THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 245, No. 1, Issue of January 10, pp. 39-45, 1970
Printed in U.S.A.

Enzymatic Synthesis of Deoxyribonucleic Acid

XXXII. REPLICATION OF DUPLEX DEOXYRIBONUCLEIC ACID BY POLYMERASE AT A SINGLE STRAND BREAK*

(Received for publication, June 30, 1969)

REGIS B. KELLY,‡ NICHOLAS R. COZZARELLI,§ MURRAY P. DEUTSCHER,¶ I. R. LEHMAN, AND ARTHUR KORNBERG

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

SUMMARY

Double stranded DNA serves as a template primer for *Escherichia coli* DNA polymerase when the DNA contains a single strand break (a nick) with a 3′-hydroxyl terminus. Initiation of replication entails covalent extension of the 3′-hydroxyl terminus and a concurrent 5′ → 3′ nuclease action by the enzyme. The primer strand is hydrolyzed at the 5′ side of the nick while the synthetic activity catalyzes the addition of nucleotides to the 3′ side. The ability of the enzyme to promote hydrolysis and synthesis simultaneously results in the translation of the nick along the DNA duplex in the 5′ to 3′ direction. The mechanism for conserving the 5′-strand and providing net synthesis of DNA in later phases of the reaction has not yet been clarified.

Replication of DNA by DNA polymerases depends critically on the structure of the template primer. We consider here the replication of six different DNA structures (Fig. 1). The simplest case is the restoration of a partially single stranded DNA to a duplex structure (Fig. 1*a*); examples are the restoration of a linear duplex from which sections had been removed by exonuclease III (3) or the filling in of the cohesive ends of bacteriophage λ (4). Replication of single stranded DNA depends on annealing that enables the 3′ end to serve as a primer and to be extended as in Fig. 1*b* (5). Copying of a single stranded, circular DNA (Fig. 1*c*) (6) usually requires an oligonucleotide primer which can be annealed to it and which is subsequently removed by the 5′ → 3′ nuclease action of DNA polymerase (6, 7); a complete duplex circle is produced when ligase is also present (8).

Does an intact duplex circle or an intact linear duplex serve as a template or primer for the purified *Escherichia coli* DNA polymerase? This report shows that such structures are not utilized by this enzyme (Fig. 1, *d* and *e*). However, it is shown that a 3′-OH single strand scission—a nick—in a duplex structure is of crucial importance. Such a nick is the region of DNA which binds to the active center of the enzyme and at which replication is initiated. This report also shows that chain growth at a nick is accompanied initially by a rapid burst of 5′ → 3′ nuclease action on the primer such that the extent of synthesis always equals the extent of hydrolysis. We postulate that, at a nick, the 5′ → 3′ nuclease action of the polymerase hydrolyzes the primer ahead of the growing strand, thus leading to a translation of the nick (Fig. 1*f*) rather than net synthesis. At later stages in the reaction, the 5′ strand is conserved and net synthesis would be expected to occur. The mechanisms of these events, while not yet understood, may involve a displacement step, as shown in Fig. 1*g*.

EXPERIMENTAL PROCEDURE

Materials

Unlabeled deoxyribonucleoside triphosphates were purchased from various suppliers and purified by ion exchange chromatography. α-^{32}P-dTTP[1] was prepared as described previously (9). ^{3}H-dTTP was purchased from Schwarz BioResearch. The preparations of α,β-dTTP methylene diphosphonate (10) and ddTTP[2] are in the cited references.

E. coli DNA polymerase (Fraction 7) was used and had a specific activity of 18,000 units per mg (11). Pancreatic and micrococcal nucleases were obtained from Worthington. Terminal deoxynucleotidyl transferase from calf thymus (12) was a gift from Dr. F. N. Hayes.

Closed circular duplex DNAs, including natural and synthetic

* This work was supported in part by grants from the National Institutes of Health (United States Public Health Service) and the National Science Foundation. The previous paper in this series is Reference 21. For recent reviews of the properties of DNA polymerase, see References 1 and 2.

‡ Fellow of the Helen Hay Whitney Foundation. Present address, Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115.

§ National Science Foundation Postdoctoral Fellow. Present address, Department of Biochemistry, University of Chicago, Chicago, Illinois 60637.

¶ Fellow of the American Cancer Society. Present address, Department of Biochemistry, University of Connecticut Health Center, Hartford, Connecticut 06105.

[1] The abbreviations used are: ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; $d(T)_{300}$ and $d(A)_{4000}$, homopolymers of approximately 300 residues of deoxyribothymidylate and 4000 residues of deoxyriboadenylate, respectively; dBrCTP, bromodeoxycytidine 5′-triphosphate; poly d(A-T), copolymer of deoxyadenylate and deoxythymidylate.

[2] M. R. Atkinson, M. P. Deutscher, A. Kornberg, A. F. Russell, and J. G. Moffatt, manuscript in preparation.

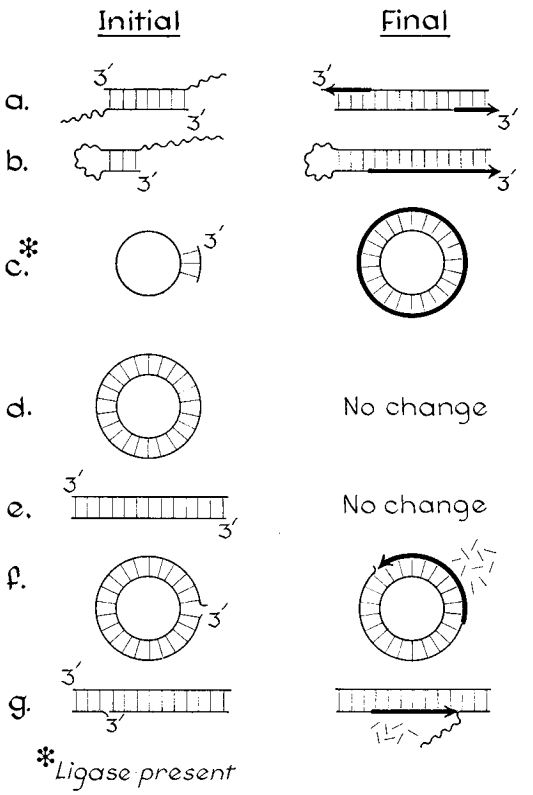

FIG. 1. Schemes for replication of various DNA structures 3′ represents a 3′-hydroxyl-terminated chain.

