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Incision of damaged versus nondamaged DNA by the *Escherichia coli* UvrABC proteins

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ABSTRACT

Incision of damaged DNA by the *Escherichia coli* UvrABC endonuclease requires the UvrA, UvrB, and UvrC proteins as well as ATP hydrolysis. This incision reaction can be divided into three steps: site recognition, preincision complex formation, and incision. UvrAB is able to execute the first two steps in the reaction while the addition of UvrC is required for the incision of DNA. This incision reaction does not require ATP hydrolysis and results in the formation of a tight UvrABC post-incision complex and the generation of an oligomer of approximately 12 nucleotides. At high UvrABC concentrations the specificity of the incision for damaged DNA is decreased and significant incision of undamaged DNA occurs. Analogous to damage specific incision, this type of incision leads to generation of an oligonucleotide, but in this case the size is approximately 9 nucleotides in length. Further evidence shows that the combination of UvrB and UvrC proteins can generate a significant amount of a similar size product on undamaged DNA. In addition, the UvrC protein alone can generate a small amount of the same product. Immunological characterization of the weak nuclease activity seen with UvrC indicates that the activity is very tightly associated with the purified UvrC protein.

INTRODUCTION

UvrABC catalyzed incision of damaged DNA requires the *E. coli* UvrA, UvrB, and UvrC proteins as well as ATP hydrolysis. The role(s) of each of these proteins in the incision reaction are only beginning to be elucidated. UvrA has been identified as an ATPase (1) and is capable of recognizing DNA damage, possibly by stabilization of a UvrA dimer at the site of damage by local unwinding of the DNA double helix (2). UvrB has recently also been identified as an ATPase whose cryptic ATPase activity is expressed only in the presence of UvrA and DNA (3). The UvrAB proteins are capable of displacing short oligonucleotides from single-stranded DNA in an ATP dependent reaction (4). In addition, the UvrAB proteins can bind tightly to damaged DNA in the presence of ATP (5-7). The formation of a UvrAB complex at a damaged DNA site seems to be the rate limiting step in the overall incision reaction, because the addition of UvrC to this preformed complex leads to very rapid incision of the DNA (5). This reaction results in two endonucleolytic breaks: one 5' to the damage site and one 3' to the damage site (6,8). The sites of incision and the spacing between breaks is essentially invariant irrespective of the type of DNA damage and always results in the generation of a 12 to 13 nucleotide product (9-13).

The UvrA, UvrB, and UvrC proteins do not turn over *in vitro* without the addition of helicase II (UvrD) and DNA polymerase I (14-16). In particular, the number of incision events is directly proportional to the amount of UvrC added (5). The exact role of UvrC in this reaction is unclear, but two simple models may explain its action: a. UvrC may change the properties of the UvrA and/or UvrB to activate their inherent nuclease activities or b. UvrC may itself encode the nuclease which can only bind to DNA through its interaction with UvrAB. A prediction of either of these models is that it may be possible to examine incision due to formation of partial UvrABC complexes.

MATERIALS AND METHODS

Enzymes

UvrA, UvrB and UvrC proteins were purified essentially as described (17) and were all >95% pure as determined by Coomassie Blue staining of SDS polyacrylamide gels. EcoRI was obtained from New England Biolabs and T4 DNA polymerase from Pharmacia.

Endonuclease Assay

The endonuclease activity was determined using a nitrocellulose filter binding assay after denaturation and renaturation as previously described (5) . The substrate used in these experiments was [^{3}H]thymidine-labeled pPYC3 DNA (10.4 kb) (6) . The reaction buffer was 85 mM KCl, 40 mM morpholinopropanesulfonate (MOPS), pH 7.6, 2 mM EDTA, 15 mM MgCl, 2 mM ATP (Pharmacia) and all reactions were carried out at 37^o in a volume of 50 μl. ATP-γ-S was obtained from Boehringer Mannheim and was used at a concentration of 0.1 mM where indicated.

