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Deoxyribonucleic Acid Polymerase: Two Distinct Enzymes in One Polypeptide

II. A PROTEOLYTIC FRAGMENT CONTAINING THE 5′ → 3′ EXONUCLEASE FUNCTION. RESTORATION OF INTACT ENZYME FUNCTIONS FROM THE TWO PROTEOLYTIC FRAGMENTS*

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SUMMARY

The small fragment (mol wt 36,000) produced by the limited proteolytic cleavage of DNA polymerase (mol wt 109,000) retains only the 5′ → 3′ exonuclease activity. The small fragment resembles the 5′ → 3′ exonuclease of the intact enzyme in degrading DNA to mono- and oligonucleotides and in its capacity to excise mismatched regions such as thymine dimers. It differs from the intact enzyme in that deoxyribonucleoside triphosphates, which support polymerization, fail to stimulate the exonuclease or to increase the proportion of oligonucleotides among the products. However with a mixture of small fragment, large fragment (mol wt 76,000; polymerase and 3′ → 5′ exonuclease functions), nicked DNA, and suitable deoxyribonucleoside triphosphates, the same influences of polymerization on 5′ → 3′ exonucleases are seen as with the intact enzyme. Thus at the locus of a nick in DNA, the two fragments bind adjacent to one another to perform the coordinated polymerization—5′ → 3′ exonuclease functions that characterize the intact enzyme.

Limited proteolysis of *Escherichia coli* DNA polymerase (mol wt 109,000) splits this single polypeptide chain into two fragments which retain the several catalytic activities of the intact enzyme (1–6). The *large fragment* (mol wt 76,000) which polymerizes deoxyribonucleotides (polymerase) and hydrolyzes DNA in the 3′ → 5′ direction (3′ → 5′ exonuclease) (1, 3) was described in the preceding paper (6). The *small fragment* (mol wt 36,000) retains only the ability to hydrolyze double-stranded DNA in the 5′ → 3′ direction (5′ → 3′ exonuclease) (3, 7) and is the subject of this report.

Unlike the intact enzyme, the small fragment has no detectable polymerase activity, and no exonuclease activity on single-stranded DNA. However, the products of the action of the small fragment on double-stranded DNA are identical with those generated by the 5′ → 3′ exonuclease of the intact enzyme since both enzymes excise mono- and oligonucleotides and also thymine dimers (8, 9). Neither enzyme exhibits detectable endonuclease activity on a variety of substrates.

The two different fragments can bind next to one another at a diester bond break (nick) in a DNA molecule, and this adjacent binding permits concomitant action of the polymerase and 5′ → 3′ exonuclease activities. Simultaneous action of the two fragments restores properties of the intact enzyme such as the stimulation of 5′ → 3′ exonuclease by concurrent DNA synthesis, and the autocatalytically primed synthesis of poly[d(A-T)].[1] However, the individual fragments show no direct affinity for one another in either the presence or absence of DNA.

EXPERIMENTAL PROCEDURE

Materials

Nucleotides and Polynucleotides—[^{14}C]dATP and [^{14}C]dCTP were purchased from Schwarz BioResearch; other deoxyribonucleotides were obtained as described previously (6). The

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[1] The abbreviations used are: poly[d(A-T)], alternating copolymer of deoxyadenylate and deoxythymidylate; $d(T)_2$, $d(T)_3$, $d(T)_5$, oligonucleotides (5′,3′) of deoxythymidylate containing 2, 3, and 5 residues, respectively, all with a 3′-hydroxyl and a 5′-phosphate terminus; $d(C)_3$, an oligonucleotide (5′,3′) of deoxycytidylate of 3 residues in length with a 3′-hydroxyl and a 5′-phosphate terminus; pppTpTpT, thymidylyl-(5′,3′)-thymidylyl-(5′,3′)-thymidine 5′-triphosphate; $d(A)_{4000}$, polydeoxyadenylate of length about 4000 residues; $d(T)_{4000}$, polydeoxythymidylate of length about 4000 residues; $d(I)_{1000}$, polydeoxyinosinic acid of length about 1000 residues; $d(T)_{200}$, polydeoxythymidylate of length about 200 residues; poly $d(A\text{-}T)_{1000}$, double-stranded, alternating copolymer of deoxyadenylate and deoxythymidylate of length about 1000 residues; $\widehat{(TT)}$, thymine dimer; $d(C)_{120}$-$d(T)_{160}$, a block copolymer containing about 120 residues of deoxycytidylate on the 5′ side of the molecule, and about 160 residues of deoxythymidylate at the 3′ side; $d(C)_{120}$ polydeoxycytidylate of length about 120 residues; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; $\overset{*}{p}\overset{*}{p}pTp(T)_{200}$, a 5′-triphosphate-terminated $d(T)_{200}$ with the β and γ phosphates labeled with [^{32}P]; pppTpT, thymidylyl-(5′,3′)-thymidine 5′-triphosphate; SDS, sodium dodecyl sulfate.

oligonucleotides $d(T)_2$, $d(T)_3$, $d(T)_5$, and $d(C)_3$ were prepared according to Weimann, Schaller, and Khorana (10) and [β, γ-^{32}P]-pppTpTpT was a gift from Dr. N. R. Cozzarelli (11).

$d(A)_{4000}$ and $d(T)_{4000}$ were prepared and purified according to Riley, Maling, and Chamberlin (12); [^{14}C]$d(A)_{4000}$ and [^{3}H]-$d(T)_{4000}$ were prepared in an identical manner using [^{14}C]dATP and [^{3}H]dTTP. $d(I)_{1000}$ was prepared and purified according to Chamberlin and Patterson (13). The synthesis of [^{3}H]$d(T)_{200}$ and the addition of a 2′,3′-dideoxythymidylate terminus to the 3′ end of an oligo(dT) have been described previously (8, 14). [β,γ-^{32}P]pppT$(pT)_{200}$ was prepared from [β,γ-^{32}P]pppTpTpT and [^{3}H]dTTP using terminal transferase according to the method of Cozzarelli, Kelly, and Kornberg (11). Activated calf thymus DNA and activated poly[$d(A\text{-}T)_{1000}$] were prepared by limited digestion with pancreatic deoxyribonuclease (15, 16). Concentrations of oligo- and polynucleotides are expressed as nucleotide residues.

