Two Forms of UvrC Protein with Different Double-Stranded DNA Binding Affinities

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The abbreviations used are: ds, double-stranded; ss, single-stranded; dTTP, deoxythymidine 5'-triphosphate; ATP, adenosine 5'-triphosphate; RF, replicative form; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; d, dalton; kd, kilo dalton; bp, base pair.
SUMMARY

Using phosphocellulose followed by single-stranded (ss) DNA cellulose chromatography for purification of UvrC proteins from overproducing cells, we found that UvrC elutes at two peaks: 0.4 M KCl (UvrC I) and 0.6 M KCl (UvrC II). Both forms of UvrC have a major peptide band (>95%) of the same molecular weight and identical N-terminal amino acid sequences which are consistent with the initiation codon being at the unusual -GTG- site. Both forms of UvrC are active in incising UV-irradiated supercoiled φX-174 RFI DNA in the presence of UvrA and UvrB proteins; however, the specific activity of UvrC II is one-fourth that of UvrC I. The molecular weight of UvrC II is four times of UvrC I based on the results of size exclusion chromatography and glutaraldehyde crosslinking reactions indicating that UvrC II is a tetramer of UvrC I. Functionally, these two forms of UvrC proteins can be distinguished under reaction conditions in which the protein/nucleotide molar ratio is above 0.06 by using UV-irradiated, 32P-labeled DNA fragments as substrates; under these conditions UvrC II is inactive in incision, but UvrC I remains active. The activity of UvrC II in incising UV-irradiated, 32P-labeled DNA fragments can be restored by adding unirradiated competitive DNA, and the increased level of incision corresponds to a decreased level of UvrC II binding to the substrate DNA. The sites of incision at the 5' and 3' sides of a UV-induced pyrimidine dimer are the same for UvrC I and UvrC II. Nitrocellulose filter binding and gel retardation assays show that UvrC II binds to both UV-irradiated and unirradiated double-stranded (ds) DNA with the same affinity (Ka = 9 x 10^8 M^-1) and in a concentration dependent manner, while UvrC I does not. These two forms of UvrC were also produced by the endogenous uvrC operon. We propose that UvrC II-DNA binding may interfere with Uvr(A)2B-DNA damage complex formation. However, because of its low copy number and low binding affinity to DNA, UvrC II may not interfere with Uvr(A)2B-
DNA damage complex formation *in vivo*, but instead through ds-DNA binding UvrCII may become concentrated at genomic areas and therefore facilitate nucleotide excision repair.
INTRODUCTION

The initial events of nucleotide excision repair in *Escherichia coli* are controlled by three *uvr* gene products: UvrA, UvrB and UvrC proteins. Our current understanding suggests that these three proteins work in a sequential manner, with UvrA first locating and dimerizing at a damaged base on double-stranded (ds)-DNA, and UvrB then binds and forms a Uvr(A)2B complex from which UvrA may dissociate. Finally, UvrC binds to the Uvr(A)2B complex and triggers dual incisions 6-8 bp 5’ and 3-5 bp 3’ to the damaged base. A diverse array of chemically and photochemically induced modifications to bases in DNA are recognized, including helix stabilizing and destabilizing damages (for review see refs. 1,2,3).

Recent studies show that UvrC is essential for 5’ and 3’ incisions and is specifically implicated in making the 5’ incision through site-directed mutagenesis studies. UvrC is probably essential for the catalysis of the 3’ incision as well, although it may catalyze this step itself (4-11).

*UvrC* differs from *uvrA* and *uvrB* in its genomic organization. It may not be under *lexA* control as *uvrA* and *uvrB* are (7). The genomic region of *E. coli* containing the *uvrC* gene is complex and contains at least one overlapping open reading frame (12,13). The *uvrC* gene itself appears to have four promoters and to be transcribed into two mRNAs (7). The translation start site for the UvrC protein is also unclear and may be the 5’-GTG- upstream of the first 5’-ATG- (13,14). The precise size of the UvrC protein is therefore unknown and may be 67,000 daltons or it may be 22 amino acids longer and 68,500 daltons.

The low natural occurrence of the Uvr proteins has necessitated their study by the use of overproducing cells containing the appropriate plasmids. This is particularly true for UvrC since it is estimated to be present at only <10 molecules per wild-type cell (2).
In this study we have purified UvrC from *E. coli* strains containing the overproducing plasmid pDR3274 (15) by several different methods, including a rapid procedure which avoids precipitation of the protein and expedites isolation of a purified protein. We find that these preparations yield two different UvrC fractions, which we call UvrCI and UvrCII according to their elution positions from ss-DNA cellulose columns. Both UvrCI and UvrCII are active in a variety of assays of UvrABC excision nuclease activity, but were found to differ in their interactions with DNA. UvrCII binds readily to ds-DNA while UvrCI does not. We present evidence which demonstrates that this UvrCII-DNA binding may affect the UvrABC excision nuclease activity. These two forms of UvrC were also isolated from wild type *E. coli* cells without containing the pDR3274 plasmids (12). The possible molecular differences between these two forms of UvrC protein and their physiological roles are discussed.
EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes *Hinf*I, *Nar*I, *EcoR*I and *Pst*I, T4 polynucleotide kinase, bacterial alkaline phosphatase, acrylamide, bis-acrylamide, agarose, and NACS Prepacs convertible columns (NACS PACS) were obtained from Bethesda Research Laboratories. The restriction enzyme *Bst*NI was obtained from DuPont New England Biolabs. Yeast tRNA was obtained from Sigma Chemical Company. All $^{32}$P-labeled nucleotides were obtained from Amersham Company or New England Nuclear. Nitrocellulose membranes (hydrophilic, 0.45 μM, HAWP) were purchased from Millipore Company.

