Replication of the lagging strand of bacteriophage T7 DNA occurs in a discontinuous fashion that requires RNA-primed DNA synthesis, the removal of the RNA primers, the replacement of the ribonucleotides with deoxyribonucleotides, and the covalent joining of adjacent DNA fragments. We have examined each of these steps as well as the whole process through the use of model substrates and partial reactions using purified proteins.

Tetraribonucleotides (pppACCC or pppACCA), synthesized by the T7 gene 4 protein on single-stranded DNA, are used as primers by T7 DNA polymerase to yield RNA-terminated DNA fragments. The removal of the RNA primers is catalyzed by the 5' to 3' hydrolytic activities of either Escherichia coli DNA polymerase I or the T7 gene 6 exonuclease. The products of hydrolysis are ppApC, ATP, and nucleoside 5'-monophosphates or ATP and nucleoside 5'-monophosphates, respectively. The requirement for DNA synthesis to fill the gap between adjacent DNA fragments can be fulfilled by Form II of T7 DNA polymerase but not by Form I. DNA synthesis catalyzed by Form II of T7 DNA polymerase eliminates gaps to create a substrate for DNA ligase whereas strand displacement synthesis catalyzed by Form I creates an aberrant structure that cannot be joined. Either the host or phage DNA ligase can effect the final covalent joining.

All steps in the replication of a lagging strand have been coupled in a model system that catalyzes the formation of covalently closed, circular, double-stranded DNA molecules using single-stranded viral DNA as template. A combination of four bacteriophage proteins, gene 4 protein, Form II of T7 DNA polymerase, gene 6 exonuclease, and DNA ligase, can accomplish this overall reaction.

The catalytic properties of DNA polymerase along with the antiparallel structure of duplex DNA molecules dictate that the leading and lagging strands be replicated differently. The replication of the lagging strand poses by far the most difficulty; the discontinuous DNA synthesis (2) that accounts for its replication requires a number of steps and proteins that are not involved in leading strand synthesis. Bacteriophage T7 DNA replication, requiring relatively few proteins, provides an opportunity to understand the enzymatic events responsible for lagging strand synthesis.

In vivo and in vitro studies have provided considerable insight into the process of discontinuous synthesis on the lagging strand of T7 DNA. Analysis of newly synthesized bacteriophage T7 DNA, isolated from phage-infected cells, reveals the presence of short fragments of DNA (Okazaki fragments) having lengths of from 1000 to 6000 nucleotides (3, 4). These nascent fragments are terminated at their 5' ends with oligoribonucleotides having the sequence pppAC(C)(N)45 in which N is mainly A and C, and the chain length is predominantly four ribonucleotides (5, 6). Mapping of initiation sites on newly replicated T7 DNA indicates that the recognition sequences for RNA primer synthesis are 3'-CTGGN-5' and 3'-CTGTN-5' (7).

From in vitro studies, it is known that the gene 4 protein of phage T7 catalyzes the synthesis of oligoribonucleotides on single-stranded DNA (8–12). This primase activity of the gene 4 protein requires the presence of a single-stranded DNA template and ribonucleoside 5'-triphosphates. The oligoribonucleotides synthesized in this reaction serve to initiate DNA synthesis when T7 DNA polymerase and the deoxyribonucleoside 5'-triphosphates are present. The chains of newly synthesized DNA are terminated at their 5' ends by covalently linked tetraribonucleotides whose sequences are, as is the case in vivo, predominantly pppACCC and pppACCA (9, 11). The primers are synthesized on the single-stranded DNA at specific sites that are complementary to the primer sequence (12). These recognition sites for the gene 4 protein share the common sequence 3'-CTGGG-5' or 3'-CTGTG-5'. Thus, a cytosine residue is required for recognition but is not copied into the primer, a finding that is consistent with the in vivo studies mentioned above.

In the preceding paper (1), a DNA molecule containing a preformed replication fork was used to define the requirements for leading strand synthesis during the replication of a duplex DNA molecule. In this phase of DNA replication, two phage proteins were found to be required: the T7 gene 4 protein and T7 DNA polymerase. Here the gene 4 protein functions as a helicase (13) to "open up" the duplex region ahead of the DNA polymerase at the replication fork. Underlying both the primase and helicase activities of the gene 4 protein is its ability to transpose unidirectionally in a 5' to 3' direction along a single strand (12), a process that requires the hydrolysis of NTPs (12, 13).

In this paper, we describe the requirements for the synthesis of a continuous lagging strand in a reaction separate from leading strand synthesis. In this process, several steps are...
required that are unique to lagging strand synthesis: RNA primers must be synthesized and extended by DNA polymerase, the RNA primers must be removed and replaced by deoxyribonucleotides, and the resulting fragments must be joined to yield an uninterrupted strand of DNA.

Of these steps, the synthesis of RNA primers by the gene 4 protein has, to date, been most thoroughly documented. In the phage-infected cell, two enzymes exist that could remove RNA primers, the 5' to 3' exonuclease activity of Escherichia coli DNA polymerase I (14) and the gene 6 protein of the phage itself. The latter enzyme is a 5' to 3' double-stranded DNA exonuclease (15, 16) that also hydrolyzes RNA in RNA/DNA hybrids (RNase H activity) (17). In the absence of a functional gene 6 protein, RNA-terminated DNA fragments accumulate in infected cells. When both the gene 6 exonuclease and E. coli DNA polymerase I are deficient, a further accumulation is observed (18). However, the major activity responsible for primer removal appears to be the gene 6 protein since, in the absence of DNA polymerase I, there is no detectable accumulation of RNA-terminated DNA fragments (18).

