Strand Displacement during Deoxyribonucleic Acid Synthesis at Single Strand Breaks

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SUMMARY

Synthesis of DNA by DNA polymerases has been studied by using circular duplex DNA templates which contain single phosphodiester bond interruptions (nicks). Escherichia coli DNA polymerase can initiate synthesis at nicks by the covalent attachment of nucleotides to the 3' end of the primer strand. An initial phase of synthesis is accompanied by the removal of nucleotides from the 3' end of the nick by the 5'-hydrolytic activity of the E. coli polymerase (nick translation). After the incorporation of 10 to 50 nucleotides, the 5' end of the primer strand is displaced by the growing 3' end of the primer strand. Elimination of the 5'-hydrolytic activity of the E. coli polymerase does not prevent this strand displacement. In contrast, phage T4 polymerase cannot initiate synthesis at nicks. In these studies polynucleotide ligase has been used to identify nicks and to measure their disappearance during strand displacement.

The DNA synthesized during the phase of strand displacement is sensitive to both exonucleases I and III of E. coli. This observation suggests that an equilibrium exists between single and double stranded forms of the newly synthesized DNA. A third phase of more extensive synthesis yields a DNA product which is resistant to exonuclease I even after denaturation, suggesting a coll complementary structure. Electron micrographs of such a product synthesized on circular PM2 DNA containing one nick per molecule reveal a single branch extending from the intact circle. Subsequent synthesis gives rise to multiple branches.

The preceding paper (1) described the repair of gaps in circular duplex DNAs by T4 DNA polymerase and by Escherichia coli DNA polymerase. Our results showed that both polymerases could initiate synthesis at gaps and replace all of the missing nucleotides so that subsequent incubation with polynucleotide ligase restored the continuity of the strands. It was also shown that the E. coli DNA polymerase, by virtue of its 5'-hydrolytic activity (2, 3), could convert a nick displaying a 5'-hydroxyl end group to one displaying a 5'-phosphomonoester through a mechanism of nick translation of the type described by Kelly et al. (4). In none of these instances, however, was there a net synthesis of DNA.

Earlier studies on net synthesis of DNA by purified E. coli DNA polymerase revealed that, when duplex DNA is used as a template, synthesis does not progress in an orderly fashion. Not only is the rate of synthesis slow (5), but the product DNA is non-denaturable (6), appears as a branched structure in electron micrographs (7), and is devoid of biological activity (8). In these early studies (6) the newly synthesized DNA could be separated from the template primer by denaturation, indicating that the product was not covalently attached to the primer. The studies described in this paper, as well as recent studies by Kelly et al. (4), show that a major part, if not all, of the synthesis observed with bihelical templates originates at 3'-hydroxyl groups which are located at nicks in the molecule. It is possible that either mechanical shear or contaminating endonucleases caused secession of the covalent bonds between the primer and the newly synthesized DNA in the earlier studies.

In an attempt to understand the mechanism by which the E. coli DNA polymerase can catalyze a net synthesis of DNA, we have examined its action at nicks in double stranded DNA. As discussed above, E. coli DNA polymerase is clearly capable of initiating synthesis at nicks in a reaction involving nick translation (Fig. 1, Reaction A). In this report we present evidence that, at a later stage in the reaction, the 5' end of the primer strand is displaced and as a result is conserved (Fig. 1, Reaction B). Synthesis subsequent to strand displacement leads to a double stranded branch. However, since the results presented in this paper suggest multiple equilibria between different structural forms of the newly synthesized DNA, the precise mechanism...
for the formation and the origin of the DNA in the branches is not known.

The present studies show that the DNA polymerase induced by phage T4 infection cannot use nicked DNA as template primer. The fact that E. coli polymerase has a 5'-hydrolytic activity which is lacking in the T4 polymerase does not explain the difference in primer requirement, since E. coli DNA polymerase, chemically altered so as to be devoid of 5'-hydrolytic activity, can still promote strand displacement and carry out a net synthesis of DNA.

As in the studies described in the accompanying paper (1), we have used circular duplex DNAs containing nicks as template primer. Poly(adenylic acid) has been used in a sensitive assay to detect strand displacement. The nicks present in Structures I or II shown in Fig. 1 can be covalently closed by ligase, but, once strand displacement has occurred as shown in Structure III, no closure can take place. The conversion of the nicked molecules to closed circular duplexes can be detected by alkaline sedimentation analysis (6).

