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Isolation and Characterization of Functional Domains of UvrA†

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ABSTRACT: The sequence of *Escherichia coli* UvrA protein suggests that it may fold into two functional domains each possessing DNA binding and ATPase activities. We have taken two approaches to physically isolate polypeptides corresponding to the two putative domains. First, a 180 base pair DNA segment encoding multiple collagenase recognition sequences was inserted into UvrA's putative interdomain hinge region. This UvrA derivative was purified and digested with collagenase, and the resulting 70-kDa N-terminal and 35-kDa C-terminal fragments were purified. Both fragments possessed nonspecific DNA binding activity, but only the N-terminal domain retained its nucleotide binding capacity as evidenced by measurements of ATP hydrolysis and by ATP photo-cross-linking. Together, the two fragments failed to substitute for UvrA in reconstituting (A)BC excinuclease and, therefore, were presumed to be unable to load UvrB onto damaged DNA. Second, the DNA segments encoding the two domains were fused to the β-galactosidase gene. The UvrA N-terminal domain–β-galactosidase fusion protein was overproduced and purified. This fusion protein had ATPase activity, thus confirming that the amino-terminal domain does possess an intrinsic ATPase activity independent of any interaction with the carboxy terminus. Our results show that UvrA has two functional domains and that the specificity for binding to damaged DNA is provided by the proper three-dimensional orientation of one zinc finger motif relative to the other and is not an intrinsic property of an individual zinc finger domain.

UvrA, which is one of the three subunits of *Escherichia coli* (A)BC excinuclease (Sancar & Sancar, 1988; Selby & Sancar, 1990), is an ATPase and a DNA binding protein with higher affinity for damaged than for undamaged DNA (Seeberg & Steinum, 1982). Analysis of the amino acid sequence of UvrA (Husain et al., 1986) has revealed 3 ATPase A and B segments interspersed with 3 zinc finger motifs each of 21–23 amino acids (Doolittle et al., 1986). It appears that the second zinc finger motif is degenerate and, therefore, has lost its zinc chelating capacity; thus, UvrA contains only 2 mol of Zn^{2+} per monomer apparently chelated by the first and third motifs (Navaratnam et al., 1989). Similarly, of the three pairs of ATPase A and B segments, B1 and A2 appear to be severely truncated (Doolittle et al., 1986). Indeed, mutagenesis of the "invariant" Lys residue in the A2 segment and the invariant Asp residue in the B1 segment had no effect on the protein's in vivo activity. This has led to the proposal that the two ATPase activities of UvrA were made up of A1–B2 and A3–B3 segments, respectively (Myles et al., 1991), and that each of these ATPase units was associated with a zinc finger, A1–B2 with Zn1 and A3–B3 with Zn3, to constitute two domains each with its own DNA binding and ATPase activities.

In this paper, we describe the use of genetic engineering methods to isolate fragments of UvrA containing A1–Zn1–B2 (70 kDa) and A3–Zn3–B3 (35 kDa) sequences. Our results show that each of these fragments constitutes a domain with nonspecific DNA binding activity but that only the A1–Zn1–B2 domain possesses ATPase activity when not physically linked to the carboxy-terminal A3–Zn3–B3 domain.

Materials and Methods

Materials. The wild-type UvrA, UvrB, and UvrC proteins were purified as described previously (Thomas et al., 1985). Collagenase from *Achromobacter iophagus* was purchased from Boehringer Mannheim Biochemicals; it was suspended at 0.12 mg/mL in TEN 7.4 and stored at 4 °C. *Nco*I was obtained from Promega; T4 kinase, bacterial alkaline phosphatase, *Bam*HI, and *Sau*3A were from Bethesda Research Laboratories; and T4 ligase and T4 DNA polymerase were from Bio-Rad.

[2,8-^{3}H]ATP (30 Ci/mmol), [α-^{32}P]ATP (3000 Ci/mmol), and [γ-^{32}P]ATP (7000 Ci/mmol) were from ICN Biomedicals, Inc.; [^{3}H]thymidine (82.4 Ci/mmol) was from New England Nuclear.

Single-strand DNA–cellulose and heparin–agarose affinity resins were from Sigma, AcA-34 gel filtration resin was from IBM Biotechnics, and DEAE-Bio-Gel was from Bio-Rad.

Silver-staining reagents were purchased from Bio-Rad, IPTG and ONPG were from Boehringer Mannheim Biochemicals, and the TLC plates (Polygram cel300 PEI) were from Brinkmann Instruments.

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The *E. coli* strains NM522 and its *uvrA*⁻ derivative UNC522 as well as CJ236 and the plasmid pUNC1940 have been described (Myles et al., 1991). The plasmid pJG200 (Germino & Bastia, 1984) was a generous gift of Dr. D. Bastia (Duke University). All the buffers have been described elsewhere (Myles et al., 1991).

Site-Directed Mutagenesis and Gene Fusions. To make an insertion derivative of UvrA with a collagenase recognition site (UvrA 60-col), we introduced, by site-specific mutagenesis (Myles et al., 1991), a *Bam*HI restriction site into the interdomain hinge coding region of *uvrA*. The mutagenesis reaction was carried out by the method of Zoller and Smith (1983) using uracil-containing pUNC1940 as template (Kunkel et al., 1987) and an oligonucleotide with the sequence 5′-CGGATTCGCCGGATCCCGTTTCTTCGG-3′. The appropriate constructs were identified by screening minipreps by restriction enzyme digestion. The new plasmid was named pUNC1940B. The 180 bp *Bam*HI–*Sau*3A collagen coding segment was isolated from pJG200 and inserted into the *Bam*HI site of pUNC1940B to obtain pUNC1940BCol. The *Nco*I–*Pst*I fragment of this plasmid, which carries the entire *uvrA* gene with the collagen insert, was ligated into pKK233-2 to obtain pUNC1941BCol which overproduces UvrA 60-col upon induction with IPTG.

