Reconstitution of an *Escherichia coli* repair endonuclease activity from the separated *uvrA*+ and *uvrB*+/*uvrC*+ gene products

*(in vitro* complementation assay/UV repair/incision/ATP)

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ABSTRACT An *in vitro* complementation assay has been used for partial purification of the *uvrA*+, *uvrB*+, and *uvrC*+ gene products from *Escherichia coli*. The *uvrB*+ and *uvrC*+ products chromatograph on DEAE-cellulose and are completely resolved from the *uvrA*+ product, which has been further purified by phosphocellulose chromatography of the nonadsorbed protein fraction from the DEAE-cellulose. Neither the *uvrB*+/*uvrC*+ nor the *uvrA*+ product shows appreciable endonuclease activity on UV-irradiated DNA when tested separately. However, these factors complement each other to yield an ATP-dependent endonuclease activity specific for UV-irradiated DNA. Gel filtration experiments with the partially purified proteins indicate that the functional *uvr*+ gene product has a molecular weight of 100,000. The *uvrB*+ gene product has an apparent molecular weight of 70,000, but it is presently unclear if this is the size of the *uvrB*+ product alone or of a complex of the *uvrB*+ and *uvrC*+ gene products.

The functions of the *uvrA*+, *uvrB*+, and *uvrC*+ genes in *Escherichia coli* are required for early steps in the excision-repair pathway of DNA repair in UV-irradiated cells (1–6). A mutation at any of these loci leads to loss of the ability to excise the major photoproduct, pyrimidine dimers, from DNA (4, 5). Excision of damaged nucleotides from DNA proceeds by the sequential action of two different nucleases (for review see ref. 6). An endonuclease catalyzes the formation of a single-strand break (incision) adjacent to the damaged nucleotide, which is then removed by an exonuclease. *In vivo* measurements of DNA strand breakage in UV-irradiated cells have suggested that the *uvr*− mutants have a defect in the endonuclease function initiating the repair (5, 7). The defect seems to be more pronounced in *uvrA* and *uvrB* than in *uvrC* mutant strains (8). Further characterization of the strand incision step in permeable cell systems has indicated that ATP is a necessary cofactor in the *uvr*+ dependent incision reaction (9, 10). A repair endonuclease activity that is ATP-dependent has recently been extracted from *E. coli* in soluble form and shown to be defective in extracts of *uvrA*, *uvrB*, and *uvrC* strains (11). Any combination of two different mutant extracts, however, showed activity similar to that observed in wild-type extracts. This observation has been used to establish an *in vitro* complementation assay for the individual *uvr*+ gene products. *In vitro* complementation assays have previously been successfully used in the purification of proteins essential for DNA replication (12, 13) but have not so far been used to characterize DNA repair functions. The principal aim of this paper is to show that functional *uvr*+ gene products can be separately purified by means of complementation in *in vitro*. The results indicate that the *uvrA*+ and *uvrB*+ products are two separate proteins of high molecular weight and argue against the concept of a *uvrA*+, *uvrB*+-coded endonuclease of low molecular weight (6, 14).

MATERIALS AND METHODS

**Bacterial Strains.** *E. coli* K-12 strains AB157 (*uvr*) and its derivatives AB1884 (*uvrC34*), AB1885 (*uvrB5*), and AB1886 (*uvrA6*) were obtained from P. Howard-Flanders, Yale University (4). The *uvr*− genotype assigned to each mutant was controlled by mating with appropriate Hfr strains. *E. coli* JC4588 (*uvr* endA) was obtained from P. Strike, Liverpool University, England, and *E. coli* SK2001 (ColE1, thy−) was from this laboratory.

**Materials.** DEAE-cellulose (DE52) and phosphocellulose (P11) were obtained from Whatman, and Ultrogel ACA34 from LKB, Sweden; ATP was grade 1 from Sigma. Pyrimidine dimer-specific endonuclease from *Micrococcus luteus* was a gift from T. Lindahl, Karolinska Institute, Stockholm, Sweden.

