Enzymatic Removal and Replacement of Nucleotides at Single Strand Breaks in Deoxyribonucleic Acid

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SUMMARY

Pancreatic DNase has been used to introduce single phosphodiester bond interruptions (nicks) into double stranded circular DNA. Such DNA substrates have been used to study the action of several exonucleases at the site of nicks. Escherichia coli exonuclease III and the exonuclease activities of phage T4 DNA polymerase (3′ → 5′-nuclease) and E. coli DNA polymerase (3′ → 5′ and 3′ → 3′-nuclease) initiate hydrolysis at these sites as well as at the termini of linear molecules. Under conditions of limited hydrolysis, these enzymes initiate hydrolysis at all nicks in the molecules. In contrast, phage λ exonuclease is unable to hydrolyze DNA containing nicks. Either the phage T4 or the E. coli DNA polymerase, in the presence of the four deoxyribonucleoside triphosphates, can completely fill in the gaps created by exonuclease action. The combined 5′, 3′-hydrolytic activity and the polymerizing activity of the E. coli DNA polymerase can account for the conversion of a nick displaying a 5′-hydroxyl end group to one displaying a 5′-phosphomonoester; i.e. the nick is translated. These studies have been facilitated by the use of polynucleotide ligase to distinguish nicks containing 5′-phosphomonoesters from gaps and from nicks containing 5′-hydroxyl groups. E. coli alkaline phosphatase does not readily distinguish 5′-phosphomonoesters at gaps from those at nicks; quantitative hydrolysis of phosphomonoesters at these sites by the phosphatase requires a higher temperature than that required for the hydrolysis of phosphomonoesters at the ends of linear duplexes.

DNA molecules containing single strand breaks may serve as intermediates in the processes of replication, recombination, repair, and restriction (see References 1 to 6). In these processes exonucleases may remove nucleotides to form single stranded regions (gaps). DNA polymerase could subsequently replace the missing nucleotides and eliminate these gaps. In each reaction the initial substrate or the final product is a DNA molecule containing a single broken phosphodiester bond (nick). Although the substrate for exonuclease and DNA polymerase action in vivo may be DNA molecules containing nicks and gaps, the majority of the previous studies on these enzymes have been concerned with their action at the termini of linear molecules.

Circular duplex DNA is a convenient substrate for studying enzymatic reactions occurring at nicks and at gaps, since the possibility of reactions at external termini is eliminated. In the studies described in this paper we have used the closed circular duplexes isolated either as the replicative form of phage φX174 DNA or as the DNA of phage PM2 (Fig. 1a). Nicks are introduced into these molecules by limited treatment with pancreatic DNase (Fig. 1b). Exonucleases which can initiate hydrolysis at single strand breaks will catalyze the formation of gaps (Fig. 1c) which can be filled in by DNA polymerases (Fig. 1d). The molecules containing nicks can be distinguished from those containing gaps by incubation with polynucleotide ligase, since the latter structure is not a substrate for ligase. The covalently closed molecules can then be identified by sedimentation analysis in alkaline (7).

This report shows that Escherichia coli exonuclease III and the hydrolytic activities of the E. coli and the phage T4 polymerases can create gaps in DNA. Both DNA polymerase activities can restore these structures to yield molecules which can be covalently closed by polynucleotide ligase. Kelly et al. (8) have carried out extensive studies on the initiation of DNA synthesis by E. coli DNA polymerase at nicks in duplex DNAs. They showed that synthesis entails the covalent extension of the 3′-hydroxyl terminus and a concurrent hydrolysis at the 5′ terminus, resulting in translation of the nick. We show that, in this process, the 5′-hydrolytic activity of polymerase can change the 5′ end from a hydroxyl to a phosphoryl group by re-opening the 5′-phosphomonoester site.

A nick refers to a single phosphodiester bond interruption in one of the two strands of a duplex DNA molecule. A gap refers to a single stranded region located internally in a duplex DNA molecule. A gap can be created by the removal of one or more nucleotides at a nick in a DNA molecule. φX174 RF DNA is the replicative form (closed circular duplex) of bacteriophage φX174 DNA. γ-P-ATP refers to ATP labeled with 32P in the γ-phosphoryl group; 3H-PM2 DNA, 3H-PM2, and 3H-T7 DNA are the DNAs isolated from bacteriophage PM2 and T7, respectively, uniformly labeled with 3H or 1H-thymine.
moving the 5'-terminal nucleoside. The following report (9) also describes synthesis of DNA by E. coli DNA polymerase at nicks in which displacement and conservation of the 5' strand occur.

**EXPERIMENTAL PROCEDURE**

**Materials**

**Enzymes**

Crystalline pancreatic DNase in standard vials (1 mg per vial) and E. coli alkaline phosphatase (chromatographically purified) were purchased from Worthington. Alkaline phosphatase was further purified as previously described (10).

Crystalline λ-exonuclease, purified and assayed by the procedure of Little, Lehman, and Kaiser (11), was a gift from Dr. A. D. Kaiser. E. coli exonuclease III was the phosphocellulose fraction purified and assayed as described previously (12).

Polynucleotide kinase was purified from E. coli infected with T4amN82 as previously described (13). The enzyme was further purified by chromatography on hydroxylapatite (14). DNA polymerase induced after the infection of E. coli B by phage T4amN82 was the hydroxylapatite fraction purified and assayed as described by Goulian, Lucas, and Kornberg (15). The preparation had a specific activity of 16,000 units per mg of protein. The exonuclease activity associated with T4 polymerase was measured as described by Oleson and Koerner (16).

E. coli DNA polymerase was the Sephadex fraction purified and assayed according to Jovin, England, and Bertsch (17). The preparation had a specific activity of 3600 units per mg of protein in the poly d(A-T)-primed assay. The exonuclease II activity was measured as previously described (18).

Phage T4 polynucleotide ligase was the phosphocellulose fraction purified from E. coli B infected with phage T4amN82 (19). The preparation had a specific activity of 17,000 units per mg of protein.

