Amplification and Purification of UvrA, UvrB, and UvrC Proteins of *Escherichia coli*

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The UvrA, UvrB, and UvrC proteins of *Escherichia coli* are subunits of a DNA repair enzyme, ABC excinuclease. In order to amplify these proteins, we have joined the artificial canonical promoter tac (Amann E., Brosius, J., and Ptashne, M. (1983) *Gene* (Amst.) 25, 167-178) to the uvr genes to obtain plasmids that express these genes under the control of the tac represor. When cells carrying the tac-uvr plasmids are induced by the gratuitous lac inducer isopropyl-β-D-galactoside the Uvr proteins are overproduced reaching a level of 10-20% of total cellular proteins after 6-8 h of induction. We have developed methods to purify the three Uvr proteins, UvrA, UvrB, and UvrC, in milligram quantities and to near homogeneity from these overproducing cells. The purified UvrA protein is an ATPase but UvrB and UvrC proteins are not. However, UvrB protein stimulates the ATPase activity of UvrA protein by a factor of 1.5 in the presence of double-stranded DNA and by a factor of about 2.6 in the presence of UV-irradiated DNA but not in the absence of DNA.

*Escherichia coli* has a potent and versatile nuclease which removes a wide variety of nucleotide adducts including pyrimidine dimers and various alkylated bases from DNA by incising the 5th phosphodiester bond and the 4th or 5th phosphodiester bond of the modified nucleotide(s) (Sancar and Rupp, 1983; Sancar et al., 1985). The enzyme is made up of three subunits, UvrA (114,000), UvrB (84,000), and UvrC (66,083) (Sancar et al., 1981a, 1981b, 1981c; Sancar et al., 1984) and has an absolute requirement for ATP and divalent cations. The ATPase activity of the enzyme is associated with the A subunit (Kacinski et al., 1981, Seeberg and Steinum, 1982) which is also a DNA binding protein (Sancar et al., 1981a) with higher affinity to UV-irradiated DNA (Seeberg and Steinum, 1982). The scarcity of the enzyme in the cell has been an obstacle in purifying it and therefore we and others have used molecular cloning techniques to amplify the individual subunits, purify them, and reconstitute the enzyme from individually purified subunits (Kacinski et al., 1981; Seeberg and Steinum, 1982; Sancar and Rupp, 1983; Yeung et al., 1983). In this paper we describe the construction of plasmids that overproduce each subunit to 10-20% of total cellular proteins and the development of relatively simple purification procedures for obtaining milligram quantities of the subunits to near homogeneity. Using the purified proteins we have investigated the ATPase activity of UvrA protein in some detail. We find that UvrA has a turnover number of about 50 ATP molecules/min and that while neither DNA nor UvrB alone have any effect on this activity the two together stimulate it about 1.5-fold if the DNA was not irradiated and about 2.6-fold when irradiated DNA was in the reaction mixture. The UvrC protein when used in an equimolar amount with UvrA protein had no effect on the activity of the latter whether or not DNA and/or UvrB protein were present during incubation.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—*E. coli* K12 strain CH296 (recA1 endA1, Flaci2) was constructed by introducing Flaci2 into a streptomycin-resistant derivative of MM294 (Lauer et al., 1981). This strain was the host for our tac-uur plasmids. The tac plasmid used in these studies, pUNC09, was constructed as described previously (Sancar et al., 1983) from the tacI2 plasmid of Amann et al. (1983). The plasmids pDR1996 (Sancar et al., 1981d), pDR1494 (Sancar et al., 1981b) and pDR3003 (Sancar et al., 1981c) were the sources of the uvrA, uvrB, and uvrC genes, respectively. By joining the uvr genes to the tac promoter in pUNC09 we obtained the Uvr protein overproducing plasmids pUNC45 (uvar), pUNC211 (uvrb), and pDR3274 (uvrc). Cultures were grown in Luria Broth which contained streptomycin (50 µg/ml) and/or tetracycline (20 µg/ml) when indicated.

**Materials**—Column materials were obtained from the following sources: Blue Sepharose and phenyl-Sepharose from Sigma, hydroxyapatite (Bio-Gel HT) and DEAE-agarose from Bio-Rad; phosphocellulose P-11 from Whatman; and single-strand DNA cellulose from P-L Biochemicals. Ethylene glycol was obtained from Fisher and isopropylthio-D-galactoside (IPTG) from Boehringer Mannheim. ATP was purchased from P-L Biochemicals and [3H]ATP (30 Ci/mmol) from ICN Radiocchemicals. Restriction enzymes, BglII nuclease, and T4 DNA ligase were purchased from New England Biolabs.

**Buffers**—The following buffers were used: Buffer A (lysis buffer) contained 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, 10% sucrose. Buffer B contained 50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 1 mM EDTA, 20% (v/v) glycerol. Buffer C is made up of 20 mM potassium phosphate, pH 6.8, 10 mM β-mercaptoethanol, 1 mM EDTA, 20% (v/v) glycerol. Buffer D is made up of 20 mM potassium phosphate, pH 7.4, 1 mM EDTA, 20% (v/v) glycerol. Buffer E is made up of 20 mM potassium phosphate, pH 7.4, 1 mM EDTA, 20% (v/v) glycerol. Buffer F is made up of 20 mM potassium phosphate, pH 7.4, 1 mM EDTA, 20% (v/v) glycerol.