Enzymes—E. coli DNA polymerase (Fraction VII) was used and had a specific activity of 4500 units per mg with activated calf thymus DNA (4). The large fragment of DNA polymerase was prepared either as described in this communication or purified from *E. coli* as described previously (6). No differences were observed between the two preparations (6). Pancreatic deoxyribonuclease was purchased from Worthington Biochemicals. Terminal deoxynucleotidyltransferase from calf thymus (17) was a gift of Dr. F. N. Hayes (Los Alamos Scientific Laboratory, Los Alamos, New Mexico), and subtilisin (Carlsberg) (18) was a gift of Dr. T. Link.

Methods

Polymerase and Exonuclease Assays—The assay of DNA synthesis on activated calf thymus DNA has been described (6), and the specific assays for 3′ → 5′ exonuclease and 5′ → 3′ exonuclease were carried out as described previously (6) except as noted in the text. Concurrent synthesis and hydrolysis of polydeoxythymidylate was measured under the conditions for assay of 5′ → 3′ exonuclease, but [^{3}H]$d(T)_{200}$ was substituted for [^{3}H]$d(T)_{300}$, and [α-^{32}P]dTTP was present at 0.5 mM.

Endonuclease Assays—Endonuclease assays were carried out in 67 mM potassium phosphate (pH 7.4) and 6.7 mM $MgCl_2$ using one of the following substrates; [^{14}C]$d(A)_{4000}$·[^{3}H]$d(T)_{4000}$; [^{14}C]-$d(A)_{4000}$·[^{3}H]$d(T)_{4000}$ (7% $\widehat{TT}$); and [^{14}C]$d(A)_{4000}$·[^{3}H]$d(T)_{200}$. The dA and dT concentrations were 30 μM and 26 μM, respectively. After 30 min at 37°, the assay mixture was made 10 mM in EDTA to stop the reaction, and 100 μl were layered on a 4-ml alkaline sucrose gradient (5 to 20% sucrose in 0.1 M NaOH, 0.9 M NaCl). The gradients were centrifuged at 50,000 rpm for 6 hours at 4° in a Spinco L-2-65B centrifuge with an SW56 rotor. Fractions of 10 drops (0.1 ml) were collected and counted in Triton-toluene scintillation fluid (19). Endonucleolytic cleavage was estimated by comparing the sedimentation profile of the polynucleotides in the assay to those of untreated polynucleotides sedimented in parallel gradients. Since the labeled polynucleotides are somewhat heterogeneous, the assay is not very sensitive, but one endonucleolytic break in 50% of the polynucleotide molecules could have been detected.

Synthesis of [^{14}*C*]$d(C)_{120}$-[^{3}*H*]$d(T)_{160}$—The block copolymer $d(C)_{120}$-$d(T)_{160}$ was prepared by synthesizing $d(C)_{120}$ first and then extending this chain with deoxythymidylate residues using terminal transferase. [^{14}C]$d(C)_{120}$ was synthesized in 1 ml of 100 mM cacodylate, 100 mM potassium phosphate (pH 6.8) containing 0.25 mM $CoCl_2$, 0.67 mM [^{14}C]dCTP, 16 μM $d(C)_3$, and 54 μg of terminal transferase. The polymerization was complete (>90% utilization of the [^{14}C]dCTP) after 8 hours at 37°, and the reaction was stopped by addition of 25 μl of 45% KOH. After 10 min at 4° the mixture was neutralized with 20 μl of 85% H_3PO_4 and dialyzed for 36 hours against 1 M NaCl, 10 mM potassium phosphate (pH 7.4), and 1 mM EDTA. This deoxycytidylate polymer (calculated to be $d(C)_{120}$) was extended with deoxythymidylate residues in a reaction mixture identical with that described above but containing 0.5 mM [^{3}H]dTTP, 0.38 mM $d(C)_{120}$, and 36 μg of terminal transferase. Polymerization was complete in 6 hours with >95% utilization of the dTTP; the reaction was stopped and the polymer purified as described above. The theoretical composition of the block copolymer is $d(C)_{120}$-$d(T)_{160}$, and this value agrees with the ratio of [^{14}C] to [^{3}H] in the final copolymer.

Further purification of the $d(C)_{120}$-$d(T)_{160}$ was effected by annealing the polymer to an excess of $d(A)_{4000}$ and sedimenting the duplex in a neutral sucrose gradient (5 to 20% sucrose in 0.1 M NaCl, 10 mM potassium phosphate buffer (pH 7.4), and 1 mM EDTA) as described above. Of the dC residues, 7% did not sediment with the $d(A)_{4000}$, but remained near the top of the gradient in a region which had a mole ratio of dC to dT of 15; these fractions were discarded. The peak fractions further down the gradient (dC to dT mole ratio = 0.75) were pooled and concentrated. The $d(C)_{120}$-$d(T)_{160}$ was separated from the $d(A)_{4000}$ by sedimentation in alkaline sucrose as described above. This is the preparation of $d(C)_{120}$-$d(T)_{160}$ used in the nuclease assay.

The copolymer was also tested for the presence of thymidylate strands which contained only a few cytidylate residues at the 5′ end by annealing to $d(I)_{1000}$ (20) and sedimenting the mixture in a neutral sucrose gradient. Of the dT residues, 6% remained at the top of the tube in a region with a molar ratio of dC to dT of 0.1. However the copolymer was not purified using this method due to our inability to separate completely all of the dI strands from the copolymer; introduction of this small amount of oligo(dI) would have complicated assays using the block copolymer.

Proteolytic Cleavage of DNA Polymerase and Separation of Fragments—DNA polymerase was split into two fragments by proteolytic cleavage as described by Klenow and Overgaard-Hansen (3). Of intact DNA polymerase, 6.0 mg were incubated in 20 ml containing 67 mM potassium phosphate (pH 6.5), 25 mM EDTA, 30 mM $(NH_4)_2SO_4$, 10% glycerol, 3 mg of BSA, 6.25 mg of activated calf thymus DNA, and 20 μg of subtilisin (Carlsberg). After 40 min at 37°, the mixture was chilled in ice and then 0.2 ml of 33 mM PMSF was added to inactivate the subtilisin (21). After 1 hour at 4°, the mixture was adjusted to 0.2 M in potassium phosphate (pH 6.8), 1 mM in β-mercaptoethanol, and DNA was removed by passage of the enzyme through a 5-ml column of DEAE-cellulose equilibrated in the same buffer. The fraction passing through the column was dialyzed for 24 hours against two changes of 20 mM potassium phosphate (pH 6.8), 1 mM β-mercaptoethanol, and then adsorbed to a 35-ml phosphocellulose column equilibrated in the dialysis buffer. The two fragments were separated by a linear gradient of 0.04 M → 0.16 M potassium phosphate (pH 6.8), 1 mM β-mercaptoethanol (100 ml of each buffer). Appropriate column

fractions were pooled and concentrated by vacuum dialysis. Total recoveries of both fragments were 70% (see Fig. 1).

