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The purification of the *Escherichia coli* UvrABC incision system

Anthony T.Yeung[1], William B.Mattes[2], Euk Y.Oh, George H.Yoakum[3] and Lawrence Grossman*

Department of Biochemistry, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD 21205, [1]Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111, [2]Laboratory of Molecular Pharmacology, and [3]Laboratory of Human Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205, USA

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ABSTRACT

The UvrA, UvrB and UvrC proteins of Escherichia coli have been purified in good yields to homogeneity with rapid three- or four-step purification procedures. The cloned uvrA and uvrB genes were placed under control of the E. coli bacteriophage λ P_L promoter for amplification of expression. Expression of the uvrC gene could not be amplified by this strategy, however, subcloning of this gene into the replication-defective plasmid pRLM24 led to significant overproduction of the UvrC protein. The purified UvrA protein, with its associated ATPase activity, has a molecular weight of 114,000, the purified UvrB is a 84,000 molecular weight protein and the UvrC protein has a molecular weight of 67,000.

INTRODUCTION

The UvrABC endonuclease, as a protein complex, is composed of three proteins, UvrA, UvrB and UvrC, with molecular weights of 114,000, 84,000 and 67,000 respectively (1,2,3). The UvrA protein was reported to possess a DNA-independent ATPase activity (4) that is able to bind to both single-stranded and double-stranded DNA (4,5). The UvrB protein, in the presence of MgATP, is able to assist the UvrA protein to form a stable protein complex on a DNA molecule possessing pyrimidine dimers (5,6). The UvrC protein binds to the UvrAB-damaged nucleoprotein complex to activate the endonucleolytic activity which generates two nicks in UV irradiated DNA seven nucleotides 5' and three to four nucleotides 3' to the same pyrimidine dimer (5,7). Excision of the pyrimidine dimer-containing DNA as a dimer-containing 12 or 13 base single-stranded fragment requires the action of the UvrD protein in the presence of DNA polymerase I and its appropriate substrates (8).

Because of the central role played by this complex in DNA repair of a wide class of DNA damaging agents, its intrinsically interesting biochemistry and the similarity of its substrate specificity with that of the putative eucaryotic repair systems, there has been a great deal of interest on the part of the community in having these proteins made available for study. It

is for these reasons that the detailed purification procedures are documented.

Most of the studies of the UvrABC endonuclease require the use of highly purified and active UvrA, UvrB and UvrC proteins. Purification of these Uvr proteins has been made difficult because their constitutive levels are extremely low (1,3) and they have unique requirements for stability in solution. The availability of the cloned uvrA, uvrB and uvrC genes has facilitated overproduction of their gene products, thereby, easing requirments for protein purification.

MATERIALS AND METHODS

Affi-Gel Blue, acrylamide, N,N'-methylene-bis-acrylamide, TEMED, ammonium persulfate and Coomassie blue R-250 were purchased from Bio-Rad; phenylagarose was obtained from BRL; DEAE-Sephacel and Sephadex G-150 were obtained from Pharmacia; bactotryptone and yeast extract were purchased from Difco laboratories; egg white lysozyme, agarose, Trizma base, ampicillin, kanamycin and chloramphenicol were from Sigma; Protein quantitation was performed by the Bradford dye-binding assay (9) as supplied by Bio-Rad Laboratories. The BA85 nitrocellulose filters were obtained from Schleicher and Schuell. The calf thymus DNA was purchased from Calbiochem and was deproteinized and dialyzed before use.

Preparation of Damaged DNA

A 15 watt General Electric germicidal lamp was used to irradiate 0.1 mg/ml of fd RF-I DNA (15) at a distance of 45 cm. The dosage at 254 nm was $2 J/M^2/sec$ producing about one pyrimidine dimer per 15 seconds per covalently closed circular replicative form I (RF-I) fd DNA molecule.

Preparation of Single-stranded DNA Sepharose Resin

One gram of calf thymus DNA (Sigma) was treated with RNase and then with Pronase CB (Calbiochem) and spooled in chloroform-isoamyl alcohol. These steps were repeated at least once. The final preparation of DNA was dialyzed versus 1 mM NaEDTA, pH 8.0 and adjusted to 10 mg/ml. One-half gram of this DNA was boiled in a microwave oven for 10 minutes and then rapidly chilled on ice. The denatured DNA was added to 300 gm of Sepharose 4B freshly activated with cyanogen bromide (Kodak) according to the procedure described by Aslam et al (10) and tumbled overnight at 4°C. The next morning, 300 ml of 0.2 M $NaHCO_3$ at pH 8.0 was added and incubated for 2 additional hours at 22°C. The supernatant fluid was removed and the unreacted CNBr-activated sites were blocked by the addition of 300 ml of 1M ethanolamine (Gallard/Schlesinger) pH 8.0 and incubated at 22°C for 2 hours. The single-stranded DNA resin was

washed exhaustively on a fritted glass funnel at 4°C with 1.5 liters of 10 mM KPO_4 pH 8.0, 1 liter of 1 M KPO_4 pH 8.0, 2 liters 1 M KCl, deionized water, 2 liters of 0.1 M NaOH, 2 liters of deionized water and 1 liter of 5 M urea.

UV-endonuclease Assay

The UvrABC endonucleolytic activity was measured by the conversion of a duplex RF-I DNA to the single-stranded species of RF-II DNA which under appropriate salt conditions binds to nitrocellulose filters (11,12). The number of pyrimidine dimers in each fd RF-I DNA molecule averaged between 1 and 6 according to the needs of the experiments. The 140 µl reaction mixture consisted of 85 mM KCl, 40 mM potassium morpholinopropanesulfonate (MOPS) at pH 7.6, 1 mM NaEDTA, 1 mM dithiothreitol, 15 mM $MgSO_4$, 2 mM ATP, 39 fmol (0.16 µg) of ^{3}H-labeled fd RF-I DNA (15), 490 fmol of UvrA protein, 535 fmol of UvrB protein and 516 fmol of UvrC protein. The mixture was incubated at 37°C and then subjected to denaturation and renaturation as described (5).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE) SDS PAGE gels were developed according to the methods described by Laemmli (13). The 16 cm x 14 cm x 1.5 mm slab gels were composed of a 9 % acrylamide separating gel at pH 9.5 and a 5 % acrylamide stacking gel at pH 6.8, with an acrylamide to bisacrylamide ratio of 40:1. The gels were run at 40 mA for 4 hours in a water-cooled gel apparatus. Because of the abundance of the UvrA protein in the gel samples, monitoring the elution profile of the UvrA protein during the purification steps can be stained in 20 minutes in a mixture of 45 % methanol, 10 % acetic acid and 1 % Coomassie blue R-250, and destained in less than two hours with several changes of 10 % methanol, 7 % acetic acid. The destaining solution can be recycled by storing the used solution in the presence of ordinary packaging yellow foam which acts as an absorbent for the blue dye.

