Enzymatic Repair of Deoxyribonucleic Acid; the Biochemical and Biological Repair Properties of a Deoxyribonucleic Acid Polymerase from *Micrococcus luteus*†

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ABSTRACT: A DNA polymerase has been purified to homogeneity from *Micrococcus luteus* with a molecular weight of 100,000–110,000. In common with the *Escherichia coli* DNA polymerase I, it has associated 3′ → 5′ (single-stranded DNA) and 5′ → 3′ (double-stranded DNA) directed exonuclease activities. It has been shown capable of carrying out both the removal of pyrimidine dimers from incised uv-irradiated DNA (5′ → 3′ activity) and a controlled reinsertion of nucleotides into cavities in the DNA strand created by excision. Controllable biochemical conditions for these steps have been established using uv-irradiated DNA incised with a uv-endonuclease from *M. luteus* and model ^{32}P-labeled DNA templates excised with exonuclease III to form such cavities. The uv-endonuclease introduced a "nick" with a 3′-OH terminus into the uv-irradiated DNA adjacent to the pyrimidine dimer. The latter was removed by the DNA polymerase in the absence of synthesis (which restricted the 5′ → 3′ nuclease activity, and hence the size of the gap) in 15 min at 30° with 2 mM Mg^{2+}. Repair synthesis was carried out at 10° in the presence of 0.5 mM Mg^{2+} and polynucleotide ligase. Biochemical repair of uv-irradiated *Bacillus subtilis* transforming DNA resulted in restoration of the single-strand molecular weight, and biological activity up to doses of 6.6 × 10^2 ergs/mm^2 (50% inactivation).

Studies of excision repair of DNA damaged by radiation and chemicals in bacteria point to sequential enzyme systems which literally remove the photochemical damage and repair the incomplete strand by replacing the nucleotides and restoring its continuity (Strauss, 1968; Setlow, 1970). One test of the validity of these model systems requires the isolation of the enzymes capable of catalyzing the individual steps and showing they are able "*in vitro*" to restore biological activity to a damaged DNA (Grossman, 1973). Specific enzymes capable of carrying out the excision stage in uv-irradiated DNA have been isolated. These include a uv-endonuclease and uv-exonuclease from *Micrococcus luteus* (Kaplan *et al.*, 1969) and a bacteriophage T4 uv-endonuclease (Friedberg and King, 1969; Yasuda and Sekiguchi, 1970). Kelly *et al.* (1969) have shown that excision of damage can also be carried out by an associated nuclease of *Escherichia coli* DNA polymerase I, which acts in the 5′ → 3′ direction after treatment of the uv-irradiated DNA with DNase I. This activity, which prefers double-stranded DNA, is stimulated by the presence of deoxyribonucleoside triphosphates and is normally involved in translation of a 3′-OH nick in the DNA strand by concurrent incorporation and breakdown (Kelly *et al.*, 1970). It was, therefore, concluded that *E. coli* DNA polymerase I could participate both in the excision and reinsertion steps of DNA repair. The ability of the polynucleotide ligase to restore the continuity of the DNA strand after DNA polymerase action has been demonstrated by Goulian and Kornberg (1967). Support for the dual function of this DNA polymerase in DNA repair has been given by the partial repair of uv-irradiated *Bacillus subtilis* transforming DNA in an "*in vitro*" system with relatively impure enzymes in which no attempt was made to disassociate the individual steps (Heijneker *et al.*, 1971).

A DNA polymerase was first isolated from *M. luteus* by Zimmerman (1966). A similarity between the specificities of the associated nuclease activities of the DNA polymerase I isolated from *E. coli* and the *M. luteus* enzyme was indicated by the studies of Miller and Wells (1972) and Litman (1970). The object of our experiments has been to characterize the repair activities of the *M. luteus* DNA polymerase, and establish biochemical conditions under which excision and polymerization can be controlled and duplicated in biological experiments to repair uv-irradiated *B. subtilis* transforming DNA. This provides a system for studies of impairment of individual steps and the effect of incorporation of base analogs on biological activity (*e.g.*, mutagenesis).

Experimental Section

Materials

Spray-dried *M. luteus* cells were obtained from Miles Laboratories, Elkhart, Ind. Radioactively labeled deoxynucleoside triphosphates [^{3}H]dTTP (56 Ci/mmol) and [^{3}H]-dATP (5.7 Ci/mmol), as well as [^{3}H]thymidine and ^{32}P-labeled sodium orthophosphate were obtained from New England Nuclear Corp. Poly(dA), poly(dT), and poly[d(A-T)] were purchased from Collaborative Research Chemicals, Waltham, Mass.

5′-(^{3}HpT)$_{200}$(pT)$_{600}$(^{14}CpT)$_{200}$ was prepared and generously provided by Barbara Garvik according to the enzymatic procedure of Bollum (1966). Poly(dA) and 5′-(^{3}HpT)$_{200}$-(pT)$_{600}$(^{14}CpT)$_{200}$ annealed in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 0.1 mM EDTA in the ratio of 1.5:1 had a melting point of 63°.

Escherichia coli polynucleotide ligase, exonuclease III, and *M. luteus* uv-exonuclease were gifts of Dr. I. R. Lehman, Dr. Andrew Braun and Barbara Garvik, respectively.

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Bacterial alkaline phosphatase, micrococcal nuclease, and pancreatic DNase I were obtained from Worthington Biochemicals.

Bovine serum albumin was a product of the Nutritional Biochemical Corp.

DNA unlabeled and radioactively labeled with tritiated thymidine and ^{32}phosphorus was prepared from *E. coli* by the method of Grossman (1967). Transforming DNA was isolated by the method of Marmur (1961) from *Bacillus subtilis* strain 168. Calf thymus DNase I "activated" template for DNA polymerase assays was prepared as described by Richardson (1966).

Methods

Ultraviolet irradiation of the DNA was carried out in 0.15 M NaCl–0.015 M sodium citrate (1 × SSC) at concentrations of 40 μg/ml in a Petri dish 3.5 cm in diameter at a liquid depth of 1–2 mm with a filtered germicidal lamp emitting 254-nm light. The dose rates were adjusted from 11 ergs/mm^2 per sec for biological studies to 1.4×10^2 ergs/mm^2 per sec for the high doses required for chemical investigation of excision of photoproducts. These conditions introduced ~0.58 pmol of pyrimidine dimers/nmol of DNA nucleotide per 10^3 ergs per mm^2, extrapolated from the high dose range, using the dimer assay of Cook (1971), modified from Carrier and Setlow (1971).[1]

Preparation of Excised DNA Templates.[1] The native DNA was first nicked with DNase I and the nicks widened into gaps by treatment with exonuclease III. *E. coli* [^{32}P]DNA (specific activity 0.8–1 × 10^5 cpm/μg of DNA) was incubated with 3×10^{-5} μg of DNase I/μg of DNA in 66 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+}, and 0.5 mM 2-mercaptoethanol for 30 min at 37°. The digestion mixture was made 50 mM with sodium chloride, 3 units of exonuclease III was added per μg of DNA and the DNA was excised at 10° for 30–120 min. The enzymes were inactivated by heating at 68° for 5 min. This resulted in a release of 8–46 nucleotides/^{32}P end (see Donelson and Wu, 1972).

Enzyme Assays. Uv-endonuclease activity was measured by the formation of bacterial alkaline phosphatase sensitive [^{32}P]phosphomonoester groups in [^{32}P]DNA as previously described (Kaplan and Grossman, 1971). Incubation was for 30 min at 37° with 0.02–0.1 unit/μg of DNA. *One unit of activity is defined as the amount of enzyme required to produce 10 pmol of hydrolyzable [^{32}P]phosphomonoester groups in 30 min at 37°.* Polynucleotide ligase was standardized according to the method described by Modrich and Lehman (1970).