Chromatography—Degradation products of [^{3}H]d(T)$_{200}$ and [^{3}H]d(T)$_{200}$ (7% $\widehat{TT}$) were separated by descending chromatography on DEAE-paper by sequential elution with 0.25 M ammonium bicarbonate and 0.3 M ammonium formate (9). Degradation products of [β,γ-^{32}P]pppTp(T)$_{200}$ ($\overset{**}{p}$ppTp(T)$_{200}$) were chromatographed for 40 hours on Schleicher and Schuell orange ribbon paper with markers of dTTP, pppTpT, and pppTpTpT. The solvent system was isobutyric acid-1 M NH_4OH-0.1 M EDTA (100:60:1.6) (11). Strips of 5 mm were cut out and counted.

Miscellaneous—Thymine dimers ($\widehat{TT}$) were formed in oligo- and polynucleotides of deoxythymidylate by irradiation with a low pressure mercury lamp. The absorbance at 267 nm was followed with time, and the percentage of thymine residues converted to dimers was calculated from the data of Deering and Setlow (22).

SDS-acrylamide gels were run as previously described (1) and protein molecular weights were determined on these gels by the method of Shapiro *et al.* (23) with the modifications of Weber and Osborn (24). DNA polymerase, bovine serum albumin, the dimer of bovine serum albumin, ovalbumin, and chymotrypsinogen were used as molecular weight markers.

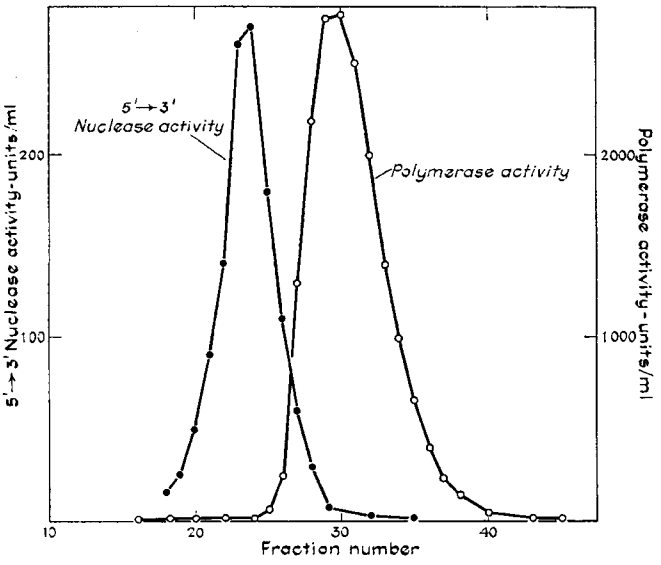

FIG. 1. Separation of the active fragments of DNA polymerase produced by proteolytic cleavage of intact DNA polymerase. Details of the cleavage and phosphocellulose chromatography are under "Methods." The gradient was started at fraction number 4, and 3-ml fractions were collected. The 5′ → 3′ exonuclease unit is 10 nmoles of nucleotide excised per hour.

TABLE I

Catalytic activities of small fragment compared with intact enzyme

DNA synthesis was measured with calf thymus DNA, and 5′ → 3′ exonuclease with concomitant DNA synthesis was assayed on d(A)$_{4000}$·[^{3}H]d(T)$_{200}$ with 0.5 mM dTTP. Enzyme concentrations were determined from the absorbance at 278 nm.

Reaction	Intact enzyme	Small fragment
	moles nucleotide polymerized or excised/mole enzyme/min	
DNA synthesis	148	<3
3′ → 5′ Nuclease	16	<0.2
5′ → 3′ Nuclease	27	37
5′ → 3′ Nuclease in the presence of deoxynucleoside triphosphates	260[a]	37[b]

[a] 5′ → 3′ Nuclease concomitant with DNA synthesis.
[b] No DNA synthesis.

RESULTS

Isolation of Small Fragment

Two laboratories have demonstrated that proteolysis of *E. coli* DNA polymerase yields an active fragment (the large fragment) of molecular weight 76,000 which retains the polymerase and 3′ → 5′ exonuclease activities of the intact enzyme (1, 2). Klenow and Overgaard-Hansen have further demonstrated that if the proteolysis is carried out in the presence of DNA, the polymerase is cleaved and one obtains not only the large fragment, but also a small fragment (mol wt 36,000) which contains only the 5′ → 3′ exonuclease activity (3). We have confirmed this result and have separated the two fragments by phosphocellulose chromatography (Fig. 1). Each of the purified fragments gave a single band on SDS-acrylamide gel electrophoresis and was stable for at least 6 months when stored in liquid nitrogen. However, the small fragment was quite labile at 37°, and lost over 50% of its activity in 25 min under assay conditions; in contrast, both the large fragment and the intact enzyme are stable under these conditions.[2]

Catalytic Activities of Small Fragment

The small fragment had full 5′ → 3′ exonuclease activity on a double-stranded DNA substrate, with even a slightly higher turnover number than that of the intact enzyme (Table I). However, the small fragment had no detectable nuclease activity on single-stranded DNA and no detectable polymerase activity (Table I).

The 5′ → 3′ exonuclease of the intact enzyme was dramatically stimulated by concomitant DNA synthesis (Table I) (8). This effect was, as expected, not seen with the small fragment (Table I).

Specificity of Small Fragment

Degradation Products of d(A)$_{4000}$·d(T)$_{200}$ and d(A)$_{4000}$·d($\widehat{TT}$)$_{200}$ (7% $\widehat{TT}$)—The 5′ → 3′ nuclease of the intact enzyme generated a characteristic mixture of mono- and oligonucleotides upon degradation of d(A)$_{4000}$·d(T)$_{200}$. The distribution of products was unaffected by the extent of hydrolysis of the substrate (Table II) (8). The small fragment also produced the same distribution of mono- and oligonucleotides and exhibited the same specificity as did the intact enzyme (Table II). The products were also similar when the 5′ → 3′ nuclease action was on a d(A)$_{4000}$·d(T)$_{200}$ in which 7% of the thymine residues were linked thymine dimers. Both the intact enzyme and the small fragment degraded this substrate to about the same extent, and excised the same amount of thymine dimers as oligonucleotides of 4 to 8 residues in length (Table II) (9). Larger oligonucleotides do not migrate from the origin in the chromatographic system used and were therefore not detected.