Buffers Used in Enzyme Purification

All buffers used in the purification were thoroughly degassed before fresh DTT or β-mercaptoethanol was added. All steps were executed as close to 0°C as possible.

Buffer 1: 0.1 M Tris-HCl, 0.3 M NaCl, 1 mM EDTA, 2 mM DTT, pH 7.5

Buffer 2: 0.1 M KPO_4, 1 mM EDTA, 2 mM DTT, 15 % glycerol pH 7.5.

Buffer 3: 0.1 M KCl, 0.1 M K+MOPS pH 7.5, 4 mM β-mercaptoethanol, 30 % glycerol

Buffer 4: 50 mM K+MOPS pH 7.5, 2 mM β-mercaptoethanol, 15 % glycerol.

Buffer 5: 2 mM K+MOPS pH 7.5, 2 mM β-mercaptoethanol, 15 % glycerol.

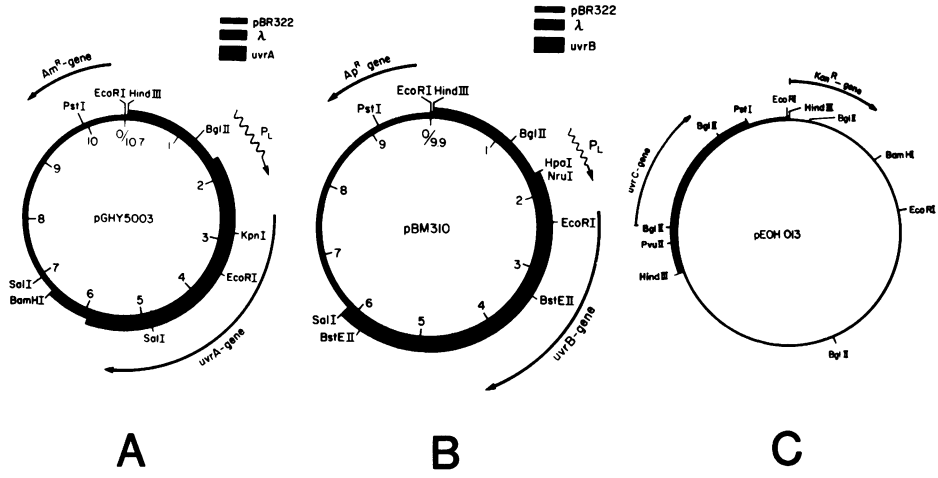

Figure 1. Plasmids used for the overproduction of the UvrA, UvrB, UvrC proteins. Panel A: pGHY5003 (14) with the uvrA gene under control of the bacteriophage λ P_L promotor. Panel B: pBM310 with the uvrB gene under the control of the P_L promotor. Panel C: pEOH013 with the uvrC gene cloned onto the 'runaway' plasmid pRLM24.

Buffer 6: 50 mM K+MOPS pH 7.5, 2 mM β-mercaptoethanol, 75 % glycerol

Buffer 7: 0.1 M Tris-HCl, 0.35 M NaCl, 1 mM EDTA, 2 mM dithiothreitol pH 7.6

Purification of the UvrA Protein

Preparation of Cell Lysate E. coli GHY8533 (14)(pGHY5003 in N99, Figure 1) was cultured in 160 liter batches in Luria Broth (10 gm/l Difco Yeast Extract, 5 gm/l Difco bactotryptone, 10 gm/l NaCl pH7.6) at 32°C in a New Brunswick Fermentor in 160 liter batches. At a cell density of $5x10^8$ cells/ml, the temperature of the culture was raised to 42°C to induce expression of the uvrA gene linked to the bacteriophage λ P_L promotor to overproduce the UvrA protein. After 4 hours, the cells were chilled to 4°C and harvested at the same temperature. The cell pellet was resuspended in an equal volume of 0.1 M Tris-HCl pH 8.0, and stored frozen at -20°C. About 700 gm of packed cells are obtained from each 160 liter culture. The recoveries of UvrA protein in these cells ranged from 2-4 % of the total soluble protein to the maximum of 7 % obtained in 1-liter cultures. About 60-100 gm of packed cells are used in a typical UvrA protein purification. This protocol has been applied to 1 liter cultures to give equally good yields of UvrA protein.

110 grams of GHY8533 frozen cell paste were suspended in 110 ml of 0.1M Tris-HCl, 4 mM dithiothreitol (DTT) pH 7.5. 10 ml of 0.5M NaEDTA pH 8.0 and 200 mg of lysozyme in 12 ml of water were mixed with the slurry and placed

on ice for 2 hours. The cell lysis and shearing of the cellular DNA was completed by sonication with a W350 Branson Sonifier at 40 watts for 15 minutes at 30 % cycle. The suspension was centrifuged for 1 hour at 27,000 x g at 4°C. The supernatant (Fraction I) contained >98 % of the UvrA protein and was immediately applied to the Affi-Gel Blue column.

Affi-Gel Blue Column Chromatography This column consisted of 150 ml of Affi-Gel Blue resin (Bio-Rad) in a 5 cm diameter column. The resin was washed in 6 M urea and 4 M NaCl, and then equilibrated in Buffer 1. Fraction I was pumped onto this column at a rate of 10 ml/min. and then washed with 100 ml of Buffer 1 at the same rate followed by 100 ml of Buffer 2. The column was then eluted with a 1-liter linear gradient of 0 to 3.5 M KCl in Buffer 2 at a rate of 3 ml/min in which 8 ml fractions were collected. A 20 µl aliquot of every fifth fraction was analyzed on SDS-PAGE. The UvrA protein usually elutes as a broad peak (Figure 2). Fractions 45-77 were pooled (Fraction II) and dialyzed two times versus 2 liters of Buffer 2. The contents inside the dialysis bag were 70 mM in KCl at the end of the dialysis. The E. coli single-stranded DNA binding protein (ssb) protein is not eluted until the application of 5M urea (data not shown).

Phosphocellulose P-11 Column Chromatography The column was 5 cm in diameter and contained 200 ml of resin. The column was washed with 500 ml of 2M KCl and 500 ml of Buffer 2 immediately before sample application. The sample, dialyzed Fraction II, was pumped onto this column at a rate of 3 ml/min followed by 300 ml of Buffer 2. The column was next eluted with a 1-liter linear gradient of 0.07 M to 0.77 M KCl in Buffer 2 at 3 ml/min, 8 ml fractions were collected and 20 µl aliquots of each fifth fraction were analyzed by SDS PAGE. The UvrA protein is eluted as a sharp peak around 0.35 M KCl (Figure 3). Fractions 37-42 were pooled (Fraction III) and dialyzed against 2 liters of 70 mM KCl in Buffer 2. In some preparations, an additional UvrA protein peak elutes after the main peak of activity. The nature of this small peak has not been investigated.

