Characterization of Strand Displacement Synthesis Catalyzed by Bacteriophage T7 DNA Polymerase*

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The DNA polymerase induced after infection of Escherichia coli by bacteriophage T7 can exist in two forms. One distinguishing property of Form I, the elimination of nicks in double-stranded DNA templates, strongly suggests that this form of the polymerase catalyzes limited DNA synthesis at nicks, resulting in displacement of the downstream strand. In this paper, we document this reaction by a detailed characterization of the DNA product.

DNA synthesis on circular, duplex DNA templates containing a single site-specific nick results in circular molecules bearing duplex branches. Analysis of newly synthesized DNA excised from the product shows that the majority of the branches are less than 500 base pairs in length and that they arise from a limited number of sites. The branches have fully base-paired termini but are attached by two noncomplementary DNA strands that have a combined length of less than 30 nucleotides. The product molecules are topologically constrained as a result of the duplex branch. DNA sequence analysis has provided an unequivocal structure of one such product molecule. We conclude that strand displacement synthesis catalyzed by Form I of T7 DNA polymerase is terminated by a template-switching reaction. We propose two distinct models for template-switching that we call primer relocation and rotational strand exchange.

Strand displacement synthesis catalyzed by Form I of T7 DNA polymerase effectively converts T7 DNA circles that are held together by hydrogen bonds in their 160-nucleotide-long terminal redundancy to T7-length linear molecules. We suggest that strand displacement synthesis catalyzed by T7 DNA polymerase is essential in vivo to the processing of a T7 DNA concatemer to mature T7 genomes.

In the previous paper, we reported the existence of two forms of purified T7 DNA polymerase (1). Although no structural differences have been found for the two forms, they can be differentiated on the basis of their activities. Form I has low specific activities of both single- and double-stranded DNA exonuclease activities relative to Form II. Equally pronounced is the lack of stimulation by T7 gene 4 protein of DNA synthesis catalyzed by Form II on nicked,1 duplex templates; gene 4 protein stimulates DNA synthesis catalyzed by Form I manyfold on such a template. In addition, evidence was presented that Form I can use a 3'-hydroxyl group at a nick as a primer to polymerize nucleotides, a reaction that leads to displacement of a 5'-terminated strand as synthesis proceeds along the duplex. In this paper, we characterize the strand displacement synthesis catalyzed by Form I of T7 DNA polymerase.

The measured differences between the two forms suggest that Form II is most appropriate for catalyzing reactions that take place at the replication fork. Form II will not polymerize nucleotides starting at nicks; it has a high level of a proofreading 3' to 5' exonuclease activity, and, as shown in the fourth paper of this series (2), DNA synthesis at gaps1 in DNA is limited to the filling of the gap, leaving a nick in the duplex.

On the other hand, strand displacement reactions may be relevant to other processes of DNA metabolism such as DNA maturation and recombination. For example, concatemers of T7 DNA are composed of a series of directly repeating monomer units, each of which contains the sequence of an entire T7 genome minus one of the 160-base pair terminal repeats (3). Watson (4) has suggested a model for the maturation of T7 concatemers in which an endonuclease makes a nick in each strand at the 5' ends of each terminal repeat to provide primers for DNA polymerase. Mature T7 DNA molecules are then generated by DNA synthesis coupled to strand displacement. Here we show that the strand displacement reaction of Form I can effect such an event.

In addition to providing information on the physiological significance of Form I of T7 DNA polymerase, the studies described here provide additional insight into the mechanisms leading to strand displacement. We show that the end product of the strand displacement reaction is, as is also the case for Escherichia coli DNA polymerase I (5), a duplex branch. Furthermore, the use of templates containing nicks at a unique sequence has made possible a detailed analysis of these branches, including the determination of the nucleotide sequence of one. Our results suggest that a duplex branch can arise as a result of either primer relocation or a rotational strand exchange.

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1 A nick in DNA is defined as a single phosphodiester bond interruption that contains a 5'-phosphoryl and a 3'-hydroxyl end group. A gap in DNA is defined as an internally located length of single-stranded DNA in a duplex DNA molecule.
**EXPERIMENTAL PROCEDURES**

**Materials**

**Bacterial Strains and Bacteriophage**—Wild type bacteriophage, T7 phage, and E. coli carrying plasmid pBR322 (7) was obtained from W. Studier (Brookhaven National Laboratories). E. coli carrying plasmid pBR322 (7) was obtained from W. Gilbert (Harvard University). The plasmid pMB9 was constructed by Rodriguez et al. (8).

DNA Preparations—plasmid DNA was prepared from cells which had been selected and grown on tetracycline-containing media (7), and an additional CsCl equilibrium centrifugation step was included after removing ethidium bromide. Micrographs were taken on a Zeiss EM10 electron microscope.