**Growth and Handling of Overproducing Cells**—Although tac plasmids are of great value in overproducing and purifying the desired proteins, special care must be taken to obtain reproducible overproduction upon induction of cells carrying these plasmids because even in Flaci2 background there is usually some overproduction of the tac controlled gene product which in the majority of cases causes slow cell growth or death thus selecting for nonoverproducing cells. To overcome this problem different approaches have been used (Amann et al., 1983; Bikle et al., 1983; DeAnda et al., 1983). By trial and error we have found that the following protocol gives reproducible overproduction (10% and higher): cell carrying tac plasmids are stored at

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1 The abbreviations used are: IPTG, isopropylthio-β-D-galactoside; SDS, sodium dodecyl sulfate.
PROTEIN CONCENTRATION WAS DETERMINED BY THE BRADFORD ASSAY (1976), OF SUPERCOILED DNA (SANCAR AND RUPP, 1983, SANCAR ET AL.). MAXICELLS WERE PREPARED BY THE METHOD OF SANCAR ET AL. (1979), AND SDS-POLYACRYLAMIDE GELS WERE RUN AND STAINED BY THE LAEMMLI METHOD (1970). THE COOMASSIE BLUE-STAINED GELS WERE SCANNED WITH A GS300 DENSITOMETER-SCANNER SYSTEM. THE DENSITOMETER-SCANNER SYSTEM WAS CAPABLE OF DETECTING ABOUT 10 NG OR MORE.

ENZYME ASSAYS—THE UVR PROTEINS WERE ASSAYED BY THE “INCISION ASSAY” OF SUPERCOILED DNA (SANCAR AND RUPP, 1983, SANCAR ET AL., 1984), BY THE “EXCISION ASSAY” WHERE THE REMOVAL OF RADIOACTIVE CISH-P STADDUCTS IS MEASURED, AND BY THE “TRANSFORMATION ASSAY” WHERE THE RESTORATION OF THE BIOLOGICAL ACTIVITY OF TRANSFORMING DNA IS MEASURED (HUSSAIN ET AL., 1985). THE ATPASE ACTIVITY OF UVR PROTEIN WAS MEASURED ESSENTIALLY AS DESCRIBED BY THOMAS AND MEYER (1982) WITH THE EXCEPTION THAT THE REACTION WAS CARRIED OUT IN ABC EXCINUCLEASE BUFFER. BRIEFLY, THE 25-µL REACTION MIXTURE CONTAINED 50 MM TRIS, pH 7.5, 100 MM KCL, 10 MM MgCl₂, 20 MM β-MERCAPTOETHANOL, 1 MM EDTA, 1% Glycerol (v/v), 50 µG/ML Bovine Serum Albumin, 0.5 MM ATP, 2 µC/ML OF PHA-T, AND ENZYME.

RESULTS

AMPLIFICATION OF UVR PROTEINS

IN ORDER TO PURIFY THE UVR PROTEINS WE JOINED THE GENES CODING FOR EACH PROTEIN TO THE TAC PROMOTER (AMANN ET AL., 1983) WHICH HAS THE CANONICAL -35 AND -10 SEQUENCES AND IS REPRESSIBLE BY THE TAC REPRESSOR. THE ECORI-PVU II “CASSETTE” OF ptt12 WAS INSERTED INTO pBR328 TO OBTAIN pUNC09 WHICH PROVIDED CONVENIENT RESTRICTION SITES FOR INSERTING THE UVR GENES. TO INSERT EACH UVR GENE INTO THIS VECTOR, PLASMIDS CARRYING THE UVR GENES WERE DIGESTED WITH RESTRICTION ENZYMES THAT CUT NEAR THE 5' TERMINUS OF EACH GENE (BAM FOR UVR A, BGl FOR UVR B, AND PVU FOR UVR C) DIGESTED WITH BamH I, NUCLEASE TO OBTAIN BOTH ENDS AND FOLLOWING INACTIVATION OF BAmH I, CUT WITH PstI TO PRODUCE FRAGMENTS CARRYING THE UVR GENES AND HAVING A BLUNT END AT 1 TERMINUS AND A PstE END AT THE OTHER. THESE FRAGMENTS WERE SUBSTITUTED FOR THE PvuII-PStI FRAGMENT “DOWNSTREAM” FROM THE TAC PROMOTER OF pUNC09 TO OBTAIN pUNC45 (uwrA’), pUNC211 (uwrB’), AND pDR3274 (uwrC’), RESPECTIVELY. PARTIAL RESTRICTION MAPS OF THESE PLASMIDS ARE SHOWN IN FIG. 1. THESE PLASMIDS WERE PICKED AS THE BEST OVERPRODUCERS FROM ABOUT 100 COLONIES SCREENED OF EACH CONSTRUCT. RESTRICTION ENZYME ANALYSIS SHOWED THAT PLASMIDS WITH LONGER DISTANCES BETWEEN THE TAC PROMOTER AND THE INITIATION CODONS OF THE UVR GENES WERE NOT GOOD OVERPRODUCERS AS A GENERAL RULE; HOWEVER, IN TWO INSTANCES PLASMIDS IN WHICH THE TAC PROMOTER WAS CLOSER TO THE INITIATION CODONS OF UVR B AND UWR C THE OVERPRODUCTIONS FROM THE RESPECTIVE PLASMIDS WERE NOT AS GOOD AS THOSE FROM pUNC211 AND pDR3274. THESE RESULTS, EVEN THOUGH SOMEWHAT PUZZLING, WERE NOT COMPLETELY UNEXPECTED AS IT HAS BEEN SHOWN BEFORE (ROBERTS ET AL., 1979) THAT SMALL CHANGES IN THE LEADER mRNA LENGTH WITHOUT AFFECTING THE RIBOSOME BINDING SITE CAN HAVE DRASTIC EFFECTS IN EITHER DIRECTION ON GENE EXPRESSION.

