An *in vitro* complementation assay for the *Escherichia coli uvrD* gene product

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ABSTRACT

An in vitro assay specific for the product of the uvrD gene of Escherichia coli has been developed. This assay, derived from properties of uvrD mutants revealed by in vivo experiments, is based on the necessity for a functional UvrD protein for complete rejoining of covalently closed circular DNA during the excision repair of UV-induced damage. Extracts prepared from gently lysed uvrD101 mutant cells are capable of restoring UV-damaged DNA to its covalently closed circular form when provided with a functional UvrD protein from other repair-deficient cell extracts or from partially purified protein fractions. This assay was employed to monitor the activity of the UvrD protein after several steps of fractionation. The partially purified UvrD protein does not complement extracts deficient in DNA polymerase I or temperature-sensitive in DNA ligase; it does, however, complement extracts from strains mutant at the uvrE and recL loci, which are considered allelic with the uvrD locus.

INTRODUCTION

Numerous studies have implicated the product of the Escherichia coli uvrD gene in several DNA metabolic functions. Mutations in the uvrD cistron, which include the uvrD, uvrE and recL mutations, affect DNA repair, mutagenesis, recombination and mismatch repair (1-11). In spite of the diversity in phenotypic responses of uvrD mutants, the precise function of the uvrD gene product remains obscure. The isolation of the original uvrD mutant was based on its deficiency in the excision repair of pyrimidine dimers (1,2). This repair pathway involves incision at the site of pyrimidine dimers, excision of the lesion, DNA synthesis to patch the excised region, and ligation to the parental DNA strand (12). UvrD mutants are deficient at pyrimidine dimer excision, show abnormal patterns of repair resynthesis, and cannot efficiently restore incised DNA to intact molecules (8). Although the available data favor a post-incision role for the uvrD gene product, recent revelations regarding the complexity of both the control and mechanism of the incision step which is mediated by the uvrA, uvrB and uvrC gene products (13-16) leave open the possibility that the uvrD product may be intimately

associated with the incision event itself.

Insight into the function of the uvrD gene product might be gained by isolating that protein in a functional form. This objective has been facilitated by the recent cloning of the uvrD gene (17,18) and the finding that this gene apparently codes for an ATPase (19). We have followed a different approach toward the isolation of the UvrD protein in our development of an in vitro complementation assay based on in vivo deficiencies associated with rejoining of incision breaks (3,8). This scheme provides the advantage of yielding a protein which is functional in at least one property expected from an in vivo phenotype characteristic of uvrD mutants. To assess the potential usefulness of this complementation assay and to learn more regarding its specificity, we employed this assay in a partial purification of the UvrD protein.

MATERIALS AND METHODS

Bacterial and Phage Strains

Strain AB2499 is an Escherichia coli K-12 strain with a $uvrB_5$ mutation (20). Strains WM280 (uvrD101), TN207 (uvrA), WM282 (uvrE156), WM284 (recL152), and D110 (polA) are thymine-requiring derivatives of E. coli K-12 and have been described (21,22). The $lig_{ts}7$ mutant employed in these studies, strain N2668 (23), was obtained from Dr. D. Billen.

Tritium-labeled covalently closed circular DNA was prepared from bacteriophage PM2 grown on Pseudomonas Bal 31 as described by Espejo and Canelo (24).

Preparation of Extracts

Our assay for the uvrD gene product is based upon the ability of extracts prepared from E. coli to introduce incisions in ultraviolet (UV)-irradiated DNA (13) and, as described below, to repair these incisions so as to yield intact DNA molecules. Extracts for these assays were prepared from E. coli wild type or from strains deficient in incision ($uvrB^-$) or deficient in the uvrD gene. To prepare extracts, cultures were grown at 37°C in M9 medium (25) supplemented with 1 µg/ml thiamine, 20 µg/ml L-tryptophan, 20 µg/ml thymine and 0.2% (w/v) vitamin-free Casamino acids (Difco) and were harvested at a cell density of about 5 x 10^8 cells per ml. Portions (20 ml) were collected by centrifugation and resuspended in 0.25 ml 2.5 M sucrose as described (13). After 5 min at 0°C, the sucrose-plasmolyzed cells were lysed by the addition of 1 ml of buffer (13) containing 100 µg/ml lysozyme. After incubation for 45 min at 0°C, the lysates were spun for 15 min at 15,000 rpm