Single-stranded DNA Sepharose Column Chromatography The column was 5 cm in diameter and contained 100 ml of single-stranded DNA Sepharose 4B resin. Immediately before sample application the column was washed with 200 ml of 0.3 M KOH, 500 ml of 2 M KCl, and then 300 ml of 70 mM KCl in Buffer 2. The sample, dialyzed Fraction III, was pumped onto the column at a rate of 3 ml/min followed by 75 ml of 70 mM KCl in Buffer 2. The column was next washed with 50 ml of 5 mM ATP, 70 mM KCl in Buffer 2 to remove several contaminating proteins and then washed with 200 ml of 70 mM KCl in Buffer 2.

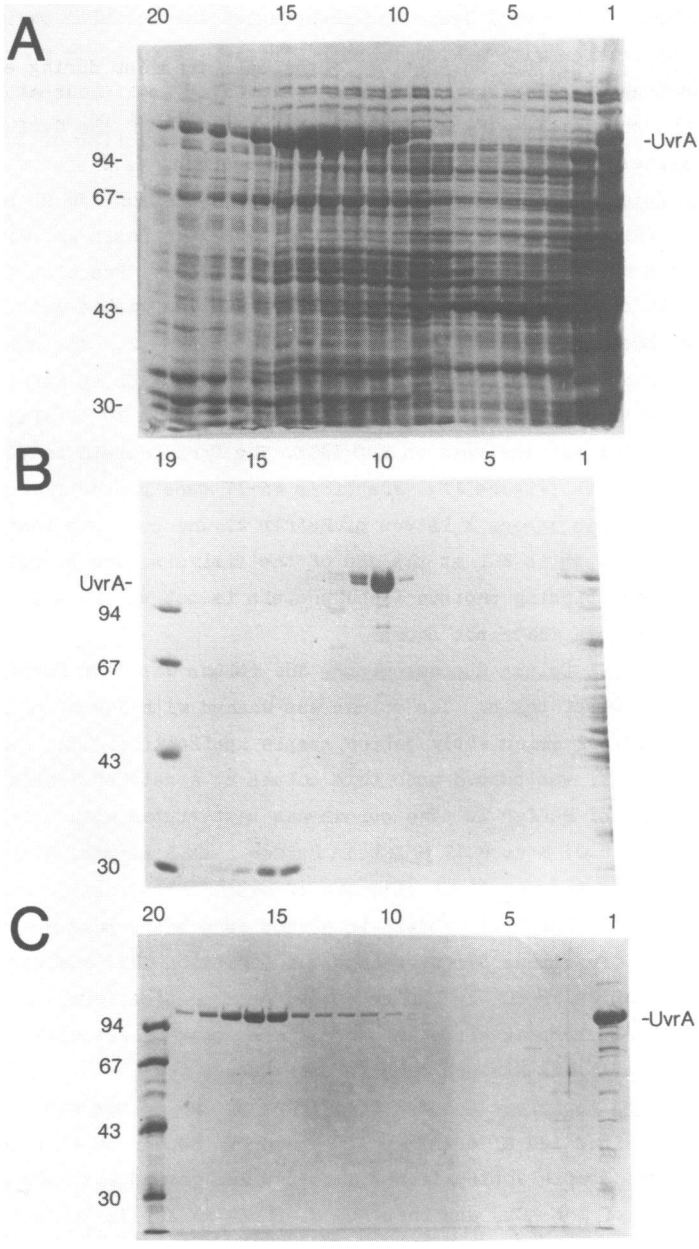

Figure 2. SDS PAGE monitoring of the purification of the UvrA protein by Affi-Gel Blue column (Panel A), phosphocellulose P-11 column (Panel B), and single-stranded DNA Sepharose column (Panel C). 20µl of every fifth fraction of the elution was analyzed on SDS PAGE by the method of Laemmli (13). Panel A, lane 1; sample applied to the column, lane 2; column flow through material

pool, lanes 3-20; every fifth fraction of the salt gradient elution profile. Lane 1, sample applied to the column, lane 2; column flow through material pool, lanes 3-18 ; every fifth fraction of the salt gradient during elution, lane 19; molecular weight standards, indicated $x10^{-3}$. Panel C, lane 1; sample applied to the column, lane 2; column flow through material pool, lane 3,4; proteins eluted by the ATP wash, lanes 5-19; every 5th fraction of the salt gradient of the elution, lane 20 molecular weight standards indicated$x10^{-3}$.

The column was eluted with a 1-liter linear gradient of 0.07 M to 0.77 M KCl in Buffer 2 in which 8 ml fractions were collected and 20 µl aliquots of each fifth fraction subjected to SDS PAGE (Figure 4). Fractions 50-72, which

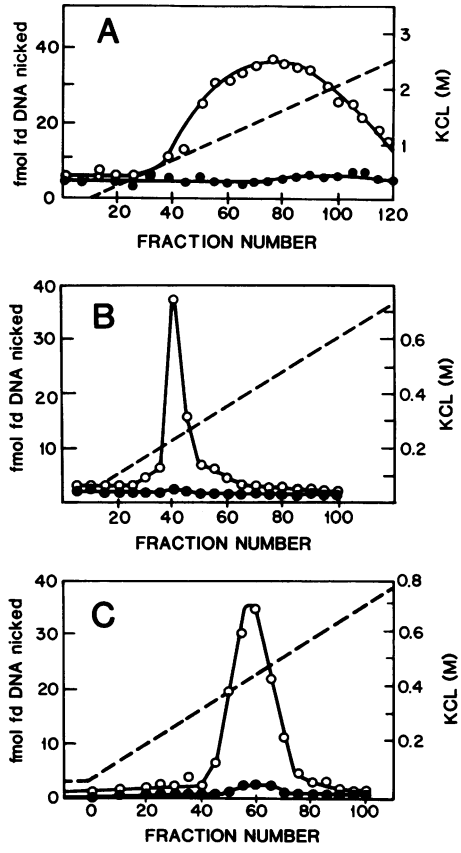

Figure 3. UvrABC complementation assay monitoring of the purification of the UvrA protein by Affi-Gel Blue Column (Panel A), phosphocellulose P-11 column (Panel B), and single-stranded DNA Sepharose column (Panel C). Each column was 5 cm in diameter and about 200 ml, 200 ml, and 120 ml in volume respectively. The fraction size was 8 ml 0.1µl of eluent was used in a typical 20 minute UvrABC complementation assay for UvrA activity (O). The dotted lines represent the KCl gradient in the elution buffer.