To construct UvrA–β-galactosidase fusion plasmids, we first modified the collagen–β-galactosidase fusion plasmid pJG200 by replacing the λ P_R promoter with the *tac* promoter from pKK233-2 to obtain pJG200–tac. The UvrA N-terminal domain–β-galactosidase fusion containing the 605 N-terminal amino acids of UvrA was constructed by ligating the *Nco*I–*Bam*HI segment from pUNC1940B into pJG200–tac linearized with *Nco*I and *Bam*HI. The resulting plasmid was named pUNC200N. To isolate a fusion containing the C-terminal domain of UvrA, first *Nco*I and *Bam*HI sites were introduced, by site-specific mutagenesis, at the interdomain hinge and 3′ end of *uvrA*, respectively, to create pUNC1940C. The resulting plasmid was digested with *Nco*I and *Bam*HI to obtain the DNA fragment encoding the C-terminal 335 amino acids of UvrA. This fragment was ligated into pJG200–tac linearized by *Nco*I and *Bam*HI to create pUNC200C. Note that both pUNC200N and pUNC200C contain the *uvrA* sequences 5′ to the collagen–β-galactosidase sequence and as a consequence the fusion proteins in both cases contain UvrA sequences at the N-termini and β-galactosidase sequences at the C-termini.

Purification of UvrA Domains and Fusion Proteins. UvrA 60-col was purified from UNC522/pUNC1940BCol cells grown at 26 °C and induced with IPTG for 8–12 h; the cells were lysed, and UvrA 60-col was purified from the 100000*g* supernatant by successive chromatography on single-strand DNA–cellulose and AcA-34 gel filtration resins. Part of the insertion protein was stored at −80 °C while the rest was digested with collagenase. To purify the two domains, 50 μg of collagenase was added to 8 mL of purified UvrA 60-col in 0.1 M KCl/core buffer and incubated at 4 °C for 1 h; the mixture was then dialyzed into 2 M KCl/core and loaded onto a 500-mL AcA-34 column equilibrated with 1 M KCl/core. The column was developed at a rate of 12 mL/h, and 4-mL fractions were collected. Peak fractions corresponding to the N- and C-terminal domains were concentrated by ultrafiltration and stored at −80 °C. The native molecular weights of the N- and C-terminal domains were determined by gel filtration chromatography on a 120-mL AcA-34 column equilibrated with 0.3 M KCl/core buffer at a concentration of 1.1 μM for both domains.

Of the fusion proteins, only the one carrying the N-terminal 605 amino acids of UvrA overproduced. This protein was purified as follows: *E. coli* UNC522/pUNC200N was grown at 26 °C and induced with IPTG, and the fusion protein was precipitated from the 100000*g* supernatant of cell-free extract with 28% saturated ammonium sulfate (Miller, 1972). The protein was then purified by sequential chromatography on DEAE-Bio-Gel and heparin–agarose columns.

Assays. In vivo complementation by the UvrA 60-col insertion mutant was tested by plating dilutions of UNC522 (*uvrA*⁻) containing the appropriate plasmids and irradiating the plates with 15 J m^{-2} from a germicidal lamp. The surviving fraction is the ratio of colonies formed after UV irradiation to colonies formed without irradiation.

ATP hydrolysis was measured as described previously (Myles et al., 1991; Scott et al., 1977).

DNA binding by UvrA derivatives was probed by gel retardation and DNase I footprinting. For gel retardation, a terminally labeled 107 bp DNA fragment (unirradiated or irradiated with 2500 J m^{-2}) was used. The fragment (1500 cpm) was incubated with the appropriate UvrA derivative in 25 μL of (A)BC excinuclease buffer supplemented with 10% glycerol and 2 mM ATP. DNA–protein complexes were separated on a 5% polyacrylamide gel which was then dried and autoradiographed. DNase I footprinting with a 137 bp DNA fragment containing a single psoralen monoadduct was conducted as described previously (Van Houten et al., 1987; Myles et al., 1991).

The incision assay for (A)BC excinuclease activity was conducted as described before (Sancar & Rupp, 1983) except the reaction mixture (160 μL) contained 10 nM UvrA, 300 nM UvrB, 70 nM UvrC, and 1.6 μg of pBR322 in (A)BC excinuclease buffer; 25-μL aliquots were taken at the specified time points.

ATP Photo-Cross-Linking. UvrA 60-col was incubated with [α-^{32}P]ATP in (A)BC excinuclease buffer (100 μL) on ice (to minimize ATP hydrolysis) and irradiated for 20 min with 150 μW/cm^2 of 254-nm light from a germicidal lamp. Following the irradiation, 20 μg of calf thymus DNA was added as carrier, and the protein was precipitated with 1 mL of ice-cold 20% trichloroacetic acid. The protein was collected by centrifugation, rinsed with 95% ethanol, suspended in 65 μL of SDS-loading dyes containing 12% glycerol and 75 mM NaOH, heated to 95 °C for 5 min, and electrophoresed on a 10% SDS–polyacrylamide gel (Laemmli, 1970). The gel was silver-stained, photographed, and then dried and autoradiographed. Where indicated, prior to the precipitation step, collagenase was added to 6 μg/mL, and the reactions were incubated at 4 °C for 20 min.