A REPRESENTATIVE PICTURE OF THE LEVELS OF UVR PROTEIN OVERPRODUCTION UPON INDUCTION CARRYING THE TAC-UVR PLASMIDS IS SHOWN IN FIG. 2. AS IS APPARENT IN THE FIGURE IN THIS PARTICULAR INDUCTION EXPERIMENT, WE OBTAINED THE UvrA PROTEIN AT 20%, THE UvrB PROTEIN AT 12%, AND THE UvrC PROTEIN AT 10% OF TOTAL CELLULAR PROTEINS. IN OTHER EXPERIMENTS WE HAVE ACHIEVED AMPLIFICATION LEVELS FOR UvrA APPROACHING 50% AND FOR UvrB 20% OF TOTAL CELLULAR PROTEINS; HOWEVER, FOR UVR C PROTEIN OUR OVERPRODUCTION IS CONSISTENTLY AND REPRODUCIBLY AROUND 10% OF CELLULAR PROTEINS. AS A RULE WHEN PURIFYING THE UVR PROTEINS WE DO NOT PROCESS A CULTURE IN WHICH THE PARTICULAR UVR PROTEIN IS LESS THAN 10% OF E. coli PROTEINS.

PURIFICATION OF UVR A PROTEIN

ALL PURIFICATION PROCEDURES FOR THE UVR PROTEINS WERE CARRIED OUT AT 0-4 °C. SINCE THE UVR PROTEINS ARE THE MOST PROMINENT CELLULAR PROTEINS IN THE OVERPRODUCING STRAINS USED HERE, SDS-POLYACRYLAMIDE GELS WERE USED TO FOLLOW THE PURIFICATION THROUGH THE CHROMATOGRAPHIC STEPS.

STEP 1: CELL-FREE EXTRACT—FROZEN CELLS FROM A 5-LITER CULTURE WERE THAWED OVERNIGHT AT 0 °C AND SONICATED 10 × 15 s WITH A BRONSON MODEL W185 SONIFIER SET AT MAXIMUM OUTPUT FOR 37 °C. THE SONICATE WAS CLEAR OF CELL DEBRIS BY CEN

Step 3: DEAE-agarose I—Fraction II was concentrated by precipitating with 55% saturated ammonium sulfate and dissolving the precipitate in 10 ml of Buffer B containing 100 mM KC1. This sample was loaded onto a 5.3 cm × 50 cm DEAE-agarose column equilibrated with Buffer B containing 100 mM KC1. The column was washed with the same buffer, collecting 4 ml fractions at 15 ml/h. The eluate was monitored with a UV monitor (280 nm) and the fractions containing UV-absorbing material were analyzed by gel electrophoresis. Figure 3 shows the protein elution profile and composition of this column. As is apparent from the figure UvrA protein eluted in the wash just ahead of most E. coli proteins. The column fractions containing the peak of UvrA protein (41-44) were pooled to constitute Fraction III.

Step 4: Blue Sepharose—Fraction III was loaded onto a 5.3
FIG. 1. The structures of the tac plasmid pUNCO9 we used in our constructions and of the tac-uur plasmids obtained by inserting the uur genes into this plasmid. The distances between the PvuII terminus of the tac fragment and the initiation codons as determined by DNA sequencing and/or restriction mapping, of \textit{uur}A (pUNC45), \textit{uur}B (pUNC211), and \textit{uur}C (pDR3274) genes were 63 ± 5, 213, and 87 base pairs, respectively. The location and direction of the tac promoter is indicated by a heavy arrow and those of \textit{uur} and \textit{tet} genes by lighter ones. The vector sequence is shown by a double line and the chromosomal insert by a single line.

A.  

B.