DNA polymerase was assayed (a) for total polymerizing activity in an incubation mixture (0.2 ml) containing 66 mM Tris-HCl (pH 8.0), 0.5 mM 2-mercaptoethanol, 4 mM Mg^{2+}, 0.025 mM of each dNTP,[1] 0.05 μCi of [^{3}H]dTTP, 225 μM "activated" DNA, and 0.05–0.2 unit of DNA polymerase. The mixture was incubated 30 min at 37° with a reagent blank in parallel, cooled to 0°, 10 μl of carrier calf thymus DNA (2 mg/ml) was added, and the DNA template was precipitated by the addition of 5 ml of cold 5% trichloroacetic acid–10 mM sodium pyrophosphate. After 15 min at 0°, the acid-insoluble DNA was filtered on a Whatman glass filter G/FC circle 2.5 cm in diameter and washed with five 5-ml portions of 5% trichloroacetic acid–10 mM sodium pyrophosphate, once with 5 ml of 95% ethanol, and dried under a heat lamp before counting in organic scintillant (Liquifluor). *One unit of activity is defined as the amount of enzyme which incorporated 10 nmol of total nucleotide into the "activated" calf thymus DNA in 30 min at 37°.* (b) For simultaneous incorporation and breakdown, the template was 5–20 μM nicked or excised *E. coli* [^{32}P]DNA and the [^{3}H]dTTP concentration increased to 0.1–0.2 μCi in the dNTP mixture. The uptake of [^{3}H]TTP and release of ^{32}P nucleotides was followed at intervals in 0.2-ml aliquots from a 1–2-ml digest. For DNase activity, a 0.2-ml aliquot was added to 0.3 ml of carrier calf thymus DNA (1 mg/ml) and precipitated with 0.5 ml of cold molar trichloroacetic acid and allowed to stand at 0° for 15 min. A 0.1-ml aliquot of the supernatant fluid after centrifuging at 5000 rpm for 20 min was counted in Aquasol. With appropriate settings, there was a ~2% ^{32}P carry-over into the tritium channel with aquasol and a 0.5–0.7% in Liquifluor.

Phosphatase activity under polymerase assay conditions was measured using the Norit assay to determine $^{32}P_i$ released from *E. coli* [^{32}P]DNA, as previously described (Kaplan and Grossman, 1971).

Transformation Experiments. The preparation and storage of competent cell cultures and transformations were carried out, as described previously (Mahler, 1968). The recipient strain BD 172 (*thr$_5^-$-trp$_2^-$*), a uv-sensitive, excision defective strain was a gift of Dr. David Dubnau.

Enzyme Purification. (i) An endonuclease specific for uv DNA was isolated from *M. luteus* by Dr. Howard Ono (1973) using a modification of the method described previously (Kaplan *et al.*, 1969). This enzyme does not adsorb to DEAE-cellulose. It is thus possible to separate it from the closely associated γ-endonuclease. (Paterson and Setlow, 1972). (ii) *Micrococcus luteus* DNA polymerase. All operations were carried out at 0–4°, unless otherwise stated. Washed spray-dried cells (50 g) were suspended in 1000 ml of 0.02 M Tris-HCl (pH 8.0)–0.2 M sucrose, 150 mg of lysozyme was added, and incubation proceeded at 37° for 10 min, at which time 0.5 ml of 0.1 M magnesium chloride was added. After 20 min lysis was complete. The lysate was diluted with an equal volume of ice–water and the magnesium concentration was increased to 5 mM. Freshly made up 10% (w/v) streptomycin sulfate was added slowly from a separatory funnel with stirring to give a final concentration of 0.9%. The stirring was continued for at least 30 min, and the streptomycin pellet was collected by centrifugation 20 min at 18,000*g*. The orange pellet was washed by resuspension in 200 ml of 0.02 M potassium phosphate (pH 6.8) and the DNA polymerase was extracted by homogenization with three 125-ml portions of 0.02 M potassium phosphate (pH 7.5). To remove DNA and RNA, the supernatant fluid was adjusted to 5 mM Mg^{2+} and 10 mM 2-mercaptoethanol and incubated at 30° with pancreatic DNase I (1 μg/ml) and RNase (2 μg/ml) until 90% of the 260-nm absorbance was rendered acid soluble. Any cloudiness was removed by centrifugation for 120 min at 68,000*g*. The enzyme was concentrated by precipitation with ammonium sulfate. Initially, 25 g of solid ammonium sulfate was added per 100 ml of extract with stirring for 1 hr followed by removal of the precipitate of nonspecific protein by centrifugation at 18,000*g* for 20 min. The DNA polymerase was precipitated by adding an additional 16 g of ammonium sulfate/100 ml of extract and stirring for 1 hr, before centrifugation. The pellet was redissolved in a minimum volume of 0.02 M potassium phosphate (pH 6.8), 10 mM 2-mercaptoethanol, and 80 mM sodium chloride. If the extract still con-

[1] Abbreviations used are: dNTPs for a mixture of the four deoxyribonucleoside triphosphates, dATP, dCTP, dGTP, and TTP. NAD^+ for nicotinamide adenine dinucleotide. The uv-irradiated DNA after uv-endonuclease treatment is referred to as "incised" DNA and after excision to form gaps as "excised" DNA. Pyrimidine dimer represents primarily the thymine-containing photoproducts.

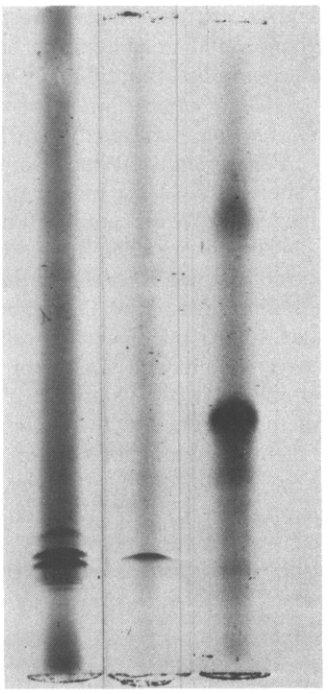

FIGURE 1: Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate of a DNA polymerase preparation eluted from phosphocellulose by 300 mM NaCl. This is compared on the left with a preparation eluted from DNA cellulose and on the right standard bovine serum albumin.

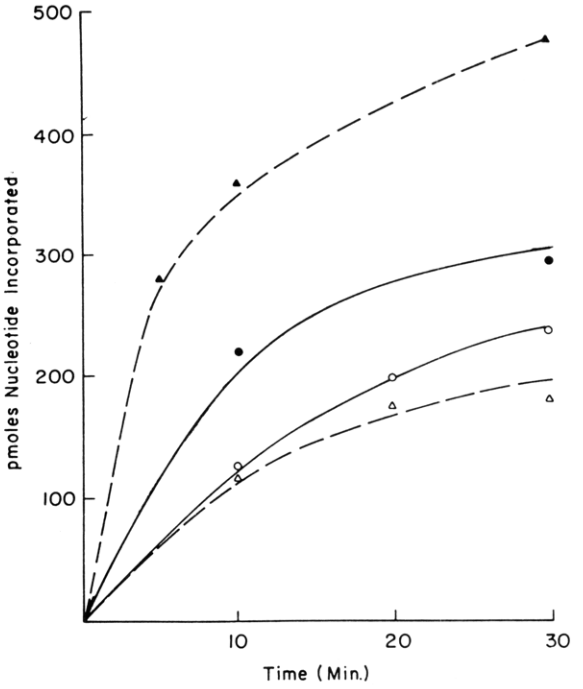

FIGURE 2: Incorporation of nucleotides into DNA treated with micrococcal nuclease, 2×10^{-4} μg/μg of DNA, 66 mM Tris-HCl (pH 8.0), and 20 mM Ca^{2+}, 5 min at 37° followed by exhaustive dialysis against buffer–0.1 mM EDTA: native (○); native + exonuclease III (●); micrococcal nuclease treated (△); micrococcal nuclease + exonuclease III (▲).

tained nucleic acids, further hydrolysis in the presence of 5 mM Mg^{2+}, DNase I (2 μg/ml), and pancreatic RNase (5 μg/ml) was carried out at 30° for 2–5 hr. This extract was dialyzed against 40 volumes of original buffer for 6 hr and adsorbed to a DEAE column dimensions 8 × 7 cm^2 preequilibrated with this buffer. The DNA polymerase was eluted batchwise with 100–150 ml of buffer containing 250 mM NaCl. The eluate was immediately diluted with an equal volume of 0.02 M potassium phosphate (pH 6.8), 10 mM 2-mercaptoethanol, and 20% glycerol and applied to a (4 cm × 7 cm^2) DNA cellulose column (Litman, 1968). The column was washed with 100 ml of buffer containing 50 mM sodium chloride, to remove nonspecific protein, followed by 50 ml of 300 mM sodium chloride which eluted some deoxyribonuclease activity. DNA polymerase was eluted with buffer–650 mM NaCl in fractions totalling 2–5% of the protein applied to the column.