The most likely enzyme that could serve to fill the gaps created by primer removal is the T7 DNA polymerase since it should be positioned at the 3' ends of the nascent DNA fragments. However, our earlier studies (10) on RNA-primed DNA synthesis, using purified proteins, suggested a requirement for additional proteins or another DNA polymerase to circumvent this problem, we found that Form I1 of T7 DNA polymerase deficient E. coli are unable to support infection by T7 ligase, defective in T7 DNA ligase, grow normally in wild type E. coli, and wild type T7 phages grow normally in E. coli strains (18).

The most likely enzyme that could serve to fill the gaps created by primer removal is the T7 DNA polymerase since it should be positioned at the 3' ends of the nascent DNA fragments. However, our earlier studies (10) on RNA-primed DNA synthesis, using purified proteins, suggested a requirement for additional proteins or another DNA polymerase. In these studies in which T7 gene 4 protein and Form I of T7 DNA polymerase were used, synthesis that had initiated at one primer site did not stop when it reached the 5' terminus of the next RNA-terminated DNA fragment. Instead, synthesis continued, resulting in the displacement of a single-stranded, RNA-terminated DNA fragment. This displaced fragment, in turn, provided additional sites for primer synthesis, leading to repeated initiation events. In this paper, we show that Form I of T7 DNA polymerase catalyzes strand displacement synthesis so rapidly that, even in the presence of DNA ligase, polymerization of nucleotides continues even after the gap is filled. Consequently, DNA ligase is unable to join adjacent fragments. In searching for activities that might circumvent this problem, we found that Form II of T7 DNA polymerase, which does not catalyze strand displacement synthesis (19), completes gap filling properly.

From in vivo studies, it is apparent that the final covalent joining of adjacent fragments can be catalyzed by either the host- or phage-encoded DNA ligase. Mutants of phage T7, defective in T7 DNA ligase, grow normally in wild type E. coli, and wild type T7 phages grow normally in E. coli strains deficient in the host ligase (3); in neither case is there any apparent defect in T7 DNA replication. However, ligase-deficient E. coli are unable to support infection by T7 ligase mutants. In this case, short fragments of newly synthesized DNA accumulate (3, 20, 21).

In this paper, we present the characterization of each of the individual partial reactions that constitute lagging strand synthesis. Based on this analysis, we have reconstituted lagging strand synthesis using four purified phage enzymes: gene 4 protein, Form II of T7 DNA polymerase, gene 6 exonuclease, and DNA ligase.

**EXPERIMENTAL PROCEDURES**

**Materials**

**Bacterial Strains and Bacteriophages—E. coli D110 Su- thy end polA1 has been previously described (22). E. coli HMS 151 (E. coli RS5004 rpa333 polAex1) was obtained from I. R. Lehman (Stanford University Medical School). E. coli 011* Su- thy, wild type T7 phage and T7 amber mutants, unless otherwise designated, were obtained from Dr. F. W. Studier (Brookhaven National Laboratory). T7 amber mutants are designated by subscript notation indicating the mutant gene only. The amber mutations used are: gene 1, am198; gene 1.3, amHA13; gene 3, am29; gene 6, am147. T7 1.132, was provided by Dr. Paul Sadowski (University of Toronto). T7 phages were grown on E. coli 011* Su- thy as described by Studier (23-25).

**Bacteriophage oX174 am5 DNA** was isolated by the procedure of Hutchison and Sinicrope (26). Duplex pMB9 DNA containing a single nick was prepared by incubation of pMB9 DNA with pancreatic DNase in the presence of ethidium bromide (27) as described in the accompanying paper (28). DNA of bacteriophage M13 was generously provided by S. W. Matson and S. Tabor (Harvard Medical School). Plasmid pBR322 DNA was a gift from Dr. Paul W. Wasmann (Harvard Medical School).

**Nucleotides—Unlabeled nucleotides were purchased from P-L Biochemicals. All labeled nucleotides were obtained from New England Nuclear.**

**Enzymes—**Gene 4 protein of bacteriophage T7 was Fraction V (50% pure) purified as previously described (30). T7 DNA ligase (95% pure) was purified by a procedure to be published elsewhere. Form I and Form II of T7 DNA polymerase and T4 polynucleotide kinase are described in the accompanying paper (19). E. coli DNA polymerase I (90-100% pure) was purchased by the procedure of Joyson and Studier (31) or purchased from New England Biolabs. E. coli exonuclease I was the DEAE fraction of Lehman (32). E. coli exonuclease III and the restriction endonuclease HaeIII were from New England Biolabs. E. coli exonuclease VII was a gift from Dr. J. Chase (Emory College of Medicine). Bacterial alkaline phosphatase was purchased from Miles Corp. and further purified as previously described (28).

**Other Materials—**Polyethyleneimine cellulose thin layer plates were purchased from Brinkmann. Ultrapure ammonium sulfate was from Schwarz/Mann.

**Methods**

**Enzyme Assays—**T7 DNA ligase was assayed using the ATP-PF exchange assay previously described for T4 DNA ligase (33). T7 DNA polymerase and gene 4 protein were assayed as described in the accompanying paper (19). E. coli DNA polymerase I was assayed as previously described (34). Gene 6 exonuclease of phage T7 was assayed essentially as described by Kerr and Sadowski (16) except that Haelll-digested SV40 [3H]DNA (1.5 nmol/reaction mixture) was used as substrate. The specific activity of the DNA was 18,000 cpm/nmol. Enzyme activity was proportional to enzyme concentration between 0.05 and 0.6 unit/reaction mixture (0.1 ml). One unit of gene 6 exonuclease activity is defined as that amount that catalyzes the removal of nmol of acid-soluble nucleotide after a 15-min incubation at 37 °C under the conditions of the standard assay. E. coli exonuclease VII was assayed as previously described (35).