FIG. 2. Overproduction of Uvr proteins. A, analysis of overproduction of Uvr proteins by SDS-polyacrylamide gel electrophoresis. Cells containing the appropriate plasmids were grown in 10-ml cultures to \( A_{600} = 0.8 \) at which time each culture was split into two and IPTG was added to one of them and incubation was continued for an additional 8 h. Lanes 1 and 2, CH296/pUNC45 uninduced and induced; lanes 3 and 4, CH296/pUNC211 uninduced and induced; lanes 5 and 6, CH296/pDR3274 uninduced and induced; lane 7, \( M \), markers with the sizes given in kilodaltons. Note that the UvrA and UvrB proteins are somewhat overproduced even in the absence of induction. B, densitometric scan of lanes 2, 4 and 6 of the gel shown in A. From these scans we find that UvrA, UvrB, and UvrC proteins constitute 20%, 12%, and 10%, respectively, of total cellular proteins in induced cells.

cm\(^2\) \times 9\) cm column of Blue Sepharose equilibrated with Buffer B + 100 mM KCl. The column was washed with 50 ml of starting buffer and developed with a 250-ml gradient of 0.1-1.5 M KCl in Buffer B. Four-ml fractions were collected at 20 ml/h. The fractions were analyzed on SDS-polyacrylamide gels. UvrA protein eluted at 0.6 M KCl. The fractions containing the protein were pooled and dialyzed against Buffer C to obtain Fraction IV.

Step 5: Hydroxylapatite—Fraction IV was loaded onto a 0.63 cm\(^2\) \times 6 cm column of hydroxylapatite equilibrated with Buffer C. The column was washed with 20 ml of the same buffer and developed with a 200-ml potassium phosphate gradient of 20–200 mM, pH 6.8. Three-ml fractions were collected at a rate of 15 ml/h and UvrA protein was localized by gel electrophoresis; the protein eluted from the column at 0.1 M potassium phosphate. The peak fractions were combined and dialyzed against 25 mM Tris-HCl + 25 mM KCl and other ingredients of Buffer B. The dialyzed material is Fraction V.

Step 6: DEAE-Agarose 2—Fraction V was loaded onto a 5.3 cm\(^2\) \times 50 cm column of DEAE-agarose equilibrated with 25
mm Tris-HCl, pH 7.5, + 25 mm KCl. Under these conditions the UvrA protein binds to the column. Following a 30-ml wash with starting buffer, UvrA protein was eluted with a 200-ml gradient of 25–250 mm KCl in Buffer B. Three-ml fractions were collected at 20 ml/h and the fractions were analyzed on an SDS-polyacrylamide gel. The fractions were also assayed for protein and ATPase to obtain the profile shown in Fig. 4. As is apparent from Fig. 4, UvrA protein elutes off this column with 100 mM KCl as a relatively sharp peak. The ATPase to protein ratio was constant from Fraction 62 through 68 and as this (a constant ratio of activity to protein) is one of the most reliable criterion of purity we considered these fractions to be pure UvrA protein, pooled them, and dialyzed them against storage buffer to obtain Fraction VI which was frozen in a dry ice-ethanol bath and stored at −80 °C.

The various purification fractions were analyzed by SDS-polyacrylamide gel electrophoresis and the percentage of UvrA protein in each fraction was determined from densitometric scans of each lane. From these values and protein determinations of each fraction a purification table was constructed. Fig. 5 shows the UvrA protein purification gel used in estimating the purity of each fraction and Table I summarizes our purification scheme and the results obtained.

**TABLE I**

| Purification of UvrA protein |
|-----------------------------|
| Fraction | Volume | Total protein | UvrA protein* | Purification factor | Yield |
| ml | mg | % | mg | % | % |
| 1. Cell-free extract | 60 | 407 | 15.2 | 61.8 | 1 | 100 |
| 2. Polymin P-NaCl extraction | 20 | 177 | 19.3 | 34.2 | 1.27 | 55.3 |
| 3. DEAE-agarose | 17 | 26.7 | 82.0 | 21.9 | 5.39 | 35.4 |
| 4. Blue Sepharose | 40 | 6.40 | 95 | 6.08 | 6.25 | 9.84 |
| 5. Hydroxylapatite | 40 | 3.68 >95 | 3.68 | 6.58 | 5.95 |
| 6. DEAE-agarose | 25 | 3.17 >98 | 3.17 | 6.58 | 5.13 |

* Determined from a densitometric scan of the gel shown in Fig. 5.

From 3 liters of cells 3.17 mg of apparently pure UvrA protein were isolated which constitutes a 5.13% yield from the starting material.

**Fig. 3.** Chromatography of UvrA protein on DEAE-agarose in high salt. Fraction II was loaded onto the column and protein eluted with Buffer B + 100 mM KCl. A, protein elution profile of the column. B, analysis of the column fractions by SDS-polyacrylamide gel. Each lane contained 10 μl of sample. The first lane shows the material (Fraction II) that was loaded onto the column and the last lane contains the M, standards. The other lanes are indicated by the column fraction numbers. The bracketed fractions were combined to obtain Fraction III.

**Fig. 4.** Chromatography of UvrA protein on DEAE-agarose in low salt. The figure shows the protein and ATPase elution profile of the DEAE-agarose column that was loaded with Fraction V and developed with a salt gradient of 25–250 mM KCl in Buffer B. The bracketed column fractions were combined to obtain Fraction VI.