in the Sorvall SS34 rotor and the supernatant collected to be used as an extract. Extracts prepared from uvrD⁻ strains are referred to as "receptor extracts." Control experiments demonstrated that these extracts maintained their activity after being frozen in liquid N_2. Frozen-thawed extracts from the uvrD⁻ strain were routinely used to test fractions recovered after chromatography for ability to complement the uvrD⁻ deficiency.

Assay for Accumulation of Breaks in DNA

Reaction mixtures included 40 mM 4-morpholinepropanesulfonic acid (Mops), pH 7.5, 87 mM KCl, 15 mM $MgSO_4$, 0.8 mM dithiothreitol, 1 mM ATP, 4 mM NMN, 150 pmol ^{3}H-PM2 DNA (specific activity 5-50 cpm/pmol), 0.010 ml receptor extract (when provided), and 0-0.025 ml of protein preparation or extract being tested in a total volume of 0.127 ml. Samples were incubated at 37° for 20 min, made 0.05 M in EDTA, and layered onto 5-20% linear alkaline sucrose gradients. Centrifugation in the SW56 rotor in a Beckman L-3 ultracentrifuge was for 50 min at 20°C. Gradients were fractionated and profiles of radioactivity were determined by liquid scintillation counting. The average number of breaks per DNA molecule was calculated by measuring the conversion of closed-circular (Form I) DNA to relaxed (Form II) and linear (Form III) molecules and assuming a Poisson distribution of single-strand breaks.

Assay for Repair of UV-Radiation-Induced Damage in DNA

Reaction mixtures were identical to those described above for measurement of breakage induction, except that 0.24 mM NAD and 0.07 mM each of dATP, dTTP, dGTP and dCTP were substituted for NMN. Complementation assays were performed by mixing 10 µl of a "receptor extract" from a uvrD⁻ strain and 10 µl of the sample being tested and incubated in the reaction mixture just described for *in vitro* DNA repair (+dNTP's + NAD). Samples were treated and analyzed as described above. For convenience we define the number of units of complementing activity as the difference between the number of single-strand breaks per DNA molecule remaining in 150 picomoles of DNA irradiated with 50 J/m^2 UV and incubated in the presence of the uvrD complementing protein plus 10 µl of receptor extract and the number of single-strand breaks remaining in a control reaction with only the receptor extract after 20 min incubation at 37°C.

Fractionation Procedure

7.2-liter batches of cells from strain AB2499 (uvrB⁻) were grown as described above, harvested, and lysed by sucrose plasmolysis and lysozyme treatment (14) to yield extracts for fractionation of proteins. Extracts

