NATURE OF THE COMPLEMENTARY STRANDS SYNTHESIZED IN VITRO UPON THE SINGLE-STRANDED CIRCULAR DNA OF BACTERIOPHAGE ϕX174 AFTER ULTRAVIOLET IRRADIATION

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ABSTRACT This paper describes experiments intended to decide whether UV lesions in DNA act as absolute blocks to chain elongation by the *Escherichia coli* DNA polymerase or only slow down the polymerization process. Ultraviolet (UV)-irradiated, single-stranded (SS) circular DNA of bacteriophage ϕX174 was used as template for the polymerase in a reaction mixture in vitro, under conditions allowing synthesis of not more than one complementary strand per template molecule. The mean length of the newly synthesized complementary strands (as determined by velocity sedimentation in alkaline CsCl gradients), as well as the over-all template activity (as measured by deoxyadenosine monophosphate [dAMP] incorporation) was found to decrease with the number of biologically lethal hits sustained by the irradiated templates. With the increase of time or temperature of reaction, the net synthesis of complementary strands increased (as a consequence of increased initiation), but their mean length remained constant. The mean length of synthesized strands was greater than would be expected if all biologically lethal hits were to block the polymerization process. The lethal hits which serve as blocking lesions are inferred to be pyrimidine dimers because it is possible to obtain synthesis of full-length complementary strands if, when heat-denatured, UV-irradiated, double-stranded replicative form (RF II) DNA of bacteriophage ϕX174 is used as a template, it is pretreated with yeast photoreactivating enzyme (YPRE) in presence of visible light.

INTRODUCTION

In spite of a large amount of work done on the photochemistry and repair of UV-irradiated DNA (see review by R. Setlow, 1968), we lack precise knowledge of how UV lesions interfere with replication of such templates. Specifically the question of whether these lesions act as absolute blocks for the polymerization enzyme or

only slow down the polymerization process has not been resolved by earlier investigations (Josse et al., 1961; Bollum and Setlow, 1963). Since precise physical data about the templates and products are needed to distinguish between these possibilities, the well characterized *E. coli* DNA polymerase reaction system (Goulian and Kornberg, 1967; Goulian, Kornberg, and Sinsheimer, 1967; Dumas et al., 1971), in which the circular single-stranded DNA of bacteriophage ϕX174 serves as the template for the in vitro synthesis of its complementary strand, offers an excellent means of clarifying the issue.

In this paper we report the results of our investigations on the nature of the complementary strand synthesized in vitro when the UV-irradiated ϕX DNA was present as the template. Most of the reactions were carried out at 15°C such that DNA synthesis was limited to one cycle of replication, and the radiation dose was never greater than 1000 ergs/mm^2 which is about 10–100 less than is usually employed in photochemical investigations. Our results indicate that although polymerization can proceed past most of the biologically lethal UV lesions on a circular single-stranded DNA template, some lesions do act as absolute blocks to chain elongation by the DNA polymerase. We also present suggestive evidence that the lesions blocking the polymerization process are pyrimidine dimers because these blocks are apparently removable through the action of yeast photoreactivation enzyme.

MATERIALS AND METHODS

Preparation of SS and RF II DNA of ϕXam3

The procedures for large-scale culture and purification of ϕX*am*3, a lysis-defective mutant of bacteriophage ϕX174, and for extraction of its SS DNA by hot phenol method have been described (Edgell et al., 1969; Dumas et al., 1971; Guthrie and Sinsheimer, 1963). Replicative form DNA of ϕX*am*3 was isolated from *E. coli* C cells infected with ϕX*am*3 in presence of chloramphenicol (cf. Komano and Sinsheimer, 1968); 84% of the DNA preparation was in the form of RFII as revealed by sedimentation in alkaline CsCl in the Spinco Analytical Ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). Viral SS DNA and RFII DNA used in this study were generously provided by Dr. L. B. Dumas and Mr. M. Eisenberg, respectively.

Irradiation

For UV irradiation, samples in watch glasses at room temperature were exposed to germicidal lamps (254 mμ) at a distance of about 25 cm, at a dose rate of about 19 ergs/mm^2 per sec. For photoreactivation, UV-irradiated DNA samples were mixed 1:1 by volume with the YPRE preparation (Muhammed, 1966) and then exposed to GE black light lamps (General Electric Co., Lamp Glass Dept., Richmond Heights, Ohio) (emission peak at 360 mμ) at a distance of about 20 cm for 30 min at room temperature. The solvent during UV irradiation was either 0.05 M Tris pH 8.1 or 0.05 M potassium phosphate, pH 7.5.

Isolation of DNA Polymerase

The DNA polymerase used in this study was isolated from *E. coli* C cells by Dr. L. B. Dumas (Dumas et al., 1971). It exhibited the same requirements for DNA synthesis, the same dependence on such variables as incubation temperature, pH, and magnesium ion concentration, and approximately the same sedimentation coefficient (5.5) as did *E. coli* DNA polymerase purified by the method of Richardson et al. (1964). The specific activity of the enzyme preparation was about 2500 units per mg of protein, one unit being defined as that amount inducing the incorporation of 1 mμmole of deoxyadenosine triphosphate (dATP) into acid-insoluble product in 30 min incubation period at 37°C in a 0.3 ml reaction mixture (see below).

