Persistence of DNA synthesis arrest sites in the presence of T4 DNA polymerase and T4 gene 32, 44, 45 and 62 DNA polymerase accessory proteins

Marc F.Charette¹, David T.Weaver² and Melvin L.DePamphilis

Department of Biological Chemistry, Harvard Medical School, Boston, MA 02115, USA

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ABSTRACT

DNA synthesis by phage T4 DNA polymerase is arrested at specific sequences in single-stranded DNA templates. To determine whether or not T4 DNA polymerase accessory proteins 32, 44, 45 and 62 eliminated recognition of these arrest sites, unique primer-templates were constructed in which DNA synthesis began at a DNA primer located at different distances from palindromic and nonpalindromic arrest sites. Nucleotide positions that caused polymerase to pause or leave the template were identified by sequence analysis of 5'-end labeled nascent DNA chains. Stable hairpin structures at palindromic sequences were confirmed by acetylation of single-stranded sequences with bromoacetaldehyde. Our results confirmed that these T4 DNA polymerase accessory proteins stimulated T4 DNA polymerase activity and processivity on natural as well as homopolymer primer-templates. However, they did not alter recognition of DNA synthesis arrest sites by T4 DNA polymerase. Extensive DNA synthesis resulted from an increased rate of translocation and/or processivity to the same extent over all DNA sequences.

INTRODUCTION

In vitro DNA synthesis by some prokaryotic and eukaryotic DNA polymerases is interrupted at specific nucleotide locations along the single-stranded DNA template (1-5). These arrest sites consist of at least two classes of sequences, palindromic and non-palindromic, that are equally effective at inhibiting DNA synthesis (6). Palindromic sequences act as arrest sites under conditions that form them into stable hairpin structures; the polymerase stops precisely at the base of the duplex DNA stem on both complementary templates, regardless of the sequence over which the enzyme travels. Non-palindromic sequences that function as arrest sites could not be explained either by formation of secondary structure or by the presence of direct or inverted repeats. DNA polymerases that recognize arrest sites appear to be those that are primarily responsible for DNA replication, indicating a possible biological role for DNA synthesis arrest sites.

In vivo DNA synthesis also pauses at specific nucleotide locations. These arrest sites are particularly prominent in the termination regions of

SV40 and polyoma virus where replication forks accumulate (6,7,29). The locations of 80% of the strongest in vivo arrest sites correlate with arrest sites recognized on the same DNA template in vitro (6). Similarly, 60% of the arrest sites observed in formation of D-loops during mitochondrial DNA replication correspond to DNA synthesis arrest sites observed in this sequence in vitro (5) and termination sites for DNA replication in plasmid R6K arrests replication forks both in vivo and in vitro (8). These results suggest that DNA synthesis arrest sites are recognized in living cells as well as in purified single-stranded DNA.

Therefore, we expected that DNA polymerases would pause at arrest sites in vitro even when DNA synthesis stimulatory proteins were present. To explore this hypothesis, phage T4 DNA polymerase was studied in the presence and absence of four proteins closely involved with the movement of this enzyme along a single-stranded DNA template. These are T4 gene products 32, 44, 45 and 62 (23,25,3,10). Gene 32 protein promotes unwinding of duplex DNA regions by binding to single-stranded DNA by preventing reannealing of complementary sequences (26,27). Gene 32 protein together with genes 44, 45, and 62 proteins stimulates the rate of T4 DNA polymerase activity on single-stranded DNA templates and increases the average length of DNA chains synthesized in a single initiation event (i.e. processivity, 13,14). Furthermore, gene 32 protein alone or together with gene 44, 45 and 62 proteins has been reported to eliminate DNA synthesis arrest sites, perhaps by melting hairpin structures (3,10). However, these results could be interpreted differently. We observed that T4 proteins 32, 44, 45 and 62 proteins did not alter the pattern of arrest sites observed with T4 DNA polymerase alone, although they did stimulate DNA synthesis on single-stranded DNA templates and increase processivity, as previously reported (3,10). Bromoacetaldehyde derivatization of single-stranded DNA (11) was adapted to demonstrate the existence of specific secondary structures at unique positions in the template sequence under the same conditions used to assay T4 DNA polymerase arrest sites. We conclude that stimulation of T4 DNA polymerase activity by these specific accessory proteins does not involve eliminating the enzyme's ability to recognize sequence-specific arrest sites in the template, but rather increases the rate and processivity of the enzyme over all DNA sequences.