**EXPERIMENTAL PROCEDURE**

**Materials**

Nucleic Acids and Nucleotides—Unlabeled deoxyribonucleoside 5'-triphosphates, ATP, S-32P-dATP (5 Ci per mmole), and S-32P-dCTP (54 mCi per mmole) were purchased from Schwarz Bioresearch. γ-32P-dATP (10 Ci per mmole) was purchased from International Chemical and Nuclear Corporation. 2-32P-dTTP (50 mCi per mmole) was prepared from 2-32P-deoxythymidine (New England Nuclear) as described by Okazaki and Kornberg (10). (Methyl-3H)-thymidine (10 Ci per mmole) was purchased from New England Nuclear. 5-Bromodeoxyuridine 5'-triphosphate was a gift of Dr. A. Kornberg.

Unlabeled and 3H-dX174 RF² DNA and unlabeled, 3H-, and 32P-P2 DNA were prepared as described in the preceding paper (1). Salmon sperm DNA was purchased from Sigma. DNA containing single strand breaks displaying 5'-phosphoryl or 5'-hydroxyl end groups was prepared as described in the preceding paper (1). Concentrations of DNA are expressed as equivalents of nucleotide phosphorus unless otherwise stated.

Enzymes—The preparations of E. coli DNA polymerase, phage T4 DNA polymerase, phage T4 polynucleotide ligase, and E. coli exonuclease I were described in the preceding paper (1). E. coli exonuclease III was the DEAE-cellulose fraction purified and assayed as described by Lehman and Nussbaum (11). Subtilisin was a gift of Dr. B. L. Vallee.

Other Materials—CsCl was purchased from the Harshaw Chemical Company. Sephadex G-100 and G-150 were purchased from Pharmacia.

**Methods**

DNA Polymerase Reactions—The incubation mixture (0.1 or 0.3 ml) for E. coli and T4 DNA polymerases contained 20 to 200 µM nicked DNA, 67 mM Tris-HCl buffer (pH 7.6), 6.7 mM MgCl₂, 10 mM 2-mercaptoethanol, 15 µM each of dTTP, dCTP, dGTP, and dATP, and 1 unit of either E. coli DNA polymerase or T4 DNA polymerase.

Normally one of the four deoxyribonucleoside triphosphates was replaced by a radioactively labeled deoxyribonucleoside triphosphate in order to measure the rate and extent of synthesis. The reaction mixture was incubated at 37° for varying periods of time as indicated in the text. The amount of DNA synthesized was determined by measuring the amount of acid-insoluble radioactivity in a sample of the reaction mixture as previously described (6).

Assay for Nicks—In several experiments DNA was incubated with polynucleotide ligase (12) to determine whether the interruptions in the DNA were nicks containing 3'-hydroxyl and 5'-phosphoryl end groups, and thus repairable by ligase. In some experiments in which the effect of DNA synthesis at the site of nicks was studied, 60 mM ATP and 0.03 unit of T4 ligase were added directly to the DNA polymerase reaction mixtures at varying times after incubation with polymerase. The reaction was allowed to proceed for an additional 20 min at 20°, then was stopped by making the mixture 15 mM in EDTA. Since all of the template primers were circular duplexes, the per cent of joining could be determined by measuring the formation of closed circular duplexes in an alkaline sucrose gradient or by a membrane filter assay as described in the preceding paper (1).

Preparation of E. coli Polymerase Lacking 5'-Hydrolytic Activity—Partial hydrolysis of E. coli DNA polymerase with subtilisin yields a fragment of 78,000 molecular weight which retains polymerizing activity and 3'-hydrolytic activity but not 5'-hydrolytic activity (13-15). E. coli polymerase was partially hydrolyzed with subtilisin according to the method of Klenow and Henningsen (14). The reaction mixture (0.3 ml) contained 200 units of purified E. coli DNA polymerase, 10 mM 2-mercaptoethanol, 65 mM potassium phosphate buffer (pH 7.5), 50 µg of crystalline bovine serum albumin (Armour), and 0.5 µg of subtilisin. After incubation for 30 min at 37°, the reaction mixture was fractionated by filtration through Sephadex G-100 as described by Klenow and Henningsen (14). The fractions containing polymerizing activity were free of 5'-hydrolytic activity (see "Results").

Electron Microscopy—Electron microscopic examination of DNA was carried out by the conventional protein monolayer method of Kleinschmidt (16). Photographs were taken by Dr. Motohiro Fuke with a Hitachi HS-7S electron microscope.

