Active Site of (A)BC Excinuclease

I. EVIDENCE FOR 5′ INCISION BY UvrC THROUGH A CATALYTIC SITE INVOLVING Asp^{399}, Asp^{438}, Asp^{466}, AND His^{538} RESIDUES*

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(A)BC excinuclease of *Escherichia coli* removes damaged nucleotides from DNA by hydrolyzing the 8th phosphodiester bond 5′ and the 5th phosphodiester bond 3′ to the modified base. The activity results from the ordered action of UvrA, UvrB, and UvrC proteins. The role of UvrA is to help assemble the UvrB·DNA complex, and it is not involved in the actual incision reactions which are carried out by UvrB and UvrC. To investigate the role of UvrC in the nuclease activity a subset of His, Asp, and Glu residues in the C-terminal half of the protein were mutagenized *in vitro*. The effect of these mutations on UV resistance *in vivo* and incision activity *in vitro* were investigated. Mutations, H538F, D399A, D438A, and D466A conferred extreme UV sensitivity. Enzyme reconstituted with these mutant proteins carried out normal 3′ incision but was completely defective in 5′ incision activity. Our data suggest that UvrC makes the 5′ incision by employing a mechanism whereby the three carboxylates acting in concert with H538 and a Mg^{2+} ion facilitate nucleophilic attack by an active site water molecule.

(A)BC excinuclease removes damaged nucleotides from DNA by incising the damaged strand both 5′ and 3′ to the lesion (Sancar and Sancar, 1988). The three subunits of the enzyme, UvrA, UvrB, and UvrC function in a partially overlapping manner in the overall repair reaction (Orren and Sancar, 1989, 1990; Bertrand-Burggraf *et al.*, 1991): UvrA which has affinity for damaged DNA (Seeberg and Steinum, 1982) associates with UvrB (which has no affinity for DNA) to form an A$_2$B$_1$ complex; this complex tracks along DNA (Koo *et al.*, 1991) and delivers UvrB to the damage site and UvrA dissociates from the complex in an ATP-dependent reaction (Orren and Sancar, 1989, 1990). The UvrB·DNA complex so-formed is extremely stable ($t_{1/2}$ 2–3 h) and constitutes a high affinity site for UvrC which upon binding to the UvrB·DNA complex triggers the dual incisions. Thus, the nuclease active site(s) must be located in UvrB, UvrC, or at the UvrB·UvrC interface. In the present study we have used site-specific mutagenesis to find out whether UvrC functions as a nuclease and to identify the active site residues in this subunit.

In our selection of targets for site-directed mutagenesis we were guided by reaction mechanisms of other nucleases. In nearly all nucleases whose reaction mechanisms are known hydrolysis of the phosphodiester bond occurs by general acid-base catalysis (Saenger, 1991). An active site water molecule which is activated either by a metal (Weber *et al.*, 1991; Volbeda *et al.*, 1991; Derbyshire *et al.*, 1991; Beese *et al.*, 1991; Nakamura *et al.*, 1991) or a histidine residue (Suck and Oefner, 1986; Suck *et al.*, 1988) attacks the phosphate, causing cleavage of the phosphodiester bond. Furthermore, in most nucleases characterized to date (excluding site-specific recombinases) His, Asp, and Glu are found in the catalytic site (Saenger, 1991). These residues are either directly involved in activating the water molecule or bind a metal ion which in turn activates the water molecule for nucleophilic attack on the phosphodiester bond. Therefore, we limited our site-specific mutagenesis to these three types of residues in UvrC. We found that mutations in 4 residues H538, D399, D438, and D466 specifically abolish the 5′ incision activity of (A)BC excinuclease without affecting the 3′ incision. We conclude that UvrC is the subunit which makes the 5′ incision.