MATERIALS AND METHODS

Unique primer-template combinations containing 5' or 3'-end labeled primers were constructed using M13 virion DNA containing various foreign DNA

inserts (1,6). Primer-templates used to map T4 DNA polymerase arrest sites were also used to optimize T4 DNA polymerase activity in the presence or absence of accessory proteins. Purified T4 DNA polymerase and its accessory proteins were generously provided by Dr. Bruce Alberts. All T4 DNA polymerase reactions were carried out under the conditions previously described (9,28) with details provided in the figure legends. Reactions were performed at 30 °C for 15 min and terminated in 20 mM EDTA. DNA was phenol extracted, precipitated with ethanol, and resuspended in 99% formamide, 0.5% bromophenol blue, 0.5% xylene cyanol FF. Samples were heated at 90 °C for 3 min to produce single-stranded DNA and then fractionated in an 8% polyacrylamide-8 M urea sequencing gel (1,6). Bromoacetaldehyde (BAA) was prepared as previously described (11).

RESULTS

Stimulation of T4 DNA Polymerase by Its Accessory Proteins on DNA

Primer Templates

Since previous studies on T4 DNA polymerase arrest sites focused attention on potential hairpin structures in the template (3,10) three unique primer-template combinations were selected which placed the initiation site for DNA synthesis 43, 18 or 1 bases upstream of a potentially stable region of duplex DNA (i.e. the stem of a hairpin structure). These unique primer-templates are referred to as I (Fig. 6A), II (Fig. 7A) and III (see below), respectively. DNA synthesis by phage T4 DNA polymerase alone and in the presence of T4 accessory proteins 45, 44/62, and 32 was optimized on primer-templates I and II by measuring the rate of incorporation of dNTPs into acid-precipitable DNA (Fig. 1). The rate of DNA synthesis increased in proportion to increased concentrations of T4 DNA polymerase. Optimum amounts of the genes 44/62 protein complex was determined in the presence of excess gene 45 protein, then gene 45 protein was varied and finally different concentrations of gene 32 protein were added. In several different experiments, the rate of DNA synthesis was increased 10 to 15-fold by addition of optimal amounts of gene 32 protein alone, while stimulation by genes 45 and 44/62 proteins together was only 2-fold. Addition of all four T4 DNA polymerase accessory proteins increased the rate of DNA synthesis 14 to 20-fold. The same results were also obtained with primer-template II. These results are consistent with previous observations on the the ability of these accessory proteins to stimulate the rate of DNA chain elongation by T4 DNA polymerase utilizing other primer-templates under the same reaction

FIGURE 1. DNA synthesis by T4 DNA polymerase (gene 43) on natural DNA primer-templates in the presence or absence of T4 DNA polymerase accessory proteins (genes 32, 44/62, and 45). T4 DNA polymerase was incubated with 80 µg/mL primer-template I (Fig. 6A) in the presence of [α-³²P]dCTP, dTTP, dGTP, and dATP (9,28). Acid-precipitable [³²P]DNA was measured over a 20 min time course at 30 °C. The concentration of T4 DNA polymerase (gene 43) was varied from 0.1 to 1.0 µg/mL (panel A, circles), and from 0.01 to 0.1 µg/mL (panel B, circles), as indicated. Some reactions contained gene 32 protein (51 µg/mL) alone (squares). Other reactions contained both genes 44/62 (0.04 µg/mL) and 45 (0.08 µg/mL) (panel B) to 0.8 (panel A) µg/mL. The high (panel A) or low (panel B) concentrations of all four accessory proteins were incubated together with 0.1 µg/mL T4 DNA polymerase (*).

conditions (9,3,10).

