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Mutations in the *Escherichia coli* UvrB ATPase motif compromise excision repair capacity

(DNA repair/incision/ultraviolet/directed mutagenesis)

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ABSTRACT **The *Escherichia coli* UvrB protein possesses an amino acid sequence motif common to many ATPases. The role of this motif in UvrB has been investigated by site-directed mutagenesis. Three UvrB mutants, with amino acid replacements at lysine-45, failed to confer UV resistance when tested in the UV-sensitive strain N364 (*ΔuvrB*), while five other mutants constructed near this region of UvrB confer wild-type levels of UV resistance. Because even the conservative substitution of arginine for lysine-45 in UvrB results in failure to confer UV resistance, we believe we have identified an amino acid side chain in UvrB essential to nucleotide excision repair in *E. coli*. The properties of two purified mutant UvrB proteins, lysine-45 to alanine (K45A) and asparagine-51 to alanine (N51A), were analyzed *in vitro*. While the K45A mutant is fully defective in incision of UV-irradiated DNA, K45A is capable of interaction with UvrA in forming an ATP-dependent nucleoprotein complex. The K45A mutant, however, fails to activate the characteristic increase in ATPase activity observed with the wild-type UvrB in the presence of UvrA and DNA. From these results we conclude that there is a second nucleotide-dependent step in incision following initial complex formation, which is defective in the K45A mutant. This experimental approach may prove of general applicability in the study of function and mechanism of other ATPase motif proteins.**

In *Escherichia coli*, Uvr-mediated repair of damaged DNA proceeds by formation of specific damaged DNA–protein complexes, followed by double endonucleolytic incision on damaged strands. Short oligomers containing damaged nucleotides are generated as a result. These early steps require MgATP, as well as UvrA, UvrB, and UvrC proteins (1, 2). Although the precise role of ATP remains unclear, nonhydrolyzable analogs of ATP fail to support incision. UvrA possesses a readily identifiable ATPase, modulated by the presence of DNA. Addition of UvrB to UvrA and DNA strongly enhances observed ATPase activity (3, 4). Recently, this laboratory reported that UvrA–UvrB nucleoprotein complexes possess a 5′ → 3′ ATP-dependent helicase-like activity (5, 6), a phenomenon that may be coupled to the ATPase activation seen under these conditions. This enhancement in ATP hydrolysis has been attributed to an activation of the UvrA ATPase by UvrB and DNA, since highly purified UvrB preparations fail to exhibit ATPase activity. It was somewhat surprising, therefore, to find that the predicted amino acid sequence of UvrB includes a sequence motif common to many known ATPases (7, 8).

This motif, common to known GTP binding proteins as well (9–12), is recognized by a segment of six amino acids highly enriched in glycine and other small side-chain amino acids such as serine, threonine, alanine, and proline. This segment is terminated by glycine, followed by a lysine and then by a serine or threonine (. . . G K T/S . . .). Unique and varied functions have been described for proteins possessing this motif. In many systems, the ability to bind and hydrolyze nucleoside triphosphates has been demonstrated, yet the specific role of hydrolysis remains unknown.

The presence of this amino acid motif in UvrB (Fig. 1) may be diagnostic of a previously uncharacterized nucleotide hydrolysis site, silent or inactive in the native protein. Study of such a cryptic UvrB activity is complicated by the fact that nucleotide hydrolysis in this system using intact proteins has been observed only when UvrA, a known ATPase, is present. To resolve this dilemma, eight mutants of UvrB in which a cryptic UvrB ATPase activity might be impaired or otherwise altered were constructed by oligonucleotide-directed mutagenesis. Recently, parallel work in this laboratory demonstrated that a cryptic UvrB ATPase activity can be measured in the absence of UvrA after proteolysis (13), further evidence that some aspects of the ATP dependence of the incision reaction directly involve UvrB. In this work, we demonstrate the successful construction of ATPase-defective UvrB mutants, which we use to further characterize the role of this activity and this protein in excision repair.