Isolation of nucleoprotein complexes

UvrAB-DNA complexes were formed by incubating 800 fmol UvrA, 800 fmol UvrB, and 10 fmol [^{3}H]DNA containing an average of 6 pyrimidine dimers per DNA molecule at 37^o for 10 min in a total volume of 100 μl with 2 mM ATP or 0.1 mM ATP-γ-S included where indicated. Stable UvrAB-DNA complexes were isolated by gel filtration chromatography on 1.5 ml Bio-Gel A15m (Bio-Rad) columns previously equilibrated with reaction buffer lacking nucleotides. The column was eluted with reaction buffer and fractions of 50 μl were collected. The leading edge of the DNA peak eluting at the void volume was used for subsequent endonuclease assays.

UvrABC-DNA complexes were formed with excess UvrA and UvrB and limiting UvrC by incubating 10 fmol of UV irradiated [^{3}H]DNA containing an average of 6 dimers per DNA molecule with 1.8 nmol UvrA, 2.4 nmol UvrB and 20 fmol UvrC at 37^o for 10 min in reaction buffer plus 2 mM ATP. The unassociated UvrC was separated from the UvrABC-DNA complex by gel filtration as above except that the column buffer contained 2 mM ATP. Additional UvrA and UvrB were added to the isolated UvrABC-DNA to ensure that these proteins were still in excess.

Preparation of [^{32}P]-Labeled Substrate DNA

EcoRI digested pPYC3 DNA was labeled by replacement synthesis using T4 DNA polymerase and [α-^{32}P]dATP (3000 Ci/mmol, ICN) and unlabeled deoxynucleoside triphosphates (18). The resulting DNA was further purified by precipitation with ethanol and column chromatography through Bio-Gel A-15m (Bio-Rad). The DNA was used within two hours of initiation of the labeling reaction.

Oligonucleotide Product Analysis

UvrABC incised [^{32}P]DNA was boiled for 5 minutes to quench the reaction and to release any bound product. Then the reaction was loaded onto 5% alkaline sucrose (0.3 M NaOH, 1 M NaCl) and centrifuged at 150,000 X G for 16 hours at 4^{o}. The uppermost 200 μl after centrifugation, containing the oligonucleotide product, was then loaded onto a 0.5 M long, 0.75 mm thick 20% or 50% polyacrylamide/urea gel (30:1 acrylamide/bis acrylamide, 7 M urea).

Oligonucleotide composition

The composition of the oligonucleotide was determined by preparation of DNA labeled to the same specific activity with either [α-^{32}P]dATP, [α-^{32}P]TTP, [α-^{32}P]dCTP, or [α-^{32}P]dGTP (3000 Ci/mmol). The amount of radioactivity in the oligonucleotide product was then calculated for each substrate preparation. Alternatively, DNA was prepared by labeling with all four labeled deoxynucleoside triphosphates and the product oligonucleotide eluted from the polyacrylamide gel was analyzed by digestion with Bal31 nuclease and separation of the nucleotides by thin layer chromatography (19).

Monoclonal antibody formation

Monoclonal antibodies to purified UvrA, UvrB and UvrC were produced after intraperitoneal and subcutaneous immunization of BALB C/J mice with 100 μg of protein per mouse in complete Freund's Adjuvant followed by two intravenous boosts of 50 μg protein on days 28 and 30. Spleens were removed on day 32 and fused to SP2/0 myeloma cells by a modification of the procedure of (20). Wells positive for growth were screened for antibody production using RIA and wells positive for antibodies against the immunized protein were cloned by dilution to obtain individual cell lines.

Monoclonal antibody purification

Antibodies from cell lines which only recognized determinants on the antigen against which they were immunized were purified from 500 ml cultures by first growing to a density of 10^6 cells/ml in RPMI (Gibco) + 10% fetal calf serum then centrifuging the cells and replacing the media with RPMI lacking fetal calf serum for 1 day. The antibodies were purified by ammonium sulfate fractionation followed by DEAE column chromatography and analyzed by SDS-PAGE.

