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A general approach for purifying proteins encoded by cloned genes without using a functional assay: isolation of the uvrA gene product from radiolabeled maxicells

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ABSTRACT

The uvrA protein (UVRA) of E. coli has been extensively purified from a strain in which UVRA is overproduced and specifically labeled with ^{35}S-methionine. This approximately 100-fold overproduction relative to normal strains is a result of having the uvrA gene present on a multicopy plasmid in a spr recA cell that makes defective lexA protein, the normal repressor of the uvrA gene, while the specific labeling of UVRA is done with maxicells. This approach facilitates the preparation of the protein since enzyme assays do not have to be carried out during the intermediate stages of purification. The purified UVRA binds to DNA and has ATPase activity but does not have intrinsic endonuclease activity. When added to extracts of uvrA$^-$ cells, the purified UVRA does promote the specific cutting of UV-irradiated DNA. Since this approach for working out rapid purification procedures by specifically labeling the proteins encoded by cloned genes does not require the use of a functional assay, it is a general one that can be applied to a wide variety of other gene products in addition to UVRA.

INTRODUCTION

The uvrA, uvrB, and uvrC gene products are required for repair of the DNA lesions produced in E. coli by a wide variety of agents which are known or presumed to produce large, bulky adducts in DNA. They have been specifically implicated in the repair of pyrimidine dimers (1-4) and the adducts produced by nitrogen mustard (5), mitomycin C (2), 4-nitroquinoline oxide (6), psoralens (7), nitrofurans (8) and the nitroimidazole hypoxic tumor cell radiosensitizer, misonidazole (9). These genes apparently have little to do with the repair of most of the major lesions produced by X-rays or methylmethanesulfonate (10).

The uvr mutants are deficient in vivo in the incision of DNA containing pyrimidine dimers (1,11), and cell-free extracts of such mutants lack the ability to specifically incise UV-irradiated DNA (12).

In order to better understand the means by which the uvrA, uvrB, and uvrC gene products mediate this step in excision repair, we have cloned all three

genes and identified their products. UVRA was found to be a polypeptide of 114 kdal with the ability to complement the UV-endonuclease deficiency of cell-free extracts of a uvrA⁻ strain (13). We have found the uvrB gene product to be a polypeptide of 84 kdal which binds to ss (single-stranded) DNA if UVRA is present (14,15) and we have recently identified the uvrC gene product as a ss DNA binding protein of 70 kdal (16).

We previously reported a radiochemical purification and initial characterization of the UVRA (13). In this work, we carry this purification further using ^{35}S-labeled UVRA which can be easily followed through the purification steps. We have also taken advantage of the observation that since the uvrA gene is normally repressed by lexA protein, UVRA is overproduced in spr (lexA repressor defective) mutants of E. coli (17,18,19). The simultaneous presence of the uvrA plasmid pDR2000 and a spr mutation leads to an approximately 100-fold enhancement of uvrA activity over that found in wild-type strains. This level of amplification has greatly facilitated the purification of UVRA from convenient volumes of maxicell cultures. Purified UVRA retains UV-endonuclease complementing activity and co-purifies with an ATPase activity which is absent from a uvrA⁻ strain.

MATERIALS AND METHODS

Bacterial strains and plasmids

AB3062 is an E. coli K-12 strain carrying endA uvrB5 thyA deo xyl thi graciously supplied by Dr. P. Howard-Flanders. The other strains used in this study, CSR603 (recA1 uvrA6 phr1) and DM1415 (recA1 lexA1 spr-1 sfi) are E. coli K-12 derivatives and have been previously described (20,21). The plasmid pBR322 and the construction of derivatives carrying the uvrA gene (pDR2000 and pDR1996) or the uvrB gene (pDR1494) have been described elsewhere (13,14,22,23).