MATERIALS AND METHODS

Strains. JM109 [*recA1, endA1, gyrA96, thi⁻, hsdR17, supE44, relA1, λ⁻*, Δ(*lac-proAB*), F′(*traD36, proAB, lacI^qZ*ΔM15)] was obtained from P. Gearhart (The Johns Hopkins University); CJ236 [*dut1, ung1, thi1, relA1*/pCJ105 (F′, Cm^r)], used to generate uracil-containing phage, was obtained from J. D. Roberts (National Institute of Environmental Health Sciences, Research Triangle Park, NC); MC1061 [Δ(*ara,leu*), *hsdR⁻*, Δ(*lacIPOZYA*)X74, *galU, galK*, strA^r], used as a high transformation efficiency and ung⁺ recipient for constructs, was obtained from F. R. Bryant (The Johns Hopkins University). The UV-sensitive strain N364 [W3110 gal⁺, sup°, F⁻, Δ(*attB-bio-uvrB*)] was obtained from M. Gottesman (Columbia University).

Molecular Cloning. pBM505 contains an *E. coli* chromosomal DNA fragment with the entire gene for UvrB in pBR325 (W. B. Mattes and L.G., unpublished work). To ease later manipulations, a subfragment was recloned by ligation of a 4.5-kilobase (kb) *Sal* I/*Nru* I pBM505 fragment to *Sma* I/*Sal* I-digested pUC19 (pUCuvrB). Similarly, ligation of a 1.8-kilobase *Bgl* II/*Eco*RI fragment internal to the UvrB gene coding sequence to *Bam*HI/*Eco*RI-digested M13mp19 vector was used to assemble the M13uvrB derivative, which was used for the *in vitro* mutagenesis experiments.

***In Vitro* Site-Specific Mutagenesis.** The simplified high-efficiency oligonucleotide-directed mutagenesis strategy of Kunkel *et al.* (14) was used. The UvrB mutants were constructed by using the following oligonucleotide primers 21 nucleotides long, synthesized on an Applied Biosystems

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380A automated synthesizer (nucleotide changes underlined): lysine-45 to alanine (K45A), GATCGGCTCAGGGGC-AACCTT; K45D, GATCGGCTCAGGGGACACCTT; K45R, GATCGGCTCAGGGCGCACCTT; N51A, CTTCACCATTGCCGCTGTCAT; N51K, CTTCACCATTGCCAAAGTCAT; V52D, CACCATTGCCAATGACATTGC; I53R, CATTGCCAATGTCCGTGCTGA; D55A, CAATGTCATTGCTGCCCTTCA. The three-nucleotide change required to generate the K45R mutant made it necessary to use template prepared from mutant K45D as an intermediate in the construction of K45R. The 158-base-pair *Nco* I/*Eco*RI DNA fragments were excised from mutant M13uvrB replicative form derivatives and were used to replace the same fragment from pUCuvrB. Plasmids encoding mutant UvrB proteins were then transferred to strain N364 (Δ*uvrB*) for UV survival experiments.

UV Survival. Survival curves were generated by irradiating stationary-phase cells. Fresh LB broth inoculated with overnight culture was shaken until cells were well into logarithmic phase. Cultures were centrifuged, resuspended in M-9 salts (no carbon source), and shaken another 1.5 hr, after which suspensions of diluted cells were irradiated with various doses of UV. Doses were determined with a digital UV meter (Hoefer). Aliquots from serial dilutions of survivors were plated, and the colonies were counted after an overnight incubation in the dark at 37°C (Fig. 2). Each data point represents a minimum of 150 colonies. Survival at zero dose was determined by mock-irradiation of cells.

Proteins. Mutant proteins K45A and N51A were purified from nalidixic acid-induced cultures of N364 (Δ*uvrB*)-derived strains. No alterations in purification strategy as described for wild-type UvrB (15) were required to purify mutant UvrB proteins. Purified mutant UvrB proteins were judged to be >90% pure as analyzed by SDS/PAGE. Wild-type UvrA, UvrB, and UvrC proteins were purified as described (15).

Nicking Assay. Endonuclease activity was measured after a 5-min incubation at 37°C with ^{3}H-labeled supercoiled DNA substrate as described (2). Reaction mixtures (140 μl) contained ^{3}H-labeled supercoiled DNA {pHE6 [3996 base pairs (16)], 10^5 cpm/μg, UV-irradiated to 720 J/m^2, 60 fmol circles per assay}, 85 mM KCl, 40 mM K$^+$-Mops (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol, 15 mM $MgSO_4$, 2 mM ATP, bovine serum albumin (50 μg/ml), and saturating levels of UvrA and UvrC. UvrB or purified mutant UvrB proteins were used to assess their ability to support the nicking reaction *in vitro* (Fig. 3). Data are expressed as fmol yield of nicks per reaction (calculated by Poisson analysis).