DNA and DNA fragment isolation. ΦX174 RFI DNA and plasmid pBR322 were isolated and purified by cesium chloride density gradient centrifugation. T7 phages were prepared by a method described by Yamamoto et al (16). T7 DNAs were prepared by removing proteins from phages by phenol and diethyl ether extractions. The 247 bp *Hinf*I-*Bst*NI single 3′ end-$^{32}$P-labeled fragments of pBR322 were isolated from a 383 bp *Bst*NI fragment that had been agarose gel purified and 3′ end labeled with $\alpha$-$^{32}$P dTTP. The 174 bp *EcoR*I-*Hae*III single 5′ end-$^{32}$P-labeled fragments of pBR322 were prepared as described previously (17). The *EcoR*I-*Pst*I 750 bp fragments of pBR322 were isolated from agarose gel electrophoresis and $^{32}$P-labeled with $\gamma$-$^{32}$P-ATP at both 5′ ends.

UV irradiation. ΦX174 RFI DNA, $^3$H-thymidine labeled T7 DNA and $^{32}$P-labeled defined DNA fragments were irradiated with a germicidal lamp (Sylvania, C15T8, major emission 254 nm) to produce eight dimers or one dimer per DNA molecule.
Purification of Uvr proteins. UvrA, UvrB, and UvrC proteins were purified from the *E. coli* K12 strain CH296 (*uvrC34*) and DR1984 (*recA1 uvrC34*) carrying plasmids pUNC45 (*uvrA*), pUNC211 (*uvrB*), or pDR3274 (*uvrC*) (16). These plasmids and *E. coli* strains were kindly provided by Dr. A. Sancar, University of North Carolina, Chapel Hill, North Carolina. The pUVC1234 plasmid construct (12) which contains the endogenous *uvrC* operon was a generous gift from Dr. R. Moses, University of Oregon, Portland, Oregon. UvrC protein was also purified from *E. coli* cells (MST 1) with the wild type *uvrC* gene (17,18).

UvrABC excision nuclease reactions. The UvrABC excision nuclease reaction was conducted in 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 100 mM KCl, 1 mM ATP, and 1 mM DTT. An aliquot of ⁴³²P-labeled DNA or φX174 RFI DNA (0.2 µg) was reacted with 15 nM UvrA, 15 nM UvrB, and 15 nM UvrC in a volume of 25 µl for 60 min at 37°C. For φX174 RFI DNA the reactions were stopped by adding 0.1 % SDS and heating at 65°C for 5 min and the DNAs were then electrophoresed in a 1% agarose gel in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) at 1 V/cm for 14 h. The gel was stained with 0.5 µg/ml ethidium bromide to visualize the supercoiled and relaxed forms of DNA. The pictures were scanned by a Bio-Image Analyzer with a Visage 100 whole band analysis software program. For ⁴³²P-labeled DNA fragments the reactions were terminated by phenol extractions. The labeled DNA was then ethanol precipitated, washed in 75% ethanol, dried and dissolved in a formamide denaturing dye mix (80% v/v formamide, 0.1% xylene cyanol and 0.1% bromophenol blue).

Sequencing gel electrophoresis. Chemical sequencing was carried out as described by Maxam and Gilbert (21) with the modifications described (17). DNA samples were heated at 90°C (2 min) and quenched in an ice bath. The samples were applied
to a sequencing gel, consisting of 8% acrylamide, 7 M urea in TBE buffer (50 mM Tris-HCl, 50 mM sodium borate, and 10 mM EDTA pH 8.3), in parallel with the Maxam and Gilbert sequencing reactions. After electrophoresis the gel was dried in a Bio-Rad gel dryer and exposed to Kodak X-Omat RP films at -70°C for various lengths of time. The intensity of bands was determined by scanning with a Bio-Image Analyzer as described above.