EXPERIMENTAL PROCEDURES

Materials—The *Escherichia coli* K-12 strain DR1984F′*lacI*^{q}(*uvrC34 recA1*) was the host for testing the complementing activities of mutant *uvrC* genes. AB1884(*uvrC34*) and UNC3024(Δ*uvrC*) (Lin and Sancar, 1991) and plasmid carrying derivatives of the latter were used for alkaline sucrose gradient analyzes. T4 polynucleotide kinase, T4 DNA ligase, and restriction enzymes were purchased from GIBCO-Bethesda Research Laboratories. Single-stranded DNA cellulose and phosphocellulose were purchased from Sigma. UvrA, UvrB, and UvrC proteins were purified as described previously (Thomas *et al.*, 1985). The following buffers were used in purification of wild type and mutant UvrC proteins. Buffer A was 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, and 10% sucrose. Buffer B was 50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 1 mM EDTA, and 20% (v/v) glycerol. The storage buffer was 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 50% (v/v) glycerol.

UV Survival—Cells were grown in Luria broth containing the appropriate antibiotics. The cells were diluted in phosphate-buffered saline, plated on Luria agar plates, and irradiated with 254 nm from a General Electric germicidal lamp at a fluence rate of 5 microwatts/cm^2. The colonies were counted after incubation at 37 °C for 24 h.

Alkaline Sucrose Gradients—The DNA of UV-irradiated cells was analyzed by sedimentation in alkaline sucrose gradients as described by Tang and Ross (1985). Briefly, cells were grown in K medium containing [^{3}H]thymidine (10 μCi/ml) and 200 μg/ml deoxyadenosine to 10^8 cells per ml, irradiated with 20 Jm^{-2} of 254-nm light. After irradiation the cells were washed with phosphate-buffered saline, resuspended in K medium and incubated for 1 h at 37 °C. Following incubation, 0.2 ml of cells were converted to spheroplasts as described and then layered on top of 3.5 ml 5 to 20% alkaline sucrose gradient (0.1 N NaOH, 0.1 M NaCl, 0.01 M EDTA). Centrifugation was at 35,000 rpm for 75 min at 18 °C in an SW-60 rotor. Fractions of 0.16 ml were collected, and DNA was precipitated with 10% trichloroacetic acid. One ml of Scintiverse II was added directly to the pellet, and the radioactivity in each fraction was quantified by scintillation counting.

Site-specific Mutagenesis—Site-specific mutants of *uvrC* gene were constructed as described by Kunkel *et al.* (1987) using the Muta-gene

M13 *in vitro* mutagenesis kit (Bio-Rad) following the manufacturer's instructions. Briefly, the *uvrC* gene was excised from the overproducing plasmid pDR3274 (Sancar and Rupp, 1983; Sancar *et al.* 1984) and inserted into M13 mp18 or M13 mp19. Mutations were generated in these constructs by oligonucleotide-directed mutagenesis, mutants were identified by single-stranded DNA sequencing, and the mutated genes were then replaced the wild type *uvrC* in pDR3274. Mutant constructs were confirmed by double-stranded DNA sequencing. Single- and double-stranded DNA sequencing were performed with the Sequenase DNA sequencing kit (United States Biochemical).

"Mini-prep" UvrC Purification—The following procedure was developed for rapid purification of small quantities of mutant UvrC proteins for preliminary characterization. Five-ml cultures were grown to A_{600} 0.8 at which time isopropyl-β-D-thiogalactoside was added to 1 mM, and incubation was continued at 37 °C for 8–12 h. Cells were collected by centrifugation, resuspended in 0.5 ml of buffer A, transferred to 1.5-ml microcentrifuge tubes, and then frozen in a dry ice-ethanol bath. The cells were thawed on ice and sonicated with a Bronson Model W185 sonifier equipped with a micro-tip. Cell debris was removed by centrifugation. The supernatant was transferred into another tube, and 40 μl of single-stranded DNA cellulose suspension in buffer B (~20-μl packed volume) was added. The tube was inverted gently several times to mix the resin and bind UvrC. The mixture was centrifuged for 5 min, decanted, and the pellet was washed twice with 0.5 ml of buffer B + 0.3 M KCl. Then, UvrC was eluted by resuspending the resin in 200 μl of buffer B + 1.0 M KCl, and the eluted protein was separated from the resin by centrifugation. A 2-μl aliquot of the protein sample was directly used to test for incision activity, and a 50-μl sample was applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to test recovery and purity. Typically 5 μg of UvrC at 40–50% purity was obtained. When necessary the mutant proteins were purified in large scale by the method of Thomas *et al.* (1985) to >90% purity.