All enzyme preparations were examined for the presence of endonucleases in an assay with ϕX174 RF² DNA as substrate as previously described (20). No detectable endonuclease activity was present in the preparations of polymerase, ligase, kinase, phosphatase, exonuclease III, or λ-exonuclease.

**Chemicals and Other Materials**

γ-32P-ATP was prepared as described previously (10). Carrier-free radioactive phosphate (32P) and 3H-thymidine (specific activity 15 Ci per mmole) were purchased from New England Nuclear. Deoxyribonucleoside triphosphates (dTTP, dGTP, dCTP, and dTTP) and ATP were purchased from Schwarz BioResearch. Thymine and deoxyadenosine were purchased from Calbiochem. Bat-T-Flex membrane filters (type B-6, BioResearch) and E. coli alkaline phosphatase (chromatographically purified) were purchased from Worthington. Alkaline phosphatase was assayed as previously described (10). Crystalline X-exonuclease, purified and assayed by the procedure of Little, Lehman, and Kaiser (11), was a gift from Dr. A. D. Kaiser. E. coli exonuclease III was the phosphocellulose fraction purified and assayed as described by Goulian, Lucas, and Kornberg (15). The preparation had a specific activity of 16,000 units per mg of protein. The exonuclease activity associated with T4 polymerase was measured as described by Oleson and Koerner (16).

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**DNA Preparations**

ϕX174 RF²—Two liters of E. coli C were infected with ϕX174λ phage in the presence of chloramphenicol, and the cells were collected and lysed as described by Sinseheimer (21).

The abbreviation used is: RF, replicative form.
DNA was identical with that described above for the unlabeled RF DNA. The specific activity of the purified $^3$H-RF was 1 to $5 \times 10^6$ cpm per pmole of DNA phosphorus.

PM2 DNA—PM2 phage were grown as described by Espejo and Canelo (25) and purified by the procedure described by Yamamoto et al. (26). $^3$H- or $^{32}$P-Labeled PM2 phage were prepared by adding $^3$H-thymidine (10 $\mu$Ci per ml) and deoxyadenosine (50 $\mu$g per ml) or $^{32}$P-inorganic phosphate (2 $\mu$Ci per ml) to the AMS nutrient broth (27) 10 min after phage infection. The DNA was extracted with phenol and sodium dodecyl sulfate as described by Espejo and Canelo (27). The DNA was dialyzed against Tris-HCl buffer (pH 8.0)-1.0 mM EDTA-10 mM NaCl.

The DNA was extracted with phenol and sodium dodecyl sulfate as described by Espejo and Canelo (27). The DNA was dialyzed against 10 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA-10 mM NaCl. The dialyzed DNA was centrifuged in a 5 to 20% neutral sucrose gradient containing 10 mM Tris-HCl buffer (pH 8.0)-10 mM NaCl-1 mM EDTA in a Spinco SW 25 rotor at 25,000 rpm for 14 hours at 4". After centrifugation, 1-ml fractions were collected from the bottom of the tube. The fractions containing closed circular duplex DNA were pooled, dialyzed against 10 mM Tris-HCl buffer (pH 8.0)-1 mM EDTA-10 mM NaCl, and concentrated with Sephadex G-200. The molar extinction coefficient at 260 nm with respect to phosphorus was 6,500. The specific radioactivity of the labeled DNAs was 2 to $5 \times 10^6$ cpm per pmole of DNA phosphorus.

$\lambda$ DNA—$^3$H-Labeled $\lambda$C857 phage were prepared by heat induction of $E$. coli W3102 ($\lambda$C857) grown in media containing $^3$H-thymidine. Phage growth, purification, and isolation of the DNA have been described previously (10). The specific activity of the DNA was $2.3 \times 10^6$ cpm per pmole of DNA phosphorus.

Hydrogen-bonded circles of $\lambda$ DNA were prepared by the procedure of Hershey, Burgi, and Ingraham (28) and concentrated by precipitation with ethanol as described by Gellert (29). $T_7$ DNA—$^3$H-Labeled $T_7$ DNA was isolated from $T_7$ phage grown and purified as previously described (29).

Concentrations of DNA are expressed as equivalents of nucleotide phosphorus unless otherwise stated. In several experiments the radioactively labeled DNAs were diluted with unlabeled DNA to lower the specific radioactivity.

Methods

Preparation of DNA Substrates

Circular DNA Containing Nicks ($5'$-Phosphomonoesters)—Circular duplexes containing nicks bearing 3'-hydroxyl and 5'-phosphoryl end groups were prepared by incubating $^3$H- or $^{32}$P-labeled $\phi X 174$ RF or PM2 DNA with pancreatic DNase. The reaction was carried out as previously described (10) with an amount of pancreatic DNase sufficient to produce one or two breaks per duplex molecule (approximately 2.5 $\mu$g of DNase in a 1-ml reaction mixture containing 0.1 mM DNA). After incubation at 20" for 30 min, the reaction was stopped by the addition of 10 mM EDTA (pH 8.0), followed by heating at 65" for 10 min.

The nicked circular DNA was purified by sedimentation through a 5 to 20% neutral sucrose gradient containing 10 mM Tris-HCl buffer (pH 8.0)-10 mM NaCl-1 mM EDTA. Sedimentation proceeded for 16 hours with $\phi X 174$ RF DNA and 14 hours in the case of the PM2 DNA, in the Spinco SW 25 rotor at 25,000 rpm at 4". A maximum of 1 pmole of DNA was layered on each 25-ml gradient. After centrifugation, 1-ml fractions were collected from the bottom of the tube. The radioactivity of each fraction was measured in 0.02-mi aliquots after precipitation with 1 x trichloroacetic acid and salmon sperm DNA (50 $\mu$g per fraction) as a carrier. The DNA was collected on a glass filter paper (Whatman GF/C, 24 mm), washed with 0.01 N HCl, and dried, and the radioactivity was measured as previously described (10). The nicked circular fractions were pooled and concentrated by dialysis against dry Sephadex G-200. The concentrated DNA was dialyzed against 10 mM Tris-HCl buffer (pH 8.0)-10 mM NaCl-1 mM EDTA.