with an approximate volume of 200 ml were made 0.8 M in KCl and applied to a 1.8 cm^2 x 20 cm DE52 (Whatman) column equilibrated with 0.8 M KCl in buffer A (40 mM Mops, pH 7.5/1 mM EDTA/10 mM 2-mercaptoethanol). The pass-through (Fraction II) was dialyzed against 0.1 M KCl in Buffer B [Buffer A containing 25% (v/v) glycerol] and applied to a 0.65 cm^2 x 15 cm DEAE-cellulose column equilibrated with 0.1 M KCl in Buffer B. The column was washed with 60 ml of Buffer B containing 0.1 M KCl and eluted with a 0.1 - 0.8 M KCl gradient in 20 ml of Buffer B. Fractions exhibiting uvrD-complementing activity were pooled (Fraction III), dialyzed against 0.1 M KCl in Buffer B, and applied to a 0.65 cm^2 x 10 cm P11 phosphocellulose (Whatman) column equilibrated with 0.1 M KCl in Buffer B. The phosphocellulose column was washed with 25 ml of Buffer B containing 0.1 M KCl and eluted with a 0.1 - 1.0 M KCl gradient in 10 ml of Buffer B. Fractions containing uvrD-complementing activity (Fraction IV) were pooled, dialyzed against 0.05 M NaCl in Buffer C [20 mM Tris, pH 7.5/1 mM EDTA/10 mM 2-mercaptoethanol/25% (v/v)glycerol] and loaded onto a 0.65 cm^2 x 4 cm DNA-cellulose column (26). After washing with 10 ml Buffer C containing 0.05 M NaCl, the column was eluted by means of a step gradient 0.05 - 4.0 M NaCl in Buffer C. Fractions containing uvrD-complementing activity (Fraction V) were pooled, dialyzed against 0.05 M NaCl in Buffer C, and applied to a 0.65 cm^2 x 5 cm DNA-cellulose column prepared from heavily UV-irradiated (>10,000 J/m^2) calf thymus DNA (27). The proteins were fractionated using a 0.05 - 4.0 M NaCl gradient in 8 ml of Buffer C. Fractions containing uvrD-complementing activity (Fraction VI, about 0.3 ml) were pooled and used immediately for further characterization or stored in 50% glycerol at -20°C.

Other Methods

Protein concentrations were determined by the method of Lowry et al. (28) using bovine serum albumin as standard.

RESULTS

Requirement for the UvrD Protein for Completion of Excision Repair

The data displayed in Table 1 show that extracts prepared from E. coli are able to repair single-strand breaks that were introduced into UV-irradiated DNA but that this restoration of the damaged and incised DNA depends on a functional uvrD gene product. PM2 DNA damaged with 50 J/m^2 of UV was incubated with extracts prepared from either an incision-deficient uvrB strain, a uvrD101 strain or a mixture of extracts from both of these strains and the number of incision breaks was measured as described in

Materials and Methods. Under normal conditions NAD, the cofactor for polynucleotide ligase, and the four dNTP's essential for repair resynthesis were included in the reactions. To measure the full extent of incision the dNTPs were omitted from the reactions and NAD was replaced by NMN in order to inhibit ligation. The data (Table 1) show that these extracts promote a low level of UV-independent endonuclease activity that occurred irrespective of the presence of either the uvrB or uvrD gene product. Irradiation of the DNA (Table 1, line 3) caused additional incisions when functional uvrB gene product was present. Since both extracts contain normal levels of the uvrA and uvrC gene products, the increased UV-dependent incision seen when extracts from both uvrB$^-$ and uvrD$^-$ strains were mixed might be due to the increased level of UvrA and UvrC proteins in the final reaction mixture. When completion of repair was allowed (Table 1, line 4) a significant number of single-strand breaks remained in the reactions that contained only the extract from uvrD$^-$ cells. These breaks presumably arise from unrepaired incisions at pyrimidine dimer sites mediated by the functional UvrA, UvrB and UvrC proteins present in these extracts. The extract prepared from the incision-deficient uvrB$^-$ strain did not accumulate single-strand breaks. A

Table 1. Complementation in unfractionated extracts

extracts reactions	uvrD$^-$ only	uvrB$^-$ only	BOTH
-UV +NAD,dNTPs	0.03	0.03	0.03
-UV +NMN	0.21	0.24	0.19
+UV +NMN	0.87	0.25	1.26
+UV +NAD,dNTPs	0.48	0.09	0.27

Breaks per DNA molecule after incubation with extracts from repair-deficient strains. Extracts (Fraction I) were prepared from strains WM280 (uvrD101) and AB2499 (uvrB) as described in Materials and Methods. Portions (10 µl) of each extract were incubated for 20 min at 37°C singly and together in standard reaction mixtures containing 150 pmol ^{3}H-PM2 DNA, either unirradiated or subjected to 50 J of UV per m^2. The accumulation of breaks after incubation in the presence of NMN and the average number of breaks per molecule after repair in the presence of NAD and dNTPs were determined from alkaline sucrose gradient analysis as described in Materials and Methods.