Filter Binding. Reaction conditions used in nicking assays were used to assemble nucleoprotein complexes in the absence of UvrC. Reaction mixtures (140 μl) containing combinations of 3.6 nM UvrA, 2 mM ATP, and 2.9 nM UvrB or mutant UvrB were assembled on ice and preincubated at 37°C for 15 min to allow complex assembly. Reaction mixtures were then transferred to ice and challenged by addition of 5 ml of 4°C 2× SSC (0.3 M NaCl/30 mM sodium citrate). After 5 min, reaction mixtures were filtered through BA85 nitrocellulose filters (Schleicher & Schuell) equilibrated in 2× SSC and washed with another two aliquots of 2× SSC. Filters were dried and DNA adsorbed to the filters was determined by counting in scintillation fluid (Table 1). Data are expressed as yield of complexes in fmol, calculated by multiplying the average number of complexes per DNA molecule (calculated by Poisson analysis) by the amount of substrate (60 fmol circles).

ATPase Assay. Purified proteins were assayed in the presence of UV-irradiated DNA. Assay mixtures (20 μl) contained 50 mM K$^+$-Mops (pH 7.5), 100 mM KCl, 50 μg of bovine serum albumin (50 μg/ml), 30 μM UV-irradiated double-stranded DNA (pUC18, UV-irradiated to 720 J/m^2, expressed as nucleotide concentration), 2 mM dithiothreitol, 15 mM $MgCl_2$, and UvrA protein as indicated (Fig. 4). Reactions were initiated by adding [^{3}H]ATP (ICN and Amersham, diluted with unlabeled ATP to specific activity of 2 Ci/mmol; 1 Ci = 37 GBq) to 108 μM final concentration. At various time points, 0.5-μl aliquots were spotted onto PEI-cellulose TLC plates (Brinkmann) and later developed in 0.5 M formic acid and 1 M LiCl. Separated ATP and ADP spots were cut out and the level of radioactivity was determined in scintillation fluid (4a20; Research Products International). Time-point data were reduced by linear regression analysis to determine initial rates. All reactions demonstrated linear increases in product formation for the range of time points used in these experiments.

RESULTS

There is increasing evidence that a conserved lysine in the ATPase motif may play a critical functional role in proteins possessing this motif. Replacement of adenylate kinase lysine-13, located within an ATPase motif and implicated in the mechanism of ATP binding to that protein (11), was found to result in severe loss of activity (17). Mutations in the glycine-rich domain of the Ha-*ras* oncogene ATPase motif can cause cells to adopt a different *in vivo* phenotype, that of cellular transformation, demonstrating the importance of this region to proper c-Ha-*ras* function (for review, see ref. 18). Mutation of lysine-16 terminating the glycine-rich segment in the ATPase motif of Ha-*ras* reduces GDP and GTP affinity by 2 orders of magnitude without affecting relative nucleotide specificity (19). We postulated that the side chain of lysine-45 in the ATPase motif of UvrB might play a similarly important role in excision repair, and we constructed three mutants at this site; replacing lysine with arginine (K45R, a conservative replacement), alanine (K45A, a relatively neutral substitution), and aspartate (K45D, a nonconservative change).

A weaker component of the ATPase motif, noted by Fry *et al.* (11), includes a few hydrophobic amino acids separated from the glycine-rich segment by about seven amino acids toward the carboxyl terminus of the protein (Fig. 1, segment 2). This region corresponds to a putative hydrophobic adenine ring binding "pocket" proposed for adenylate kinase (11) and to a region of known α-helical secondary structure deduced from the x-ray structures of elongation factor Tu (20), Ha-*ras* (21), and adenylate kinase (22). The role of this region in UvrB was investigated by constructing five additional mutants at selected sites; changing asparagine-51 to alanine and lysine (N51A, N51K), valine-52 to aspartate (V52D), isoleucine-53 to arginine (I53R), and aspartate-55 to alanine (D55A), including both conservative and nonconservative substitutions.