ΦX RF (replicative form) and *E. coli* 15 plasmid DNA, were prepared as before (13). Phage T7 DNA, judged by alkaline velocity sedimentation to contain an average of 0.5 nick per molecule, was a gift of Dr. Rolf Sternglanz. ^{3}H-Labeled $^{2}H^{15}N$ T7 DNA was a gift of Dr. Zoltan Lucas. Exonuclease III treatment of DNA (14) and the preparation of ^{3}H-labeled (15) and ^{32}P-labeled (16) *E. coli* DNA have been described. $d(A)_{4000}$ was prepared according to Riley, Maling, and Chamberlin (17). ^{3}H-Labeled $d(T)_{300}$ was prepared with terminal deoxynucleotidyl transferase. A 1-ml reaction contained: 8 μM $d(T)_3$ as initiator, 2 mM ^{3}H-dTTP (50 μC per μmole), 0.1 M potassium cacodylate (pH 7.0), 0.1 M potassium phosphate (pH 7.0), 0.5 mM $CoCl_2$, 1 mM mercaptoethanol, and 40 μg of terminal transferase. The reaction, monitored by adsorption to DEAE paper, was complete in 6 hours at 37° (95% of the dTTP polymerized). Purification of the oligomer and further details on the preparation are given elsewhere (18).

Concentrations of oligo- and polynucleotides are given in terms of molarity of polymer; DNA concentrations are expressed in terms of nucleotide residues.

Methods

Assays—Assays of both synthesis and hydrolysis by DNA polymerase were performed in 50 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, and 1 mM mercaptoethanol. The reaction volume, enzyme and triphosphate concentrations, incubation time, and temperature are indicated in the legends to the figures and tables. For DNA-primed assays, incorporation of α-^{32}P-labeled dTTP was measured by precipitation with 7% perchloric acid (19). Since $d(T)_{300}$ is poorly precipitated by acid, synthesis and hydrolysis of deoxythymidylate polynucleotides were measured by adsorbing the polymerized materials to DEAE paper (Whatman DE-81) and eluting the mononucleotides and small oligonucleotides with 0.3 M ammonium formate, pH 8.0 (18). The measurement of hydrolysis of DNA has been described (15).

Nicked DNA Duplex—To introduce nicks into closed, circular DNA duplexes with pancreatic DNase, 220 pmoles of DNA nucleotide in 30 μl containing 50 mM Tris-hydrochloride-5 mM $MgCl_2$ (pH 7.4), were incubated for 2 hours at 37° in the presence of 0.2 to 2 ng of pancreatic DNase. The number of nicks was determined by velocity sedimentation of a sample on an alkaline sucrose gradient (5 to 20% sucrose, 0.3 M NaOH, 0.8 M NaCl, 1 mM EDTA) for 2 hours at 60,000 rpm in the SB-405 rotor of an International B-60 ultracentrifuge. Under these conditions the intact material travels approximately 2.5 times faster than the nicked DNA. The number of nicks (n) per molecule in the DNA preparation was calculated with the equation: fraction of intact molecules $= e^{-n}$.

Density Gradient Sedimentation—A density label, in addition to a radioactive one, was introduced into the product under standard reaction conditions except that dBrCTP replaced dCTP. The buoyant densities of the reaction products were measured before and after denaturation with alkali. After stopping the reaction with 25 mM EDTA, a 100-μl sample was denatured by the addition of 20 μl of 1 M NaOH. After 5 min at 0° the sample was neutralized by adding 20 μl of 1 M HCl and 300 μl of 1 M Tris buffer, pH 8.1. To the undenatured control sample (100 μl) only the Tris buffer was added. To each sample were added 3 g of CsCl, 25 μmoles of EDTA, 200 μg of bovine serum albumin, and water to a final volume of 3 ml. The bovine serum albumin was added to improve recoveries from the gradients, especially that of denatured DNA. Polyallomer centrifuge tubes were also soaked in 10 mg per ml of bovine serum albumin prior to use to minimize sticking of DNA to the walls of the tube. One milliliter of paraffin oil was layered over the sample in the centrifuge tube and the latter was centrifuged for 40 hours at 30,000 rpm at 15° in the SB 405 rotor of an International B-2 ultracentrifuge. After centrifugation, the fractions were collected into tubes containing 0.1 ml of 0.25 mg per ml of calf thymus DNA, precipitated with 5% trichloracetic acid, and filtered on a glass filter; the filter was counted in a scintillation counter.

To calculate the cesium chloride density gradient under these conditions, T7 DNA, poly d(A-T), and $^{2}H^{15}N$ T7 DNA of known densities were centrifuged to equilibrium under the conditions described above; the gradients were calculated from the known densities. Each experimental gradient contained a reference density marker and the density of any DNA relative to that marker was calculated assuming a constant density gradient. The denaturation procedure outlined above caused an increase in density in ΦX RF of 0.021 g cm^{-3} relative to a poly d(A-T) marker and may therefore be assumed to be effective.