Monoclonal antibody column

Purified monoclonal antibody from cell line C9.1 was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia) in 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3

by incubation at 4° for 16 hours. The gel was transferred to 1 M ethanolamine for 2 hours at 25° then washed with 0.1 M acetate buffer pH 4 and equilibrated with phosphate buffer (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.6).

Immunoprecipitation

UvrC antigen was bound to antibody in solution in phosphate buffer at 37° for 30 min. Then fixed *Staphylococcus aureus* cells (Miles) which were washed twice with phosphate buffer were added and incubated at 37° for 15 min with occasional mixing. The cells and any bound antibody-antigen complexes were removed by centrifugation at 15,000 X G for 5 min. The supernatant, containing any protein not bound by the added antibody, was analyzed for endonuclease activity. The nuclease activity from washed cells was negligible.

RESULTS

ATP requirement

The *in vitro* reconstituted UvrA, UvrB, and UvrC proteins have been shown to lead to selective endonucleolytic cleavage of damaged DNA. The overall reaction requires the presence of all three proteins and ATP hydrolysis. This reaction can be broken down into two major steps: the recognition of the damage by UvrAB and DNA incision after the addition of UvrC (5).

It has previously been shown that the formation of a stable UvrAB complex requires ATP hydrolysis (5). In order to investigate whether the final incision reaction required nucleoside triphosphate hydrolysis, UvrAB-DNA complexes were formed in the presence of ATP and then the free ATP was removed by gel filtration chromatography. UvrC was then added to the isolated UvrAB nucleoprotein complexes in the absence of nucleotide, in the presence of ATP or ATP-γ-S. Incision of the DNA was seen in all cases (Table 1). The increased incision seen when ATP was omitted or replaced with ATP-γ-S in the second stage of the reaction may be due to stabilization of the active UvrAB complex on DNA as determined by nitrocellulose filter

	Nicks/molecule Stage 2		
Stage 1	none	ATP	ATP-δ-S
none	0.03	0.05	0.10
ATP	0.75	0.50	1.03
ATP-δ-S	0.01	0.16	0.00

Table 1. ATP requirement for UvrABC incision. UvrAB complexes were formed (see Methods) either with no nucleotide, ATP, or ATP-γ-S, then the free nucleotides and unbound proteins were removed by gel filtration. UvrC was then added either with no nucleotide, ATP, or ATP-γ-S and DNA incision was measured by the denaturation/renaturation assay.

binding (data not shown). No incision was seen if the UvrAB complex was formed in the absence of a hydrolyzable nucleotide. Thus, the formation of the UvrAB complex requires nucleoside triphosphate hydrolysis but the incision reaction does not.

UvrC is part of the UvrABC complex

It has been implied that UvrC is part of a stable post-incision UvrABC-DNA protein complex and that UvrC can turn over after the action of UvrD (14). In order to demonstrate that the additional incision reaction seen after UvrD action is due to the actual turn over of UvrC, UvrABC post-incision complexes were isolated by gel filtration chromatography (Fig. 1). Addition of UvrD led to incision of additional DNA molecules only when UvrC was present in the original reaction along with UvrA and UvrB, thereby demonstrating that UvrC must have been part of the post-incision complex and that it can turn over and act on other DNA molecules when pulsed with UvrD protein. UvrC alone did not bind to DNA in the absence of UvrA and UvrB under these conditions.

UvrABC incision of undamaged DNA

The selectivity of damaged versus non-damaged DNA is dependent upon the concentrations of the enzyme subunits and on the concentrations of the substrate DNA. With concentrations of enzymes greater than 50 nM the combination of UvrA, UvrB, UvrC and ATP can lead to incision of nondamaged DNA when measured by nitrocellulose filter binding following denaturation/renaturation (Fig. 2). A unique characteristic of damaged DNA incision by UvrABC is the generation of an oligonucleotide fragment of 12-13 nucleotides which results

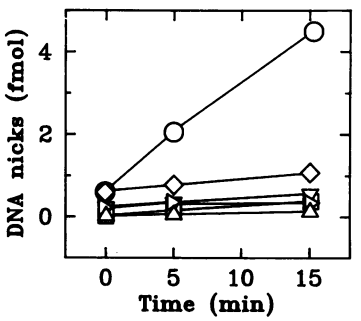

Figure 1. Turn over of UvrC bound to UvrABC complex. Protein-DNA complexes with UvrA and UvrB and limiting UvrC where indicated were isolated by filtration as previously described. Additional 800 fmol UvrA, 800 fmol UvrB and 400 fmol UvrD were added to the isolated protein-DNA complexes in second stage of the reaction.