**DNA binding assay.** $^{32}$P-labeled pBR322 DNA fragments or linearized φX174 RFI DNA, and $^{3}H$-thymidine labeled T7 DNA were incubated with UvrC or UvrA at different protein/DNA ratios in UvrABC excision nuclease reaction buffer for 60 min at 37°C. At the end of incubation the mixtures were chased with an excessive amount of calf thymus DNA for 10 s, filtered through a membrane (0.45 µM, HAWP, Millipore) and washed with UvrABC excision nuclease reaction buffer without ATP for 10 s, or electrophoresed in a 0.5% agarose gel in TAE buffer (Tris, 50 mM, pH 7.9, acetate, 50 mM, and EDTA, 1 mM). The radioactivity in the dried membranes was counted in a LKB 1219 scintillation counter. The agarose gels were air dried and exposed to Kodak X-Omat RP films at -70°C for various lengths of time. The intensity of bands was determined by scanning with a Bio-Image Analyzer.

**N-terminal amino acid analysis.** To determine the N-terminal amino acid sequence of UvrC, the proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The UvrC band was eluted and the first 12 N-terminal amino acids were determined by Beckman amino acid sequences according the method as described by Matsudaira (22).

**UvrC crosslink reactions.** Aliquots containing 1 µg of UvrC were incubated with 0.1% of glutaraldehyde at room temperature for 10 min. Immediately after reaction
the proteins were denatured by boiling in SDS solution for 5 min, separated by 7% SDS-PAGE, and then transferred to a nitrocellulose membrane.

**Immunoblotting.** A 7 or 10% acrylamide/0.32% bis-acrylamide/SDS was employed as described by Lammeli (23). After electrophoresis the proteins were electrotransferred to a nitrocellulose membrane, reacted with polyclonal UvrC antiserum (18); the antigen-antibody was further conjugated with horseradish peroxidase-labeled antibodies and then detected by chemilluminescence method (24).
RESULTS

Purification of two forms of UvrC by ss-DNA cellulose chromatography.

Several purification schemes have been reported for UvrC purification and they have in common two steps: phosphocellulose and ss-DNA cellulose chromatography (15,25,26). UvrC proteins can be precipitated with polymin P before going through chromatographic separation (25). In these procedures UvrC is lastly eluted from a ss-DNA cellulose column with 0.65 M KCl. We found that substituting a KCl gradient for the more usual step gradient to elute the ss-DNA cellulose column results in the elution of two distinct peaks of A280 material and both peaks have UvrC activity (Figure 1A, details are described in the following sections).

Since the two-peak elution profile of UvrC protein has never been reported before, we then explored the possibility whether the multistep purification procedure for UvrC was responsible for this striking pattern by simplifying the purification scheme. UvrC was purified from a cell sonicate (CH296 or DR1984 cells containing pDR3274 and induced with 0.5 mM IPTG) prepared in 0.3 M KCl. The supernatant after centrifugation was purified through a phosphocellulose and then a ss-DNA cellulose column, without going through a polymin P precipitation step. Elution of the ss-DNA cellulose column with a KCl gradient gives a similar profile as in Figure 1A, showing two peaks of protein (Figure 1B). Both peaks contain a major (>95 %) UvrC band of a molecular weight of 65,000 dalton when separated by SDS-PAGE (Figure 1C); the amount of UvrC collected in the first peak fractions is approximately twice as much as in the second peak fractions. We refer to the two fractions of UvrC according to their elution position as UvrCI (0.4 M KC1) and UvrCII (0.6 M KC1).
UvrCI and UvrCII are active in incising UV-irradiated supercoiled φX174 RFI DNA. The activity of these two forms of UvrC in incising UV-induced DNA damage was tested. Various amounts of UvrC proteins were reacted with UV-irradiated φX174 RFI DNA (2.2 nM) in the presence of UvrA (15 nM) and UvrB (15 nM) (we term this reaction condition which has a relatively high nucleotide/Uvr ratio as condition I). The results in Figure 2 show that both forms of UvrC were active in incising UV-irradiated supercoiled DNA. However, the specific activity of UvrCII is lower than that of UvrCI; the former is one-fourth as active in incising UV-irradiated φX174 RFI DNA as the latter.

Since the majority of proteins present in both peaks have a molecular weight (denatured by boiling in SDS solution) corresponding to that of UvrC, their N-terminal amino acid sequences were determined. We have found that the first 12 of the 13 N-terminal amino acids of both forms of UvrC are the same, starting from the second amino acid the sequences are:
AspGlnPheAspAlaLysAlaPheLeuLysThrVal.