Incision Assay—The DNA substrate was a 3'- or 5'-labeled 137-mer duplex with a centrally located furan-side thymine-psoralen monoadduct. The substrate (HMT·DNA)[1] was constructed as described previously (Van Houten *et al.*, 1987) using the psoralen (HMT) adducted dodecamer kindly provided by Dr. J. E. Hearst (University of California, Berkeley, CA). The (A)BC excinuclease reactions were performed in 25-μl ABC buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl$_2$, 2 mM ATP, 5 mM dithiothreitol, and 50 μg/ml bovine serum albumin) containing 0.1 μg of undamaged plasmid DNA, ~2000 cpm of ^{32}P-labeled 137-mer, 5 nM UvrA, and 80 nM UvrB. The reaction mixture was incubated at 37 °C for 30 min, then 2 μl of UvrC Mini-prep or 40 nM UvrC was added, and incubation was continued for another 30 min. The reaction was stopped by adding 1 μl of oyster glycogen (10 mg/ml) and 60 μl of ice-cold ethanol. The DNA was collected by centrifugation, dried, resuspended in a formamide/dye mixture, and analyzed on an 8% polyacrylamide sequencing gel.

RESULTS

Isolation and in Vivo Characterization of uvrC Mutants— We have previously shown that the *Bacillus subtilis uvrC* gene (Chen *et al.*, 1988) complements *uvrC* mutations in *E. coli* (Lin and Sancar, 1990) and that the active site of UvrC is within the carboxyl-terminal half of the protein (Lin and Sancar, 1991). Therefore, we restricted our mutagenesis to the Asp, Glu, and His residues within the carboxyl-terminal half of *E. coli* UvrC that are homologous to the *B. subtilis* UvrC. The mutant proteins were tested for *in vivo* complementation and *in vitro* incision activities. The results are summarized in Tables I and II. Using a qualitative spot test it was found that mutations in D399, D438, D466, and H538 inactivated UvrC. Furthermore, this loss of activity was not due to unfolding of the protein, because all mutant proteins were overproduced to levels comparable to that of wild type UvrC, were soluble, and behaved identical to wild type protein on several chromatographic resins.

Quantitative UV survival tests were conducted on the UV-sensitive mutants to evaluate the level of deficiency in each

[1] The abbreviations used are: HMT, 4'-hydroxymethyl-4,5',8-trimethylpsoralen; UvrC·H538F, etc., UvrC protein containing a change (His, Phe, etc.) at amino acid 538.

TABLE I
Properties of UvrC His mutants

Mutant	UV survival[a]	Incisions	
		5'	3'
	5 erg/mm^2		
WT[b]	4.8×10^{-1}	+	+
H112F	5.1×10^{-1}	+	+
H377F	2.1×10^{-2}	+	+
H377F	2.4×10^{-1}	+	+
H402F	2.4×10^{-1}	+	+
H490F	6.4×10^{-1}	+	+
H528F	9.9×10^{-2}	+	+
H532F	8.4×10^{-2}	+	+
H538F	4.0×10^{-5}	−	+
H540F	1.4×10^{-1}	+	+
H545F	2.2×10^{-1}	+	+
H610F	1.7×10^{-1}	+	+
H538D	1.3×10^{-1}	±	+
H538N	6.5×10^{-1}	±	+
H538Y	1.7×10^{-5}	−	+

[a] *E. coli* K-12 strain DR1984 F'laci^q (*uvrC*⁻, *recA*⁻) was used as the host, the UV survival of this strain at 5 erg/mm^2 is 3.0×10^{-6}. H538A failed to complement *in vivo*; however the mutant protein was only marginally overproduced and therefore was not investigated *in vitro*.
[b] WT, wild type.