	Stage 1	Stage 2
□	UvrA + UvrB	UvrA + UvrB
△	UvrA + UvrB	UvrA + UvrB + UvrD
◇	UvrA + UvrB + UvrC	UvrA + UvrB
○	UvrA + UvrB + UvrC	UvrA + UvrB + UvrD
▽	UvrC	UvrA + UvrB
▷	UvrC	UvrA + UvrB + UvrD

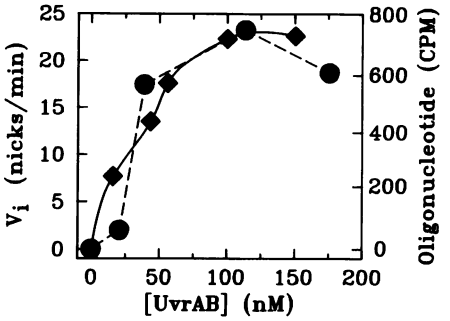

Figure 2. UvrAB concentration dependency of undamaged DNA incision. DNA incision of nondamaged DNA was assayed with 50 nM UvrC and RFI [^{3}H]-pPYC3 DNA in the denaturation/renaturation assay (◆) and linear [^{32}P]-pPYC3 DNA in the oligonucleotide formation assay (●).

from incision on both the 5' and 3' sites of the damaged nucleotide(s). In order to verify that the non-specific DNA incision is related to the UvrABC system, and not to some minor contaminant of the proteins which becomes significant at high concentrations, the product of this incision was analyzed on a 50% acrylamide gel (Fig. 3). The product generated by incision of non-damaged DNA also migrates as an oligonucleotide but its mobility is consistent with an oligomer 9 nucleotides in length. This same product can be seen in reactions with damaged DNA in previously published figures, but was ignored at the time (16). Such a product is likely to have arisen from undamaged regions of the substrate DNA. The dependence of the formation of this oligonucleotide product on the concentration of UvrAB follows the dependence of DNA nicking as measured previously. Thus, the nuclease activity appears to be related to nuclease activity by the UvrABC proteins. The nucleotide composition of the oligonucleotide was determined to be 12% G, 17% C, 31% T, 40% A.

Protein requirement for oligonucleotide generation from undamaged DNA

The oligonucleotide product was not formed in the presence of UvrA, UvrB, or UvrAB in the absence of UvrC (Fig. 4). The most active endonucleolytic activity was seen when UvrA, UvrB and UvrC were present; however, a significant stimulation in product formation was seen when UvrBC was assayed. Oligonucleotide formation was completely dependent on the presence of ATP. No product was formed in the absence of ATP or in the presence of ATP-γ-S. High levels of UvrC alone (90 nM) led to observation of a small amount of endonuclease activity which resulted in the formation of an oligonucleotide product which migrates as described above. The addition of UvrA and/or ATP did not have any significant effect on the level of product formed. In addition, there was no specificity for damaged DNA when incision was measured with UvrAC, UvrBC, or UvrC alone. Two possible explanations for this DNA damage independent nuclease activity are: a) UvrC has an inherent, but weak,