**RESULTS**

Inability of T4 Polymerase to Initiate Synthesis at Nicks

In the preceding paper (1) it was shown that T4 polymerase can completely fill in gaps in circular duplexes, a reaction similar
to the repair of single stranded regions at the termini of linear DNA molecules (17). In contrast, Goulain, Lucas, and Kornberg (17) have presented evidence that native duplex DNAs, such as T7 DNA, have little or no template activity. As shown in Table I, T4 polymerase, in contrast to E. coli polymerase, was unable to initiate synthesis at nicks in circular duplexes as measured by the formation of acid-insoluble radioactive nucleotides. The presence of a 5'-hydroxyl or a 5'-phosphoryl end group at the nick did not permit synthesis to occur. The lack of synthesis at a nick displaying a 5'-hydroxyl end group makes it highly unlikely that the nick has been eliminated by contaminating T4 ligase which can use dATP as a cofactor.3

The results given above indicate that T4 polymerase cannot catalyze an extensive synthesis on nicked circular templates. However, the polymerization of a few nucleotides at the nick would escape detection by the radioactive assay. A more sensitive assay makes use of the fact that T4 DNA ligase is highly specific for a nick. If one or more nucleotides are added to the 5' side of the nick, the resulting displacement of the 5' end of the strand (see Fig. 1) eliminates the nick and prevents joining. When T4 ligase and ATP were added to nicked circular DNA which had been previously incubated with T4 DNA polymerase and the four deoxynucleoside triphosphates, there was no decrease in the number of closed circular duplexes formed (Table II). In a control incubation it was found that the T4 DNA polymerase preparation alone can catalyze the covalent joining of 6% of the nicked circles. This suggests that T4 ligase, which uses ATP as cofactor, contaminates the polymerase preparation. When dATP was omitted from the polymerase reaction, repair activity was eliminated. However, the incorporation of a few nucleotides in a "limited reaction" (18) can still be expected to occur in the presence of three deoxynucleoside triphosphates. As shown in Table II, even with the inclusion of the deoxynucleoside triphosphates, there is no significant inhibition of joining. These results demonstrate that T4 DNA polymerase cannot initiate synthesis at nicks.

The standard T4 polymerase reaction mixture contained 25 µM 3H-PM2 DNA (two nicks per molecule), 20 µM deoxynucleoside triphosphates as indicated, and 0.6 unit of T4 polymerase. After incubation for 30 min, the DNA was precipitated, and the amount of acid-insoluble radioactivity was measured. As indicated below, the nicks in PM2 DNA displayed either 5'-phosphoryl (5'-P) or 5'-hydroxyl (5'-OH) end groups.

Table II

<table>
<thead>
<tr>
<th>Treatment prior to ligase</th>
<th>Closed circular duplexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>No T4 polymerase, plus dTTP, dCTP, dGTP, dATP</td>
<td>%</td>
</tr>
<tr>
<td>T4 polymerase plus dTTP, dCTP, dGTP, dATP</td>
<td>40</td>
</tr>
<tr>
<td>T4 polymerase plus dTTP, dCTP, dGTP</td>
<td>48</td>
</tr>
<tr>
<td>T4 polymerase plus dTTP, dCTP, dGTP, dATP</td>
<td>37</td>
</tr>
</tbody>
</table>

3 dATP, a competitive inhibitor of T4 ligase (12), although much less effective, can replace ATP as a cofactor for the enzyme (C. L. Harvey and C. C. Richardson, unpublished results).

The standard reaction mixtures for T4 and E. coli polymerase contained 8 µM 3H-PM2 DNA (two nicks per molecule) and 7 units of T4 polymerase and 4 units of E. coli polymerase, respectively. α-32P-dATP (6 × 106 cpm per µmole) was used as the labeled deoxynucleoside triphosphate. After incubation for 30 min, the DNA was precipitated, and the amount of acid-insoluble radioactivity was measured. As indicated below, the nicks in PM2 DNA displayed either 5'-phosphoryl (5'-P) or 5'-hydroxyl (5'-OH) end groups.

Table I

<table>
<thead>
<tr>
<th>Polymerase</th>
<th>5' End groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'-P</td>
</tr>
<tr>
<td>T4</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>E. coli</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Fig. 2. Measurement of hydrolysis of nicked PM2 DNA during the course of synthesis by Escherichia coli polymerase. The standard incubation mixture contained 7 µM α-32P-PM2 DNA (four nicks per molecule), 66 µM four deoxynucleoside triphosphates, and 1 unit of E. coli DNA polymerase. α-32P-dATP (3 × 106 cpm per µmole) was used as the labeled deoxynucleoside triphosphate. The reaction mixture was incubated at 37° and samples were removed for the determination of acid-soluble 32P (O- - -O)-labeled material (PM2 DNA) and acid-insoluble 3H (●●●●●)-labeled material (newly synthesized DNA).