**Fig. 5.** Purification of UvrA protein. Samples from each step of the purification procedure were separated on a 10% SDS-polyacrylamide gel which was stained with Coomassie Blue and photographed. Lanes 1 and 2 contain cells from 200 μl of CH296/pUNC45 cultures that were grown without and with IPTG induction. The other lanes contained samples from the successive purification fractions as indicated. Lanes F1 and F2 contained about 50 μg, F3 through F5 about 6 μg, and F6 contained 4.75 μg of protein. The last lane shows the molecular weight markers. This is a photograph of an SDS-polyacrylamide gel that was stained with Coomassie Blue.
Purification of UvrB Protein

The UvrB protein overproducing cells were grown, induced, and stored as described for the UvrA protein. Similarly the purification of this protein was followed by gel electrophoresis and only in the final step the protein was tested for activity.

Step 1: Cell-Free Extract—In purifying UvrB protein we faced two problems that are relatively frequently encountered during protein purification via overproduction by recombinant DNA techniques: specific cleavage and precipitation. Fig. 6A shows that the majority of UvrB protein is cleaved to a radioactive band at 78,000 Mₚ, protein (UvrB*) upon sonication of thawed cells as a densitometric scan of the Coomassie Blue-stained gel demonstrates (data not shown) the increase in the amount of the Mₚ 78,000 protein closely matches the decrease in the UvrB protein band. That there is indeed a precursor-product relationship between the UvrB protein and the UvrB* is demonstrated by labeling UvrB protein in maxicells. After two cycles of freeze-thaw the maxicells lysed and in addition to the UvrB band a radioactive band at Mₚ 78,000 appeared (Fig. 6B), confirming our observation that the Mₚ 78,000 band seen in Coomassie Blue-stained gels following sonication is a proteolytic product of UvrB protein. Addition of a combination of protease inhibitors to the cell suspension did not have any effect on the cleavage. A second problem we encountered in preparation of the cell-free extract was that a significant amount of UvrB pelleted with the membrane, perhaps due to precipitation. Usually the greater the overproduction the more UvrB protein was lost to precipitation and less to cleavage and vice versa. Because of these two problems the frozen UvrB cells were thawed quickly in a 23°C water bath, sonicated, and the cell debris was removed with a single centrifugation step of 27,000 x g for 20 min. Even though about 90% of the UvrB protein was lost to cleavage and/or precipitation during this step there is still a significant amount of intact protein left (about 12 mg from a 4-liter culture) in soluble form to proceed to the next step.

Step 2: Phenyl-Sepharose—Solid ammonium sulfate was added to Fraction I to reach 20% saturation and then the sample was loaded onto a 0.63 cm² x 20 cm phenyl-Sepharose column equilibrated with Buffer B containing 20% saturated ammonium sulfate. The column was washed with 30 ml of starting buffer and developed with a 200-ml linear gradient of Buffer B containing 100 mM KC1 and 20% saturated ammonium sulfate to 50% ethylene glycol in Buffer B + 100 mM KC1. Four-ml fractions were collected at 20 ml/h and analyzed on SDS-polyacrylamide gels. The UvrB protein eluted at about 40% ethylene glycol and the peak fractions were pooled and dialyzed overnight against Buffer B + 100 mM KC1 to obtain Fraction II.

Step 3: DEAE-Agarose—Fraction II was loaded onto a 5.3 cm² x 50 cm DEAE-agarose column equilibrated with Buffer B + 100 mM KC1. The column was washed with 30 ml of equilibration buffer and developed with a 200-ml linear gradient of 0.1–0.5 M KC1 in Buffer B. Four-ml fractions were collected at 20 ml/h and UvrB protein which eluted with about 300 mM KC1 was located by gel electrophoresis. The peak fractions were pooled and dialyzed against Buffer B + 100 mM KC1. The dialyzed sample is Fraction III.

Step 4: Blue Sepharose 1—Fraction III was loaded onto a 2 cm² x 8 cm Blue Sepharose column equilibrated with Buffer B + 100 mM KC1, the column was washed with 30 ml of the same buffer and developed with a 200-ml linear gradient of 0.1–1.0 M KC1 in Buffer B. Four-ml fractions were collected at 20 ml/h. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis and assayed for protein by the Bradford method. The protein elution profile of the column is shown in Fig. 7. The major contaminants eluted in the wash whereas UvrB protein eluted in a highly pure form at 0.22 M KC1. The peak fractions were pooled and dialyzed against Buffer B + 100 mM KC1 to constitute Fraction IV.

Step 5: Blue Sepharose 2—Fraction IV was rechromatographed on Blue Sepharose to remove minor contaminants that eluted early in the gradient of the first Blue Sepharose column but trailed into the UvrB peak. The sample was loaded onto the same column as in the previous step but a shallower
Purification of Uvr Proteins

0.1-0.5 M KCl gradient was applied. The UvrB protein was well separated from the contaminants but eluted in a larger volume. The peak fractions were pooled and concentrated to 4 ml using a Centricon-30 unit (Amicon), dialyzed against storage buffer, and finally frozen and kept at -80 °C (Fraction V).

