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Involvement of helicase II (*uvrD* gene product) and DNA polymerase I in excision mediated by the uvrABC protein complex

(DNA repair/pyrimidine dimer/"repairosome")

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ABSTRACT **The bimodal-incision nature of the reaction of UV-irradiated DNA catalyzed by the *Escherichia coli* uvrABC protein complex potentially leads to excision of a 12- to 13-nucleotide-long damaged fragment. However, the oligonucleotide fragment containing the UV-induced pyrimidine dimer is not released under nondenaturing *in vitro* reaction conditions. Also, the uvrABC proteins are stably bound to the incised DNA and do not turn over after the incision event. In this communication it is shown that release of the damaged fragment from the parental uvrABC-incised DNA is dependent upon either chelating conditions or the simultaneous addition of the *uvrD* gene product (helicase II) and the *polA* gene product (DNA polymerase I) when polymerization of deoxynucleoside triphosphate substrates is concomitantly catalyzed. The product of this multiprotein-catalyzed series of reactions serves as a substrate for polynucleotide ligase, resulting in the restoration of the integrity of the strands of DNA. The addition of the uvrD protein to the incised DNA–uvrABC complex also results in turnover of the uvrC protein. It is suggested that the repair processes of incision, excision, resynthesis, and ligation are coordinately catalyzed by a complex of proteins in a "repairosome" configuration.**

The removal of ultraviolet light-induced pyrimidine cyclobutane dimers from damaged DNA in bacteria is catalyzed by two essentially different mechanisms. In *Micrococcus luteus* (1–3) and T4 phage-infected *Escherichia coli* (4, 5) the 5′-pyrimidine *N*-glycosylic linkage of the pyrimidine dimer is initially acted upon by a dimer-specific endonuclease. This 17-kDa protein has two activities: A pyrimidine dimer DNA glycosylase activity initially hydrolyzes the *N*-glycosyl linkage of the 5′-pyrimidine moiety of the dimer, generating an apyrimidinic site juxtaposed to a thymine-thymidylate dimer. A 3′-apyrimidinic acid endonuclease activity associated with the same enzyme generates a 3′-hydroxylated apyrimidinic terminus and a 5′-phosphorylated thymine-thymidylate dimer terminus (3). Although these are seemingly different activities associated with the same polypeptide, a common modified Michael addition reaction can be invoked to explain both activities (unpublished data).

The incision reaction in uninfected *E. coli*, which requires the *uvrA*, *uvrB*, and *uvrC* gene products (6–8), results in two endonucleolytic breaks: one break occurs seven nucleotides 5′ to a pyrimidine dimer and a second site of hydrolysis is three to four nucleotides 3′ to the same dimer (9–11). Preliminary evidence suggests a sequential mechanism in which the 5′ break is made first. Although the size of the incised fragment in the DNA suggests that incision may be coordinated with excision reactions, neither fragment release under nondenaturing conditions nor turnover of the uvrABC proteins could be demonstrated (9–11). The implication of these findings is that the mixture for the *in vitro* reaction using purified uvrA, uvrB, and uvrC proteins lacks important components limiting the repair process to uncoupled repair reactions. The uvrABC-incised DNA complex is, therefore, amenable to an examination of those other proteins that may facilitate the excision reaction.

Although the *uvrA*, *uvrB*, and *uvrC* genes are required for incision of damaged DNA *in vivo*, additional gene products have been implicated in the excision-repair process. Those that have been implicated include the products of the *uvrD* (12, 13), *polA* (14, 15), *polC* (15), *recA* (12, 14), *recB* (12, 14), *lexA* (16), and *lig* (17) genes. The last four genes control steps beyond the excision step or are the regulatory genes influencing the expression of the *uvr* system. The gene product of *uvrD* has been identified by a number of laboratories as helicase II (18–22). DNA polymerase I, the product of the *polA* gene, participates in excision repair by virtue of its coordinated polymerase and 5′- to -3′ exonuclease functions (23) as well as its strong affinity for nicked sites on damaged DNA. The involvement of these two proteins in assisting in the release of the damaged fragment and the uvrABC complex from irradiated incised DNA is described in this manuscript.