Preparation of cell-free extracts of ^{35}S-labeled maxicells

Radiolabeled UVRA was prepared using a modification of the published maxicell procedure (13,24). 2 l of logarithmic phase DM1415 carrying either pDR2000 or pDR1996 grown at 37°C in K-medium (25) supplemented with 5 µg/ml tetracycline and 200 µg/ml ampicillin were irradiated with a fluence of 36-50 J/m^2 from a germicidal UV lamp. After irradiation, cycloserine (200 µg/ml), carbenicillin (100 µg/ml) and methicillin (100 µg/ml) were added and the incubation was continued with shaking at 37°C for 12-14 hrs.

The cells were harvested by centrifugation and washed once with 100mM NaCl in 20mM Tris-HCl buffer (pH 7.5) at room temperature. The cell pellet

was resuspended in 400 ml sulfur-free Hershey salts (26) supplemented with 10 mg/ml glucose, 0.2 µg/ml thiamine, 150 µg ml l-leucine, proline, histidine, threonine and arginine and 40 µg/ml all other "standard" l-amino acids except cysteine, methionine, and valine. The cultures were incubated for 45-60 min at 37°C with shaking after which ^{35}S-l-methionine was added to 1 µCi/ml. The cells were further incubated with shaking at 37°C for another hour after which they were chilled on ice, pelleted by centrifugation, washed with successive volumes of 200 and 20 ml of ice-cold 40mM Tris-HCl (pH 8.0) and then resuspended in 4 ml ice-cold 86% sucrose, 40mM Tris-HCl (pH 8.0), 10mM Na_2EDTA (ethylenediaminetetraacetate) and 10mM Na_2EGTA (ethylene glycol bis(β-aminoethyl ether) tetraacetate) (12).

The mixture was kept on ice for 5-10 min after which 16 ml of ice-cold Buffer Y [50mM K-MOPS (morpholinopropanesulfonic acid adjusted to pH 7.5 with KOH), 100mM KCl, 1mM Na_2EDTA, 1mM (dl)dithiothreitol, 10mM ME (β-mercaptoethanol)], and 125 µg/ml egg white lysozyme was added and the mixture returned to ice for 2-3 hours . The plasmolysed cells were pelleted at 0°C for 5 min at 27,000 g and the clear supernatant decanted. The latter cell-free extract was dialyzed against Buffer A (25% glycerol, 50mM K-MOPS(pH 7.5), 100mM KCl, 1mM Na_2EDTA, 10mM ME) at 0°C overnight and stored at 0°C(12,27).

DEAE Biogel and ss DNA cellulose chromatograpny

DEAE Biogel A was obtained from BioRad and denatured DNA cellulose (1.5 mg DNA/ml bed volume) from PL Biochemicals. Both resins were equilibrated with Buffer A and were used as specified by the suppliers.

UV-endonuclease assay

This assay was performed essentially as described by Seeberg et al. (12, 27). Cell free extract of AB3062 was used as a "receptor" for uvrB complementing activity. For the receptor of uvrA complementing activity, a cell-free extract of CSR603 carrying pDR1494 was made and partially purified by gel-filtration chromatography. 1-10 µl UVRA, 10-15 µl receptor extract, and 50 ng ^{3}H-labeled øx174 RF DNA [70-80% ccc (covalently-closed circular and superhelical)] UV-irradiated with 0 - 50 J/m^2 were mixed with 100 µl Buffer Y, 2 µl 100mM Na_2ATP and 1 µl 1M $MgSO_4$ and incubated at 37°C for 15 min. The reaction was terminated with 20 µl 0.25M Na_2EDTA and the samples were layered onto 5 ml 0.3N NaOH, 0.7N NaCl, 5-20% alkaline sucrose gradient and sedimented for 90 min at 45,000 rpm and 20-25° in an SW50.1 rotor. 0.4 ml samples were collected, neutralized, and assayed for radioactivity by scintillation counting.

The fraction of ccc DNA converted to nicked circles was determined for

UV-irradiated and unirradiated DNA and breaks/molecule were calculated from the formula:

- ln(fraction ccc after incubation) + ln(fraction ccc, no treatment).

The excess breakage of UV-irradiated DNA over unirradiated DNA defines the UV-endonuclease activity (12).