Analysis of the purification steps by SDS-polyacrylamide gel electrophoresis is shown in Fig. 8. The lanes of the gel shown in this figure were scanned with a densitometer to determine the percentage of UvrB protein in each fraction. Based on this data a purification table (Table II) was obtained. From 4 liters of cells, 0.75 mg of apparently pure protein was recovered with a 6.3% yield relative to the cell-free extract. However, as about 90% of the protein is lost to cleavage and/or precipitation during the making of the cell-free extract the actual yield (based on the amount of UvrB protein in intact cells) is about an order of magnitude lower than that reported in the table.

Purification of UvrC Protein

The UvrC protein is relatively unstable and therefore its purification should be carried out around the clock. Table III summarizes the purification scheme.

Step 1: Cell-free Extract—This step was exactly as described for UvrA protein except the starting material was induced from cells) is about an order of magnitude lower than that reported for UvrA protein except the starting material was induced

Fig. 8. Purification of UvrB protein. The lanes marked J and 2 contain total cellular proteins from 200 µl of CH296/pUNC211 that were grown without and with IPTG induction, respectively. Lanes F1-F5 contain samples of the corresponding purification fractions. The amount of protein in F5 lane was 3.75 µg. The last lane contains the M, markers.

| Step | Name | Protein contents | Yield (factor) |
|------|------|-----------------|----------------|
| 1    | Cell-free extract | 0.75 mg | 100 |
| 2    | Polymin P-NaCl extraction | 0.75 mg | 100 |
| 3    | Dialysis precipitation | 20 ml | 87 |
| 4    | Ammonium sulfate | 7 ml | 18 |
| 5    | AcA44 column | 20 ml | 12.1 |
| 6    | Phosphocellulose | 30 ml | 8.5 |

*From densitometric scan of a Coomassie Blue-stained gel. Although Fractions 5 and 6 appeared gel-pure they contained a nonspecific enzymatic activity (endonuclease, topoisomerase I) which converted superhelical DNA to relaxed form.

TABLE II

| Fraction | Volume | Total protein | UvrB protein | Purification factor | Yield (factor) |
|----------|--------|--------------|--------------|------------------|--------------|
| 1. Cell-free extract | 30 ml | 3.0 | 1.18 | 1 | 100 |
| 2. Phenyl-Sepharose | 35 ml | 31.5 | 7.8 | 2.46 | 2.56 | 20.8 |
| 3. DEAE-agarose | 30 ml | 6.90 | 18.9 | 1.30 | 6.28 | 11.0 |
| 4. Blue Sepharose 1 | 25 ml | 0.90 | >0.96 | 0.90 | 33.2 | 7.6 |
| 5. Blue Sepharose 2 | 4 ml | 0.75 | >0.75 | 0.75 | 33.2 | 6.3 |

*From densitometric scan of the gel shown in Fig. 8.

CH296/pDR3274 cells obtained from a 4-liter culture. The cell-free extract is Fraction I.

Step 2: Polymin P Precipitation-Salt Extraction—Polymin P (10%, pH 8.0) was added to Fraction I over 10 min, with constant stirring, to 0.5%. Following an additional 30 min of stirring the precipitate was collected by centrifugation at 12,000 × g for 10 min. The pellet was dissolved in 20 ml of Buffer A containing 0.5 M NaCl by gentle stirring over a 30-min period, after which the sample was subjected to centrifugation at 12,000 × g for 10 min; the supernatant was saved and constituted Fraction II.

Step 3: Dialysis Precipitation—Fraction II was dialyzed against 1 liter of Buffer B + 100 mM KCl for 6 h, with occasional inversion of the dialysis bag. The UvrC protein preferentially precipitates out during this procedure and is collected by centrifugation at 12,000 × g for 10 min. The pellet was resuspended in 20 ml of Buffer A containing 0.5 M NaCl. This is Fraction III.

Step 4: Ammonium Sulfate Precipitation—Solid ammonium sulfate was added to Fraction III to 55% saturation, with constant stirring over 30 min. After an additional 30 min of stirring, the precipitate was collected by centrifugation as above and resuspended in 7 ml of Buffer B + 0.5 M KCl to constitute Fraction IV.

Step 5: AcA44 Chromatography—Fraction IV was loaded onto a 2 cm × 65 cm AcA44 gel filtration column equilibrated with Buffer B + 0.5 M KCl. The column was washed with the same buffer at 15 ml/h, collecting 2-ml fractions. The protein eluted at 50-62 ml. The peak fractions were pooled and diluted with Buffer B to reduce the KCl concentration to 0.3 M KCl. The diluted sample represents Fraction V.

Step 6: Phosphocellulose—Fraction V was loaded onto a 2 cm × 15 cm column of phosphocellulose P-11 equilibrated with 0.3 M KCl in Buffer B. The column was washed with 50 ml of starting buffer and developed with a 500-ml linear gradient of 0.3-1.2 M KCl in Buffer B. Three-ml fractions were collected at 80 ml/h and assayed for UvrC protein by gel electrophoresis and for protein by the Bradford assay. The protein elution profile is shown in Fig. 9. This profile coincides very closely to that of UvrC protein as after the AcA44 step the preparation contains UvrC at greater than 99% purity as is apparent in Fig. 10. The UvrC protein eluted at 0.55 M KCl from the phosphocellulose column, the peak fractions were pooled and dialyzed against 0.3 M KCl in Buffer B to constitute Fraction 6. At this step as in the previous one the protein appears pure on an SDS-polyacrylamide gel but it has an activity (endonuclease, topoisomerase I) associated with it that converts superhelical DNA to open circles as measured
Chromatographic behavior. The fractions of the major peak indicated and at present we have no definitive explanation for its different behavior. The minor peak centered at fraction 20 was also mainly UvrC protein.