[2] P. Setlow and A. Kornberg, unpublished results.

TABLE II

Products of hydrolysis of $d(A)_{4000}\cdot d(T)_{200}$ or $d(A)_{4000}\cdot d(T)_{200}$ (7% $\widehat{TT}$)

Degradations were carried out at 37° in 0.4 ml of 67 mM potassium phosphate (pH 7.4), 6.7 mM $MgCl_2$, and 67 μg per ml of BSA containing 32 nmoles of $d(A)_{4000}$ and 28 nmoles of either [^{3}H]-$d(T)_{200}$ or [^{3}H]$d(T)_{200}$ in which 7% of the residues were thymine dimers. Intact polymerase (40 pmoles) or the small fragment (48 pmoles) was added to start the reaction; 100-μl samples were removed and hydrolysis was halted by addition of 3 μl of 0.5 M EDTA. The digest was chromatographed on DEAE-paper with appropriate markers. The percentage of degradation of the $d(T)_{200}$ was determined by absorption of a small sample (10 μl) of the digest to DEAE-paper, and elution of mononucleotides and small oligonucleotides with 0.3 M ammonium formate (pH 8.5).

Products	$d(A)_{4000}\cdot d(T)_{200}$		$d(A)_{4000}\cdot d(T)_{200}$ (7% $\widehat{TT}$)	
	Intact enzyme with 10 or 100 % hydrolysis	Small fragment with 10 or 100 % hydrolysis	Intact enzyme with 78% hydrolysis	Small fragment with 70 % hydrolysis
	%	%	%	%
$d(T)_1$	79	79–81	72	71
$d(T)_2$	17–18	16–17	16	13
$d(T)_3$	3–4	3	<1	<1
$d(T)_{4:8}$	<1	<1	13[a]	16[a]

[a] These oligonucleotides contained thymine dimers (9).

Degradation of 5′-Triphosphate-terminated Polynucleotide—The 5′ → 3′ exonuclease action on $d(A)_{4000}\cdot\overset{**}{p}ppT(pT)_{200}$ released >85% of the termini as the dinucleoside tetraphosphate, $\overset{**}{p}ppTpT$ (Fig. 2). Inasmuch as the small fragment does not contain the triphosphate binding site of DNA polymerase (6), orientation of the triphosphate terminus of the polynucleotide in this site cannot be invoked as in earlier studies (11, 25) to explain the preferential release of a dinucleoside tetraphosphate. Instead it seems more reasonable to suggest that the 5′ → 3′ exonuclease site fails to bind the highly charged triphosphate terminus and therefore displaces the strand by one nucleotide and as a result cleaves at the subterminal diester bond.

These results with the small fragment make it necessary to re-examine the interpretation of previous data on the cleavage by intact DNA polymerase which also releases a dinucleoside tetraphosphate from a 5′-triphosphate-terminated polynucleotide (Fig. 2) (11). It had been suggested that binding of the terminus in the triphosphate site oriented the 5′ → 3′ exonuclease attack at the subterminal diester bond. Now the alternative possibility offered above to explain the action of the small fragment may also apply to the intact enzyme.

Lack of Endonuclease Activity in Intact Enzyme and Small Fragment—Although the 5′ → 3′ hydrolytic activity of both the intact enzyme and the small fragment is termed an exonuclease, it is evident that this activity has some endonucleolytic character since oligonucleotides of up to 8 residues in length are excised (Table III) (9). Highly purified intact DNA polymerase has no detectable endonuclease activity toward double-stranded DNA (4), but it is possible that the intact enzyme and the small fragment could act as endonucleases as shown in Scheme I (*a*, *b*, *c*) either by cleaving the strand opposite a nick in a nicked DNA, or by producing a nick adjacent to a nonbase paired region in a double-stranded DNA such as the mismatched region caused by a thymine dimer (26). These types of endonuclease reactions

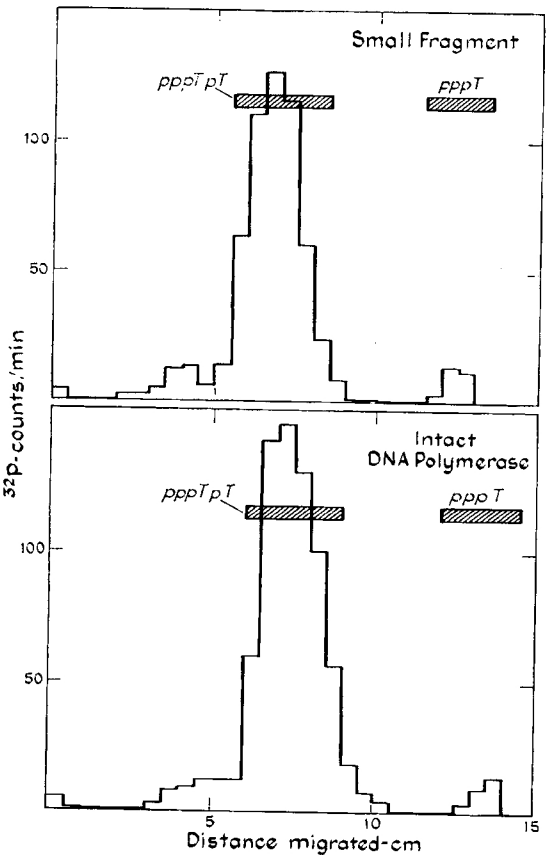

FIG. 2. A dinucleoside tetraphosphate as the degradation product of $d(A)_{4000}\cdot\overset{**}{p}ppTp(T)_{200}$. The reaction was carried out in 70 μl of 67 mM potassium phosphate (pH 7.4), 6.7 mM $MgCl_2$, 67 μg per ml of BSA containing 32 nmoles of $d(A)_{4000}$, 28 nmoles of $\overset{**}{p}ppTp(T)_{200}$, and either 22 pmoles of intact polymerase of 18 pmoles of the small fragment. After a 40-min incubation at 37° the reaction was halted by addition of 2 μl of 0.5 M EDTA, and then 50 μl were chromatographed in isobutyric acid-1 M NH_4OH-0.1 M EDTA (100:60:1.6).