**Identification and Isolation**—The nucleotide sequence of the snapback DNA in the band labeled G4ATG in Fig. 6 was determined as follows. EcoRI-nicked pBR322 DNA (10 µg) was incubated with Form I of T7 DNA polymerase (2.5 units) in a 0.4-ml reaction mixture containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM 2-mercaptoethanol, and 150 µCi of each dNTP. After incubation for 45 min at 30 °C, EDTA was added to a final concentration of 10 mM, and the DNA was extracted with phenol followed by chloroform/isooamyl alcohol (24:1). The extracted DNA solution was then concentrated by centrifugation and passed over a Sephadex G-100 column. The DNA in the pooled void volume of the column eluate was digested to completion with endonuclease HindIII which cleaves 29 bases distal to the EcoRI site. The DNA was then treated with 3.6 µl of a solution containing 0.3 M NaCl, 0.03 mM sodium citrate, and then incubated at 65 °C for 40 min. Finally, the annealed DNA was dialyzed extensively at 4 °C against 10 mM Tris-HCl (pH 7.9) and 1 mM EDTA.

**Electron Microscopy**—DNA samples were mounted for electron microscopy by the formamide-cytochrome c method of Davis et al. (19). In the experiments shown in Table I and Fig. 2, the spreading solution (60 µl) contained either 14.5 µl of a reaction mixture after exonuclease III treatment (see Fig. 2) or 10 µl containing 9 ng of pBR322 DNA (either panhandle or supercoiled) in 40 mM Tris-HCl (pH 7.8), 5 mM sodium acetate, 1 mM EDTA, brought to 40% formamide, 50 µg/ml cytchrome c, 0.1 M Tris-HCl (pH 8.6), 10 mM EDTA, and 5 µg/ml ethidium bromide. The reaction was heated in a boiling water bath for 2 min and chilled, ssucrose and bromphenol blue were added, and the reaction was photographed on a micrograph taken on a Zeiss EM10 electron microscope.

**RESULTS**

**Identification and Isolation of the Product of DNA Synthesis at Nicks**

In the preceding paper (1), we proposed that Form I of T7 DNA polymerase could catalyze the polymerization of nucleo-
tides using the 3'-hydroxyl group at a nick as a primer. We interpreted the ability of Form I to destroy a nick in DNA as a substrate for DNA ligase as indicating that this form of the enzyme can catalyze such a reaction since this reaction was dependent upon the addition of dNTP's, thus providing evidence for the displacement of the 5' terminus at a nick from its normal double helical conformation. Form II, on the other hand, does not catalyze such a reaction and thus resembles T4 DNA polymerase, a well documented example of a polymerase that does not catalyze strand displacement synthesis (5). As shown in the previous paper and as will be documented more quantitatively below, the amount of DNA synthesis catalyzed by Form I at nicks is very low. However, even very limited strand displacement synthesis on singly nicked, circular templates markedly changes their physical properties. We have taken advantage of this observation to separate the product of strand displacement synthesis from nicked templates in order to study its structure and properties.

**Detection of the Product by Gel Electrophoresis in the Presence of Ethidium Bromide.**—The topological constraint in covalently closed circular DNA molecules prevents a change in the number of rotations of one strand about the other. Thus, ethidium bromide causes a positive shift in the superhelical winding number of these DNA molecules since it unwinds the DNA helix upon intercalation. It has been shown that at subsaturating concentrations of ethidium bromide the relative electrophoretic mobilities of covalently closed circular SV40 DNA molecules having different numbers of superhelical turns before binding ethidium bromide are largely dependent upon the number of superhelical turns that result after binding of ethidium bromide (20). In the experiments described here, the ethidium bromide concentration (0.06 µg/ml) causes binding of less than 0.01 mol of dye/mol of nucleotide to nicked circular DNA and relaxed covalently closed DNA and less than 0.10 mol of dye/mol of nucleotide to negatively supercoiled DNA of less than 50 superhelical turns (21). Under these conditions, as shown in Fig. 1, negatively supercoiled pMB9 DNA migrates considerably faster than circular pMB9 DNA containing a single nick (lane 1). Relaxed covalently closed pMB9 DNA, prepared by incubation of nicked pMB9 DNA with DNA ligase, migrates faster than supercoiled DNA (lane 2).

When the nicked pMB9 DNA is used as a template for Form I of T7 DNA polymerase, one product of the DNA synthesis reaction can be identified by its mobility during electrophoresis on agarose gels in the presence of ethidium bromide. This product is the band labeled X in lane 3 of Fig. 1. It migrates slightly slower than relaxed, covalently closed circles, but faster than naturally occurring, negatively supercoiled DNA. The sample shown in lane 3 was incubated with DNA ligase prior to electrophoresis. Therefore, all of the nicked DNA must have been used as template-primer by Form I of T7 DNA polymerase since no DNA is observed at the position of relaxed covalently closed molecules, the product of ligase sealing of nicked circles. The product of DNA synthesis by Form I is produced in the same amounts even if DNA ligase and ATP are present at the onset of the reaction (data not shown).

Form II of T7 DNA polymerase, as expected from the results presented in the previous paper (1), does not produce a product that can be identified by gel electrophoresis. After incubation with Form II, the nicked pMB9 DNA is largely converted to covalently closed circles by incubation with DNA ligase (Fig. 1, lane 4); no species X is observed. Although not shown in Fig. 1, in the absence of ligase, no unique DNA product is observed and the addition of an equal number of units of Form II to a reaction mixture containing Form I did not greatly reduce the production of species X.