MATERIALS AND METHODS

Enzymes. The purified uvrA, uvrB, and uvrC proteins were isolated by methods briefly described previously (10). The helicase II (uvrD) from *E. coli* was purified according to already described methods (19, 24) and the *E. coli* DNA polymerase I, the Klenow fragment of DNA polymerase I, and bacterial alkaline phosphatase were purchased from Bethesda Research Laboratories. T4 DNA ligase was obtained from Collaborative Research and the *E. coli* DNA ligase was obtained from New England Biolabs. *E. coli* DNA polymerase III was purified by a modification of a described procedure (25) and was a gift of Jonathan LeBowitz (The Johns Hopkins University). Exonuclease III, T4 DNA polymerase, and T4 polynucleotide kinase were purchased from P-L Biochemicals.

Nicking-Reclosing Assay. The uvrABC endonuclease activity was followed by a nitrocellulose filter binding assay after denaturation and renaturation essentially as described (10) with the exception that the reactions were contained in a 100-μl volume and where indicated 1 μM each dATP, dCTP, dGTP, and dTTP and 5 μM NAD^+ were included in the reaction mixture. The DNA used in the assay was covalently closed circular DNA (ccc DNA), the double-stranded replicative form from bacteriophage fd, tritium labeled to a specific activity of 1.6×10^5 cpm/μg and containing between 0 and 12 pyrimidine dimers per DNA molecule as indicated. Each reaction mixture contained 50 ng of fd [^{3}H]DNA and, where indicated, 880 fmol of uvrA protein, 800 fmol of uvrB protein,

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Abbreviation: ccc DNA, covalently closed circular DNA.

220 fmol of uvrC protein, 200 fmol of uvrD protein, 270 fmol of DNA polymerase I, and 1400 fmol of T4 DNA ligase.

Preparation of ^{32}P-Labeled Substrate DNA. Double-stranded fd DNA was digested with *Bam*HI (New England Biolabs) and labeled by replacement synthesis using T4 DNA polymerase and [α-^{32}P]dATP (3000 Ci/mmol, Amersham; 1 Ci = 37 GBq) and unlabeled deoxynucleoside triphosphates (26). The resulting DNA (specific activity = 10^8 cpm/μg) was further purified by precipitation with ethanol and column chromatography through Bio-Gel A-5m (Bio-Rad).

Electrophoretic Identification of the Excised Oligonucleotide Fragment. ^{32}P-labeled DNA (10 ng) was allowed to react with the uvrABC protein complex under the standard conditions for 30 min at 37°C, followed by precipitation with the addition of 100 μl of 500 mM ammonium acetate/10 mM magnesium acetate/10 μg of tRNA per ml and 0.5 ml of isopropyl alcohol at −70°C for 20 min (27). The pellet after centrifugation was resuspended in water and brought to 50 mM ethylenediaminetetraacetic acid (EDTA). The sample was loaded on a 20% polyacrylamide/7 M urea gel (0.075 × 35 × 50 cm) after addition of an equal volume 98% formamide and heating to 95°C for 5 min. Electrophoresis was carried out for 3 hr at 2000 V. The molecular weight standards used were d(GTTAAC) (*Hpa* I linker), $(dT)_9$, and $(dT)_{10}$ obtained from P-L Biochemicals and were labeled by using polynucleotide kinase and [γ-^{32}P]ATP (Amersham).

Fragment Release Assay. ^{32}P-labeled DNA was allowed to react with uvrABC according to procedures described in the previous section and loaded directly onto a 0.8-ml DEAE-Sephacel (Pharmacia) column previously equilibrated with 0.2 M NaCl/50 mM potassium 4-morpholinepropanesulfonate (Mops), pH 7.6. The column was washed with 1 ml of 0.2 M NaCl/50 mM Mops, pH 7.6, which was found to completely remove mononucleotides from the column. Oligonucleotides were eluted with a 3-ml gradient from 0.2 M to 0.5 M NaCl in 50 mM Mops, pH 7.6, and 300-μl fractions were collected. A $(dT)_{10}$ standard eluted in fraction 4 and high molecular weight DNA remained bound to the column in 0.5 M NaCl (data not shown).