**Isolation of Circular DNA.** *E. coli* SK2001 was grown for several generations in K-medium (8) containing [3H]thymidine (2 μCi/ml) at a specific activity of 1.7 μCi/μg. While the cells were still in the exponential growth phase chloramphenicol was added (150 μg/ml) and the incubation was continued for 4 hr. The bacteria were washed and concentrated 40-fold in 50 mM phosphate buffer (pH 7.0), containing 20 mM EDTA, 15% sucrose, and 0.1 M NaCl. The cells were then treated with lysozyme (1 mg/ml) for 10 min on ice, and lysed by adding NaCl to 1 M and sodium dodecyl sulfate to 1% and incubating at 60° for 10 min. The lysate was incubated overnight at 0°, and chromosomal DNA and cell debris were removed by centrifugation (45 min at 27,000 × g). Covalently closed ColE1 DNA in the supernatant was purified by phenol extraction and repeated banding in CsCl/ethidium bromide (15). The ethidium bromide was removed by several extractions with isopropanol and the purified DNA was dialyzed extensively against 10 mM Tris-HCl, pH 8.0/1 mM EDTA. Less than 7% of the DNA prepared in this way contained a ribonucleotide sequence, as judged by stability to alkali treatment (16). The ColE1 DNA had a specific activity of 16,000 cpm/μg.

Covalently closed PM2 [3H]DNA (18,000 cpm/μg) was a gift from S. Ljungquist, Karolinska Institute, Stockholm, Sweden.

**Preparation of Cell Extracts.** Mutant extracts for complementation assays, referred to as receptor extracts, were prepared by a combination of sucrose plasmolyis (17) and lysozyme treatment as previously described (11), except that lysates were centrifuged at 27,000 × g for 15 min to remove cell debris and DNA. The receptor extracts can be stored for a few

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days at 0° or for weeks at -90° after being frozen in liquid nitrogen. For purification of individual uvr gene products by column chromatography, bacteria were grown in K-medium (9) in 8-liter batches to approximately 2 x 10^8 cells per ml, washed once in 40 mM Tris-HCl (pH 8.0), and resuspended at 0° in 12 ml of 2.4 M sucrose/10 mM ethylene glycol bis(2- aminoethyl ether)-N,N' -tetraacetate (EGTA)/40 mM Tris-HCl, pH 8.0. The suspension was diluted with 48 ml of 100 mM KCl/50 mM 4-morpholinepropanesulfonate (Mops)/1 mM EDTA/1 mM dithiothreitol, at pH 7.5, containing 125 μg of lysozyme per ml. The cells were lysed by incubating for 45 min at 0°, and the lysate was centrifuged at 27,000 x g for 15 min. The supernatant, referred to as crude extract, was applied directly to ion-exchange cellulose columns. The extracts prepared in this way contained 3–5 mg of protein per ml and were virtually free of DNA (11).

Endonuclease Assays. Standard assay mixtures contained 0.05 μg of DNA, 2 mM ATP, 85 mM KCl, 15 mM MgSO4, 1 mM EDTA, 1 mM dithiothreitol, 40 mM Mops at pH 7.5, and protein extract as indicated, in a total of 0.14 ml. The standard UV dose for irradiation of DNA was 180 J/m^2 of 254 nm light, which induces about 30 dimers per ColEl DNA molecule of M_r = 4.2 x 10^6 (ref. 18), as determined by titration of pyrimidine dimers in DNA exposed to lower UV doses of pyrimidine dimer-specific endonuclease from Micrococcus luteus. Samples were incubated for 10 min at 37° unless otherwise indicated and the reaction was stopped by adding 2.5 ml of denaturation buffer (1 M NaCl/20 mM EDTA/50 mM phosphate at pH 11.9) followed by neutralization with 0.8 ml of 0.5 M HCl. The samples were then filtered through nitrocellulose filters (Millipore HAWP) and the radioactivity bound to the filter was estimated as nicked DNA (14, 19, 20). The total amount of DNA in the reaction mixtures was determined by denaturation of both nicked and closed DNA at pH 13.0, followed by neutralization and filtration (20). The number of breaks per DNA molecule was calculated as described by Kuhnlein et al. (20), assuming a Poisson distribution of breaks in the circle population. This number was converted to breaks per unit size DNA to facilitate comparison of results obtained with ColEl DNA and PM2 DNA. UV-endonuclease activity was determined by subtracting breakage observed in nonirradiated DNA from that observed in UV-irradiated DNA in parallel incubations.