TABLE II
Properties of UvrC Asp and Glu mutants

Mutant	UV survival[a]	Incisions	
		5'	3'
	5 erg/mm^2		
WT[b]	4.8×10^{-1}	+	+
D350A	2.9×10^{-1}	+	+
D399A	2.0×10^{-6}	−	+
D399N	1.1×10^{-6}	−	+
D437A	4.8×10^{-2}	±	+
D438A	7.3×10^{-5}	−	+
D438N	5.8×10^{-6}	−	+
D461A	2.1×10^{-1}	+	+
D466A	6.9×10^{-6}	−	+
D466N	9.3×10^{-7}	−	+
D501A	2.2×10^{-2}	+	+
D535A	2.2×10^{-1}	+	+
E316A	3.4×10^{-2}	+	+
E424A	2.4×10^{-1}	+	+
E481A	1.6×10^{-1}	+	+
E516A	4.1×10^{-2}	+	+
E536A	4.3×10^{-1}	+	+
E588A	1.0×10^{-1}	+	+

[a] *E. coli* K-12 strain DR1984 F'laci^q (*uvrC*⁻, *recA*⁻) was the host strain.
[b] WT, wild type.

mutant. The results are shown in Fig. 1. Although cells carrying UvrC·D399A, UvrC·D438A, UvrC·D466A, and UvrC·H538F(Y) were very sensitive to UV, all were slightly more resistant than cells lacking the protein. UvrC·H538N complemented the mutant to wild type level, while cells carrying UvrC·H538D were slightly more UV-sensitive than wild type. Thus, the H → N and H → D changes at position 538 might be considered conservative substitutions.

Incision Activities of Mutant Proteins—When the four UvrC mutants unable to complement *uvrC34 in vivo* were purified and tested in a plasmid-nicking assay (Lin and Sancar, 1989) a paradoxical result was obtained. All mutants, when mixed with UvrA and UvrB, were as active as wild type UvrC in converting UV irradiated superhelical plasmid into open circle form (data not shown). We suspected that this apparent discrepancy between the *in vitro* and *in vivo* results might be due to abnormal incision(s) made by enzyme formed

with mutant UvrC subunit which does not lead to adduct removal. Therefore, we investigated the incision pattern of (A)BC excinuclease reconstituted with mutant UvrC proteins using a uniquely adducted DNA. The results are shown in Fig. 2. All mutant proteins deficient in complementing activity *in vivo* were totally lacking the 5′ incision activity while retaining normal 3′ incision. The simplest interpretation of this data is that UvrC makes the 5′ incision and that the 3′ incision alone leads to a negligible level of adduct removal by $3′ \to 5′$ exonucleases and therefore has only a marginal effect on cell survival.
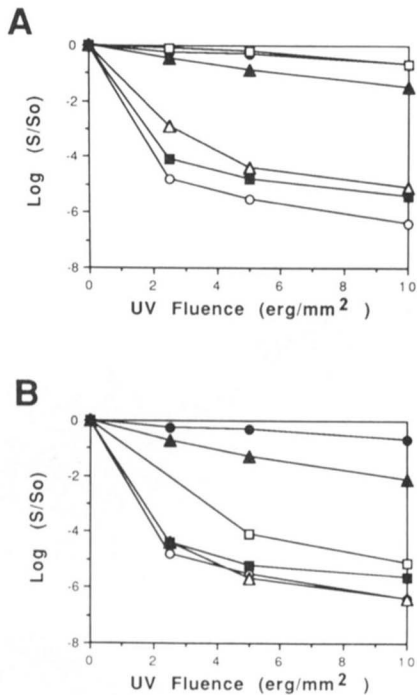

FIG. 1. **UV survival of DR1984(*uvrC34 recA1*) containing pDR3274(*tac uvrC⁺*) or derivative plasmids with mutations in *uvrC*.** *A*, His mutants: ○, no plasmid; ●, *uvrC⁺*; △, H538F; ▲, H538D; □, H538N; ■, H538Y. *B*, Asp mutants: ○, no plasmid; ●, *uvrC⁺*; △, D399A; ▲, D437A; □, D438A; ■, D466A. Cultures were grown to stationary phase in Luria broth, and cells were diluted, plated on Luria agar plates, and irradiated with 254-nm light from a germicidal lamp. The surviving colonies were counted after incubation at 37 °C for 24 h.