Lysine-45 of UvrB Is Essential to UV Resistance. Plasmids encoding either wild-type or mutant UvrB were introduced into N364, a UV-sensitive *E. coli* strain in which the entire *uvrB* gene is deleted. Plasmids encoding the five segment 2 UvrB mutants (N51A, N51K, V52D, I53R, D55A) did not differ appreciably from plasmids encoding wild-type UvrB in their ability to complement the chromosomal *uvrB* deletion in N364. The other three mutants, all at lysine-45, failed entirely to enhance UV survival and were indistinguishable from N364 carrying either pUC19 vector or no plasmid (Fig. 2). To rule out changes in stability or expression as a mechanism for the acute loss of function in the lysine-45 mutants, extracts of N364-derived cells were examined for the presence of UvrB or mutant UvrB proteins by Western blotting (data not shown). Mutant proteins were found to be overexpressed to similar steady-state levels. Differential expression or stability of the mutant proteins is judged to be an insufficient explanation for the dramatically altered *in vivo* phenotypes of the three lysine-45 UvrB mutants.

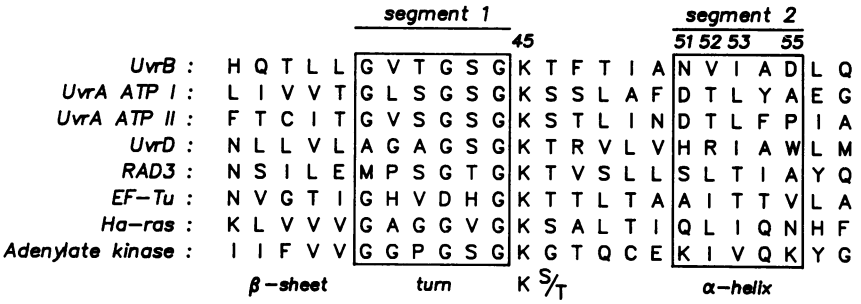

FIG. 1. The ATPase motif. Selected proteins possessing an ATPase motif are aligned as suggested by Fry *et al.* (11). Residues targeted for mutagenesis in UvrB are numbered. Segment 1 denotes a small side-chain-rich region, while a region of weaker homology described in the text is denoted by segment 2. The x-ray crystal structures of elongation factor Tu (20), Ha-ras (21), and adenylate kinase (22) have been solved. These three proteins possess strikingly similar secondary structure in this region. Sequence references are as follows: UvrB residues 34–57, refs. 7 and 8; UvrA ATP I 36–49 and ATP II 635–658, ref. 23; UvrD 24–47, ref. 24; RAD3 37–60, ref. 25; elongation factor Tu (EF-Tu) 13–36, ref. 26; Ha-ras 5–28, ref. 18; adenylate kinase 10–33, ref. 27. Amino acids are designated by the single-letter code.

K45A Is Inactive in Incision. The mutant proteins N51A and K45A were purified from extracts of N364-derived strains. In purifying the mutant proteins, no modifications in UvrB purification strategy were required. When incision of UV-irradiated DNA was measured *in vitro* (Fig. 3), wild-type UvrB and N51A mutant proteins were able to support incision to similar extents. In contrast, the K45A protein failed to support incision of UV-irradiated DNA. Levels as high as 135 ng of K45A caused no increase in incision when included in the assay. No increase in nicking was detected under these conditions when unirradiated DNA was used as a control.

K45A Interacts with UvrA on DNA. Assay conditions have been described that allow for the discrimination of UvrA–DNA complexes from UvrA–UvrB nucleoprotein complexes (28). In this assay, cold 2× SSC rapidly dissociates UvrA–DNA complexes, while the addition of UvrB allows formation of a stable 2× SSC-resistant UvrA–UvrB complex on UV-damaged DNA. Nucleoprotein complexes were assembled and challenged by addition of cold 2× SSC (Table 1). Under conditions in which wild-type UvrB and N51A protein formed nucleoprotein complexes on UV-irradiated DNA, the K45A mutant protein also formed complexes, although with a reduced yield. Controls showed that under all conditions both UvrA and ATP are required to generate 2× SSC-resistant complexes and that stable complexes did not form in the absence of UvrB or UvrB mutant protein.