ATPase assay (modified from Ref. 28)

^{3}H-labeled ATP (24 Ci/mmol in 50/50 water/ethanol) obtained from Amersham was dried down under vacuum and resuspended in 10 µl of Buffer A containing UVRA, 50 µg/ml nuclease free BSA (bovine serum albumin; BRL) and 20mM $MgSO_4$. After 10 min at 37°C, the reaction was terminated with 5 µl 0.25M Na_2EDTA(pH 8.0). 1-2 µl was spotted onto polyethyleneimine coated chromatographic plates (Brinkmann) and the spots were overlaid with 1 µl of a solution containing ATP, ADP, and AMP (3 mg/ml each in 0.25M Na_2EDTA, pH 8.0). The chromatograms were developed first with ethanol and then with 0.75 M Na-phosphate buffer (pH 3.75). While damp, the spots were visualized under UV and scraped into scintillation vials containing 0.5 ml 1N HCl. The samples were stored overnight at room temperature, mixed with 7 ml liquid scintillation cocktail and assayed for radioactivity by scintillation counting to determine the amount of ATP converted to ADP during the 10 min reaction.

Other Methods

Binding of UVRA to DNA was measured by neutral sucrose gradient sedimentation as described before (13). Sucrose gradient solutions contained 5 or 20% (w/v) sucrose, 50mM K-MOPS(7.5), 100mM KCl, 1mM Na_2EDTA, and 10mM β-ME. SDS-PAGE (Sodium dodecylsulfate-polyacrylamide gel electrophoresis) and autoradiography have also been described (13) except that DATD (diallylditartaramide) was used as a crosslinker in the place of N,N'methylenebisacrylamide in a 10:1 ratio of acrylamide:DATD. Protein concentrations were determined by the method of Bradford (29) using BioRad Coomassie Blue Reagent concentrate. Photoreactivation was carried out by the method of Sancar and Rupert (30) with partially-purified *E. coli* photolyase (AS60P fraction (31) generously provided by B. Sutherland). Briefly, 100 µl DNA at 100 µg/ml in Tris-HCl (pH 7.4) 50mM, 1mM Na_2EDTA was mixed with 5 µl partially-purified photolyase, incubated at room temperature for 5 min and exposed on ice to black-light for 1 hour. DNA was phenol/ether extracted prior to UV-endonuclease assay.

RESULTS

Preparation of maxicells and cell-free extracts

We chose maxicells of DM1415 carrying either pDR2000 or pDR1996 for the purification of UVRA for the following reasons: 1. The presence of the uvrA gene on a multicopy plasmid increases the amount of UVRA in a cell. 2. The spr allele of the lexA gene results in an inactive lexA protein, and consequently, because it is the normal repressor of the uvrA gene, the level of UVRA is higher than it would be in a $lexA^+$ strain. Thus, in this strain the level of UVRA is approximately 100 times higher than in a normal strain without a plasmid (17) making it an excellent source of UVRA (Figure 1). 3. UVRA is selectively labeled in maxicells of this strain and the protein can be followed readily through the purification steps by assay of radioactivity by scintillation counting or by SDS-PAGE and autoradiography.

Maxicells from a 2-liter culture were labeled as described in Materials and Methods and were plamolysed by the method of Seeberg et al. (12) to yield 20 ml of cell-free extract (Fraction I). We have found that this procedure efficiently releases nearly all the cellular radiolabeled UVRA into the cell-free extract, but that most of the cellular protein and DNA remain with the precipitate (12). Thus, this procedure is superior to other, less specific means of lysis (such as sonication) for the preparation of UVRA.

Chromatography of UVRA and SSB on DEAE Agarose

The 20 ml cell-free extract from the previous step was applied to a DEAE Biogel A column (100 ml bed volume) and eluted with Buffer A. Both UVRA (27) and SSB (13) eluted in the void volume (Fraction II) as determined by SDS-PAGE analysis and autoradiography (Figure 3).

