Reconstitution of Nucleotide Excision Nuclease with UvrA and UvrB Proteins from Escherichia coli and UvrC Protein from Bacillus subtilis*

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Recently, an open reading frame which has a deduced amino acid sequence that shows 38% homology to Escherichia coli UvrC protein was found upstream of the aspartokinase II gene (ask) in Bacillus subtilis (Chen, N.-Y., Zhang, J.-J., and Paulus, H. (1989) J. Gen. Microbiol. 135, 2931–2940). We found that plasmids containing this open reading frame complement the uvrC mutations in E. coli. We joined the open reading frame to a tac promoter to amplify the gene product in E. coli and purified the protein to near homogeneity. The apparent molecular weight of the gene product is 69,000, which is consistent with the calculated molecular weight of 69,378 for the deduced gene product of the open reading frame. The purified gene product causes the nicking of DNA at the 8th phosphodiester bond 5' and the 5th phosphodiester bond 3' to a thymine dimer when mixed with E. coli UvrA and UvrB proteins and a DNA substrate containing a uniquely located thymine dimer. We conclude that the gene product of the open reading frame is the B. subtilis UvrC protein. Our results suggest that the B. subtilis nucleotide excision repair system is quite similar to that of E. coli. Furthermore, complementation of the UvrA and UvrB proteins from a Gram-negative bacterium with the UvrC protein of Gram-positive B. subtilis indicates a significant evolutionary conservation of the nucleotide excision repair system.

Nucleotide excision repair is the major pathway for removing DNA damage in Escherichia coli. This pathway repairs DNA damage ranging from covalently modified bases (Sancar and Sancar, 1988) to noncovalent drug-nucleotide adducts (Lambert et al., 1989) to apurinic/apyrimidinic sites (Lin and Sancar, 1989). They suggested that the B. subtilis gene which maps close to the B. subtilis uvrB gene was actually the homologue of E. coli uvrC and suggested the uvrC designation for the gene previously referred to as uvrB in B. subtilis. We will, hereafter, follow their recommendation and refer to the uvr gene adjacent to ask in B. subtilis as uvrC.

In this study, we have attempted to complement the E. coli uvrC mutations with the B. subtilis gene in vivo and to reconstitute (A)BC excinuclease activity in vitro with E. coli UvrA and UvrB proteins plus B. subtilis UvrC protein. Our studies show that the B. subtilis UvrC protein can substitute for its E. coli homologue both in vivo and in vitro. We conclude that B. subtilis has an excision nuclease quite similar to the E. coli (A)BC excinuclease.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The E. coli K12 strain DR1984 (uvrC4 recA1) was used as the host (Sancar et al., 1984) for propagation of plasmids, complementation of uvrC mutation, and over-

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1 Q. Shi and A. Sancar, unpublished observation.
2 The abbreviations used are: kbp, kilobase pair; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside; bp, base pair(s).
production of \textit{B. subtilis} UvrC protein. In addition, we used \textit{E. coli} K12 strains AB2405 (wurC53) and N3024 (wurC79::Tn10) for in vivo complementation. All strains were obtained from Dr. B. J. Bachmann (Yale University College of Genetic Science). The plasmids pDR3274 and p6935, which carry the \textit{E. coli} and \textit{B. subtilis} wur genes, respectively, have been described elsewhere (Thomas et al., 1985; Bondak and Sancar, 1989). The \textit{B. subtilis} UvrC protein expression plasmid pUCN6935 was constructed by ligating the EcoRI-NruI fragment carrying wurC from p6935 into the EcoRI-Smal sites of pKK223-3 (Pharmacia LKB Biotechnology Inc.). Following ligation and transformation into \textit{DH}10B, ampicillin-resistant clones were isolated, and the appropriate construct was identified by restriction enzyme analysis of DNA minipreps. The \textit{E. coli} cultures were grown in Luria Broth, which contained ampicillin (50 \text{mg/mL}) or tetracycline (20 \text{mg/mL}), when indicated.