TABLE III

Lack of endonuclease activity

The assay conditions were described under "Methods," and both endonuclease and exonucleolytic hydrolysis were measured. The intact enzyme and the small fragment were both assayed at 240 pmoles per ml. This gives a ratio of enzyme to 3′ or 5′ ends of 1.8 to 1 with the substrate $d(A)_{4000}\cdot d(T)_{200}$, and a ratio of 36 to 1 with the other substrates.

Substrate	Nuclease breaks			
	Intact enzyme		Small fragment	
	Exonuclease	Endonuclease	Exonuclease	Endonuclease
	pmoles/100 μl reaction		*pmoles/100 μl reaction*	
[^{14}C]$d(A)_{4000}\cdot$[^{3}H]$d(T)_{200}$	2300[a]	<0.3[b]	2100[a]	<0.25[b]
[^{14}C]$d(A)_{4000}\cdot$[^{3}H]$d(T)_{4000}$	480[c]	<0.15[d]	300[c]	<0.15[d]
[^{14}C]$d(A)_{4000}\cdot$[^{3}H]$d(T)_{4000}$ (7% $\widehat{TT}$)	270[c]	<0.15[d]	180[c]	<0.15[d]

[a] Greater than 90% of the hydrolysis was of the dT strand.
[b] Measured only on dA strand.
[c] Approximately 50% of the hydrolysis was of the dT strand.
[d] Measured on both dA and dT strands.

Scheme I: Possible Sites for Endonuclease Cleavage

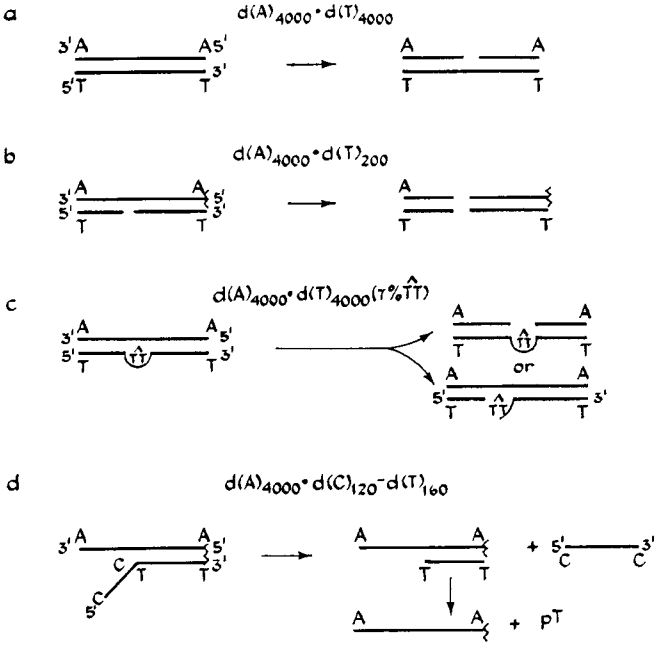

TABLE IV

Lack of hydrolysis of $d(A)_{4000}\cdot d(C)_{120}$-$d(T)_{160}$

The reactions were carried out at 37° in 200 μl of 67 mM sodium phosphate (pH 8.0), 67 mM $MgCl_2$ containing 30 nmoles of $d(A)_{4000}$, 13.4 μg of BSA, and either 2.0 nmoles of $[^3H]d(T)_{200}$ or 2.8 nmoles of $[^{14}C]d(C)_{120}$-$[^3H]d(T)_{160}$. Prior to addition of the BSA, the mixture was heated to 80° for 5 min and then quickly cooled in ice to destroy the secondary structure of the deoxycytidylate strands; 14 pmoles of small fragment were added to each assay and 1.4 nmoles of $[^{14}C]d(C)_{120}$ were added where noted.

Substrate	Hydrolysis	
	dC	dT
	%/30 min	
$d(A)_{4000}\cdot d(C)_{120}d(T)_{160}$	<1	1.8
$d(A)_{4000}\cdot d(T)_{200}$		85
$d(A)_{4000}\cdot d(T)_{200} + d(C)_{120}$	<2	80

might be more frequent with the small fragment which may have lost the ability to orient itself through binding to a 3′-OH terminus. Although these endonucleolytic reactions are only possibilities, the importance of endonuclease action in proposed models of DNA replication and repair (5, 27, 28) make their assay in both the intact enzyme and the small fragment important.

The endonuclease activity of both the small fragment and the intact enzyme was therefore tested on the several different substrates shown in Scheme I (*a*, *b*, *c*). The ratio of enzyme to available nicks or ends was varied from 2 to 36, but no endonuclease was detected. The ratio of measured exonucleolytic cleavage to the upper limit of endonucleolytic cleavage was always greater than 10^3 in experiments in which the extent of exonucleolytic degradation of the substrates varied from 2 to 80% (Table III).

Lack of Degradation of Long Displaced Oligonucleotide—The data in the previous section suggest that whereas the 5′ → 3′ exonuclease activities of both the intact enzyme and the small fragment can excise small oligonucleotides, they are unable to excise large oligonucleotides. This was tested directly using the substrate $d(A)_{4000}\cdot d(C)_{120}$-$d(T)_{160}$ (Scheme I*d*). When the small fragment was assayed on this substrate under conditions where poly (dC) has no secondary structure (20) there was no detectable degradation of the dC strand; also <2% of the dT was solubilized (Table IV). The rate of solubilization of thymidylate residues from this substrate was also more than 40 times slower than the same reaction with the substrate $d(A)_{4000}\cdot d(T)_{200}$[3] (Table IV). Similar results were obtained using the intact enzyme, but the experiment was complicated by the action of the 3′ → 5′ exonuclease.

The data in this and the previous section suggest two conclusions concerning 5′ → 3′ exonuclease action: (*a*) the 5′ → 3′ exonuclease of both the intact enzyme and the small fragment cannot act as an endonuclease since there is an upper limit to the size of the oligonucleotide that can be excised, and (*b*) the specificity of the 5′ → 3′ exonuclease is similar in both the intact enzyme and the small fragment.