The combined DNA cellulose fractions containing 2000 units of activity were dialyzed for two 16-hr periods against 0.02 M potassium phosphate (pH 6.8)–10 mM 2-mercaptoethanol buffer containing 40% glycerol and applied to a phosphocellulose (P-11) column 6 cm × 0.8 cm^2 preequilibrated with this buffer. The column was washed with four column volumes of buffer and then batchwise with buffer containing 150, 300, and 500 mM sodium chloride. Approximately 60% of the DNA polymerase was eluted in the 300 mM sodium chloride fractions and was stored in buffer containing 40% glycerol at −20°. The specific activity of the pure fractions varied between 2000 and 5650 units per mg of protein using an "activated" calf thymus DNA template (Table I).

Results

Purity of the DNA Polymerase. As shown in Figure 1, the purified enzyme migrated as a single protein band on sodium dodecyl sulfate polyacrylamide electrophoresis at pH 7.2 (Weber and Osborn, 1969), when 20 μg of protein was applied to 5 and 7.5% gels. The molecular weight of this protein was 100,000–110,000 when compared with bovine serum albumin. All preparations were tested for nonspecific endonucleases by incubation with native and uv-irradiated *B. subtilis* transforming DNA (1 unit/0.2 μg of DNA). Their presence can thus be detected by loss in biological activity.

TABLE I: Purification of DNA Polymerase from *Micrococcus luteus*.

Fraction	Total Units	Protein (mg/ml)	Sp Act. of CT DNA Poly [d(A-T)]		% Yield
Lysate	30,500	21.7	1.6		100
Streptomycin sulfate	20,250	7.1	23		66.6
Ammonium sulfate II	16,450	29.7	37	179	54
DEAE-cellulose[a]	7,544	2.9	109		24.8
DNA cellulose[a]	3,635	0.2	750	2350	11.8
Phosphocellulose[a]	2,710	0.07	2130	4740	8.9
	(1,130)[b]	(0.02)[b]	(5650)[b]		(4.7)[b]

[a] Specific activities from peak fractions. [b] Most recent preparations.

TABLE II: Assay of Phosphomonoesterase Activity in DNA Polymerase Preparations.

Assay Conditions	pmol Liberated/μg of [^{32}P]DNA[c]	
	$^{32}P_i$	[^{32}P]-Nucleotides
Bacterial alkaline phosphatase[a]	10	5
DNA polymerase[b]	0	58
DNA polymerase + dNTPs	<1	1750

[a] Incubation with 0.25 unit of bacterial alkaline phosphatase 15 min at 65°. [b] Incubation 30 min at 37° under standard polymerase conditions. [c] *E. coli* [^{32}P]DNA specific activity 2.5×10^5 cpm/μg of DNA nucleotide) prepared as described in methods.

The action of the DNA polymerase in DNA repair is controlled by the nature of the end group produced at the initial incision by the uv-endonuclease adjacent to the pyrimidine dimer. 3′-Phosphomonoester groups do not provide priming sites for polymerase action, shown in the experiment summarized in Figure 2, in which 3′-phosphomonoester groups were introduced into DNA by treatment with micrococcal nuclease. Incorporation at these ends occurred only after pretreatment with *E. coli* exonuclease III which releases P_i and generates a 3′-OH group. This showed, in addition, that the purified DNA polymerase preparation did not contain a phosphatase able to remove this block. The absence of a phosphomonoesterase is confirmed in Table II, which shows no release of P_i when the DNA polymerase was incubated with *E. coli* [^{32}P]DNA under standard assay conditions.

Uv-Irradiated Template for the M. luteus DNA Polymerase. Incubation of uv-irradiated DNA with the uv-specific endonuclease at 37° in 30 mM Tris-HCl (pH 8.0), 0.5 mM Mg^{2+}, 0.5 mM 2-mercaptoethanol, and 50 mM sodium chloride introduced 3′-OH priming sites for the DNA polymerase (incised DNA). Figure 3 shows the increased incorporation of nucleotides into DNA irradiated with doses of 3.3, 6.6, and 13.2 ergs per mm^2, and incised with 0.14 unit of uv-endonuclease for 60 min at 37°, as a function of DNA polymerase concentration. At each dose a plateau in the incorporation was reached at an approximate equivalence of DNA polymerase molecules to pyrimidine dimers introduced by irradiation. For example, with a specific activity of DNA polymerase of 5650 units/mg of protein, one unit represents 1.065×10^{12} molecules. Thus, approaching saturation the ratios of DNA polymerase:pyrimidine dimers at 3.3, 6.6, and 13.2×10^2 ergs per mm^2 were 0.8, 0.86, and 0.87, respectively.

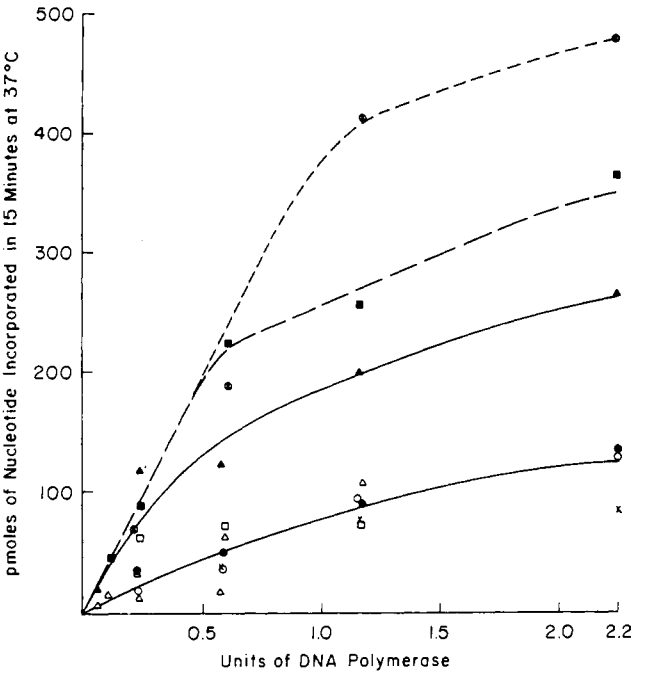

FIGURE 3: Dose response of incorporation of nucleotides into uv-irradiated *B. subtilis* transforming DNA incised with uv-endonuclease 0.14 unit/μg of DNA in 30 mM Tris-HCl (pH 8.0), 0.5 mM 2-mercaptoethanol, 50 mM NaCl, and 0.5 mM Mg^{2+} for 60 min at 37°. Incorporation at 37° with 0.1–2.2 units of DNA polymerase under standard assay conditions: unirradiated (○); unirradiated + uv-endonuclease (●). The effect of uv irradiation: 3.3×10^2 ergs/mm^2 (△); 3.3×10^2 ergs/mm^2 + uv-endonuclease (▲); 6.6×10^2 ergs/mm^2 (□); 6.6×10^2 ergs/mm^2 + uv-endonuclease (■); 1.32×10^3 ergs/mm^2 (×); 1.32×10^3 ergs/mm^2 + uv-endonuclease (⊗).

This indicated that the number of 3′-OH end groups introduced by the uv-endonuclease was directly related to the ultraviolet damage. The absence of 3′-monophosphodiester groups was shown experimentally by treatment of incised irradiated DNA (4×10^4 ergs/mm^2) with bacterial alkaline phosphatase (0.1 unit/μg of DNA) for 15 min at 65°. The incorporation of nucleotides by the DNA polymerase in 60 min at 20° before and after phosphatase action of 521 and 391 pmol, respectively, showed no enhancement which would have been expected if dephosphorylation released 3′-OH end groups.

Excision of Pyrimidine Dimers by DNA Polymerase. The presence of associated DNase activities active in the 5′ → 3′ direction against double-stranded DNA and 3′ → 5′ requiring single-stranded DNA has been described by Miller and Wells (1972) and Litman (1970). Activities in our preparation of DNA polymerase against *E. coli* [^{32}P]DNA and $(^3HpT)_{200}$-$(pT)_{600}(^{14}CpT)_{200}$ alone and annealed to poly(dA) are summarized in Table III. In experiments with the synthetic poly-

TABLE III: Nuclease Activities of *Micrococcus luteus* DNA Polymerase.