Results

Isolation of N- and C-Terminal Domains of UvrA. The strategy for isolation of functional domains of UvrA is illustrated in Figure 1. The plasmid pJG200 constructed by Germino and Bastia (1984) carries a *Bam*HI–*Sau*3A fragment which encodes a 60 amino acid peptide from the chicken collagen gene; this peptide contains several collagenase cleavage sequences. A *Bam*HI site was created in the interdomain region of UvrA by site-directed mutagenesis in pUNC1940. The collagen gene segment isolated from pJG200 was ligated into this site (between the 1815 and 1816 bp of the coding sequence), and the entire *uvrA* gene was subcloned into the expression vector pKK233-2. The insertion protein, UvrA 60-col, was overproduced and purified by two chromatographic steps (Figure 2). The purified protein was cleaved with collagenase into two fragments of 70 and 35 kDa (Figure 2,

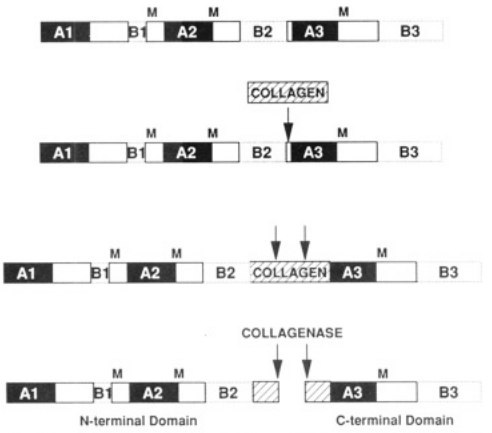

FIGURE 1: Strategy for the isolation of UvrA's functional domains. The top figure shows schematically the structural organization of the UvrA protein: three nucleotide binding motifs consisting of ATPase A and B segments (which contain Walker's A and B sequence, respectively) are interspersed with three metal (zinc) binding motifs (M) of which the middle one is truncated. A *Bam*HI restriction site was incorporated, by site-directed mutagenesis, within the coding segment for UvrA's putative interdomain hinge region. The 180-nucleotide *Bam*HI–*Sau*3a fragment from the chicken collagen gene was isolated from pJG200 and ligated into UvrA's *Bam*HI site to encode UvrA 60-col. This protein is uniquely susceptible to collagenase digestion at UvrA's nonfunctional hinge region. Such protease treatment yields two discrete polypeptide fragments; the amino-terminal domain is ~70 000 Da while the carboxy terminus is ~35 000 Da.

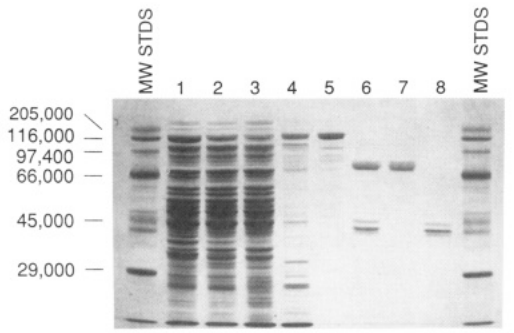

FIGURE 2: Overproduction and purification of UvrA 60-col and UvrA's two functional domains. Samples from major steps in the purification of UvrA 60-col and the isolation of its constituent domains were electrophoresed on a 10% SDS–polyacrylamide gel which was stained with Coomassie blue. Lane 1 is 5 μL of cells taken immediately after collecting and sonicating. Lanes 2 and 3 are 5 μL each of supernatant from 12 000 and 35 000 rpm spins, respectively. Lane 4 is 2 μL of the peak fractions from single-strand DNA–cellulose chromatography. Lane 5 is 3 μL of the peak fractions from gel filtration on AcA-34. Lane 6 is protein from lane 5 after treatment with collagenase. Lanes 7 and 8 are 10 μL of the two protein peaks from a second AcA-34 gel filtration performed immediately following collagenase treatment of the protein peak from lane 5. MW STDS, protein molecular weight standards.

lane 6), and the two polypeptides were separated by gel permeation chromatography on AcA-34 resin to obtain the pure N- and C-terminal fragments of UvrA (Figure 2, lanes 7 and 8, respectively). Collagenase cleavage is specific for the insertion protein UvrA 60-col. Under the conditions used to achieve total cleavage of the insertion protein, no detectable cleavage of the wild-type UvrA protein was observed (data not shown). It is also significant that the effective separation of the two domains by gel permeation chromatography suggests that there is minimal noncovalent association between the N-terminal and C-terminal halves of the protein.

DNA Binding of the Domains. The UvrA 60-col protein complemented the *uvrA*⁻ mutations to near wild-type level,