Activity on Nicked DNA—In contrast to T4 DNA polymerase the E. coli polymerase can use nicked circular duplexes as primer templates. As shown in Table I, the enzyme can initiate synthesis at nicks bearing either 5'-phosphoryl or 5'-hydroxyl end groups. A more detailed examination of the kinetics of synthesis revealed that, given sufficient time, the polymerase could catalyze a net synthesis on DNA. As shown in Fig. 2, when 2 nmoles of nicked α-32P-PM2 DNA were incubated with DNA polymerase and the four deoxynucleoside triphosphates, there was a progressive synthesis of DNA. After 2 hours, 2.6 nmoles of DNA had been synthesized. Furthermore, only 20% of the input primer template was hydrolyzed to acid-soluble material during the incubation, the major portion of the hydrolysis oc-
currying during the initial 15 min of incubation. The early phase of synthesis, which is accompanied by the hydrolysis of DNA, probably reflects nick translation of the type described by Kelly et al. (4). Nevertheless, in this prolonged incubation synthesis far exceeds hydrolysis.

Cova lent Attachment of Product to Primer—The DNA synthesized on a nicked circular DNA template is covalently attached to the primer template as shown by pycnographic and sedimentation analysis.

Fig. 3. Pycnographic analysis of primer product: a, unfracti onated; b, rerun of hybrid; c, denatured. 3H-θX174 RF DNA containing one nick per molecule was used to prime a standard reaction (0.3 ml) containing dCTP, dGTP, 8-C-dATP, and 5 bromodeoxyuridine 5'-triphosphate and 1 unit of Escherichia coli DNA polymerase. Samples of the reaction mixture were removed during incubation at 37° and the extent of replication was measured as described under "Methods." When synthesis had progressed to 0.06 replication, EDTA was added to a concentration of 0.01 M and the reaction was diluted to 3.6 ml with 0.01 M Tris-HCl buffer (pH 7.5) containing 0.01 M NaCl and 0.001 M EDTA and then concentrated with Sephadex G-150 as described in the preceding paper (1). The DNA was then sedimented through a 5 to 20% CsCl sucrose gradient at 30,000 rpm for 16 hours at 4° in the type SW 50.1 Spinco rotor and analyzed as previously described (1).

Fig. 4. (left). Alkaline sedimentation analysis of primer product. The 0.06 replication product isolated by CsCl density gradient centrifugation (Fig. 3b) was dialyzed against 0.01 M Tris-HCl buffer (pH 7.5) containing 0.01 M NaCl-0.001 M EDTA and then concentrated with Sephadex G-150 as described in the preceding paper (1). The DNA was then sedimented through a 5 to 20% alkaline sucrose gradient at 30,000 rpm for 16 hours at 4° in the type SW 50.1 Spinco rotor and analyzed as previously described (1).

E. coli DNA polymerase was used to catalyze a 0.06 replication of 3H-θX174 RF DNA containing one nick per molecule. The newly synthesized DNA contained both a density label arising from 5-bromodeoxyuridine 5'-triphosphate and a radioactive label from 32P-dATP. The densities of the product and primer were determined by centrifugation to equilibrium in a CsCl density gradient both before and after alkaline denaturation (Fig. 3). The product DNA, before denaturation (Fig. 3a), had a significantly higher density than that expected for fully light DNA. The DNA was isolated and recentrifuged to equilibrium in CsCl before (Fig. 3b) and after (Fig. 3c) denaturation. Even after denaturation, the product containing 5-bromodeoxyuridine was significantly heavier than that expected for fully heavy single strands. After 1.0 replication with 5-bromodeoxyuridine 5'-triphosphate, the product DNA, although heterogeneous, banded at the density expected for hybrid molecules; the product banded at the same density after denaturation, suggesting a covalent attachment of the product to the primer even after extensive synthesis. Kelly et al. (4) arrived at a similar conclusion with an extensively replicated sample of nicked θX174 RF DNA.

Sedimentation analysis in alkali confirmed that the product was covalently attached to the primer. The DNA isolated from the neutral CsCl density gradient (Fig. 3a) was sedimented through an alkaline sucrose gradient. As shown in Fig. 4, the product had sedimentation properties identical with those of the primer. Since the size of the product would be only 12% (0.06 replication) of that of the primer DNA if DNA synthesis occurred to the same extent at all nicks, our findings suggest that the product is indeed covalently attached to the primer.

The number of replications is defined as the ratio of moles of synthesized DNA phosphorus to the number of moles of phosphorus in the template primer DNA.
Effect of ligase on DNA synthesis at nicks

The standard Escherichia coli polymerase incubation mixtures (0.3 ml) contained 4 μM PM2 DNA (two nicks per molecule), α-32P-dTTP as the labeled precursor, and 2 units of polymerase. The separate reaction mixtures were incubated at 37°C for 15 min with ligase and ATP was added at the times indicated as described under "Methods." The amount of DNA synthesized was determined by measuring the amount of acid-insoluble radioactivity.