UvrCII is a tetramer and UvrCI is a monomer. Since the monopeptide of UvrCI and UvrCII not only have the same molecular weight but also have the same N-terminal amino acid sequence, and no other peptides have been found in association with these two forms of protein (Figure 1), the results raise the possibility that these two forms of UvrC could result from different folding and/or that the native form of these two proteins may be composed of different numbers of monopeptide. To test these possibilities we have determined the molecular weight of these two forms UvrC proteins by glutaraldehyde crosslink reaction and size exclusion chromatography (27). Figure 3 show that treatment of UvrCI with 0.1% of glutaraldehyde resulted in one additional band corresponding to molecular of 130 kd besides the 65 kd band. In contrast, the same glutaraldehyde treatment of UvrCII resulted in two additional
bands; one corresponding to 130 kd the same as observed in UvrCI treatment, and
the other has a molecular weight larger than 240 kd. Results from size exclusion
chromatography show that UvrCI eluted at a major peak corresponding to the bovine
albumin position (68 kd). In contrast, UvrCI and UvrCII mixture eluted at two peaks
corresponding to ~ 68 kd and >232 kd, respectively (Figure 3B). Together, these
results suggest that UvrCI is a monomer and UvrCII is a tetramer.

**UvrCI and UvrCII react to UV-irradiated linear DNA differently.** It has been well
established that the UvrABC excision nuclease makes dual incisions 6-8 bp 5' and
3-5 bp 3' to UV-induced pyrimidine dimers and other chemical-DNA adducts (for
review see refs. 1, 2, 3). However, it has been found that occasionally the 5' and 3'
incisions induced by UvrABC may uncouple, and the uncoupled incision occurs in
UV, bulky chemical carcinogen and CC-1065 modified DNA (for review see ref. 2).
Selby and Sancar (28) have reported that "aged" UvrC proteins may lose their 5'
incision ability. In order to determine whether UvrCI and UvrCII, in combination
with UvrA and UvrB (we term the collective function of Uvr proteins as UvrABCI
and UvrABCII excision nuclease, respectively), would have the same dual incision
activity. UV-irradiated single 3'-end-\(^{32}\)P-labeled HinfI-BstNI 247 bp pBR322 DNA
fragments (1 nM) or single 5'-end-\(^{32}\)P-labeled EcoRI-HaeIII 174 bp pBR322 DNA
fragments were reacted with 15 nM of these proteins (we term this reaction
condition which has a relatively low nucleotide/Uvr ratio as condition II). The results
are shown in Figure 4; while UvrABCI makes the expected dual incisions at
pyrimidine dimers, under the same reaction conditions UvrABCII does not incise the
same UV-irradiated linear DNA fragments (compare lane 11 to lane 15 in Figure
4A and lane 12 to lane 17 in Figure 4B).

There are two possible explanations for these unexpected results; one is that
UvrABCII is inactive in incising photodimers in linear DNA and the other
explanation is that UvrCII may interfere with Uvr(\(A\))\(_2\)B-photodimer complex formation. It has been reported that the presence of excessive UvrA protein inhibits UvrABC excision activity, and the inhibition has been speculated to result from the binding of excessive UvrA proteins to damaged bases which consequently prevents proper Uvr(\(A\))\(_2\)B-DNA damage complex formation (29,30). The inability of UvrABCII to incise the small quantity of UV-irradiated linear DNA fragments shown in Figure 4 cannot be due to an excessive amount of UvrA proteins since the active UvrABCII reaction conditions contain the same amount of UvrA proteins as in the inactive UvrABCII reaction conditions. One of the major differences between conditions I and II is that while the nucleotide/UvrC ratio is 770 in condition I it is between 10 to 17 in condition II; the difference between these two conditions is 45 to 77 fold. If the UvrCII protein is able to bind to ds-DNA and UvrCI is not, then the formation of a proper Uvr(\(A\))\(_2\)B-DNA damage complex may be affected by excessive amounts of UvrCII (but not UvrCI), similar to the effect of excessive UvrA proteins. To test this possibility we added different amounts of unirradiated linearized \(\phi X174\) RF DNA (competitive DNA) in condition II to reduce the protein/nucleotide ratio. The results in Figure 4 show that while additional DNA, ranging from 0.05 to 0.2 \(\mu\)g, enhances UvrABCII excision activity, it affects UvrABCII incision activity more drastically, restoring the incision activity of UvrABCII to a level comparable to that of UvrABCII. These results also show that UvrABCII, in the presence of competitive DNA, makes dual incisions 6-8 bp 5' and 3-4 bp 3' of a photodimer in the substrate DNA in the same fashion as UvrABCII does. It is worth noting that in the presence of excessive competitive DNA the incision activities of both UvrABCII and UvrABCII are reduced (lanes 14 and 18 in Figure 4A; lanes 16 and 21 in Figure 4B).