Determination of the Number of Nucleotides Incorporated per Repair Site. The number of nucleotides incorporated per repair site was determined by first allowing 10 ng of ^{3}H-labeled fd cccDNA containing an average of one dimer per molecule to react with uvrA, uvrB, and uvrC proteins under the standard reaction conditions at 37°C for 5 min. An aliquot was removed and the amount of endonuclease activity was determined as above. Helicase II, DNA polymerase I, and T4 DNA ligase were then added along with all four α-^{32}P-labeled deoxynucleoside triphosphates at a final specific activity of 92 Ci/mmol and incubated for an additional 5 min at 37°C. The total amount of incorporation into both nicked and covalently closed molecules was determined by trichloroacetic acid precipitation, while the amount of incorporation into nicked molecules was measured by using the endonuclease assay. From these data the amount of incorporation into the covalently closed molecules resulting from resealing of molecules nicked by the uvrABC endonuclease was calculated. Incubation for longer periods of time did not lead to an increase in UV-dependent nicking or nucleotide incorporation.

RESULTS

Generation of a DNA Fragment as the Result of Double Incision by uvrABC. The generation of the predicted product, as a consequence of the initial two-site incision catalyzed by the uvrABC complex, is shown in Fig. 1. This fragment is released after denaturation of the protein-damaged DNA complex. The 12- to 13-nucleotide-long DNA product predicted from analysis of incision sites on DNA of defined

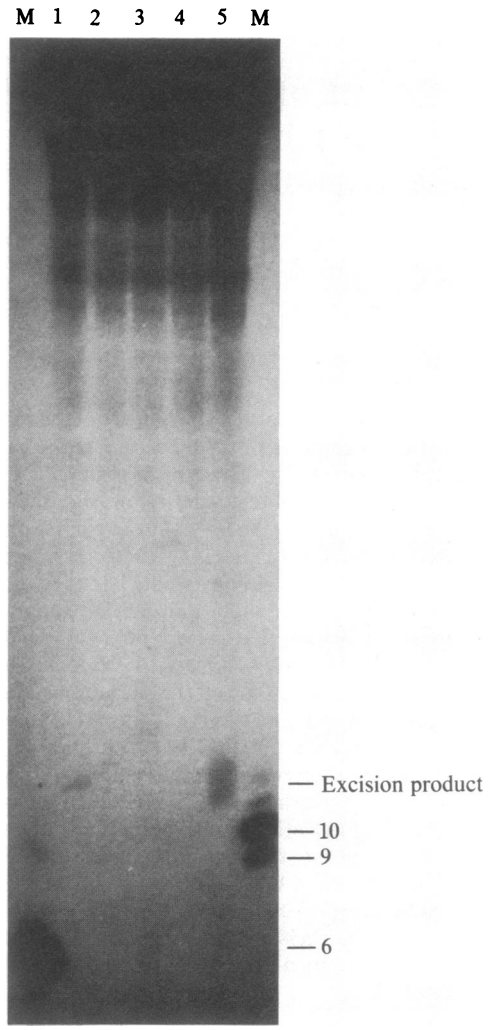

FIG. 1. EDTA-assisted release of fragment generated by uvrABC nuclease on UV-damaged DNA. ^{32}P-labeled DNA was allowed to react with uvrABC, precipitated, treated with 50 mM EDTA, loaded onto a 20% polyacrylamide/7 M urea gel, electrophoresed, and autoradiographed. Lane 1, (no UV) DNA; lane 2, (no UV) DNA + uvrAC proteins; lane 3, (no UV) DNA + uvrABC endonuclease; lane 4, (UV) DNA + uvrAC proteins; lane 5, (UV) DNA + uvrABC endonuclease; lanes M, markers of *Hpa* I linker (6 nucleotides), $(dT)_9$, and $(dT)_{10}$. (UV) DNA samples contained an average of 12 dimers per fd DNA molecule.

sequences (9–11) can be identified on a 20% polyacrylamide gel using generally labeled DNA as a substrate. This fragment is generated only when the uvrA, uvrB, and uvrC proteins are present and when the DNA used as a substrate has been damaged by UV irradiation (lane 5). When the DNA is undamaged or when one of the protein components is withheld from the reaction mixtures, the fragment is not generated (lanes 1–4). This fragment appears polydisperse rather than as two sharp bands because it is composed of a population of many different sequences that have significant sequence-dependent mobility differences in this gel system (28). This fragment can also be end-labeled by using polynucleotide kinase only after alkaline phosphatase treatment (data not shown) and thus must have a phosphorylated 5′ terminus. A free 3′ hydroxyl on the parental DNA strand is inferred because it provides a priming site for DNA polymerase I (see below); similar results were obtained by Sancar and Rupp (9). It should be noted that in Fig. 1 the release of the fragment is due to the denaturing conditions under which the samples are prepared and the gel is run and is independent of the EDTA-stimulated release (see below).