The 5' to 3' hydrolytic activities of E. coli DNA polymerase I and of T7 gene 6 exonuclease were, in some experiments, determined in a reaction containing 50 mM Tris-HCl (pH 7.5), 15 mM MgCl2, 33 /&gmcyan;M each dATP, dTTP, dCTP, and dGTP, and the indicated amounts of E. coli DNA polymerase I. The reaction mixture (0.1 ml) for the assay of DNA polymerase I contained T7 [5'-32P]DNA (1.8 pmol of 5' terminal phosphorus; 6.1 × 107 cpm/nmol), 70 mM potassium phosphate buffer (pH 7.4), 7 mM MgCl2, 1 mM d-mercaptoethanol, 33 /&gmcyan;M each dATP, dTTP, dCTP, and dGTP, and the indicated amounts of E. coli DNA polymerase I. The reaction mixture (0.1 ml) for the assay of gene 6 exonuclease contained T7 [5'-32P]DNA (1.8 pmol of 5' terminal phosphorus; 6.1 × 107 cpm/nmol), 50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 1 mM dithiothreitol, 20 mM KCl, and the indicated amounts of T7 gene 6 exonuclease. In both assays, after incubation for 10 min at 37 °C, 0.1 ml of salmon sperm DNA (2.5 mg/ml) and 0.5 ml of 0.7 N trichloroacetic acid were added. After centrifugation at 10,000 × g for 10 min, an aliquot of the supernatant fluid was removed and the radioactivity was determined. For both enzymes, one unit of 5'-terminal nucleotide activity is defined as the amount of enzyme catalyzing the conversion of 1 pmol of 5'-terminal phosphate to an acid-soluble form in 10 min at 37 °C.

**Preparation of Radioactively Labeled DNA and RNA-DNA Substrates—**T7 [5'-32P]DNA was prepared as described by Chase and Richardson (35). T7 DNA was partially degraded by sonic irradiation, dephosphorylated by incubation with bacterial alkaline phosphatase at 65 °C, and then phosphorylated using T4 polynucleotide kinase in the presence of [γ-32P]ATP (6.1 × 107 cpm/nmol). The [5'-32P]DNA was generously provided by M. J. Engler and C. C. Richardson, unpublished results.
was purified on a benzoylated DEAE column as previously described (35).

Fragments of T7 DNA bearing radioactively labeled tetraribonucleotides at their 5' termini were synthesized in a reaction containing T7 gene 4 protein and T7 DNA polymerase as previously described (10). The tetraribonucleotide primers synthesized by the T7 gene 4 protein were radioactively labeled with 32P using [γ-32P]ATP and (10). The tetraribonucleotide primers were separated on a T7 DNA polymerase (Form I), 2 units of gene 4 protein, and 5 μg of E. coli DNA-binding protein in 10 mM Tris-HCl (pH 7.5), 10 mM dithiothreitol, 0.5 mg/ml of bovine serum albumin. After incubation at 30 °C for 10 min, the reaction was stopped and the RNA-DNA product was isolated as previously described (10). The average length of the RNA-prime DNA was 400 nucleotides, calculated on the basis of pppACCC being the predominant primer synthesized in this reaction (11).

**Assay for Removal of RNA Primers**—The assay for enzymatic removal of a gene 4 protein tetraribonucleotide primer from the 5' terminus and radiolabeling the reaction mixture was measured by the conversion of acid-extractable radioactivity in the primer into an acid-soluble form. The tetraribonucleotide primers contained both [γ-32P]ATP at their 5' termini and [32P]CMP internally. The reaction mixture (0.2 ml) for assay of removal of primers by E. coli DNA polymerase I contained [32P]CMP, 50 pmol of 3H-dTTP, 7 mM MgCl2, 1 mM 2-mercaptoethanol, 33 μM each dATP, dGTP, dTTP, and dCTP, and 0.18 unit (5'-terminal nucleotide units) of E. coli DNA polymerase I.

The reaction mixture (0.2 ml) for assay of removal of primers by T7 gene 6 exonuclease contained [32P]RNA·[3H]DNA (1.6 pmol of primer), 50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 1 mM dithiothreitol, 20 mM KCl, and 2.2 units (5'-terminal nucleotide units) of T7 gene 6 exonuclease.

After incubation for the indicated times at 37 °C, 25-μl aliquots were removed and added to 10 μl of salmon sperm DNA (2.5 mg/ml) in a 0.4-ml microcentrifuge tube at 0 °C. After the addition of 15 μl of 1 N trichloroacetic acid, the solution was centrifuged for 5 min in an Eppendorf microcentrifuge. The supernatant fluid was removed and the radioactivity was determined.