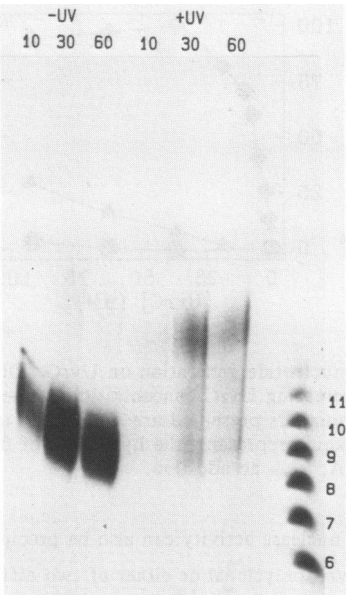

Figure 3. Product formation on damaged versus nondamaged DNA. UvrA (200 nM), UvrB (200 nM), UvrC (50 nM) were incubated with [^{32}P]-pPYC3 DNA for various lengths of times. The oligonucleotide products were analyzed by 50% polyacrylamide gel electrophoresis following sucrose gradient fractionation. Lanes 1-3 nonirradiated DNA, Lane 4-6 DNA irradiated at 1000 J/m^2. UvrABC reaction time in minutes is indicated above the lanes. Markers in the last lane are from oligo d(T).

nuclease activity stimulated by UvrAB, or b) a minor contaminant in the UvrC protein preparation is an endonuclease which generates an oligomer which happens to be the same size as that generated by UvrABC on nondamaged DNA.

Comparison of physical properties of the UvrC nuclease and the UvrABC nuclease

Attempts to affect the weak UvrC nuclease activity by partial thermal denaturation while maintaining the ability of UvrC to stimulate the normal UvrABC incision of damaged DNA were unsuccessful. Both UvrAB dependent and UvrAB independent endonuclease activities are inactivated within 1 min at 65^o or above and both activities are decreased to about 50% after 1 hour at 53^o.

Monoclonal antibodies to UvrC

In order to determine if the UvrC protein itself or a contaminant is responsible for the UvrAB independent nuclease activity, the purified UvrC was passed through a monoclonal antibody column made with a monoclonal antibody against UvrC. As seen in Figure 5, the UvrAB dependent and independent nuclease activities coeluted.

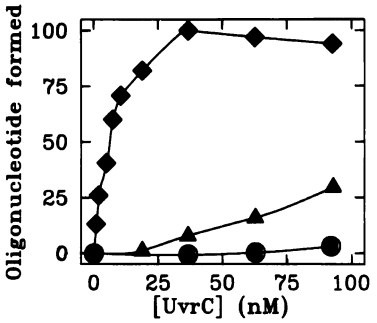

Figure 4. Dependence of oligonucleotide generation on UvrC. Oligonucleotide formation was quantitated as a function of increasing UvrC concentration in the presence of UvrA (300 nM) and/or UvrB (300 nM). The numbers presented are normalized to the maximum amount of product formed with UvrAB which represents the hydrolysis of 0.2% of the input DNA. ◆ - UvrAB, ▲ - UvrB, ■ - UvrA, ○ - no addition.

The UvrAB independent nuclease activity can also be precipitated from solution by protein A cells when either UvrC polyclonal or either of two different monoclonal antibodies against UvrC is used (Table 2). A monoclonal antibody made against UvrA (A22.1) does not lead to precipitation of the nuclease.

DISCUSSION

The UvrABC incision of DNA can be divided into two major steps: i) the localization of the damage and formation of a UvrAB-preincision DNA complex and ii) the dual incision of

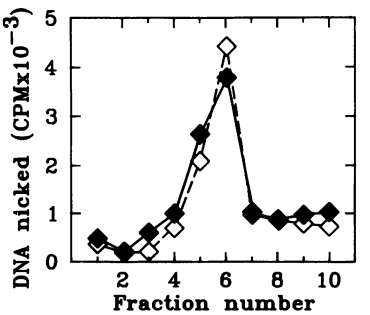

Figure 5. Monoclonal antibody affinity chromatography. UvrC (5 ug) was loaded onto a 2 ml C9.1 monoclonal antibody column. The column was washed extensively with 150 mM NaCl, 100 mM $NaPO_4$, pH7.6, then the UvrC was eluted with 500 mM NaCl in phosphate buffer and 100 μl fractions were collected. Endonuclease activity was measured by nitrocellulose filter binding after denaturation/renaturation using 10 fmol [^{3}H]-pPYC3 DNA. UvrAB dependent nuclease activity (◆) was determined by assaying 1 μl of each fraction with 50 nM UvrAB and DNA containing an average of 4 pyrimidine dimers per molecule. UvrAB independent nuclease activity (◇) was assayed using 50 μl of each fraction.

