., 1982), an intermediate capacity to progress through template barriers was expected.

Results from the analysis of the ability of all four forms of DNA polymerase III to extend four different *Sau3A1* primers and to progress through pause sites on these primer-template combinations are summarized in Figure 5. The pattern of pausing indicates that the addition of auxiliary subunits to the catalytic core enzyme increases the ability of each stable form of the polymerase to progress through regions of the template which would otherwise halt DNA polymerase III. Some pause sites are, however, unique to each enzyme form. In all cases, the pause sites are irregularly spaced, indicating that barriers to the progression of DNA polymerase III enzyme forms are template specific. The inclusion of SSB and/or spermidine in reactions using DNA polymerase III' and DNA polymerase III* has effects similar to those observed with the holoenzyme (data not shown).

Correlation of Pauses in Synthesis to Template Secondary Structure. Since the entire sequence of fd DNA is known (Beck et al., 1978), a computer-directed search of a 300-nucleotide region directly adjacent to the primer termini made possible the identification of potential local hairpin structures. A criterion for computer-predicted hairpin structures was chosen such that at least 22 hydrogen bonds were formed in a 12-nucleotide section of the stem of each hairpin structure.⁴ These structures, as compiled in Figure 5, represent potential

barriers to synthesis catalyzed by DNA polymerase III forms. Indeed, out of a total of 232 pause sites observed on the four *Sau3A1* primed fd templates, approximately 65% of these occurred within 15 nucleotides of the stems of computer-predicted local secondary structures. Pause sites at which the largest fraction of ³²P-labeled nascent DNA chains accumulated [(▽) Figure 5] on each of the primer-template combinations (e.g., at positions 5559, 1559, and 1193) correlated with specific template regions which possessed a high degree of base pair homology. Other, less complementary helical structures also partially block synthesis by the polymerases. In many cases, these weak barriers were stabilized by spermidine, as indicated by an increased number of termination sites at and around these regions.

The proximity of polymerase pause sites to hairpin structures also correlated with the size of each enzyme used. In most cases, pausing occurred on the primer-proximal side of the base of each hairpin (Figure 8). Since several potential hairpin structures can exist within a particular region of a template strand, the grouping of pause sites within the hairpin structures shown in Figure 8 is based upon the frequency of pausing by each enzyme form and the degree of base pair homology present in each computer-predicted hairpin structure. When pause sites and hairpin structures are paired in this way, pauses characteristic of the core form of DNA polymerase III are typically located closer to the base of hairpin structures than those of the larger holoenzyme. This suggests that the active site for nucleotide addition in the holoenzyme, a much larger molecule than the core enzyme, is a greater distance away from the portion of the enzyme which encounters and is blocked by the hairpin structure. DNA polymerase III' and DNA polymerase III*, both intermediate in size with respect to the core and holoenzyme, frequently paused at sites which were between those seen for the core and holoenzyme.

Correlation of Pauses to Long-Range Interactions. Approximately one-third of synthesis pause sites did not correlate with regions of potential local hairpin structure in the 300 nucleotides adjacent to the primer termini. We considered that these pause sites may result from long-range base pairing of the template region adjacent to the pause site with distinct DNA sequences.

In an effort to find possible sites for such long-range interactions, a computer search of the entire fd DNA sequence was undertaken to correlate pause sites to potential duplex

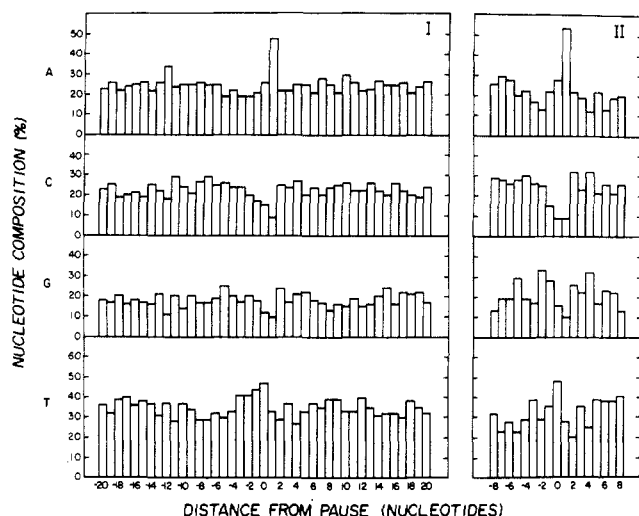


FIGURE 9: (I) Correspondence of polymerase pausing to template base composition. The percent nucleotide composition in template regions surrounding pause sites was determined by computer analysis of pooled sites (180 sites) observed on *Sau3A1*-A, -C, and -D fragment primed fd DNA templates by all forms of DNA polymerase III. (II) The base composition of pause sites not associated with potential hairpin structures (81 sites which occurred in template regions other than the 15 nucleotides immediately preceding the base of computer-predicted hairpin structures or in the 3'-proximal side of the stem of these structures up to the unpaired region of the loop structure) is shown for this subclass of pause sites. Pause sites observed on *Sau3A1*-A, -C, and -D primed fd DNA templates by all forms of DNA polymerase III were pooled. Polymerase progression is from right to left, with the position of enzyme pausing labeled as 0.