Measurement of DNA Synthesis

The reaction mixtures for DNA synthesis usually contained per ml: 67 μmoles of potassium phosphate, pH 7.5, 6.7 μmoles of $MgCl_2$, 1.0 μmoles of β-mercaptoethanol, 160 mμmoles each of deoxynucleoside triphosphates, 53–266 mμmoles of φX viral DNA, 1.6 mμmoles of degraded calf thymus DNA and 10–20 units of DNA polymerase, unless otherwise indicated. DNA concentrations in all reaction mixtures are expressed as concentration of nucleotides in DNA.

The degraded calf thymus DNA, which supplied short oligonucleotides needed for initiation of the in vitro polymerization reaction ("initiators"), was prepared by enzymatic degradation of high molecular weight calf thymus DNA as follows: the reaction mixture contained (per ml) 2 mg of calf thymus DNA, 50 μmoles of Tris-HCl buffer, pH 8.0, 5 μmoles of $MgCl_2$, and 67 μg of pancreatic DNase. The mixture was incubated for 2 hr at 37°C. The DNase activity was destroyed by heating 10 min in boiling water bath. The solution was stored at −20°C.

The reaction mixture (0.3 ml) for the assay of the activity of DNA polymerase consisted of 20 μmoles of potassium phosphate, pH 7.5, 2 μmoles of $MgCl_2$, 0.3 μmoles of β-mercaptoethanol, 10 μmoles each of dCTP, dGTP, TTP,[1] and α-^{32}P-dATP, 70 mμmoles of φX viral DNA, 20 mμmoles of degraded calf thymus DNA, and 0.2–20 units of DNA polymerase.

The progress of a reaction was followed by measuring the accumulation of acid-insoluble radioactivity with time. Aliquots of 0.02 ml were transferred into 1 ml of cold 15% trichloroacetic acid (TCA). 100 μg denatured calf thymus DNA was added as carrier. After at least 30 min at 0°C, the precipitates were collected on glass fiber filters, washed with cold 5% TCA, followed by methanol, dried, and counted in a gas flow counter. Aliquots of reaction mixtures to be further characterized by zone sedimentation were adjusted to 0.01 M EDTA and stored at −20°C until used.

Zone Sedimentation in CsCl

The techniques used were similar to those of Burton and Sinsheimer (1965). For centrifugation at alkaline pH, 0.15 volumes of 1.0 N KOH were added to reaction mixture aliquots. These were then layered onto preformed linear gradients of CsCl (ρ = 1.20–1.35 g/ml) in 4.8 ml of 0.1 N KOH. The samples were centrifuged at 4°C for the desired time. For centrifugation at neutral pH, aliquots of reaction mixtures were layered onto preformed linear

[1] dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; TTP, thymidine triphosphate.

gradients of CsCl (ρ = 1.20–1.35 g/ml) in 48 ml 0.05 M potassium phosphate, 0.01 M EDTA, pH 7.2. These were centrifuged at 15°C for the desired time.

Fractions (10 drops) were collected from the bottoms of the tubes onto 2.3 cm Whatman 3 MM filters. The filters were soaked in cold 5% TCA, washed with the same on a Buchner funnel, washed with methanol, and dried. Radioactivity was measured in a liquid scintillation spectrometer.

In all zone sedimentation experiments ^{3}H-labeled, single-stranded viral DNA of ϕX*am*3 was used as marker.

Infectivity Assay of Viral DNA

For infectivity assay of viral DNA's spheroplasts of *E. coli* K12W6 (for use with single-stranded DNA), or *E. coli* K12 AB1887, an *hcr*$^-$ strain (for use with double-stranded replicative form RFII DNA), were prepared and infected as described by Guthrie and Sinsheimer (1963). After a growth period of 90–120 min at 35°C, the infected spheroplasts were lysed by means of 10-fold dilution and 30 min incubation at room temperature in 0.05 M sodium tetraborate and the total yield of liberated free phage was assayed by the conventional agar layer method (Adams, 1956), using the permissive strain, *E. coli* K12 HF4714 as plating bacteria.

The concentrations of single-stranded and RF DNA's were in the range of 10^5–10^9 molecules/ml, while those of the spheroplasts were about 0.5–1.0 $\times$ 10^9/ml in the growth tubes during infectivity assays (the DNA concentrations were estimated with reference to a standard preparation of viral SS DNA). Within this range of (DNA):(protoplast) ratios the phage yield per input DNA molecule was constant and varied from 0.1 to 1.0 for SS DNA and from 0.001 to 0.01 for RFII DNA in different experiments. It was established in each experiment that infectivity of irradiated DNA samples of both kinds (SS and RFII) decreased linearly upon dilution exactly as did an unirradiated standard; i.e., irradiated samples behaved simply as populations of molecules which had a reduced number of infective particles, without any interference from damaged molecules. Therefore, the assay in the inactivation experiments was not a complete dilution curve, but an assay in duplicate or triplicate of the irradiated samples. All assays were carried out in dim yellow light to avoid any possible photoreactivation.

Materials

Unlabeled deoxyribonucleoside triphosphates were purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.). α-^{32}P-dATP was purchased from International Chemical and Nuclear Corp. (ICN), Burbank, Calif. YPRE was the generous gift of Dr. J. K. Setlow of Oak Ridge National Laboratory.