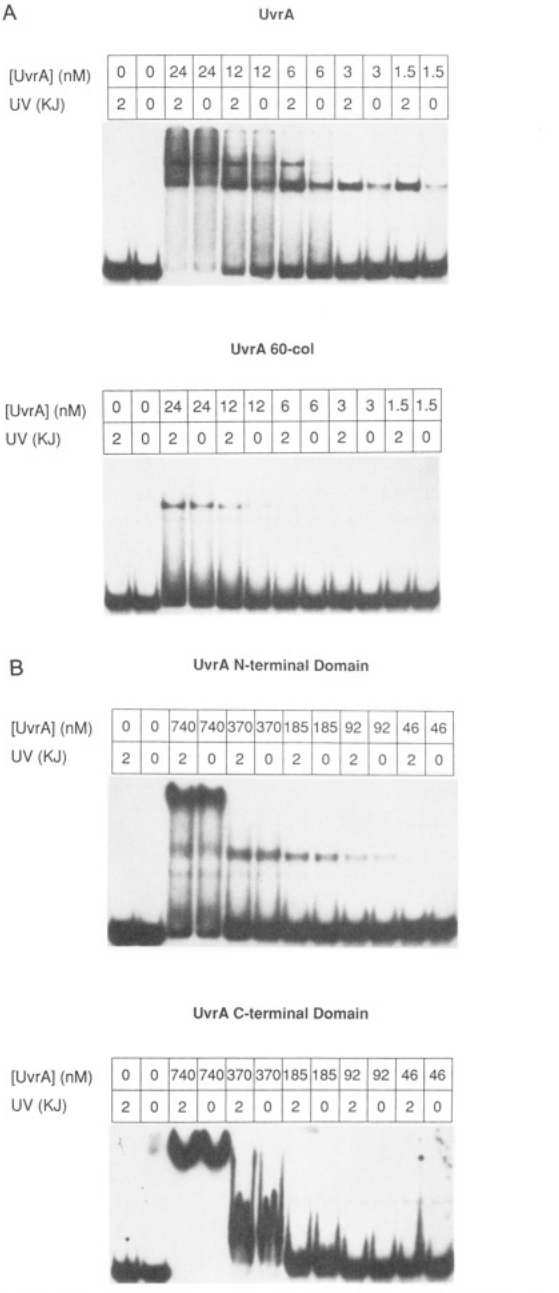

FIGURE 3: (A) Gel retardation analysis of UvrA and UvrA 60-col. 1500 cpm of 5′-^{32}P-end-labeled fragment DNA was incubated at room temperature for 15 min with UvrA or UvrA 60-col at the concentrations indicated (1.5–24 nM). Twenty-five-microliter reactions were in (A)BC excinuclease buffer plus 10% glycerol and 2 mM ATP. The entire sample was loaded onto a prerun 5% nondenaturing polyacrylamide gel run at 100 V for 2.5 h at room temperature. The gels were dried and autoradiographed overnight at −80 °C. (B) Gel retardation analysis of UvrA's amino- and carboxy-terminal domains. Reactions were performed and gels run as described in (A) with the exception that protein concentrations range from 46 to 740 nM. KJ, kilojoules per square meter.

an observation which is consistent with our prediction that the insertion is not in a functionally important active site. Surprisingly, however, in vitro this protein had a substantially compromised DNA binding affinity as compared to wild-type UvrA. Figure 3A shows a comparison of DNA binding of wild-type UvrA with UvrA 60-col by gel retardation. The insertion protein has about 5-fold lower nonspecific DNA binding affinity than does the wild-type protein. Also, UvrA 60-col has virtually identical affinity for damaged and undamaged DNA. This is not the result of an adverse effect of the insertion on the overall structure of UvrA as the mutant

protein has ATPase K_m and V_{max} values nearly identical with those of wild-type UvrA (data not shown). Also, the insert is far removed from each of the three Zn fingers and, therefore, does not directly perturb any of these motifs. A possible explanation for the selective effect of the insertion on DNA binding is that the relative orientation of the two zinc fingers is important for binding to DNA with high affinity and specificity; the insertion could adversely affect this property of the protein by acting at a distance to alter the alignment of the Zn fingers.

Such an explanation is entirely consistent with the DNA binding activities of the isolated domains (Figure 3B). Both polypeptides bind to DNA nonspecifically and at approximately $^1/_{20}$th the affinity of wild-type UvrA to undamaged DNA. The quality of the binding interactions of the two domains does differ, however, in that the N-terminal domain apparently binds with a defined stoichiometry as suggested by the appearance of a band of intermediate migration. At high protein concentrations, the N-terminal domain coats the DNA, resulting in a band near the gel origin. The C-terminal domain causes a smearing of the DNA band at low concentrations apparently because of the high off-rate of complexes formed with this domain; at high concentrations, this fragment too causes severe retention of the DNA band by cooperative binding. Thus, the experiments with the N- and C-terminal fragments lead us to conclude that these UvrA protein fragments (each of which contains one zinc finger) constitute functional domains capable of folding properly and of binding to DNA with a reasonably high affinity.

ATPase Activity of the Domains. The data described thus far are consistent with our model that UvrA is comprised of two functional domains. Having demonstrated that both domains bind to DNA, we next wished to determine whether each domain has an ATPase activity as well. First, UvrA 60-col was digested to completion with collagenase, and the activity of the cleaved protein was compared to that of the undigested enzyme (Figure 4A). Cleavage of the protein into two domains resulted in about 3–4-fold reduction in the rate of ATP hydrolysis. Under the assay conditions, the amount of intact protein was less than 1% of the control (data not shown), and, therefore, we consider the residual ATPase to be intrinsic to one or both of the domains. To quantify the contribution of the individual domains to this activity, the purified polypeptides were tested separately. The results shown in Figure 4B suggest that the N-terminal fragment does have an intrinsic ATPase activity. Although a low level of ATP hydrolysis was observed with the C-terminal fragment as well, we cannot eliminate the possibility of ATP hydrolysis by a minor ATPase contaminant in the C-terminal domain preparation. We conclude, therefore, that only the N-terminal domain is an ATPase. The low level of ATP hydrolytic activity by the N-terminal domain is in apparent contradiction of the data shown in Figure 4A where the collagenase-digested UvrA 60-col yielded much higher ATPase activity compared to the purified N-terminal fragment. We considered the possibility that following the collagenase digestion, the two domains remain associated noncovalently and that such an association might be responsible for the ATPase function of the C-terminal domain or for increased catalytic efficiency of the N-terminal fragment. This was tested by conducting the ATPase assay with a mixture of the two domains. However, the mixture had no greater than additive activity (Figure 4A,B); therefore, the greatly reduced activity in the N-terminal domain compared to the unfractionated domains cannot be explained by activation through noncovalent association with the C-terminus.