Circular DNA Containing $5'$-Hydroxyl End Groups—The nicked DNAs prepared by incubation of the circular duplexes with pancreatic DNase were dephosphorylated by incubation with $E$. coli alkaline phosphatase at 65" as described previously (10). Under these conditions the internal $5'$-phosphomonoesters are hydrolyzed to yield DNA containing nicks bearing 3'- and 5'-hydroxyl end groups. The reaction was terminated by the addition of potassium phosphate buffer (pH 7.5) to a concentration of 1.4 mM (10). A portion of the dephosphorylated DNA was used without further purification; another portion was phosphorylated in the polynucleotide kinase reaction as described below.

Circular DNA Containing $5'$-$^{32}$P-Phosphomonoesters—The circular DNA containing 5'-hydroxyl end groups was radioactively labeled in the polynucleotide kinase reaction containing $\gamma$-$^{32}$P-ATP as described previously (10). The DNA containing internal $5'$-$^{32}$P-Phosphomonoesters was purified by sedimentation through a neutral sucrose gradient as described above for the nicked DNA. End group analysis (10) of the purified DNA revealed that 40% of the 5'-hydroxyl end groups were phosphorylated in the kinase reaction.

$T_7$ DNA Containing External $5'$-$^{32}$P-Phosphomonoesters—Terminal labeled $^3$H-$T_7$ DNA was prepared by incubation of the DNA with alkaline phosphatase and then with polynucleotide kinase and $\gamma$-$^{32}$P-ATP as previously described (10). The labeled DNA (0.7 pmole) was isolated by sedimentation through a 5 to 20% neutral sucrose gradient containing 10 mM Tris-HCl buffer (pH 8.0) 10 mM NaCl-1 mM EDTA for 15 hours in the SW 25 type Spinco rotor at 22,000 rpm at 4". After centrifugation, 1-ml fractions were collected from the top of the gradient to minimize shearing, and the radioactivity in an aliquot of each fraction was measured as described above. The fractions containing the intact molecules of $T_7$ DNA were pooled and dialyzed, first against 10 mM Tris-HCl buffer (pH 8.0)-1.0 mM NaCl-1 mM EDTA, and then against 10 mM Tris-HCl buffer (pH 8.0)-10 mM NaCl-1 mM EDTA. The dialyzed DNA was concentrated by dialysis against dry Sephadex G-200. The efficiency of labeling was more than 85%. $5'$-$^{32}$P-Phosphoryl $T_7$ DNA Containing $5'$ Single Stranded Termini—$^3$H-$T_7$ DNA containing external $5'$-$^{32}$P-Phosphomonoesters was incubated with exonuclease III until 5% of the $^3$H-radioactivity was released as mononucleotides. The procedure for incubation and assay of the extent of reaction has been described previously (31).

$5'$-$^{32}$P-Phosphoryl $T_7$ DNA Containing $5'$ Single Stranded Termini—$^3$H-$T_7$ DNA was incubated with $\lambda$-exonuclease until 12% of the $^3$H radioactivity was released as mononucleotides. The DNA was then dephosphorylated with alkaline phosphatase and labeled in the polynucleotide kinase reaction with $\gamma$-$^{32}$P-

$^{32}$P-DNA indicates that the DNA molecules bear $5'$-$^{32}$P-labeled phosphomonoesters at their 5' termini. If the molecules are circular, this implies that the $5'$-$^{32}$P-phosphomonoester is located at a nick or gap.
ATP. The reaction mixture (0.5 ml) contained 0.6 mM "H-T7 DNA, 67 mM glycine buffer (pH 9.2), 3 mM MgCl₂, 3 mM 2-mercaptoethanol, and 3 units of λ-exonuclease. The reaction mixture was incubated for 30 min at 37°C. The extent of hydrolysis was determined by measuring the release of acid-soluble radioactivity. The reaction mixture was then adjusted to pH 8.0 by adding Tris-HCl buffer (pH 9.0). Bacterial alkaline phosphatase (0.2 unit) was added, and the incubation was continued at 65°C for 30 min. Additional alkaline phosphatase (0.2 unit) was added after 15 min. The 5'-hydroxyl-terminated DNA was then labeled with 32P in the polynucleotide kinase reaction as described previously (10). The DNA was purified by sedimentation through a sucrose gradient and then concentrated as described above.

**Repair of Nicks**

**Incubation with Polynucleotide Ligase**—We incubated DNA with polynucleotide ligase to determine whether the interruptions in the DNA were nicks containing 3'-hydroxyl and 5'-phosphoryl end groups and thus would be repairable by ligase. The incubation mixture (0.1 ml) contained 10 to 100 μM 32P ϕX174 RF DNA or PM2 DNA containing one to two breaks per molecule, 67 mM Tris-HCl buffer (pH 7.6), 6.7 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.06 mM ATP, and 0.05 unit of T4 polynucleotide ligase. After incubation at 20°C for 15 min, the reaction was stopped by chilling and the addition of 20 mM EDTA (pH 8.0).

**Assay of Covalent Closure**—The extent of covalent closure was measured in one of three ways. (a) When the substrate contained internal 5'-32P-phosphomonoesters, the number of phosphodiester bonds formed was measured in the standard ligase assay (19). (b) Closed circular duplexes are more stable to heat than the nicked circular duplexes. The two forms can be distinguished by the ability of the closed duplexes to pass through a nitrocellulose membrane filter after a denaturing treatment (20). (c) The rapid sedimentation rate of closed circular duplexes in alkaline relative to that of the nicked molecules (7) provides a sensitive measure of the relative amounts of these two species in a reaction mixture. The DNA (less than 20 nmoles) was sedimented in a 5 to 20% sucrose gradient containing 50 mM potassium phosphate buffer (pH 12.5)-1.0 mM MgCl₂, 10 mM 2-mercaptoethanol, and X-exonuclease (0.001 to 0.1 unit). After incubation at 37°C for 30 min, the mixture was made 5 mM with EDTA and heated at 65°C for 10 min to inactivate the enzyme.