**Identification of Products of RNA Primer Hydrolysis**—[γ-32P]ATP- or [32P]CMP-RNA·[3H]DNA (0.9 pmol of [γ-32P]ATP or [3P]CMP-RNA·[3H]DNA) containing [3P]CMP, in separate reaction mixtures (20 μl), were incubated with either E. coli DNA polymerase I (0.075 5'-terminal nucleotide unit) or T7 gene 6 exonuclease (1.45 5'-terminal nucleotide units) for 20 min at 37 °C under the conditions used for assay of hydrolysis of RNA primers. The reaction was stopped by the addition of 50 μl of a solution containing 7 M urea, 0.05% xylene cyanol, and 0.05% bromphenol blue. One-half (40 μl) of each sample was applied to a 23% polyacrylamide gel containing 7 M urea and electrophoresed as previously described (11). The radioactive species were cut from the gel, crushed, and soaked in water to remove the products. The products were adsorbed to Norit, washed, and eluted. The eluted products were then analyzed, along with markers of ATP, CTP, ADP, CDP, AMP, and CMP, by chromatography on polyethyleneimine cellulose thin layer plates developed with 0.5 M LiCl, 1 M formic acid.

**Assay of Repair of Gaps and Nicks**—The extent of gap filling by T7 DNA polymerase was measured by determining the amount of covalently closed circular DNA molecules when circular, duplex DNA containing a gap was incubated with T7 DNA polymerase and T7 DNA ligase. The reaction mixture (10 μl) contained 50 mM Tris-HCl (pH 8.0), 50 mM MgCl2, 150 μM ATP, GTP, UTP, CTP, [γ-32P]ATP (100 pmol), dGTP, dCTP, and dTTP, 20 mM KCl, 10 mM dithiothreitol, and 0.25 nmol of single-stranded circular DNA (M13 or dX174 DNA as indicated), and the indicated amounts of T7 gene 4 protein, T7 DNA polymerase, T7 gene 6 protein, and T7 DNA ligase. After incubation at 30 °C for 1 h, the reaction was stopped and subjected to electrophoresis on an 8.8% agarose gel containing ethidium bromide, and the gel was photographed as described above for the assay of repair of gaps and nicks. The agarose gel was dried onto a sheet of Whatman 3MM filter paper and radiographographed. The dried gel was cut into strips and the radioactivity in covalently closed duplex circles was measured in a liquid scintillation counter.

- **Exonuclease Assay**
- **Repair Assay**
- **Products Assay**
- **Ligation Assay**

**Identification**

**Results**

**Discussion**

**Conclusion**

**Table I**

**Purification of T7 gene 6 exonuclease**

| Fraction | Purification of T7 gene 6 exonuclease |
|----------|---------------------------------------|
|          | Protein Units | Recovery % |
| Fraction  | Units/mg | % |
| I. Extract* | 1,500 | 1,250 | 1,200 | 100 |
| II. Polynucle P | 1,250 | 300 | 4,500 | 90 |
| III. Ammonium sulfate | 1,450 | 210 | 6,900 |
| IV. DEAE-cellulose | 720 | 31 | 3,300 | 48 |
| V. Phosphocellulose | 680 | 6 | 93,300 | 37 |

*Extract prepared from 26 g of T7 DNA-infected E. coli HMS151.
The supernatant fluid was collected and the A260 was adjusted to 200 by the addition of the appropriate amount of 50 mM Tris-HCl (pH 7.5), 10% sucrose, 0.1 M NaCl. The resultant volume of 146 ml was designated Fraction I.

To Fraction I (146 ml), 4.4 ml of 10% Polymyx P was added dropwise with stirring over a 10-min period and stirring was continued for an additional 10 min. The precipitate was collected by centrifugation at 10,000 g for 10 min and suspended, with the aid of a Dounce homogenizer, in 75 ml of 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (Buffer A) containing 50 mM (NH₄)₂SO₄. The precipitate was collected again by centrifugation and resuspended in 75 ml of Buffer A containing 0.25 M (NH₄)₂SO₄. After centrifugation, the supernatant fluid (74 ml) was collected as Fraction II.

Ammonium sulfate (22 g) was added, with stirring, to the supernatant fluid over a 20-min period. After an additional 20-min period of stirring, the resulting precipitate was collected by centrifugation and dissolved in 30 ml of 50 mM potassium phosphate buffer (pH 7.4), 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol (Buffer B). The conductivity of the solution was adjusted, by the addition of Buffer B, to a value equal to that of 0.1 M (NH₄)₂SO₄ in Buffer B to yield Fraction III (50 ml).

A column of Whatman DE52 DEAE-cellulose (12.5 cm × 18 cm) was prepared and equilibrated with Buffer B containing 0.1 M (NH₄)₂SO₄. Fraction III (49 ml) was applied to the column and then the column was washed with 500 ml of Buffer B containing 0.1 M (NH₄)₂SO₄. Next, the column was washed with 350 ml of 0.15 M (NH₄)₂SO₄ in Buffer B and finally, gene 6 exonuclease activity was eluted with 0.2 M (NH₄)₂SO₄ in Buffer B. Fractions of 15 ml were collected and assayed for exonuclease activity. The peak fractions, comprising approximately 80% of the activity applied to the column, were combined and dialyzed against two changes (12 h each) of 2 liters of 20 mM potassium phosphate buffer (pH 6.5), 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol (Buffer C) to yield Fraction IV (225 ml).

A column of Whatman P-11 phosphocellulose (3 cm × 16 cm) was prepared and equilibrated with Buffer C. Fraction IV was applied to the column and then washed with 100 ml of Buffer C. Proteins were eluted with a linear gradient (400 ml) from 0 to 0.4 M (NH₄)₂SO₄ in Buffer C. The flow rate of the column was 25 ml/h and fractions of 6 ml were collected. Exonuclease activity eluted in a sharp peak at approximately 0.1 M (NH₄)₂SO₄. The pooled fractions were concentrated by dialysis against dry Sephadex G-200 and then against 50% glycerol (Fraction V).