Roles of Active Site Residues in Catalysis—The identification of His and Asp residues in the active site of UvrC raised the possibility that His[538] and 1 of the 3 Asp residues required for activity along with a Ser residue or a water molecule in the active site might constitute a catalytic triad (Stryer, 1988). Amino acid arrangements reminiscent of a catalytic triad (originally described for serine proteases) have recently been discovered in a number of nucleases, most notably DNase I (Suck and Oefner, 1986; Suck *et al.*, 1988; Lahm *et al.*, 1991). In current models for the functioning of a catalytic triad, His, aided by the electrostatic (hydrogen bonding) effect of Asp, facilitates the nucleophilic attack by H-OH or Ser-OH, first by acting as a general base to take up the liberated proton, and then to facilitate the decomposition of the pentahedral intermediate formed by donating the proton (general acid).

To find out if H538 had a similar function in UvrC, we constructed UvrC·H538N, and UvrC·H538D mutations and tested them *in vivo* and *in vitro*. The former was as active as wild type *in vivo*, while the latter had slightly reduced activity (Fig. 1). *In vitro* analysis showed that both mutants were capable of 5′ incision (Fig. 2*B*, *lanes 7* and *8*). A kinetic experiment was carried out to find out if there were any differences between the rate enhancements conferred by His[538] and its functionally competent replacements. The results are shown in Fig. 3. The rate of incision with UvrC·H538N is approximately 20% and that of UvrC·H538D is about 10% of that obtained with the wild type protein. Since similar mutations in enzymes presumed or known to act by a "catalytic triad mechanism" reduce k_{cat} by 10^3–10^5 (see Saenger, 1991; Emmerich *et al.*, 1992), we conclude that H538 functions in a different capacity, perhaps by aiding the orientation and polarization of the phosphate by direct hydrogen bonding to the oxygens of the scissile phosphodiester bond. In contrast, the 3 Asp residues appear to play a more direct role in catalysis as $D \to N$ substitution in any of the three sites proved to be as detrimental as the less conservative $D \to A$ substitutions. Thus, it appears that UvrC has a mechanism more akin to RNase H or Klenow $3′ \to 5′$ exonuclease, where D and E residues play prominent roles (see Saenger, 1991), rather than DNase I, RNase A, and RNase T1, where histidines play pivotal roles in catalysis (see Nishikawa *et al.*, 1987). However, $D \to N$ substitutions in all three positions in UvrC, like $D \to A$ substitutions, eliminated activity completely, and therefore, it was not possible to differentiate between the 3 Asp residues with regard to the specific func-
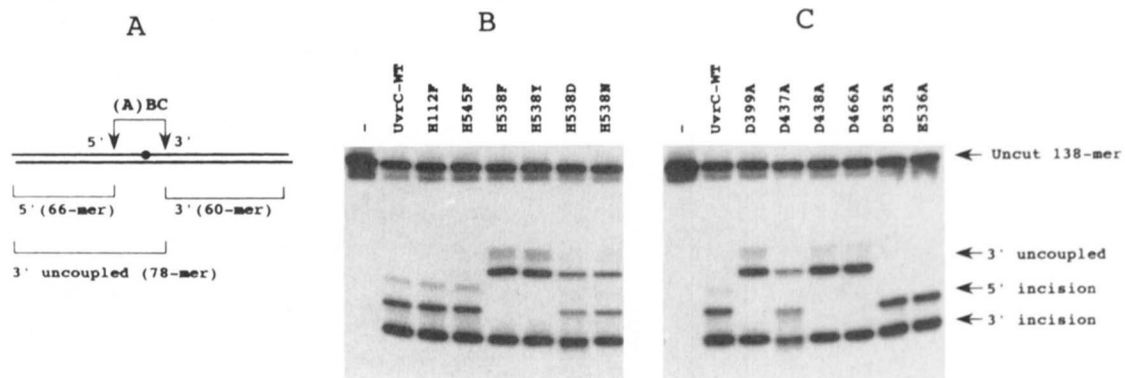