**E. coli DNA Polymerase**—E. coli DNA polymerase, in addition to its polymerizing activity, catalyzes the hydrolysis of DNA from both 3' and 5' termini (18, 32, 33). The reaction mixture (0.1 ml) contained 10 to 100 μM nicked DNA, 67 mM Tris-HCl buffer (pH 7.6), 6.7 mM MgCl₂, 10 mM 2-mercaptoethanol, and 1 unit (polymerase unit) of E. coli exonuclease II. The reaction mixture was incubated for 10 min at 37°C.

**T4 DNA Polymerase**—T4 DNA polymerase also catalyzes the hydrolysis of DNA, but only from 3' termini (34). The reaction mixture (0.1 ml) contained 10 to 100 μM nicked DNA, 67 mM Tris-HCl buffer (pH 8.0), 6.7 mM MgCl₂, 10 mM 2-mercaptoethanol, and 1 unit (polymerase unit) of T4 polymerase. The reaction mixture was incubated at 37°C for 10 min.

**λ-Exonuclease**—The reaction mixture (0.1 ml) contained 10 to 100 μM nicked DNA, 67 mM glycine-KOH buffer (pH 9.2), 6.7 mM MgCl₂, 10 mM 2-mercaptoethanol, and 10 units of λ-exonuclease. After incubation for 15 min at 37°C, 100 mM NaCl and 15 mM Tris-HCl (pH 4.0) to yield pH 7.6 were added to diminish the activity of λ-exonuclease. Since λ-exonuclease hydrolyzes DNA exclusively from 5' termini (35), DNA substrates bearing 5'-32P-phosphomonoesters were used in a specific assay. The reaction mixture is identical with that described above, except that circular DNA containing 5'-32P-phosphomonoesters was used as substrate. In some experiments the DNA was first treated with exonuclease III in order to create gaps.

**Treatment of DNA with Phosphatase**

The reaction mixture (0.5 ml) contained ϕX174 RF DNA with either nicks (9 μM) or gaps (15 μM) (exonuclease III treatment) and bearing 5'-32P-phosphomonoesters, 45 mM Tris-HCl buffer (pH 8.0), 60 mM MgCl₂, and 0.15 unit of bacterial alkaline phosphatase. The reaction mixtures were incubated at the temperatures indicated in the text for 30 or 60 min, with an addition of 0.1 unit of enzyme after 15 min. The DNA was precipitated with trifluoroacetic acid in the presence of acid-soluble radioactivity.
Calculation of Number of Nicks per Molecule

The average number of nicks introduced by pancreatic DNase was calculated from the amount of DNA remaining in the closed circular duplexes. For example, the relative amount of RF in
in an alkaline sucrose gradient for a length of time sufficient to resolve partially single stranded circles and single stranded linear molecules. Fig. 4 shows that the DNA was distributed between two overlapping peaks, as measured by the H radioactivity. However, the 32P location at the 5' termini of the linear strands was present only in the more slowly sedimenting peak. This result is in agreement with the earlier observation that circular single strands sediment faster than do linear single strands of the same molecular weight (7). It is also clear that the nicked duplex molecules from which these single strands were derived contain approximately one break per duplex molecule.

When the nicked DNA containing 5'-32P-phosphomonoesters was incubated with polynucleotide ligase, 70% of the DNA was covalently joined to yield closed circular duplexes (Fig. 5). Approximately 20% of the 32P was found in these closed circular duplexes. The low yield of closed circular duplexes is in part due to the inefficient phosphorylation of end groups at single strand breaks (10). In addition, about 50% of the 32P was present in small heterogeneous DNA (Fig. 4). If the 32P in this fragmented DNA, which could not be converted to closed circles, is taken into consideration, then approximately 40% of the 32P could be recovered as covalently closed molecules after treatment with ligase. When joining was assayed by measuring the incorporation of 32P into phosphodiesters, 70% of the 32P was found in such linkage.

**Action of Exonucleases at Nicks**

*E. coli* Exonuclease III

*E. coli* exonuclease III catalyzes a stepwise hydrolysis of linear duplex DNA starting at the 3' end of each strand and producing 3'-mononucleotides (40). When exonuclease III was incubated with 3H-φX174 RF DNA containing one nick per molecule, the rate of hydrolysis as measured by the release of acid-soluble mononucleotides was identical with that observed when linear T7 DNA was used as a substrate. As shown in Fig. 6, the nicked φX174 RF DNA can no longer be repaired by incubation with ligase after it has been incubated with exonuclease III. However, as shown below, these molecules can be joined by ligase if they are first incubated with DNA polymerase and the four deoxyribonucleoside 5'-triphosphates. These results, considered together, show that exonuclease III can initiate hydrolysis at nicks at a rate comparable to that observed at the termini of linear molecules. A more detailed study of the action of exonuclease III on nicked φX174 RF is shown in Fig. 7. An amount of exonuclease sufficient to hydrolyze 0.05% of the DNA resulted in an 80% inhibition of joining by ligase. Thus, on an average, after only five nucleotides have been removed from a nick, joining is prevented, suggesting that exonuclease III initiates hydrolysis at all nicks in the molecule.

