Volume 13 Number 5 1985 Nucleic Acids Research

Stimulation of the UvrABC enzyme-catalyzed repair reactions by the UvrD protein (DNA helicase II)

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Received 3 January 1985; Accepted 12 February 1985

ABSTRACT

An in vitro assay system was constructed using highly purified preparations of UvrA, UvrB, UvrC, UvrD proteins and DNA polymerase I, the objective being to analyse the role of UvrD protein in excision repair of UV-induced DNA damage. UvrABC enzyme-initiated repair synthesis was greatly enhanced by the addition of UvrD protein to the reaction mixture. Further analysis revealed that UvrD protein stimulated introduction of strand breaks in irradiated DNA by UvrABC enzyme but had no effect on the DNA polymerase I reaction. Thus, the site of action of UvrD protein is probably at the incision-excision step and not in later steps in excision repair.

INTRODUCTION

Incision is the first step in excision repair of ultraviolet-damaged DNA, and is controlled by at least three genes, uvrA, uvrB and uvrC, in Escherichia coli (1-3). The products of these genes are components of an enzyme that induces strand breaks at both sides of a pyrimidine dimer, thereby releasing a damaged portion of nucleotides from the DNA (4, 5).

The uvrD has been regarded as a gene that is also involved in excision repair (6, 7). Like other uvr mutants, the uvrD mutant is sensitive to UV and is defective in host cell reactivation for UV-irradiated phages. Incision of irradiated DNA occurs normally, but excision and later steps are retarded in the uvrD mutant (8-12). Recently, the uvrD gene has been cloned and the gene product identified as DNA helicase II (DNA-dependent ATPase I) (13-17).

We attempted to elucidate the role of UvrD protein in the excision repair process and we obtained evidence that UvrD

protein stimulates the incision reaction catalyzed by UvrABC enzyme.

MATERIALS AND METHODS

Enzymes

UvrA, UvrB and UvrC proteins were purified from E. coli strain, as described (4, 18). UvrD protein was purified from E. coli W3623(pKO201) as described (16). One unit of activity of UvrD protein was defined as the activity that hydrolyses 1 nmol of ATP in 20 min at 35°C. Specific activity of the UvrD protein preparation used (Fraction V) was 1.7×10^6 units/mg. E. coli DNA polymerase I was purchased from New England Biochemicals Inc., and was checked for the absence of endonuclease activity.

DNA

Closed circular pBR322 DNA was prepared from E. coli HB101(pBR322). After treating growing culture with chloramphenicol (a final concentration, 170 µg/ml), the cells were collected and lysed by lysozyme. Polyethyleneglycol 6000 (a final concentration, 10%) and NaCl (0.5 M) were added to the clear lysate and DNA was precipitated. From the precipitate, closed circular DNA was purified in a CsCl-ethidium bromide density gradient (19).

To prepare nicked DNA, pBR322 DNA (1 µg) was treated with 0.1 µg of DNase I in 50 mM Tris•HCl(pH 7.2)-10 mM $MgCl_2$-2.5 µg bovine serum albumin-15 mM dithiothreitol (total volume, 50 µl) at 37°C for 20 min. The reaction was terminated by adding phenol saturated with Tris-EDTA. After deproteinisation, phenol in aqueous solution was removed by shaking with chloroform-isoamyl-alcohol (24:1 v/v). DNA was precipitated with ethanol and dissolved in water. With this treatment, about 90% of DNA was converted to nicked molecules, as estimated by 1% agarose gel electrophoresis.

UvrABC Enzyme-Initiated Repair Reaction

The reaction mixture contained 50 mM Tris•HCl (pH 7.2), 10 mM $MgCl_2$, 15 mM dithiothreitol, 2 mM dATP, 2 mM dTTP, 2 mM dGTP, 0.033 mM α-[^{32}P]dCTP (1×10^5 c.p.m.), 0.5 µg bovine serum albumin, 100 ng of UV-irradiated, closed circular pBR322 DNA (50 J/m^2), 12.2 ng of UvrA protein, 50 ng UvrB protein, 6.67 ng of

UvrC protein, 0.25 unit of DNA polymerase I and various amounts of UvrD protein, in a total volume of 10 µl. The reaction was performed at 13°C to avoid a snap back reaction (20). The reaction was terminated by the addition of 50 mM EDTA, and then 10 µg of heat-denatured calf thymus DNA and 5% trichloroacetic acid were added successively. The precipitate was washed once with 5% trichloroacetic acid and then dissolved in 1 M ammonia. Radioactivity in both acid-soluble and acid-precipitable fractions was determined in a low-background gas-flow counter.