If the density of fully dBrC-substituted DNA is assumed to be 0.085 g cm^{-3} greater than that for unsubstituted DNA (20), then the fractional density increase is defined as the increase in density relative to unsubstituted DNA, divided by 0.085. If the extent of replication (moles of product per mole of primer) is E, but only a fraction, f, of the primer molecules is actually involved in replication, then the real extent of replication is E/f. For undenatured material, the fractional density shift is $E/(f + E)$. If the primer and product are not covalently attached and the

complex is denatured, the fractional density shift will be 1; if they *are* covalently attached, calculation of the density of the product from the extent of replication depends on the average molecular weight of the primer after denaturation and the fraction of the single stranded breaks in the molecule which can participate in replication. For the simplest case, a singly nicked ΦX RF preparation in which all nicks are active in replication and no hydrolysis of the primer occurs, the fractional density increase is $2E/(f + 2E)$. In fact, hydrolysis of the DNA primer is known to occur during synthesis (*vide infra*). In practice, the small amount of hydrolysis with this primer ($\leq 25\%$) would cause an error in this expression less than or comparable to those in E and f.

Chromatography—The products of exonuclease digestion were analyzed by descending chromatography on Schleicher and Schuell Orange Ribbon paper with a solvent containing isobutyric acid, 1 M ammonium hydroxide, and 0.1 M EDTA in the proportions 100:60:1.6.

RESULTS

3′-OH-terminated Nick is Essential for Replication—Preparations of closed, circular DNA duplexes had very low activity as primers for *E. coli* DNA polymerase. However, even the best preparations were not inert, since they contained small amounts of the nicked form of the duplex. The importance of a 3′-OH-terminated nick in replication is based on three lines of evidence. (*a*) 3′-OH termini, introduced by scissions with pancreatic DNase, increased priming activity linearly in proportion to the number of nicks (Fig. 2). By extrapolation, we judge that a duplex with no nicks has insignificant priming activity. Since the replication rate is expressed in Fig. 2 as residues incorporated per residue of primer per unit time, and since the plasmid DNA molecule is four-tenths as large as ΦX RF (13), this molecule appears to be twice as active as a primer (Fig. 2). Actually, all duplex forms were equally active when assessed on the basis of number of residues incorporated per nick. (*b*) Physical studies have already shown that DNA polymerase does not bind to intact duplex regions of DNA but rather to nicked forms; 1 molecule of enzyme binds per nick (21). (*c*) Under conditions of replication, the growing chain was found attached exclusively to the nicked form in a mixed population of native closed and nicked molecules. Nicked and closed molecules can be separated either by the differences in their sedimentation velocities on a neutral sucrose gradient or by the differences in their buoyant densities in CsCl density gradients containing 100 μg per ml of ethidium bromide. In both cases, a short incubation (10 min at 37°) with the use of a labeled circular duplex primer under the usual conditions showed that the product had the same velocity and density as the nicked forms. We conclude, therefore, that the primer must have a terminus.

To be active in replication, the nick must have a 3′-OH terminus. Introduction by micrococcal nuclease of 3′-P-terminated nicks into a preparation of ΦX RF molecules which already contained an approximately equal number of 3′-OH-terminated nicks caused little change (7% decrease) in the priming activity of this preparation under conditions of *enzyme excess*. Conversion of termini from 3′-P to 3′-OH by the phosphatase action of exonuclease III increased the rate of DNA synthesis by 110%, but had no effect on the starting material. These results indicate that 3′-P-terminated groups are inactive for replication and can be fully activated by the action of phosphatase.

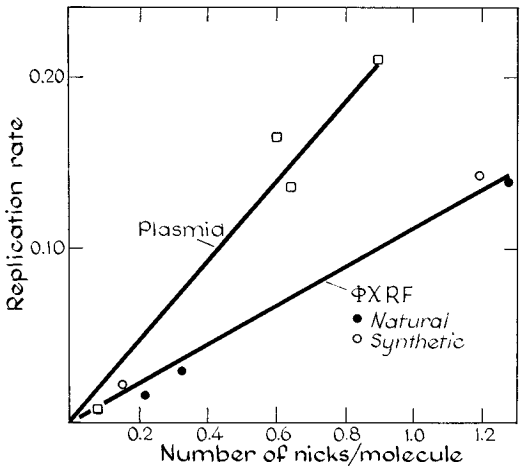

FIG. 2. Influence of nicks on the priming ability of circular DNA duplexes. Nicks were introduced into preparations of ΦX RF (natural or synthetic) and *E. coli* 15 plasmid DNA with the use of pancreatic DNase. The number of nicks was measured by velocity sedimentation (see "Methods"). The nicked circles were then incubated with excess DNA polymerase (1 nmole per ml) and labeled triphosphates (100 μM) under standard conditions. The replication rate represents the number of nucleotide residues synthesized per nucleotide residue of primer, measured in 15 min at 37°.

Results with linear duplexes were identical with those cited with circular duplexes. Phage T7 DNA, an intact linear duplex, was essentially inert for replication; template primer activity appeared in proportion to the number of 3′-OH nicks introduced by nuclease.[3]

Covalent Attachment of Product to Primer—The strict requirement of DNA polymerase for a 3′-OH terminus arises because the enzyme can catalyze only the addition of nucleotide residues to such a terminus to give chain extensions with a covalent linkage between product and primer.

Such covalent linkage was shown with ^{3}H-labeled ΦX RF DNA (67% intact duplex) as primer for DNA polymerase in a standard reaction mixture containing α-^{32}P-labeled dTTP but with dCTP replaced by dBrCTP. After 0.2 replication, the densities of product and primer were determined by centrifugation to equilibrium in a CsCl density gradient, both before and after alkaline denaturation (Fig. 3). Before denaturation (Fig. 3*a*) the product was intermediate in density between fully heavy and fully light; as expected, the majority of the primer molecules remained in the light region. Although 0.2 replication had occurred, the ratio of product to primer in the intermediate density region (Fractions 10 to 13) was, on the average, approximately 0.6. From these results it appears that two-thirds of the primer DNA is not involved in replication because of the large fraction of closed circles in the ΦX RF preparation. Thus the *real* extent of replication (see "Methods") is 0.6, not 0.2. The predicted fractional density increase in this case (see "Methods") is 0.38, in close agreement with a measured average of 0.37.