As shown in Table I, this procedure resulted in an 80-fold purification of the activity over the starting material, with an overall yield of 40%. The specific activity of the purified enzyme was 93,000 units/mg. Electrophoresis of the denatured and reduced gene 6 exonuclease (Fraction V) through polyacrylamide gels containing sodium dodecyl sulfate revealed a single band corresponding to a protein of M₅₀ = 31,000.

RESULTS

Removal of RNA Primers

T7 DNA isolated from the virion is composed of two uninterrupted strands, neither of which contains any detectable ribonucleotides (37, 38). Furthermore, E. coli DNA ligase, an enzyme that can replace the T7 DNA ligase in vivo (3), cannot catalyze the joining of a 5'-RNA-terminated fragment to a DNA fragment (39) even if the 5' terminus is converted to a monophosphate ester. Therefore, an efficient mechanism must be available in T7-infected cells to remove the many 5'-tetraribonucleotides that terminate lagging strand Okazaki fragments.

T7 Gene 6 Exonuclease and E. coli DNA Polymerase I Catalyze the Hydrolysis of RNA Primers—Two enzymes have been implicated in the removal of RNA primers from T7 DNA in vivo (18). E. coli DNA polymerase I and the gene 6 exonuclease encoded by phage T7, both of which are known to hydrolyze RNA in RNA/DNA duplex hybrid molecules (14, 17). In addition, the 5' to 3' hydrolytic activity of E. coli DNA polymerase I has been shown to be essential for the processing of Okazaki fragments generated during the replication of the bacterial chromosome in vivo (40, 41).

In order to examine the removal of RNA primers from the 5' termini of DNA, we have chosen for a model substrate the newly synthesized DNA made in a reaction in which T7 gene 4 protein and T7 DNA polymerase act on a duplex T7 DNA template in the presence of radiolabeled dNTPs and dNTPs. In this reaction, DNA fragments 5000 to 6000 nucleotides in length are produced. Each of these has a tetraribonucleotide (pppACC or pppACCA) covalently linked to its 5' terminus, the result of multiple priming events catalyzed by the primase activity of the gene 4 protein (10, 11). In order to follow the removal of the primers they were radioactively labeled with [32P]CMP; the DNA was labeled with 3H. As shown in Fig. 1, both E. coli DNA polymerase I and T7 gene 6 exonuclease render the radioactively labeled RNA primers acid-soluble. The addition of 2-fold additional E. coli DNA polymerase I resulted in greater than 90% removal in a 40-min incubation. However, the addition of 2-fold more gene 6 exonuclease did not result in the removal of more than 75% of the primer. In addition, considerable hydrolysis of the 3H-labeled, newly synthesized DNA accompanied primer removal by the gene 6 exonuclease (Fig. 1). The resistant fraction of primers and the surprisingly high level of DNA hydrolysis observed are most likely a result of an unusual structure of a portion of the DNA primed fragments synthesized in the reaction (Ref. 11; see "Discussion"). The 5' to 3' hydrolytic activity of E. coli DNA polymerase I, on the other hand, was capable of effecting primer removal without significant hydrolysis of newly synthesized DNA.

Products of Primer Hydrolysis—In order to identify the products of primer hydrolysis catalyzed by E. coli DNA polymerase I and the gene 6 exonuclease, the tetraribonucleotides were labeled at their 5' termini with [γ-32P]ATP or internally with [32P]CMP. After partial digestion of the radioactively labeled primers by each of the enzymes, the acid-soluble products were separated by electrophoresis through a poly-
acrylamide gel containing urea; the autoradiogram of such a gel is shown in Fig. 2.

When \(^{32}P\)ATP-labeled primers were incubated with *E. coli* DNA polymerase I, two radioactive products were observed (Fig. 2). The major, slower moving product is the triphosphate-terminated dinucleotide pppApC; the minor product is ATP (see Fig. 2 for identification). Two radioactively labeled products were also found when the \(^{32}P\)CMP-labeled primers were used as substrate. Again, one is pppApC while the other is CMP. Thus, the 5’ to 3’ hydrolytic activity of *E. coli* DNA polymerase I hydrolyzes the primer by cleaving the first or second phosphodiester linkage from the 5’ terminus, releasing ATP or pppApC, respectively. The analogous products have also been observed when the enzyme hydrolyzes a DNA molecule bearing a 5’-triphosphate (42). In these earlier studies, kinetic data indicated that the dinucleotide observed represented the initial cleavage product of the 5’ to 3’ hydrolytic activity and was not the product of cleavage of a larger oligonucleotide by the 3’ to 5’ hydrolytic activity of DNA polymerase I. The ATP observed must be a hydrolysis product of the 5’ to 3’ activity since pppTpTpT is not a substrate for the 3’ to 5’ hydrolytic activity of DNA polymerase I (42). However, the CMP residues observed may be subsequent cleavage products of the 5’ to 5’ hydrolytic activity, or the products of the combined activities of the 5’ to 3’ and 3’ to 5’ hydrolytic activities.

When the analysis was carried using gene 6 exonuclease, only a single radioactive product, \(^{32}P\)CMP, was produced when \(^{32}P\)CMP-labeled primer was used as substrate. Similarly, only a single radioactive product, \(^{32}P\)ATP, was released from the \(^{32}P\)ATP-labeled primer. We conclude that gene 6 exonuclease removes single nucleotide residues exclusively even if a 5’-terminal triphosphate is present. A similar result was obtained by Shinozaki and Okazaki (17) using a 5’-triphosphate-terminated RNA transcript covalently attached to DNA.