**Hydrolytic Activity of T4 DNA Polymerase**

Bacteriophage T4 induces a new DNA polymerase after infection of *E. coli*. The purified enzyme has been shown to possess a hydrolytic activity in addition to its polymerizing activity (15). The purified T4 polymerase catalyzes a stepwise hydrolysis of nucleotides from the 3' ends of DNA strands in single stranded or duplex configuration. Like exonuclease III, the T4 polymerase can initiate hydrolysis at nicks; the rate of hydrolysis of nicked φX174 RF was found to be identical with that observed with linear T7 DNA. As shown in Fig. 8, the nicked φX174 RF could not be joined by ligase after treatment with T4 polymerase. However, as discussed below, repair could be achieved if the polymerizing activity was permitted to act in the presence of the four deoxyribonucleoside triphosphates. Similar results were obtained when joining was measured in the standard ligase assay with 5'-32P-φX174 RF DNA (Table I). The incorporation of the 5'-32P-phosphomonoesters into phosphodiester bonds was markedly inhibited by prior incubation of the DNA with T4 polymerase. This inhibition could be completely overcome by the addition of the four deoxyribonucleoside triphosphates, indicating that the inhibition was due to a gap and not to strand breakage.

**Hydrolytic Activities of E. coli DNA Polymerase**

Purified *E. coli* DNA polymerase catalyzes the hydrolysis of polydeoxyribonucleotides from both the 3' and 5' termini of the molecule (18, 32, 33). Studies similar to those described above for exonuclease III and T4 polymerase reveal that the *E. coli* polymerase can initiate hydrolysis at nicks (Table II). When nicked circular PMP DNA is incubated with DNA polymerase, the molecules can no longer be joined by incubation with ligase alone. Including the four deoxyribonucleoside triphosphates in the reaction mixture, however, allows the polymerizing activity to function and covalent joining to occur. Although these experiments do not distinguish between 3'→5' and 5'→3' hydrolytic activities of *E. coli* polymerase, experiments described below, as well as those carried out by others (8, 32-34), demonstrate that both activities function at nicks.
Formation and repair of gaps by T4 DNA polymerase

The reaction mixtures (0.1 ml) contained 10 μm nicked 5'-32P-φX174 RF DNA (two nicks per molecule). After incubation with T4 DNA polymerase (1.5 units), the DNA was then incubated with T4 ligase, either in the presence or absence of the four deoxynucleoside triphosphates (dXTP), as indicated below. A control incubation was carried out with no polymerase. The incubations and the assay for the incorporation of 32P-phosphomonoesters into phosphodiester bonds are described under "Methods."

<table>
<thead>
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<th>Treatment prior to ligase</th>
<th>Phosphate-resistant 32P</th>
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<tr>
<td>None</td>
<td>74</td>
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<tr>
<td>T4 polymerase</td>
<td>14</td>
</tr>
<tr>
<td>T4 polymerase + dXTP</td>
<td>82</td>
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</tbody>
</table>

Formation and repair of gaps by Escherichia coli DNA polymerase

The reaction mixtures (0.1 ml) contained 20 μm nicked 32P-PM2 DNA. Eight per cent of the molecules in the preparation were closed circular duplexes. After incubation with E. coli DNA polymerase (2.0 units), the DNA was then incubated with T4 ligase, either in the presence or absence of the four deoxynucleotides triphosphates (dXTP), as indicated below. Control incubations were carried out with the omission of all enzymes or DNA polymerase. The incubation and sedimentation assay for closed duplexes are described under "Methods."

<table>
<thead>
<tr>
<th>Treatment prior to ligase</th>
<th>Closed circular duplexes</th>
</tr>
</thead>
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<tr>
<td>None</td>
<td>65</td>
</tr>
<tr>
<td>E. coli polymerase</td>
<td>8</td>
</tr>
<tr>
<td>E. coli polymerase + dXTP</td>
<td>60</td>
</tr>
</tbody>
</table>

Inability of Phage λ-Exonuclease to Initiate Hydrolysis at Nicks

The exonuclease induced by phage λ has been shown to initiate hydrolysis from the 5' end of a duplex DNA, producing nucleoside 5'-monophosphates (11, 35). Genetic studies have shown that the enzyme is important in the recombination system of phage λ (41). We find that, unlike the three exonucleases described above, λ-exonuclease is unable to hydrolyze nicked circular DNA duplexes. A similar observation has been made by Carter and Radding (42). As shown in Fig. 9, when an amount of exonuclease sufficient to hydrolyze 100 mmoles of linear T7 DNA was added to an incubation mixture containing 10 mmoles of nicked φX174 RF DNA, there was no hydrolysis at nicks, as measured in the ligase assay. This result was confirmed by measuring the extent of joining of 5'-32P-phosphomonoesters in nicked φX174 RF DNA before and after incubation with λ-exonuclease. Incubation of 15 mmoles of the ligase substrate with the exonuclease (1.5 to 30 units) had no effect on its subsequent closure by T4 ligase. The amount of exonuclease added is sufficient to degrade 300 mmoles of linear DNA, and the removal of a single nucleotide would have prevented joining in these assays.

When 3H-T7 DNA and 5'-32P-φX174 RF DNA were incubated together with λ-exonuclease, there was no significant release of the 32P under conditions in which 50% of the 3H was rendered acid-soluble (Fig. 10). This experiment shows that the exonuclease is fully active in the reaction mixture containing the nicked φX174 RF.

In order to determine whether λ-exonuclease can hydrolyze circular duplexes containing 5'-32P-φX174 RF DNA, T4 DNA polymerase was treated with exonuclease III prior to incubation with λ-exonuclease. The introduction of a gap did permit λ-exonuclease to initiate hydrolysis at some of the 5' termini (Fig. 11). However, when all of the nicks had been converted to gaps as judged by the lack of repair in the ligase assay, only 30% of the 5'-terminal nucleotides could be hydrolyzed with an amount of λ-exonuclease sufficient to hydrolyze completely the 5'-terminal nucleotides of linear DNA. The explanation for this observation is not known, although it may be related to the fact that λ-exonuclease has a tendency to “stick” to a given DNA molecule, particularly at low DNA concentrations (42).