RESULTS

Evidence for Ultraviolet-Induced Decrease of Mean Chain Length of Newly Synthesized Complementary Strands

As a result of UV irradiation at 254 mμ, both free phages as well as the viral SS DNA exponentially lost their biological activity at the same rate, one lethal "hit" corresponding to approximately 95 ergs/mm^2. This is in accordance with the earlier finding of Yarus and Sinsheimer (1967). To investigate the effect of UV irradiation

on the template activity, aliquots of viral SS DNA which had previously received doses of UV irradiation corresponding to 0, 1, 2, and 8 lethal hits were incubated separately in identical DNA polymerase reaction mixtures at 15°C. Fig. 1 shows the results of this experiment. The rate of dAMP incorporation which is the measure of rate of polymerization of the complementary strands, was found to decrease progressively with increasing dose. After 6 hr, total incorporation by the templates harboring 8 lethal hits was about half of that by the unirradiated template. Under these experimental conditions (with limiting concentration of initiator) it is evident that net polymerization had ceased at about 0.4-fold synthesis in the case of unirradiated templates, i.e., when the moles of nucleotide incorporated into the product were equal to about 40% of the moles of nucleotides in the template.

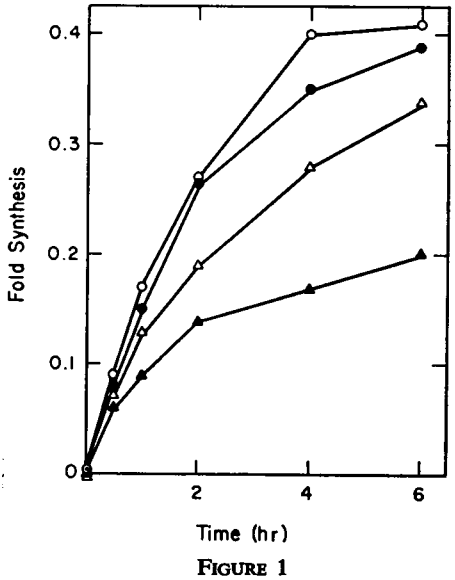

FIGURE 1

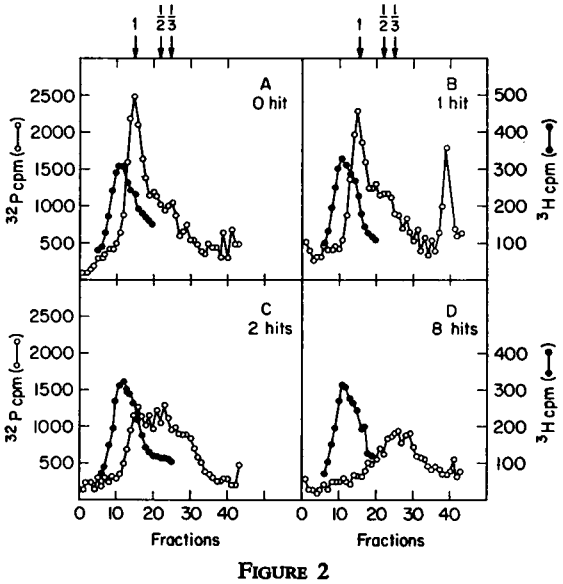

FIGURE 2

FIGURE 1 Time course of DNA synthesis at 15°C with UV-irradiated single-stranded $\phi X am3$ DNA as template. Each reaction mixture (0.6 ml) contained 100 mμmoles each of dCTP, dGTP, TTP, and α-^{32}P-dATP (4.5×10^6 cpm/μmole), 40 mμmoles of $\phi X am3$ DNA, 1.0 mμmole of degraded calf thymus DNA (initiator), and components listed in Materials and Methods. $\phi X am3$ viral DNA was mixed with ^{3}H-labeled $\phi X am3$ DNA marker, UV-irradiated with various doses equivalent to 0 hit (○———○), 1 hit (●———●), 2 hits (△———△), and 8 hits (▲———▲), and then added to the respective reaction mixtures.

FIGURE 2 Zone sedimentation in alkaline CsCl gradients of the products of DNA synthesis reactions, as described in Fig. 1, after 7 hr of incubation at 15°C. To 0.3 ml aliquots of each of the reaction products, A (0 hit), B (1 hit), C (2 hits), and D (8 hits), 0.04 ml 1 N KOH and 0.02 ml 0.5 mg/ml $\phi X am3$ DNA as carrier were added. The solutions were then layered onto alkaline CsCl gradients and centrifuged for 3 hr at 45,000 rpm in an SW 50.1 rotor (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) at 4°C. The positions expected for DNA molecules of fractional lengths as compared to the full-length complementary strand of the ϕX viral DNA were estimated according to Studier (1965) and Martin and Ames (1961), and are indicated by arrows on top of the diagram. (○———○) ^{32}P cpm; (●———●) ^{3}H cpm.

To investigate the nature of the newly synthesized strands, aliquots of the above reaction mixtures, taken after 7 hr, were denatured in alkali and sedimented through alkaline CsCl density gradients (Figs. 2 A–D). The ^{32}P-labeled complementary strands were found to sediment at gradually decreasing rates as the number of lethal hits in the templates increased. The sedimentation profile of the complementary strands synthesized from unirradiated templates or from templates with one lethal hit has a peak corresponding to the position of one full-length linear ϕX DNA (compared to the position of the ^{3}H-labeled ϕX DNA marker) (Figs. 2 A and B). Templates with two hits produced complementary strands which sedimented as a very broad band ranging in size from about one-third to one full length linear molecules; while templates with 8 hits gave rise to complementary strands sedimenting with a broad radioactivity peak corresponding to about $\frac{1}{3}$ the size of full-length complementary strands (Fig. 2 C and D).