**Electron Microscopic Analysis of the Product.**—The properties of the product of synthesis catalyzed by Form I on nicked circles suggested that it was topologically constrained, yet it seemed unlikely that the molecule could be covalently closed since it could be formed in the absence of DNA ligase. In order to further characterize the product, the DNA labeled X in Fig. 1 was eluted from a gel and examined by electron microscopy after removal of ethidium bromide. All of the molecules observed were circular duplexes, and approximately 40% had a single branch arising from the circle; the lengths of the branches were too short for accurate measurement but were most less than 5% of the length of the 4362-bp pBR322 molecule. Two examples of typical branched circular molecules are shown in Fig. 2A. We refer to the branch as a "panhandle."

In order to confirm that the product molecules isolated from the gel are indeed topologically constrained, the DNA was also prepared for electron microscopy in the presence of an amount of ethidium bromide known to be sufficient to

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2 The abbreviation used is: bp, base pair.
create positive supercoiling of covalently closed, circular DNA molecules (22). Of the molecules observed, 76% appeared to be supercoiled molecules as shown in Fig. 2B. These topologically constrained, circular molecules are not covalently closed since the constraint is removed by incubation with E. coli exonuclease III. Exonuclease III is specific for double-stranded DNA and hydrolyzes phosphodiester bonds sequentially from the 3' end (23). After limited treatment with bromide (Fig. 2C, Table I). Incubation with exonuclease III was without effect on the covalently closed supercoiled pBR322 DNA molecules.

These results, taken together, show that the product molecules arising from DNA synthesis at a nick on a circular DNA molecule are not covalently closed. We conclude that the DNA circles are topologically closed by virtue of having a double-stranded branch. This branch, which we call a pan-handle, connects the 3' and 5' termini of the interrupted DNA strands in these molecules, thereby establishing a constant topological winding number (21).

**FIG. 2.** Electron microscopic analysis of the product of DNA synthesis catalyzed by Form I of T7 DNA polymerase. pBR322 DNA (0.6 μg) containing approximately 1 nick/molecule was incubated in a 60-μl reaction mixture containing 40 mM Tris-HCl (pH 7.5), 20 mM MgCl2, 20 mM 2-mercaptoethanol, 300 μM each of dGTP, dATP, dCTP, and dTTP, and 0.15 unit of Form I of T7 DNA polymerase. After incubation at 30 °C for 50 min, electrophoresis of the products of the reaction was carried out in a 0.8% agarose gel and dialyzed extensively to remove ethidium bromide (Sample B). The most likely structure that would account for the properties of the product synthesized by Form I of T7 DNA polymerase at nicks. A, pBR322 DNA singly nicked with EcoRI serves as a primer-template for Form I of T7 DNA polymerase. DNA synthesis along the circular template strand gives rise to DNA having the sequence c b a'. B, incubation of the product molecules with EcoRI eliminates the covalent attachment of newly synthesized DNA to the circular template (Fig. 3, Structure A). C, subsequent denaturation by heat or alkali disrupts the hydrogen bonding and releases the newly synthesized DNA. The self-complementary sequences in the newly synthesized DNA allow the DNA to renature rapidly to form a hairpin structure (snapback DNA).

**Analysis of the Structure of the Newly Synthesized DNA**

The most likely structure that would account for the properties of the product synthesized by Form I of T7 DNA polymerase is a duplex branch on the circular template (Fig. 3, Structure A). Such a branch may arise by strand displacement during DNA synthesis from the nick followed by strand switching and polymerization on the displaced single strand. In order to characterize the product further, we have taken
advantage of the single EcoRI restriction site in pBR322 DNA. In these experiments, pBR322 dimeric DNA was nicked with the restriction endonuclease EcoRI and then used as a primer-template for Form I of T7 DNA polymerase. The newly synthesized DNA, radioactively labeled using \([\alpha^{32}P]dNTP\) substrates, can then be excised from the primer-template by incubation with EcoRI endonuclease since DNA synthesis will regenerate the EcoRI restriction site (Fig. 3).

**Newly Synthesized DNA Can Be Separated from Primer-Template by Site-specific Cleavage followed by Denaturation—** The newly synthesized DNA can be converted to a single-stranded linear form by denaturation of the product, confirming the presence of ends. Singly nicked DNA circles were incubated with Form I of T7 DNA polymerase and \([\alpha^{32}P]dATP\) to generate panhandles as described above. As shown in Fig. 4, lane 2, denaturation of the product molecules gave rise to a wide radioactively labeled region in the gel. At a lower exposure, two bands are observed in this region, each representing one of the two complementary nicked strands of pBR322.

When the product molecules were cleaved with EcoRI and then analyzed on a gel, nearly all of the radioactive newly synthesized DNA was found in duplex, linear monomers of pBR322 (Fig. 4, lane 3). A small portion of the newly synthesized DNA was released from the template by this treatment and may be derived from “hairpin” branches (see “Discussion”). The majority of this newly synthesized DNA is released from the template only if the EcoRI cleavage is followed by denaturation (Fig. 4, lane 4); the radioactive label is found in a collection of bands corresponding to small DNA fragments (labeled nascent in Fig. 4) whose length and structure are analyzed in more detail in the following sections. In this experiment, T7 DNA polymerase was not inactivated prior to EcoRI treatment and as a consequence, the recessed ends of pBR322 unit length restriction fragments were end-labeled and the separated strands of these molecules appear in lane 4.