<table>
<thead>
<tr>
<th>Effect of ligase addition at</th>
<th>DNA synthesized in 15 min (pmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>1.5</td>
</tr>
<tr>
<td>0.5 min</td>
<td>15</td>
</tr>
<tr>
<td>1.0 min</td>
<td>25</td>
</tr>
<tr>
<td>2.0 min</td>
<td>26</td>
</tr>
<tr>
<td>15 min</td>
<td>24</td>
</tr>
</tbody>
</table>

Displacement of 5' End of Primer Strand—The results described above suggest that E. coli DNA polymerase can initiate hydrolysis at nicks, and that the product of synthesis is covalently linked to the primer. Furthermore, since the amount of DNA synthesized exceeds the extent of hydrolysis (Fig. 2), a mechanism must exist for conservation of the primer strand. The experiments described in this section show that a nick is essential for the initiation of DNA synthesis, but that the nick is eliminated shortly thereafter, most likely by displacement of the 5' end of the primer strand (see Fig. 1).

Nicked PM2 DNA serves well as a primer for E. coli DNA polymerase. However, if the nicks are repaired by incubation with T4 polynucleotide ligase prior to incubation with polymerase, synthesis is almost completely eliminated (Table III). If ligase is instead added to the polymerase reaction mixture after synthesis is under way, there is no inhibition of subsequent synthesis (Table III). If ligase is added 30 sec after synthesis has started, there is a 60% inhibition, but after 60 sec ligase is ineffective.

The inhibitory effect of ligase is directly related to its ability to convert the nicked molecules to covalently closed circles (Fig. 5). During a 3-min period of synthesis primed by nicked PM2 DNA, there was a rapid decrease in the percentage of molecules which could be converted to closed circles by T4 ligase. By the time that 50 nucleotides had been polymerized, none of the molecules could be repaired.

A plausible explanation for the effect of DNA synthesis on ligase action is that the 5' end of the primer strand is displaced by synthesis occurring at the 3' side of the nick. When nicked PM2 DNA, which had served as a primer for the incorporation of 70 nucleotides, was incubated with ligase, no covalently closed circles were detected (Fig. 6a). Incubation of this primer product with exonuclease III in the presence of ligase leads to the conversion of approximately 50% of the molecules to closed circular duplexes (Fig. 6b). As shown below, all of the newly synthesized DNA was made acid-soluble by incubation with exonuclease III. A similar result was obtained when the DNA was incubated with the exonuclease activity of the T4 DNA polymerase (Fig. 6c). Since both of these exonucleases remove nucleotides from the 3' termini of DNA molecules (17, 19), our findings support the assumption that DNA synthesis at the 3' side of the nick is responsible for the inhibition of ligase action. These results also eliminate the possibility that contaminating endonucleases converted the circular duplexes to linear molecules or to a form which could not serve as a substrate for ligase activity.

Secondary Structure of Product—It has been previously shown that the product of extensive synthesis by E. coli polymerase with PM2 DNA (6 μM) containing one nick per molecule was incubated at 37°C in a standard reaction mixture containing α-32P-dATP as the labeled precursor and 2 units of Escherichia coli DNA polymerase in order to obtain 0.05 (B) and 0.7 replication (C). A separate incubation was carried out for 30 min at 37°C in the absence of DNA polymerase (A). The extent of synthesis was measured on samples of the reaction mixture by determining the amount of acid-insoluble radioactivity. The reactions were stopped by making the solution 15 mM in EDTA. The solutions were then examined in the electron micrograph as described under "Methods."
Fig. 8 (for legend see p. 2096)
either linear (5, 6) or circular duplexes (20) is resistant to E. coli exonuclease I, an enzyme highly specific for single stranded DNA bearing a 3'-hydroxyl end group (11). On the basis of the product's susceptibility to exonuclease I, DNA synthesis at nicks can be divided into two phases (Fig. 7). Initially all of the newly synthesized DNA can be hydrolyzed by exonuclease I after denaturation. However, when approximately 1000 nucleotides have been incorporated at each nick, the product is resistant to the enzyme after denaturation.

Surprisingly, a significant percentage (10 to 30%) of the first 100 nucleotides polymerized were susceptible to exonuclease I prior to any denaturing treatment (Fig. 7). Although not shown in Fig. 7, this exonuclease I-sensitive DNA could be rendered resistant to exonuclease I by continued synthesis (15 min) in the presence of unlabeled deoxynucleoside triphosphates. In such a pulse-chase experiment less than 5% of the labeled DNA was sensitive to the action of exonuclease I.