It is worthwhile to mention here that ^{3}H-labeled single-stranded DNA of ϕX*am*3 was added as marker prior to irradiation and so was present during both irradiation and the DNA synthesis reactions. Figs. 2 A–D indicate that no changes such as chain breaks, cross-linkage, etc., were produced in the template molecules up to an irradiation dose of 8 lethal hits.

The concentrations of short oligonucleotide initiators in the above reaction mixtures were low enough to insure that more than one initiation per template was extremely unlikely (Dumas et al., 1970), and thus limited the net synthesis. As a consequence the template-product complexes should sediment in neutral CsCl gradient as does ϕX174 replicative form DNA (16S) when the templates are unirradiated, but more like ϕX174 SS viral DNA (24S) when the template contains many hits, because in the latter case the complementary strands of the template-product complexes would be much less than full-length molecules. Fig. 3 (A–C) shows the sedimentation profiles of the products (after 6 hr) of three 15°C reactions, containing, respectively, unirradiated templates in the presence of low initiator concentration and templates harboring 8 lethal hits with low, as well as more than 100 times higher, concentrations of initiators. It is evident that the template-product complexes with unirradiated templates or with irradiated templates in the presence of very high initiator concentrations (allowing multiple initiations), sedimented as expected as a narrow band slower than the ^{3}H-labeled, marker ϕX single-stranded DNA (Figs. 3 A and C). The sedimentation rate of this complex is about 17S, similar to that of ϕX174 RFII (16S), the nicked, double-stranded DNA from ϕX174-infected *E. coli* (Burton and Sinsheimer, 1965). But the sedimentation profile of template-product complexes obtained from irradiated templates in the presence of low initiator concentration (where more than one initiation was unlikely) was like that expected from a heterogeneous population of partially double-stranded molecules composed of viral SS DNA templates associated with pieces of less than full-length product strands (Fig. 3 B).

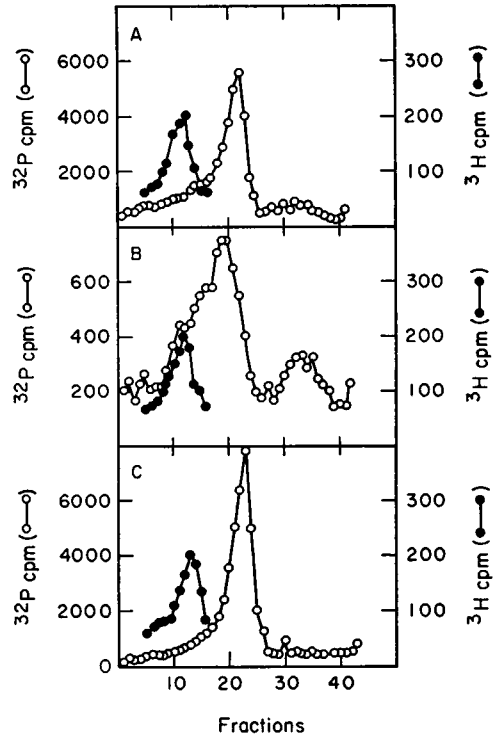

FIGURE 3 Comparative zone sedimentation analysis of reaction products in neutral CsCl gradients. All reaction mixtures (0.3 ml) contained 50 mμmoles each of dCTP, dGTP, TTP, and α-^{32}P-dATP (1.2 × 10^7 cpm/μmole), and components listed in Materials and Methods. Amounts of templates (φX*am*3 viral DNA) and initiators (degraded calf thymus DNA) in the reaction mixtures were as follows: A, 80 mμmoles of unirradiated templates and 0.5 mμmoles of initiators; B, 80 mμmoles of UV-irradiated templates containing 8 hits and 0.5 mμmoles of initiators; and C, 80 mμmoles of UV-irradiated templates containing 8 hits and 68 mμmoles of initiators. After 6 hr of incubation at 15°C, 0.02 ml φX*am*3 DNA carrier, 0.02 ml ^{3}H-labeled φX*am*3 DNA marker, and 0.025 ml 0.05 M potassium phosphate, pH 7.5, 0.01 M EDTA, were added to 0.05 ml aliquots of the reaction mixtures. The solutions were then layered onto neutral CsCl gradients in the same buffer and centrifuged at 15°C in an SW 65 rotor at 55,000 rpm for 100 min. (O———O) ^{32}P cpm; (●———●) ^{3}H cpm.

Evidence that UV Irradiation Generates Lesions which Absolutely Block Chain Elongation

Experiments described in the previous section demonstrated that the use of UV-irradiated circular single-stranded DNA of bacteriophage φX174, bearing many lethal hits, as a template for in vitro DNA synthesis resulted in the synthesis of product strands of less than full length under conditions in which unirradiated templates synthesized complete complementary strands. Evidently the UV lesions on the template strands interfered with the polymerization of the complementary strand. To decide whether such interference amounted only to slowing down of the rate, or to

complete blocking of the chain elongation process, the following experiments were carried out.