Release of the DNA Fragment by Protein Factors. Identification of the fragment as it is released under nondenaturing conditions is limited to the use of those techniques in which fragments of defined sizes are resolvable under extremely benign conditions such that fragment release when facilitated by other protein factors can be followed. The entire reaction mixture was passed through a DEAE-Sephacel column equilibrated and developed as described in *Materials and Methods*. In Fig. 2 it can be seen that only the simultaneous addition of both uvrD protein and DNA polymerase I resulted in release of an oligonucleotide peak, and the magnitude of this peak was identical to that of the fragment released by EDTA treatment. Neither uvrD alone (Fig. 2) nor DNA polymerase I alone (data not shown) stimulated release of radioactivity after uvrABC incision of damaged DNA. The background peak is seen with the DNA substrate alone and is not the result of UV irradiation or uvrABC action. The net amount of radioactivity associated with an oligomeric species that is released by EDTA and by the combined action of uvrD and polymerase I corresponds to the removal of a 12- to 13-nucleotide oligomer at approximately 25% of the damaged sites.

Requirement of Helicase II (uvrD) for Polymerase I Incorporation of dNTPs. The incorporation of deoxynucleoside triphosphates into an acid-precipitable DNA when catalyzed by DNA polymerase I is dependent on prior incision of the UV-irradiated DNA by the uvrABC endonuclease and on the addition of helicase II (Fig. 3). In the absence of incision due to either the lack of a damaged DNA substrate or the lack of one of the components of the uvrABC endonuclease there is only a minimal stimulation of DNA polymerase I nucleotide incorporation by uvrD into endogenous nicks in the DNA.

Nicking-Reclosing. The ordered sequence of excision repair reactions terminates with the restoration of the integrity of the repaired strands by ligation after incision, excision, and resynthesis. It is to be expected, therefore, that the requirements for ligation should be internally consistent with the helicase II–DNA polymerase I-mediated excision–resynthesis reaction. To determine which protein factors were necessary to complete the repair reaction and restore the DNA to a covalently closed molecule, the nicking-reclosing assay described in *Materials and Methods* was used. It can be seen in Table 1 that the addition of helicase II, DNA polymerase I, and DNA ligase leads to almost complete resealing of the nicked DNA. The addition of DNA ligase alone leads to a small but significant resealing of the incised DNA, suggesting that at least some of the sites are susceptible to ligation reactions. Furthermore, T4 DNA ligase and *E. coli* DNA ligase were found to be completely interchangeable in this assay, whereas DNA polymerase I could not be substituted for by the Klenow fragment, DNA polymerase III holoenzyme, or T4 DNA polymerase.

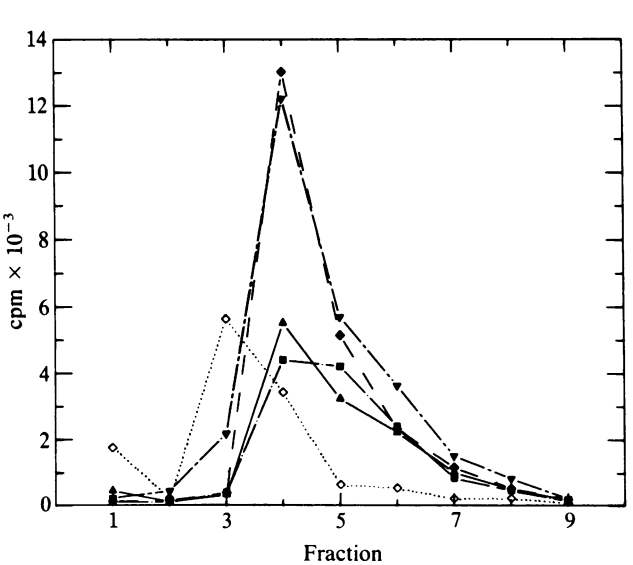

FIG. 2. Helicase II and DNA polymerase I effect on fragment release as detected by DEAE-Sephacel column chromatography. Unirradiated (◇) or UV-irradiated (12 dimers per fd molecule, all other symbols) ^{32}P-labeled DNA (10 ng) was allowed to react with the uvrABC endonuclease at 37°C for 30 min under standard conditions with the addition of the deoxynucleoside triphosphates followed by subsequent treatment at 37°C for 15 min as follows: ▲, no addition; ◇ and ◆, brought to 50 mM EDTA; ■, uvrD protein; ▼, uvrD protein and DNA polymerase I.