After denaturation, again the bulk of the primer was in a sharp peak, slightly denser than the original DNA (Fig. 3*b*), and presumably is denatured, intact ΦX RF. About 15% of the

[3] Under conditions (10 min at 37°) such that ΦX RF containing 0.4 nick per molecule gave 4.6% replication, T7 DNA, 8 times as long as ΦX RF and containing 0.5 nick per molecule, gave 0.5% replication.

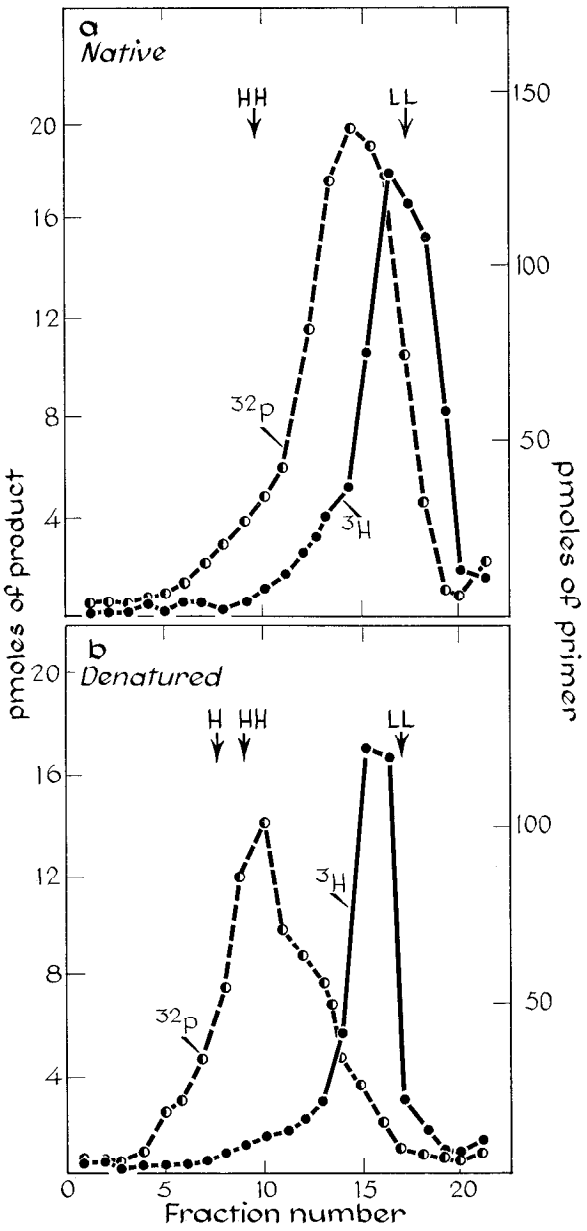

FIG. 3. Linkage between ΦX RF primer and its product: *a* before and *b*, after denaturation. ^{3}H-Thymidine-labeled ΦX RF, 2.2 nmoles (3,600 cpm per nmole), was used to prime a standard reaction (200 μl) containing dGTP, dATP, α-^{32}P-dTTP and dBrCTP (100 μM), and 100 pmoles of DNA polymerase. After 30 min at 37°, the reaction was stopped by adding EDTA and chilling to 0°. Of the sample, 100 μl were denatured as described under "Methods." The denatured sample and the remaining undenatured material were centrifuged in separate tubes to equilibrium in a CsCl density gradient, with ^{14}C-poly d(A-T) as a density reference (not shown). The calculated positions relative to the poly d(A-T) marker of light, double stranded DNA (*LL*), heavy, double stranded DNA (*HH*), and heavy, single stranded DNA (*H*) are indicated by *arrows*.

primer DNA appears to have increased in density by addition of product DNA. The ratio of product to primer in the intermediate density positions varied from 0.2 to 1.6; the average of 1.2 agreed with a real extent of replication of 0.6, assuming covalent attachment of product to the nicked (primer) strand. The average fractional density increase of 0.55 was likewise in reasonable agreement with calculation (see "Methods"). We therefore conclude that more than 90% of the product was covalently attached to the primer.

Inasmuch as a circular DNA primer template was used in this last experiment, it seemed possible that noncovalent initiation might occur if linear duplexes with "squared off" ends were tested. To check this, unlabeled T7 DNA (150 nmoles, 0.5 nick per molecule) was used as primer in a standard reaction mixture except that dCTP was replaced by dBrCTP. After 5% and 37% replication (10 and 90 min at 37°, respectively), the density of the product before denaturation was 0.004 and 0.040 g cm^{-3}, denser than the native primer DNA. After denaturation, the product was 0.019 and 0.053 g cm^{-3} denser than denatured primer DNA, compared to a value of 0.085 g cm^{-3} expected for fully bromocytosine-substituted DNA. The presence of DNA with an intermediate density value again supports covalent linkage.

A similar result was obtained when ^{3}H-labeled ^{2}H^{15}N T7 DNA was used to prime a standard reaction with unmodified "light" triphosphates. In this experiment, material of intermediate density, lighter than the primer was observed. Only with extensive replication with a T7 DNA containing 10 to 20 breaks per strand was a product obtained which appeared to be fully light. Thus the discrepancy between this result and that of Richardson, Schildkraut, and Kornberg (3) is probably due to the presence of endonuclease activity in the earlier enzyme preparations or to inactive nicks in the preparations of DNA or to both causes.

Degradation of Template Primer Is Associated with Synthesis—Chain growth at a nick by extension of the 3′-OH-terminated chain (Fig. 1*f*) should entail the displacement or removal of the 5′-terminated chain that lies in the path. The experiments which follow show that, in the initial phase of replication at a nick, there is a marked increase in the rate of hydrolysis and that this rate matches the rate of chain growth.

Degradation of *E. coli* DNA was greatly enhanced by the presence of deoxyribonucleoside triphosphates (Fig. 4*a*), thus confirming earlier observations (22). All four triphosphates were required (Table I); analogues which bind in the triphosphate site but do not substitute in replication, such as ATP and the α,β-dTTP methylene diphosphonate (10), did not support the enhanced degradation of DNA.