Single-stranded $\phi X am3$ DNA, UV-irradiated with a dose equivalent to 8 lethal hits, was used as template in a DNA polymerase reaction mixture at 15°C in the presence of low initiator concentration. Aliquots of reaction products were removed at 3, 6, and 12 hr and sedimented in alkaline CsCl gradients (Figs. 4 A–C). The sedimentation profiles of the newly synthesized ^{32}P-labeled complementary strands are almost similar in all three cases, with broad peaks corresponding to about 0.27

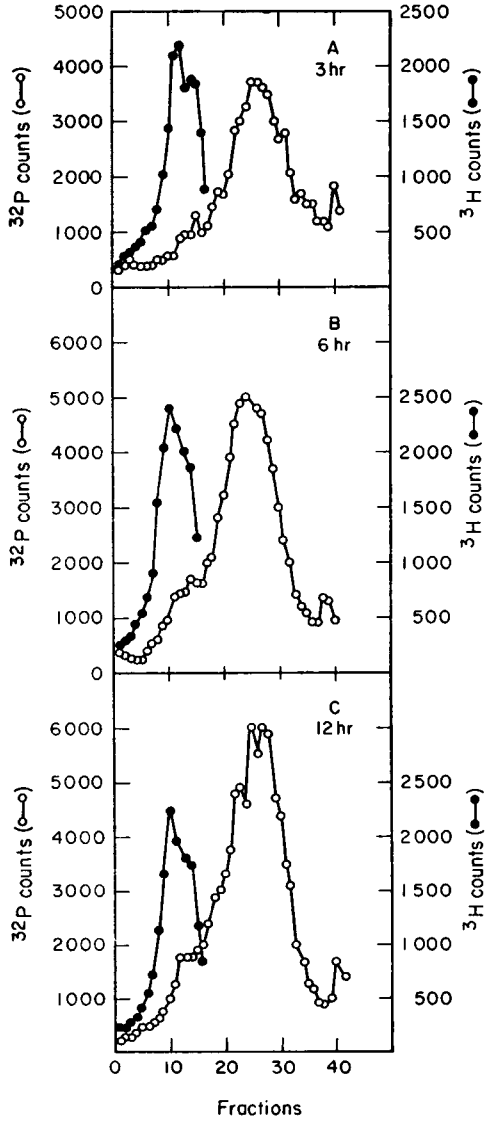

FIGURE 4 Zone sedimentation in alkaline CsCl of the reaction products after 3, 6, and 12 hr of DNA synthesis. The reaction mixture (0.6 ml) contained 50 mμmoles each of dCTP, dGTP, TTP, and α-^{32}P-dATP (1.8×10^7 cpm/μmole), 32 mμmoles of UV-irradiated $\phi X am3$ DNA containing 8 hits, 1.0 mμmole of degraded calf thymus DNA, and the components listed in Materials and Methods. To each of 0.1 ml aliquots of the reaction mixture after 3, 6, and 12 hr of incubation at 15°C, 0.02 ml 0.5 mg/ml $\phi X am3$ DNA carrier, 0.02 ml ^3H-labeled $\phi X am3$ DNA (marker), and 0.025 ml 1 N KOH were added. The solutions were then layered onto alkaline CsCl gradients and centrifuged at 4°C in a SW 50.1 rotor at 45,000 rpm for 3 hr. ^{32}P (○———○) and ^3H (●———●) counts for 4 min intervals are shown.

full-length linear ϕX DNA. There is no evidence of chain elongation of the product strands during as long as 9 hr of incubation, indicating that at least some of the UV lesions in the template molecules irreversibly block the polymerization process. The increase in DNA synthesis during the incubation period, viz., 0.15-fold at 3 hr, 0.24-fold at 6 hr, and 0.31-fold at 12 hr, must represent new initiations.

Aliquots of reaction products of a similar experiment were removed at 3 and 12 hr, and sedimented in neutral CsCl gradients. No change was observed in the size distribution of template-product complexes formed, with the time of incubation of the reaction mixture.

In order to ascertain whether these blocks to chain elongation could be removed at a higher temperature the following experiment was carried out: samples of unirradiated and UV-irradiated ϕX*am*3 viral DNA containing 8 lethal hits were added as templates to separate DNA polymerase reaction mixtures at 15°C without any extraneous initiator. (Under these conditions, the endogenous initiator activity associated the viral DNA templates would allow a slow rate of DNA synthesis to a final extent

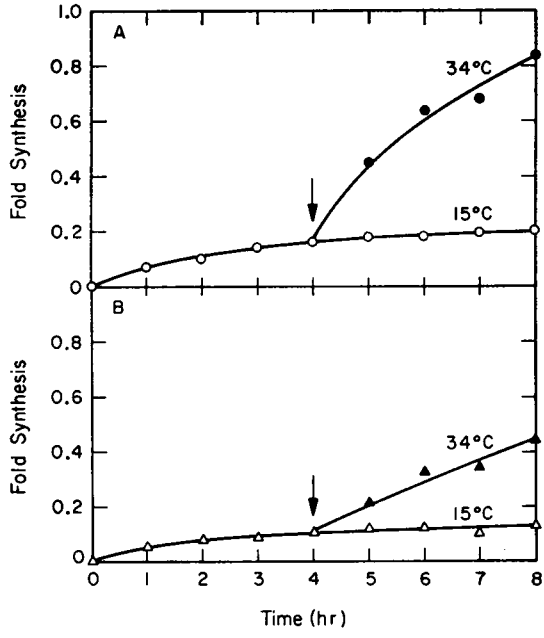

FIGURE 5 Time course of DNA synthesis with and without temperature shift-up. The reaction mixture (0.6 ml), contained 100 mμmoles each of dCTP, dGTP, TTP, and α-^{32}P-dATP (3.7×10^6 cpm/μmole in A and 7.5×10^6 cpm/μmole in B), 160 mμmoles of unirradiated ϕX*am*3 DNA (A) or 160 mμmoles of UV-irradiated ϕX*am*3 DNA containing 8 lethal hits (B), and the components listed in Materials and Methods. Both the reaction mixtures were incubated at 15°C for 4 hr, after which 0.25 ml aliquots of each of A and B were shifted to 34°C, and all four reactions were followed for a further period of 4 hr. DNA synthesis with unirradiated ϕX DNA: 15°C (○———○), 34°C (●———●). DNA synthesis with UV-irradiated ϕX DNA: 15°C (△———△), 34°C (▲———▲).