structures that may arise by either (a) intrastrand base pairing between sequences adjacent to the primer terminus and other sequences separated by great distances in the template or (b) interstrand base pairing between regions adjacent to the primer terminus and the plus or minus strand of the restriction fragment used to prime synthesis. The 35-nucleotide sequence immediately following each pause site on the template strand was matched with other possible complementary 35 nucleotide long sequences chosen at 5-nucleotide intervals on the fd template and primer strands. The number of sites with hydrogen-bond interactions ranging from 0 to 105 were compared with interactions observed when randomly generated sequences (35 nucleotides long) were substituted for the pause site region on the fd DNA. Our analysis demonstrated that all of the computer-predicted long-range hydrogen-bonding interactions at pause sites were similar (with respect to hydrogen-bonding capacity) to the interactions observed with randomly generated fragments. Therefore, long-range base pair interactions do not appear to be a significant factor in determining sites of DNA polymerase III pausing.

Correlation of Pause Sites to the Nucleotide Sequence and Base Composition of the Template. The specific nucleotide sequence and base composition at and around pause sites were also investigated. Examination of di- and trinucleotide sequences at all pause sites, including those sites not related to hairpin structures, showed no specific sequence at which pausing occurs. These data are not tabulated but can be derived from Figure 5. However, the base composition of the template strand at pause sites does appear to be a unique feature of polymerase pausing. The data, presented in Figure 9 (I), illustrate the percentage of each nucleotide averaged for each pause site in the template (labeled 0) and a region spanning 20 nucleotides in the 5' (minus) and 3' (plus) directions from each site. These data were obtained by pooling all pause sites (180 sites) observed on *Sau3A1*-A, *Sau3A1*-C,

and *Sau3A1*-D primed fd DNA templates, in the absence of SSB and spermidine, for each enzyme form. Results indicate that there is a relatively low concentration at pause sites of dC and dG residues (about 13% each) and an exceptionally high concentration of dA (48%) at a position which corresponds to the last nucleotide incorporated (position 1) by each of the polymerases. A comparison of the concentrations of dC, dG, and dA in the entire fd DNA molecule (20% dC, 21% dG, and 24% dA) to the concentrations of these residues in 200-nucleotide regions directly adjacent to each primer terminus (21% dC, 20% dG, and 26% dA) indicates that these regions are representative of the entire molecule and not particularly deficient in these nucleotides.

Most striking, however, was the unusual propensity of dT to be the last nucleotide incorporated at pause sites. We originally thought that the combination of high template dA concentration and secondary structure might be distinguishing features of sites which promoted the pausing of DNA polymerase III containing enzymes. To investigate this possibility, the nucleotide composition at replication pause sites of each of the DNA polymerase III enzyme forms on *Sau3A1*-A, *Sau3A1*-C, and *Sau3A1*-D primed fd templates which occurred in regions of the template lacking the potential for secondary structural interactions was determined (Figure 9, II). The base composition at and around this class of pause sites was essentially identical with that seen when all of the pause sites were pooled together. It would appear, therefore, that pausing of DNA polymerase III containing enzymes may be independently promoted by template base composition in the absence of significant secondary structure.

Discussion

Sites on an fd DNA template which block the progress of each of four forms of *E. coli* DNA polymerase III have been located at single nucleotide resolution. The progression of each enzyme form was shown to be highly sensitive to both template secondary structure and DNA base composition. A total of 232 specific pause sites of DNA synthesis catalyzed by each of the polymerases were detected on the four primer-template combinations used in this study. Approximately 65% of the sites at which pausing was observed were within 15 nucleotides of potential template hairpin structures. The remainder of the pause sites were detected in regions of the template which lack the potential to form such structures.