Primer-template III consisted of the template from primer-template II annealed with a 26 base primer (24) whose 3'-end was at position 19 in figure 7A. This primer was radiolabeled with 2 residues of [α - 32 P]dATP using AMV reverse transcriptase (1) moving its 3'-terminus to position 21, the entrance to a 14-15 bp duplex region in the template. Primer-template III was essentially inactive with T4 DNA polymerase. Little DNA synthesis was detected and at least 95% of a [$5'$ - 32 P]primer remained unused. Addition of genes 44/62, 45 and 32 proteins had no effect, even when T4 DNA polymerase, primer-template and accessory proteins were preincubated in the absence of dNTPs. In contrast, *E. coli* DNA polymerase I (Klenow fragment) utilized this primer-template efficiently. Therefore, T4 DNA polymerase, with or without its accessory proteins, was unable to initiate synthesis when the primer was adjacent to the stem of a potentially stable hairpin.

T4 DNA polymerase accessory proteins were also able to stimulate T4 DNA polymerase on a homopolymer homopolymer primer/template that lacked the capacity to form hairpin structures. Using oligo(rA₁₀):poly(dT), low concentrations of T4 DNA polymerase were stimulated about 2-fold by T4 genes 45 and 44/62 proteins, while addition of gene 32 protein stimulated DNA synthesis 3-fold (data not shown). Interestingly, high concentrations of genes 45 and 44/62 alone were stimulatory whereas addition of gene 32 protein caused inhibition, indicating that an optimal ratio of these proteins was required.

Processivity of T4 DNA Polymerase In the Presence or Absence of Its Accessory Proteins

T4 proteins 32, 44/62 and 45 have been reported to increase the processivity of T4 DNA polymerase, i.e. the average number of nucleotides incorporated per enzyme initiation event (22,10,3,13,14). A relatively simple processivity assay involving oligo(5'- 32 P-rA₁₀):poly(dT) as the primer-template has been previously described (12,16). In this assay, the relative number of initiation events that resulted in DNA chains of specific lengths is observed under conditions where a large excess of primer remains unused, confirming that DNA polymerase molecules reinitiated on radiolabeled primers rather than newly extended DNA chains. For example, low concentrations of *E. coli* DNA polymerase I (Klenow fragment) revealed an average processivity of 19 bases under these conditions (Fig. 2A, lane 4), and low concentrations of T4 DNA polymerase revealed an average processivity of 11 bases (Fig. 2A, lane 1). However, addition of all four T4 DNA

FIGURE 2. Length of DNA products synthesized by T4 DNA polymerase in the presence or absence of accessory proteins on oligo(5'-³²P-rA₁₀):poly(dT). Reaction conditions are the same as in Figure 1 except that the primer was radiolabeled instead of the dCTP substrate, and reactions were terminated at 10 min. DNA products were then fractionated in either 23% polyacrylamide-8M urea gel (panel A) or 6% polyacrylamide-8M urea gel (panel B) as previously

described (12). Lane 0 - poly(dA) chain length standards generated by incorporation of [α - 32 P]dATP into this primer-template with 0.1 μ g/mL T4 DNA polymerase. Lane 1 - 0.01 μ g/mL T4 DNA polymerase. Lane 2 - 0.01 μ g/mL T4 DNA polymerase, 51 μ g/mL gene 32 protein, 20 μ g/mL gene 45 protein, and 10 μ g/mL gene 44/62 proteins. Lane 3 - 0.1 μ g/mL T4 DNA polymerase. Lane 4 - 0.005 units/mL Klenow fragment.

polymerase accessory proteins to this concentration of T4 DNA polymerase resulted in a smaller number of initiation events but in chains that were about 100 times longer. Note the absence of radioactivity in the region of 1 to 20 residues, but the presence of [32 P]DNA at the top of the gel in figure 2A, lane 2, compared with the series of strong [32 P]DNA bands 6 to 14 residues in length in lane 1 and the absence of radioactivity at the top of lane 1.