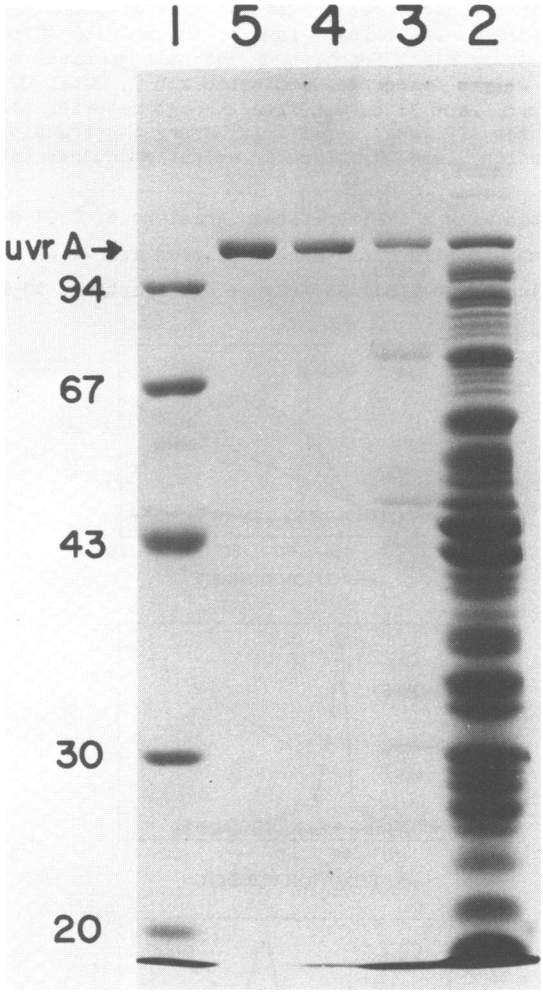

Figure 4. Summary of the purification of UvrA protein. Purification procedure for the UvrA protein was monitored by SDS PAGE according to the method of Laemmli (13). Lane 1, molecular weight standards, labeled x 10^{-3}; lane 2, crude extract of 42°C-induced GHY8533; lanes 3,4 and 5, pooled UvrA protein peak fractions from the Affi-Gel Blue column, phosphocellulose P-11 column, and single-stranded DNA Sepharose column, respectively.

contained most of the UvrA protein, were pooled (Fraction IV) and stored in 200 µl aliquots under liquid nitrogen.

Purification of UvrB Protein (Figure 5)

Cell Growth and Lysis Six-600 ml cultures of WM1731 (pBM310 in E.coli N99, Figure 1) were grown at 30°C in a rotary shaking incubator to a density of 4

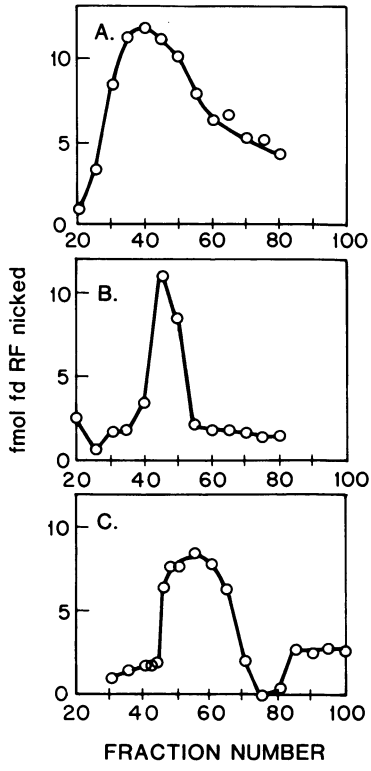

Figure 5. Purification of the UvrB protein. (a), Elution profile of the UvrB during the purification presented in Table II. 1µl of each sample was incubated for 5 minutes at 37°C in the presence of 1.3 pmol of UvrA, 0.5 pmol of UvrC, 4 µg tRNA and 12 fmol ^{3}H-fd RF-I DNA containing an average of six pyrimidine dimers per fd DNA molecule. The level of endonuclease activity that was not specific for UV damage was also measured for each sample and has been subtracted before the UV-specific activity is presented. This non-UV specific activity is usually less than 1.5 fmol of fd RF-I DNA nicked per five minutes because the presence of tRNA in the assay buffer. Panel A: Affigel blue column, panel B; DEAE-sephacel column, panel C; phenyl agarose column. The elution profile of the sephadex G-150 column was not assayed. Instead the UvrB elution from the sephadex G-150 column was followed by SDS PAGE to facilitate the pooling of the fractions of the highest purity.

x10^{8} cells/ml. The cultures were rapidly shifted to 42°C by the addition of 400 ml Luria broth prewarmed to 55°C. The cultures were shaken for three hours at 42°C and then harvested by centrifugation at >25°C. The cells should not be chilled until the next step or autolysis will occur. An equal weight of Buffer 3 was added to the wet cell pellet and the cell suspension was stirred at 0°C overnight. During this time autolysis occurred and the suspension became viscous. The viscous lysate was passed through a French

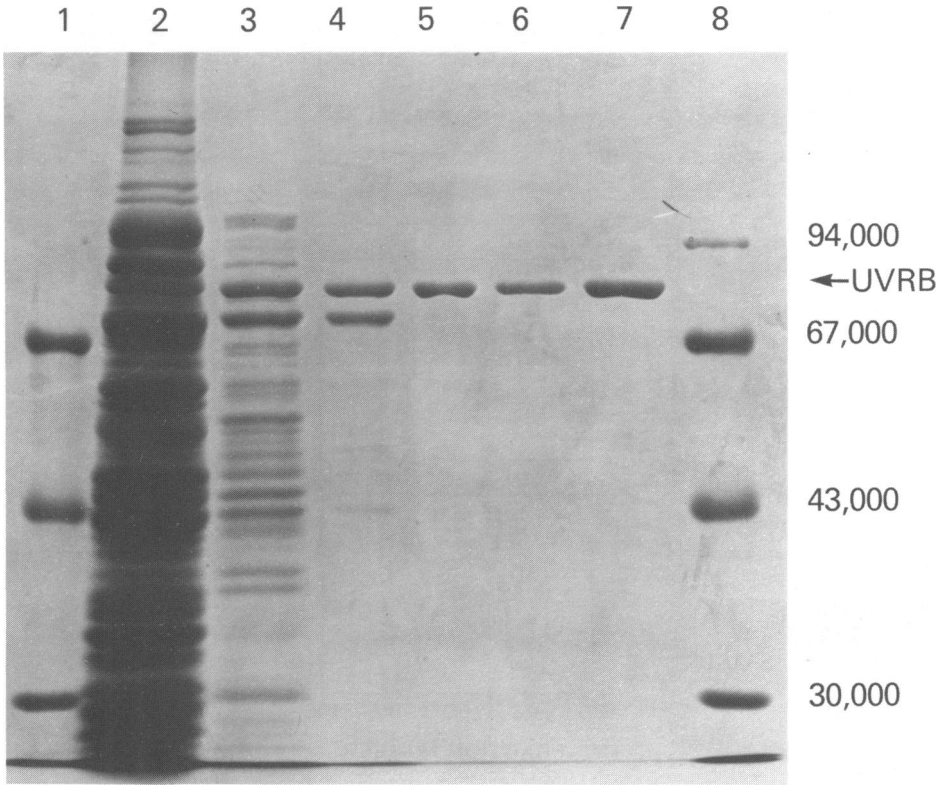

Figure 6. SDS PAGE analysis of the purification of UvrB protein. This 7 % gel was performed by the method of Laemmeli (13). Lanes 1 and 8 contain the molecular weight markers. Referring to UvrB fractions described in Table II; lanes 2 to 7 contain fractions I, II, III, IV, V and V of volumes 10, 40.2, 18.4, 5.1, 8.4 and 25 µl respectively.

pressure cell twice and then centrifuged at 27,000 x g for 35 min at 4°C to prepare the high-speed supernatant (Fraction I).