Our analysis of the pattern of pausing by each of the DNA polymerase III enzyme forms revealed that the presence of auxiliary subunits to the core form of the enzyme changes the recognition of pause sites. Sites on the template which were very effective in halting the progression of DNA polymerase III were often less effectively recognized by the holoenzyme as pause sites. Similarly, variation in the pattern and intensity of pausing by DNA polymerase III' and DNA polymerase III* were observed. Clearly, the subunits needed to generate each of the larger forms of the enzyme have a distinct functional effect on primer elongation.

A specific effect of auxiliary subunits to DNA polymerase III is the proximity of pause sites to the base of stable hairpin structures. The progression of the core form of DNA polymerase III was typically arrested at sites which were closer to the base of hairpin structures than the higher molecular weight enzyme forms. Possibly a portion of the enzyme away from the active site first contacts the template secondary structure, and this contact discourages further progression of the enzyme. The distance between the active site and this portion of the enzyme would be expected to be the greatest in the holoenzyme form.

Hairpin structures with high levels of G-C base pairing in the stem functioned as the strongest pause sites for each of the polymerases. However, modification in hairpin structures as a consequence of spermidine or SSB interaction with the template strand can induce changes in the relative pattern and intensity of pausing.

All of the intense pause sites observed in the presence of spermidine could be attributed to local hairpin structures. Other pause sites, not associated with local hairpins, faded in the presence of spermidine. Pausing at these sites should have been enhanced if it were resulting from long-range interactions. Analysis of the 35 nucleotide long sequence adjacent to pause sites not associated with local hairpins indicated that these sequences have no unusual homology with any potential long-range interaction sites. These results suggest that pause sites can be classified into two categories based on the proximity of each site to regions of local secondary structure in the template.

Template strands which were coated with SSB provided few, if any, barriers to the progression of the higher molecular weight DNA polymerase III enzyme forms. This result is consistent with the ability of the more complex DNA polymerase III containing enzymes to synthesize DNA products which are severalfold larger in the presence of SSB compared to products made in the absence of this protein.

The analysis of template base composition indicated that the last nucleotide incorporated prior to pausing of DNA polymerase III enzyme forms occurred at template sites which have uncharacteristic levels of dC, dG, and dA residues (9%, 10%, and 48%, respectively). Furthermore, the template composition at the pause site was also unusually low in dC and dG. This correlation of base composition and pausing was observed at all pause sites regardless of the proximity of each site to stable hairpin structures. In contrast, Weaver & DePamphilis (1982) had previously shown that mammalian DNA polymerase α was typically arrested in synthesis at GC-rich sites on a ϕ X174 template. The recognition and response of DNA polymerases in general to potential pause sites must be dependent on characteristics of the enzyme.

Significant differences were observed in comparison of the ability of each form of DNA polymerase III to utilize primer-templates for synthesis and progress through template-specific barriers. As expected, the most striking differences were observed when the core and holoenzyme forms of the polymerase were compared. The amount of synthesis observed with excess levels of core polymerase on *Sau*3A1 fragment primed fd templates after 30 min at 30 °C was approximately 10% of that observed in holoenzyme-catalyzed reactions, partially reflecting the efficiency with which these two polymerases find and productively interact with 3'-OH termini. Movement of the core polymerase through synthetic barriers was a slow but constant process, characterized by the clustering of pause sites adjacent to helical structures in the template. This may reflect a requirement for multiple cycles of rebinding of the core polymerase before movement through hairpin-related pause sites can be accomplished. The holoenzyme appeared to move rapidly through some potential pause sites, only to stall at later sites on the template, or terminate synthesis as a result of enzyme inactivation. At sites where primer extension terminated, attempts to reactivate synthetic activity through the addition of fresh holoenzyme, excess β subunit, ATP, or SSB were unsuccessful. Significant additional movement through these sites required both SSB and fresh holoenzyme. This result indicates that the loss of holoenzyme activity observed in these experiments is directly related to the

process of pausing and enzyme inactivation at template secondary structures.

It has been our intention in this study to view the average product size determinations for forms of *E. coli* DNA polymerase III made earlier (Fay et al., 1981; 1982) at high resolution by examining the structural features of pause sites for DNA synthesis. Viewed within the constraints of the number of data points examined, we have obtained results which suggest that the exact positions where synthesis pauses are determined by the size of the enzyme and the presence of specific protein subunits, the presence of potential secondary structure in the template, and the nucleotide composition of the template.