Polyacrylamide gels and were therefore combined to yield Fraction VI.

The other lanes contain samples from the purification fractions as indicated. The F7 lane contained 20 mg of protein. In the last lane are the Mr markers.

Fig. 9. Chromatography of UvrC protein on phosphocellulose. The profile of the KCl and that of protein elution are shown. The minor peak centered at fraction 20 was also mainly UvrC protein and at present we have no definitive explanation for its different chromatographic behavior. The fractions of the major peak indicated by the bracket showed no contaminating protein bands on SDS-polyacrylamide gels and were therefore combined to yield Fraction VI.

Fig. 10. Purification of UvrC as analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 1 and 2 contain total cellular proteins from uninduced (1) and induced (2) CH296/pDR3274 cells. The other lanes contain samples from the purification fractions as indicated. The F7 lane contained 20 μg of protein. In the last lane are the Mr markers.

Activities Associated with the Uvr Proteins

The UvrA protein is an ATPase and UvrA and UvrC proteins are DNA binding proteins. The purest fractions of all three proteins lack endonuclease (measured by conversion of superhelical DNA to open circles) and exonuclease (measured by the release of radioactivity from terminally labeled DNA in an acid-soluble form) as well as phosphatase (measured with 5' terminally labeled DNA) activities. Furthermore, the UvrB and UvrC proteins were devoid of ATPase activity. Mixture of the three proteins converts superhelical DNA damaged by a variety of agents to open circles (Sancar and Rupp, 1983) as expected.

Stability of the Uvr Proteins

We have not conducted a systematic quantitative study on the stability of the purified Uvr proteins. However, from our studies with these proteins over the past few years the following statements can be made. All three proteins are stable for at least 1 year when stored at -80°C in the storage buffer. During this period UvrA retains full ATPase and incision complementing activity and UvrB retains fully its incision complementing activity, while UvrC protein may have lost about 50% of its complementing activity. The UvrA protein is relatively stable during purification and can be obtained in fully active form after a 2-week purification procedure and it is stable at -20°C for at least 6 months when stored in 20% or 50% glycerol. The UvrB protein is cleaved severely at the 10-day period, the protein tends to aggregate in buffers containing less than 0.3 M KCl unless the buffer contains 5% glycerol. In the storage buffer at -20°C UvrC protein loses its activity completely (as determined by the incision assay) in 6-8 weeks.

Effect of UvrB and UvrC Proteins on the ATPase Activity of UvrA Protein

The ABC excinuclease is an ATP-dependent enzyme and its A subunit is an ATPase (Kacinski et al., 1981; Seeberg and Steinum, 1982) whose activity is unaffected by its other substrate, DNA. Since the subunits of ABC excinuclease act in concert and not sequentially (Sancar and Rupp, 1983) they must be either assembling in solution to make an ABC nuclease complex or assembling on the DNA at the damage site. In either case it is likely that interaction of the A subunit with the other two will have a modifying effect on its ATPase activity. To test for such an effect we measured the rate of ATP hydrolysis by UvrA protein under a variety of conditions and obtained the results summarized in Table IV. In agreement with previous results the UvrA protein is an ATPase that is not stimulated by double-stranded single-stranded, or UV-irradiated DNA. The most striking finding is that UvrB protein stimulates the ATPase activity in the presence of nonirradiated DNA by about 50% and by 150% when the DNA is irradiated. The fact that there is no stimulation in the absence of DNA suggests that the enzyme complex is assembled on DNA. We have at present no satisfactory explanation for the small but reproducible decrease in ATPase activity caused by UvrB protein in the absence of DNA and a similar but smaller effect of UvrC protein under most conditions.
This has been due to several factors: in the case of UvrA protein we have not been able to find a chromatographic resin that will bind the enzyme strongly. In the case of UvrB the majority of the protein is lost to cleavage and/or precipitation during preparation of the cell-free extract. Both of these problems have been reported in the literature before with other overproduced proteins; in the case of T7 phage RNA polymerase (Davanloo et al., 1984) the major problem was proteolytic cleavage and it was prevented by adding substrate to the cell-free extract. In the cases of P22 repressor (Deanda et al., 1983) and Salmonella Hin proteins (Bruist and Simon, 1984) the overproduced proteins precipitated and while this precipitation was preventable by carrying out all the purification steps of P22 repressor in 0.5 M NaCl, the precipitation of Hin protein was irreversible and could not be prevented. We have tried a combination of protease inhibitors and the inclusion in lysis buffer of substrate and the UvrA and UvrC proteins to prevent the proteolytic cleavage of UvrB protein, and high salt to prevent precipitation, but have not been able to overcome these problems reproducibly yet. We hope further experimenting with the lysis conditions will help solve the problem. The UvrC protein binds to several column resins tightly and therefore it is easy to purify; however, due to its instability we have to proceed through the purification steps rapidly and chose the steps that give the quickest results but not necessarily high yields. Even with these problems we have been able to purify substantial quantities of all three proteins and start a systematic study of their characterization. Furthermore, the purification procedures we have developed are very reproducible and can be scaled up easily to obtain quantities of Uvr proteins sufficient for physical studies. Indeed we have obtained large quantities of UvrA protein by scaling up the procedure described here and crystallized it and hope to obtain crystals of sufficient size to study it by x-ray diffraction in the near future.