mixture of the two extracts repaired more incisions than the extract from the uvrD$^-$ strain was able to do, and this occurred in spite of the increased incisions seen with both extracts present and normal ligation inhibited. This correction of a uvrD$^-$ deficiency at DNA strand rejoining by extracts prepared from strains that are incision-deficient but uvrD$^+$ provides a potential means for purifying the uvrD gene product by in vitro complementation. The in vitro situation is similar to the inefficiency of DNA strand break restoration seen with uvrD$^-$ strains in vivo (8).

To obtain some information regarding the specificity of the complementation assay described above we investigated the possibility that the assay might indirectly reflect DNA polymerase or ligase activity. An experiment like the one shown in Table 1 was performed using an extract from the uvrD101 strain mixed with an extract from a strain that was either polA1 (deficient in DNA polymerase I), $lig_{ts}7$ (temperature sensitive for polynucleotide ligase), uvrA (incision deficient), uvrE (mutant in uvrD cistron) or recL (mutant in uvrD cistron). The data (not shown) indicated that the extracts from polA, uvrA and $lig_{ts}7$ (assayed at restrictive temperature) could all complement the uvrD101 deficiency in vitro. However, neither the extract from the uvrE or the recL strain could correct the uvrD-related defect in rejoining of DNA single-strand breaks. Collectively, these results provide added confidence that the complementation assay shown in Table 1 is probably a direct measure of the uvrD gene product.

Fractionation of Extracts Containing the UvrD Protein

To determine whether the complementation effect seen in Table 1 would persist after partial fractionation of the crude extracts we attempted to partially separate the uvrD complementing activity from other cellular components. The incision deficient uvrB$^-$ strain was used as the source for large preparations of extract which, after removal of endogenous DNA, were fractionated by column chromatography as described in Materials and Methods. Samples recovered from the columns were mixed with portions of "receptor extracts" prepared from a uvrD101 strain as described in Materials and Methods and assayed for ability to complement the in vitro repair deficiency characteristic of extracts from uvrD$^-$ strains. Those fractions containing the uvrD-complementing activity which eluted from the various columns were pooled, dialyzed to an appropriate salt concentration, and subjected to successive steps of chromatography as described in Methods. In this way uvrD complementing activity was eluted successively from DEAE-cellulose, phosphocellulose, and DNA-cellulose columns. Figure 1 shows the profile of com-

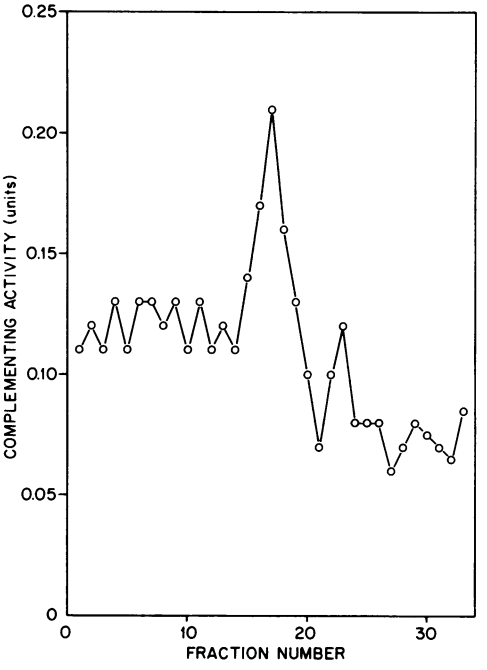

Figure 1. Fractionation by phosphocellulose column chromatography. Fractions from DE52 chromatography containing uvrD-complementing activity were pooled, dialyzed to lower salt concentration, and applied to a P-11 phosphocellulose column. Fractionation was accomplished by elution with a linear gradient of 0.1 to 1.0 M buffered KCl. A portion of each fraction (12 µl) was used for determination of ability to complement the uvrD101 repair defect (o——o) as described in Materials and Methods.

plementation activity recovered from a phosphocellulose column. As an additional control, a parallel purification procedure was performed using an extract made from the uvrD101 mutant. In this "mock" purification no complementation activity was found in the fractions recovered from a phosphocellulose column identical to the one shown in Fig. 1 (data not shown). This result reduces the concern that the complementing activity could be artifactual or ascribable to salt or high protein concentration.