**Materials**—Single-stranded DNA cellulose was purchased from Sigma, Ultrafuge AcA34 from Pharmacia LKB Biotechnology Inc., IFTG from Boehringer Mannheim, and restriction enzymes and T4 DNA ligase from Bethesda Research Laboratories. The following buffers were used in purification and characterization of UvrC protein: Buffer A, 50 mM Tris-\text{HCl}, pH 8.0, 100 mM NaCl, 10 mM \text{\beta}-mercaptoethanol, 1 mM EDTA, 10% sucrose; Buffer B, 50 mM Tris-\text{HCl}, pH 7.5, 10 mM \text{\beta}-mercaptoethanol, 1 mM EDTA, and 20% (v/ v) glycerol; storage buffer, 50 mM Tris-\text{HCl}, pH 7.4, 100 mM KCl, 1 mM EDTA, 50% (v/v) glycerol, and 50% (v/v) reagent grade sucrose; Buffer C, 50 mM Tris-\text{HCl}, pH 7.5, 100 mM KCl, 10 mM MgCl\text{\textsubscript{2}}, 10 mM dithiothreitol, 2 \text{M} ATP, and 50 \mu g/mL bovine serum albumin as standard (Bradford, 1976). SDS-polyacrylamide gels were run and stained by the Laemmli method (Laemmli, 1970). The Coomassie Blue-stained gels were scanned with a GS300 transmission/reflectance scanning densitometer (Scientific Instruments, San Francisco, CA), and the peaks were integrated using the Application Integrator from Dynamic Solution Corporation. The densitometer-scanner system was capable of detecting protein bands of about 10 \text{ng} or more.

**UV Survival Test**—All tests for UV survival shown in this paper were performed using \textit{E. coli} DR1984 as the host. DR1984 and its plasmid-containing derivatives were grown to stationary phase in Luria Broth or Luria Broth containing the appropriate antibiotic. The overnight culture was thawed at 0 \degree C and sonicated 10 X 10 \text{sec} with a Branson model W105 sonifier set at maximum output for the small tip. The sonicate was cleared of cell debris by centrifugation at 12,000 \text{x} g for 10 min, followed by centrifugation at 100,000 \text{x} g for 60 min. The supernatant (fraction 1, 25 \text{ml}) was loaded directly onto a 7-mL single-stranded DNA cellulose column equilibrated with Buffer B + 0.1 M KCl, at a flow rate of 10 \text{ml} /h. The column was washed with 12 \text{ml} of the same buffer. Bound proteins were eluted with a step gradient of 12 \text{ml} of Buffer B + 0.3 M KCl, 12 \text{ml} of Buffer B + 0.5 M KCl, and 30 \text{ml} of Buffer B + 1.2 M KCl. Three-mL fractions were collected, and 20 \mu l of each fraction was loaded onto an SDS-PAGE to locate UvrC protein. Fractions containing the UvrC protein were collected and dialyzed against Buffer B + 0.1 M KCl (fraction 2, 15.0 mL).

Fraction 2 was loaded onto a 10-mL phosphocellulose F-11 column equilibrated with Buffer B + 0.1 M KCl. The column was washed with 16 \text{ml} of the same buffer and developed with a step gradient of 15 \text{ml} of Buffer B + 0.3 M KCl, 15 \text{ml} of Buffer B + 0.5 M KCl, and 30 \text{ml} of Buffer B + 1.2 M KCl. Three-mL fractions were collected, and UvrC was located by SDS-PAGE. The fractions containing UvrC protein were dialyzed against Buffer B + 0.3 M KCl (fraction 3, 3.0 mL).

Fraction 3 was loaded onto a 3-mL single-stranded DNA cellulose column equilibrated with Buffer B + 0.3 M KCl. The column was washed with 12 \text{ml} of the same buffer and developed with a 30-mL continuous gradient of 0.5-1.2 M KCl in Buffer B. Two-mL fractions were collected, and UvrC was located by SDS-PAGE. The fractions containing UvrC were combined and dialyzed against storage buffer (fraction 4, 2.2 mL). Fraction 4 was divided into 25-\mu l aliquots, frozen in a dry ice-ethanol bath, and stored at -80 \degree C.

**RESULTS**

**Purification of \textit{B. subtilis} UvrC Protein**—All purification steps were carried out at 0 \degree C. Samples of the chromatographic fractions were analyzed by SDS-PAGE to locate the UvrC protein.

**Protein Chemistry**—Amino-terminal sequencing and amino acid composition were determined at the Yale Protein Chemistry Facility by Dr. Ken Williams.