Cooperative Interaction of Mixture of Small and Large Fragments

There are three reactions in which the catalytic properties of the intact enzyme are strongly influenced by concomitant action of both the 5′ → 3′ exonuclease and polymerase activities: (*a*) stimulation of 5′ → 3′ exonuclease by concurrent DNA synthesis (8), (*b*) the excision of oligonucleotides greater than 3 residues in length (8), and (*c*) kinetics of primed net synthesis of the copolymer poly[d(A-T)] (1, 6). The following experiments show that these properties of the intact enzyme can be reconstituted in mixtures of the small and large fragments of DNA polymerase.

Stimulation of 5′ → 3′ Exonuclease by DNA Synthesis—The stimulation in 5′ → 3′ exonuclease activity by DNA synthesis has been attributed to concomitant action of the polymerase and 5′ → 3′ exonuclease activities of a single enzyme molecule (8). If the two separate fragments bind next to one another at the same nick in a double-stranded DNA, the adjacent fragments might then act as a single enzyme. Therefore the 3′ and 5′ termini of the substrate $d(A)_{4000}\cdot d(T)_{200}$ were saturated with the large and small fragments, respectively, and 5′ → 3′ exonuclease activity was measured (Table V). Under these conditions, the small fragment was stimulated by simultaneous DNA synthesis by the large fragment. The turnover number of the stimulated exonuclease was only slightly less than the same reaction catalyzed by the intact enzyme (Table V). Furthermore, under these conditions of saturating levels of both fragments, nick translation in which hydrolysis of DNA matches synthesis was observed (Fig. 3) as with the intact enzyme previously (8).

Pattern of Oligonucleotide Excision during DNA Synthesis—Additional evidence for the coordinated action of the catalytic activities of the two fragments was observed when the products

[3] This number may be even higher, since the slow rate of solubilization of thymidylate from the block copolymer may be due to degradation of the small percentage of thymidylate strands which have only a few dC residues at the 5′ end (see "Methods").

TABLE V

5′ → 3′ Exonuclease activity during concomitant DNA synthesis

The assays were carried out in 0.2 ml as described under "Methods." To each assay, 3.0 nmoles of $d(A)_{4000}$ and 2.7 nmoles of $[^3H]d(T)_{200}$ were added; 14 pmoles of the small fragment were present and 18 pmoles of the large fragment were added. dTTP was added to 0.5 mM.

Enzymes and addition	5′ → 3′ Exonuclease
	moles nucleotide excised/ mole enzyme/min
Small fragment	
None	37
dTTP	37
Small fragment + large fragment	
None	33
dTTP	188
Intact enzyme	
None	27
dTTP	260

TABLE VI

Products of hydrolysis of $d(A)_{4000}\cdot d(T)_{200}$ during concomitant DNA synthesis

The degradation and the chromatography of the digests were carried out as described in the legend to Table II, but with only 16 nmoles of $d(A)_{4000}$ and 14 nmoles of $[^3H]d(T)_{200}$. The intact enzyme was present at 150 pmoles per ml, the small fragment at 220 pmoles per ml, and the large fragment at 270 pmoles per ml; 0.5 mM dTTP was added.

Products	Intact enzyme + dTTP		Large fragment + small fragment + dTTP	
	35% hydrolysis	100% hydrolysis	25% hydrolysis	98% hydrolysis
	%		%	
$d(T)_1$	55	71	47	75
$d(T)_2$	22	29	23	25
$d(T)_3$	10	<1	11	<1
$d(T)_{4-8}$	13	<1	19	<1

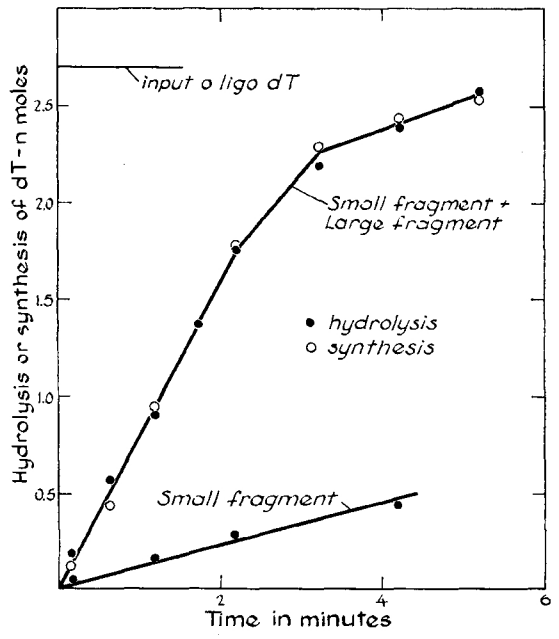

FIG. 3. Nick translation, the simultaneous synthesis and hydrolysis of DNA, catalyzed by a mixture of small and large fragments. The incubations were carried out as described in the legend to Table V, but with [α-^{32}P]dTTP at 0.5 mM. Twenty-five-microliter samples were withdrawn from the assays with time, and adsorbed to squares of DEAE-paper for measurement of both synthesis and hydrolysis of polydeoxythymidylate.

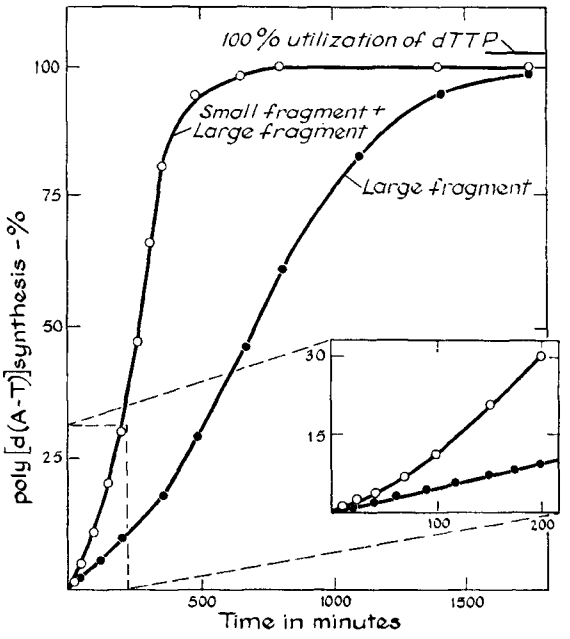

FIG. 4. Primed poly[d(A-T)] synthesis by the large fragment or a mixture of the two fragments. The reaction was carried out at 37° in 2.5 ml of 67 mM potassium phosphate (pH 7.4), 67 mM $MgCl_2$, 75 μM dATP and 75 μM [^{3}H]dTTP. BSA was present at 100 μg per ml and poly[d(A-T)$_{1000}$] at 0.7 μM. The reaction was started by addition of the large fragment (18 pmoles) or a mixture of the large fragment (18 pmoles) and the small fragment (20 pmoles). Poly[d(A-T)] synthesis was measured by adsorbing a 40-μl sample of the reaction mixture to squares of DEAE-paper and eluting unreacted triphosphates and oligonucleotides with 0.3 M ammonium formate (9). The *symbols* in the *inset* are the same as those in the main figure.