The kinetics of degradation coincided with that of synthesis (Fig. 4*b*). A homopolymer duplex of d(A)$_{4000}$ matched by short segments of ^{3}H-d(T)$_{300}$ served as a template primer for ^{32}P-dTTP incorporation. Synthesis proceeded up to the level of the input d(A)$_{4000}$ with a coincident degradation of the input ^{3}H-d(T)$_{300}$. Thus in the presence of homopolymer synthesis, supported by a single triphosphate, hydrolysis and synthesis are equimolar and have similar kinetics, and both cease when the d(T)$_{300}$ chains have been completely solubilized. In the absence of synthesis, the initial rate of hydrolysis was about 20 times slower.

Synthesis-stimulated Degradation of Template Primer Occurs Only at 3′-OH Nicks—Synthesis with DNA which had been treated with exonuclease III (as in Fig. 1*a*) or with denatured DNA (as in Fig. 1*b*) showed little or no enhancement of degradation (50% and −30%, respectively) compared to that for samples of the "native" DNA (290% to 610%). The synthesis-stimulated degradation, observed with so-called "native" duplex DNA, varied greatly from one DNA preparation to another, presumably a function of the abundance of 3′-OH-nicked regions

TABLE I

Requirement for all triphosphates to stimulate hydrolysis of DNA

Hydrolysis was measured in the usual reaction mixture containing 20 nmoles of ^{32}P-*E. coli* DNA (6×10^6 cpm per μmole of nucleotide), 3 pmoles of DNA polymerase, and each nucleoside triphosphate at 33 μM. Incubations were at pH 7.4 for 30 min at 37°.

Additions	Nucleotide made acid-soluble
	nmoles
None	0.13
dATP, dTTP, dGTP, dCTP	1.85
dTTP, dGTP, dCTP	0.13
dGTP, dCTP	0.10
dATP, dTTP	0.12
ATP, dTTP, dGTP, dCTP	0.13

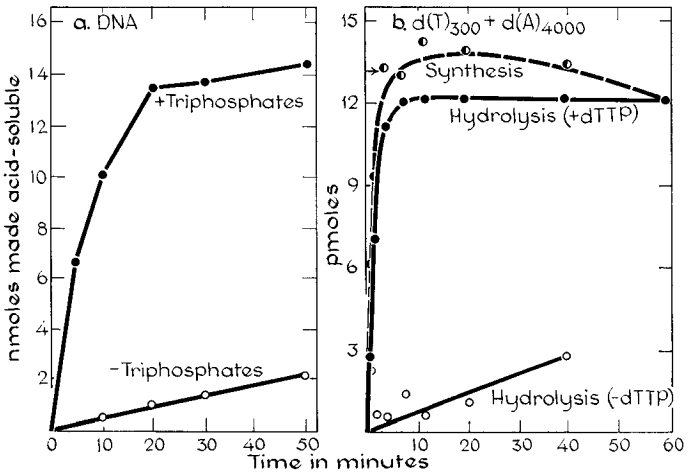

FIG. 4. Triphosphate stimulation of DNA hydrolysis and relative rates of synthesis and hydrolysis. *a*, hydrolysis was measured under the usual conditions (see "Methods") at pH 7.4 with 46 nmoles of ^{3}H-*E. coli* DNA (6×10^5 cpm per μmole). The DNA was previously incubated in the usual reaction mixture (0.3 ml) for 20 min at 37° with 0.17 μg per ml of pancreatic DNase followed by the addition of 10 pmoles of DNA polymerase to all mixtures and 33 μM deoxyribonucleoside triphosphates to half of the mixtures and an equivalent volume of water to the other mixtures. Incubations with DNA polymerase were performed for the indicated times at 37°. In *b*, the mixture contained, in 0.05 ml, 1.2 pmoles of $d(A)_{4000}$, 12 pmoles of ^{3}H-labeled $d(T)_{300}$ (4×10^7 cpm per μmole of nucleotide), 70 mM phosphate (pH 7.4), 5 mM $MgCl_2$, and 10 pmoles of DNA polymerase. The concentration of dTTP, where present, was 0.4 mM (1.2×10^8 cpm per μmole). Samples were removed at intervals during the incubation at 21° and the radioactivity which adsorbed to DEAE paper was measured. The *arrow* on the *ordinate* corresponds to the input concentration of poly dA.

in the DNA relative to the total number of exonuclease sites. To confirm that 3′-OH nicks in native DNA were the active sites of degradation, *E. coli* DNA was nicked to a limited extent by pancreatic DNase.[4] The initial rate of solubilization of the primer by DNA polymerase was increased 4-fold by this DNase treatment, in the absence of synthesis, and 50-fold in the presence of all four triphosphates. No such effect was obtained with

[4] ^{3}H-*E. coli* DNA (92 nmoles, 6×10^5 cpm per μmole) in 0.3 ml was incubated with pancreatic DNase (0.17 μg per ml, 30 min at 37°) under standard assay conditions. No DNA was rendered acid-soluble under these conditions.

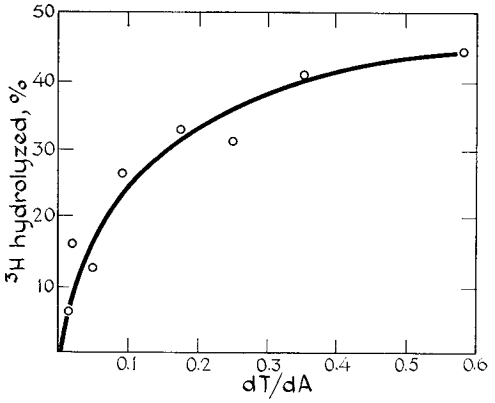

FIG. 5. Influence of $d(T)_{300}$:$d(A)_{4000}$ ratio on hydrolysis of the $d(T)_{300}$. Each tube contained, in 0.1 ml, 7 pmoles of ^{3}H-$d(T)_{300}$, 500 μM dTTP, 4 pmoles of DNA polymerase and 0.5, 0.7, 1, 2, 3, 4, 6, or 8 pmoles of $d(A)_{4000}$. The samples were incubated 3 min at 21°. The insoluble material was determined by adsorption to DEAE paper. In control tubes with no dTTP, no hydrolysis was observed (<4%). *dT/dA* is the molar ratio expressed in concentrations of nucleotide residues.