Complementation Assays. Receptor extract from 10^8 cells (10 μl) was mixed with an aliquot of the protein fraction to be tested and assayed for endonuclease activity both on nonirradiated and UV-irradiated DNA. The receptor extract and the protein fraction were also incubated separately with DNA to make sure that the activity observed was due to complementation. The activity of DNA endonuclease I, which was sometimes significant in DEAE-cellulose fractions derived from endI+ cells, is suppressed by the receptor extract, presumably due to the presence of trNA in the extract (21), and does not interfere with the complementation assay. Complementing activity, referred to as uvrA+, uvrB+, or uvrC+ activity, is defined as net UV-endonuclease activity due to complementation, i.e., total UV-specific activity in complemented reaction mixtures minus the background value observed with the receptor extract alone (see Fig. 1).

Non-specific endonuclease activity, which accounted for the nicking of 25–40% of the DNA, was reproducibly observed in different extract preparations irrespective of whether ColEl DNA or PM2 DNA was used as substrate. The background value of UV-endonuclease activity observed in uvr mutant extracts was usually somewhat higher in uvrC− than in uvrA− or uvrB− extracts (11). This may be analogous to the situation in vivo, in which uvrC strains show an intermediate response between wild-type and uvrA or uvrB strains with respect to strand break formation in UV-irradiated cells (8).

RESULTS

In vitro complementation assay for uvrA+, uvrB+, and uvrC+ gene products

Cell extracts from uvrA, uvrB, and uvrC strains are deficient in an ATP-dependent UV-endonuclease activity which is present in extracts from wild-type cells (11). The defective endonuclease in the mutant extract can be restored, however, by addition of the missing gene product in the form of a protein fraction. Thus, the presence of a uvr+ gene product in a protein fraction can be detected by its unique and selective ability to restore the endonuclease activity in extracts from the corresponding mutant. The basic properties of the in vitro complementation system are shown in Fig. 1, exemplified by the assay of uvrA+ activity present in an extract from a uvrC strain. Addition of increasing amounts of uvrA+ activity to receptor extract from uvrA cells causes a gradual increase in the amount of UV-endonuclease activity until maximum complementation is reached. The amount of protein needed to yield 50% of maximum complementation can be taken as a measure of the relative concentration of uvrA+ activity in the protein fraction tested. Results similar to those shown in Fig. 1 are obtained when receptor extracts from uvrB and uvrC extracts are used to assay appropriate protein fractions for uvrB+ and uvrC+ activity, respectively (data not shown).

Ion-exchange chromatography of uvrA+, uvrB+, and uvrC+ gene products

In preliminary experiments, extracts from wild-type E. coli

![Fig. 1. Complementation assay for uvrA+ activity. Receptor extract (10 μl) from AB1886 (uvrA) was supplemented with increasing amounts of extract from AB1884 (uvrC) (open symbols) or AB1886 as control (filled symbols), and assayed in triplicate for endonuclease activity on unirradiated (triangles) or UV-irradiated ColEl DNA (circles). Assays for endonuclease activities in the extract from AB1884 alone (30 μg of protein) showed that 40% of the nonirradiated and 56% of the UV-irradiated DNA was nicked. Protein was determined by the method of Lowry et al. (22). Bars indicate standard deviations.](image-url)
respectively, chromatography fractions as appropriate uvr
under those uvrB wild-type + uvrA extract wild-type lusophosphate cellulose was inactivated then tested by salt complementation. Neither were applied for tested for uvrA (O), uvrB (O), and uvrC (a) activities by complementation (10 µl of receptor extract and 10 µl of fraction). The recovery of the uvrA + and uvrC + activities obtained by the DEAE-cellulose chromatography described was determined from another experiment similar to that shown. Pooled peak fractions were diluted and assayed for complementing activities to measure the relative concentration of uvrB + and uvrC + in this fraction compared to that which is normally observed when an appropriate crude mutant extract is tested (e.g., uvrA +). The estimated recoveries were 55% and 30% for uvrB + and uvrC + activities, respectively, in a total volume of 4 ml.