of about 0.25-fold; i.e., about one-fourth of the unirradiated template molecules would be converted completely to double-stranded RFII DNA [Dumas et al., 1970.]) After 4 hr of incubation at 15°C, portions of the reaction mixtures were warmed to 34°C and all the reactions were allowed to proceed for another period of 4 hr. Aliquots of reaction mixtures were removed every hour to monitor the amount of DNA synthesis (Fig. 5).

At 15°C polymerization reactions tended toward saturation values of 0.21-fold and 0.14-fold synthesis, respectively, for the unirradiated and the irradiated templates. On shift-up to 34°C after 4 hr, DNA synthesis continued to increase in both cases; after 8 hr there was about 0.85-fold synthesis of the unirradiated templates, and about 0.44-fold synthesis of irradiated ones. (This is believed to represent increased chain initiation at the higher temperature). Aliquots of 8 hr reaction products at 34°C were then sedimented in alkaline CsCl gradients (Figs. 6 A–B). Comparison of the sedimentation profiles of ^{32}P-labeled product strands from unirradiated and irradiated templates revealed that the latter were still mostly less than full-length viral complementary strands, with a peak at about ⅓ length, while the former extended to and beyond full length.

These experiments indicate that prolonged incubation at either 15°C or at 34°C could not remove the block to polymerization present in the UV-irradiated viral SS

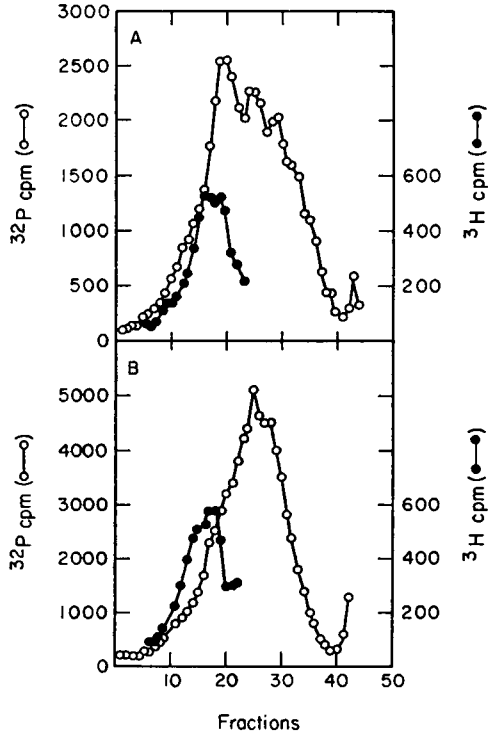

FIGURE 6 Zone sedimentation in alkaline CsCl gradients of the reaction products after temperature shift-up. After 4 hr at 15°C followed by 4 hr at 34°C, 0.1 ml aliquots of reaction mixtures of A and B described in Fig. 5 were removed and 0.02 ml 0.12 M EDTA, 0.02 ml 0.5 mg/ml $\phi X am3$ DNA (carrier), 0.02 ml ^{3}H-labeled $\phi X am3$ DNA (marker), and 0.025 ml 1 N KOH were added. The solutions were then layered on alkaline CsCl gradients and centrifuged at 4°C in an SW 50.1 rotor at 45,000 rpm for 3 hr. (O———O) ^{32}P cpm; (●———●) ^{3}H cpm.

DNA templates so as to allow chain elongation of the newly synthesized complementary strands; with the increase of time or temperature of reaction, the net synthesis of complementary strands increased, as a result of new initiation, but their mean length remained approximately constant.

Evidence that the Absolute Blocks in the UV-Irradiated φXam3 DNA towards Polymerization can be Removed by Yeast Photoreactivation Enzyme (YPRE)

The physicochemical nature of lesions produced in the native double-stranded DNA by UV irradiation at 254 mμ has been intensively investigated. The major portion—about 70% (Castellani et al., 1964) or even 90% (Witkin, 1967)—of these lesions have been postulated to be pyrimidine dimers and are believed to be predominantly responsible for the lethal inactivation, especially of bacteria and phages, caused by UV irradiation (see reviews by J. Setlow, 1967; R. Setlow, 1968). However, the nature of UV lesions in single-stranded DNA has not yet been so well characterized as in the case of double-stranded DNA. Efficiency of production (quantum yield) of pyrimidine dimers in denatured DNA has been claimed to be about twice that for native DNA (Wacker et al., 1962; Setlow, Carrier, and Bollum, 1964, 1965). On the other hand, David (1964) showed that only about one in three lethal hits of UV-irradiated bacteriophage φX could be correlated to the thymine dimers (cytosine-containing dimers were not investigated) produced in its DNA, and was unable to detect any difference in quantum yields between φX DNA and T4 DNA.