We conclude from these data that the newly synthesized DNA is all covalently attached to primer-template at the EcoRI site. Although the restriction endonuclease EcoRI can cleave all of the newly synthesized DNA from its primer, cleavage is not sufficient to release the newly synthesized DNA from its template. Denaturation after cleavage releases the newly synthesized DNA, indicating that it is hydrogen-bonded to the template. The results are then consistent with the branched DNA structure shown in Fig. 3, A and B.

**The Duplex Branches Have Fully Base-paired Termini—** The relatively high concentration of T7 DNA polymerase in these experiments ensures that all of the branches are fully base paired at their termini as depicted in Fig. 3A. According to the model shown in Fig. 3, release of the newly synthesized DNA from the template by cleavage with EcoRI and denaturation should be followed by the rapid formation of a hairpin structure once the denaturing condition is removed (Fig. 3C). Such a structure arises because the two halves of the newly synthesized DNA are complementary to one another.

The pattern shown in Fig. 5, EcoRI-nicked circles were used as a primer-template for Form I of T7 DNA polymerase in a reaction containing \([\alpha^{32}P]dNTPs\). After digestion with EcoRI, the product molecules were denatured and analyzed on non-denaturing 8% polyacrylamide gels. The newly synthesized DNA appears as a number of discrete bands ranging in length from approximately 106 to 500 bp (Fig. 5, lane 2). This is the same set of fragments that was less resolved in the experiment shown in Fig. 4.

In order to determine if the 3’ and 5’ ends of the newly synthesized DNA are flush as depicted in Fig. 3, we incubated a portion of the EcoRI-digested and denatured DNA with T4 DNA ligase. T4 DNA ligase will catalyze the covalent joining of two duplexes provided they have perfectly matched base pairs at their termini (24). The majority of the newly synthesized DNA fragments were substrates for the end-joining reaction of T4 DNA ligase (Fig. 5, lane 2). The pattern of the slow migrating DNA product obtained from the ligase reaction and shown in lane 2 is not affected by incubation with either T7 gene 6 exonuclease or E. coli exonuclease III, thus indicating the absence of any 3’ or 5’ termini (data not shown). From these data, we conclude that the duplex branches have fully base-paired or flush termini. In the subsequent analysis of the product, we refer to the rapidly renaturable newly synthesized DNA obtained after EcoRI cleavage as snapback DNA.

**Length and Duplex Content of Snapback DNA—** Since most of the newly synthesized or snapback DNA is released from its primer-template only after denaturation, we conclude that it is hydrogen-bonded to both strands of the primer-template as diagrammed in Fig. 3. In order to determine if the duplex structure of the newly synthesized DNA is interrupted at the branch point, we have used S1 nuclease to test for the presence of internal single-stranded regions in snapback DNA.
Strand Displacement Synthesis by T7 DNA Polymerase

Radioactively labeled snapback DNA, prepared as described in the previous section, was incubated in the presence and absence of S1 nuclease and then analyzed by electrophoresis through an 8% polyacrylamide gel. Densitometer tracings of autoradiograms of these gels are shown in Fig. 6A. Treatment with S1 nuclease reveals two important properties of snapback DNA. First, the DNA is predominantly duplex since the DNA in both reaction mixtures was precipitated with ethanol after the addition of carrier tRNA. The precipitate was redissolved and then analyzed by electrophoresis on an 8% acrylamide gel (see “Experimental Procedures”). The other half of the reaction mixture containing heat-denatured product was precipitated and analyzed in an identical manner except that it received no T4 DNA ligase (lane 1). DNA length markers (lane m) were prepared by digesting plasmid pAR111 DNA with Hinfl and radioactively labeling the products in an end-filling reaction with Form II of T7 DNA polymerase under the same conditions as the experimental reaction. L and R, leftward and rightward bands described under “Discussion.”

Radioactively labeled snapback DNA, prepared as described in the previous section, was incubated in the presence and absence of S1 nuclease and then analyzed by electrophoresis through an 8% polyacrylamide gel. Densitometer tracings of an autoradiogram of these gels are shown in Fig. 6A. Treatment with S1 nuclease reveals two important properties of snapback DNA. First, the DNA is predominantly duplex since S1 nuclease has only a slight effect on the total radioactivity. Second, S1 treatment does produce a general shift toward a smaller size. For example, two peaks, one at a position of 132 base pairs, and the other at 107 base pairs, appear to be shifted by treatment with S1 to positions of 121 and 102, respectively (Fig. 6A). In control experiments (not shown), we have demonstrated that the snapback DNA shown in Fig. 6A, if denatured after S1 treatment, does not renature and migrates slower on these same gels than does a duplex fragment of the same length.