K45A Fails to Activate a Characteristic ATPase. Both wild-type UvrB and N51A protein were successful in ATPase activation in the presence of UvrA and UV-damaged DNA, saturating at similar concentrations (Fig. 4). In contrast, K45A failed to cause an increase in ATPase activity, instead resulting in a saturable inhibition of UvrA-associated ATPase activity. A 50% suppression of activity was observed at approximately stoichiometric protein ratios of UvrA to K45A, suggesting that K45A is a tightly bound component of inhibited complexes.

DISCUSSION

We hypothesized that the "ATPase motif" common to the amino acid sequences of known nucleoside triphosphatases (Fig. 1) serves a common and specific function in these proteins. In this model, specific amino acid residues in the ATPase motif would contribute directly to interaction with ATP and therefore to the ultimate function of these proteins as well. Although purified UvrB preparations lack ATPase activity, the amino acid sequence of UvrB includes an ATPase motif. Recently, this laboratory reported the fortuitous observation that UvrB possesses a cryptic ATPase activity, revealed in the absence of other Uvr proteins after limited proteolysis (13). It was assumed that any cryptic nucleotide hydrolysis activity should prove critical to the requirement for UvrB in incision. An ATPase-deficient UvrB mutant would therefore be quite useful in the determination of the role of nucleotide and UvrB in nucleotide excision repair. Consequently, the region of UvrB possessing an ATPase motif was subjected to site-directed mutagenesis to determine its role, if any, in excision repair.

Substitution of UvrB lysine-45 by the structurally neutral amino acid alanine (K45A) would not be expected to disrupt local structure in this region, yet an acute effect on *in vivo*

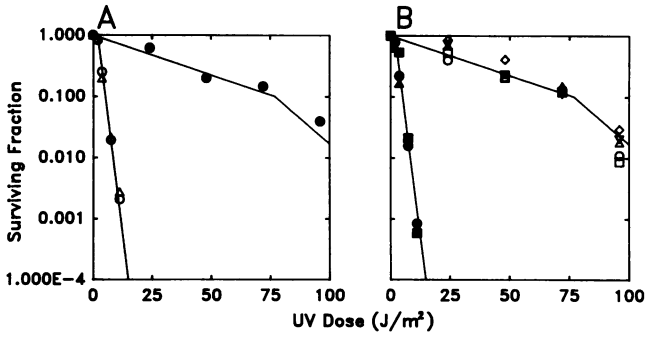

FIG. 2. UV survival in Δ*uvrB* background of N364. Data are expressed as surviving fraction at a given dose. (*A*) Controls: ●, pUCuvrB; ○, pUC19; △, no plasmid. (*B*) Mutants: ●, pK45A; ▲, pK45D; ■, pK45R; ○, pN51A; □, pN51K; △, pV52D; ▽, pI53R; ◇, pD55A.

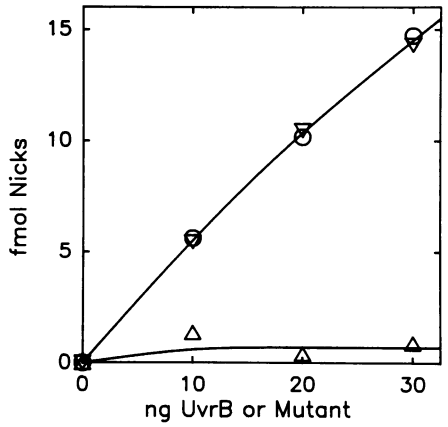

FIG. 3. *In vitro* nicking assay. Endonuclease activity was measured after incubating purified proteins with UV-damaged substrate. To reaction mixtures containing UvrA, UvrC, and ATP, various amounts of either UvrB (○), N51A (▽), or K45A (△) proteins were added as indicated. Data are expressed as fmol of nicks per reaction mixture.

Table 1. DNA binding assay

Protein	2× SSC-resistant nucleoprotein complex formation, fmol observed			
	+ ATP, + UvrA	− ATP, + UvrA	+ ATP, − UvrA	− ATP, − UvrA
UvrB	20.7	0.2	0.3	0.1
N51A	17.4	0.1	0.2	0.1
K45A	7.9	0.3	0.3	0.1
None	0.4	NT	NT	NT

Ability to form 2× SSC-resistant nucleoprotein complexes was assayed with UV-damaged substrate. UvrA protein, ATP, and UvrB, or mutant UvrB, were added as indicated. Data are expressed as fmol yield of complexes per reaction mixture. NT, not tested.

function was observed (Fig. 2). Similarly, loss of function was also observed in the nonconservative lysine-45 mutant in which the negatively charged amino acid aspartate was substituted (K45D). Because the highly conservative substitution of arginine at this site (K45R) also results in synthesis of a gene product that fails to complement UvrB deficient cells, we believe that the amino acid side chain of lysine-45 is critical to the mechanism of action of UvrB.