DNA previously treated with micrococcal nuclease to produce 3′-P-terminated nicks.

The importance of nicked regions for the synthesis-stimulated degradation was also shown by lowering the molar ratio of $d(T)_{300}$ to $d(A)_{4000}$ from the level of about 10 (complete matching as in Fig. 4*b*) to a ratio of about 1 while keeping the number of thymidylate termini constant. With a decrease in ratio there is a decrease in the frequency of nicks. A corresponding decrease in synthesis-stimulated degradation was observed (Fig. 5), despite the constancy of the number of thymidylate termini.

Template Primer Degradation Associated with Synthesis Progresses in a 5′ → 3′ Direction—DNA polymerase binds a nicked region of DNA in its active center (21). Distinctive exonuclease sites within the center enable the enzyme to catalyze hydrolysis of the 3′-terminated chain (3′ → 5′) or of the 5′-terminated chain (5′ → 3′) (1). The following experiments support the conclusion that the degradation of the template primer associated with synthesis is predominantly, if not exclusively, of the 5′-terminated chain, the chain which lies in the path of the growing strand.

^{3}H-Polythymidylate chains, of about 200 residues, were extended by terminal transferase at the 3′-hydroxyl end with an average of 20 ^{32}P-labeled deoxythymidylate residues. Such chains were thus labeled distinctively with ^{32}P for one-tenth of their length at the 3′-OH end and with ^{3}H in the remainder. The assignment of this structure is verified by the data in Fig. 6, *a*, *b*, and *d*. When the labeled chains were degraded directly by DNA polymerase in the absence of $d(A)_{4000}$ (Fig. 6*a*), virtually all of the ^{32}P was released before the ^{3}H was liberated.[5] Inasmuch as single stranded DNA is attacked exclusively 3′ → 5′ (18), this is exactly the result expected. In the presence of $d(A)_{4000}$, degradation occurs at both ends (18). As seen in Fig. 6*b*, this observation was confirmed, although with some preference for 5′ → 3′ degradation; these measurements, made at 21°, illustrate the sharply reduced 3′ → 5′ exonucleolytic rates for

[5] In molar terms, the rates were almost equal, probably because of asynchronous hydrolysis. Thus the ^{3}H-labeled regions of some chains were probably hydrolyzed before the hydrolysis of the ^{32}P-labeled regions of other chains was complete.

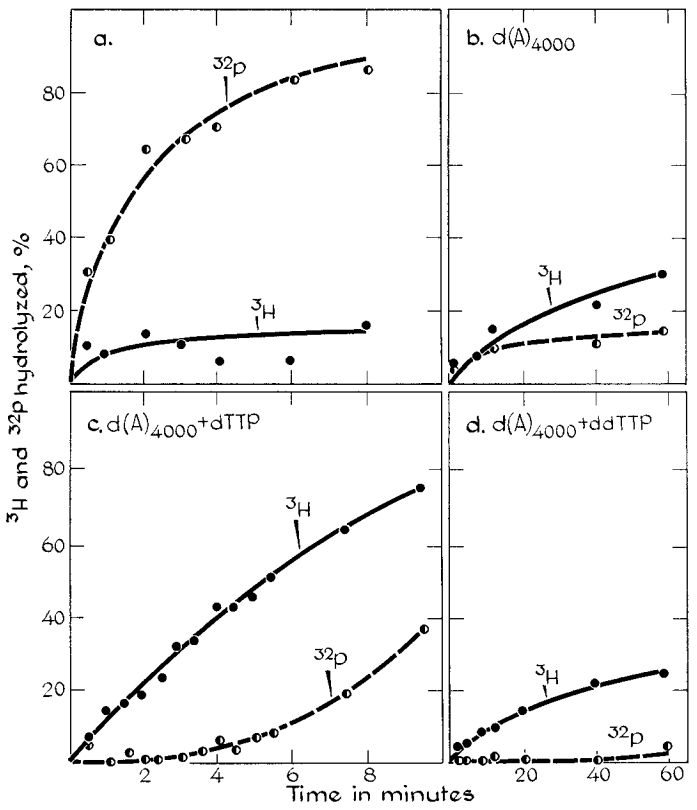

FIG. 6. Rate of degradation by DNA polymerase of ^{3}H-d(T)$_{200}$ with 20 ^{32}P-thymidylate residues at the 3′-end. Each tube contained, in 0.1 ml, 35 pmoles of ^{3}H, ^{32}P-d(T)$_{220}$ (^{32}P, 1×10^8 cpm per μmole; ^{3}H, 2×10^7 cpm per μmole), 20 pmoles of DNA polymerase, 63 mM phosphate (pH 7.4), and 5 mM $MgCl_2$. Added, as indicated, were 2.5 pmoles of d(A)$_{4000}$, 0.5 mM dTTP, and 0.5 mM ddTTP. Samples were removed at intervals and the amount which adsorbed to DEAE paper was measured. Note that the time scales in *a* and *c* are 10 times those in *b* and *d*.

TABLE II

Products of hydrolysis of d(T)$_{300}$

Each experiment contained, in 0.1 ml, 50 pmoles of ^{3}H-d(T)$_{300}$ (3×10^7 cpm per μmole of nucleotide) and 20 pmoles of DNA polymerase. Where indicated, additions were 3 pmoles of d(A)$_{4000}$, 50 nmoles of dTTP, or 50 nmoles of ddTTP. Samples were incubated at 37° for 4 min. Identification of products was by descending chromatography.