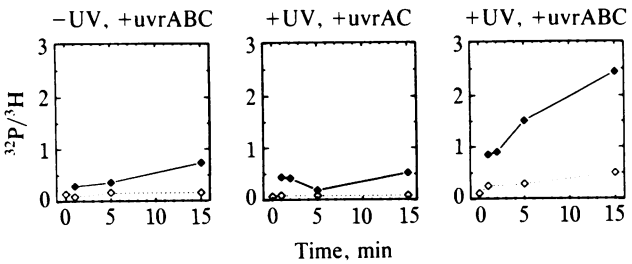

FIG. 3. DNA polymerase I-catalyzed incorporation of dNTPs into trichloroacetic acid-precipitable DNA. ^{3}H-labeled fd DNA (10 ng, 6 dimers per molecule) was allowed to react with uvrABC, then DNA polymerase I was added along with [α-^{32}P]dATP (500 nCi, 3000 Ci/mmol), dGTP, dCTP, dTTP, and where indicated uvrD protein. ◇, No uvrD protein; ◆, 200 pmol of uvrD protein was added.

The incorporation of nucleotides into the DNA as a result of the repair process was determined by quantitating the total number of nucleotides incorporated and the number of sites repaired as shown in Table 2. The net results, 3.02 fmol of dNTP incorporated into 0.24 fmol of ccc DNA resynthesized, indicate incorporation of 12.6 nucleotides per dimer site, in good agreement with the size of the gapped DNA that is expected to result after release of the DNA fragment containing the damaged site.

Turnover of the uvrC Protein. The nicking of UV-irradiated DNA was found to be directly related to the amount of the uvrC protein added in the endonuclease assay under those circumstances in which excess amounts of the proteins uvrA and uvrB were included with the DNA substrate (Fig. 4*A*). The further addition of uvrC protein after the initial reaction had reached completion led to a rapid increase in the nicking of damaged DNA. This catalytic enhancement was not observed in those reactions augmented with either uvrA or uvrB proteins or under prolonged incubation conditions.

Table 1. Completion of repair reaction as determined by the nicking-reclosing assay

Proteins added	ccc DNA formed, %
uvrABC	0.0
uvrABC + uvrD	3.8 ± 10.5
uvrABC + pol I	13.3 ± 11.7
uvrABC + ligase	28.9 ± 12.4
uvrABC + uvrD + pol I	8.5 ± 14.5
uvrABC + uvrD + ligase	7.0 ± 2.0
uvrABC + pol I + ligase	5.1 ± 4.1
uvrABC + uvrD + pol I + ligase	81.4 ± 15.3
uvrABC + uvrD + Klenow + ligase	20.7 ± 9.5
uvrABC + uvrD + pol III + ligase	1.7 ± 4.7
uvrABC + uvrD + T4 pol + ligase	32.1 ± 13.1

All protein components were mixed together at 0°C with ^{3}H-labeled DNA (6 dimers per molecule) then transferred to 37°C for 5 min. The numbers in the table represent mean ± 2σ of at least six determinations. pol, DNA polymerase; Klenow, Klenow fragment of DNA polymerase I.

Table 2. Nucleotide incorporation after uvrABC incision

Proteins added	Nicked DNA, fmol	ccc DNA, fmol
uvrABC	1.12	1.24
uvrABC + uvrD + pol I + ligase	0.85	1.48
Net ccc [^{3}H]DNA resynthesized		0.24
uvrD + pol I + ligase	9.20	1.88
uvrABC + uvrD + pol I + ligase	9.36	4.90
Net [^{32}P]dNTP incorporation into ccc DNA		3.02
12.6 nucleotides incorporated per dimer site		

The DNA contained an average of one pyrimidine dimer per molecule, and 500 nCi of [α-^{32}P]dNTP (92 Ci/mmol) was included. The radioactivity in the nicked molecules was determined from nitrocellulose filter binding after denaturation and renaturation. The radioactivity in the ccc DNA molecules was determined by subtracting the total radioactivity in the nicked DNA species from the amount in the acid-precipitable DNA. The numbers in the table represent means of duplicate determinations. Similar results were obtained when the experiment was repeated several times. Abbreviations as in Table 1.