Failure to generate phenotypically distinct mutants with respect to UV survival by alterations in neighboring amino acids, characterized by the five segment 2 UvrB mutants, further emphasizes the importance of lysine-45 of UvrB. While some segment 2 mutations were specifically selected as highly nonconservative substitutions (V52D, I53R), the *in vivo* repair activity of these mutants was not compromised. From these data, we conclude that the amino acid side chains substituted in this region of UvrB do not play critical roles in excision repair. Similar results were obtained when UV sensitivity was assayed in the *uvrB* wild-type genetic background of JM109. Plasmids encoding the lysine 45-UvrB mutants (K45A, K45D, K45R) were able to confer UV sensitivity to this strain (data not shown). This suggests that the products of the lysine-45 mutants express partial function *in vivo* by competing with wild-type UvrB for other excision repair components, perhaps by forming nonproductive complexes that could interfere with normal repair processes.

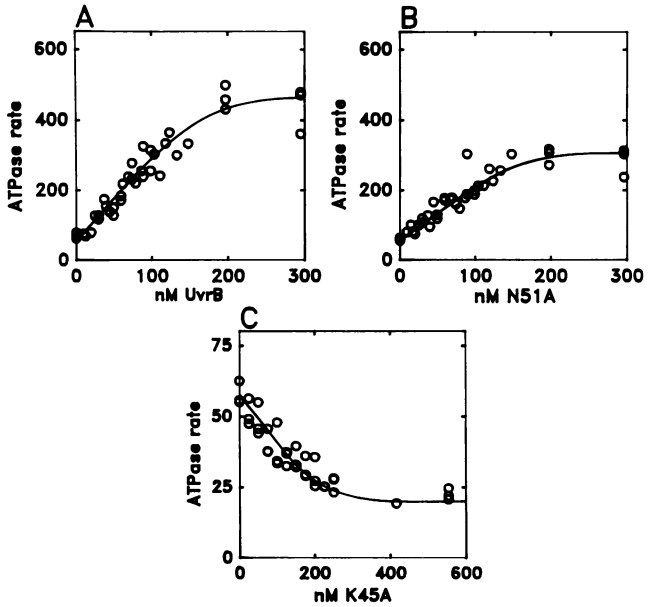

FIG. 4. ATPase assay. ATPase rates, stated as molecules of ATP hydrolyzed per min per UvrA molecule, were measured in the presence of UvrA and UV-damaged DNA after addition of various amounts of UvrB (*A*), N51A (*B*), or K45A (*C*) protein. Reaction mixtures contained 12 nM UvrA (*A* and *B*) or 71 nM UvrA (*C*).

The *in vivo* determination of the ability of the mutants to interact with excision repair components is complicated by the fact that the level of plasmid-encoded mutant protein far exceeds that expected from a single chromosomal UvrB wild-type gene. As a consequence, the properties of purified mutant proteins were examined *in vitro*, selecting for further analyses one mutant representative of each of the two phenotypes identified *in vivo*. The protein products of the lysine to alanine mutant (K45A), conferring no UV resistance, and the asparagine to alanine mutant (N51A), conferring wild-type levels of UV resistance, were purified from N364 cell extracts and compared to wild-type UvrB protein.

DNA damaged by a number of agents, including UV, can be specifically incised in the presence of UvrA, UvrB, UvrC, and MgATP. *In vitro*, the mutant N51A protein showed activity similar to wild-type UvrB protein in incision of UV-irradiated DNA, while the mutant K45A protein was totally inactive (Fig. 3). The UV-survival defect observed in cells producing K45A *in vivo* therefore results from an inability to support incision of damaged DNA. The failure of K45A to function in incision was further investigated by nitrocellulose filter binding assays (Table 1). The N51A protein behaved similarly to wild-type UvrB protein, efficiently forming nucleoprotein complexes with UvrA and UV-damaged DNA. K45A protein also formed complexes under the conditions used, although only ≈40% yield of complexes was observed as compared to wild-type UvrB protein. Formation of these complexes, like those formed with wild-type UvrB, required UvrA and added nucleotide. These findings suggest that the mechanism by which K45A interferes with JM109 excision repair *in vivo* is through successful interaction with UvrA on DNA, forming a nonproductive nucleoprotein complex that fails to function in incision.