The size of the [32 P]DNA at the top of the gel in figure 2a was estimated by electrophoresis of the same samples in a lower percentage of polyacrylamide (Fig. 2B). The small number of chains made by T4 DNA polymerase plus its accessory proteins (Fig. 2A, lane 2 top) were 800 to 1000 bases long (Fig. 2B, lane 2). Chains of this size were not made by high concentrations of T4 DNA polymerase alone. A 10-fold increase in the concentration of T4 DNA polymerase resulted in complete utilization of [32 P]rA₁₀ primer, synthesis of chains 10 to 50 residues long (Fig. 2A, lane 3), as well as a fraction of chains at the top of the gel (Fig. 2A, lane 3) which were up to 70 bases in length (Fig. 2B, lane 3). These data confirm the ability of T4 DNA polymerase accessory proteins to dramatically increase the processivity of T4 DNA polymerase, at least in the absence of potential hairpin structures.

Arrest of T4 DNA Polymerase at Specific Template Sequences in the Presence or Absence of Its Accessory Proteins

T4 DNA polymerase (1,3,10), like other replicative DNA polymerases (1,2,4-6), recognizes specific DNA sequences in the template as DNA synthesis arrest signals. To determine whether or not the T4 DNA polymerase accessory proteins eliminated the enzyme's ability to detect these arrest signals, the DNA products synthesized on several different primer-templates were examined at the level of nucleotide sequence. The primers were radiolabeled at their 3'-ends so that the intensity of the [32 P]DNA bands observed following gel electrophoresis reflected the number, rather than the length, of nascent DNA molecules. To insure that the rate limiting step in our measurements was elongation of DNA chains rather than initiation of synthesis at the primer, T4 DNA polymerase and its accessory proteins were

FIGURE 3. T4 DNA polymerase arrest sites on primer-template I. The primer in primer-template I (Fig. 6A) was extended with a single [α - 32 P]dTTP in the presence of AMV reverse transcriptase (1). Stimulation of T4 DNA polymerase by its accessory proteins was analyzed under conditions optimized for DNA synthesis on this primer-template, and the products fractionated by electrophoresis in a 8% polyacrylamide-8M urea gel. The relative concentrations of T4 DNA polymerase (p43; 1 = 0.01 μ G/mL), gene 32 protein

(p32; 1 = 10.2 μ G/mL), and genes 45, 44/62 proteins (p45,44/62; 1 = 0.004 and 0.002 μ G/mL) are given for gel lanes 2-9. Lane 1 shows the position of the radiolabeled primer. Primer-template I was sequenced using Klenow fragment and the dideoxynucleotide method of Sanger et al. (1978)(18). Lanes C,T,A,G identify the nucleotides in the TEMPLATE sequence where ddGTP, ddATP, ddTTP and ddCTP were used, respectively. Nucleotide positions 1, 12, 17, 47 and 64 are indicated as distances from the M13 primer where DNA synthesis was initiated. Nucleotide positions near the bottom of this gel were identified from other experiments in which this region was better resolved.

preincubated for 20 min at 30 °C with the primer-template in the absence of dCTP. This allowed formation of initiation complexes that incorporated the first 7 residues; subsequent addition of dCTP allowed chain elongation to proceed. Denatured [32 P]DNA products were fractionated according to length over a range of 150 nucleotides by gel electrophoresis, and the nucleotide template positions of their 3'-ends determined by comparison with positions where similar chains were terminated with dideoxynucleotides in the presence of *E. coli* DNA polymerase I (18,1,6). *E. coli* DNA polymerase I did not recognize any arrest signals in these templates (1,6).