FIG. 2. **Incision pattern of (A)BC excinuclease reconstituted with His or Asp mutants of UvrC.** *A*, substrate and predicted reaction products. The location of the psoralen adduct is indicated by ●, and the (A)BC excinuclease incision sites are indicated by *arrows*. The damaged strand was labeled either at the 5′ or the 3′ end. The labeled fragments expected to be generated form the various incision patterns are indicated. *B*, incision pattern of UvrC·his mutants. A mixture of 3′- or 5′-labeled DNAs was incubated with no enzyme (*lane 1*) or with 5 nM UvrA, 80 nM UvrB, and 40 nM wild type or mutant UvrC as indicated for 30 min at 37 °C, and the reaction products were analyzed on an 8% polyacrylamide sequencing gel. The faint bands above the 66-mer in *lanes 2–6* and above the 78-mer in *lanes 5–8* are produced from the 5′-labeled DNA (data not shown) by aberrant incisions perhaps by secondary action of the enzyme on already-incised DNA. These bands are variable in intensity from experiment to experiment and are not always observed. *C*, incision pattern of UvrC·Asp and Glu mutants. The reaction conditions were as described in *B* except for the UvrC mutants used.
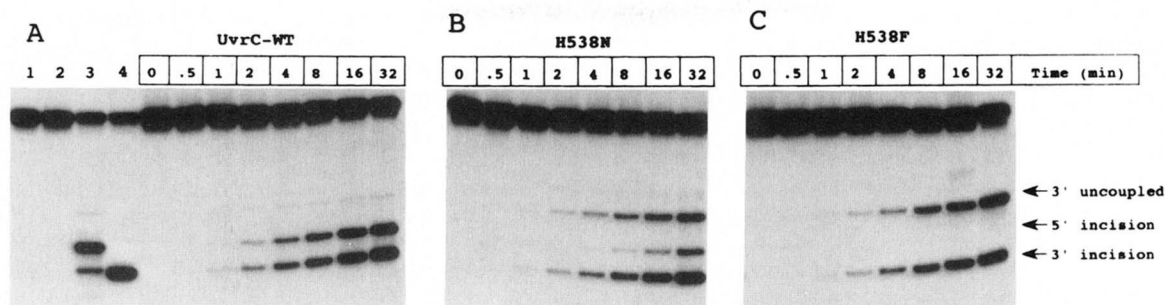
FIG. 3. **Kinetics of 3′ and 5′ incision with wild type and mutant UvrCs.** The mixture of equal cpm of 3′- or 5′-labeled DNAs was incubated with 5 nM UvrA and 80 nM UvrB for 30 min at 37 °C in 250 µl of ABC buffer. At "zero time" UvrC was added to 40 nM, and 25-µl samples were taken at the indicated times, the reaction was stopped by adding 60 µl of ethanol and 10 µg of oyster glycogen, the DNA was collected by centrifugation, and the products were analyzed on 8% polyacrylamide sequencing gels. In *A lanes 1* and *2* contain 5′- and 3′-labeled DNAs, respectively, and *lanes 3* and *4* contain 5′- and 3′-labeled DNAs treated with (A)BC excinuclease. *A*, UvrC·WT; *B*, UvrC·H538N; *C*, UvrC·H538Y.

tions (metal chelating, polarizing nonester oxygen, activating water) they may carry out in catalysis.

The Phenotype of UvrC⁻ Mutants—The biochemical properties of the noncomplementing *uvrC* mutants provided us the opportunity to explain an unusual feature of *uvrC⁻* cells. In contrast to *uvrA⁻* or *uvrB⁻* cells, which fail to nick DNA after UV irradiation, *uvrC⁻* mutants accumulate single-strand breaks following exposure to UV (Ogawa *et al.*, 1968; Rupp and Howard-Flanders, 1968; Deutsch *et al.*, 1976; Seeberg *et al.*, 1980; Tang and Ross, 1985).