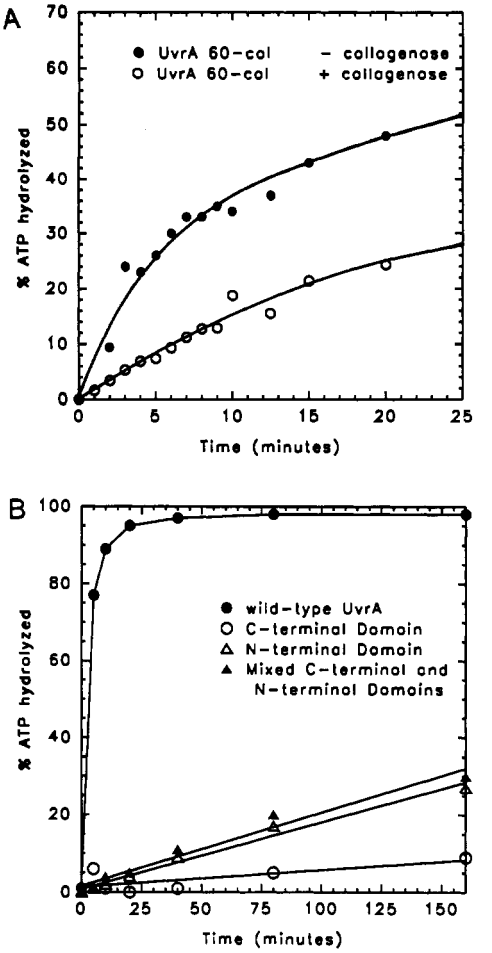

FIGURE 4: (A) ATPase activity, before and after treatment of UvrA 60-col with collagenase. 6.3 μg of UvrA 60-col was incubated for 90 min on ice in 42 μL of collagenase or TEN 7.4. Ten microliters of each reaction was adjusted to 50 μL with ABC buffer and 1 μL of [^{3}H]ATP; 2.5 μL of each reaction was spotted on PEI–cellulose at the times indicated. (B) ATPase activity of amino-terminal and carboxy-terminal domains before and after mixing. Fifty-five-microliter reactions containing ~840 nM each of UvrA, UvrA amino terminus, and UvrA carboxy-terminus or ~840 nM each of UvrA amino terminus and carboxy terminus, 1 μCi of [^{3}H]ATP, 50 μg/mL BSA, and (A)BC excinuclease buffer were incubated at room temperature. Two-microliter aliquots were removed at the times indicated, spotted on TLC plates, and processed as described under Materials and Methods.

We believe that the ATPase activity in the digested UvrA 60-col is exclusively due to the N-terminal domain and that the decreased activity in the purified domain is the result of inactivation during preparative steps.

We have previously shown (Myles et al., 1991) that dimerization is essential for the ATPase activity of the UvrA protein. We, therefore, would predict that because the N-terminal domain has ATPase activity it must also be a dimer. Indeed, when the native molecular weight was determined by gel filtration chromatography, we obtained a value of 126 000. This value is in excellent agreement with the expected molecular weight of 140 000 for a dimer of the N-terminal domain (data not shown). Interestingly, under identical experimental conditions, the C-terminal fragment behaved as a monomer.

To provide further evidence for the proposed assignment of ATPase active site(s), we conducted photo-cross-linking experiments. UvrA 60-col was mixed with [α-^{32}P]ATP, irradiated at 4 °C, and digested with collagenase. The two fragments were then separated on an SDS–polyacrylamide gel which was silver-stained, photographed, dried, and autoradiographed (Figure 5). UvrA 60-col was cross-linked to