	E. coli [^{32}P]DNA[a]				Poly(dA)·Poly(dT)[b]				Poly(dT)[b]			
	Double Stranded		Single Stranded[c]		5′ → 3′ ^{3}H		3′ → 5′ ^{14}C		5′ → 3′ ^{3}H		3′ → 5′ ^{14}C	
dNTPs (0.1 mM)	−	+	−	+	−	+	−	+	−	+	−	+
% breakdown of template	0.8	26.6	1.8	1.2	14	32	0	0	0	0	12.5	12

[a] Incubation under standard polymerase conditions ± dNTPs for 30 min at 37°. [b] 5′-$(^3HpT)_{200}(pT)_{600}(^{14}CpT)_{200}$. [c] DNA denatured by heating at 100° for 15 min and immediate cooling in ice.

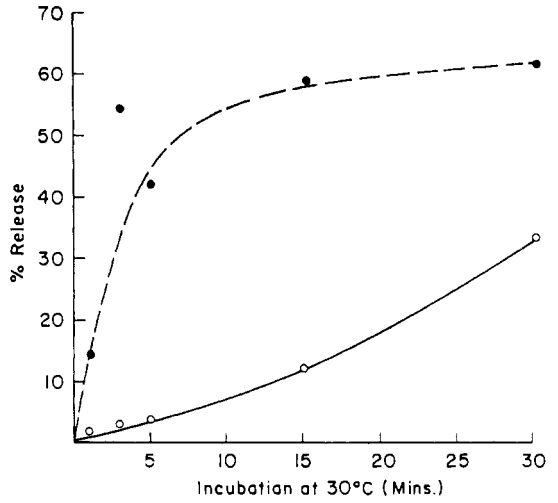

FIGURE 4: Time course of release of pyrimidine dimers from incised uv-irradiated DNA (3.4×10^4 ergs/mm^2) by *M. luteus* DNA polymerase. 2.4 nmol of *E. coli* [^{3}H]DNA (0.5×10^5 cpm/nmol), 0.12 unit of DNA polymerase/nmol of DNA, 2 mM Mg^{2+}, 0.1 mM deoxynucleoside triphosphates incubated at 30° for 0–30 min: release of pyrimidine dimers (●); and nucleotides (○).

nucleotide, the 3′ end of the poly(dT) strand was labeled with ^{14}C-labeled nucleotides and the 5′ end with ^{3}H-labeled nucleotides allowing detection of release in either direction. The activity 5′ → 3′ was almost exclusively against double-stranded DNA and stimulated by the dNTPs in contrast to the 3′ → 5′ nuclease, which preferred single-stranded DNA. In common with the similar activity in *E. coli* DNA polymerase I, the 5′ → 3′ nuclease released pyrimidine dimers from incised, uv-irradiated DNA, measured by a decrease in the dimer content of the incised DNA on incubation with the DNA polymerase. Specific removal from incision sites was shown by immediate release of dimers coupled with minimal

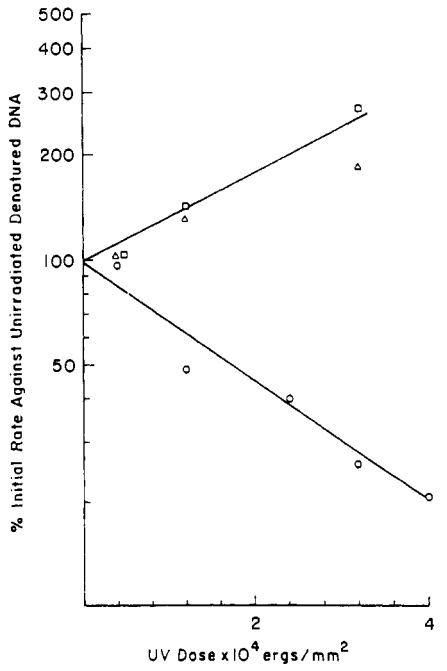

FIGURE 5: Inhibition by uv irradiation of breakdown of heat-denatured *E. coli* [^{32}P]DNA by DNA polymerase: heat-denatured uv-irradiated DNA + DNA polymerase (○); heat-denatured uv-irradiated DNA + uv endonuclease + DNA polymerase (□); and heat-denatured uv-irradiated DNA + uv-exonuclease (△).

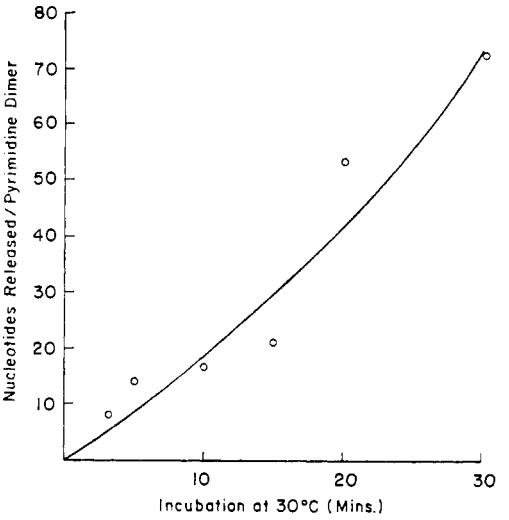

FIGURE 6: The number of nucleotides released for each pyrimidine dimer during incubation with DNA polymerase at 30° under standard assay conditions.

DNA breakdown. Figure 4 summarizes experiments with DNA irradiated with 3.4×10^4 ergs/mm^2, treated with uv-endonuclease, and incubated with DNA polymerase at 30°. This showed at 5 min a release of 40–60% of the pyrimidine dimers accompanied by 1–2% of the total nucleotides. Dimer excision did not require the dNTPs although the rate was stimulated by their presence in accord with the action of the 5′ → 3′ nuclease.

That the 3′ → 5′ exonuclease is not involved in excision can be demonstrated by its sensitivity to the presence of photoproducts in irradiated single-stranded DNA. This is shown in Figure 5, in which the dose dependence of the inhibition of the 3′ → 5′ nuclease activity against uv-irradiated single-stranded DNA is compared with an independent exonuclease isolated from *M. luteus* active against single-stranded DNA, but not inhibited by pyrimidine dimers (Kaplan and Grossman, 1971). Treatment of the irradiated DNA with the *M. luteus* uv-endonuclease prior to denaturation resulted in an increased initial rate of breakdown. This reflected the increased number of 3′-OH end groups produced by the uv-endonuclease, and the seeming release of dimers only after complete digestion from the opposite end of the incised strand to the limit 5′-damaged end (Hamilton, unpublished data).

Figure 6 shows that the number of nucleotides released for each pyrimidine dimer increased with time of incubation. For repair of the DNA strand, such nucleotides would have to be replaced totally and with great fidelity. The excising capacity of the *M. luteus* DNA polymerase was further examined after incision of DNA irradiated with varying doses. In these experiments, summarized in Figure 7, the irradiated DNA was treated with uv-endonuclease under saturating conditions, which ensured that the majority of the dimers resulted in nicks with 3′-OH end groups. It is clear that the efficiency of excision by the DNA polymerase decreased with increasing uv dose.

Reinsertion. Repair of the cavity in the DNA strand created by the excision of photochemical damage requires two distinct biochemical steps; reinsertion of correctly base paired nucleotides and closure of the resultant single-stranded break by a polynucleotide ligase. For ligase action, reincorporation of the nucleotides must either be juxtaposed to the existing 5′ end of the chain (*i.e.*, be stoichiometric), or proceed by "nick translation" (Kelly *et al.*, 1970), without displacement

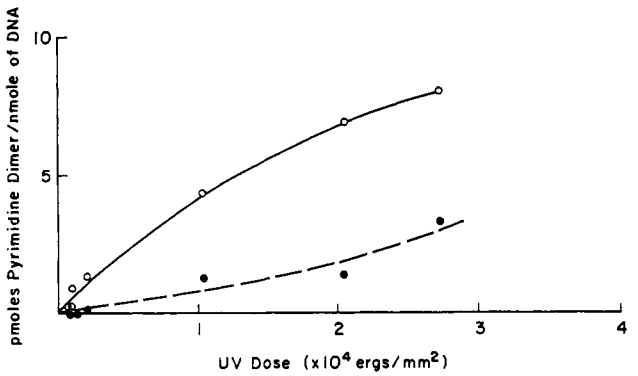

FIGURE 7: The excision of pyrimidine dimers from uv-irradiated DNA as a function of dose. Total pyrimidine dimers introduced into the DNA by uv irradiation (○); pyrimidine dimers remaining after incubation with DNA polymerase 15 min at 30° under standard assay conditions (●).

of the strand. The latter involves the concurrent activities of polymerization and breakdown which were followed in our experiments with [^{32}P]DNA by the double-labeling technique described in Methods. To standardize the conditions of reincorporation, preliminary experiments were carried out with model templates in which cavities were formed by incubation of DNase I treated DNA with exonuclease III under controlled conditions at 10° (Methods). To quantitate reinsertion, two factors were varied; temperature of incubation and divalent Mg^{2+} concentration. Repair synthesis by the *E. coli* DNA polymerase I has been shown to predominate at temperatures below 20° in repair of single-stranded areas of λ phage (Wu and Kaiser, 1968) and φXRF (Dumas *et al.*, 1971). Higher temperatures favor the formation of artifacts in the double-stranded DNA (Richardson *et al.*, 1964).