Although the DNA synthesized early in the reaction is susceptible to exonuclease I prior to denaturation, it is also susceptible to exonuclease III, an enzyme specific for duplex DNA (19). As shown in Fig. 6, exonuclease III could remove the nucleotides that had been incorporated at the nick, restoring the DNA to a form which could be covalently joined by ligase. The ability of exonuclease III to hydrolyze the newly synthesized DNA was confirmed by incubating the three samples described in Fig. 7 in a standard exonuclease III reaction mixture (19) containing 10 units of exonuclease III; more than 99% of the 3H in the product was made acid-soluble in each case.

Electron Microscopic Analysis of Primer Product—More than 90% of the PM2 DNA preparation containing one nick per molecule appeared as either twisted or open circles in electron micrographs (Fig. 8A). The open circles exhibited a sharp distribution of molecular lengths (Fig. 9A) with an average length of 3.2 μ. This value is in good agreement with the value of 3.0 μ obtained by Espejo, Canello, and Sinsheimer (21). Detailed examination of the open circles failed to reveal any ends (branches).

Micrographs of primer product complexes isolated from an E. coli DNA polymerase reaction after 0.05 replication revealed the presence in 80% of the molecules of a single branch arising from an otherwise intact open circle (Fig. 8B). Since single stranded DNA could not be visualized under the conditions used in these experiments, the branches must represent duplex nucleotide chains. As shown in Fig. 9B, the branched circles have an average length of 3.1 μ, indistinguishable from that of the unreplicated samples. Although the average length of branches (0.15 μ, Fig. 9B) is in fairly good agreement with the extent of synthesis, a theoretically calculated length must be based on the percentage of nicked circles in the preparation and must account for the mechanism of branch formation (see "Discussion").

After 0.7 replication, the circular PM2 molecules are still present (Fig. 8C) and have an average length of 3.1 μ (Fig. 9C). Now, however, multiple long branches with a heterogeneous length distribution extended from the circular structures. When this DNA was denatured with alkali, the circles could no longer be detected in electron micrographs, but linear molecules with branches were present. These findings support our earlier conclusion that the product of extensive synthesis renatures readily.

Sedimentation Analysis of Product of 0.7 Replication—The product primer complex isolated after 0.7 replication sedimented 1.38 times faster in alkali than did the nicked PM2 DNA primer (Fig. 10a). Since single stranded DNA could not be visualized under the conditions used in these experiments, the branches must represent duplex nucleotide chains. As shown in Fig. 10b, the branched circles have an average length of 3.1 μ, indistinguishable from that of the unreplicated samples. Although the average length of branches (0.15 μ, Fig. 10B) is in fairly good agreement with the extent of synthesis, a theoretically calculated length must be based on the percentage of nicked circles in the preparation and must account for the mechanism of branch formation (see "Discussion").

Sedimentation Analysis of 0.7 Replication—The product primer complex isolated after 0.7 replication sedimented 1.38 times faster in alkali than did the nicked PM2 DNA primer (Fig. 10a). This change in sedimentation rate indicates a molecular weight 2.2 times that of a single strand of PM2 DNA (22). When the primer product was sedimented at neutral pH, it sedimented 1.45 times faster than did the open circular PM2 DNA (Fig. 10b). The molecular weight calculated from this value is 2.9 times that of PM2 DNA. These results are discussed below.
Inhibition of ligase action by synthesis catalyzed by Escherichia coli polymerase lacking 5'-hydrolytic activity

The standard E. coli polymerase reaction mixture (0.1 ml) contained 16 μM 5'H-PM2 DNA (one nick per molecule) and either 0.15 unit of untreated E. coli DNA polymerase or 0.15 unit of the fragment (mol wt 75,000) lacking 5'-hydrolytic activity (see "Methods"). After incubation at 37° for 5 min, T4 polynucleotide ligase and ATP were added, and the percentage of closed circles found was measured by sedimentation in alkali (see "Methods").

<table>
<thead>
<tr>
<th>Addition</th>
<th>Closed circular duplexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>%</td>
</tr>
<tr>
<td>Intact untreated polymerase</td>
<td>85</td>
</tr>
<tr>
<td>Polymerase lacking 5'-hydrolytic activity</td>
<td>13</td>
</tr>
</tbody>
</table>

in terms of possible mechanisms for extensive synthesis by E. coli DNA polymerase.