UvrCII is a double-stranded DNA binding protein and UvrCI is not. The above results suggest that UvrCII may be a ds-DNA binding protein while UvrCI is not. To
test this possibility the conventional nitrocellulose filter binding assay for two forms of UvrC, and UvrA proteins was performed. Different amounts of Uvr proteins were added to a fixed amount of $^3$H-labeled T7 DNA (7.3 fmol) with or without UV-irradiation to produce 8 dimers per DNA fragment. The protein-DNA mixtures were incubated in the standard UvrABC reaction solution for 60 min at 37°C and then were chased with an excessive amount of cold DNA before filtering through nitrocellulose membranes. Results in Figure 5 show that UvrCII indeed binds to ds-DNA, while UvrCI does not, and UvrCII-DNA binding is linearly proportional to UvrCII concentrations. Furthermore, it appears that there is no significant difference between the binding affinities of UvrCII to UV-irradiated and unirradiated DNA. These two features are in great contrast to UvrA-DNA binding, which is exponentially proportional to UvrA concentrations and shows higher affinity towards UV-irradiated DNA than non-irradiated DNA (31). Consistent with these filter binding assay results, Figure 6 shows that the presence of UvrCI proteins does not affect the mobility of the DNA fragments; in contrast, the presence of UvrCII proteins retard the mobility of the labeled DNA fragments significantly. Scatchard plot (with the assumption that UvrCII is a tetramer with molecular weight of 272 kd on the basis of crosslinking reaction results) renders the binding constant of UvrCII–dsDNA $9 \times 10^8$ M$^{-1}$ (Figure 6) which is one tenth to one twelfth of UvrA. It is worth noting that a significant portion of the retarded DNA fragments distribute in a smear (in an overly exposed film, data not shown); the smear may result from dissociation of UvrCII from DNA during electrophoresis.

The relationship between UvrCII-DNA binding and UvrABCII incision activity. In order to determine the effect of UvrCII-DNA binding on UvrABCII incision activity, 3'-end-32P-labeled UV-irradiated HinFI-BstNI 247 bp DNA fragments (1 nM) were incubated with 15 nM UvrCII in standard reaction conditions for 60 min at
37°C, different amounts of competitive DNA (nonlabeled linearized φX174 RF DNA) were added and the mixtures were further incubated for another 60 min at 37°C with or without UvrA and UvrB proteins. At the end of the incubation the sites and the extent of incision of these DNAs by UvrABCII (Figure 7) and the percent of labeled DNA binding to UvrCII (Figure 7) were determined by sequencing gel electrophoresis and filter binding assay, respectively. The incision activity of UvrABCII increases as the amount of the additional competitive DNA increases (Figure 7). Conversely, the results in Figure 8 show that the fraction of damaged DNA bound with UvrCII decreases exponentially as a function of the concentrations of the competitive DNA. The relationship between the reduction of damaged DNA bound with UvrCII and the increase of UvrABCII incision is better demonstrated in Figure 8, which shows that the incision reaches a plateau level when UvrCII-damaged DNA binding is reduced to a few percentile and further addition of competitive DNA slightly reduces incision. These results are consistent with the interpretation that the UvrCII-DNA binding hinders Uvr(A)2B-damaged DNA complex formation and consequently reduces UvrABC incision activity.

Isolation of UvrCI and UvrCII from *E. coli* cells with and without plasmids containing the endogenous *uvrC* operon. Since pDR3274 is a recombinant plasmid with the *uvrC* structural gene sequence linked to *tac* promoter (15) it was possible that the two forms of UvrC proteins produced by this plasmid may be different from that produced by the native endogenous *uvrC* operon. To test this, we isolated UvrC, from *E. coli* cells without harboring the pDR3274 plasmid. Cell lysates were chromatographed through phosphocellulose and ss-DNA cellulose columns following the procedures described above. Although the wild type cell lysates did not render two clear A280 absorption peaks corresponding to 0.4 and 0.6 – 0.7 M KCl, Western blotting results show that two peaks of UvrC protein were separated and eluted at
these two KCl concentrations (Figure 9). Together, these results suggest that two forms of UvrC are produced by the endogenous \textit{uvrC} operon.
DISCUSSION

The functions of uvrC are the least understood among the three uvr genes-uvrA, uvrB and uvrC- which are involved in the initial incision step of nucleotide excision repair. Although in vitro it has been demonstrated that the addition of UvrC proteins to a Uvr(A)2B-damaged DNA complex induces dual incisions 5' and 3' to damaged bases, several laboratories have reported that UV-irradiation induces DNA single-strand breaks in uvrC34 mutant cells, but not in uvrA and uvrB mutant cells, even though these three uvr mutant cells are unable to excise cyclobutane pyrimidine dimers (19,32-34). Using a viral-E. coli transfection system we have found that only uvrC mutant cells show low transfectivity for viral DNA containing N-(guanosin-8-yl)-2-aminofluorene adducts; uvrA and uvrB cells show the same transfectivity as wild-type cells. In contrast, these three mutant cells have the same low transfectivity for UV-irradiated, or N-acetoxy-2-acetylaminofluorene and anti- and syn-benzo(a)pyrene diol epoxide modified viral DNA (20,35). These results indicate that uvrC gene products may function in a manner more complicated than simply participating in the incision step and may interact with proteins other than UvrA and UvrB in vivo.