![Fig. 2. Products of RNA primer hydrolysis.](image)

**Fig. 2. Products of RNA primer hydrolysis.** DNA fragments bearing radioactively labeled tetranucleotides at their 5’ termini were hydrolyzed with either *E. coli* DNA polymerase I or T7 gene 6 exonuclease. The RNA primers were radioactively labeled with \(^{32}P\)ATP or \(^{32}P\)CMP as described under “Experimental Procedures.” Radiolabeled products were analyzed by electrophoresis through a 23% polyacrylamide gel containing 7 M urea and located by autoradiography. The identity of the indicated products was determined as described under “Experimental Procedures.” The species containing both ATP and CMP was identified as the terminal pppApC by alkaline hydrolysis followed by thin layer chromatography; greater than 90% of the radioactivity was found in pppAp. Lanes 1 and 2, RNA primers labeled at their 5’ termini with \(^{32}P\)ATP; lanes 3 and 4, RNA primers labeled internally with \(^{32}P\)CMP; lanes 1 and 3, hydrolysis by *E. coli* DNA polymerase I; lanes 2 and 4, hydrolysis by T7 gene 6 exonuclease.

**Elimination of Gaps and Resulting Nicks**

In the accompanying papers (1, 19, 28), we showed that Form I of T7 DNA polymerase catalyzes limited strand displacement synthesis at a nick, with subsequent strand switching and additional synthesis giving rise to branched duplex structures (panhandles). Such an aberrant structure prevents joining of adjacent DNA fragments by DNA ligase (19). If Form I of T7 DNA polymerase catalyzes a similar strand displacement reaction after filling in the gap between adjacent Okazaki fragments, then gap filling could specifically require Form II of T7 DNA polymerase, a form that does not catalyze strand displacement synthesis.

Although it seemed likely that Form I of T7 DNA polymerase would also catalyze strand displacement synthesis at the nick generated by gap filling, one cannot a priori assume that such a reaction would occur. For example, the blunt ends generated by the 5’ to 3’ hydrolytic activity of *E. coli* DNA polymerase III acting on duplex DNA molecules bearing 5’-single-stranded tails are not recognized as such by the enzyme (43). The enzyme continues its hydrolysis into the duplex region even though it cannot initiate hydrolysis at blunt ends normally. Thus, Form I of T7 DNA polymerase, once engaged in the process of DNA synthesis, might not recognize the nick it creates.

In the experiment shown in Fig. 3, plasmid pMB9 DNA containing a single nick, was incubated with sufficient *E. coli* exonuclease III to create a gap several hundred nucleotides long. The ability of Forms I and II of T7 DNA polymerase to eliminate the gap without subsequent strand displacement synthesis was measured by determining the number of covalently closed circular molecules produced by DNA synthesis in the presence of DNA ligase. The covalently closed circular DNA can be readily separated from the molecules containing...
nicks and gaps by electrophoresis through an agarose gel containing ethidium bromide (Fig. 3, lanes 1 and 2). As shown in the accompanying paper (28), molecules containing duplex branched structures (panhandles) can be identified by their slightly lower mobility relative to covalently closed duplex circles. DNA synthesis catalyzed by Form I of T7 DNA polymerase leads to the formation of panhandles (Fig. 3, lanes 5, 7, and 8); few covalently closed circular molecules formed. On the other hand, DNA synthesis catalyzed by Form II of DNA polymerase completely fills the gaps created by exonuclease III; the resulting nicked DNA is a substrate for T7 DNA ligase (Fig. 3, compare lanes 2 and 11). Finally, a mixture of the two forms of T7 DNA polymerase yields both products, molecules containing panhandles and covalently closed duplex circles (Fig. 3, lanes 12 and 13).

It is particularly noteworthy that panhandles are generated by Form I of T7 DNA polymerase even in the presence of T7 DNA ligase. Thus, the presence of DNA ligase during gap filling does not prevent strand displacement catalyzed by Form I of T7 DNA polymerase. Such is not the case for preparations of E. coli DNA polymerase I (44); the large fragment of this enzyme catalyzes strand displacement synthesis (1).

Complementary Strand Synthesis of Single-stranded Circular DNAs: a Model System for Lagging Strand Synthesis

Our earlier attempts to reconstitute lagging strand synthesis (10) using duplex DNA as a template were complicated not only by the simultaneous leading strand displacement, but also by the occurrence of aberrant strand displacement and strand switching that gave rise to excessive DNA synthesis (see the Introduction). In this section, we report the conversion of single-stranded, circular DNA to covalently closed, duplex circular DNA as an in vitro system for the synthesis and processing of Okazaki fragments. As depicted schematically in Fig. 4, the initial step in the synthesis of the complementary strand by T7 protein is the synthesis of a tetraribonucleotide at one of 13 sites on the φX174 DNA molecule (12). T7 DNA polymerase then uses the tetraribonucleotide as a primer to initiate DNA synthesis, a reaction that is analogous to the synthesis of RNA-primed DNA on the lagging strand at a replication fork. The removal of the RNA primer, the completion of DNA synthesis (gap filling), and the ligation of the adjacent ends are equivalent to the stages involved in the processing of Okazaki fragments during replication. Although E. coli DNA polymerase I can also catalyze the removal of the RNA primers (see above), in these studies we have used the T7 gene 6 exonuclease since in vivo studies suggest that it is the major activity responsible for primer removal (18).