were applied to DEAE-cellulose and the eluted fractions were tested for ATP-dependent UV-endonuclease activity without complementation. Neither the flow-through fraction nor a high salt eluate from the DEAE-cellulose showed any activity of this type, as if the endonuclease originally present in the extract had been inactivated during chromatography. Eluted fractions were then tested for the presence of the individual uvr + gene products by the complementation system. As shown in Fig. 2, the uvrB + and uvrC + activities cochromatograph on DEAE-cellulose and were eluted by a salt gradient after the major protein peak. The uvrA + activity does not bind to DEAE-cellulose but can be purified by phosphocellulose chromatography of the flow-through fraction from the DEAE-cellulose column (Fig. 3). The uvrA + activity binds to phosphocellulose and is eluted by a salt gradient ahead of the major protein peak. A small but significant peak of uvrC + activity was also detected in the eluate from the phosphocellulose column. The uvrA + activity is clearly separated from the uvrB + activity in the chromatography system used, and the loss of the ATP-dependent UV-endonuclease activity in the wild-type extract after DEAE-cellulose chromatography is accounted for by the physical separation of these factors. The uvrA + activity was also separated from the uvrB + activity when wild-type extracts were applied directly to phosphocellulose without prior passage through DEAE-cellulose, since the uvrB + gene product did not adsorb to the phosphocellulose under those conditions (data not shown).

Complementing activities have been isolated from appropriate uvr mutants in addition to wild-type cells, by the same procedure as described in the legends to Figs. 2 and 3 (data not shown). The uvrA + and uvrC + activities were absent from chromatography fractions derived from uvrA and uvrC cells, respectively, but the other complementation activities were isolated in normal amounts from these mutants. Similarly, no uvrB +, but normal uvrA +, activity was recovered in fractions derived from uvrB cells. However, very little uvrC + activity could be detected at the usual elution position when an extract from uvrB cells was chromatographed on DEAE-cellulose. Assays for uvrC + activity in crude cell extracts from uvrB cells indicated the presence of normal amounts of activity, and the uvrB mutation therefore seems to affect the chromatographic behavior of the uvrC + product. This observation can possibly be explained by a molecular interaction between functional uvrB + and uvrC + gene products.

Reconstitution of UV-endonuclease from separated uvrA + and uvrB +/uvrC + gene products

Neither the uvrA + product nor the uvrB +/uvrC + gene products show appreciable UV-endonuclease activity by itself under standard assay conditions. However, when these factors are combined, a major endonuclease activity is recovered which is specific for UV-irradiated DNA and completely dependent upon ATP. This endonuclease activity apparently corresponds to the uvr + -dependent endonuclease present in crude extracts (11), while an ATP-independent UV-endonuclease and a nonspecific endonuclease activity also present in the crude extract (11) have been largely removed during purification of the uvrA + and uvrB + gene products. The amount of UV-endonuclease activity observed in complemented reaction mixtures increases with the amount of protein added, suggesting enzyme-limited conditions. These results are obtained with DNA exposed to a heavy UV dose (180 J/m²) containing a large number of dimers per molecule. Fig. 4 right shows dose-response curves for the reconstituted endonuclease on lightly irradiated DNA. The amount of breaks induced by the E. coli enzyme is close to that obtained with optimal amounts of purified pyrimidine dimer-specific endonuclease from M. luteus. This implies that purification of the uvr + gene products has yielded preparations of the ATP-dependent UV-endonuclease that are 20 times more efficient per dose unit for the DNA than observed for the same activity in the crude extracts (24). Even
for the purified preparations, a relatively large amount of protein is required for optimal activity, suggesting that very few active enzyme molecules are normally present per *E. coli* cell.

**Gel filtration of uvrA<sup>+</sup> and uvrB<sup>+</sup> products**

The *uvrA<sup>+</sup>* and *uvrB<sup>+</sup>/uvrC<sup>+</sup> activities purified by ion-exchange chromatography were separately subjected to gel filtration on a column that had been calibrated with a set of reference proteins to obtain further purification and characterization of these factors (Fig. 5). The *uvrA<sup>+</sup>* and the *uvrB<sup>+</sup>* activities eluted as single peaks corresponding to proteins with molecular weights of about 100,000 and 70,000, respectively. The *uvrC<sup>+</sup>* activity seems labile and most of it could not be recovered after gel filtration. It has therefore been difficult to determine the peak fraction(s) of *uvrC<sup>+</sup>* activity, but the gels showed that the *uvrC<sup>+</sup>* product has a molecular weight similar to that of the *uvrB<sup>+</sup>* product (data not shown). Thus, it is not clear at this point if the *M<sub>e</sub>* of 70,000 reflects the *uvrB<sup>+</sup>* product by itself or a complex of the *uvrB<sup>+</sup>* and *uvrC<sup>+</sup>* products.