Nick Translation

Nicked pBR322 DNA was incubated with 0.25 unit of DNA polymerase I and various amounts of UvrD protein in the same reaction mixture used for UvrABC enzyme-initiated repair reaction except that irradiated pBR322 DNA and UvrABC proteins were omitted. All other conditions and procedures were the same as described above.

Incision Reaction

The reaction mixture (10 µl) contained 50 mM Tris•HCl (pH 7.2), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 100 ng of closed circular pBR322 DNA (non-irradiated or irradiated at 50 J/m^2), 0.5 µg of bovine serum albumin, 12.2 ng of UvrA protein, 50 ng of UvrB protein, 6.67 ng of UvrC protein and various amounts of UvrD protein. The mixture was incubated at 37°C for 20 min and then chilled. After addition of EDTA and NaCl, DNA was precipitated with ethanol. The precipitate was dissolved in the xylenecyanol-bromophenol blue-glycerol and electrophoresed in a 1% agarose gel. The gels were photographed with a short wave UV lamp and the film was scanned using a densitometer.

Polyacrylamide Gel Analysis of Products of the Incision Reaction

The reaction mixture (5 µl) was the same as that for the incision reaction except that pBR322 DNA (25 µg) was labeled with $\alpha[^{32}P]$-dCTP and irradiated with 500 J/m^2 of UV, and 14.4 ng of UvrA protein, 100 ng of UvrB protein, 13.3 ng of UvrC protein were used. After incubation at 37°C for 20 min, the reaction was terminated by adding EDTA. Part of the preparation was mixed in an equal volume of formamide-xylencyanol-bromophenol blue, heated at 90°C for 3 min, then loaded on a 20% polyacrylamide gel containing 8M urea, as described by Maxam and Gilbert (21).

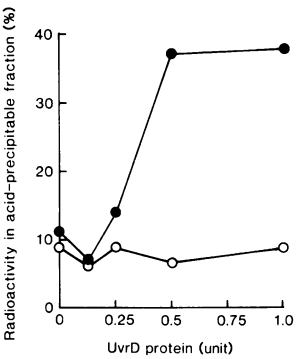

Fig. 1. Effect of UvrD protein on UvrABC enzyme-initiated repair synthesis. UV-irradiated pBR322 DNA was incubated with or without UvrABC enzyme in the presence of DNA polymerase I and various amounts of UvrD protein, under the conditions described in Materials and Methods. The reaction was terminated at 6 hr of incubation. ●, with UvrABC enzyme; o, without UvrABC enzyme.

Electrophoresis was carried out at 2000 V for 2h, and the gel was fluorographed at -80°C.

RESULTS

Stimulation of Repair Synthesis by UvrD Protein

Figure 1 shows effects of UvrD protein on UvrABC enzyme-initiated repair synthesis. Rate of incorporation of labeled nucleotides into nicked or gapped sites, produced by UvrABC enzyme in UV-irradiated DNA, increased with increasing concentrations of UvrD protein. The rate was maximum at 0.5 unit/assay mixture (10 µl) and then leveled off. This amount of UvrD protein corresponds to 0.1 molecule per molecule of DNA. No such distinct effect of UvrD protein was observed when UvrABC enzyme was not present in the reaction mixture. Similarly, UvrD protein showed no effect on the incorporation when the substrate DNA was non-irradiated, even in the presence of UvrABC enzyme (data not shown).

Figure 2 shows the time-course of the reaction in the presence and absence of UvrD protein. The rate of reaction in the presence of UvrD protein (0.5 unit/10 µl) was about 3 times higher than that of the control sample not containing UvrD protein. The reaction proceeded linearly until about 70% of

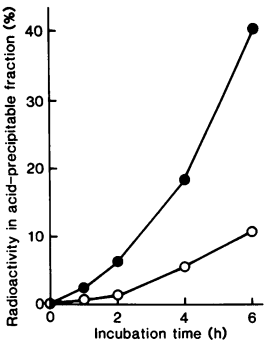

Fig. 2. DNA repair synthesis in the presence or absence of UvrD protein. UV-irradiated pBR322 DNA was incubated with or without UvrD protein in the presence of UvrABC enzyme and DNA polymerase I, under the conditions described in Materials and Methods. o, without UvrD protein (control); ●, with 1.0 unit of UvrD protein.

labeled nucleotides had been incorporated.