Chromatography of UVRA and SSB on denatured DNA cellulose

Fraction II which contained both UVRA and SSB was applied to a column of denatured DNA cellulose (15 ml) equilibrated with Buffer A. The column was washed with 50 ml Buffer A and then eluted with [KCl] and [NaCl] step gradients as described in Figure 2. Aliquots of the fractions were assayed for radioactivity by scintillation counting and for protein composition by SDS-PAGE analysis and autoradiography. As is seen in Figure 2, two distinct peaks of radioactivity were eluted from DNA cellulose. The first (0.2-0.5M KCl) contained the 114 kdal uvrA polypeptide (13) while the second peak (2.25M NaCl) contained the 18.5 kdal ssb polypeptide (13). Upon Coomassie blue staining, the 0.4M ss DNA cellulose column fraction (Fraction III) appears free of stainable contaminating protein bands and is estimated to be at least 95% pure. The purification steps are summarized in Table I and Figure 3. As

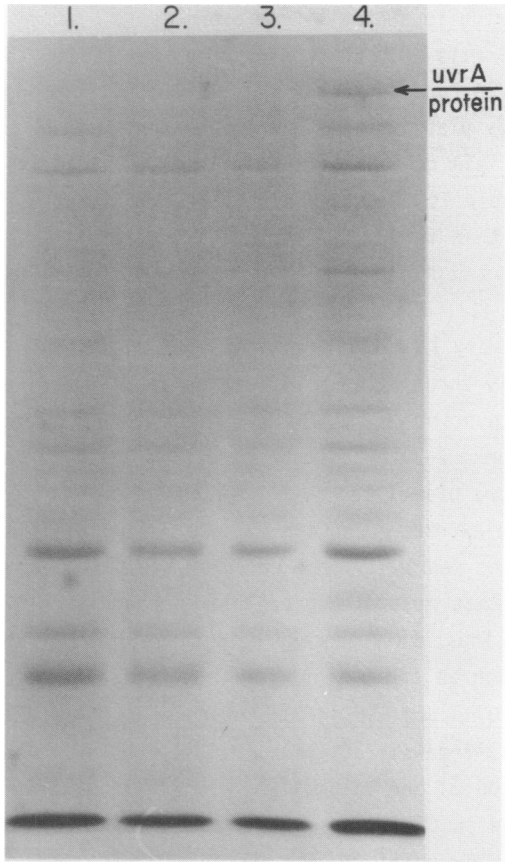

Figure 1. Amplification of UVRA in DM1415/pDR1996. 25 µl of logarithmic phase cultures were lysed and the proteins were analyzed by SDS-PAGE. The 12% gel was stained with Coomassie Blue. Lane 1, CSR603; Lane 2, CSR603/pDR1996; Lane 3, DM1415; Lane 4, DM1415/pDR1996. The arrow indicates the position of radioactive UVRA.

shown in the table, approximately 20% of the total maxicell radiolabeled UVRA was obtained in a highly-purified form after a 60-fold purification.

Previous reports (13,32) indicated that UVRA in cell free extracts did bind to DNA. However, these reports could not distinguish direct binding of UVRA to DNA from indirect DNA binding mediated by other proteins present in the crude preparations. Therefore, we tested our purified UVRA (Fraction III) for DNA binding activity. As shown in Figure 4, UVRA binds to ss and ccc DNA in the presence and absence of Mg^{++} but to ds (double-stranded) linear DNA