Action of Bacterial Alkaline Phosphatase at Nicks and Gaps

At temperatures below 37° alkaline phosphatase will quantitatively hydrolyze external phosphomonoesters (those located at either end of the duplex molecule), but will not hydrolyze phosphomonoesters located at nicks (10). However, at elevated temperatures both types of phosphomonoesters are hydrolyzed by phosphatase (10). As shown in Table III, the external phosphomonoesters of T7 DNA are quantitatively hydrolyzed by alkaline phosphatase at 20° as well as at 65°, while the internal phosphomonoesters of nicked φX174 RF are susceptible to phosphatase only at 65°.

When a gap is introduced into the nicked φX174 RF, the phosphomonoesters are still resistant to hydrolysis by phosphatase, only 10% being hydrolyzed at 20° (Table III). Apparently a single stranded region adjacent to the phosphomonoester inhibits the action of phosphatase, since 5'-phosphomonoesters on linear T7 molecules bearing 3' or 5' single stranded regions were not quantitatively hydrolyzed at 20°, whereas they were hydrolyzed at 65° (Table III). These substrates were prepared by partial digestion of T7 DNA by exonuclease III or λ-exonuclease.

Synthesis at Gaps by DNA Polymerases

The studies described above show that exonuclease III and the hydrolytic activities of E. coli and phage T4 polymerase can initiate hydrolysis at a nick. Their continued action leads to the formation of a gap in one of the two strands of the duplex. These DNA molecules in turn can be used to study the polymerizing activity of T4 and E. coli DNA polymerase at gaps.

T4 DNA Polymerase

T4 DNA polymerase can initiate synthesis at a gap created by exonuclease III, replace the missing nucleotides, and leave a nick which can then be covalently joined by ligase (Figs. 6 and 7). Similarly, after the hydrolytic activity of T4 polymerase has created a gap in nicked DNA, the addition of the four deoxynucleotide triphosphates enables the polymerizing activity to fill in the gap (Fig. 8, Table I). Since T4 polymerase has no 5'-hydrolytic activity (34), the nicks remaining after synthesis by polymerase are located at the sites where they were originally introduced. This is clearly shown in the experiments in Fig. 7 and Table I in which the nick displays a 5'-32P-phosphomonoester.
**E. coli DNA Polymerase**

E. coli DNA polymerase in the presence of the four deoxynucleoside triphosphates can also fill in the gaps in DNA. When the preparation of exonuclease III-treated φX174 RF shown in Fig. 6 was incubated with E. coli polymerase (2 units), the four deoxynucleoside triphosphates, and T4 ligase (0.05 unit) as described under "Methods," 77% of the DNA sedimented as closed circles in alkaline sucrose gradient in the Spinco type SW 50.1 rotor at 40,000 rpm for 3 hours. One-half of the control reaction mixture lacking ligase was centrifuged.

**Conversion of 5'-Hydroxyl Termini to 5'-Phosphoryl Termini by E. coli DNA Polymerase**

DNA bearing 5'-hydroxyl group at nicks cannot be covalently closed by polynucleotide ligase (43). However, the combined action of a 5'-exonuclease and a DNA polymerase can effectively convert a nick bearing a 5'-hydroxyl terminus to one bearing a 5'-phosphoryl terminus. Hence, E. coli DNA polymerase which has a 5'-hydrolytic activity in addition to its polymerizing activity theoretically should be able to catalyze both of these reactions. Klett, Cerami, and Reich (32) have shown that the E. coli polymerase can hydrolyze 5'-hydroxyl-terminated, as well as 5'-phosphoryl-terminated, polynucleotides. In Fig. 12 a scheme is shown for the repair of nicks bearing 5'-hydroxyl end groups by E. coli DNA polymerase and by ligase. In the first reaction the 5'-hydrolytic activity of DNA polymerase excises at least the terminal 5'-nucleoside, and perhaps several other bases. The excision of 5'-nucleosides or 5'-mononucleotides, however, exposes a 5'-phosphoryl group. In the second reaction repair synthesis by polymerase closes the gap created by the excision. The end product contains a nick displaced from its original position, but now having a 5'-phosphoryl and 3'-hydroxyl end group. Kelly et al. (8) have shown that E. coli DNA polymerase can translate a nick bearing a 5'-phosphoryl end group in such a manner. In the final step ligase catalyzes the covalent closure of the molecule.

In this section we show that E. coli DNA polymerase and the ligase can convert nicked PM2 DNA bearing 5'-hydroxyl groups and 5'-hydroxyl-terminated hydrogen-bonded circles of phage λ DNA to closed circular duplexes, and that both the polymerizing activity and the 5'-hydrolytic activity of DNA polymerase are essential in the reaction.

**5'-Hydroxyl Nicked PM2 DNA**

When PM2 DNA containing nicks displaying 3'- and 5'-hydroxyl groups was incubated with E. coli DNA polymerase, the four deoxynucleoside triphosphates, polynucleotide ligase, and ATP, up to 50% of the DNA molecules were converted to...
closed circles (Table IV). In the absence of E. coli DNA polymerase or ligase, there was no significant joining. Furthermore, T4 polymerase, lacking a 5'-hydrolytic activity, could not replace E. coli polymerase in the reaction mixture. It is possible to isolate two fragments of E. coli DNA polymerase after limited hydrolysis with subtilisin (44-46). Furthermore, one of the fragments contains the polymerizing activity and 3'-hydrolytic activity and the other the 5'-hydrolytic activity. When the fragment of E. coli DNA polymerase lacking 5'-hydrolytic activity was incubated with 5'-hydroxyl nicked PM2 DNA in the presence of ligase, no covalent joining was observed (Table IV).