Effect of UvrD Protein on DNA Polymerase I Reaction

The repair synthesis which we measured above is composed of two steps; an incision-excision reaction catalyzed by UvrABC enzyme and a nick translation reaction by DNA polymerase I. Thus, the stimulation of the repair synthesis may be caused by stimulation of either one or both of the reactions.

For elucidation, we examined the effect of UvrD protein on the nick translation reaction. pBR322 DNA was briefly treated with DNase I to produce a few nicks per DNA molecule and nick translation reaction by DNA polymerase I was performed in the presence of various amounts of UvrD protein. Figure 3 shows that the rate of incorporation of nucleotides by DNA polymerase I was not significantly affected by the addition of UvrD protein (up to 1 unit/10 µl). A similar experiment was performed with higher concentrations of UvrD protein (2 and 4 units/10 µl) and no distinct enhancement was observed (data not shown). Thus, DNA polymerase I reaction *per se* does not seem to be affected by UvrD protein.

Stimulation of Incision by UvrD Protein

We next investigated whether UvrD protein stimulates incision of UV-irradiated DNA by UvrABC enzyme. Supercoiled pBR322 DNA was irradiated with UV, treated with UvrABC enzyme in

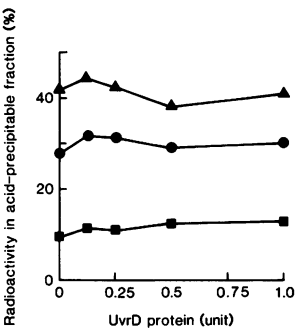

Fig. 3. Effect of UvrD protein on incorporation of nucleotides by nick translation. pBR322 DNA treated with DNase I was incubated with DNA polymerase I and various amounts of UvrD protein. The reaction was terminated at 1 hr (■), 4 hr (●) and 6 hr (▲).

the presence of various amounts of UvrD protein, and ratio of nicked DNA to supercoiled DNA was determined by electrophoresis on agarose gel. The result is shown in Figure 4. With increasing amounts of UvrD protein, the rate of conversion of supercoiled DNA to nicked DNA increased, and the increase leveled off at 0.5 unit/10 µl of UvrD protein added. In the absence of

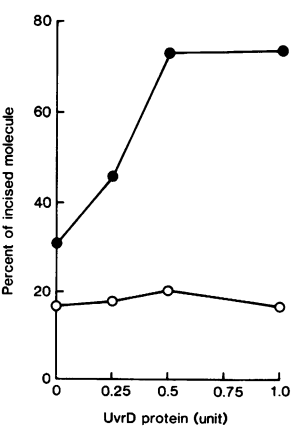

Fig. 4. Stimulation of UvrABC enzyme-catalyzed incision reaction by UvrD protein. UV-irradiated DNA was incubated with or without UvrABC enzyme in the presence of various amounts of UvrD protein, under the conditions described in Materials and Methods. The reaction was performed at 37°C for 20 min. o, without UvrABC enzyme; ●, with UvrABC enzyme.