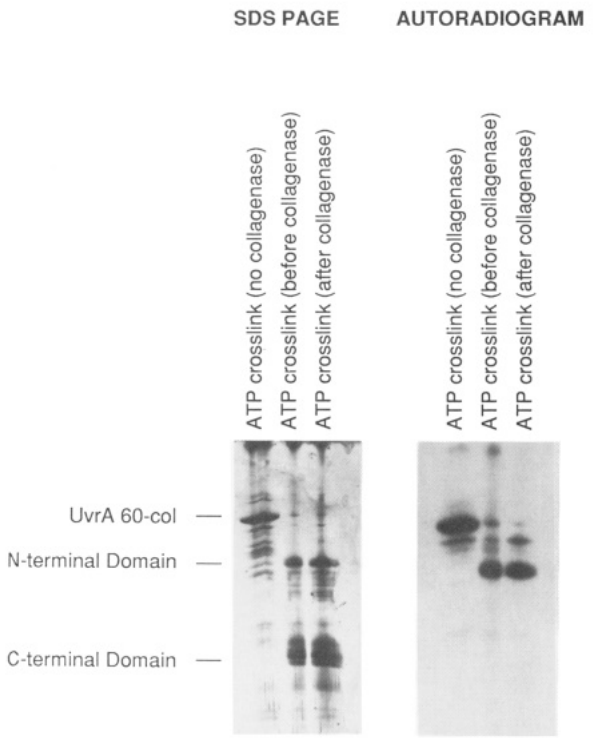

FIGURE 5: [^{32}P]ATP photo-cross-linking of UvrA 60-col before and after collagenase treatment. Three identical 100-μL reactions contained 15 μg of UvrA 60-col and 50 μCi of [α-^{32}P]ATP. Reactions 1 (left) and 2 (middle) were irradiated with 150 μW/cm^2 of 254-nm light from a germicidal lamp after which 0.6 μg of collagenase was added to reaction 2 only and incubated for 30 min on ice. To reaction 3 (right) was added 0.6 μg of collagenase; the reaction was incubated on ice for 30 min followed by UV irradiation as above for reactions 1 and 2. Protein was precipitated with 1 mL of 20% TCA using 20 μg of calf thymus DNA as carrier. The precipitate was suspended in SDS loading dyes (plus NaOH and glycerol) and electrophoresed on a 10% SDS–polyacrylamide gel. The left panel is the silver-stained SDS gel, and the right panel is an autoradiogram of the same gel.

[α-^{32}P]ATP efficiently, and upon cleavage with collagenase, the majority of the label was found to be associated with the N-terminal domain while no ^{32}P was incorporated within the C-terminal fragment. If UvrA 60-Col was digested prior to [α-^{32}P]ATP photo-cross-linking, the N-terminal domain was still labeled and with equivalent efficiency as compared to the uncut protein. Furthermore, cross-linking was inhibited by low concentrations of cold ADP, indicating that binding of ATP to the N-terminal domain is specific both in the intact protein and in the isolated fragment (data not shown). Thus, we conclude that under conditions which favor binding but not hydrolysis (i.e., 4 °C) ATP binds exclusively to the N-terminal domain.

These data are of particular significance in light of our finding that while UvrA has two apparent ATPase active sites, ADP binds with a stoichiometry of only 1 mol/mol of UvrA dimer (Myles et al., 1991). The experiments described in that report did not, however, unambiguously reveal to which of the two potential sites ATP and ADP bound. The results presented in this study clarify this uncertainty by showing that under nonhydrolytic conditions, ADP binds only to the N-terminal domain. Furthermore, inhibition of labeling of the N-terminal domain by ADP does not result in labeling of the C-terminus (data not shown), indicating that binding of ADP to the N-terminus is not sufficient to induce ATP binding to the C-terminus.

Nuclease Complementation. To determine whether either or both of the domains together can function in complementing the (A)BC excinuclease activity, we conducted incision assays

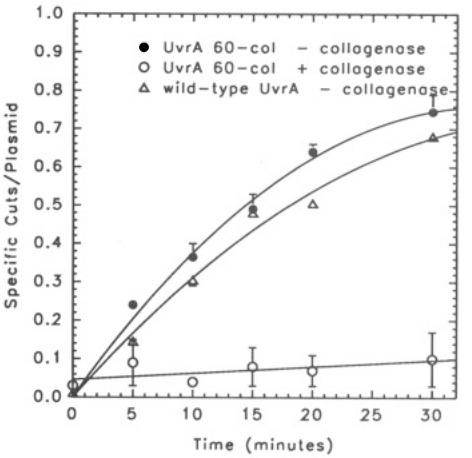

FIGURE 6: Plasmid incision activities of UvrA 60-col before and after treatment with collagenase. Three 42-μL reactions containing (1) 7.6 μg of UvrA 60-col in (A)BC excinuclease buffer, (2) 7.6 μg of UvrA 60-col in (A)BC excinuclease buffer and 0.25 μg of collagenase, and (3) 7.6 μg of UvrA in (A)BC excinuclease buffer were incubated at 4 °C for 90 min. Twenty-five microliters of each reaction was mixed with SDS-loading dyes and electrophoresed on a 10% SDS–polyacrylamide gel to ensure complete collagenase digestion (data not shown). Six 160-μL plasmid incision reactions containing 9 nM UvrA 60-col (+collagenase treatment) or 9 nM UvrA (−collagenase), 300 nM UvrB, 70 nM UvrC, 10 μg/mL ^{3}H-pBR322 (irradiated or nonirradiated), and 2 mM ATP in (A)BC excinuclease buffer were incubated at room temperature. At the times indicated, 25 μL of each reaction was quenched by the addition of loading dyes containing 0.2% SDS and held at −80 °C until electrophoresed on a 0.87% agarose gel. The bars represent the standard deviation of three experiments.

with UV-damaged plasmid DNA. The results shown in Figure 6 indicate that the insertion mutant, in contrast to its DNA binding activity, is virtually indistinguishable from the wild-type protein in the incision assay. This is in agreement with other data from our laboratory which suggest that the damage recognition unit of (A)BC excinuclease is the $(UvrA)_2(UvrB)_1$ complex and not the UvrA dimer (Bertrand-Burggraf et al., 1991). Apparently, the diminished affinity of the UvrA insertion mutant for damaged DNA had little effect on the ability of the true recognition complex to bind to damage, and to load UvrB. Treatment of UvrA 60-col with collagenase, however, completely abolished the nuclease complementing activity of this protein. This allows us to make two conclusions. First, the ATPase activity in collagenase-treated UvrA 60-col was not due to uncleaved protein. Second, neither of the two domains is capable of loading UvrB onto a damage site, an essential step for incision. Thus, it is clear that UvrA can be separated into two structural domains: the N-terminal one having DNA binding and ATPase functions and the C-terminal one having only DNA binding activity. However, covalent association of the two domains is essential to achieve UvrA-mediated loading of UvrB onto damaged DNA.