In summary, the data show that the snapback DNA fragments derived from panhandled circles have internally located, hinge-like, single-stranded regions that connect two complementary sequences. The data of Fig. 6 have been used to construct the frequency versus length distribution shown in Fig. 7. From the converted data, we calculate median “base pair” lengths of 156 before, and 134 after treatment with S1 nuclease. The overall distribution is indicative of the amount of strand displacement synthesis that takes place prior to template switching. This may be determined by the extent of synthesis before dissociation of the DNA polymerase from its primer-template (see “Discussion”). If there is one single-stranded region per snapback fragment, then the median length of this single-stranded region is less than 30 nucleotides.

Nucleotide Sequence of a Branch—In order to establish
definitively the structure of a branch, we have isolated a species of snapback molecule from an acrylamide gel and determined a portion of its nucleotide sequence. For this determination, we chose the easily isolatable snapback species corresponding to 132 base pairs (see Fig. 5, lane 1). A partial sequence, 44 nucleotides, is shown in Fig. 8. The nucleotides of the newly synthesized DNA are numbered starting from the EcoRI cut site which provided the 3'-hydroxyl-terminated primer. An interesting feature is the pentanucleotide sequence 5'-GGATG-3' at position 133 which we have labeled as the switch site. The pentanucleotide sequence 3'-CCTAC-5' at position 113 is complementary to the intact circular template at which synthesis switched complementarity is also present at position 116 in the displaced strand. Notice the boxed-in sequence 5'-CATCC-3' on the newly synthesized DNA which is the same as that on the displaced strand. We propose two models of template-switching that require a sequence such as that shown here that occur on both template strands. Such potential switching sites occur quite frequently in the DNA sequence (see “Discussion”).

Conversion of Hydrogen-bonded T7 Circles to Linear Molecules by Form I of T7 DNA Polymerase

The T7 DNA genome is a linear molecule of 39,936 base pairs terminated at each end by a directly repeated sequence of 160 base pairs (26). The terminal repeat provides the link in assembly of immature T7-length DNA molecules to form head to tail concatamers. In vitro, partial digestion of the 3'-hydroxyl termini of mature T7 DNA with E. coli exonuclease III leaves the complementary 5' termini free to anneal to one another (12). These molecules have essentially the same structure as the hydrogen-bonded concatamers which have been isolated from T7 infected-cells (27) and which have been postulated to be intermediates in the synthesis of mature T7 DNA molecules (4).

We have demonstrated that Form I of T7 DNA polymerase alone, by virtue of its strand displacement reaction, can synthesize through the 160-base pair repeat in hydrogen-bonded concatamers and thereby generate mature T7 DNA molecules. As shown in Table II, when hydrogen-bonded circles of T7 DNA are incubated with Form I of T7 DNA polymerase under conditions of DNA synthesis, most of the circular molecules (88%) are converted to linear molecules as scored by electron microscopy. Such a reaction occurs even in the presence of DNA ligase due to the efficiency of the strand displacement reaction.

The conversion of hydrogen-bonded circles to linear molecules by DNA synthesis catalyzed by Form I of T7 DNA can

![Fig. 7. Molecular frequency distribution of snapback DNA fragments before and after treatment with S1 nuclease. Both mass-weighted distributions of Fig. 6 were converted to molecular frequency distributions as follows. Partitions were drawn at equal intervals from 50 to 500 base pairs. The area of each partition was divided by its average base pair length and the quotient was plotted in a histogram after normalization to 100%. The resulting distributions represent the population: A, of snapback fragments before S1 nuclease digestion; and B, of snapback fragments after S1 nuclease digestion has removed single-stranded nucleotides. Our analysis indicates that the measured median, molecular length was decreased 22 base pairs by S1 nuclease digestion which is equivalent to approximately 30 nucleotides of single-stranded DNA (see Fig. 6).](image)

![Fig. 8. The nucleotide sequence of one panhandle branch. The product of synthesis catalyzed by T7 DNA polymerase Form I on EcoRI-nicked pBR322 DNA was prepared with unlabeled dNTP precursors and purified by passage over a Sephadex G-100 column. The DNA was digested with HindIII, denatured, and 5' end labeled using [γ-32P]ATP and T4 polynucleotide kinase. Next, the DNA was heated and rapidly chilled. The resulting snapback DNA was fractionated by polyacrylamide gel electrophoresis. A well separated DNA fragment, approximately 100 base pairs in length, was electroeluted and sequenced by the method of Maxam and Gilbert (Ref. 18; see "Experimental Procedures"). The markers above and below the sequence indicate the base pair position to the right of the EcoRI site in pBR322 (25).](image)

| Treatment | No. of molecules | Conversion* |
|-----------|----------------|-------------|
|           | Circular       | Linear      | %            |
| None      | 58             | 47          | 0            |
| DNA polymerase − dTTP | 65 | 43 | 0 |
| DNA polymerase + dTTP | 7 | 89 | 88 |