5'-Hydroxyl-terminated Hydrogen-bonded Circles of λ DNA

Repair by Purified E. coli Polymerase and T4 Ligase—The purified T4 ligase catalyzes the conversion of hydrogen-bonded circles of λ DNA to covalently closed molecules (19, 47), a form which can be readily identified by a 4-fold increase in sedimentation rate in alkali (48). As shown in Table V, T4 polynucleotide ligase could catalyze the covalent closure of 17% of the molecules in a preparation of hydrogen-bonded λ circles. If the DNA was dephosphorylated prior to incubation with ligase, no covalent circles were formed. However, incubation of the 5'-hydroxyl-terminated hydrogen-bonded circles with E. coli DNA polymerase and the four deoxynucleoside 5'-triphosphates converted them to a form which could be covalently closed by T4 polynucleotide ligase (Table V). As in the case of the 5'-hydroxyl nicked PM2 DNA discussed above, T4 DNA polymerase could not replace E. coli DNA polymerase.

Role of DNA Polymerase in Repair by Extracts—The results shown in Table VI show that extracts of wild type E. coli can catalyze the covalent closure of 5'-hydroxyl terminated circles of λ DNA and that DNA polymerase is an essential component of this reaction. When an extract of E. coli K12 endonuclease I-negative cells, supplemented with DPN (the co-factor of the E. coli DNA ligase), was incubated with 5'-phosphoryl hydrogen-bonded circles, 11% of the DNA was converted to covalently closed circles. On the other hand, only 2% of the 5'-hydroxyl-terminated circles could be covalently closed (Table VI). The addition of the four deoxynucleoside 5'-triphosphates not only increased the joining of 5'-phosphoryl terminated circles 3 fold, but also made possible the joining of a comparable amount of the 5'-hydroxyl-terminated circles. Aiding antiserum to E. coli DNA polymerase to the extract caused a marked decrease in the joining of both forms of λ DNA (Table VI).

DeLucia and Cairns (49) have isolated a mutant of E. coli, E. coli PolAI, which has less than 1% of wild type DNA polymerase activity in cell-free extracts. When the λ circles described above were incubated with extracts of a strain of E. coli PolAI, also lacking endonuclease I (50), the results were similar to those obtained with wild type extracts containing antiserum to DNA polymerase; there was no detectable joining of 5'-hydroxyl-terminated λ DNA circles and only 3% of 5'-phosphoryl-terminated circles (Table VI). The addition of purified E. coli DNA polymerase initiates hydrolysis at the 5' terminus of the nick releasing the terminal base as a nucleoside or oligonucleotide. In the process, a 5'-phosphoryl end group is exposed. Repair synthesis by polymerase closes the gap, and the nick is repaired by ligase.
The reaction mixtures (0.1 ml) contained either 20 μM 5'-P-nicked PM2 DNA or 20 μM 5'-OH-nicked PM2 DNA, 67 mM potassium phosphate buffer (pH 7.6), 6.7 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.03 mM dATP, dCTP, dGTP, and dTTP, and 0.06 mM ATP. T₄ ligase (0.05 unit), T₄ DNA polymerase (10 units), or Escherichia coli DNA polymerase (2 units) was added as indicated below. After incubation at 20°C for 20 min, EDTA was added to a concentration of 20 mM. The percentage of closed circular duplexes was measured by the membrane filter method previously described (20). The 5'-P- and 5'-OH-nicked PM2 DNA uniformly labeled with ³²P were prepared as described under "Methods." The E. coli polymerase lacking 5'-hydrolytic activity was prepared by the method of Klenow and Henningsen (45) as described in the accompanying paper (9).

### TABLE IV

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>Closed circular duplexes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-P-nicked PM2 DNA</td>
<td>1&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Minus T₄ ligase</td>
<td></td>
</tr>
<tr>
<td>Plus T₄ ligase</td>
<td>55</td>
</tr>
<tr>
<td>5'-OH-nicked PM2 DNA</td>
<td>1&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Minus T₄ ligase</td>
<td></td>
</tr>
<tr>
<td>Plus T₄ ligase</td>
<td>3</td>
</tr>
<tr>
<td>Plus E. coli polymerase</td>
<td></td>
</tr>
<tr>
<td>Plus E. coli polymerase lacking 5'-hydrolytic activity and T₄ ligase</td>
<td>1&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plus E. coli polymerase and T₄ ligase</td>
<td>50</td>
</tr>
<tr>
<td>Plus T₄ polymerase and T₄ ligase</td>
<td>1&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

### TABLE V

Covalent closure of 5'-hydroxyl-terminated circles of λ DNA by *Escherichia coli* DNA polymerase and T₄ ligase

The reaction mixtures (0.15 ml) contained 10 mM Tris-HCl buffer (pH 7.6), 4 mM MgCl₂, 0.5 mM EDTA, 0.05 mM ATP, 5 units of T₄ ligase, and 1 mole of 5'-phosphoryl (5'-P)- or 5'-hydroxyl (5'-OH)-terminated hydrogen-bonded circles of λ DNA. As indicated, the reaction mixtures were supplemented with 0.5 mM dATP, dCTP, dGTP, and dTTP (dXTP), 7 units of T₄ DNA polymerase, and 7 units of *E. coli* DNA polymerase. After 15 min at 30°C, the reactions were stopped and the percentage of covalently closed circles was determined by centrifugation in 5 to 20% alkaline sucrose for 67 min at 40,000 rpm in a Spincor type SW rotor. The stimulation in joining seen with 5'-phosphoryl-terminated circles of λ DNA when deoxynucleoside triphosphates are present and the decrease in joining when polymerase is removed suggest that repair synthesis is necessary to counteract the action of exonucleases in the extract. The complete absence of joining of 5'-hydroxyl-terminated circles of λ DNA by extracts in which polymerase is inactive can be explained by the strict requirement for a 5'-hydroxyl activity to remove the 5'-hydroxyl terminus in addition to a polymerizing activity to fill in the resulting gap.

### DISCUSSION

The exonucleases and DNA polymerases used in these studies have already been thoroughly characterized (6) although mainly in regard to their action on the termini of linear molecules. In this report we have systematically examined their action on circular duplex DNA molecules containing nicks and gaps. The use of such DNA substrates has several advantages for this type of study. (a) The relatively low molecular weight and the homogeneity of some of the circular duplexes, such as φX174 RF and PM2 DNA, facilitate their manipulation. (b) The marked effect of a single interruption in their strands on their physical properties makes possible an accurate analysis of the number of interruptions. (c) The absence of external termini eliminates concern over reactions occurring at these sites. In our studies DNA ligase has been useful in distinguishing between nicks and gaps, since ligase can repair only the former.