The eluates from the gel filtration experiments were also tested for UV-endonuclease activity without complementation (Fig. 5). Although the phagecellulose fraction containing *uvrA<sup>+</sup>* activity did not exhibit UV-endonuclease activity under the standard assay conditions used here (Fig. 4, left), assays of this fraction without magnesium in the reaction buffer showed some activity. However, this Mg<sup>2+</sup>-inhibited endonuclease was completely separated from the *uvrA<sup>+</sup>* activity by gel filtration (Fig. 5 upper) and therefore does not seem to be related to the *uvrA<sup>+</sup>* product. This activity may correspond to endonuclease III described by Radman (26).

**DISCUSSION**

An *in vitro* complementation assay has allowed the partial purification of the products of the *uvrA<sup>+</sup>*, *uvrB<sup>+</sup>*, and *uvrC<sup>+</sup>* genes in *E. coli*. It is concluded that the complementing activities reflect functional *uvr* gene products because (i) the complementation is specific for the different types of *uvr* mutant extracts (Figs. 2 and 3); (ii) the proteins purified in this way, which are inactive by themselves, can jointly restore the *uvr<sup>+</sup>* dependent UV-endonuclease activity present in wild-type extracts (Fig. 4); and (iii) no complementation is observed between a given receptor extract and proteins from the same mutant.

The endonuclease activity reconstituted from the separated *uvrA<sup>+</sup>* and *uvrB<sup>+</sup>/uvrC<sup>+</sup>* gene products has characteristics corresponding to the activity responsible for incision at pyrimidine dimers in UV-irradiated whole *E. coli* cells. First, the endonuclease activity depends on functional *uvr* gene products which are known to control excision of dimers *in vivo* (2). Second, the endonuclease activity requires ATP as cofactor, as has been shown for the *uvr<sup>+</sup>*-dependent strand incision preceding dimer excision in permeabilized cells (9, 10, 27). Third, the amount of breaks induced by the endonuclease in DNA exposed to low UV doses is approximately the same as that obtained with dimer-specific endonuclease from *M. luteus*. All together, these characteristics suggest that the reconstituted endonucleases act at the site of pyrimidine dimers in the DNA.
but additional experiments are needed to obtain direct evidence of this assumption.

Braun et al. (14, 28) have previously characterized a UV-endonuclease from E. coli of low molecular weight (Mø of 12,000) that appeared to be absent from extracts of uvrA and uvrB. It has been suggested that this enzyme might be the product of the uvrA and uvrB genes (6). It seems difficult to reconcile those data with the present study, which indicates that the uvrA+ and uvrB+ products can be completely separated by ion-exchange chromatography and that both these factors are recovered in high molecular weight form. However, it could be that the uvrA+ and uvrB+ products were recovered in an ATP-dependent oligomeric form here, whereas the procedure used by Braun et al. (14, 28) yields active subunit structures. The results described here suggest that the uvrB+ and uvrC+ products may exist as a complex because they copurify on DEAE-cellulose and apparently also on gel filtration.

ATP is clearly required as a cofactor for the uvr+-coded endonuclease activity investigated here (Fig. 4). The ATP dependency has not been found for other enzymes that incise damaged DNA and therefore appears to be characteristic for the uvr+-coded endonuclease. Previous experiments with cells made permeable and crude cell extracts have shown that neither GTP, nor ADP, nor AMP can substitute for ATP in this reaction (9, 24). The role of ATP is now known, but two possibilities are presently being investigated. One hypothesis is that ATP is needed to form a repair enzyme complex composed of the three uvr+ gene products. An obligatory role for ATP in complex formation has been reported by Wickner and Hurwitz (29) for the dnaB+ and dnaC+ proteins. The second possibility, which does not exclude the first one, is that one of the uvr+ gene products acts as an ATPase. Enzymes have recently been isolated that use ATP as an energy source to unwind the DNA duplex (30, 31), and local DNA unwinding catalyzed by enzyme activity of this kind may be needed for excision of pyrimidine dimers in E. coli.

Endonuclease responsible for incision at pyrimidine dimers in UV-irradiated DNA have been extensively purified from M. luteus (32) and from E. coli infected with bacteriophage T4 (33, 34) by assaying for single proteins that show endonuclease activity on UV-irradiated DNA. This approach may not be feasible in the purification of the uvr+-dependent incision activity from E. coli since this requires three different gene products and these are likely to be separated during purification. The use of an in vitro complementation assay avoids this difficulty, and it seems possible that reconstitution of the endonuclease activity from pure components will ultimately allow an elucidation of the detailed mechanism of initiation of excision repair in UV-irradiated E. coli.

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