Similarly, purification to near homogeneity of all three proteins now makes an extensive biochemical study of the enzyme feasible without interference from nonspecific nucleases or other E. coli ATPases. With regard to the last point, having UvrB and UvrC proteins free from ATPases has enabled us to study the effect of these subunits on the ATPase function of UvrA protein and obtain some evidence of how these proteins assemble. We find that UvrB protein stimulates the ATPase activity in the presence of DNA by a factor of 1.5 and in the presence of UV-irradiated DNA by a factor of about 2.5 but not in the absence of DNA, strongly suggesting that these proteins and perhaps the whole ABC excinuclease complex assemble on the substrate, and that the excinuclease does not exist as such free in solution. In line with this idea are the report by Kacinski and Rupp (1982) that UvrB protein does not bind to DNA but binds to DNA-UvrA protein complex and the finding by Yeung et al. (1983) that UvrB protein increased the specific binding to UV-irradiated DNA of the UvrA subunit by a factor of three. Furthermore, our own studies aimed at demonstrating a complex formation in the absence of DNA by centrifugation of the subunit mixture through sucrose gradients or in an Airfuge by the sedimentation equilibrium method have failed to reveal any interaction (at biologically relevant concentrations) between the subunits while electron microscopic studies show that the A and B subunits make a complex on DNA that contain a single monomer of each. We expect further work in these lines will help clarify the problem of assembly and subunit structure of ABC excinuclease.

### Table IV

| Additions* | ATPase pmol hydrolyzed | Relative activity^6 |
|-----------|------------------------|---------------------|
| None      | 2020                   | 100                 |
| ssDNA     | 1810                   | 89.6                |
| dsDNA     | 2120                   | 105                 |
| UV-DNA    | 2010                   | 99.5                |
| B         | 1510                   | 74.7                |
| B + daDNA | 3600                   | 153                 |
| B + UV-DNA| 5440                   | 269                 |
| C         | 1920                   | 95.0                |
| C + daDNA | 2050                   | 101                 |
| C + UV-DNA| 1580                   | 95.1                |
| B + C     | 1430                   | 70.8                |
| B + C + daDNA | 2810               | 139                 |
| B + C + UV-DNA | 4950              | 245                 |

* The reactions were done as described under "Experimental Procedures." All reaction mixtures contained 0.70 pmol of UvrA, and 0.83 and 0.75 pmol of UvrB and UvrC proteins where indicated. The DNA used was the pBR322 plasmid and it was present at 0.07 pmol in the reaction mixture. dsDNA, double-stranded plasmid DNA (about 50% superhelical); ssDNA, single-stranded plasmid DNA obtained by boiling linear pBR322; UV-DNA, UV-irradiated pBR322 (about 5 pyrimidine dimers/molecule).

^6 The activities are the rates relative to that obtained with UvrA protein alone in the standard reaction mixture.

^1 The amounts of UvrB and UvrC proteins used in these experiments contained less than 1% of ATPase activity observed with UvrA protein whether the assays were conducted in the presence or absence of DNA.

### DISCUSSION

The ABC excinuclease is an important DNA repair enzyme because it recognizes base adducts produced by many physical and chemical agents and removes them from DNA. The enzyme is made up of three subunits, UvrA, UvrB, and UvrC, and because of the large number of adducts that are removed it is difficult to imagine that the enzyme can recognize these various adducts and bind to them directly. It is more likely that the enzyme recognizes a common helical deformity and acts on it. In line with this concept is the finding that the excinuclease hydrolyzes the 8th phosphodiester bond 5' to the adducted base thus making it possible for the enzyme to contact the adduct containing region from the "back" (with regard to the adduct) of the helix and make its incisions. While the recent cloning of the containing region from the "back" (with regard to the adduct) of the helix and make its incisions. While the recent cloning of this concept enabled us to study the effect of these subunits on the ATPase activity of UvrA, and the finding by Yeung et al. (1983) that UvrB protein increased the specific binding to UV-irradiated DNA of the UvrA subunit by a factor of three. Furthermore, our own studies aimed at demonstrating a complex formation in the absence of DNA by centrifugation of the subunit mixture through sucrose gradients or in an Airfuge by the sedimentation equilibrium method have failed to reveal any interaction (at biologically relevant concentrations) between the subunits while electron microscopic studies show that the A and B subunits make a complex on DNA that contain a single monomer of each. We expect further work in these lines will help clarify the problem of assembly and subunit structure of ABC excinuclease.

^6 C. W. Carter and A. Sancar, unpublished results.

^1 M. Levy and A. Sancar, unpublished results.
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