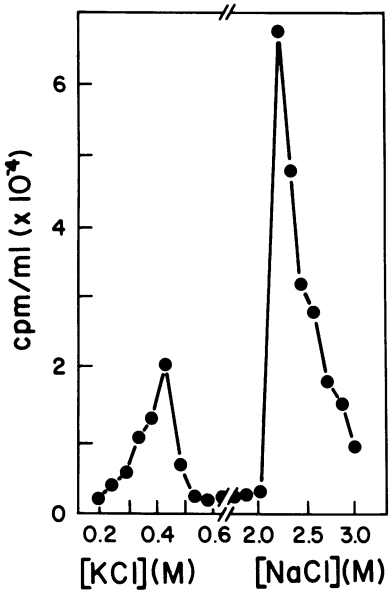

Figure 2. Chromatography of maxicell extract of DM1415/pDR2000 on ss DNA cellulose. 5 ml of extract (equivalent to Fraction I in Table 1) was applied to a 15 ml bed-volume ss DNA cellulose column. The column was washed with 40 ml Buffer A and elution was carried out with a [KCl] step gradient to 0.7M (0.1M increments, 1.5 ml each) and an [NaCl] step gradient (0.25M increments, 3 ml each). 1.3-1.5 ml fractions were collected and 0.1 ml of each was assayed for radioactivity by scintillation counting.

only when Mg^{++} was present. We also observed that the non-hydrolyzable ATP analogue ATP[S] (Adenosine 5'-O-(3-thiotriphosphate), tetralithium salt) strongly stimulated the binding of uvrA protein to ds linear DNA (Fig. 4). ATP also stimulated binding to ds linear DNA, while UV-irradiation of both ss and ds DNA led to increased binding of radiolabeled UVRA (data not shown).

UV endonuclease complementing activity of purified UVRA.

When tested in the UV-endonuclease complementation assay of Seeberg et al. (12), purified UVRA promoted cutting of UV-irradiated DNA when added to a cell-free extract of uvrA$^-$ cells but had no effect when added to a similar extract prepared from uvrB$^-$ cells (Fig. 5). The purified protein was devoid of endonuclease activity on its own on either UV-irradiated or unirradiated DNA and the UV-endonuclease cutting was prevented by pretreatment of the DNA with E. coli photolyase and near-UV light to show that the specific cutting is at or near pyrimidine dimers (Fig. 5).

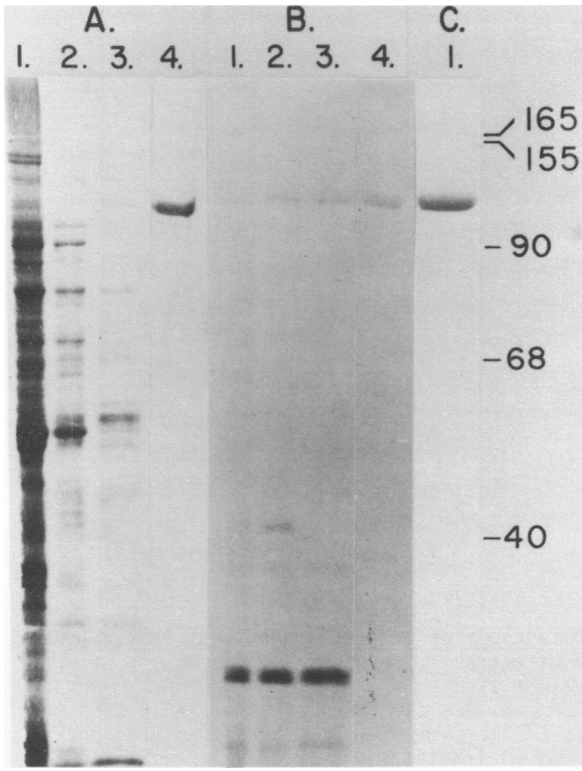

Figure 3. UVRA purification. Panel A: Coomassie blue stained SDS-polyacrylamide gel (10% acrylamide) showing samples from various stages of the purification described in Table 1. Lane 1, 25µl maxicells; Lane 2, 25µl Fraction I; Lane 3, 100µl Fraction II; Lane 4, 50µl Fraction III. Panel B: Autoradiogram of gel in Panel 1. Panel C: Lane 1, Coomassie blue-stained gel of 100 µl of Fraction III. E. coli RNA polymerase (165, 155, 90 and 40 kdal) and BSA (68 kdal) were the molecular weight markers.