The understanding of UvrC function has been hampered by the scarcity of these proteins in cells and their instability. Purification of UvrC proteins has been achieved by using λ lysogens with the uvrC gene (36,37) or cells with a plasmid containing the uvrC gene (12). The method of purification is mainly based on the ability of UvrC proteins bind to ss-DNA. Yeung et al (26) have eluted purified UvrC protein at 0.45 M KCl; however, using a step gradient Thomas et al (15) have reported that UvrC proteins were eluted completely at 0.65 M KCl. Our modified method further separated the UvrC into two peaks, one at 0.4 M KCl and another at 0.6 M KCl. It appears that the quantity of ss-DNA cellulose used for column
chromatography and the elution rate are the two critical factors for separating these two forms of UvrC proteins; we observed a single UvrC peak which contained both UvrCI and UvrCII activities when the purification procedure involved a small quantity of ss-DNA cellulose and fast elution rate (data not shown).

We have found that these two forms of UvrC proteins can be further purified by ds-DNA cellulose chromatography but with low recovery (20%); the reason for this low recovery is not clear. However, we also have found that the two forms of UvrC prepared by ss-DNA cellulose chromatography and those further purified by ds-DNA cellulose chromatography have the same mobility in SDS-PAGE, the same PI (native form: 7.48, denatured form: 9.4), and in the presence of UvrA and UvrB, both showed a dual incision pattern on UV-irradiated DNA. The sources causing the differences in ds-DNA binding and elution pattern in ss-DNA chromatography are unknown. Contrary to the published results, we have found that the N-termini of these two forms of UvrC proteins we have purified are not blocked and the first 12 of the 13 N-terminal amino acids, starting from the second amino acid are consistent with the result reported by Moolenaar et al (13) that the initiation codon for of \textit{uvrC} gene starts at the 5'-GTG- of positions 882-884 rather than at the 5'-ATG- of positions 772-774 (14). The source of this discrepancy is unclear.

Since the peptide in the two forms of UvrC have the same molecular weight and N-terminal amino acid sequence, it is possible that UvrCII may have resulted from aggregation of UvrCI. Although we are unable to exclude this possibility entirely, several lines of evidence suggest this may not be the case. Firstly, so far we are unable to convert either form of UvrC to the other by treatments such as oxidation and reduction. Secondly, we show that only monomeric and tetrameric, but no dimeric and trimeric, UvrC proteins are obtained in size exclusion chromatography. Thirdly, in the electrophoretically separated products of UvrCII treated with the cross-linking reagent glutaraldehyde we observed mainly the
monomeric and tetrameric, but no trimeric forms of UvrC. Furthermore, we observe the existence of these two forms of UvrC in wild type cells, even though the copy number of the UvrC proteins in these cells is very low (~10 copies per cell).

It is possible that the difference in ds-DNA binding resides in post-translation modification and/or in the association of cofactors. If one UvrC form is modified or associated with some cofactors while the other is not, then the two UvrC proteins most likely would be observed in overproducing cells for the simple reason that overproduced UvrC proteins oversaturate the post-translation modification capacity or the amount of cofactors present in the cells. However, using the same purification protocol we have isolated UvrC forms which eluted at 0.4 M KCl as well as at 0.6 – 0.7 M KCl from wild type *E. coli* cells harboring no recombinant *uvrC* plasmid, and *E. coli* cells with pUVC1234 plasmids containing endogenous *uvrC* operon (12). Due to the minute quantity of UvrC purified from these cells we were unable to determine their activities. However, since even in cells producing minute amounts of UvrC proteins we were able to isolate UvrC which had an elution profile identical to that of to UvrCI and UvrCII, it is likely that these two forms of UvrC proteins are the normal *uvrC* gene products and are not the results of overproduction.

We speculate that UvrCII is a major form existing in wild-type cells for two reasons: one is that UvrCII should be active in incision *in vivo* because the cellular nucleotide/protein molar ratio is relatively large. The second reason is that since each cell has only <10 molecules of UvrC, and UvrCII does not form a complex with the free forms of UvrA or UvrB protein, to account for the fast and efficient excision repair in vivo the UvrC proteins must be concentrated in the genome area. The loose association of UvrCII to DNA would fulfill this critical requirement.
REFERENCES


FIGURE LEGENDS

Figure 1. Separation of two forms of UvrC by ss-DNA cellulose chromatography. The UvrC proteins from cell lysates of CSH296/pDR3274 were either precipitated with polymin P (0.5%) (A) or without this precipitation (B). Partially purified UvrC collected from combined peak fractions of phosphocellulose chromatography was diluted to 0.2 M KCl in buffer A, and applied to a 1.5 x 14 cm column containing 0.5-1 g ss-DNA cellulose. The column was washed and developed with a linear gradient of KCl (0.3 - 1 M KCl; dotted lines) at a flow rate of 0.5 ml/min and the 4 ml fractions were collected. (A) and (B), the wavelength 280 nm absorbance profile of the eluted fractions (solid lines), and (C), the SDS-PAGE analysis of proteins in the different fractions from (B). The molecular standards are in lane 1 and their molecular masses (kilo dalton) are indicated at the left side of the panel.