Earlier studies (10) on *E. coli* alkaline phosphatase revealed that results obtained for the action of enzymes at external termini of linear molecules cannot always be extended to their action at internal sites. While the phosphatase can hydrolyze external phosphomonoesters at 25°C, significantly higher tempera-
ures are required to hydrolyze internal phosphomonoesters. In the present report we show that elevated temperatures are required to hydrolyze phosphomonoesters at gaps as well as at nicks. It is evident from our studies that similar considerations apply to the action of exonucleases at nicks and gaps. Thus, while E. coli exonuclease III and the hydrolytic activities of the E. coli and phage T4 polymerases can initiate hydrolysis at nicks, the phage λ exonuclease cannot do so.

E. coli exonuclease III was found to hydrolyze nicked DNA at a rate comparable to that observed with linear duplex DNA. Furthermore, the fact that an average of only five nucleotides need be removed from a nick to inhibit joining by ligase suggests that the enzyme initiates hydrolysis at all of these sites. Similar results were found with the hydrolytic activities of the T4 and E. coli polymerases. Sadowski and Hurwitz (51) have shown that T4 exonuclease IV can create gaps in nicked DNA, and it seems likely that this activity is identical with that associated with the T4-induced DNA polymerase. From our results it is clear that the E. coli polymerase can create gaps, and that hydrolysis can occur in a 5' → 3' direction. Although these studies do not establish whether or not simultaneous 3' → 5' hydrolysis is occurring, the work of Cozzarelli, Kelly, and Kornberg (34) indicates that both hydrolytic activities function at nicks. Furthermore, England, Kelly, and Kornberg (52) have shown that E. coli DNA polymerase can bind to nicks and that there is only a single DNA-binding site.

The inability of λ-exonuclease to initiate hydrolysis at nicks was shown in assays involving either the closing of circles in the presence of ligase or the hydrolysis of 5'-32P-nicked circular DNA. Both assays are capable of detecting the removal of a single nucleotide from the 5' terminus, the known site of initiation for the exonuclease (35), and the former assay could detect the removal of a single nucleotide from the 3' terminus as well. The lack of activity on such substrates was due specifically to the structure of a nick, since circular DNA containing gaps was also resistant to its action.

The gaps produced by exonuclease action at nicks are readily filled in by either the T4 or the E. coli DNA polymerase. There are several experiments which show that the single stranded ends produced by exonuclease III can be filled in by DNA polymerase (31, 53). A more extensive type of repair synthesis involves the synthesis of the complementary strand of M13 or φX174 DNA. Both assays are capable of detecting the removal of a single nucleotide from the 5' terminus, the known site of initiation for the exonuclease (35), and the former assay could detect the removal of a single nucleotide from the 3' terminus as well. The lack of activity on such substrates was due specifically to the structure of a nick, since circular DNA containing gaps was also resistant to its action.

The formation and repair of gaps are phenomena which probably occur in vivo in a number of biological processes including the repair of ultraviolet-induced lesions in DNA and genetic recombination. Since the 5' → 3'-hydrolytic activity of the E. coli DNA polymerase can excise thymine dimers (58), this activity in conjunction with the polymerizing activity of the enzyme could account for the removal of such lesions and their replacement by normal nucleotides. The sensitivity of E. coli PolA1, a mutant lacking this DNA polymerase in extracts, to ultraviolet light (59) could be due to the absence of one or both of these activities.

We also report a special, but related, type of repair synthesis which occurs through a mechanism of nick translation of the type shown by Kelly et al. (8). Although nicked DNA bearing 5'-hydroxyl end groups cannot be covalently joined by polyuridylate ligase alone, it can be joined in a reaction mixture containing E. coli DNA polymerase, the four deoxynucleoside 5'-triphosphates, and ligase. Our results suggest that the joining process involves the removal of nucleotides from the 5' terminus by the 5' → 3'-hydrolytic activity, and their replacement by DNA polymerase. Since the polymerase can hydrolyze 5'-hydroxyl-terminated polymers (32), it will excise the 5' terminus, simultaneously exposing a 5'-phosphoryl group. Wu and Kaiser (60) found that 5'-phosphoryl- and 5'-hydroxyl-terminated λ DNA were equally infectious in a transfection assay with helper phage. If covalently closed circles are a prerequisite for replication or lysogeny (or both), then a mechanism for the conversion of 5'-hydroxyl-terminated λ DNA to closed circular duplexes must exist. The combined actions of the 5'-hydrolytic activity and the polymerizing activity of E. coli polymerase provide such a mechanism. Our results show that extracts of wild type E. coli can catalyze the joining of 5'-hydroxyl-terminated circles of λ DNA and that DNA polymerase is an essential component of this reaction.

The role of exonucleases and DNA polymerases in recombination is more difficult to evaluate. In the case of phage T4, it has been shown that hydrogen-bonded recombinant molecules produced in cells infected with phage defective in the synthesis of ligase and polymerase can be subsequently covalently joined by polymerase and ligase in vitro (61, 62). This result suggests that the recombination process in phage T4 may involve the repair of gaps. Recombination in phage λ also appears to involve limited synthesis of DNA (63). λ-Exonuclease is an essential component in the recombination system of λ phage (41) as is the β-protein (64). Carter and Radding (42) have reported that the λ-exonuclease cannot initiate hydrolysis at single strand breaks even in the presence of the β-protein whose function has not yet been established. The finding that the exonuclease is unable to hydrolyze DNA containing nicks or gaps suggests that an early step in recombination in vivo might be the introduction of double strand breaks or the exposure of single strand regions by strand separation rather than by hydrolysis.

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