* Per cent conversion = 100 × (F1 − F3)/(1 − F3) where F1 is the fraction of linear molecules after treatment with T7 DNA polymerase and F3 is the fraction of linear molecules before treatment.
Strand Displacement Synthesis by T7 DNA Polymerase

be described as occurring in two steps. First, the polymerase extends the 3'-hydroxyl terminus generated by exonuclease III treatment, using the single-stranded gap as template. When polymerization reaches the terminal redundancy, the enzyme encounters duplex template. Form I of T7 DNA polymerase catalyzes strand displacement synthesis on nicked, duplex DNA templates, a reaction that is characteristic of other, but not all, DNA polymerases. For example, DNA polymerase I of E. coli catalyzes strand displacement synthesis at nicks, but only after its 5' to 3' exo activity has removed 20 to 50 nucleotides (5). The 5' to 3' hydrolytic activity is not, however, a prerequisite for strand displacement since elimination of the exo activity does not prevent this reaction (5). Bacteriophage T5 DNA polymerase also catalyzes strand displacement synthesis (28), but it incorporates far more nucleotides (approximately 10,000/nick) than does Form I of T7 DNA polymerase. On the other hand, DNA polymerases II and III of E. coli, in the absence of other proteins, are limited to gap filling as is the T4 DNA polymerase (5).

The mechanistic features required for strand displacement to occur during synthesis catalyzed by a DNA polymerase are not known. However, T7 DNA polymerase offers a unique opportunity to study this reaction since the enzyme can exist in two forms, only one of which can catalyze strand displacement synthesis. Since the secondary structure of duplex DNA in solution is dynamic (29), it does not appear unreasonable to assume that either form of T7 DNA polymerase would have a propensity to synthesize DNA from a nick. Instead of forcing open the helix during polymerization, the polymerase might wait for thermally induced hydrogen bond breakage and/or base pair unstacking. The high rate of branch migration gives credence to this idea (30).

As shown in the following paper (31), Form II of T7 DNA polymerase cannot catalyze strand displacement synthesis even if presented with a preformed displaced strand. If strand displacement synthesis can occur without actively forcing open the DNA duplex, then it seems likely that Form II has a mechanism by which it prevents this reaction. In this regard, its double-stranded 3' to 5' DNA exo activity could be an important part of a mechanism designed to prevent strand displacement. By the same logic, Form I of T7 DNA polymerase, whose exo activity has been subdued, would be capable of catalyzing strand displacement. Both the single- and double-stranded DNA exo activities of Form II are at least 30-fold higher than those of Form I. As yet we do not know what chemical change converts Form II to Form I. Relevant to this topic is the fact that the gene 5 protein holds the active site of the 3' to 5' single-stranded DNA-specific exo activity (32, 33). We do not know, however, whether the double-stranded DNA-specific exo activity, which apparently involves both subunits, shares part of its active site with the single-stranded DNA exo activity.

**Template Switching**—The fact that duplex branches are the final product of strand displacement synthesis implies that a switch from the original template strand to the complementary displaced strand has occurred. The duplex branches found with Form I of T7 DNA polymerase are similar to, albeit shorter than, those found in the product synthesized by E. coli DNA polymerase I (5) and T5 DNA polymerase (28). The studies we have described here with T7 DNA polymerase provide two new facts regarding the mechanism of template switching. First, from the nucleotide sequence, we find that a short stretch of single-stranded DNA exists at the branch point. This observation is not consistent with the model suggested by Kornberg (34) in which the polymerase transcribes from one strand to another without a contortion of the DNA. Second, the point at which synthesis switches from one strand to another is not random. Rather, it occurs at a site where a sequence on the original template strand is repeated on the displaced strand.

Our data are consonant with either of two alternative models for template switching which we shall call primer relocation and rotational strand exchange. Essential to both mechanisms depicted in Fig. 9 is the presence of a pair of short inverted repeat sequences separated by a variable number of nucleotides. The number of nucleotides between the inverted repeats determines the length of the single-stranded region at the branch point. The molecular mechanism by which strand switching occurs is different between the two models. In the case of primer relocation, the sequence of one inverted repeat at the 3' end of the growing strand anneals to the complementary sequence of the other inverted repeat on the displaced strand. In rotational strand exchange, the appropriate DNA intermediate arises by annealing of the sequences of the two inverted repeats within the displaced strand alone.

The individual steps involved in strand switching in the primer relocation model (Fig. 9) are as follows. 1) DNA polymerase binds to the 3'-hydroxyl terminus at a nick and initiates the polymerization of nucleotides. 2) DNA polymerase proceeds through the two inverted repeats, but dissociates from the growing strand after copying the second inverted repeat sequence. It should be noted that T7 DNA polymerase, in the absence of other replication proteins, is not highly processive and we assume that the dissociation event occurs randomly during synthesis. 3) Branch migration occurs, unwrapping the growing strand. 4) The inverted repeat at the 3' end of the now single-stranded growing strand anneals to the complementary sequence on the 5'-displaced strand to form a panhandle. Alternatively, it could anneal to the same sequence on its own strand to form a hairpin branch. 5) Finally, the reannealed 3'-hydroxyl end of the growing strand is used as a primer for T7 DNA polymerase where it completes the displaced branch. In Fig. 10A, we have depicted the structure of the final intermediate of the primer relocation reaction using the sequence of the branch determined in this study.