generated by 5′ → 3′ exonuclease action during DNA synthesis were analyzed. Under these conditions the intact enzyme initially excised a high percentage (13%) of oligonucleotides of 4 to 8 residues in length (Table VI) (8), while in the absence of DNA synthesis less than 1% of the products were large oligonucleotides (Table II). The small fragment also produced no large oligonucleotides (Table II), and the product distribution was not affected by addition of either the large fragment or deoxynucleoside triphosphates.[2] However a mixture of both fragments plus deoxynucleoside triphosphates produced a high percentage (19%) of large oligonucleotides. After 3 hours at 37°, when hydrolysis was complete, both the intact enzyme and the mixture of fragments eventually degraded the large oligonucleotides to a mixture of mono- and dinucleotides (Table VI) (8).

Kinetics of Primed Poly[d(A-T)] Synthesis—Another reaction which involves the participation of both polymerase and 5′ → 3′

exonuclease is the primed synthesis of poly[d(A-T)]. The kinetics of the reaction catalyzed by the intact enzyme is autocatalytic early in the synthesis. By contrast the reaction catalyzed by the large fragment is relatively linear and also less rapid (Fig. 4) (1). Since poly[d(A-T)] synthesis is carried out with a 20-fold excess of polymerase molecules over 3'-OH primer ends, a possible explanation for the autocatalytic kinetics with the intact enzyme is the generation of poly[d(A-T)] oligonucleotides by action of the 5′ → 3′ exonuclease. These oligonucleotides might act as new primer strands, which the large excess of polymerase molecules could utilize for synthesis of poly[d(A-T)]. Such a mechanism for increasing the rate of poly[d(A-T)] synthesis would not be available to the large fragment for lack of 5′ → 3′ exonuclease activity.

In agreement with these observations, the more rapid autocatalytic mode of poly[d(A-T)] synthesis was restored to the large fragment by addition of an equimolar amount of the small fragment (Fig. 4). Presumably concomitant action of the two fragments caused excision of oligonucleotides of poly[d(A-T)] (see Table VI), which acted as new primer strands. In poly[d(A-T)] synthesis catalyzed by the intact enzyme, the 5′ → 3′ exonuclease eventually degrades all the product. This was not the case with the mixture of the two fragments presumably because the lability of the small fragment at 37° led to denaturation of the 5′ → 3′ exonuclease activity early in the reaction.[2]

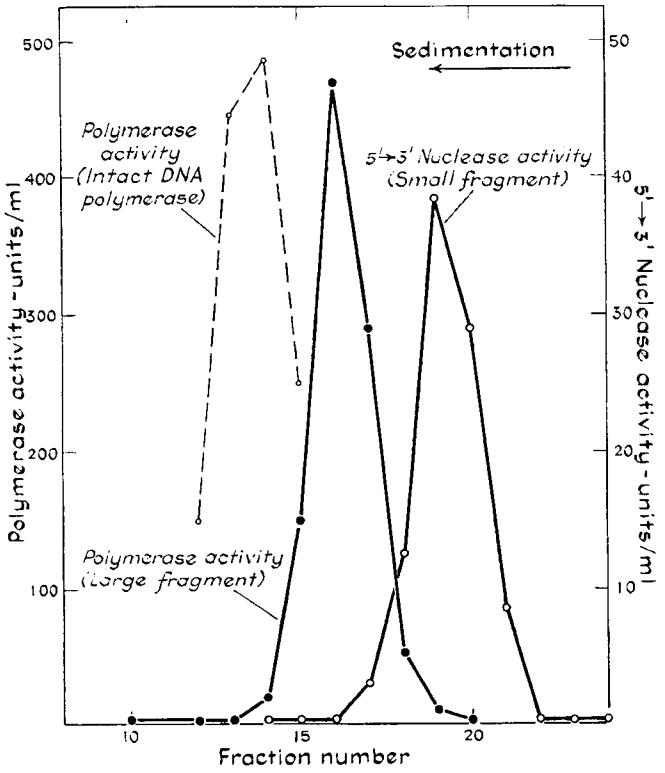

FIG. 5. Sedimentation of a mixture of the large and small fragments in a sucrose gradient. The two fragments were mixed in 67 mM potassium phosphate (pH 7.4), 0.2 mM β-mercaptoethanol, and incubated at 37° for 10 min and then at 4° for 50 min. One hundred microliters of this solution containing 300 pmoles of the large fragment and 100 pmoles of the small fragment were layered on a 4-ml sucrose gradient (5 to 20% sucrose, 67 mM potassium phosphate (pH 7.4), 0.2 mM β-mercaptoethanol) and centrifuged at 4° in a Spinco model L2 65B centrifuge with a SW 56 rotor at 56,000 rpm. After 12 hours, 20-drop fractions were collected and assayed for polymerase and 5′ → 3′ exonuclease activities. Intact enzyme (300 pmoles), the large fragment, and the small fragment were also sedimented as markers in three parallel gradients.

Lack of Direct Binding between Small and Large Fragments

The preceding experiments demonstrated that a mixture of the small and large fragments of DNA polymerase in the presence of DNA restores the properties of the intact enzyme. This result might be caused by reassociation of the two fragments on the DNA or independently of it. Binding of one fragment to the DNA may or may not favor binding of the other fragment adjacent to it.

Inasmuch as the large and small fragments are readily separated by Sephadex (3) or phosphocellulose chromatography (Fig. 1), affinity between them cannot be strong. This was demonstrated more directly by preparing a mixture of the fragments and then sedimenting them on a sucrose gradient (Fig. 5). The small and large fragments sedimented independently of one another; their positions in the gradient were identical when they were sedimented alone or together.