One means of testing whether the pyrimidine dimers on the φX*am*3 DNA are the blocks to chain elongation observed in the experiments described in the previous sections would be to monomerize these dimers by visible light irradiation in the presence of a photoreactivation enzyme (Wulff and Rupert, 1962) and, then use these reactivated molecules as templates in DNA polymerase reaction mixtures.

Unfortunately, the efficiency of monomerization of pyrimidine dimers by photoreactivation in denatured DNA has been shown to be low (Setlow and Carrier, 1964), as is also evident from out experimental results (Fig. 7), showing a photoreactivable sector of only about 0.2 for SS DNA of φX*am*3 while that for RF DNA was about 0.87. Even when single-stranded viral DNA UV-irradiated with a dose of 760 ergs/mm^2 (equivalent to 8 lethal hits) was first converted in vitro to double-stranded RFII with the help of DNA polymerase, visible light irradiation in the presence of YRPE produced only slight reactivation of about 15%. On the other hand, heat-denatured DNA single strands, derived from RF DNA which had been irradiated with the same dose and then photoreactivated by means of visible light irradiation in the presence of YPRE, showed about 90% of the plaque-forming ability of similar molecules obtained by heating unirradiated RF molecules.

These results suggested that pyrimidine dimers which were produced in the native RF molecules by UV irradiation, causing loss of infectivity, continued to remain on the heat-denatured RF molecules, but were monomerized, if the irradiated native RF were first photoreactivated.

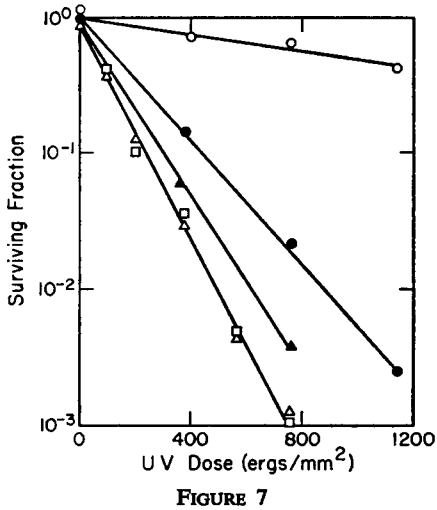

FIGURE 7

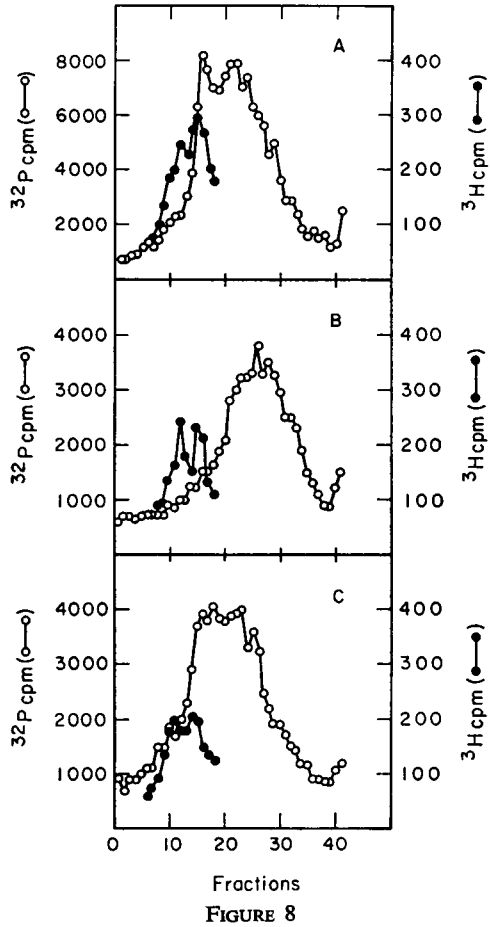

FIGURE 8

FIGURE 7 Inactivation curves of UV-irradiated RFII DNA with (○———○) and without (●———●) photoreactivation in vitro (see Materials and Methods). (▲———▲) and (△———△) represent, respectively, the survival of UV-irradiated single-stranded *φXam3* DNA with, and without, prior photoreactivation treatment. (□———□) denotes the survival of UV-irradiated *φXam3* virus without photoreactivation.

FIGURE 8 Zone sedimentation in alkaline CsCl gradients of products of reaction mixtures containing denatured *φXam3* RFII DNA as templates. Three samples, each consisting of 43 mμmoles of RFII DNA in 0.05 M Tris pH 8.1, were treated as follows: The control sample (A) was mixed with 1 volume of YPRE solution and kept in the dark; the second sample (B) was irradiated with an UV dose of 760 ergs/mm², equivalent to 8 lethal hits for single-stranded *φXam3* DNA, then mixed with 1 volume of YPRE solution as before and kept in dark. The remaining sample (C) was UV-irradiated with the same dose as B, mixed with 1 volume YPRE solution, and subjected to photoreactivation treatment as described in Materials and Methods. All three samples were then denatured in a boiling water bath for 5 min and then rapidly chilled in ice. Each of the three heat-denatured RFII DNA samples was used as a template for DNA synthesis in a reaction mixture (0.33 ml) consisting of 50 mμmoles each of dCTP, dGTP, TTP, and α-^{32}P-dATP (6.9×10^7 cpm/μmole), 0.5 mμmoles of degraded calf thymus DNA, and the components listed in Materials and Methods. After 6 hr of incubation at 15°C, 0.025 ml 0.12 M EDTA, 0.02 ml 0.5 mg/ml *φXam3* DNA (carrier), 0.02 ml ^{3}H-labeled *φXam3* DNA (marker), and 0.025 ml 1 N KOH were added to 0.1 ml aliquots of the reaction mixtures. The solutions were then layered onto alkaline CsCl gradients, and centrifuged at 4°C in a SW 50.1 rotor at 45,000 rpm for 3 hrs. (○———○) ^{32}P cpm; (●———●) ^{3}H cpm.