Reinsertion into Model Excised DNA Templates. Both the incorporation and release of nucleotides during polymerase action reached a similar dNTP substrate saturation optimum at 0.1 mM, and no inhibition of polymerization occurred when 10 mM *N*-ethylmaleimide was added to the assay mixture. Incorporation at both 10 and 37° in the presence of excess template is shown in Figure 8a,b to have a similar optimum Mg^{2+} concentration of 2–4 mM. At low Mg^{2+} concentrations (0.3–1 mM), however, the breakdown of the excised template did not parallel the incorporation of nucleotides. This was decreased at 37°, and at 10° no release of nucleotides could be detected up to 150-min incubation. This suggested that under these conditions of slow incorporation in template excess, release of nucleotides only occurred after the cavities were filled in and "nick translation" could proceed. This was supported by experiments in which the incubation temperature was varied, shown in Figure 9. With 0.5 mM Mg^{2+}, replacement of nucleotides excised from *E. coli* [^{32}P]DNA by exonuclease III (80 pmol/1.2 nmol of DNA) occurred in a stoichiometric fashion only at 10°, but this was accompanied by a slow release of template ^{32}P-labeled nucleotides. At the higher temperatures, quantitative gap filling was masked by "nick translation" and displacement synthesis. This approximate stoichiometry of reinsertion of tritium-labeled nucleotides at 10° was confirmed in experiments in which *E. coli* [^{32}P]DNA was treated sequentially with pancreatic DNase I at 37° and exonuclease III at 10° to release 47.5 and 107 pmol of nucleotide/1.12 nmol of DNA nucleotide. This represented gaps in the DNA strand 12 and 27 nucleotides in length. The kinetics of reinsertion at 10° are shown in Figure 10. A low level of presumably terminal incorporation of nucleotides, occurred in the nicked DNA. Incorporation into the

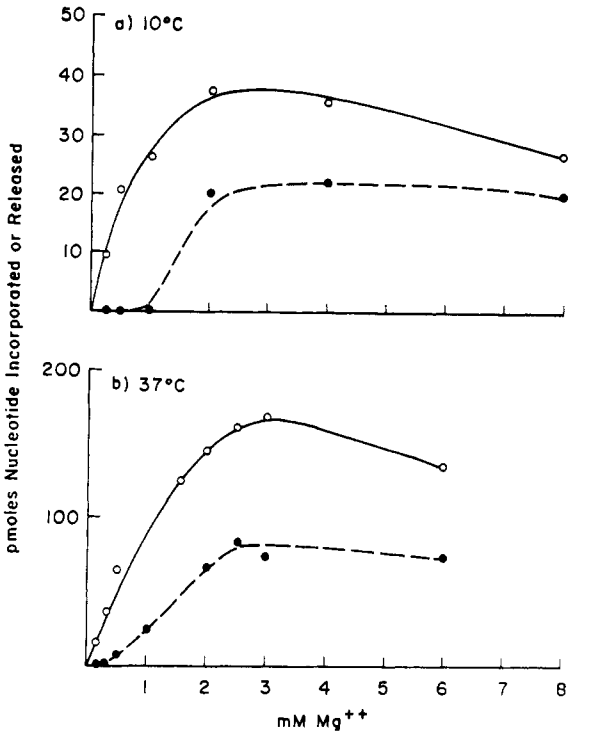

FIGURE 8: The simultaneous release of ^{32}P nucleotides from excised *E. coli* [^{32}P]DNA during incorporation of [^{3}H]TMP as a function of Mg^{2+} concentration. Excision was with exonuclease III and incorporation under standard assay conditions with 0.02 unit of DNA polymerase/nmol of DNA: incorporation (○); breakdown (●); (a) 150 min at 10°; (b) 20 min at 37°.

excised templates reached a plateau at 90–150 min, which represented 80–85% replacement of the excised nucleotides and was unaffected by the addition of fresh DNA polymerase. Again, detectable release of nucleotides occurred only at 90–150 min, suggesting this followed gap filling.

Excised Uv-Irradiated DNA. A similar pattern was found using the excised irradiated DNA template. *B. subtilis* transforming DNA irradiated with 2×10^3 ergs/mm^2 was incised with uv-endonuclease and incubated 30 min at 30° with DNA polymerase under standard assay conditions with 2 mM Mg^{2+} in the absence of dNTPs. In a parallel experiment with ^{3}H-labeled DNA, this resulted in excision of 100 pmol of nucleotide/nmol of DNA or ~90 nucleotides for each pyrimidine dimer. Incubation at 10° after addition of dNTPs and dilution

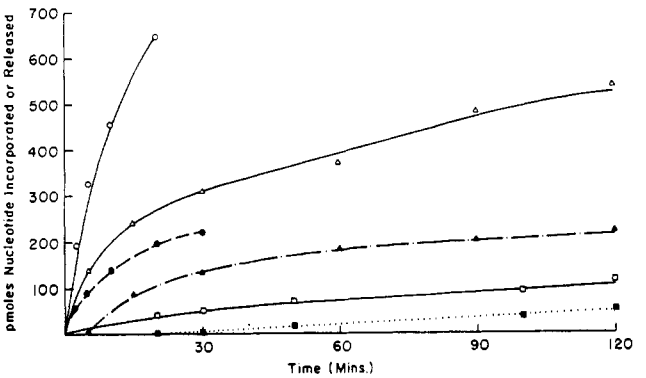

FIGURE 9: The effect of temperature on the incorporation and simultaneous release of nucleotides by DNA polymerase from *E. coli* [^{32}P]DNA excised with exonuclease III. Standard assay conditions with 0.5 mM Mg^{2+}. Incorporation and breakdown respectively at 37° (○,●), 20° (△,▲), and 10° (□,■).

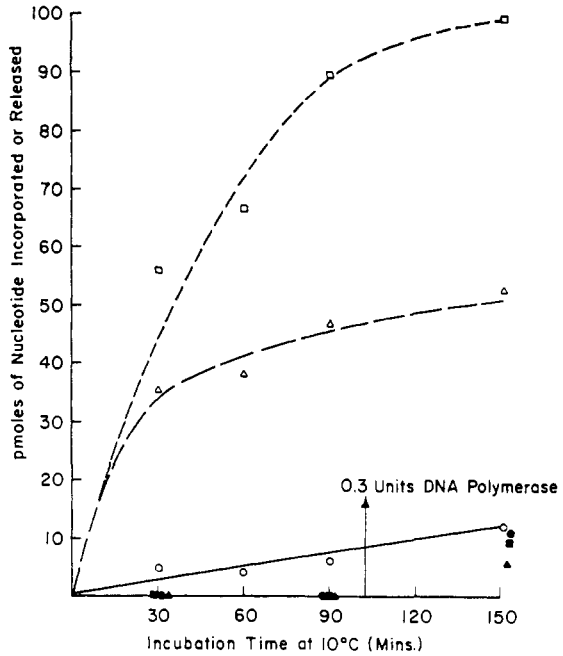

FIGURE 10: Stoichiometry of reinsertion of nucleotides into gaps in double-stranded DNA by DNA polymerase at 10°. *E. coli* [^{32}P]-DNA (0.8×10^5 cpm/nmol) was treated sequentially with DNase I (3×10^{-5} μg/μg of DNA) in 66 mM Tris-HCl (pH 8.0), 5 mM Mg^{2+} 30 min at 37°, and exonuclease III (3 units/μg of DNA) for 60 and 120 min at 10°. Both enzymes were inactivated by heating for 5 min at 68°. Incorporation and release of nucleotides was then followed under standard assay conditions with 0.5 mM Mg^{2+} for 90–180 min at 10°, as described in methods: incorporation (open symbols) and breakdown (filled symbols), respectively; DNase I treated DNA (○,●); excised DNA 47.5 pmol of nucleotide released (△,▲); and 107 pmol of nucleotide released (□,■).