Affi-Gel Blue Column Chromatography The high-speed supernatant (54 ml) was loaded onto an Affi-Gel Blue column (2.5 x 5.3 cm) which had been equilibrated with 50 mM KCl in Buffer 4. The column was washed with 300 ml of 50 mM KCl in Buffer 4 and eluted with a linear gradient of 0.05 M to 1 M KCl in Buffer 4 at approximately 1 ml/min in which 8.6 ml fractions were collected. The UvrB protein eluted as a broad peak at 0.26 M KCl. Fractions 30-54 were pooled and dialyzed against enough Buffer 4 to bring the KCl concentration to 50 mM (Fraction II).

DEAE-Sephacel Column Chromatography The dialyzed Affi-Gel Blue pool

(Fraction II-219 ml) was loaded onto a DEAE-Sephacel column (2.5 x8.1 cm) previously equilibrated with 50 mM KCl in Buffer 4. After loading, the column was washed with 50 mM KCl in Buffer 4 and eluted with a linear gradient of 0.05 - 1 M KCl in Buffer 4 at a flow rate of approximately 1 ml/min in which 9 ml fractions were collected and assayed by PAGE and the UvrABC endonuclease complementation assay. The peak of UvrB protein and activity eluted at 0.25 M KCl (fractions 42-52). The pool was adjusted to 0.5 M KCl by the addition of 2 M KCl to give a final volume of 116 ml (Fraction III).

Phenyl agarose column chromatography This column (2.5 x 4.2 cm) was first washed with 15 % glycerol, then 5 M urea and finally equilibrated with 0.5 M KCl in Buffer 4. Fraction III was loaded onto it and then the column was washed with 320 ml of 0.5 M KCl in Buffer 4. The UvrB protein was eluted with 720 ml of a decreasing salt gradient of 25 to 1 mM KCl in Buffer 5 in which 8.2 ml fractions were collected during the wash and the gradient. The UvrB protein eluted shortly after the start of the gradient but in a broad peak (fractions 49-80, 264 ml) at 0.022 M KCl. To concentrate the pooled fractions they were loaded onto a small DEAE-Sephacel column (2.5 x 6 cm) previously washed with 2 M KCl and equilibrated with 50 mM KCl in Buffer 4. The column was eluted by a steep 60 ml gradient of 0.05 - 1 M KCl in Buffer 4, and the pool of fractions (1.1 ml/fraction) containing UvrB protein was further concentrated by dialysis against two volumes of Buffer 6 to give Fraction IV (27.5 ml).

Sephadex G-150 Column Chromatography This column (2.5 x 94 cm plus a 1.0 cm layer of Sephadex G-10 on top) was first equilibrated with 0.25 M KCl in Buffer 4. 20 ml of Fraction IV was loaded onto the column at a flow rate of 0.22 ml/ min and eluted with 0.2 M KCl in Buffer 4. Fractions 48-66 (4.6 ml/ fraction) contained UvrB protein and were pooled. This pool was concentrated by dialysis against 1.59 volumes 0.2 M KCl in Buffer 6 to give Fraction Va (21.0 ml, fractions 48-58) and Vb (14.7 ml, fractions 59-66). The SDS PAGE patterns for each step in purification is shown in Figure 6.

Purification of UvrC Protein(Figure 7)

Preparation of Cell Lysate E. coli cells EOH-013 harboring the uvrC containing plasmid, pEOH-013 (Figure 1) was cultured in 160 liter batches in Luria Broth (10 gm/l Difco Yeast Extract, 5 gm/l Difco bactotryptone, 10 gm/l NaCl pH7.6) at 37°C in a New Brunswick Fermentor. The cells were harvested by centrifugation at a cell density of about $8x10^8$ cells per ml culture at 4°C, resuspended in equal volumes of 0.1 M Tris-HCl pH 8.0, and stored frozen at -20°C. About 700 gm of packed cells are obtained from each 160 liter

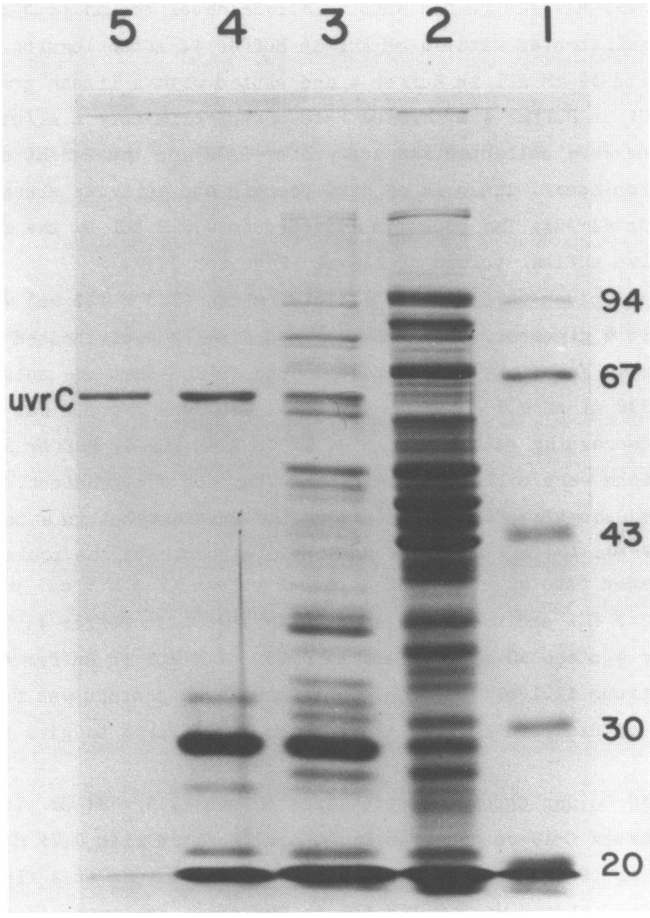

Figure 7. Purification of the UvrC protein. Purification procedure of the UvrC proteins was evaluated by SDS PAGE. Lane 1; molecular weight standards indicated $x10^{-3}$, lane 2: 1.5 µl crude extract of EOH-013, lanes 3,4,5; 60 µl each of the pooled UvrC protein peak fractions from the Affi-Gel Blue column, phosphocellulose P-11 column, and single-stranded DNA Sepharose column, respectively. The samples were precipitated with 10% trichlorcacetic acid for 10 minutes at 0°C and pelleted by centrifugation for 30 minutes at 9000xg at 4°C. The pellets were washed with acetone, dried, and dissolved by boiling for 5 minutes in 50mM Tris-HCl pH 6.8, 300mM β-mercaptoethanol and 2% SDS to prepare for electrophoresis.