In light of results presented in this paper it is likely that the *uvrC⁻* mutants investigated in the previous studies (*uvrC34* and *uvrC56*) are active site mutants which induce the 3′ nick only by interacting with the UvrB·DNA complex. This model would predict that Δ*uvrC* mutants should not accumulate single-strand nicks but that introduction of a plasmid carrying any of the four mutations D399A, D438A, D466A, and H538F should restore a *uvrC34*-like behavior *in vivo*. Fig. 4 shows alkaline sucrose gradient profiles of DNA of *E. coli* with either *UvrB5* or *uvrC34* mutation, or Δ*uvrC*, and its derivative carrying a plasmid with *uvrC*-H538F, following UV irradiation. In agreement with the literature, no nicks are formed in *E. coli uvrB5* (*A*) and nicks do accumulate in *uvrC34* (*B*). In contrast, *E. coli* Δ*uvrC* behaves nearly identically to *E. coli uvrB5* (*C*). As predicted, introduction of a plasmid carrying *uvrC*-H538F mutation into the Δ*uvrC* strain results in a *uvrC34*-like DNA profile (*D*). It is reasonable to conclude, then, that *uvrC34* and *uvrC56* mutants make proteins (Tang *et al.*, 1991) which induce the 3′ incision by binding to UvrB.

DISCUSSION

Regarding the reaction mechanism of (A)BC excinuclease two questions are of interest: Which subunit(s) makes the incisions, and which amino acids are involved in catalysis? The results presented in this study and recapitulated below provide provisional answers to both questions and also provide a possible explanation for a curious phenomenon associated with *uvrC⁻* mutants.

1) Mutations in 4 residues of UvrC specifically inhibit the 5′ incision without affecting the 3′ incision. The mutations apparently do not cause a gross conformational change in UvrC because the mutant proteins have the same solubility and chromatographic properties as the wild type. Furthermore, the mutations must not affect the binding site because the 3′ incision which requires specific binding of UvrC to the UvrB·DNA complex occurs at a normal rate. Although, because of its sequential reaction mechanism and lack of catalytic turnover in the absence of PolI and helicase II, it would
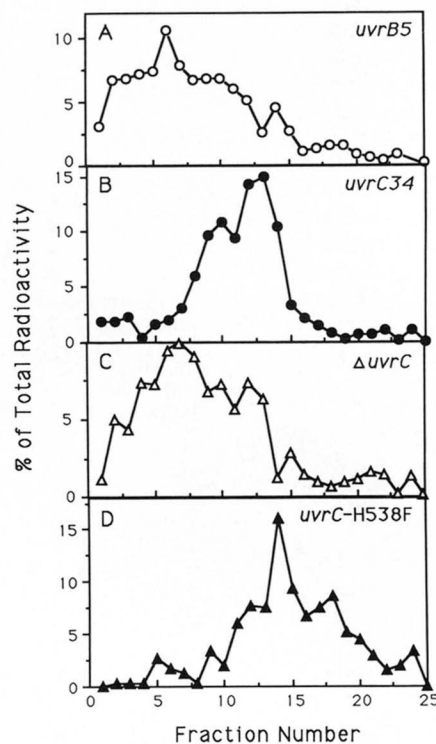

FIG. 4. **Alkaline sucrose gradient profiles of DNA from various *uvrC* mutant.** Following UV irradiation with 20 J/m² cells were incubated at 37 °C for 1 h and then lysed on top of an alkaline sucrose gradient. Following centrifugation, fractions were collected from the bottom, and radioactivity in each fraction was quantified and expressed as the percentage of total radioactivity. *A*, AB1885(*uvrB5*); *B*, AB1884(*uvrC34*); *C*, UNC3024(Δ*uvrC*); *D*, UNC3024(*uvrC*-H538F).

be difficult to apply standard Michaelis-Menten formalism to (A)BC excinuclease, normal 3′ incision might be interpreted to reflect wild type K_m for binding to UvrB·DNA by mutant UvrCs, and the four mutations might justifiably be called UvrC V_{max} mutations. Thus, we propose that UvrC makes the 5′ incision and that these 4 residues are directly involved in catalysis. The possibility that some UvrB residues may also participate in 5′ incision cannot be eliminated based on currently available data alone. However, considering that 4 amino acid residues of UvrC are required for the reaction we think the direct participation of additional amino acids in catalysis to be unlikely. Similarly, we consider unlikely a "straw man" model, whereby the UvrC mutants that abolish 5′ nicking catalyzed by UvrB do so by keeping UvrB from attaining proper conformation, because many UvrC mutants

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with demonstrable deficiencies in their interactions with UvrB interfered with both 3' and 5' nicking but never with 5' nicking alone (Lin and Sancar, 1991).