Extent and products of hydrolysis	Additions			
	None	d(A)$_{4000}$ + ddTTP	d(A)$_{4000}$	d(A)$_{4000}$ + dTTP
Hydrolysis (%)	15	4	6	15
Products (%)				
d(T)$_1$	>97	75	82	56
d(T)$_2$	<2	20	15	21
d(T)$_3$	<1	5	3	11
d(T)$_{4\text{-}6}$	0	0	0	12[a]
Exonuclease action	3′ → 5′	5′ → 3′	Both	—[b]

[a] Tentative identifications of these compounds indicate the following percentage distribution: d(T)$_4$, 4.8; d(T)$_5$, 3.7; d(T)$_6$, 3.4.

[b] Object of inquiry in this experiment.

duplex as compared with single stranded chains (compare Fig. 6, *a* and *b*). With ddTTP present, chains which add such a residue become almost insusceptible to 3′ → 5′ exonuclease action, as well as being inert to further growth (23). We may conclude from Fig. 6*d* that the addition of a dideoxynucleotide residue to the labeled chains was favored over 3′ → 5′ degradation and, when achieved, effectively blocked such degradation. By contrast, ^{3}H liberation, indicative of 5′ → 3′ degradation, proceeded as in the absence of ddTTP (compare Fig. 6, *b* and *d*).

Finally, when degradation was measured in the presence of sustained synthesis, made possible by the presence of dTTP, there was an initial rate of release of ^{3}H more than 40 times greater than that of ^{32}P (Fig. 6*c*). Although the ^{32}P was liberated eventually, the pronounced lag in its release signifies that the chains were degraded from 5′ → 3′ to begin with; only after extensive removal of the ^{3}H-labeled residues were the ^{32}P-labeled regions at the 3′ ends attacked.

Another line of evidence favors the contention that synthesis-stimulated degradation is chiefly 5′ → 3′. In the absence of d(A)$_{4000}$, hydrolysis is exclusively 3′ → 5′ and the products of hydrolysis are mononucleotides (24) (Table II, Column 1); in the presence of d(A)$_{4000}$ and the chain growth terminator, ddTTP, hydrolysis is exclusively 5′ → 3′ and the products in this case include dinucleotides (18) and trinucleotides (Table II, Column 2). This characteristic difference between the 3′ → 5′ and 5′ → 3′ exonucleolytic actions can be used to determine which of the two nuclease actions is stimulated by synthesis (Table II). In the presence of d(A)$_{4000}$ and dTTP (and, therefore, of synthesis) the dinucleotide abundance (Table II, Column 4) resembled that of the 5′ → 3′ exonuclease and therefore supports the conclusion that it is this exonuclease which is active during synthesis.

A much larger frequency of oligonucleotides was observed among the products of 5′ → 3′ hydrolysis in the presence than in the absence of synthesis. The reason for this is not yet clear. The distribution of products was the same for 4% or for 60% hydrolysis, and is thus independent of the degree of hydrolysis.

When hydrolysis was permitted from both ends (Table II, Column 3), there was a high proportion of dinucleotides. This result implies that, under such conditions, the 5′ → 3′ exonuclease is considerably more rapid than the 3′ → 5′ exonuclease (compare Fig. 6*b*).

DISCUSSION

We have presented evidence here that *E. coli* DNA polymerase can use a double stranded DNA as a primer for DNA synthesis only if that DNA contains a single chain scission with a 3′-OH terminus. The enzyme will not initiate synthesis either at the ends of a linear duplex or at a sequence of nucleotides along an intact DNA helix. The local distortion and denaturation of a DNA helix which may be present in the vicinity of a nick are not sufficient by themselves to allow the initiation of DNA synthesis, since the nick must also contain a 3′-OH terminus. Replication occurs by extension of the primer strand at the nick rather than by initiation of a chain, *de novo*. Replication of nicked T7 DNA or of nicked ΦX RF in which bromocytosine was incorporated into the product DNA as a density label enabled us to demonstrate that at least 90% of the product was covalently attached to DNA of primer density after alkaline denaturation. Since initiation of synthesis on a circular, single stranded ring seems also to require a 3′-OH-terminated oligonucleotide as initiator

(6), no case has yet been clearly documented in which DNA polymerase can initiate synthesis *de novo* except, perhaps, for the synthesis "*de novo*" of poly d(A-T) (2).

During synthesis in which a duplex is restored (Fig. 1*a*), the primer chain is extended by covalent attachment to the 3′-hydroxyl end. During replication at a nick (Fig. 1*f*), we have postulated that the same pattern is followed. Since, in the latter case, the 5′-ended chain lies in the path of elongation of the 3′ chain, some mechanism or mechanisms must exist for removing the 5′ chain. That the 5′ → 3′ exonuclease activity of the polymerizing molecule is initially responsible for hydrolyzing the 5′-ended chain is apparent from the observations that the onset of replication at a nick is associated with a burst of hydrolysis of the primer, equimolar with synthesis, and in a 5′ → 3′ direction. In support of this model, we have presented evidence (18) that the 5′-exonuclease site is oriented relative to the polymerizing site so as to permit one enzyme to synthesize and hydrolyze simultaneously. The alternative explanation that synthesis causes displacement of the 5′-end, which is then degraded by the 5′ → 3′ nuclease activity of another polymerase molecule, seems unlikely since this exonuclease activity has an absolute requirement for a double stranded template (18). It is interesting to note that the T4 polymerase, which cannot utilize nicked DNA as a template primer, has no 5′ → 3′ nuclease activity (18).

The mechanism by which the 5′-exonuclease action releases oligonucleotides of varying sizes has not been settled. It seems more probable that there is a single 5′-exonuclease site on the enzyme rather than several. Movement of the 5′-ended chain through this site may permit subterminal diester bonds to be cleaved with a decreasing frequency.[6]

Since the frequency distribution of oligonucleotides is maintained during the synthesis-coupled hydrolysis, it is tempting to believe that this hydrolytic activity is an integral part of the enzyme function and may be associated *in vivo* with a repair function whereby damaged regions of DNA, resistant to hydrolysis, are excised as oligonucleotides and replaced. For example, the initial step in the excision of thymine dimers produced by ultraviolet light may be the introduction of a 3′-hydroxyl nick to the 5′ side of a dimer by an endonuclease (for a review, see Reference 25). The *E. coli* DNA polymerase, by translating the nick, could excise the dimer and replace that region. The chains could then be sealed by the polynucleotide ligase (26). Recent studies have shown that the 5′ → 3′ nuclease function of *E. coli* polymerase can indeed excise oligonucleotides containing thymine dimers from ultraviolet-irradiated DNA (27).