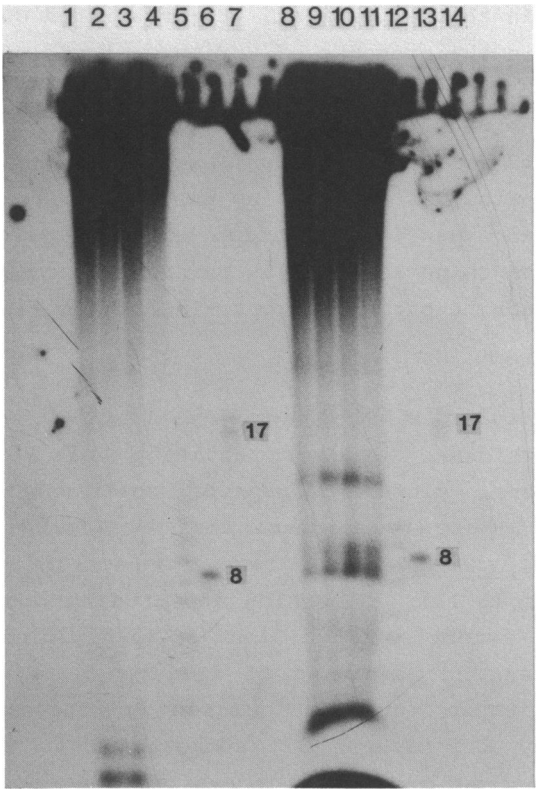

Fig. 5. Polyacrylamide gel analysis of incision products. ^{32}P-labeled pBR322 DNA was incubated with or without UvrABC enzyme in the presence of various amount of UvrD protein. The samples were heated at 90°C for 3 min in the presence of 45% formamide, then applied to a 20% polyacrylamide gel. Lanes 1-4, without UvrABC enzyme, lanes 8-11, with UvrABC enzyme. The following amounts of UvrD protein were added to the reaction mixture: lanes 1 and 8, 0 unit; lanes 2 and 9, 0,5 unit; lanes 3 and 10, 1.0 unit; lanes 4 and 11, 1.5 units. Lanes 5-6 and 12-14 are markers; lanes 5 and 12, poly(dT) ladder; lanes 6 and 13, 8-bases oligomer; lanes 7 and 14, 17-bases oligomer.

UvrABC enzyme, only a small amount of DNA was converted to nicked molecules and no stimulation with UvrD protein was observed. It is to be noted that this pattern of stimulation is similar to that observed for UvrABC enzyme-initiated repair synthesis, and that in both cases the saturation occurred at almost the same level (0.5 unit/10 µl) of UvrD protein.

The result shown in Figure 5 further confirms the above

conclusion. In this experiment, UV-irradiated DNA treated with UvrABC enzyme in the presence of various amounts of UvrD protein, was denatured and subjected to polyacrylamide gel electrophoresis. As expected from the mode of action of UvrABC enzyme (4, 5, 18), 12 to 13-nucleotides length fragments were produced and this production was enhanced by the addition of UvrD protein. There was an accompanying formation of smaller fragments (6 to 8-nucleotide length nucleotides), perhaps due to cleavage of the excised fragments at pyrimidine-cytosine lesion site.

DISCUSSION

Excision repair of UV-damaged DNA involves incision at the site of pyrimidine dimers, excision of oligonucleotides containing dimers, closure of gaps by repair synthesis and final joining (3). There is evidence that *uvrD* mutants, sometimes referred to as *uvrE* and *recL*, have a defect in some step(s) in excision repair (8-11). Incision appeared to proceed normally, but rate and extent of excision of pyrimidine dimers were markedly reduced and rejoining of incision breaks was slow and incomplete in these mutants. Based on these *in vivo* data Kuemmerle, Ley and Masker (12) suggested that the *uvrD*$^+$ gene product acts during or immediately after the incision event to modulate the structure or conformation of the DNA molecule being repaired.

It has been shown that extracts prepared from wild type strains of *E. coli* are able to repair single-strand breaks that were introduced into UV-irradiated DNA but that this restoration of the damaged and incised DNA depends on a functional *uvrD*$^+$ gene product (22). Thus, the *in vitro* situation is similar to the inefficiency of DNA strand break restoration seen with *uvrD* mutants *in vivo*.

To obtain further insight into the function of UvrD protein, we constructed an *in vitro* system, consisting of purified preparations of UvrABC enzyme, DNA polymerase I and UvrD protein. Using this system we found that UvrABC enzyme-initiated repair synthesis is greatly enhanced by the addition of UvrD protein. Further analysis revealed that this enhancement is caused by stimulation of the UvrABC enzyme-catalyzed reaction but not the

DNA polymerase I reaction.

With regard to the molecular mechanism(s) involved in stimulation of UvrABC-catalyzed incision reaction, we assume that UvrD protein interacts with a UvrABC enzyme complex to promote the incision reaction, per se. It is also possible that UvrD protein promotes the release of a UvrABC enzyme complex which is otherwise bound to the DNA molecule or the release of a oligonucleotide fragment to which the enzyme complex is bound. In such cases, accelerated rate of release would lead to a higher turnover of the enzyme. Evidence has been obtained that UvrD protein greatly promotes the release of damaged oligonucleotides from the DNA (E. Seeberg et al., unpublished), providing an experimental basis for the last possibility. However, since these possibilities are not mutually exclusive, the other mechanisms presented above are still possible.

ACKNOWLEDGMENTS

We thank M. Ohara (Kyushu University) for comments on the manuscript. This work was supported by Scientific Research and Cancer Research Grants from the Ministry of Education, Science, and Culture of Japan.

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