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Poly(ADP-ribose) Polymerase Inhibitors Preserve Nicotinamide Adenine Dinucleotide and Adenosine 5'-Triphosphate Pools in DNA-Damaged Cells: Mechanism of Stimulation of Unscheduled DNA Synthesis[†]

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ABSTRACT: Inhibitors of poly(ADP-ribose) polymerase stimulated the level of DNA, RNA, and protein synthesis in DNA-damaged L1210 cells but had negligible effects in undamaged L1210 cells. The poly(ADP-ribose) polymerase inhibitors stimulated DNA repair synthesis after cells were exposed to high concentrations of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (68 and 136 μ M) but not after exposure to low concentrations (13.6 and 34 μ M). When the L1210 cells were exposed to 136 μ M *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, the activation of poly(ADP-ribose) polymerase resulted in the rapid depletion of oxidized nicotinamide adenine dinucleotide (NAD⁺) levels and subsequent depletion of adenosine 5'-triphosphate (ATP) pools. After low doses of

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (13.6 μ M), there were only small decreases in NAD⁺ and ATP. Poly(ADP-ribose) polymerase inhibitors prevented the rapid fall in NAD⁺ and ATP pools. This preservation of the ATP pool has a permissive effect on energy-dependent functions and accounts for the apparent stimulation of DNA, RNA, and protein synthesis. Thus, the mechanism by which poly(ADP-ribose) polymerase inhibitors stimulate DNA, RNA, and protein synthesis in DNA-damaged cells appears to be mediated by their ability to prevent the drastic depletion of NAD⁺ pools that occurs in heavily damaged cells, thereby preserving the cells' ability to generate ATP and maintain energy-dependent processes.

Poly(ADP-ribose)¹ polymerase, a chromatin-bound enzyme, cleaves NAD⁺ to yield nicotinamide and ADP-ribose, and then the same enzyme polymerizes successive ADP-ribose residues to synthesize poly(ADP-ribose) (Hayaishi & Ueda, 1977). While the function(s) of poly(ADP-ribose) is (are) not yet clearly established, a number of studies have shown that its synthesis is stimulated by DNA-damaging agents such as MNNG, Me₂SO₄, MNU, and UV, γ , or X irradiation (Berger et al., 1979, 1980; Jacobson, M. K., et al., 1980; Durkacz et al., 1980; Sudhakar et al., 1979; Skidmore et al., 1979; Benjamin & Gill, 1980). It has also been shown that the increase in poly(ADP-ribose) polymerase activity stimulated by DNA damage is associated with a decrease in cellular NAD⁺ levels and an increase in intracellular poly(ADP-ribose) levels (Jacobson, M. K., et al., 1980; Durkacz et al., 1980; Skidmore et al., 1979; Jaurez-Salinas et al., 1979; Sims et al., 1982). The possibility that poly(ADP-ribose) synthesis is required during the DNA repair process is suggested by the observations

that NAD⁺-starved 3T3 cells lose their ability to repair MNNG-induced DNA damage (Jacobson, E. L., et al., 1980), that poly(ADP-ribose) polymerase inhibitors retard the rejoining of DNA strand breaks in Me₂SO₄-treated L1210 cells (Durkacz et al., 1980), and that the cytotoxicity of some DNA-damaging agents is enhanced by poly(ADP-ribose) polymerase inhibitors (Durkacz et al., 1980).

In contrast to some of the studies outlined above, we have recently shown that the unscheduled DNA synthesis that occurs in normal human lymphocytes after treatment with DNA-damaging agents such as UV irradiation, Me₂SO₄, or MNNG can be stimulated by the addition of nicotinamide analogues to the culture medium (Berger & Sikorski, 1980). Miwa et al. showed that similar stimulation of unscheduled DNA synthesis occurred when DNA-damaged lymphocytes were treated with a series of nicotinamide analogues (Miwa et al., 1981), and we showed that this stimulation was dependent on the concentration of the agents in the culture medium (Berger & Sikorski, 1980) and on their potency as inhibitors of poly(ADP-ribose) polymerase (Sims et al., 1982). Stimulation of DNA repair synthesis by poly(ADP-ribose) polymerase inhibitors has also been observed in DNA-damaged rat hepatocytes (Althaus et al., 1980). Since lymphocytes and hepatocytes are resting intermitotic cells with negligible levels of replicative DNA synthesis, it seemed possible that the ability of poly(ADP-ribose) polymerase inhibitors to stimulate un-

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¹ Abbreviations: ADP-ribose, adenosine 5'-diphosphate ribose; dThd, thymidine; dTMP, thymidine 5'-phosphate; Leu, leucine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosourea; NAD⁺, nicotinamide adenine dinucleotide (oxidized); UMP, uridine 5'-phosphate; Urd, uridine; ϵ RAdo, 1,*N*⁶-etheno-2'-ribosyladenosine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.