Another important question which this work points to but leaves unclear is the mechanism of the several-fold net synthesis of DNA observed in earlier studies (28). In the replication of $d(A)_{4000}\cdot d(T)_{300}$ (Fig. 4*b*), synthesis stops at one replication when all of the oligo dT primer has been hydrolyzed. More extensive synthesis, including that of branched or readily renaturable polymers (29), could not occur since only one triphosphate, dTTP, was present in the reaction mixture. With DNA as primer, synthesis-stimulated hydrolysis usually ceased after about 25% hydrolysis. Achievement of net synthesis is influenced by the number of nicks in the DNA and perhaps by factors which affect the 5′ → 3′ nuclease action and lead to a displacement step, as illustrated in Fig. 1*g*. It is known that, for DNA, nick translation is an early event in the reaction (Fig. 4*a*) and also that the synthesis of branched DNA occurs after extensive synthesis (30). It is pertinent now to investigate how net synthesis and the synthesis of branched DNA may be related (1).

[6] The distribution of products can be explained simply if we ascribe a fixed probability of cleavage (p) to each bond, and assume that the frequency of producing n-mers is p times the probability $[1 - p]^{n-1}$ of not cleaving the preceding bonds.

REFERENCES

1. Kornberg, A., *Science*, **163**, 1410 (1969).
2. Englund, P. T., Deutscher, M. P., Jovin, T. M., Kelly, R. B., Cozzarelli, N. R., and Kornberg, A., *Cold Spring Harbor Symp. Quant. Biol.*, **33**, 1 (1968).
3. Richardson, C. C., Schildkraut, C. L., and Kornberg, A., *Cold Spring Harbor Symp. Quant. Biol.*, **28**, 9 (1963).
4. Wu, R., and Kaiser, A. D., *J. Mol. Biol.*, **34**, 523 (1968).
5. Goulian, M., Lucas, Z. J., and Kornberg, A., *J. Biol. Chem.*, **243**, 627 (1968).
6. Goulian, M., *Proc. Nat. Acad. Sci. U. S. A.*, **61**, 284 (1968).
7. Klett, R. P., Cerami, A., and Reich, E., *Proc. Nat. Acad. Sci. U. S. A.*, **60**, 943 (1968).
8. Goulian, M., and Kornberg, A., *Proc. Nat. Acad. Sci. U. S. A.*, **58**, 1723 (1967).
9. Okazaki, R., and Kornberg, A., *J. Biol. Chem.*, **239**, 269 (1964).
10. Englund, P. T., Huberman, J. A., Jovin, T. M., and Kornberg, A., *J. Biol. Chem.*, **244**, 3038 (1969).
11. Jovin, T. M., Englund, P. T., and Bertsch, L. L., *J. Biol. Chem.*, **244**, 2996 (1969).
12. Kato, K., Gonçalves, J. M., Houts, G. E., and Bollum, F. J., *J. Biol. Chem.*, **242**, 2780 (1967).
13. Cozzarelli, N. R., Kelly, R. B., and Kornberg, A., *Proc. Nat. Acad. Sci. U. S. A.*, **60**, 992 (1968).
14. Deutscher, M. P., and Kornberg, A., *J. Biol. Chem.*, **244**, 3029 (1969).
15. Deutscher, M. P., and Kornberg, A., *J. Biol. Chem.*, **244**, 3019 (1969).
16. Lehman, I. R., *J. Biol. Chem.*, **235**, 1479 (1960).
17. Riley, M., Maling, B., and Chamberlin, M. J., *J. Mol. Biol.*, **20**, 359 (1966).
18. Cozzarelli, N. R., Kelly, R. B., and Kornberg, A., *J. Mol. Biol.*, in press.
19. Mitra, S., Reichard, P., Inman, R. B., Bertsch, L. L., and Kornberg, A., *J. Mol. Biol.*, **24**, 429 (1967).
20. Goulian, M., Kornberg, A., and Sinsheimer, R. L., *Proc. Nat. Acad. Sci. U. S. A.*, **58**, 2321 (1967).
21. Englund, P. T., Kelly, R. B., and Kornberg, A., *J. Biol. Chem.*, **244**, 3045 (1969).
22. Lehman, I. R., *Annu. Rev. Biochem.*, **36**, 645 (1967).
23. Atkinson, M. R., Huberman, J. A., Kelly, R. B., and Kornberg, A., *Fed. Proc.*, **28**, 347 (1969).
24. Lehman, I. R., and Richardson, C. C., *J. Biol. Chem.*, **239**, 233 (1964).
25. Takagi, Y., Sekiguchi, M., Okubo, S., Nakayama, H., Shimada, K., Yasuda, S., Nishimoto, T., and Yoshihara, H., *Cold Spring Harbor Symp. Quant. Biol.*, **33**, 219 (1968).
26. Olivera, B. M., and Lehman, I. R., *Proc. Nat. Acad. Sci. U. S. A.*, **57**, 1426 (1967).
27. Kelly, R. B., Atkinson, M. R., Huberman, J. A., and Kornberg, A., *Nature*, **224**, 495, (1969).
28. Bessman, M. J., Lehman, I. R., Simms, E. S., and Kornberg, A., *J. Biol. Chem.*, **233**, 171 (1958).
29. Inman, R. B., Schildkraut, C. L., and Kornberg, A., *J. Mol. Biol.*, **11**, 285 (1965).
30. Schildkraut, C. L., Richardson, C. C., and Kornberg, A., *J. Mol. Biol.*, **9**, 24 (1964).