ATPase activity associated with UVRA

Since the in vitro UV-endonuclease assay of Seeberg et al. (12) required ATP, it was conceivable that one or more of the three uvr proteins might have an associated ATPase activity. Therefore, we tested our purified UVRA preparations for ATPase activity and found that an ATPase activity co-chromatographed with the radiolabeled UVRA on DEAE Biogel and ss DNA cellulose. The absence of this ATPase activity from a uvrA$^-$ strain (Figure 6) provided further evidence that the activity was due to UVRA itself rather than to an unrelated contaminant.

Table 1. Purification of UVRA

	Fraction	Volume	Total protein	Radiochemical recovery	Purification
	Maxicells	2 l	4 x 10^{11} maxicells	----	----
I.	Extract	20 ml	18 mg	100%	1
II.	DEAE Agarose (run through)	2 ml	7.4 mg	90%	2.2
III.	ss DNA Cellulose (0.4M KCl)	1.3 ml	60 µg	21%	63

Total protein was determined by the method of Bradford (29). Radiochemical recovery was calculated by densitometric scanning of the autoradiograms of PAGE-SDS analyses of each fraction where the peak areas and volumes were used to calculate the recovery of radiolabeled UVRA.

$$\text{Purification} = \frac{(\text{Total protein in Fraction I})(\text{Radiochemical recovery})}{\text{Total protein}}$$

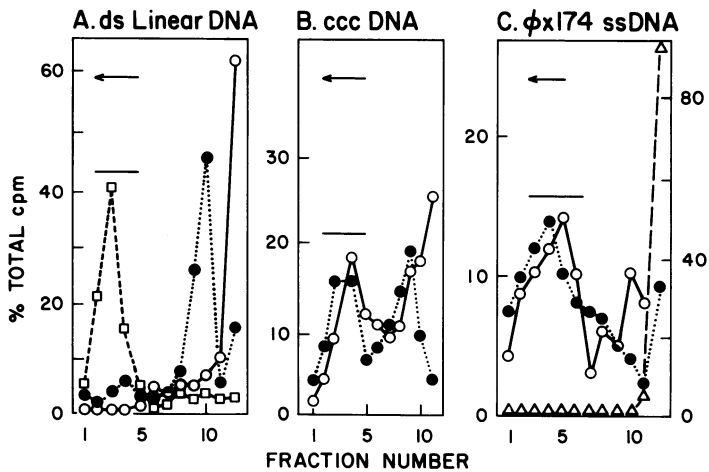

Figure 4. Binding of purified UVRA to DNA. Fraction III UVRA (27 µg/ml, 3300 cpm/µg) was mixed with DNA and sedimented through sucrose gradients as described previously (13). The reaction mixtures contained 100 µg/ml DNA, 4.5 µg/ml UVRA, and Buffer Y salts. Panel A: Binding to Adenovirus 2 ds linear DNA (90 min, 25°C, 45K) in the presence (□,●) and absence (o) of 20mM $MgSO_4$. For (□), 2.5mM ATP[S] is present in the sample but not in the sucrose gradient. Panel B: Binding to pBR322 ccc DNA (150 min, 45K, 25°C, 90% ccc) in the presence (●) and absence (o) of 20mM $MgSO_4$. Panel C: Binding to øx174 ss virion DNA (175 min, 25°C, 45K) in the presence (●) and absence (o) of 20mM $MgSO_4$. Control without DNA (Δ, right ordinate).