antibody	activity soluble
none	100
UvrC polyclonal	7.7
C9.1 monoclonal	4.4
C11.3 monoclonal	0.0
A22.1 monoclonal	96.3

Table 2. Immunoprecipitation of UvrC. UvrC (0.5 μg) was incubated with various antibodies which were subsequently precipitated using *S. aureus* cells. The percent of UvrAB independent nuclease remaining in the supernatant fractions was determined by the denaturation/renaturation assay using [^{3}H]-pPYC3 DNA. UvrC polyclonal is a rabbit polyclonal antibody raised against UvrC. C9.1 and C11.3 are two different monoclonal antibodies against UvrC. A22.1 is a monoclonal antibody which recognizes UvrA.

DNA after addition of UvrC. ATP hydrolysis is required in the reaction for the formation of the UvrAB-preincision complex. Removal of the ATP or replacement with ATP-γ-S after formation of this complex does not inhibit incision after UvrC addition.

UvrC binds to UvrAB and becomes part of a stable post-incision DNA complex which does not turn over. The addition of UvrD to this UvrABC post-incision complex leads to the release of UvrC from the complex and allows this protein to incise at other sites.

The ability of the UvrABC proteins from *E. coli* to recognize a wide variety of damaged DNA structures may result from relatively poor discrimination between damaged and undamaged DNA. At high concentrations of DNA or UvrAB, nondamaged as well as damaged DNA can be incised. UvrA-DNA binding studies indicate that there is only a 10^3-10^4 difference in the equilibrium binding constants between damaged DNA and nondamaged DNA (7,S. Mazur and L. Grossman, unpublished observations). Given the large excess of nondamaged sites versus damaged sites in the cell, this discrimination factor is rather poor. If these *in vitro* reactions mimic the reactions catalyzed by UvrABC *in vivo*, then tight regulation of the intracellular UvrABC protein concentrations is predicted to be crucial in order to avoid incision of nondamaged DNA.

The incision of nondamaged DNA requires the addition of UvrC and leads to the generation of an oligonucleotide which migrates as a nanomer on denaturing polyacrylamide gels. Low concentrations of a similar product is formed by the UvrC protein in the absence of UvrA and UvrB. Hence the UvrC preparation used in these studies represents a nuclease specifically stimulated by UvrA and UvrB leading to production of a nanomer. Nondamaged DNA incision was significantly stimulated by the addition of UvrB to UvrC which suggests a physical interaction between UvrB and UvrC. This may be similar to the copurification properties of UvrB and UvrC previously seen by Seeberg (21).

Confirmation that the nuclease activity seen with UvrC preparations was indeed due to the UvrC protein itself came from the similarity in physical properties of the UvrAB

dependent and UvrAB independent nuclease activities. In addition, both activities are bound by monoclonal antibodies directed against UvrC.

The evidence presented demonstrates that UvrC nuclease activity can effectively be turned off in the absence of UvrAB. Only with the high concentrations used here can this activity be detected in the absence of UvrAB. Thus, the multiprotein requirement for incision of damaged DNA by the UvrABC system *in vivo* reflects the subdivision of the task for damage localization, assembly of the complex, preparation of the site, and then the addition of the nuclease which normally acts only at preformed complexes. These observations are consistent with the DNAse I footprinting data of the UvrAB and UvrABC protein complexes with psoralen modified DNA in which a DNAse I hypersensitive site is generated by UvrAB near the expected UvrABC incision site (7). It is possible that in certain circumstances, the lack of a requirement for UvrC in UvrAB damage specific incision is due to the ability of other nucleases in the extract to substitute for UvrC and selectively incise at this hypersensitive site (22-24).

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Abbreviations ATP-γ-S adenosine-5'-O-(3-thiotriphosphate)
MOPS morpholinopropanesulfonate

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