Primer-templates I and II were used to determine the effect of T4 gene products 32, 45, and 44/62 on the ability of T4 DNA polymerase to traverse a major hairpin structure predicted on the basis of DNA sequence analysis. At low concentrations of T4 DNA polymerase, DNA synthesis did not proceed much past the first major arrest site at positions 12 and 13 in primer template I (Fig. 3, lane 2). At a 10-fold higher concentration of polymerase, DNA synthesis did not proceed much past the second major arrest site at positions 47 and 48 (Fig. 3, lane 9; Fig. 6A). Addition of gene 32 protein to the lower concentration of polymerase increased the lengths of nascent DNA chains, but did not diminish the effects of arrest sites (lanes 3 and 4). Higher concentrations of gene 32 protein inhibited DNA synthesis (lane 5), as previously observed with homopolymers. Addition of genes 32, 45 and 44/62 proteins stimulated DNA synthesis as indicated by increased chain sizes extending to the top of the gel, but the major arrest sites remained (lanes 6-8). Gene 45 and/or genes 44/62 proteins had little effect on DNA chain elongation in the absence of gene 32 protein (data not shown). Longer exposures of the gel in figure 3 revealed a large number of minor arrest sites that were also expressed in both the absence or presence of T4 DNA polymerase accessory proteins.

Similar results were obtained with primer-template II which contained two major arrest sites at the bases of hairpin structures (positions 17, 18 and positions 37, 38) in addition to the non-hairpin arrest site at position

LANE	1	2	3	4	5	6	7	8
p43		1	1	1	1	1	1	10
p32					1	5	25	5
p45, 44/62			25				25

FIGURE 4. DNA synthesis arrest sites on primer-template II. The primer of primer-template II was radiolabeled and DNA synthesis carried out as described in figure 3. The relative concentrations of T4 DNA polymerase (p43; 1 = 0.01 μ G/mL), gene 32 protein (p32; 1 = 10.2 μ G/mL), and genes 45,

44/62 proteins (p45,44/62; 25 = 0.08 and 0.04 $\mu\text{g/mL}$) are given for the gel lanes 2-9. Lane 1 shows the migration of the radiolabeled primer. The TEMPLATE sequence was determined as described in figure 3. Nucleotide positions 1, 7, 13, 17 and 37 designate distances from the M13 primer.

13, 14 (Fig. 7A). Increasing concentrations of gene 32 protein stimulated DNA synthesis and increased the average lengths of DNA chains, but did not eliminate arrest sites (Fig. 4, lanes 4,5). Higher concentrations of gene 32 protein inhibited DNA synthesis, although it also appeared to promote unfolding of the first hairpin structure (lane 6). Addition of genes 45 and 44/62 proteins, with or without gene 32 protein, stimulated DNA synthesis but most DNA chains were still arrested at the first hairpin (lanes 3 and 7). Accessory proteins did help the polymerase intrude into the hairpin about 6 bases, and did increase the number of chains that were arrested at the second hairpin site.

Primer-templates I, II and III were constructed primarily to test the effects of stable hairpins on DNA synthesis from a primer located varying distances upstream. Two additional primer-templates were examined that contained several strong arrest sites that bore no obvious relationship to stable hairpin structures. In primer-template IV, the first 25 bases were the same as those shown in primer-template II (Fig. 7A) while the template sequence from position 26 to 220 was the ϕX174 Hae III(8) DNA fragment (M13/ ϕX in ref. 1). The only duplex structure extends from position 19 to 25. T4 DNA polymerase encountered the same arrest sites at positions 13 and 17 as it did in primer-template II as well as 10 additional arrest sites which could not be correlated with a hairpin structure (Fig. 5, lanes 1 and 5). Addition of T4 gene 32, 45, and 44/62 proteins to T4 DNA polymerase had no effect on this pattern of arrest signals, although they did increase the distance the enzyme traveled (Fig. 5, lanes 2-4). Similar results were obtained with an M13mp5-parvovirus H1 recombinant DNA (Rsab9C2; ref. 6) that contained a very strong nonpalindromic arrest site (data not shown). The pattern of arrest signals observed by T4 DNA polymerase on these two primer-templates was the same as those recognized by DNA polymerase α (6).