The results of chromatography from DNA cellulose prepared with UV-irradiated DNA are shown in Fig. 2. After this DNA cellulose chromatography step, fractions containing uvrD-complementing activity were pooled (Fraction VI). The complementation activity was found to be approximately proportional to the amount of this protein fraction added to the repair

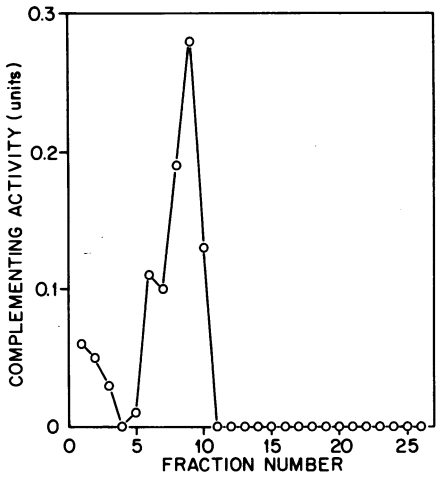

Figure 2. Fractionation on DNA-cellulose. Fractions from DNA cellulose chromatography containing uvrD-complementing activity were pooled, dialyzed against Buffer C containing 0.05 M NaCl, and loaded onto a column packed with DNA-cellulose made with UV-irradiated DNA. Fractionation was achieved by elution with a gradient of 0.05 M - 4.0 M buffered NaCl. UvrD-complementing activity (23 µl portions; o—o) was determined as described in Materials and Methods.

assay reaction mixture (Figure 3). Controls (not shown) indicated that the uvrD-complementing activity was labile to heat inactivation.

A protein preparation recovered from an experiment like that shown in Fig. 3 was examined for ability to complement repair deficiencies in the uvrD protein, DNA polymerase I and DNA ligase. As shown in Table 2, this protein fraction corrected the repair defect in extracts prepared from uvrD101 and uvrE mutant strains. In accord with the in vivo properties of polA mutants (29) the extract prepared from a strain deficient in DNA polymerase I showed an inability to complete in vitro repair. The addition of the uvrD$^-$ complementing activity to the extract from the polA1 strain did not reduce the single-strand breaks to the normal "background" level. The mixture of the uvrD complementing activity and an extract from a ligase-deficient strain showed complex behavior. The putative UvrD protein actually seemed to amplify the effect of the ligase deficiency, resulting in increased breakage of the UV-damaged DNA, although by itself it induced only minimal DNA breakage in the presence of NAD. Although the reasons for this behavior are not clear, the data do indicate that the uvrD complementing activity cannot overcome the deficiencies caused by the polA1 and $lig_{ts}7$ mutations but

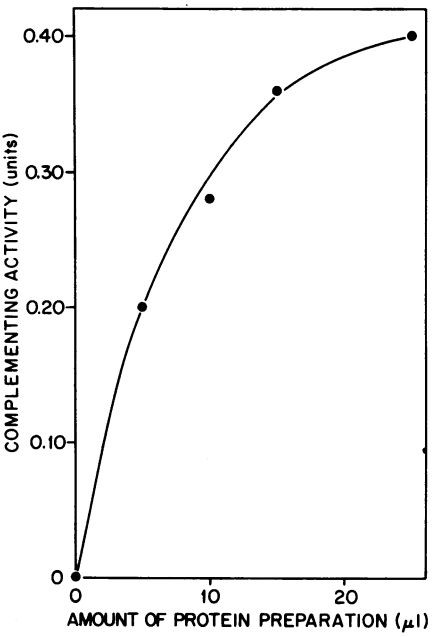

Figure 3. Complementation of the uvrD101 repair deficiency depends on the concentration of UvrD protein. Portions of the peak fractions of complementing activity recovered from chromatography on DNA-cellulose made with UV-irradiated DNA were incubated in the standard repair assay mixture containing NAD and dNTPs and 150 pmol UV-irradiated ^{3}H-PM2 DNA. UvrD-complementing activity is shown as a function of the amount of partially purified protein included in the reaction mixture (o——o).

instead shows at least some specificity for correction of the repair defect posed by the uvrE and uvrD101 mutations.