to give a 0.5 mM Mg^{2+} concentration is shown in Figure 11 to result in an incorporation plateau of 130 pmol in 180–300

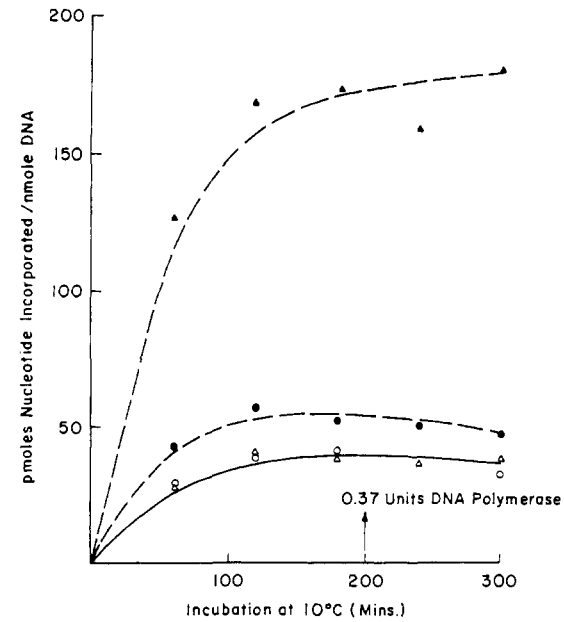

FIGURE 11: Reinsertion of nucleotides into irradiated *B. subtilis* DNA (2×10^3 ergs/mm^2) after excision of pyrimidine dimers by DNA polymerase as described in the text. Incorporation was under standard conditions at 10° with 0.5 mM Mg^{2+}, 3 nmol of DNA, and 1.12 units of DNA polymerase. After incubation for 180 min, a further 0.37 unit of DNA polymerase was added: native unirradiated (○); native incised-excised (●); uv-irradiated (△); uv-irradiated excised (▲).

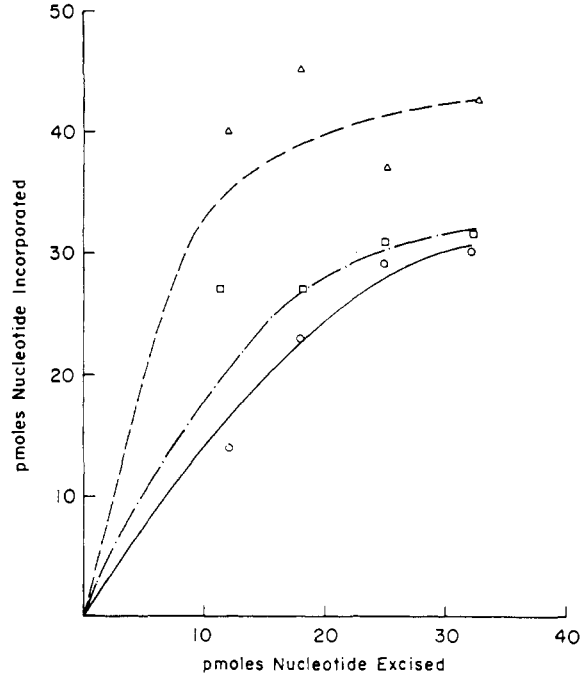

FIGURE 12: The termination of the reinsertion process by polynucleotide ligase. Excised irradiated DNAs (6.6×10^2 and 1×10^3 ergs per mm^2) with varying lengths of gaps were prepared as described in the text. Reinsertion of nucleotides was carried out at 10° in the presence and absence of polynucleotide ligase. Continued incorporation which occurred on raising the temperature to 30° and adding fresh DNA polymerase reflect the presence of 3′-OH priming sites. Control reinsertion at 10° (○); incorporation on raising the temperature to 30° after incubation at 10° with (1) NAD^+ (△), (2) polynucleotide ligase + NAD^+ (□).

min, which was not affected by the addition of fresh DNA polymerase. For optimal repair, 0.3 unit of DNA polymerase was required for each pmol of pyrimidine dimer, as indicated earlier in Figure 3.

Requirement for Polynucleotide Ligase for Stoichiometric Reinsertion of Nucleotides. B. subtilis DNA was irradiated with 6.6×10^2 and 1×10^3 ergs/mm^2, incised and excised for varying times at 30° to produce gaps of different lengths. The temperature was lowered to 10° and the replacement of nucleotides followed for 150 min in the presence of 0.1 mM dNTPs containing 0.2 μCi of [^{3}H]TTP. At the end of this time, aliquots were taken and incubated with and without polynucleotide ligase in the presence of 26 μM NAD^+ for a further 60 min at 10°. The temperature was raised to 30° and any additional incorporation representing the continued presence of 3′-OH ends measured after 20-min incubation. The results summarized in Figure 12 show that presence of the polynucleotide ligase in the assay at 10° terminated the incorporation of nucleotides by the DNA polymerase, as would be expected after closure of the final 3′-OH terminated single-stranded break.

Restoration of Molecular Weight after Biochemical Repair. One biochemical criterion that these changes have been effective is the restoration of the single-stranded molecular weight of the damaged DNA. This was followed by sedimentation of the ^{3}H-labeled *B. subtilis* transforming DNA during repair on alkaline sucrose gradients (McGrath and Williams, 1966), using the incorporation of [^{14}C]TTP to identify the repaired region. The initial treatment of uv-irradiated *B. subtilis* transforming DNA with polynucleotide ligase resulted in a slight increase in biological activity. This suggested the introduction of some ligasable single-strand breaks under our irradiation

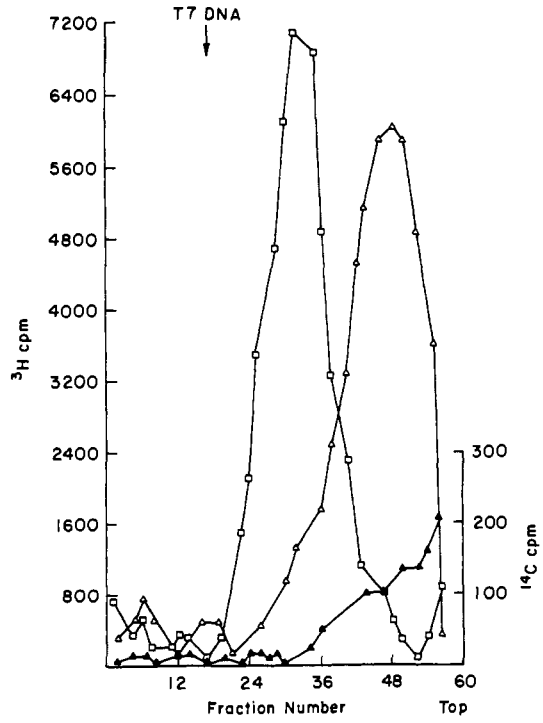

FIGURE 13: Alkaline sucrose gradient sedimentation of *B. subtilis* transforming [^{3}H]DNA (0.6 × 10^5 cpm/nmol) irradiated with 6.6 × 10^2 ergs/mm^2 and incised with uv-endonuclease, 60 min at 37° in 66 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.036 unit of uv-endonuclease/μg of DNA: uv-irradiated DNA + polynucleotide ligase (□); uv-irradiated DNA + uv-endonuclease + DNA polymerase uv-irradiated DNA + uv-endonuclease + DNA polymerase + [^{14}C]TTP only (△); incorporated [^{14}C]TTP (40 Ci/mol) (▲). [^{14}C]T7 DNA of single-strand mol wt 1.26 × 10^7 was used as a standard.