In order to investigate the nature of the complementary strands of DNA synthesized with such molecules, heat-denatured samples of unirradiated RFII, UV-irradiated RFII (with a dose of 760 ergs/mm², i.e. equivalent to 8 lethal hits on SS DNA) and similarly irradiated but photoreactivated RFII molecules, were added to DNA polymerase reaction mixtures at 15°C in the presence of low initiator concentration, and aliquots of reaction products after 6 hr incubation period were sedimented in alkaline CsCl gradients (Fig. 8). The sedimentation profiles of the ^{32}P-labeled product strands of denatured unirradiated RFII and those of denatured UV-irradiated but photoreactivated RFII are very similar; in both cases, half- to full-length complementary strands accounted for about 50% of radioactivity (Figs. 8 A and C). On the other hand, the product strands from denatured irradiated RFII, which had not been photoreactivated, sedimented as a broad band with a peak corresponding to about one-third the size of full-length complementary strands; half to full-length complementary strands accounted for only about 10% of the radioactivity in this case (Fig. 8 B). These results thus indicate that denatured UV-irradiated RFII molecules in which pyrimidine dimers had been previously monomerized by photoreactivation treatment are capable of acting as templates for synthesis of full-length complementary strands—a capacity unattainable if the dimers persist on the templates during the polymerization reaction.

DISCUSSION

We have presented evidence to show that UV-irradiated, circular, single-stranded DNA bears lesions which act as blocks for the DNA polymerase thereby preventing chain elongation of the complementary strands during in vitro polymerization. Our evidence does not exclude the possibility of slow, subsequent reinitiation beyond such blocks. As a result partially double-stranded replicative form (RFII) type products are formed from which smaller than full-length complementary strands can be dissociated by sedimentation in alkaline CsCl gradients.

The mean length of the majority of these newly synthesized strands is about one-third of a full-length complementary strand, when the single-stranded viral DNA template contains 8 lethal hits. If all the lethal hits produced lesions equally effective in blocking polymerization, the length distribution of product strands would peak at one-eighth of full length; such a distribution would give rise to a radioactivity peak in its sedimentation profile at a position corresponding to 1.62×0.125 (Rupp and Howard-Flanders, 1968) i.e. at about one-fifth, instead of, as observed, one-third of the full-length linear ϕX DNA marker (Fig. 2 D). It is likely that more than one type of lesion is produced by UV irradiation of single-stranded DNA and that not all types prevent the formation of complete, full-length complementary strands.

When a population of mature single-stranded DNA virus such as ϕX174 is irradiated extracellularly with a low dose of UV, the genetic material of a part of the irradiated populations would then be expected to contain only nonblocking hits; these, in turn, would not prevent intracellular conversion of the DNA into the

regular double-stranded replicative forms in proper host cells under suitable growth conditions. This expectation has already been shown to be justified by Datta and Poddar (1970) who have demonstrated that the essential precondition for the partial repair in vivo of UV-irradiated bacteriophage ϕX174 is intracellular formation of RF.

It is of course our assumption that this *E. coli* polymerase is involved in the conversion of single-stranded viral DNA to RF (however, cf. Knippers and Strätling, 1970). The properties of other DNA polymerases with respect to UV-induced lesions are unknown.

Two major types of photoproducts, dimers and hydrates of pyrimidines, have been isolated from UV-irradiated DNA. In UV-irradiated single-stranded ϕX DNA, as has already been mentioned, a minority, about one-third, of the photoproducts are thymine dimers (cytosine-containing dimers might increase this fraction slightly). Using calf thymus DNA polymerase and denatured DNA as templates, Bollum and Setlow (1963) have earlier shown that irradiation of denatured DNA inhibits the polymerization reaction in vitro; such inhibition produced by long wavelengths was found to be partially reversed by short wavelength UV irradiation which decreases the equilibrium content of dimers. On the other hand, Grossman (1968) has demonstrated that cytosine hydrates are more readily formed in denatured DNA than in native DNA. It is also possible that some as yet undiscovered photoproducts, or some isomeric dimers (Pearson et al., 1965; Wang and Vargese, 1967) are formed in UV-irradiated single-stranded DNA. It is therefore difficult to be absolutely certain as to which types of photoproducts actually act as the blocks which we observe for DNA polymerase.

Our study of the reaction products, when using denatured RFII molecules as templates, however, strongly suggests that these blocks may be pyrimidine dimers, because of the observed effect of prior photoreactivation on such templates. In spite of some very indirect indication that pyrimidine hydrates may be acted upon by photoreactivation enzyme (Tao et al., 1967), the cyclobutane type of pyrimidine dimers have been demonstrated to be the predominant class of substrates for these enzymes (Setlow, Boling, and Bollum, 1965). The observations that thymine hydrates are unstable under most experimental conditions (Johns et al., 1965) and cytosine hydrates are reversed by heat (Ono et al., 1965) are additional arguments against the possibility that the blocking lesions for DNA polymerase are pyrimidine hydrates.

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