Requirements for Complementary Strand Synthesis—Synthesis of the complementary strand of φX174 single-stranded viral DNA to yield a covalently closed, circular duplex is shown in Fig. 5. The products of the reaction are separated by electrophoresis through an agarose gel containing ethidium bromide. The appearance of covalently closed, circular DNA is seen only in the presence of all four enzymes: T7 gene 4 protein, Form II of T7 DNA polymerase, T7 gene 6 exonuclease, and T7 DNA ligase (Fig. 5, lane 4). In the absence of gene 4 protein or T7 DNA polymerase, no DNA synthesis occurs; only the input φX174 single-stranded template is observed. Both gene 6 exonuclease (lane 3) and DNA ligase (lane 5) are required for the formation of covalently closed, circular molecules; in the absence of either, nicked duplex, circular molecules accumulate. Since the gene 6 exonuclease is needed, sealing by T7 DNA ligase must require that RNA primers first be removed. No DNA containing duplex branches (panhandles) is present in any of the reaction mixtures, attesting to the absence of Form I of T7 DNA polymerase. Panhandle DNA is generated by Form I of T7 DNA polymerase in a reaction requiring neither DNA ligase nor gene 6 exonuclease (data not shown).

In order to obtain a more quantitative evaluation of the data presented in Fig. 5, the quantity of newly synthesized DNA in each species of product molecule was determined by
Form I of T7 DNA Polymerase—Our initial attempts to reconstitute lagging strand synthesis involved the use of Form I of T7 DNA polymerase. In light of the studies described in the accompanying papers (1, 19, 28) and those presented in the section on gap filling above, we can now explain our inability to obtain covalent closure of the product molecules in the four-enzyme system just described. However, our attempts to identify factors that would allow the formation of covalently closed circles, perhaps by preventing panhandle formation, by Form I of T7 DNA polymerase led to the purification of Form II of T7 DNA polymerase and to the discovery that another protein, E. coli exonuclease VII, could partially overcome aberrant strand displacement and strand-switching reactions by Form I of T7 DNA polymerase.

In order to facilitate distinguishing between covalently closed DNA molecules and topologically restrained molecules containing panhandles, we have used the assay described in Fig. 6. The combination of Form I of T7 DNA polymerase, gene 4 protein, gene 6 exonuclease, and T7 DNA ligase results in extensive synthesis of DNA on single-stranded, circular DNA templates (Fig. 6, lane 1). Although a significant portion of the newly synthesized DNA has a mobility similar to but not identical with that of covalently closed, circular molecules in ethidium bromide, it can be distinguished from the latter form by its susceptibility to a combination of E. coli exonuclease I and III prior to electrophoresis through an agarose gel. After photograph, the gel was prepared for fluorography by first soaking in 95% ethanol for 2 h and then in 3% 2,5-diphenyloxazole in 95% ethanol for 2 h. The gel was transferred to H2O, soaked for 12 h at 4 °C, and then dried onto Whatman 3MM filter paper after which it was analyzed by fluorography. A concentrated fraction of protein was prepared from T7-infected E. coli D110. Lysis and removal of debris were carried out as previously described (19). A portion of the extract (0.35 ml) was passed through a DE52 column (0.75 ml) equilibrated with 0.25 M (NH4)2SO4, 50 mM Tris-HCl (pH 7.5), and 1 mM dithiothreitol in order to remove the bulk of the nucleic acids. The protein passing through the column was collected as a single fraction and assayed for its ability to promote covalent closure of the newly synthesized DNA in the assay described above. Lane 1, no exonuclease treatment following the DNA synthesis reaction; lane 2, treatment with exonucleases following the DNA synthesis reaction; lane 3, extract of T7-infected cells (2 μl of DE52 fraction added to DNA synthesis reaction, no exonuclease treatment following DNA synthesis reaction; lane 4, extract of T7-infected cells (2 μl of DE52 fraction added to DNA synthesis reaction, treatment with exonucleases following DNA synthesis reaction.

**Table II**

**Requirements for synthesis of covalently closed, circular duplex DNA**

| Conditions | Products of DNA synthesis | Nicked circles | Linear duplex | Covalently closed circles | Single-stranded circles | pmol |
|------------|---------------------------|----------------|---------------|-------------------------|-------------------------|-----|
| Complete   |                           | 34             | 10            | 61                      | <0.1                    |     |
| -Gene 4    |                           | 0.1            | 0.8           | 0.2                     | <0.1                    |     |
| -Ligase    |                           | 130            | 11            | 0.3                     | <0.1                    |     |
| -Gene 6    |                           | 160            | 16            | 0.1                     | <0.1                    |     |
| -Gene 6, ligase |                 | 180            | 15            | 0.1                     | <0.1                    |     |
| -Gene 6, ligase, -gene 4 |   | 0.2            | 0.6           | 0.1                     | <0.1                    |     |