Determination of Secondary Structure Using Bromoacetaldehyde

In an effort to establish that the predicted hairpin structures shown in figures 6A and 7A actually existed in solution under T4 DNA polymerase assay conditions, bromoacetaldehyde (BAA) modification of single-stranded DNA was adapted from the procedure described by Kohwi-Shigematsu and coworkers (11) to identify duplex regions in the template. BAA specifically acetylates N1

FIGURE 5. DNA synthesis arrest sites on primer-template IV. The structure of primer-template IV was the same as that for primer-template II except that ØX174 Hae III fragment 8 was inserted into the M13mp7 Hinc II site (1). Reaction conditions are described in figure 3.

and N6 of adenine and N3 and N4 of cytosine residues in single-stranded DNA under physiological conditions (11,17). We discovered that BAA modified single-stranded DNA templates arrested the progress of *E. coli* DNA polymerase I (Klenow fragment), one of the few DNA polymerases that ignore sequence specific arrest signals in natural DNA templates (1,6). Therefore, BAA was used to map regions of secondary structure in these templates in the following way. A unique 3'-end labeled primer-template such as the ones described in figures 6A and 7A was treated with limiting concentrations of BAA under the same conditions used to determine DNA synthesis arrest sites in order to randomly derivatize A and C residues. Ideally, one A or C residue per template would be modified. These modified primer-templates were used as a substrate for *E. coli* DNA polymerase I in the presence of all four deoxyribonucleoside triphosphates so that the enzyme synthesized DNA from a unique starting point through a specific DNA region. DNA synthesis terminated at modified A or C residues, presumably because these residues were not involved in base-pairing. The locations of modified A and C residues were determined by comparing the lengths of nascent DNA chains produced on BAA-modified templates with the sequence of nascent DNA synthesized by *E. coli* DNA polymerase I (Klenow fragment) on the same, unmodified primer-template in the presence of one of the four dideoxyribonucleotide substrates (18).

The hairpin structure predicted in primer-template I was confirmed by treating this DNA with increasing concentrations of BAA under T4 DNA polymerase assay conditions. The unmodified template revealed no DNA arrest sites for *E. coli* DNA polymerase I under these conditions (Fig. 6B, lane 1); all of the radiolabeled primer was extended to chains longer than 300 bases and thus remained at the top of gel (not shown). As the BAA concentration was increased, DNA synthesis was terminated at many nucleotide locations (Fig. 6B, lanes 2-5), all of which corresponded to A or C residues in the template. The intensity of termination sites increased with increasing BAA, indicating that a greater fraction of A and C residues were modified.

Not all of the available A and C residues in the template region examined were modified (Fig. 6). Primer-template I could be folded to accommodate most of the modified A and C residues in single-stranded regions of the template and the unmodified A and C residues in duplex regions of the template (Fig. 6A), revealing the hairpin structure predicted from inspection of the DNA sequence using parameters previously described (1,6). The base of this hairpin corresponded to the strong T4 DNA polymerase arrest site (Fig.

FIGURE 6. Structure of Primer-Template I. Panel (A): Primer-template I was constructed by annealing M13mp2 circular, single-stranded virion DNA with a 15 base primer (New England BioLabs) that was complementary to the sequence 18 bases from the Eco RI cloning site (15). This provided a primer-template