Addition of the uvrD protein led to an effect similar to the further addition of uvrC protein, suggesting that the uvrC protein was then turning over. Addition of uvrD protein in control reactions in which only the uvrA and uvrB proteins were preincubated with the DNA did not lead to an increase in incision, suggesting that the uvrD preparation contained no uvrC-like activity. Furthermore, those reactions including uvrD protein proceed with linear kinetics, as shown in Fig. 4*B*.

DISCUSSION

The current perception of excision repair mechanisms in *E. coli* and in lower eukaryotes (29, 30) must take into account the large number of genes participating in the incision reaction of DNA containing "bulky adducts" such as pyrimidine dimers. The initial studies by Seeberg and his colleagues (6–8) gave evidence from enzyme complementation studies that three proteins, in the presence of ATP, are required to effectively incise UV-damaged DNA. It was implicit in these and subsequent *in vitro* studies that the *E. coli uvr* system represented an order of molecular complexity not observed for other repair systems. It is now apparent that the uvrA protein in the presence of ATP finds a damaged site on DNA (7, 10) as well as provides a site for the binding of the uvrB protein (31). Catalysis is, however, dependent on the subsequent binding of the uvrC protein to the uvrAB–DNA complex (10, 11).

From protein–DNA binding studies it is known that this complex is stable after catalysis and, unlike a typical enzyme system, protein turnover is not observed (10, 11), suggesting that there is a partial repair reaction in the presence of limiting protein species. The release of the 12- to 13-nucleotide oligomer product of the incision reaction can be demonstrated by protein-denaturing conditions, chelating conditions, or the action of helicase II and DNA polymerase I. When the presence of a protein complex at the damaged site was examined by diagnostic use of *E. coli* exonuclease III sensitivity, using linear damaged DNA substrates, the results indicated protection rendered by the postincision uvrABC complex (data not shown). This protection was partially relieved by helicase II and completely relieved by the combination of helicase II and DNA polymerase I in the presence of deoxynucleoside triphosphates, suggesting that the combined action of helicase II and DNA polymerase I