Figure 2. UvrABCI and UvrABCII nuclease incisions of UV-irradiated supercoiled DNA. Supercoiled ΦX174 RFI DNA containing 8 pyrimidine dimers per DNA molecule (U8) was reacted with various amounts of either UvrCI or UvrCII (1 represents 15 nM; the molar concentration was calculated based on the assumption that the molecular weight of both UvrC forms is 65 kd according to the results shown in Figure 1C) in combination with UvrA (15 nM) and UvrB (15 nM) under standard reaction conditions at 37°C for 60 min and the resultant DNA was separated by electrophoresis in a 1% agarose gel. (A), represents a typical gel. The top panel indicates individual Uvr proteins used in the incision assay. U0, unirradiated ΦX174 RFI DNA; +, Uvr protein presence; -, Uvr protein absence; CCC: covalently closed circle; OC : open circle. (B), effect of UvrC concentration on the incision of U8 DNA. The fraction of U8 incised was quantified from densitometer scanning of bands corresponding to CCC and OC in (A).
Figure 3. Determinations of UvrCI and UvrCII molecular weight by size exclusion chromatography and glutaraldehyde crosslinking reactions. (A), A typical gel filtration profile of UvrCI and UvrCII. UvrCI and UvrCII purified from ss-DNA cellulose column were separated by Sephacryl S-300 HR gel chromatography (eluted with 0.55 M KCl); (a), separation of a mixture of UvrCI and UvrCII, (b), UvrCI only, and (c), UvrCII only. The positions of the molecular weight standards are indicated by arrows. (B), A typical Western blotting profile of UvrCI and UvrCII after glutaraldehyde treatment. UvrCI and UvrCII proteins were reacted with glutaraldehyde, denatured as described, separated in a 7% PAGE and transferred to a nitrocellulose membrane. The membrane was reacted with UvrC antibodies followed by horseradish peroxidase antibodies. The antigen-antibody reactions were detected by chemilluminescence method (24). Lane 1, UvrCI mock treated; lane 2, UvrCI treated with 0.1% glutaraldehyde at room temperature for 10 min; lane 3, UvrCII mock treated; lane 4, UvrCII treated with glutaraldehyde as in lane 2. The positions of the molecular standards (in kd) are indicated.

Figure 4. (A) UvrABCI and UvrABCII incisions of UV-irradiated (one pyrimidine dimer per DNA fragment, lanes 11 to 18) and unirradiated (lanes 1 to 6) 3' end-32P-labeled 247-bp BstNI-HinfI pBR322 DNA fragments. The DNA fragments were incubated in the standard reaction mixture containing 15 nM UvrA, 15 nM UvrB and 15 nM UvrCI or UvrCII, and with different amount of competitive DNA (unirradiated, nonlabeled linear ΦX174 RF ds-DNA as indicated at the top of the panel); 0 µg for lanes 11 & 15, 0.1 µg for lanes 12 & 16, 1 µg for lanes 13 & 17 and 2 µg for lanes 14 & 18. Lanes 1 to 6 are reactions using unirradiated DNA fragments. The mixtures were incubated at 37°C for 60 min, and the resultant DNAs were electrophoresed in a sequencing gel. The Maxam and Gilbert A+G, G, T+C and C sequencing reactions are indicated. Uvr protein treatments were as follows: Lane 1, no enzyme, lane 2, UvrA only; lane 4, UvrCI; lane 3, UvrCII; lanes 5 & 11 to 14, UvrABCI; and lanes 6 & 15-18, UvrABCII.
(B) UvrABCI and UvrABCII incisions of UV-irradiated (one pyrimidine dimer per DNA fragment, lanes 12 to 21) 5’ end-\(^{32}\)P-labeled 174 bp EcoRI-HaeIII pBR322 DNA fragments. The conditions for UvrABC reactions and the sequencing gel electrophoresis are the same as described in Figure 4 (A). The amounts of competitive DNA added are indicated at the top of the panel. The pyrimidine tracts (1 to 16) which contain two or more contiguous pyrimidines and which have the potential to form photodimers are indicated in the left panel. The photodimers induced corresponding UvrABC incision bands (u1 to u16) are indicated in the right panel.

Figure 5. DNA binding of UvrCI (△, ▲), UvrCII (□, ■) and UvrA (○, ●). Different concentrations of proteins were incubated with a fixed amount of \(^{3}\)H-thymidine labeled T7 DNA (7.3 fmole) with (solid symbols) or without (open symbols) UV-irradiation (to produce 8 dimers per DNA molecule) in the standard UvrABC reaction solution for 60 min at 37°C. At the end of incubation an excessive amount of calf thymus DNA was added for 10 s and the mixture was filtered through a 0.45 µm nitrocellulose membrane. DNA retained on the membrane was measured and is interpreted to be protein bound. All data points are the average of duplicate assays.