β-Galactosidase Fusions. Although we ascribe the ATPase activity in collagenase-treated UvrA 60-col to the N-terminal domain, there is much less activity associated with the purified domain as compared to the wild-type protein, thus raising the possibility that the ATPase activity in the collagenase digest was due to the N-terminal domain associated noncovalently with the C-terminal domain. Although the C-terminus does not stimulate the N-terminus' ATPase activity in mixing experiments, we wished to verify a bona fide ATPase activity associated with the N-terminal domain and perhaps the C-terminal domain by independent methods. Toward this goal, the gene segments corresponding to each domain were fused to the β-galactosidase gene as shown in Figure 7. The C-terminal domain–β-galactosidase fusion protein was not ov-

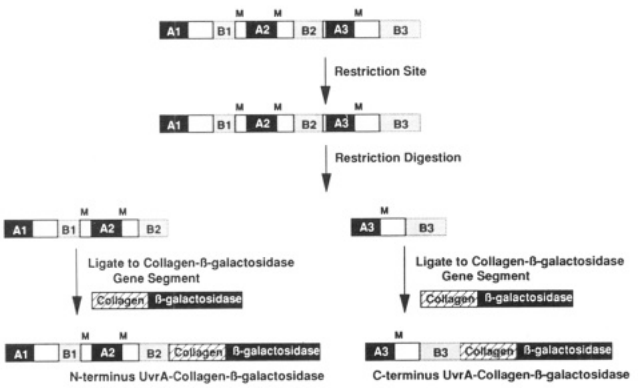

FIGURE 7: Schematic representation of the construction of UvrA domain–collagen–β-galactosidase fusion proteins. A restriction site (*Bam*HI for the amino-terminal domain fusion and *Nco*I for the carboxy-terminal domain fusion) was incorporated into the coding region of the UvrA gene by site-directed mutagenesis. Also, in the case of the carboxy-terminal domain fusion, a *Bam*HI site was inserted precisely at the 3′ end of UvrA's coding region. For both the amino-terminal and carboxy-terminal domain constructs, the respective *Nco*I–*Bam*HI gene segments were ligated in frame 5′ to the collagen–β-galactosidase coding region of pJG200-tac (see Materials and Methods).

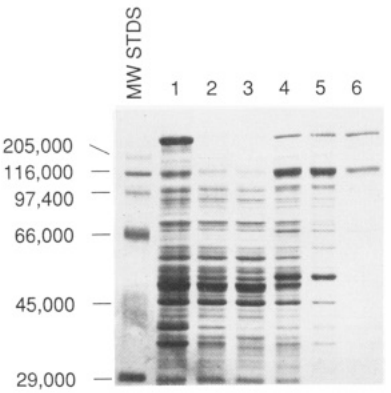

FIGURE 8: Overproduction and purification of the amino-terminal domain–collagen–β-galactosidase fusion protein. Samples at major steps in the purification of the amino-terminal domain fusion protein were electrophoresed on a 10% SDS–polyacrylamide gel which was stained with Coomassie blue. Lane 1 is 5 μL of overproducing cells immediately after sonication. Lanes 2 and 3 are 5 μL each of the supernatants after 30-min centrifugation at 12 000 rpm and 60-min centrifugation at 35 000 rpm, respectively. Lane 4 is 5 μL of 28% ammonium sulfate precipitate suspended in 0.1 M KCl/core. Lane 5 is 10 μL of pooled peak fractions off DEAE-Bio-Gel. Lane 6 is 20 μL of pooled peak fractions off heparin–agarose. The top band in lanes 1 and 4–6 is the fusion protein, and the band at 116 kDa is β-galactosidase.

erproduced and, therefore, could not be analyzed. However, the N-terminal fusion protein was overproduced and soluble to a level which permitted its purification as shown in Figure 8. Because of the affinity between β-galactosidase monomers and the N-terminal domain–β-galactosidase fusion protein, approximately equal moles of β-galactosidase and the fusion protein copurify in our purification scheme which is based on chromatographic properties of β-galactosidase (Figures 8, lane 6).

The ATPase activity in the purified protein was comparable to that obtained with the collagenase-digested insertion protein. To demonstrate that this ATPase activity was intrinsic to the fusion protein and not due to an ATPase which copurifies with the UvrA β-gal fusion peptide, we wished to demonstrate that the fusion protein elution profile closely matched the ATPase elution profile in a quantitative manner. The purified protein was passed through a gel filtration and an ion-exchange