Synthesis at Nicks by E. coli DNA Polymerase Lacking 5'-Hydrolytic Activity

Unlike T4 polymerase, E. coli polymerase is able to initiate synthesis at nicks. The two enzymes differ in that the former does not have a 5'-hydrolytic activity, whereas the latter does (1-3, 17). It was therefore of interest to see whether E. coli polymerase, lacking 5'-hydrolytic activity, could nevertheless initiate synthesis at nicks. This can be experimentally determined because it has been shown that E. coli polymerase can be separated into two fragments after limited hydrolysis with subtilisin (13-15). The polymerizing activity and the 2'-hydrolytic activity are contained in one of the fragments, whereas the 5'-hydrolytic activity is present in the other.

E. coli DNA polymerase was separated from its 5'-hydrolytic activity according to the method of Klenow and Henningens (14). Fragments (mol wt 75,000) containing polymerase activity were devoid of 5'-hydrolytic activity, as measured by their inability to convert nicks bearing 5'-hydroxyl end groups to nicks bearing 5'-phosphoryl end groups (see Table V of preceding paper (1)).

When the E. coli polymerase lacking 5'-hydrolytic activity was used in a reaction primed by nicked PM2 DNA, it catalyzed synthesis at a rate indistinguishable from that observed with the normal E. coli DNA polymerase. The rate of synthesis with nicked PM2 DNA in a standard reaction mixture, such as that described in Fig. 1, was 6% of that observed in the standard d(A-T)-primed assay for both enzymes. Since the nick translation cannot occur in the absence of the 5'-hydrolytic activity, then synthesis by the altered enzyme must proceed by a mechanism of strand displacement. In support of this interpretation DNA synthesis on nicked PM2 DNA catalyzed with polymerase treated with subtilisin eliminated the nick as efficiently as did synthesis by the untreated polymerase (Table IV).

**DISCUSSION**

E. coli DNA polymerase, in contrast to T4 DNA polymerase, can catalyze extensive DNA synthesis in the presence of a duplex DNA primer (6, 23, 24). However, the product of such a net synthesis has been found to have structural aberrations (6, 7) and to be devoid of biological activity (8). The work of Englund, Kelly, and Kornberg (25) has shown that E. coli polymerase can bind to nicks in DNA. Studies by Kelly et al. (4) and those described in the preceding paper (1) show that E. coli DNA polymerase can initiate hydrolysis at the 5' side of a nick and polymerization at the 3' side. Such simultaneous hydrolysis and synthesis results in nick translation, but not in net DNA synthesis. The present report, however, shows that net synthesis of DNA is also initiated at nicks in DNA. Although nick translation usually precedes the onset of net synthesis, it is not a prerequisite, since E. coli polymerase lacking 5'-hydrolytic activity can catalyze a net synthesis of DNA.

Our interpretation of earlier results, as well as those described in this and the accompanying paper (1), is summarized schematically in Fig. 11. E. coli polymerase initiates synthesis at a nick on a circular duplex by adding nucleotides covalently to the 3' terminus of the primer strand while concurrently removing nucleotides from the 5' terminus. The product (I) is identical with the original primer, except that the nick has been translated.

After the addition of approximately 50 nucleotides the 5' end of the primer strand is displaced and 5'-hydrolysis ceases. Further synthesis results in continued displacement of the 5' end of the primer strand to result in Structure II. Since the original nick has been eliminated by strand displacement, the molecule can no longer be repaired by ligase. However, exonuclease II can remove nucleotides from the 3' end of the newly synthesized DNA and restore Structure I which can then be repaired by ligase. The newly synthesized DNA in Structure II is susceptible to hydrolysis by exonuclease I, an enzyme specific for single stranded DNA. This result suggests that Structure II is in equilibrium with Structure III in which the original displaced strand has in turn displaced the newly synthesized DNA. Recent experiments by Lee, Davis, and Davidson (26) suggest that this type of equilibrium is established during the annealing of phage DNAs. Even if such an equilibrium involved only a few nucleotides at the 3' end of the newly synthesized DNA, exonuclease I could shift the equilibrium in the direction of Structure III by eliminating the 3' single stranded ends as they were displaced.

At some point subsequent to strand displacement an event occurs which converts the newly synthesized DNA to a form which is resistant to exonuclease I even after denaturation. A single duplex branch can also be observed in electron micrographs at this stage of synthesis. A duplex branch can arise by one of two different mechanisms. If an equilibrium exists between Struc-
structures II and III as discussed above, then the single stranded 3' end of the newly synthesized DNA in Structure III could loop back on itself and serve as its own primer template to yield Structure V. This duplex branch would renature readily after denaturing treatments by virtue of its hairpin structure. This type of reaction is known to occur during the copying of a single stranded DNA by E. coli DNA polymerase (6).