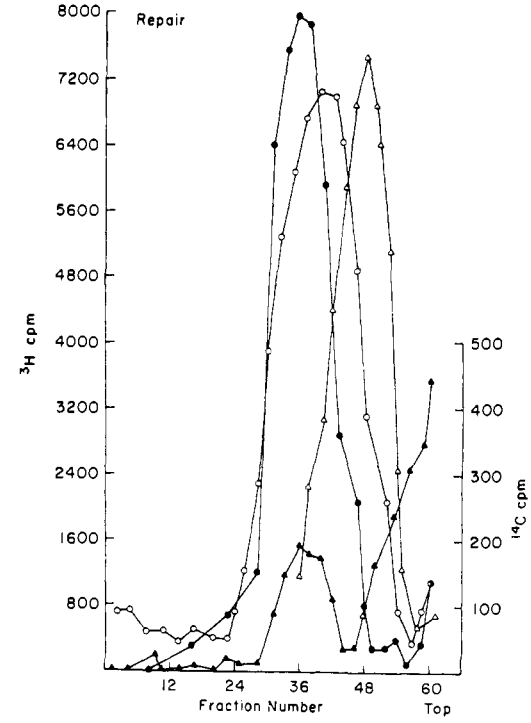

FIGURE 14: Alkaline sucrose gradient sedimentation of *B. subtilis* transforming [^{3}H]DNA irradiated with 6.6 × 10^2 ergs/mm^2. Restoration of molecular weight after reinsertion of nucleotides and ligase action: native DNA (○); incised uv-irradiated DNA + polynucleotide ligase (△); and incised uv-irradiated DNA with complete repair conditions (○); incorporated [^{14}C]TTP (40 Ci/mol) (▲).

conditions. Uv-induced single-strand breaks which are manifest under alkaline conditions have also been reported (Gaudin and Lemone Yielding, 1972). Figure 13 compares alkaline sucrose gradients of a polynucleotide ligase-treated uv-irradiated DNA, before and after, treatment with uv-endonuclease and DNA polymerase in the presence of a single triphosphate ([^{14}C]TTP). Molecular weights calculated by the method of Burgi and Hershey (1963) showed a fall in the single-stranded molecular weight from 4.6 × 10^6 daltons to 1.7 × 10^6 daltons. In the absence of the 3 dNTPs, no biological repair occurred; however, as indicated by the gradients in Figure 13, some incorporation of the [^{14}C]TTP occurred as end labeling into low molecular weight components. Incubation of the excised DNA under repair conditions (Figure 14) gave a significant, but not complete restoration of single strand molecular weight (3.3 × 10^6). In contrast to the excised DNA shown in Figure 13, the alkaline sucrose gradients in Figure 14 show a coincidence of the incorporated [^{14}C]TTP with the DNA molecules of restored molecular weight suggesting specific gap repair. Although all molecules of DNA have obviously not been repaired, it will be apparent in the next section, that those regions of the molecule containing the specific gene marker tested have been faithfully repaired by this procedure.

Biological Repair of Uv-Damaged B. subtilis Transforming DNA. The biological experiments, based on the biochemical data, were designed according to the following schedule. (1) Irradiation of *B. subtilis* 168 DNA. Single-stranded breaks sealed with polynucleotide ligase, 0.02 unit/μg of DNA in 7 mM Tris-HCl (pH 8.0), 2 mM Mg^{2+}, and 26 μM NAD^+ for 20 min at 30°. (2) Incision with *M. luteus* uv-endonuclease, 0.07 unit/pmol of pyrimidine dimer in 66 mM Tris-HCl (pH 8.0), 50 mM sodium chloride, and 0.5 mM Mg^{2+} for 60 min at 37°. The enzyme was inactivated by heating for 5 min at 68°. (3) Excision: incubation with *M. luteus* DNA polymerase, 0.3 unit/pmol of pyrimidine dimer in 66 mM Tris-HCl (pH 8.0) and 2 mM Mg^{2+} for 5–15 min at 30°. (4) Reinsertion and repair: the temperature was lowered to 10°. The four dNTPs were added (each 0.025 mM) with [^{3}H]TTP and incubated in the presence of 0.5 mM Mg^{2+}, 26 μM NAD^+, and polynucleotide ligase (0.02 unit/μg of DNA) for 90–240 min. The enzymes were inactivated 5 min at 68°. (5) Transformation assays: the host was *B. subtilis* BD172 uv-sensitive *thr*$_5^-$-*trp*$_2^-$. The transformation using wild-type DNA was from tryptophan$^-$ to tryptophan$^+$ and transformants were selected. The stepwise repair of DNA irradiated with 4.45 × 10^2 ergs/mm^2 compared with native DNA is summarized in Table IV. It is clear that the specific breaks introduced at the pyrimidine dimer sites by the highly purified endonuclease did not significantly decrease transforming activity. However, extension of these nicks into gaps during excision by the DNA polymerase resulted in additional inactivation of the marker. Under the complete sequential repair conditions in which the nucleotides were reincorporated at 10°, a 96% restoration of biological activity was obtained. Repair of biological activity also occurred when the incorporation step was carried out at 30°, although at this temperature less repair (70–80%) was achieved than at 10° (Table V). In this experiment, the number of nucleotides incorporated at 30° greatly exceeded those excised by the DNA polymerase. This enhanced incorporation probably represented displacement synthesis, preventing the action of the polynucleotide ligase.

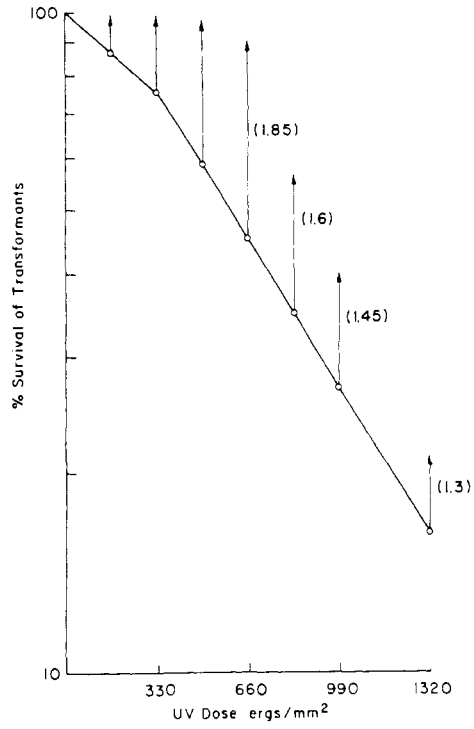

FIGURE 15: Uv dose dependence of repair "*in vitro*" of *B. subtilis* transorming DNA. Transforming activity remaining after uv irradiation (○); and extent of repair (▲). Enhancement of uv-irradiated activity shown in parentheses.

Influence of Uv Dose on the Final Level of Repair. The extent of repair of irradiated *B. subtilis* DNA possible with this enzyme system decreased with increasing uv dose (Figure 15). The survival curve shows that only with doses which resulted in survival exceeding 50% (*i.e.*, up to 6.6×10^2 ergs/mm^2) was it possible to attain the original native transforming activity. With increased exposure of the DNA to uv irradiation, restoration of biological activity decreased dramatically until at 1.3×10^3 ergs/mm^2 only 21% of the original native activity could be obtained. One possible explanation for the decreased repair is a consequence of "*in vitro*" repair of pyrimidine dimers in opposite strands as a result of increased irradiation. Since the 5′ → 3′ nuclease of the DNA polymerase is acting in opposing directions on the two strands, this could produce an effective double strand break. In fact, in experiments in which *E. coli* [^{3}H]DNA was exposed to doses of 0.7×10^3 and 1.92×10^3 ergs per mm^2, then treated with uv-endonuclease, there was a fall in double strand molecular weight measured in neutral sucrose gradients, from 2.36×10^7 to 2.03×10^7 and 1.44×10^7, respectively. Excision without synthesis was further shown to increase this strand breakage. This is similar to the results of Laipis *et al.* (1969), in which transforming DNA was inactivated by treatment with pancreatic DNase I and exonuclease III. At levels below 40% survival, the concerted actions of the *E. coli* DNA polymerase I and polynucleotide ligase were not able to restore full biological activity to this excised *B. subtilis* DNA.

TABLE IV: Transforming Activities of Native and Uv-Irradiated *Bacillus subtilis* DNA at the Individual Steps of DNA Repair.

Conditions	% Transformation	
	Native	Uv-Irradiated (4.4 × 10^2 ergs/mm^2)
No treatment	100	43
+ ligase		60
+ uv-endonuclease	96	39
+ DNA polymerase	93	43
+ uv-endonuclease + ligase	100	60
+ uv-endonuclease + DNA polymerase		30
+ DNA polymerase + ligase		60
+ uv-endonuclease + DNA polymerase + ligase[a]	96	96

[a] Two-step repair process described in the text.

TABLE V: Repair of *B. subtilis* Transforming DNA at 10 and 30°.[a]

Temp Incorp (°C)	Incubn Time (min)	Nucleotide Reinserted/ Pyrimidine Dimer	% Repair
10	75	11	70
	150	13.2	96
30	15	31	74
	30	37.7	64

[a] Radiation dose 4.4×10^2 ergs/mm^2. 20 nucleotides excised for each pyrimidine dimer by the DNA polymerase in 15 min at 30°.