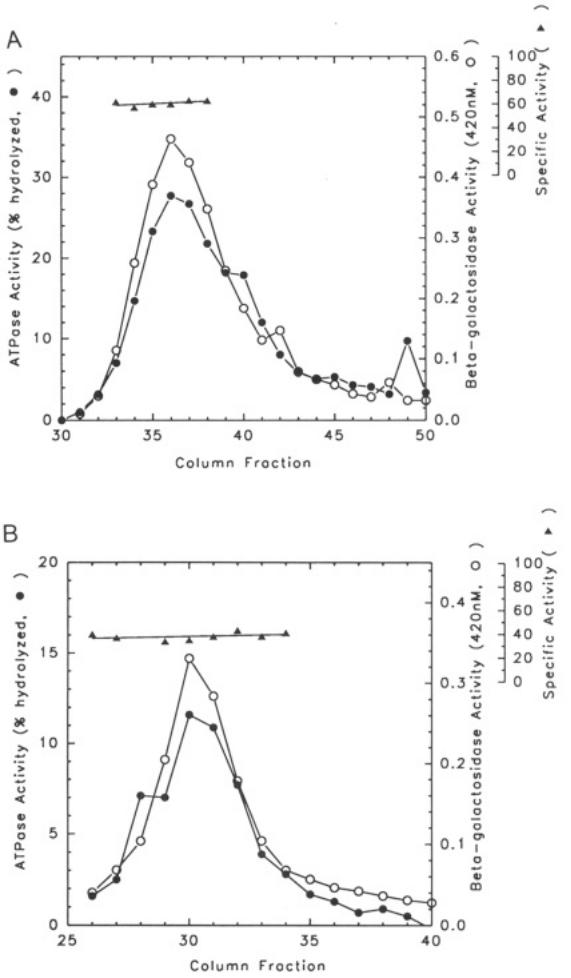

FIGURE 9: Correlation of the amino-terminal domain–collagen–β-galactosidase's ATPase and β-galactosidase activities. (A) A 30-mL column of AcA-34, equilibrated with 0.5 M KCl/core at 4 °C, was loaded with 80 μg of the amino-terminal domain–collagen–β-galactosidase fusion protein dialyzed against the same buffer. The column was pumped at 1.5 mL/h, and 0.5-mL fractions are collected. ATPase activity was assayed in a 75-μL reaction containing 50 μL of each fraction, ATPase buffer, 10 μM ATP, and 1 μCi of [^{3}H]ATP (30 Ci/mmol). Reactions were incubated at room temperature for 60 min and chromatographed on PEI–cellulose. β-Galactosidase activity was measured by mixing 50 μL of each fraction with 200 μL of 4 mg/mL *o*-nitrophenyl β-D-galactopyranoside (ONPG) and incubating at room temperature for 45 min. Each reaction was quenched by the addition of 0.5 mL of 1 M Na_2CO_3, and color development was assayed spectrophotometrically by measuring the A_{420}. (B) Eighty micrograms of amino-terminal domain fusion protein was adsorbed to a 10-mL DEAE-Bio-Gel column equilibrated with 0.1 M KCl/core and washed with 50 mL of 0.1 M KCl/core. Protein was eluted by a series of 5-mL steps from 0.1 to 1.0 M KCl/core; 625-μL fractions are collected, and 50 μL of each was assayed as described for the AcA-34 fractionation. Specific activities for both (A) and (B) were determined by dividing the percent ATP hydrolyzed by the absorbance at 420 nm. Note that since only the fusion protein and its cleavage product, the full-length β-galactosidase, are present in the enzyme preparations analyzed, β-galactosidase activity resulting from α-complementation would not interfere with our assay. Also, neither the ion-exchange nor the gel permeation column separates β-galactosidase tetramers (native form) from the tetramers or dimers of the fusion protein. Therefore, the ATPase might actually be that of fusion protein tetramer in which the UvrA N-terminal domain is arranged in a head-to-head fashion.

column, each fraction was tested for ATPase and β-galactosidase activities, and an aliquot of each fraction was electrophoresed on an SDS–polyacrylamide gel to identify the protein peak for quantitative densitometry. The ATPase activity in each fraction was divided by the β-galactosidase activity to obtain values for specific activities throughout the elution

profile. We found excellent correlation between the β-galactosidase elution profile and the ATPase profile on two chromatography resins, i.e., AcA-34 gel filtration and DEAE anion-exchange resins (Figure 9A,B, respectively). The specific activity for ATP hydrolysis is constant over the peak of the elution profiles for each column. Therefore, we conclude that the N-terminal domain possesses an intrinsic ATPase activity independent of any interaction with the C-terminal domain.

Discussion

Sequence analysis by Doolittle et al. (1986) and our experiments with site-directed mutants (Myles et al., 1991) suggested that UvrA may be comprised of two functional domains each possessing DNA binding and ATPase activities. In this investigation, we have tested this prediction directly by isolating and characterizing polypeptides corresponding to the two putative domains.

Both the N-terminal domain of 70 kDa and the C-terminal domain of 35 kDa bind to DNA with high affinity but without the capacity to discriminate between damaged and undamaged DNA. Each domain is expected to have one zinc finger, and it is reasonable to assume that these zinc fingers confer the DNA binding affinity to each half of the UvrA protein and that the relative orientation of the zinc fingers in the intact protein confers the specificity for damaged DNA.

Of the two domains, only the N-terminal one appears to be able to function as an independent ATPase. This domain was found to hydrolyze and to cross-link to ATP when physically separated from the C-terminal domain. Further, when fused to β-galactosidase, the N-terminal domain retains its ATPase activity. In contrast, the purified C-terminal domain lacked ATPase activity, and when intact UvrA 60-col was cross-linked to ATP by UV irradiation, no label was incorporated into this domain. Furthermore, ADP binding to the N-terminal ATPase site also failed to make the C-terminal domain amenable to photo-cross-linking to ATP even though our kinetic data suggest that ADP has an allosteric effect on this protein (Myles et al., 1991) presumably by activating the C-terminal ATPase site. When considered in light of the results with the site-specific mutagenesis studies, these data are entirely consistent with UvrA having an N-terminal domain and a C-terminal domain each of which has the ability to bind to DNA by virtue of its zinc fingers and each having the potential to hydrolyze ATP via their Walker and B sequence containing segments.

Finally, our finding that the N-terminal domain is a dimer demonstrates that UvrA dimerizes in a head-to-head fashion. The observation that the C-terminus behaves as a monomer indicates that the N-terminal 605 amino acids carry the structural determinants necessary and sufficient for dimerization of the UvrA protein.

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