Template switching by rotational strand exchange is also shown schematically in Fig. 9. This mechanism involves the following steps, some of which are shared by the previous model. 1) As before, DNA polymerase initiates polymerization from the 3'-hydroxyl terminus of the nick. 2) After proceeding through the second inverted repeat, the polymerase dissociates from the growing strand. 3) At this point, the nucleotide sequences of both inverted repeats on the displaced strand have been exposed and can anneal to form a hairpin-like structure. 4) Rotation of the helical arms may make possible the alignment of the growing 3'-hydroxyl-terminated strand with the displaced strand at the point of the inverted repeat annealing. 5) Finally, the 3'-hydroxyl end of the growing strand is extended, using the displaced strand as template to create a crossed strand exchange between two homologous duplex DNA segments. Such an intermediate structure is depicted in Fig. 10B using the nucleotide sequence of the branch determined in this study. In the vicinity of the cross-
FIG. 9. Schematic representation of mechanisms of switching. As discussed in the text, thermally induced opening of the DNA helix may facilitate strand displacement synthesis at a nick. T7 DNA polymerase Form I cycles on and off the DNA, incorporating one or more nucleotides per cycle. Primer relocation (pathway on the left) involves the formation of two DNA intermediates. The first intermediate, which has two branches protruding from the same site, is formed by rewinding of the original displaced strand with concomitant unwinding of the 3' end of the newly synthesized strand. The second intermediate is formed by annealing the terminal nucleotide sequence of the 3' end of the newly synthesized DNA strand to any single-stranded complementary sequence. If annealing occurs to the 5'-terminated displaced strand, DNA polymerase could use it as a primer to synthesize a stable panhandle. Alternatively, the second intermediate could be formed by reannealing the 3'-terminal nucleotide sequence to a complementary sequence located internally on the same strand. This structure, if used as a primer by T7 DNA polymerase Form I, would give rise to a stable hairpin structure. Rotational strand exchange (pathway on the right) involves self-annealing of the original displaced strand between the sequence located at the replication fork and any complementary sequence closer to the 5' end. The duplex in the intermediate thus formed can stack with the unreplicated duplex by a rotational movement which simultaneously brings the displaced strand into a position adjacent to the hydrogen-bonded 3' end. Finally, the polymerase rebinds and extends the 3' end using the displaced strand as a template, a step which requires transversal of the strand crossover point. This gives rise to a fully hydrogen-bonded bihelical DNA structure with a cross-strand exchange (37) that is topologically equivalent to the panhandle derived by primer relocation (see Fig. 10 for an example).

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over point, this structure is essentially identical with the classic recombination intermediated proposed by Holliday (36). Studies with space-filling models have indicated that strands crossing from one double helix to the other can exist in a fully base-paired configuration (37). While this does not mean that they exist in a suitable configuration for DNA polymerase primer-template activity, the alignment of primer and template is reasonable.

In considering either model for strand switching, it is interesting to consider the role of the 3' to 5' single- and double-stranded DNA exonuclease activities of Form I of T7 DNA polymerase. Although these two activities are present in relatively low amounts in Form I, they probably contribute to either primer relocation or rotational strand exchange by removing any unpaired 3'-terminal nucleotides, thus providing DNA polymerase with a suitable hydrogen-bonded primer. As a result, greater flexibility in dissociation of the polymerase is possible. Studies with E. coli DNA polymerase I (38) and T4 DNA polymerase (39) have shown that such unpaired termini are removed quantitatively by the 3' to 5' exonucleases of these enzymes even in the presence of dNTPs.

Both models of template switching invoke the use of inverted repeat sequences in formally the same way. One can therefore predict, using either model, the location of all possible branches. Electrophoretic analysis of the newly synthesized DNA revealed the presence of a limited set of discrete bands corresponding to branches of discrete lengths (Fig. 5). To demonstrate that all of these bands could have arisen through a DNA intermediate involving an inverted repeat, we have made a computer-assisted search of the nucleotide sequence of pBR322 (25) 550 bases on either side of the EcoRI site. Template switching was simulated according to simple annealing rules which could apply to both primer relocation and rotational strand exchange. Our analysis, based upon a
the gel tracings in Fig. 6A. The DNA branch that was sequenced, and which arose as a consequence of the existence of denaturation. Some of the predicted snapback fragments are predicted branches can be tentatively identified with peaks in closely spaced, making their identification less certain, but it of the inverted repeat GGATG, is well separated from other is compelling that spacing between predicted groups of frag-

length scale of the gel tracings according to the length of the probability of specific annealing events. Most of the 18 possible switchover sites which could give rise to branches in the DNA intermediate proposed in the rotational strand exchange scheme (see Fig. 9). Note that this structure can be converted to the structure A by "rotary diffusion" (35) of the cross-connection point, a distance of five nucleotides to the left.