Discussion

The DNA polymerase purified from *M. luteus* by the procedure described resembles the *E. coli* DNA polymerase I, in its molecular weight of 100,000–110,000, associated nuclease activities, ability to excise pyrimidine dimers from "incised" uv-irradiated DNA and lack of inhibition by *N*-ethylmaleimide. Its retention by phosphocellulose conflicts with the observation of Zimmerman (1966). This is either due to (1) the exhaustive treatment with DNase I and RNase subsequent to the ammonium sulfate step to remove nucleic acids, or (2) an aggregation phenomenon favored by the buffer and dialysis conditions prior to the phosphocellulose column, which is being investigated. It is significant that the level of the 5′ → 3′ nuclease when measured by simultaneous release of nucleotides during polymerization under optimal conditions resulted in nuclease:polymerase ratios of approximately 0.5. This is not greatly different from what would be expected with *E. coli* DNA polymerase I during "nick translation." The isolation from *M. luteus* of DNA polymerases with decreased nuclease activities and lower molecular weights could be due to protease action during purification. It has been shown, for example, that treatment of *E. coli* DNA polymerase I with a protease extract from *B. subtilis* results in two subunits, one of mol wt 75,000 containing 3′ → 5′ nuclease and polymerase activities, and the other of mol wt 35,000, the 5′ → 3′ nuclease (Brutlag *et al.*, 1969).

The biochemical conditions for the action of the DNA polymerase in DNA repair are the introduction of a 3′-OH priming site, control of excision and correct reinsertion of nucleotides

prior to termination by a polynucleotide ligase. Our experiments, using highly purified enzymes, show that the first requirement is provided by a uv-endonuclease. Based on work in this laboratory (Ono and Braun, 1973) and the work of Paterson and Setlow (1972), it must be assumed that there are at least two endonucleases specific for uv-irradiated DNA in *M. luteus* cells, two of which can be distinguished by their behavior on DEAE-cellulose, and the termini of the nick introduced adjacent to the dimer. In contrast to the endonuclease used in these experiments, the one adsorbed by DEAE-cellulose yields 3′-PO_4 ends (Kushner *et al.*, 1971), and requires phosphatase action to produce a 3′-OH priming site for the DNA polymerase.

Under our experimental conditions, treatment of uv-irradiated transforming DNA with purified endonuclease resulted in a slight loss of activity. If one considers that this enzyme is nicking at already damaged sites, the slight losses we observe might be attributed to reduction in molecular size below that required for integration into the bacterial chromosome (Morrison and Guild, 1972). Unlike the situation in *Haemophilus influenzae* transformation (Setlow *et al.*, 1970), "*in vitro*" endonuclease treatment of irradiated *B. subtilis* DNA does not result in intracellular repair. Since the repair enzymes have not been identified and isolated in *Bacillus subtilis*, we have no means of knowing if the uv-endonuclease is the missing enzyme in our uv-sensitive host.

The size of the excised gap in the DNA strand is dependent on the 5′ → 3′ directed nuclease activity, which continued to release nucleotides after removal of the pyrimidine dimer. Excision in the presence of the dNTPs resulted in a four- to sixfold enhancement of the release of nucleotides compared with 1.3–1.8 stimulation of the rate of dimer excision. This activity can, therefore, be restricted by omitting the dNTPs without great loss of efficiency of dimer excision. An interesting aspect of pyrimidine dimer excision by the DNA polymerase is its decreased efficiency at high uv doses. It is possible that this may reflect the introduction of dimers into regions of the DNA strand in which they are resistant to DNA polymerase and require the action of an independent uv-specific endonuclease.

The *in vitro* experimental device of incorporation at low temperature with suboptimal Mg^{2+} concentration which favors "repair synthesis" allows the termination of reinsertion to be determined biochemically. There is an approximate stoichiometry between the nucleotides incorporated and the number excised. The slow release of nucleotides, which occurs at this point, probably represents a slow "nick translation" (Dumas *et al.*, 1971). The validity of this biochemical criterion has been confirmed by the ability of the polynucleotide ligase to seal the strand and restore the molecular weight ot a value approaching the untreated DNA.

The biological repair of *B. subtilis* transforming DNA reflects a high fidelity of replacement of correct base pairing nucleotides by the DNA polymerase.

The problem still unresolved is the decreased repair "*in vitro*" of DNA exposed to high uv doses. Incomplete excision of pyrimidine dimers from incised-irradiated DNA may be a contributory factor. A more probable explanation is the formation of double-strand breaks which occur during the excision step "*in vitro*." This could result from concentration of the pyrimidine dimers in (A + T)-rich regions of the irradiated DNA (Shafranovskaya *et al.*, 1973; Brunk, 1973), which at high doses form gaps in opposing strands close enough for the formation of double-strand breaks (Freifelder and Trumbo, 1969). In a system under normal temperature conditions, in which the steps are not separated, the proximity of the excision and polymerizing activities in the DNA polymerase molecule would decrease the time between excision and resinsertion, as in "nick translation," minimizing the presence of sensitive single-stranded gaps. The DNA polymerase is, therefore, a unique enzyme in which all of its enzymatic functions can be directed toward maintaining the integrity of the DNA. To implicate this system further in DNA repair "*in vivo*" requires investigation of the necessary controls which allow repair over the whole biological range, combined with studies "*in vivo*," involving mutants of *M. luteus* with defects in one or another of these associated enzyme activities. The uv sensitivity of the *E. coli polA*$^-$ mutant (Delucia and Cairns, 1969) with markedly reduced polymerase activity exhibits nearly normal excision properties (Boyle *et al.*, 1970), since the 5′ → 3′ exonuclease activity is unaffected (Lehman and Chien, 1973).

The possibility of alterations in the efficiency of the 3′ → 5′ nuclease, thereby altering its capacity for monitoring reinsertion is indicated by the experiments of Muzyczka *et al.* (1972) with the mutator and antimutator polymerases of T4 bacteriophage.

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Transcription of *Xenopus* Chromatin by Homologous Ribonucleic Acid Polymerase: Aberrant Synthesis of Ribosomal and 5S Ribonucleic Acid†

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ABSTRACT: Both homologous DNA-dependent polymerases I and II can transcribe the genes for ribosomal RNA and for 5S RNA when given frog (*Xenopus laevis*) chromatin as a template. Both polymerases, however, transcribe these genes aberrantly. They transcribe the wrong strand and the spacer sequences which are not read *in vivo*. We conclude that still other factors are necessary for accurate *in vitro* transcription to occur.

Complete understanding of the control mechanisms for gene transcription in eukaryotes will require reproduction of those controls *in vitro*. With this goal in mind we have isolated the two major DNA-dependent RNA polymerases from the frog, *Xenopus laevis*, and have studied their ability to transcribe the genes for ribosomal RNA and for 5S RNA using homologous chromatin as a template.

Materials and Methods

Isolation of RNA Polymerase. RNA polymerases I and II were purified from *X. laevis* tissue culture cells and separated from each other as described by Roeder *et al.* (1970) with a slight modification. Polymerases I and II were eluted stepwise from DEAE-Sephadex by 0.19 and 0.28 M ammonium sulfate, respectively. The two polymerase fractions were purified to be free of each other by their α-amanitin sensitivity. Polymerase I thus purified was totally insensitive to α-amanitin, while polymerase II was inhibited completely by the toxin, suggesting very little contamination by polymerase I or III.

Preparation of Chromatin. Chromatin was prepared from *X. laevis* liver as described previously by Reeder (1973) and used within 6 hr after its preparation.

Preparation of DNA and RNA. Purified ribosomal DNA and unlabeled 5S RNA were gifts of Dr. D. D. Brown (Dawid *et al.*, 1970). Purified 5S DNA, strand-separated 5S DNA, and ^{3}H-labeled 5S RNA complimentary to either H or L strand of 5S DNA were gifts of Dr. K. Sugimoto (Brown and Sugimoto, 1973). The strands of rDNA[1] were separated by centrifugation in CsCl gradients as previously described (Dawid *et al.*, 1970). DNA from each fraction of such a strand separation gradient was caught on a separate nitrocellulose filter. A stack of filters from a single gradient was hybridized with various RNA preparations to assess the strand selectivity of transcription under different conditions. Labeled and unlabeled 18S and 28S rRNAs were prepared according to Brown and Weber (1968).

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[1] Abbreviations used are: rDNA, DNA containing the genes for 18S and 28S ribosomal RNA plus spacer sequences; 5S DNA, DNA containing the genes for 5S RNA plus spacer sequences; rRNA, 18S and 28S ribosomal RNA.