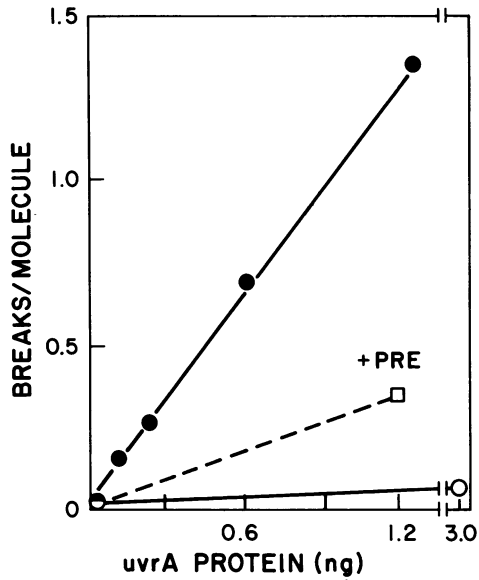

Figure 5. UV-endonuclease complementing activity of UVRA. Fraction III UVRA was added to uvrA⁻ (●,□) or uvrB⁻ (o) receptor extracts together with øx174 RF DNA which had been irradiated with UV fluences of 30 (●), 40 (o) or 50 (□) Jm^{-2}. Backgrounds subtracted from the experimental values include 0.3 breaks/molecule present in irradiated and unirradiated DNA plus 0.2 breaks/molecule introduced by the uvrA⁻ extract or 0.4 breaks/molecule by the uvrB⁻ extract and 0.1 breaks/molecule resulting from the photoreactivation treatment with E. coli photolyase (PRE) and black light. No increased breaks were seen after incubation with UVRA alone.

This uvrA-associated ATPase hydrolyzed ATP and dATP but not GTP or dGTP (data not shown). It was competitively inhibited by ADP (Figure 7) with a K_I of 25 ± 5μM. Similar competitive inhibition was also observed (data not shown) with dADP and ATP[S]. Seeberg and Steinum (33) have also observed ATPase activity in purified UVRA.

DISCUSSION

The first step in nucleotide excision repair in E. coli is the incision of the damaged DNA by a UV-endonuclease activity which is constituted from the products of at least the uvrA, uvrB, and uvrC genes (12). The location of the endonucleolytic incision relative to the lesion is not known at present, and it will probably be necessary to obtain all three uvr proteins in order to understand the specificity and mechanism of this reaction. In this report, we have described a simple method of purifying one of these proteins, the uvrA

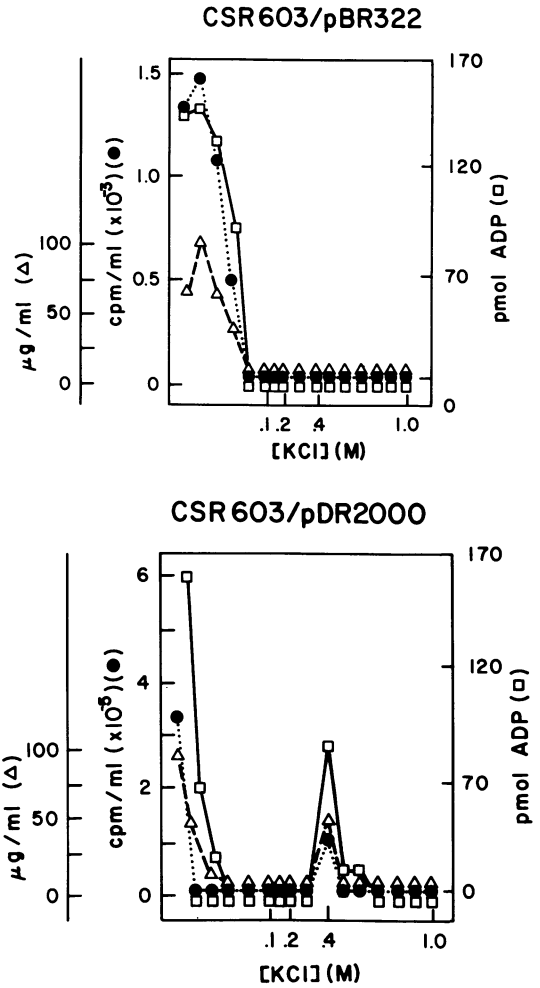

Figure 6. Chromatography of UVRA-associated ATPase activity on ss DNA cellulose. Maxicell extracts of 500 ml of either CSR603/pBR322 (Top) or CSR603/pDR2000 (Bottom) were loaded onto a 100 ml DEAE Biogel column and the run through material was then applied to a 15 ml ss DNA cellulose column as described in Materials and Methods. The DNA cellulose column was eluted with a step gradient of 0.1 to 1.0 M KCl (0.1M increments, 1.5 ml each). 10 μl of each fraction was assayed for ATPase (□) as described in Materials and Methods, 0.1 ml was assayed for radioactivity (●) by scintillation counting and 0.5 ml was assayed for protein (Δ) by the Coomassie Blue protein assay of Bradford (29).