DISCUSSION

Our results show that incisions introduced into UV-irradiated DNA by E. coli uvrA, uvrB and uvrC gene products can be repaired in vitro by extracts that contain wild-type uvrD gene products. The reduced restoration of incised DNA seen with extracts from uvrD^{-} cells could mean that the UvrD protein is essential for a post-incision function or that an aberrant protein synthesized in the uvrD^{-} cells prevents or aborts normal DNA repair. A component present in extracts prepared from wild-type E. coli could complement the in vitro uvrD^{-} deficiency either by providing a functional UvrD protein or by diluting an aberrant UvrD protein that might be present in extracts from uvrD^{-} cells.

Table 2. Ability of Fraction VI to complement extracts from strains mutant in different repair capacities

Source of extract	Breaks remaining per DNA molecule		Complementing activity, units
	Extract only	+Fraction VI	
WM280 (uvrD101)	0.32	0.11	0.21
WM282 (uvrE)	0.44	0.18	0.26
D110 (polA)	0.91	0.34	-
N2668 ($lig_{ts}7$,42°)	1.19	3.20	-
None (control)	-	0.35	-

Extracts (10 µl) were incubated alone and with 25 µl Fraction VI in the standard assay mixture containing NAD and dNTPs as well as 150 pmol UV-irradiated PM2 DNA. The average number of breaks per molecule of DNA was determined as described in Materials and Methods. Extracts from polA strains perform apparently normal UV-dependent incision but show more strand-breaks in unirradiated DNA than do extracts from uvr^- strains (data not shown). This accumulation of UV-dependent and UV-independent incisions is probably responsible for the larger number of breaks seen (lines 3 and 4) with extracts deficient in the final steps of repair. The incision seen in line 5 is UV-independent. A more thorough characterization of this apparent nicking activity and the effects seen with the polA and lig^- strains in lines 3 and 4 would best be performed after additional steps of purification.

The in vitro deficiency at rejoining single strand breaks in irradiated DNA is in accord with in vivo properties of $uvrD^-$ mutants (8). Any model seeking to explain the molecular mechanism of the UvrD protein must take account of the likelihood that accumulation of interruptions in the DNA of $uvrD^-$ mutants is a major factor in the sensitivity of these strains to DNA damage. Several suggestions have been made regarding the mode of action of the uvrD gene product. The fact that uvrD mutants are deficient at dimer excision but do perform DNA repair synthesis (6,8,30) prompted our speculation (8) that in uvrD mutants the DNA strand that contains the dimer is displaced from its complementary strand without excision of the dimer. This would allow a DNA polymerase to perform DNA synthesis using the DNA strand complementary to the damaged strand as a template but could retard or even block rejoining of the repair patch to the contiguous DNA molecule. A role for the UvrD protein in altering the post-incision conformation of the incised DNA molecule is compatible with this notion. Other authors have suggested that the uvrD gene product might act as a helicase (19). Experiments with strains carrying a cloned $uvrD^+$ gene have led to the hypothesis that the uvrD gene may play some regulatory role in the cell (18). The data

shown here can also be accommodated within the framework of a model in which the uvrD gene product directly or indirectly modulates the activity of ligase or DNA polymerase. However, serious consideration of the UvrD protein's mechanism of action must await its isolation in a more purified form. Hopefully, the complementation assay we describe, perhaps in conjunction with the molecular cloning of the uvrD gene, may permit purification and eventual characterization of the UvrD protein.

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