In the presence of UvrA and DNA, the addition of UvrB protein causes an increase in observed ATPase activity. The ability of mutant proteins to cause this characteristic ATPase activation was therefore assayed (Fig. 4). K45A protein was totally defective in the ATPase activation but revealed its ability to interact with UvrA by allosterically inhibiting the UvrA-associated ATPase. These data are consistent with the unmasking of a cryptic UvrB ATPase activity in the presence of UvrA and DNA, an activity that is defective in the K45A mutant. Since saturating levels of the N51A protein produced activation to only 65% of that seen with UvrB wild type, we propose that amino acid side chains in this region, while capable of influencing ATPase activity, act indirectly through subtle alterations in nearby secondary structure rather than by forming a part of the active site.

In summary, the side chain of lysine-45 in the ATPase motif of UvrB was found to be essential to *in vivo* function. In investigating the mechanism of the defect in lysine-45 mutants, we found in all cases that formation of 2× SSC-resistant nucleoprotein complexes requires both UvrA and ATP. The K45A–UvrA complex, however, fails to activate a characteristic ATPase in the presence of damaged DNA and fails to proceed to incision. On the basis of these results, we propose a simple model (Fig. 5), in which UvrA, UvrB, and damaged DNA are assembled into an initial complex in a nucleotide-dependent reaction. A second nucleotide-dependent step, thought to be defective in the K45A mutant, is required for transition into a productive preincision complex, reacting with UvrC at the damaged site to produce incision. Continuing characterization of these UvrB mutants will clarify the specific point at which a UvrB ATPase becomes essential for further steps leading to incision. Because the conditions under which the K45A ATPase is defective are analogous to those used for the UvrA–UvrB helicase (6), it is possible that the defect in K45A may represent the failure of UvrA–K45A complexes to translocate to sites of damage. In agreement with

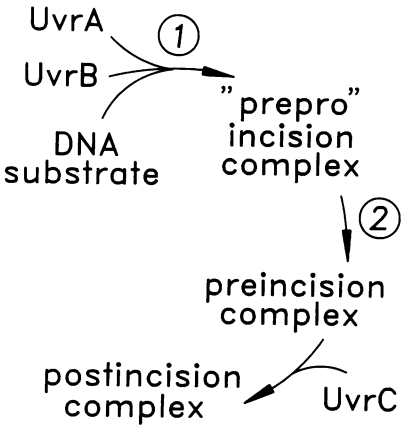

FIG. 5. Model incorporating two nucleotide-dependent steps (labeled 1 and 2) leading to incision. The UvrA ATPase may fulfill the nucleotide requirement for the initial "prepro" nucleoprotein complex. The nucleotide requirement of the second step, leading to a mature preincision complex, has an absolute requirement for the side chain of lysine-45 on UvrB.

this model, preliminary work suggests that the arrested nucleoprotein complex formed with K45A represents a nonspecifically bound nucleoprotein intermediate in nucleotide excision repair.

We have raised the question of whether the presence of an ATPase motif can be useful in identifying those proteins whose activity is dependent on ATP or related cofactors. Our success in isolating an ATPase-deficient mutant of UvrB suggests the strategy described here may be of general use in the study of other ATPase motif proteins. A similar strategy was recently reported in the construction of an ATPase-defective yeast excision repair protein, RAD3 (29).

Purified UvrA was prepared by Dr. E. Y. Oh (University of California at San Francisco), in conjunction with S. Thiagalingam (The Johns Hopkins University) and Dr. S. Mazur (American University). The tritiated substrate used in nicking and binding assays was prepared by L. Claassen. The comments of Dr. F. R. Bryant were greatly appreciated. This work was supported by grants from the National Institutes of Health (GM 22846, GM 31110) and the Department of Energy (DE-FGOL-86ER 60396).

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