culture. All steps were performed at 0-4°C unless otherwise noted and all buffers used in the purification were thoroughly degassed. It is important to use at least 100 gm of packed cells of EOH-013 in a UvrC protein purification in order to stabilize the UvrC activity. Cell pellets

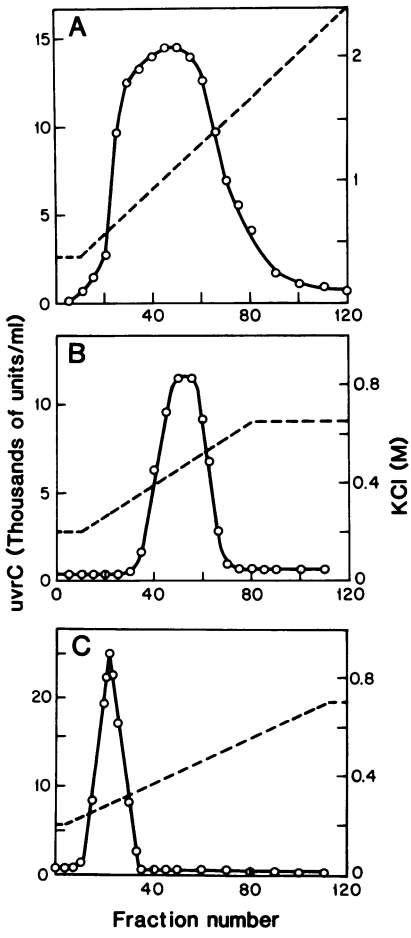

Figure 8. Elution profile of the UvrC protein from Affi-Gel Blue Column (Panel A); phosphocellulose P-11 column (Panel B), and single-stranded DNA Sepharose column (Panel C) during the three purification steps. Each column was 100 ml in volume and 5 cm in diameter. The fraction size was 8 ml 0.5µl of eluent was used in a typical 10 minute UvrABC complementation assay for UvrC activity (O). The dotted lines represent the KCl gradient in the elution buffer.

accumulated from 1 liter size cultures are also useful. A 200 gm portion of cell paste was sliced into thin sections to which was added 100 ml of 25°C 0.1M Tris-HCl pH 7.2 and the mixture to thaw in a water bath at 0°C. After thawing 10 ml of a 0.5 M NaEDTA (pH 8.0) solution was added to bring the mixture to 17 mM EDTA. The mixture was adjusted to 2 mM in dithiothreitol and a fresh solution of 200 mg lysozyme in 12 ml of deionized water was

mixed in. The mixture was kept on ice for 2 hours after which 19.2 ml of 5M NaCl was added to bring the mixture to 0.3 M in NaCl. The mixture was sonicated at 40 Watts at 30% duty cycle with a Branson Sonic disrupter for a total of 15 minutes. Cell lysis was complete by the end of 10 minutes. The cell lysate was centrifuged for 1.5 hours at 27,000 x g. The supernatant fraction was collected and quickly frozen at -20°C in 24-15 ml polypropylene tubes.

The following three purification steps each used 5 cm diameter columns containing 100 ml of resin:

Affi-Gel Blue Column Chromatography (Figure 8a) The column was washed with 5 M urea and 2 M KCl and equilibrated in Buffer 7 before use. Twenty four tubes of the supernatant fraction of the centrifuged cell lysate (Fraction I) were thawed in ice water and pumped onto the Affi-Gel Blue column at a rate of 6 ml/min. The column was washed with 200 ml of Buffer 7, and then with 300 ml of 0.2 M KCl in Buffer 2. The column was then eluted with a 1 liter linear gradient of 0.2 to 2.5 M KCl in Buffer 2. Fractions of 8 ml were collected at a rate of 3 ml/minute and the UvrC activity assayed for after each fraction was collected. As seen in Figure 3 the UvrC protein eluted as a broad peak (Fraction II). Fraction 25 to 80, which represented about 80% of the UvrC activity, was pooled and dialyzed against about 1.4 liter of Buffer 2 such that the equilibrium KCl concentration was 0.15-0.20M.

Phosphocellulose P-11 Column Chromatography (Figure 8b) The column was washed with 2 M KCl in Buffer 2 and equilibrated in Buffer 2 containing 0.2 M KCl. The dialyzed Fraction II was pumped onto the phosphocellulose column at a rate of 3 ml/min. The column was washed with 200 ml of 0.2 M KCl in Buffer 2, and then eluted with a 1 liter linear gradient of 0.2 to 0.7 M KCl in Buffer 2 at a rate of 3 ml/min. The fractions were assayed for UvrC activity as each fraction was collected. The UvrC activity eluted at about 0.4M KCl as a fairly sharp peak. The UvrC activity peak was pooled (Fraction III) and immediately dialyzed *versus* 2 liters of 0.15 M KCl in Buffer 2 overnight such that the KCl concentration at equilibrium was 0.2 M.

Single-stranded DNA Agarose Column (Figure 8c) This column was washed in 300 ml of 5 M urea and 200 ml of 2 M KCl and equilibrated in 0.2 M KCl in Buffer 2. The dialyzed Fraction III was pumped onto the column at a rate of 3 ml/min. The column was washed with 100 ml of 0.2 M KCl in Buffer 2 and then eluted with an 1 liter linear gradient of 0.2 to 0.7 M KCl in Buffer 2 at a rate of 3 ml/min. Each 8 ml fraction was mixed by inversion 4-times and then quickly stored frozen in liquid nitrogen as 100 µl aliquotes. A limited

Table I
Purification of UvrA Protein

Fraction		Volume (ml)	Total protein (mg)	Total UvrA (mg)	Yield (%)
I	Crude extract	270	7,020	130	100
II	Affi-Gel Blue	280	792	108	83
III	Phosphocellulose P-11	95	74	60	46
IV	ssDNA Sepharose	184	42	42	33

number of assays can be fitted into this time frame to locate the center of the UvrC peak. The column was eluted at 6 ml/minute after the first 35 fractions were collected.

RESULTS

Purification and Properties of the UvrA Protein

The UvrA protein tends to aggregate during the purification procedure and storage. Its solubility at less than 0.3 M salt concentration at pH7.6 is dependent on the presence of at least 10 mM KPO_4 in the buffer (data not shown). However both PO_4 and high salt are not needed if UvrA is kept in a solution at pH greater than 8.5. Although UvrA activity is stable to pH as high as 10, pH 7.5 is used throughout this study in order to maintain UvrA in a more physiological environment. Because the exposed sulfhydryls of UvrA protein are sensitive to oxidation 2 mM dithiothreitol is used throughout the purification. The intermolecular crosslinking of UvrA molecules to form long filaments upon standing in solution can only be reversed by boiling the filaments in a buffer containing dithiothreitol or β-mercaptoethanol (data not shown). The oxidation of small volumes of UvrA protein can be a rapid process at 0°C with up to 20 % loss in activity in 24 hours. Thus purified UvrA protein is routinely stored in small aliquots in liquid nitrogen. The oxidation of UvrA protein during SDS PAGE can also cause the UvrA protein band to appear as a doublet in the gel (data not shown). This can be prevented by pre-electrophoresis of the gel before sample application.