**FIG. 6. Assay for factors that promote covalent closure of the DNA synthesized by Form I of T7 DNA polymerase.** The reaction mixture for assay of factors that promote the overall conversion of single-stranded circles to covalently closed duplex circles in the presence of Form I of T7 DNA polymerase, T7 gene 4 protein, gene 6 exonuclease, and T7 DNA ligase is described under "Experimental Procedures." In the reaction, [H3]dTTP (61 cpm/pmol) replaced dTTP. Newly synthesized DNA not covalently joined into a circle was hydrolyzed by a combination of E. coli exonuclease I and III prior to electrophoresis through an agarose gel in the presence of ethidium bromide. After photograph, the gel was prepared for fluorography by first soaking in 95% ethanol for 2 h and then in 3% 2,5-diphenyloxazole in 95% ethanol for 2 h. The gel was transferred to H2O, soaked for 12 h at 4 °C, and then dried onto Whatman 3MM filter paper after which it was analyzed by fluorography. A concentrated fraction of protein was prepared from T7-infected E. coli D110. Lysis and removal of debris were carried out as previously described (19). A portion of the extract (0.35 ml) was passed through a DE52 column (0.75 ml) equilibrated with 0.25 M (NH4)2SO4, 50 mM Tris-HCl (pH 7.5), and 1 mM dithiothreitol in order to remove the bulk of the nucleic acids. The protein passing through the column was collected as a single fraction and assayed for its ability to promote covalent closure of the newly synthesized DNA in the assay described above. Lane 1, no exonuclease treatment following the DNA synthesis reaction; lane 2, treatment with exonucleases following the DNA synthesis reaction; lane 3, extract of T7-infected cells (2 μl of DE52 fraction added to DNA synthesis reaction, no exonuclease treatment following DNA synthesis reaction; lane 4, extract of T7-infected cells (2 μl of DE52 fraction added to DNA synthesis reaction, treatment with exonucleases following DNA synthesis reaction.

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At present, we consider our understanding of lagging strand synthesis only partially complete. First, the reconstituted system lacks the tight regulation necessary for complete conversion of single-stranded circular DNA into covalently closed duplex circles. One problem is the potent activity of gene 6 exonuclease on duplex DNA. DNA hydrolysis invariably accompanies primer removal and suggests the presence of other factors or perhaps a complex of replication enzymes that are under tighter regulation. In this regard, it is important to note that the lagging strand synthesis characterized in this paper is uncoupled from leading strand synthesis. The fact that the helicase activity of the gene 4 protein is specific for T7 DNA polymerase (1, 30) suggests a physical association of at least two enzymes at the replication fork. This in turn raises the possibility that leading and lagging strands are replicated DNA polymerase used in these earlier studies could itself catalyze strand displacement synthesis.

Another enzymatic activity was also found that enabled the Form I of T7 DNA polymerase to fill gaps to yield nicks that could be repaired by T7 DNA ligase. Upon purification, this activity was identified as E. coli exonuclease VII and it functioned equally well in the presence or absence of gene 6 exonuclease. We propose that the single strand-specific exonuclease VII hydrolyzes the displaced single strand before strand switching by the polymerase occurs. Thus, the RNA primer is removed by exonuclease VII, and DNA ligase can seal the duplex generated by the complete excision of the displaced strand. Whether this is ever a physiological role of exonuclease VII is not known. However, it is interesting to note that mutants defective in exonuclease VII are phenotypically hyper-Rec (45) as are other E. coli mutants perceived to increase the occurrence of single-stranded tails (46). That exonuclease VII mutants support normal infection by bacteriophage T7 map indicate that this repair pathway is seldom needed when Form II of T7 DNA polymerase is present.

Based on the known activities of the T7 gene 4 protein, T7 DNA polymerase, T7 gene 6 exonuclease, and T7 DNA ligase, we initially expected that a combination of these proteins would readily carry out the multiple steps of lagging strand synthesis. However, the first difficulty encountered arose when only two of the proteins, T7 gene 4 protein and T7 DNA polymerase, were used to carry out the first steps in lagging strand synthesis, i.e. RNA primer synthesis and the extension of the primer by polymerization of deoxyribonucleotides. We found that extensive synthesis occurred on the lagging strand, the result of strand displacement synthesis and multiple priming events (10, 11). We erroneously attributed this aberrant reaction to the ability of the helicase activity of gene 4 protein to allow the T7 DNA polymerase to catalyze strand displacement synthesis at a nick. We now know that Form I of T7 DNA polymerase and Form II of T7 DNA polymerase are present. The results of this study are summarized in Fig. 7. An extract of T7-infected cells, after preliminary fractionation with streptomycin sulfate and ammonium sulfate, is chromatographed on DEAE-cellulose. E. coli exonuclease VII and T7 DNA polymerase activities were measured and, as can be seen in A, the stimulatory activity spans the fractions encompassing both enzymatic activities. Furthermore, the DEAE fraction of Form II of T7 DNA polymerase is most effective in promoting the synthesis of covalently cloned molecules when Form I is omitted from the reaction mixture (Fig. 7B).

**DISCUSSION**

**Fig. 7.** DEAE chromatography of factors that promote covalent closure of DNA synthesized in the presence of Form I of T7 DNA polymerase. An extract of T7-infected E. coli D110 was prepared, fractionated with streptomycin sulfate and (NH₄)₂SO₄, and then applied to a column of DE52 as described in the accompanying paper (19). The protein was eluted from the column as described (19) except that KCl replaced NaCl. The column fractions were assayed for exonuclease VII (O- - O) and T7 DNA polymerase (O- - O) as described under "Experimental Procedures." Fractions were assayed for their ability to promote the formation of covalently closed duplex circles as described in Fig. 6. A is a photograph of an ethidium bromide-stained agarose-ethidium bromide gel of reaction mixtures containing Form I of T7 DNA polymerase and fractions (2 μl) of the column eluate. B is a photograph of an ethidium bromide-stained agarose-ethidium bromide gel of reaction mixtures that lack Form I of T7 DNA polymerase but to which fractions (2 μl) of the column eluate were added.

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concurrently with DNA synthesis occurring within the protein complex (48, 49). In this structure, the use of potential primer recognition sites could be regulated, obviously a parameter of lagging strand synthesis not addressed in this study.

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