in which the 3'-end of the primer was 43 bases from a 13 base-pair duplex DNA region that would result if the hairpin structure existed under our assay conditions. The 3'-end of this primer was labeled by incorporation of a single [^{32}P]dTMP using [α - ^{32}P]dTTP and AMV reverse transcriptase (T^{*}; ref.6). In the absence of dCTP, preincubation of this primer extended it to position 7 (arrow). Closed circles indicate C and A residues that were modified by BAA, open circles indicate C and A residues that were protected from BAA. Major arrest sites are enclosed in boxes. Only that portion of the sequence that could be accurately read from the gel is shown. Panel (B): The TEMPLATE sequence was determined as described in the legend to figure 3. Lane 6 shows another aliquot of primer-template I that was incubated with 10 $\mu\text{g}/\text{mL}$ of T4 DNA polymerase as described in figure 3. Lanes 1 to 5 contain primer-template I that was reacted with 0, 1, 8, 16 and 32 mM BAA, respectively, for 15 min at 30 $^{\circ}\text{C}$ in the same solution used for T4 DNA polymerase assays, except that deoxyribonucleotides were omitted. BAA-modified primer-templates were isolated by precipitation in ethanol, washed with 70% ethanol, and resuspended in 40 mM KPO_4 (pH 7.5), 6.6 mM MgCl_2 , 1 mM 2-mercaptoethanol, and 50 μM each of dATP, dGTP, dCTP and dTTP. The primer was then extended by addition of 1 unit of *E. coli* DNA polymerase I (Klenow fragment, New England BioLabs) for 10 min at 37 $^{\circ}\text{C}$, adjusted to 20 mM EDTA, extracted with phenol, precipitated with ethanol, and then resuspended in 5 μL 99% formamide, 0.5% bromophenol blue, and 0.5% xylene cyanol FF. The sample was heated at 90 $^{\circ}\text{C}$ for 3 min to denature the DNA, and then fractionated by electrophoresis in an 8% polyacrylamide-8 M urea gel (1,6).

6B, lane 6) described above. Since the A and C residues in the duplex stem region of the hairpin structure were unmodified at high, as well as low, concentrations of BAA, no significant transient melting of these regions appeared to occur under the conditions of the T4 DNA polymerase assay. However, four A residues that resisted modification were not associated with a readily identifiable secondary structure (positions 23, 26, 45, and 61 in Fig. 6). These anomalies may reflect abnormal base pairing (30) or perturbations in single-stranded DNA that enhance the accessibility of some bases while diminishing the accessibility of others.

Primer-template II was also examined for secondary structure with increasing concentrations of BAA under the same conditions used for T4 DNA polymerase assays (Fig. 7B, lanes 1-5). Unmodified A and C residues defined three regions of the template involved in secondary structure, nucleotides 18 to 35, 38 to 44, and 53 to 59 (Fig. 7A). The first region could be represented as duplex DNA by base pairing with a sequence 224 bases away. This structure results from the M13 polylinker region present at each end of the ϕX174 DNA insert (15). The formation of this region of secondary structure in single-stranded m ϕX03 virion DNA solution was confirmed by excising the insert DNA by cleavage with Bam HI restriction endonuclease (6). Previous workers have shown that recombinant M13 phage DNA containing this

FIGURE 7. Structure of Primer-Template II. Panel (A): Primer-template II was constructed by annealing m0X03 with a 15 base primer (Fig. 6) complementary to the M13 sequence 18 bases from the Eco RI cloning site. The 3'-end of this primer was 18 bases from a potential duplex DNA region. Symbols are the same as in figure 6. Panel (B): Aliquots of primer-template

II were treated as described in figure 6B. Lanes 1 to 5 reveal the A and C residues that were modified by BAA and thus arrested *E. coli* DNA polymerase I. Lane 6 shows the two major arrest sites for 10 $\mu\text{g/mL}$ T4 DNA polymerase on unmodified primer-template II. Lanes C, T, A and G reveal the TEMPLATE sequence as described in the legend to figure 3.

polylinker can be cut within the linker by the appropriate restriction endonuclease (19,20). Since the 17 bp region of duplex DNA beginning at position 18 actually exists under the T4 DNA polymerase assay conditions, it was most likely responsible for the strong T4 DNA polymerase arrest site at position 17-18 (Fig. 7A, and 7B, lane 6). The second and third protected regions can form a second hairpin with a 7 bp stem and 8 base loop. The 5 A and 1 C residues in the loop (positions 46-51) are readily modified (Fig. 7B), demonstrating the single-stranded nature of the loop. Only the adenine at position 18, which should be available for BAA modification, does not appear to fit the pattern. However, proximity to the strong signal at cytosine 17 may have affected detection of a signal at position 18.