The elution profiles of UvrA purification are shown in Figures 2, 3 and

Table II

Purification of the UvrB protein

Fraction		Volume mg/ml	Protein[2] mg/ml	UvrB[1] mg/ml	Total UvrB	Yield %
I	27,000xg Supernatant	54	52.4	0.77	41.5	100
II	Affigel-blue	215	0.83	0.1	21.3	51
III	DEAE-Sephacel	99.2	0.47	0.16	15.6	38
IV	Phenyl Agarose	27.5	0.53	0.49	13.4	32
V	Sephadex- G-150	45.8	0.26	0.26	11.7	28

1 Determined by densitometric analysis of Coomasie Blue stained SDS PAGE using the purest UvrB preparations as reference .
2 Determined by the Bradford dye binding assay using bovine serum albumin as a standard.

4. Figure 2 and 3 represented data from two separate purification attempts using identical conditions. The Affi-Gel Blue resin was chosen for the first step of UvrA protein purification because of the efficiency of separation of DNA from the UvrA protein and because the UvrA protein is greater than 50% pure by using this step. The cell extracts were maintained in NaCl buffer instead of the KCl buffer because the UvrA protein binds less tightly to this resin in the presence of KCl. The ability to apply the sample to the column in 0.35 M NaCl allowed the UvrA to be separated from the DNA which does not bind to the Affi-Gel Blue resin under these conditions. The typical absorbance ratio at 260:280 nm of the UvrA peak from this column is 0.68, indicating that little nucleic acids, if any, are present. About 10 % of the UvrA protein remains bound the Affi-Gel Blue resin at the end of the elution and can be washed out with 5 M urea.

Purification and Properties of the UvrB Protein The results of the purification scheme are summarized in Table II and Figures 5 and 6. We have observed that the use of the λ N99 host is critical for a high yield of purified protein. The uvrB gene product was highly amplified in N4830 (34) at 42°C, and lacking the λ lysozyme gene this strain is not predisposed to autolysis as is the N99 strain after 42°C induction. However, in induced cultures of N4830/pBM310 the uvrB gene product was quantitatively sequestered into material that sedimented at 27,000 x g, when followed by SDS PAGE (data not shown). This putative UvrB polypeptide could not be extracted into an

Table III
Purification of UvrC Protein

Fraction	Total Volume (ml)	Units ($x10^{-6}$)	Protein (mg)	Specific activity (units/μg)	Purification (fold)	Recovery (%)
I. Crude Extract	330	2.6[b]	11,055	0.00024	1	
II. Affi-Gel Blue	475	5.2	228	0.023	96	100
III. Phospho-cellulose	240	2.6	67	0.037	154	50
IV. ssDNA-sepharose	170	2.4	1.9	1.25	5208	46

[a] Typical purification from 100 grams of packed cells.
[b] The measured UvrC activity in the crude extract approximately equaled half of the uninhibited UvrC activity. The Fraction I in which there was no inhibition by cellular DNA and in which the UvrC activity was stable was taken as 100% yield.

enzymatically active soluble form by treatment with 0.4 M KCl, 0.5 % Brij 58, 0.1 % octyl D-glucoside, 20 % sucrose, and 2 M urea. The behavior of the uvrB gene product on phenyl agarose columns suggests that it is a very hydrophobic protein. Hence, in cell extracts from strains other than N99, perhaps the amplified UvrB protein is irreversibly associated with the cell membrane. The pool of fractions from the Affi-Gel Blue column containing the uvrB gene product (Fraction II) contained about 5 % of the total nucleic acid present in Fraction I, as monitored by absorbances at 260 nm and 280 nm. Chromatography on DEAE-Sephacel serves to concentrate the uvrB gene product and separate it from the remaining nucleic acid.

On both Affi-Gel Blue and DEAE-Sephacel a 70 Kd protein copurifies with the 80 Kd uvrB gene product (Figure 6). This lower molecular weight species is possibly a proteolytic breakdown product of the UvrB protein since its amount as judged by SDS polyacrylamide electrophoresis increases in dialyzed Fraction II upon prolonged storage at 4°C, whereas, the amount of the 80 Kd uvrB gene product decreases. At 0.5 M KCl it does not bind to phenyl agarose while the UvrB protein binds tightly to this resin until the salt is lowered to less than 25 mM KCl. The loss of a strongly hydrophobic domain may explain why this 70,000 dalton polypeptide cannot replace UvrB protein in the UvrABC UV-endonuclease complementation assay.

After chromatography on Sephadex G-150 and concentration by dialysis against 75 % glycerol the uvrB gene product is homogeneous as determined by

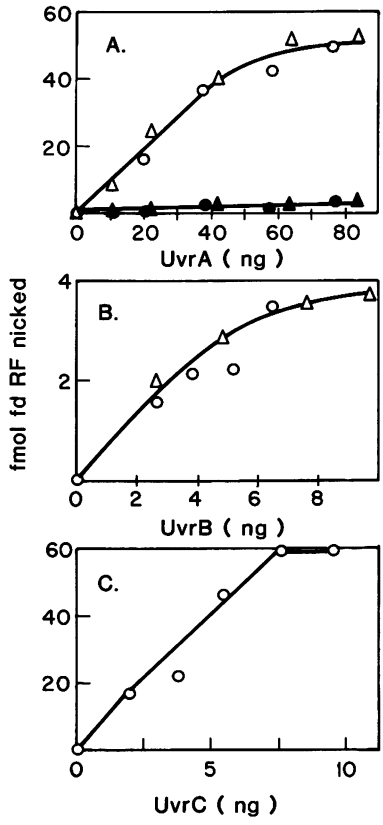

Figure 9. Dependence of the UvrABC endonucleolytic acitivity on the concentrations of the UvrA, UvrB and UvrC proteins. Panel A; varying amounts of UvrA protein was incubated with 500 fmol of UvrB protein, 500 fmol of UvrC protein, 60 fmol of ^{3}H-fd-RF-I DNA for 20 minutes at 37°C in a typical UvrABC endonuclease assay. The triangles and circles each represent the activity of two different UvrA preparations made six months apart. Panel B; initital velocity measurements of the UvrABC endonuclease nicking of fd RF-I DNA containing an average of six pyrimidine dimers per molecule. Varying amounts of UvrB protein were incubated with 500 fmol of UvrA and 500 fmol of UvrC and the substrate fd DNA for 5 minutes at 37°C. Triangles; UvrB of fraction IV, circles ; UvrB of fraction V. Panel C; varying amounts of UvrC protein was incubated in the presence of 640 fmol of UvrA, 754 fmol of UvrB and 60 fmol of ^{3}H-fd-RF-I DNA for 20 minutes at 37°C in a typical UvrABC endonuclease assay.