Alternatively, at some point after displacement of the 5' end of the primer strand (Structure II), synthesis switches to the complementary strand to form a fork such as that shown in Structure IV. This mechanism has been used previously to explain the nondenaturability of product DNA and the presence of branches (6). The enzyme need not mediate the switch to the displaced strand, since it could be accomplished by a rewinding of the 5' end of the primer strand to create a single stranded growing end. This single stranded region could then anneal sufficiently to the displaced strand and serve as a primer with the displaced strand serving as template.

However, Structure IV, if it is an intermediate, must be converted in this process to a structure such as Structure V if extensive synthesis is to occur with the formation of multiple dangling branches. Structure IV could be converted to Structure V by a process in which the newly synthesized DNA, which is self-complementary, folds back on itself. Further synthesis on Structure V could occur by extension of the 3' end, strand displacement, and a repetition of the above reactions.

Both pycnographic and sedimentation analysis indicate that all of the newly synthesized DNA is covalently attached to the primer. After 0.7 replication, the product primer had an apparent molecular weight 2.2 times that of a single strand of PM2 DNA as measured by its sedimentation rate in alkaline relative to nicked PM2 DNA. If the 0.7 replication sample were similar to Structure IV or V, then the theoretical molecular weight of the product primer would be 2.4 times that of a single strand of PM2. However, at neutral pH the 0.7 replicated primer product had an apparent molecular weight 2.9 times that of PM2 DNA, while the theoretical value for Structure IV or V would be only 1.7. The higher value obtained at neutral pH may reflect a more compact structure of the branched molecules seen in electron micrographs.

Electron microscopy has been successfully used in earlier studies on the repair of single stranded termini in DNA (5), the synthesis of complementary strands of circular DNA (20), and the net synthesis of DNA on linear duplexes (7). In the present study, as in the work of Mitra et al. (20), it was possible to visualize branches arising from the circular primer molecules. In the early stages of synthesis these branched molecules are readily analyzed, since on the average only a single branch is present per circular duplex. In this instance a good correlation exists between the extent of synthesis as measured by the incorporation of radioactive precursor and that obtained by measuring the average lengths of the branches. After 0.05 replication, the average length of the branches was 0.15 \mu. Molecules having a structure similar to that of Structure IV or V in Fig. 11 would be expected to have a branch length of 0.16 \mu, in good agreement with our experimental result. The complex nature of the 0.7 replication sample, on the other hand, makes such an analysis difficult, although continued synthesis on Structure V should lead to longer branches by a repetition of the steps leading to this structure.

These studies do not elucidate the mechanism by which strand displacement can occur during synthesis by E. coli polymerase but not during that catalyzed by T4 polymerase. One possibility is that the 5'-hydrolytic activity, by continuously hydrolyzing ahead of the growing strand, occasionally creates a gap which results in a destabilization of the 5' end of the primer strand, permitting synthesis to invade the region before the secondary structure can be restored. However, our studies with E. coli DNA polymerase, separable from its 5'-hydrolytic activity, reveal that strand displacement occurs equally well during DNA synthesis with this modified enzyme.

It is not known whether E. coli DNA polymerase in vivo catalyzes a reaction at nicks leading to strand displacement and the formation of duplex branches. Some recent models of DNA replication involve in part such a strand displacement. For example, the "rolling circle" model described by Gilbert and Dressler (27) proposes that synthesis is initiated at nicks and results in strand displacement. Another model (23, 24, 28) attempts to account for the unidirectional replication of bacterial and phage chromosomes and the origin of small fragments of DNA originally observed by Okazaki et al. (29) by proposing that structures such as Structure IV in Fig. 11 are formed by E. coli polymerase.

The fork is then cleaved by a specific endonuclease and the process is repeated. If replication proceeds by this process, then a mechanism must exist in the cell to prevent the formation of the structures (III and V) found in the current studies.

Several lines of evidence suggest that E. coli DNA polymerase is involved in repair processes. The 5'-hydrolytic activity of the purified enzyme can excise thymine dimers from DNA (30) while the polymerizing activity can fill in gaps (1). A mutant of E. coli, E. coli PolA (31), whose extracts lack this polymerase activity, grows normally but is sensitive to ultraviolet light and to nuclease (32). Furthermore, this mutant is unable to excise thymine dimers after irradiation with ultraviolet light (33). The finding (34) that there is an additional DNA polymerase activity in wild type E. coli raises questions as to the specific role of each of these enzymes in DNA metabolism.

T4 DNA polymerase, on the other hand, has no 5'-hydrolytic activity and is unable to initiate synthesis at nicks, yet this enzyme is essential for phage DNA synthesis (35, 36).

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