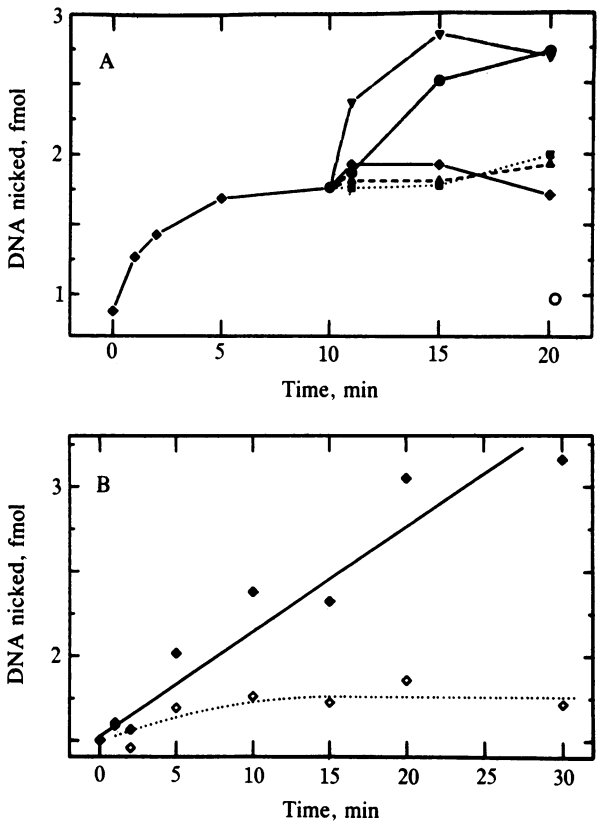

FIG. 4. Turnover of uvrC by helicase II. (*A*) UV-specific endonuclease activity of the purified uvrABC proteins was followed by the nicking assay as described in *Materials and Methods*. Forty femtomoles of fd [^{3}H]DNA containing an average of six pyrimidine dimers per molecule was incubated with 880 fmol of uvrA, 800 fmol of uvrB, and 50 fmol of uvrC proteins (♦), or 880 fmol of uvrA, 800 fmol of uvrB, and 200 fmol of uvrD (○). After 10 min at 37°C, 880 fmol of uvrA (■), 800 fmol of uvrB (▲), 44 fmol of uvrC (▼), or 200 fmol of uvrD protein (●) was added. (*B*) As in *A* except that 80 fmol of fd [^{3}H]DNA was used and only 22 fmol of uvrC protein was added at time 0. ◇, No uvrD; ♦, 200 fmol of uvrD was added at time 0.

facilitated the release of the uvrABC complex. Restoration of the covalently closed double-stranded DNA molecule requires the action of polynucleotide ligase. Furthermore, by using limited amounts of the *uvrC* gene product in the incision reaction, it is possible to demonstrate that the addition of helicase II leads to turnover of the uvrC protein. A similar conclusion was reached from *in vivo* studies in which Ben-Ishai and Sharon found that in *uvrD* mutants incision is saturated at significantly lower fluences than in wild-type strains (32).

This evidence, and the data reported here for *in vitro* studies, leads to the suggestion that in the absence of DNA polymerase I the uvrD protein is capable of interacting with the uvrABC protein complex to carry out a partial reaction in which an intermediate in the excision repair mechanism can accumulate. The *uvrD* gene product is believed to act via protein–protein interactions with the uvrABC complex rather than simply binding at one of the nicks in the DNA created by the incision step, because other studies have shown that uvrD protein requires a 12-nucleotide single-stranded region of DNA to bind (33). DNA polymerase I is also incapable of initiating nucleotide incorporation into this nicked DNA in the absence of uvrD protein, presumably because the nicks are protected by the uvrABC protein complex. The physical association between helicase II and DNA polymerase I has been observed, as well as the inhibition of polymerization by helicase II (unpublished results).

This evidence for a series of multiprotein interactions leads to the proposition of a "repairosome" complex by analogy to other protein complexes such as the primosome involved in DNA replication (34). In the absence of protein architectural studies the suggestion of the repairosome complex is, however, based on indirect evidence, just as the primosome and replisome models are. Because these *in vitro* systems are amenable to complementation experiments it is anticipated that structure–function relationships can be more firmly established.

In addition to the potential role of such a complex facilitating coordinated reactions, the persistence of the uvrABC complex binding to the DNA after incision may also be important *in vivo* in reducing the vulnerability of nicks and single-strand gaps to various nucleases within the cell, thereby protecting the genome until the rest of the repair machinery arrives. It is not believed that the uvrA, uvrB, or uvrC proteins are covalently attached to the DNA after incision because the fragment can be released by treatment with EDTA (this work) or by phenol extraction (9). *uvrD* mutants exhibit normal levels of DNA incision after UV irradiation but are defective in the excision step of DNA repair and in postincision rejoining of repaired regions (35, 36). Neither *uvrD* nor *polA* mutants are as sensitive to UV light as the individual *uvr* mutants, implying alternative pathways for the repair of UV-damaged DNA that can bypass the requirement for helicase II or DNA polymerase I. In addition, a number of other genes have been identified that are implicated in the excision repair pathway but whose *in vitro* roles in the uvrABC reaction have yet to be studied. The ability of other DNA polymerases to carry out excision repair in *polA* mutants may require additional protein factors besides uvrA, uvrB, uvrC, and uvrD. The use of purified proteins makes the excision reaction amenable to further study by reconstitution analysis. For example, exonuclease VII of *E. coli* is capable of excising pyrimidine dimers from UV-irradiated DNA preincised with the T4 or *M. luteus* dimer-specific DNA glycosylase–apyrimidinic acid endonuclease (37). That such an enzyme can participate in "back-up" excision mechanisms *in vivo* is suggested from the increased excision deficiency of *polAex xseA* mutants (37) when compared to single mutants in either of these genes. Whereas incision is dependent on each of the *uvrABC* genes, the excision process has the potential for the action of a number of different genes.

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