SDS PAGE (Figure 6). The total yield for the purification was 7.6 mg from 6 liters of culture (Table II).

Purification and Properties of the UvrC Protein The results of the purification is shown in Table III and Figure 8. The procedure resulted in a

purification of 5,208-fold with an overall yield of about 46 %. The purified UvrC protein appeared to be 100 % active (15). The UvrC protein was electrophoretically pure after the cell extract was resolved by chromatography on Affi-Gel Blue, phosphocellulose P-11, and single-stranded DNA Sepharose resins. The result of UvrC protein purification is presented in Figure 7. While the purification procedures for the UvrA and UvrB proteins are best followed by SDS gel electrophoresis, the UvrC protein is barely overproduced and unstable, hence its purification is best followed by the UvrABC complementation assay (Figure 9). Measurement of UvrC activity in the crude extract was about 50% efficient because of inhibition by the DNA in the cell extract. The UvrC activity is stable in the crude extract at -20°C. The UvrC protein binds to Affi-gel Blue column even in the presence of 1M NaCl while DNA does not. Thus, the Affi-Gel Blue chromatography step effectively removed greater than 99 % of the cellular DNA from the UvrC protein fractions. In the Affi-Gel Blue fractions UvrC activity decayed with a half-life of 7 days at 0°C (data not shown). The total UvrC activity from the pooled fractions of the Affi-Gel Blue column is, as a consequence, assigned as 100% activity (Table III) so that the final yield of UvrC activity is not distorted. Endonuclease I activity partly co-purified with the UvrC activity in both the Affi-Gel Blue step and the phosphocellulose P-11 step (data not shown). The endonuclease I activity will result in high background in the assay unless yeast tRNA (Boehringer Mannheim) is included in the assay buffer to inhibit the endonuclease I activity. tRNA showed no effect on the UvrABC endonuclease activity at up to 140 μg/ml (data not shown). We have chosen not to use endonuclease I-defective host cells because it is preferable to be able to detect the presence of endonuclease I during purification. The main cause of UvrC inactivation after the Affi-Gel Blue step, which removed most of the nucleic acids and greater than 80 % of the protein, is the precipitation of the UvrC protein. The UvrC protein will form a fine precipitate rapidly unless it is in the presence of KPO_4 and at least 0.2 M KCl. The UvrC protein constituted a significant portion of the precipitate and could not be reactivated. Hence, care must be taken to prevent the salt concentration from decreasing much below 0.2 M during dialysis steps. The significant loss of UvrC activity at the phosphocellulose P-11 step was partly the result of deleting the trailing part of the UvrC activity peak in the elution of the Affi-Gel Blue column, and partly because the pooled UvrC activity was dialyzed against a calculated volume of KCl-free buffer instead of several changes of buffer containing 0.2 M KCl.

The UvrC protein binds tightly to the phosphocellulose P-11 resin and was not eluted until about 0.4 M KCl concentration. The pooled UvrC activity from this step decayed with a half-life of five days at 0°C and was not totally stable at -20°C (data not shown). The tightness of binding of UvrC protein to single-stranded DNA Sepharose resin was dependent on the number of times the resin has been used for UvrC purification. When a freshly prepared batch of single-stranded DNA Sepharose is used, the UvrC activity is eluted by about 0.3 M KCl in the elution buffer. A good batch of single-stranded DNA Sepharose resin is essential because the UvrC protein fractions applied to the column had to be in 0.2 M KCl to prevent UvrC protein precipitation. With a batch of single-stranded DNA Sepharose resin which has been used repeatedly, UvrC protein may have difficulty binding to the resin even at 0.2 M KCl in the buffer. The UvrC activity is extremely unstable once the elution from the single-stranded DNA Sepharose column has begun. Thus the fractions were frozen in small aliquots in liquid nitrogen as each fraction was collected from the single-stranded DNA Sepharose column and later used to determine the UvrC activity peak. To further improve the yield and specific activity of the UvrC protein, the whole purification effort was completed in just a little over two days. The specific activity of the resultant UvrC protein was 1250 units per microgram protein. One unit of UvrC activity is defined as that amount of UvrC activity which will lead to incision of 9.8 fmol of fd RF-I DNA in 5 minutes at 37°C when incubated in the presence of 500fmol of UvrA and UvrB proteins and 39 fmol of fd RF-I DNA which contain an average of 6 pyrimidine dimers per fd molecule in the assay buffer described above (Figure 9). Thus, one unit of purified UvrC activity equals 0.7 nanogram protein (10 fmol) as judged by either the protein assay method of Lowry (16) or the Bio-Rad Bradford dye-binding assay (9), using Bovine Serum Albumin Fraction V (Boehringer Mannheim) as standard.

The UvrC activity from the single-stranded DNA agarose column decayed with a half-life of 7.5, 11 and 5 hours when stored at 0, 22, and 37°C respectively (data not shown). The stability of the UvrC activity at this step was not improved by the addition of bovine serum albumin to 0.2 mg/ml, or the presence of UvrA and UvrB proteins, Triton X-100, or the non-ionic detergents CHAPSO and CHAPS (CalBiochem), or 50% glycerol, or freezing at -20°C (data not shown). Nevertheless, the UvrC activity can be stored indefinitely when frozen in liquid nitrogen. The half-life of the UvrC activity in the fractions from the single-stranded agarose step can be as short as 2 hours at 0°C when less UvrC protein is present. The inactivation

of UvrC protein in this step is not due to protein precipitation, and it was not uncommon in our early attempts of UvrC purification to obtain an electrophoretically pure UvrC protein preparation which requires 50 times more UvrC protein to produce the same amount of UvrC activity as the UvrC protein from a well executed purification effort. Although UvrA and UvrC are purified with the same sequence of procedures, there is little detectable cross-contamination of these proteins at the end of the purification attempts because each protein is selectively amplified for expression, and because they are eluted by very different salt concentrations from the phosphocellulose P-11 and the single-stranded DNA Sepharose columns.

The UvrABC endonuclease activity is unchanged by the use of either Tris-HCl, HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid), or MOPS (morpholinopropanesulfonate) as the buffer for the maintenance of pH in the assay (data not shown). The assay can be totally inhibited by 40 mM of inorganic PO_4 in the assay buffer as expected for an ATPase that hydrolyses ATP to form ADP and PO_4 (data not shown). The assay is also inhibited by about 50% by the presence of 10 µg of either RF-I, RF-II or linear duplex DNA in the assay (data not shown).

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*To whom reprint requests should be sent

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