requirement for an inverted repeat sequence capable of forming a duplex containing at least 12 hydrogen bonds and separated by less than 30 bases (see Fig. 6), indicated 42 possible switchover sites which could give rise to branches with total lengths of 500 base pairs or less. This number of branches is more than sufficient to be consistent with our data since we see approximately 15 prominent bands ranging in length from 50 to 500 bp (Fig. 5). Other factors would certainly affect the abundance of specific branches. For example, if one assumes that competition with other sequences could slow annealing of the inverted repeats (see Fig. 6B), then the number of possible branches predicted is reduced to 18. Similarly, it seems likely that secondary structure would inhibit branch migration and that this in turn would reduce the probability of specific annealing events. Most of the 18 predicted branches can be tentatively identified with peaks in the gel tracing of Fig. 6 (compare A and B). Each predicted branch has been marked by a lettered square on the base pair length scale of the gel tracings according to the length of the DNA which would result after EcoRI cleavage and denaturation. Some of the predicted snapback fragments are closely spaced, making their identification less certain, but it is compelling that spacing between predicted groups of fragments, or individual ones, is closely reflected by the peaks of the gel tracings in Fig. 6A. The DNA branch that was sequenced, and which arose as a consequence of the existence of the inverted repeat GGATG, is well separated from other predicted panhandle fragments. Also well separated is the branch DNA which is predicted to have arisen from annealing of the inverted repeat of CCCC (Fig. 6B, square a). In fact, it is the only one predicted to have a duplex stem shorter than 115 bp with a loop length of less than 30 bases even if competitive annealing is not invoked as a limitation to our search. This predicted species matches well with the first prominent band seen in the autoradiograms (Fig. 6A). Finally, we must mention that, as indicated by the number-length distribution (Fig. 7), the short snapback fragments are a numerically larger fraction of the population, a result that is consistent with both models. It is possible that some of the small molecular weight peaks observed in the region between 50 and 100 nucleotides are the result of strand switching at sites having less than 12 hydrogen bonds. Although these sites would form less stable intermediates, they would, according to the model, form duplexes more often than those located further from the EcoRI site.

There are only two heavily labeled bands in the region from 450 to 500 base pairs (L and R in Fig. 5). Band L (leftward) could have arisen from relocation of either of both of the four-base inverted repeats, GGCG and CCCG, located at positions 457 and 490, respectively. Similarly, band R (rightward) could have arisen from the repeat of GGCG at position 506. It is interesting to note that, although there are some bands corresponding to molecules of lengths greater than 500 base pairs, none of these approaches the intensity of these two bands. Inspection of the sequence of pBR322 reveals that this higher molecular weight region is rich in GC base pairs. We suggest that Form I of T7 DNA polymerase makes slower progress through GC-rich duplex because of the unforced nature of its strand-displacing action. This effect, coupled with a low level of single strand-specific 3' to 5' exonuclease activity, could increase the frequency of strand switching in, or just preceding, a GC-rich region.

Role of Form I in vivo—Clearly, Form II of T7 DNA polymerase is an appropriate enzyme for most of the polymerization reactions occurring on both leading and lagging strands. In fact, under proper conditions, it polymerizes nucleotides faster than Form I and with higher fidelity (1, 31). It does not displace the 5' end of Okazaki fragments, allowing proper sealing by DNA ligase (2). On the other hand, there is no reason at present to conclude that Form I does not have a function in vivo. Both forms of the enzyme exhibit high fidelity (1) and, more important, some steps in T7 DNA replication would be best served by a polymerase that catalyzes limited strand displacement synthesis such as Form I. For example, a single-stranded tail such as that generated by the strand displacement reaction catalyzed by Form I could serve as an intermediate in recombination. Furthermore, from the DNA sequence of concatemers, it is almost certain that a strand displacement reaction such as the one proposed by Watson (4) must occur in order to generate the second copy of the terminal repeat sequence found at each end of a mature T7 genome. In fact, we demonstrated here that, in vitro, intramolecular (circular) hydrogen-bonded T7 DNA whose structure mimics that of a concatemer can be converted to a unit-length linear T7 DNA molecule by the strand displacement activity of Form I of T7 DNA polymerase. However, it is important to emphasize that we have not analyzed the detailed structure of the ends of the unit-length DNA generated in this reaction. Nucleotide sequence analysis of the ends of the T7 genome reveals the presence of at least seven inverted repeats that could potentially give rise to template switching prior to the completion of the strand displacement reaction. Clearly, further studies are necessary to investigate the effects of DNA-binding proteins or other replication proteins on the rate of strand displacement and template strand
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switching.

If both forms of T7 DNA polymerase do have physiological roles, it is likely that there are mechanisms to regulate, or minimize, the action of Form I at nicks and at gaps on the lagging strand side of the replication fork since strand displacement would be counterproductive. Two general mechanisms come to mind. First, the conversion of Form II to Form I might be timed to occur late in infection, when the maturation of concatemers occurs. Second, Form II may be converted to Form I only at the site where the activity of Form I is required. If this type of mechanism existed in the extreme, Form I would never be released as such from the DNA. If this were the case, the preparations of Form I could be considered artifactual even though its activity and basic structure were the case, the preparations of Form I could be considered artifactual even though its activity and basic structure are physiologically important.

Even if Form I does not play an essential role in T7 DNA metabolism, the presence of a small proportion of Form I in the T7-infected cell could give rise to a number of infrequent alterations, i.e., mutations in the viral DNA. Template switching catalyzed by Form I could facilitate both frame shift and template switching. The latter mechanism could, if fact, be a general one to give rise to new combinations of gene structure in both prokaryotic and eukaryotic cells.

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