Our data indirectly implies that UvrB makes the 3' incision for the following reason. First, when incision reactions are carried out with aged UvrC which has greatly diminished 5' incision activity, near-normal 3' incision is observed (Selby and Sancar, 1988) indicating that oxidation (perhaps of H538) or denaturation that caused UvrC to lose its 5' incision activity did not interfere with its binding to the UvrB·DNA complex and triggering the 3' incision by inducing a conformational change in one or both components of the complex. Second, mutagenesis of all conserved amino acids in UvrC with general acid-base properties (D, E, H) failed to affect the 3' incision site. Finally, the UvrB mutant (E639A) and UvrB* (Arikan *et al.*, 1986) with about 0.1% of wild type 3' and 5' incision activity can be loaded onto 3'-incised DNA with UvrA; upon addition of UvrC to these pre(3')-incised DNA· UvrB(UvrB*) complexes normal 5' incision takes place, suggesting that the defect in these mutants is the formation of the 3' incision which is a prerequisite for the conformational change in the UvrB·UvrC·DNA complex that enables UvrC to make the 5' incision (Lin *et al.*, 1992).

2) Assuming that UvrC makes the 5' incision it is desirable to know the precise roles of the active site residues. Of the 4 such residues we have identified H538 appears to be the least important as the nonconservative H → D change at this position resulted only in a 10-fold decrease in activity. This result excludes the possibility of a general acid-base catalysis role for this residue. Considering that 2 polar residues at this position were compatible with activity it is possible that H538 contributes to catalysis by hydrogen bonding to substrate or the transition state either directly or through water molecules and that substitutions which do not exclude water from the active site would be compatible with activity.

In contrast, nonconservative changes in any of the three positions D399, D438, and D466 completely abolish activity. Based on crystal structures of nucleases, three not necessarily exclusive roles have been ascribed to acidic residues in nucleases: hydrogen bonding to His in a catalytic triad such as in DNase I (Lahm *et al.*, 1991), acting as a general acid-base catalyst by directly activating a water molecule such as in RNase H (Nakamura *et al.*, 1991), or liganding metal ions in the active site and thus enabling them to interact with water to generate the attacking hydroxide ion as in 3' → 5' exonuclease of Klenow fragment and P1 nuclease (Beese and Steitz, 1991; Volbeda *et al.*, 1991). The behavior of H538D mutation makes the first mechanism unlikely for UvrC. With regard to the latter two mechanism; it is known that UvrC does not contain zinc (data not shown) but incision by UvrB·UvrC requires ATP (or ATPγS) and Mg^{2+} (Orren and Sancar, 1990). Thus, it is conceivable that Mg^{2+} is involved in catalysis by UvrC as is the case for RNase H. However, our data do not have the resolution to discriminate between mechanisms involving metal-activated or directly carboxylic acid-activated water molecules. Regardless of the details of the actual functions of carboxylate groups in UvrC, it is becoming quite evident that two to three carboxylate groups in active sites of nucleases are a recurring theme (Xu and Schildkraut, 1991; Thielking *et al.*, 1991; Saenger, 1991) and therefore we are

quite confident that D399, D438, and D466 are involved in catalysis by UvrC.

3) Finally, the identification of UvrC mutants which lack the 5' incision activity but which enable (or in association with) UvrB to make the 3' incision has helped explain the slow accumulation of nonproductive nicks in *uvrC34* and *uvrC56* mutants following UV irradiation without measurable increase in cell survival (Tang and Ross, 1985). Our results suggest that these two mutations inactivate the 5' incision activity of UvrC without interfering with its binding to UvrB· DNA and promoting the 3' incision which does not result in adduct removal. Isolation of the two mutant proteins in sufficient quantities for *in vitro* assays would be necessary to test this prediction.

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