DISCUSSION

Phage T4 DNA polymerase accessory proteins, the products of T4 genes 32, 44, 45 and 62, were found to stimulate T4 DNA polymerase activity and processivity, as previously reported by others. The optimal amounts of accessory proteins were determined on each template examined, and stimulation of total incorporation of dNTPs ranged from 14 to 20-fold (Fig. 1), similar to previous experiments (3,10). These accessory proteins also stimulated synthesis on oligo(rA₁₀):poly(dT) about 3-fold, demonstrating that their function was not solely to eliminate base-paired secondary structure in the template. With this primer-template, T4 DNA polymerase incorporated an average of 11 dNTPs per initiation event, as previously reported for natural DNA templates (21), and processivity was increased 80-100 fold by addition of the accessory proteins (Fig. 2), as previously reported for natural DNA templates (13,14). Increased processivity was also apparent with primer-templates I and IV; 0.01 $\mu\text{g/mL}$ of T4 DNA polymerase in the presence of accessory proteins synthesized longer DNA chains than did 0.1 $\mu\text{g/mL}$ of T4 DNA polymerase alone (Figs. 3 and 5).

Using a low ratio of T4 DNA polymerase to primer-template in order to prevent reinitiation on the same nascent DNA chain, the same palindromic (Figs. 3,4) and nonpalindromic (Fig. 5) arrest sites were recognized by T4

DNA polymerase in the presence or absence of T4 gene 32, 44, 45, and 62 proteins. The presence of a stable hairpin structure downstream from the primer did not interfere with DNA synthesis or stimulation by accessory proteins unless the primer was located at the base of the hairpin (e.g. primer-template III). The relative frequency at which polymerase paused at each arrest signal was the same in the presence or absence of these accessory proteins, although the enzyme traveled further on the same template in a shorter amount of time in the presence of accessory proteins. This was apparently accomplished by an increase in processivity, resulting in the enzyme pausing at arrest sites without falling off the template, and/or by increasing to the same extent the rate of translocation of the enzyme from one nucleotide to the next, regardless of sequence.

Similar experiments have led other investigators to the conclusion that these T4 accessory proteins specifically eliminate DNA synthesis arrest sites (3,10). However, two important differences exist between previous experiments and the ones described above that could explain the contradictory conclusions. First, the ratio of T4 DNA polymerase to primer-template was about 8000 times greater in previous experiments. Thus, the rate of reinitiation on the same template was about 8000 times faster, producing much longer DNA chains in the same period of time and thus allowing polymerase to pass through regions of arrest sites more quickly. Second, nascent DNA chains were uniformly labeled with [α - ^{32}P]dNTPs. This means that the total amount of [^{32}P]DNA increased with time and amount of radiolabel per DNA chain increased in proportion to chain length. Thus, it is difficult to compare the heavy bands of [^{32}P]DNA that appear at the top of the gels in longer incubations with the light bands that appear lower in the gels at shorter incubations. These problems make it difficult to determine from these data whether accessory proteins specifically suppressed arrest signals or simply allowed synthesis to proceed more rapidly over the entire template, as suggested above.

Although T4 DNA accessory proteins 32, 45 and 44/62 failed to eliminate sequence-specific arrest signals from a single-stranded template, it is possible that additional phage or cellular proteins that associate with the replication complex are necessary for a sequence-independent DNA elongation reaction (31). However, since DNA replication forks pause at specific template sites in vivo (see Introduction), our results are consistent with the hypothesis that DNA synthesis pauses at specific sequences in vivo as well as in vitro.

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Present addresses: ¹Creative Biomolecules, 35 South Street, Hopkinton, MA 01748 and ²Whitehead Institute and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142, USA

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