Figure 6.(A), Gel electrophoresis of DNA fragments reacted with UvrCI and UvrCII. Aliquots containing \(^{32}\)P-end labeled EcoRI-PstI 750 bp pBR322 DNA fragments (14 fmole) were incubated with different amounts of UvrCI (0, 84, 168, 336, and 672 fmole) or UvrCII (0, 84, 168, 252, 336, 420, 504 and 672 fmole) under the same conditions as described in Figure 5. At the end of the incubation the protein-DNA mixtures were immediately electrophoresed in a 0.5 % agarose gel in TBE buffer. The autoradiographs were scanned and the binding data were plotted as shown in (B). Data points were obtained from results of both the gel retardation assay and the filter binding assay. Scatchard plot of the binding data (from the gel retardation assay) is shown in the insert. The average number of UvrCII
bound per DNA fragment \( (\nu) \) was calculated from the distribution of DNA in the various bands as a direct average:

\[
\nu = \frac{\sum n f_n}{\sum f_n}
\]

where \( f_n \) is the fraction of DNA fragments that has \( n \) proteins bound (38). The percentage of DNA bound in the filter binding assay was normalized by using the highest binding as 100%.

Figure 7. The effect of unirradiated, competitive DNA on the incision of UV-irradiated substrate DNA by UvrABCII excision nuclease. The 3′-end-labeled UV-irradiated 247 bp BstNI-HinfI pBR322 DNA fragments (1 nM) were reacted with UvrCII (15 nM) in the standard UvrABC reaction solution in the presence of different amount of competitive DNA (as indicated at the top of the panel) for 60 min at 37°C. At the end of the incubation the mixtures were either filtered through a nitrocellulose membrane as described in Figure 5 or further incubated for 60 min following the addition of UvrA (15 nM) and UvrB (15 nM). The resultant DNAs were separated by electrophoresis in a sequencing gel as described in Figure 4. The symbols and lanes description are the same as in Figure 4.

Figure 8. The relationship between UvrCII-DNA binding and UvrABCII incision of UV-irradiated substrate DNA. The percent of \( ^{32}P \)-labeled UV-irradiated DNA fragments retained in the filter due to UvrCII binding was calculated as described in Figure 6. The percent of incision was calculated from the densitometer scanning results of Figure 7 (total intensity of u1 to u16) using the highest cutting as 100% (lane 17).

Figure 9. Purification of two forms of UvrC from wild type E. coli cells harboring no uvrC plasmids. A typical immunoblotting result is shown. The purification procedures were the same as described in Figure 1. The proteins eluted from ss-DNA cellulose column by a KCl gradient were separated by electrophoresis using a 12% SDS-PAGE,
electroblotted to a nitrocellulose membrane, and detected using UvrC antibodies as by described in Figure 3. UvrC proteins were eluted at fractions around 0.4 M KCl and at fractions between 0.6-0.7 M KCl with no protein detected in the intermediate salt range.
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Figure 1A & 1B

![Graph](image-url)
Figure 1C
Figure 2
Figure 3A

232 kD  67 kD

a) 

b) 

c)
Figure 3B
Figure 4A

A   A   ABCI
B   B   ABCII
CI   C   0  0.1  1.0  2.0
CI   CII  0  0.1  1.0  2.0
competitive-DNA (µg)
Figure 4B

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competitive-DNA (µg)
Figure 5

![Graph showing the percent DNA bound by different proteins (UvrA, UvrCII, UvrCI) across various protein/DNA molar ratios (0 to 24). The graph includes lines for each protein type, indicating their binding efficiency at different molar ratios.](image-url)
Figure 6A
Figure 6B

![Graph showing the relationship between protein concentration (nM) and percent DNA bound with UvrCII](image-url)
Figure 7

[Image of a gel electrophoresis pattern with bands labeled A, B, CII, CI, G, T, M, and numbers 0, 11, 22, 44, 66, 88, 101, 321, 196. There are lanes labeled 'Cont', 'UV', and 'ABCII'.]
Figure 8

![Graph showing percent incision or binding against competitive DNA (ng). The graph has two lines: one for incision and one for DNA-UvrCII binding. The x-axis represents competitive DNA in ng, ranging from 0 to 132, and the y-axis represents percent incision or binding, ranging from 0 to 100. The incision line starts near 100 at 0 ng, decreases to about 50 at 22 ng, and then increases to about 70 at 132 ng. The DNA-UvrCII binding line starts near 0 at 0 ng, increases to about 20 at 22 ng, and then decreases to about 10 at 132 ng.](link_to_graph)
Figure 9

Wild-type UvrC

Fraction # 2 4 6 8 10 12 14 16 18 20

0.4 M KCl 0.6 - 0.7 M KCl
Two forms of UvrC protein with different double-stranded DNA binding affinities
Moon-shong Tang, Michael Nazimiec, Xiangcang Ye, Ganesh H. Iyer, Jamie Eveleigh, Yi Zheng, Wenjing Zhou and Yen-Yee Tang

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