Similarly, the separate fragments exhibited no physical affinity for one another on DNA (Table VII). The turnover number of the small fragment was measured in the presence of deoxyribonucleoside triphosphates and with several ratios of large and small fragments to 3′ and 5′ ends on the DNA. At a high level of saturation of the 3′ and 5′ ends, the two fragments bound adjacent to one another at the same nick, and the result was similar to that seen in Table V. The turnover number of the small fragment was increased by the concomitant DNA synthesis of an adjacent large fragment. However, the turnover number

TABLE VII

Lack of affinity between small and large fragments on DNA

Assays were carried out as described in Table V with different amounts of both fragments as noted and with 0.5 mM dTTP.

Ratio of fragments to 3′ or 5′ ends		Turnover number of 5′ → 3′ exonuclease activity[a]	
Large fragments	Small fragments	Experimental	Theoretical value if binding to DNA is random[b]
0	0.80	35[c]	
2.50	0.80	196[d]	
0.50	0.30	123	116
0.16	0.12	70	61

[a] Nanomoles of nucleotide excised per mole of small fragment per min.

[b] These values are calculated assuming that the small and large fragments bind both very tightly to 5′ and 3′ ends, respectively, and that the binding is uninfluenced by the presence of another fragment. A value of 196 was found for a small fragment adjacent to a large fragment and 35 for a small fragment alone. Thus with a 0.50 ratio of large fragment, one-half of the small fragments would have a neighboring large fragment (196/2 = 98) and one-half would not (35/2 = 18).

[c] Specific activity of small fragment alone.

[d] Specific activity of small fragment adjacent to large fragment during DNA synthesis.

of the small fragment decreased as the ratio of fragments to 3′ and 5′ ends decreased; the experimental values were almost identical with values calculated assuming random binding of the fragments to DNA. If the fragments had had a strong affinity for one another on DNA, a decrease in the ratio of fragments to 3′ or 5′ ends would not have resulted in a corresponding decrease in the turnover number of the small fragment.

DISCUSSION

Effects of DNA Synthesis on Excision Function—Studies of the action of the separate fragments of DNA polymerase suggest a mechanism whereby concomitant DNA synthesis could both stimulate the rate and alter the specificity of the 5′ → 3′ exonuclease. The large fragment is fully active in catalyzing DNA synthesis on a nicked double-stranded DNA and probably displaces the strand ahead of the 3′-OH growing point (6). It is therefore probable that DNA synthesis by the intact enzyme may also cause some fraying at the 5′ end of the strand ahead of a growing point. This frayed strand might be more susceptible to degradative attack by the same enzyme molecule (8) resulting in an increased rate of 5′ → 3′ exonuclease. The fraying of the 5′ end will also explain the excision of oligonucleotides (Table VI) since both the intact enzyme and the small fragment are known to excise large oligonucleotides from a DNA duplex containing frayed 5′ ends produced by thymine dimer formation (Table II) or the introduction of mismatched bases (9). The reason for the apparent upper limit on the size of the oligonucleotides produced by 5′ → 3′ exonuclease action is, however, not clear.

Concomitant Action of Small and Large Fraction—The stimulation in the exonucleolytic rate of the small fragment by concurrent DNA synthesis catalyzed by the large fragment can best be explained by concomitant action of two different fragments which have bound adjacent to one another at a single nick in a DNA molecule. The alternative explanation that DNA synthesis by the large fragment causes displacement of the 5′ end which is then attacked by an unbound small fragment seems less likely since the small fragment shows an absolute specificity for a double-stranded substrate, although the 5′ end at which the small fragment acts may have some single stranded character, *i.e.* may be frayed due to a thymine dimer or some other cause. Previous work of Kelly *et al.* indicated that the stimulation in 5′ → 3′ exonuclease by DNA synthesis in the intact polymerase also involved concomitant action of the polymerase and 5′ → 3′ exonuclease activities of the same molecule (8).

Lack of Affinity between Small and Large Fragment—The experiment demonstrating the lack of affinity between the two fragments in the absence of DNA is quite conclusive (Fig. 5), but demonstration of their lack of affinity in the presence of DNA (Table VII) is based on two assumptions: (*a*) that the two fragments bind tightly to DNA, and (*b*) that the binding of the fragments to DNA is reversible and allows formation of thermodynamically favored states. Sedimentation studies have demonstrated that intact DNA polymerase binds tightly to both DNA and poly[d(A-T)] oligomers (29), and we have similar data for the binding of the large fragment to $d(A)_{4000} \cdot d(T)_{200}$.[2] The small fragment also binds tightly to DNA, since the rate of 5′ → 3′ exonuclease on $d(A)_{4000} \cdot d(T)_{200}$ was the same when the ratio of small fragment to 5′ ends was reduced from 3 to 1 to 1 to 1. There are no data on the dissociation rates of the individual fragments from DNA, but the binding of the intact enzyme to DNA is reversible (29). There are no data on the velocity of this dissociation, but it takes place at 4°, while our experiments were performed at 37° where the dissociation would be expected to be more rapid.

Structure of DNA Polymerase—The cleavage of DNA polymerase into two active fragments which show no affinity for one another, and the similarity of their catalytic reactions to the analogous reaction catalyzed by the intact enzyme indicates that within the single polypeptide chain of DNA polymerase are two distinct enzymes, one enzyme containing the polymerase and 3′ → 5′ exonuclease function and the other enzyme containing the 5′ → 3′ excision function. The two enzymes are held together by a polypeptide link (susceptible to proteases), which ensures that both the polymerase and excision function act simultaneously at the same nick in a DNA molecule.

Possible Importance of Two Enzymes—Single Polypeptide Structure—The coupling of the polymerase and excision functions in a single enzyme may be of significant advantage in recombinational events and in the *in vivo* repair of lesions in DNA such as thymine dimers. As a thymine dimer is excised by the excision function, concurrently the polymerase function fills in the gap caused by the excision. Therefore excision repair by DNA polymerase at no time leaves a single strand gap which might be attacked at the 3′ end by exonucleases such as exonuclease III, but rather leaves only a nick with a 3′-OH and a 5′-P from which the polymerase can be displaced by DNA ligase. Possibly the extensive DNA degradation observed after ultraviolet irradiation of *E. coli* mutants which lack DNA polymerase (pol A mutants (30)) is the result of the production of single strand gaps during the excision of thymine dimers by secondary repair systems (31, 32). The lifetime of these gaps may be long enough to enable enzymes such as exonuclease III to attack these single strand gaps and thereby cause extensive degradation of the DNA.

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