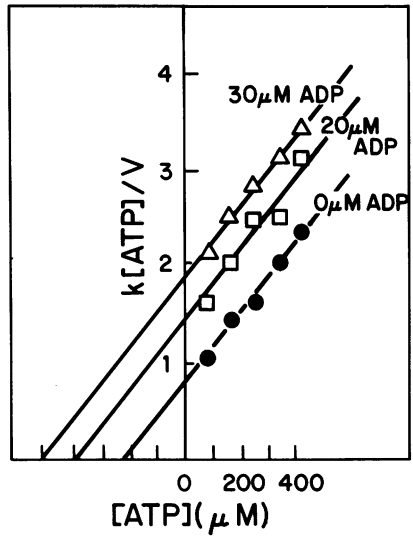

Figure 7. Kinetics of the UVRA-associated ATPase activity: inhibition by ADP. 25 ng of UVRA Fraction III was assayed for ATPase in presence of 0, 20 and 30 μM ADP; and plots of k[ATP]/v vs [ATP] are presented. For k[ATP]/v vs [ATP] plots, competitive inhibition is indicated by a family of parallel lines where the x intercept is $-K_m(1 + [I]/K_I)$ and $k = 10^6$min/l.

gene product. Two factors contributed to our success. First, the use of a lexA repressor-defective mutant carrying a multicopy plasmid bearing the uvrA gene provided about 100 times the amount of UVRA present in wild-type E. coli cells (17). Second, our choice of maxicells as a source of uvrA protein allowed us to specifically radiolabel UVRA which could then be followed through purification by simple assays of radioactivity.

We found three activities associated with purified UVRA: UV-endonuclease complementation, DNA-binding, and ATPase activity. In theory, any of these could have been used to follow UVRA during purification. UV-endonuclease complementation, used by Seeberg et al. to purify UVRA by conventional methods (12,27,32,33), is the most specific but is time consuming and difficult to adapt for convenient, routine use. The other two activities, ATPase and DNA binding, are much less specific and are more laborious than scintillation counting verified by SDS-PAGE and autoradiography. Using the latter, we are able to prepare purified UVRA in 1-2 days from radiolabeled maxicells.

Purified UVRA was found to bind to ss and ds DNA indicating that the DNA binding activities reported earlier (13,32) are intrinsic to the uvrA protein.

The binding to ss and UV-irradiated ds linear DNA was markedly stronger (Figure 4 and unpublished observations) than to unirradiated ds linear DNA; this suggests that UVRA is a "melting" protein and recognizes locally-denatured sites in ds DNA produced by UV-photoproducts.

An ATPase activity copurified with UVRA; and although we have not rigorously excluded that this activity may be due to contaminants, its absence from a uvrA$^-$ strain strongly suggests to us that it is due to UVRA. Additional data (not shown) indicate that the ATPase, the radiolabeled uvrA polypeptide and the uvrA UV-endonuclease complementing activity also co-chromatograph on phosphocellulose as well as on ss DNA cellulose, again strongly suggesting that this ATPase activity is an intrinsic activity of UVRA. Furthermore, the stimulation by ATP of the binding of UVRA to DNA demonstrates the interaction of UVRA and ATP, while the even greater stimulation by ATP[S] suggests that the ATPase may modulate the strength of the interaction between UVRA and DNA.

In this communication, we have described the purification of the uvrA protein by following the radioactive label in a maxicell preparation without having to use an assay for functionality at any intermediate step. This approach is highly useful since a functional assay is not used during purification. It can be applied to a wide variety of proteins and should be particularly advantageous for those gene products without a convenient functional assay.

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