**Complementation by \textit{B. subtilis} UvrC in \textit{E. coli}**—We first examined whether the \textit{B. subtilis} UvrC protein, which is adjacent to the uvrC and highly homologous to \textit{E. coli} wurC, encodes a protein which can substitute for the \textit{E. coli} uvrC gene product in \textit{E. coli} cells. The plasmid carrying ORF598, p6935, was inserted into \textit{E. coli} wurC strains. Three mutant alleles of wurC were tested, wurC33, wurC34, and wurC79::Tn10. The plasmid complemented all three mutations to the same level. The result obtained with DR1984 (wurC34) is shown in Fig. 1. The \textit{B. subtilis} gene complements the \textit{E. coli} mutation somewhat less efficiently than the corresponding \textit{E. coli} gene. Whether this is due to a low level of expression from the \textit{B. subtilis} promoter or to suboptimal interaction of \textit{B. subtilis} UvrC protein with the \textit{E. coli} UvrB-DNA complexes (Orren and Sancar, 1989) cannot be ascertained from these data. Never-
B. subtilis UvrC Protein

![Fig. 1. UV survival of DR1984 (uvrC34 recA1) containing various plasmids.](image)

![Fig. 2. Partial restriction map of the tac-uvrC plasmid used to purify UvrC of B. subtilis.](image)

![Fig. 3. Purification of B. subtilis UvrC.](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total UvrC protein</th>
<th>UvrC protein*</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cell-free extract</td>
<td>310.0</td>
<td>11</td>
<td>34.7</td>
</tr>
<tr>
<td>2. Single-stranded DNA cellulose I</td>
<td>7.8</td>
<td>45</td>
<td>3.5</td>
</tr>
<tr>
<td>3. Phosphocellulose</td>
<td>4.0</td>
<td>57</td>
<td>2.3</td>
</tr>
<tr>
<td>4. Single-stranded DNA cellulose II</td>
<td>2.2</td>
<td>98</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* Determined from a densitometric scan of the gel shown in Fig. 3.

Nevertheless, these findings provide very strong evidence that ORF598 is the *B. subtilis* uvrC gene, and hereafter we will refer to it as such.

**Overproduction of *B. subtilis* UvrC Protein**—To obtain pure *B. subtilis* UvrC protein for *in vitro* characterization, an overproducing plasmid was constructed by joining the *B. subtilis* gene to an *E. coli* promoter. The 3.25-kbp *B. subtilis* EcoRI-NruI fragment from p6935 was inserted into the *toe* promoter. Cultures were grown to stationary phase in Luria Broth, and dilutions were plated on Luria Broth agar plates that were irradiated with UV light from a germicidal lamp at a rate of 0.5 erg mm⁻² s⁻¹. The surviving colonies were counted 24 h after irradiation. The data points are the mean values of two experiments.

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were analyzed by SDS-polyacrylamide gel electrophoresis, stained with Coomassie Blue, and scanned by a densitometer to estimate the relative amount of the UvrC protein on the gel. The amounts are expressed relative to the highest fraction. Cl, 4 ~1 from each fraction was assayed by plasmid-nicking activity as described in the text; the number of incisions/plasmid was calculated and plotted. The data points plotted are mean values from two experiments. With nonirradiated DNA, we obtained incisions ranging from 0.01 nick/plasmid at 30 s to 0.35 nick/plasmid at 60 min. These background values have been subtracted from the total nicks to calculate the UV damage-specific nicks plotted in this graph.

To determine the mode of incision of enzyme reconstituted with B. subtilis UvrC, a 48-mer duplex containing a single thymine dimer at unique location was used as substrate. Fig. 6 shows the incision pattern of excision nuclease reconstituted with E. coli UvrA and UvrB. A, internally labeled 48-bp duplex with thymine dimer was digested by 10 nM E. coli UvrC or 80 nM B. subtilis UvrC in the absence or presence of 20 nM UvrA and 200 nM UvrB at 37 °C for 30 min. Reaction products were run on a 12% sequencing gel and autoradiographed. The scission products 19-mer (3' incision) and 13-mer (5' incision) are indicated. B, schematic representation of the substrate and the labeled single-stranded incision products. The asterisks indicate the positions of the 32P label, and the two arrows indicate the incision sites of (A)BC excinuclease.

reconstituted with the B. subtilis UvrC is less efficient. Nevertheless, it is clear from this figure that the sites of incision of enzyme reconstituted with the B. subtilis UvrC are the same as the natural E. coli (A)BC excinuclease.

DISCUSSION

Our results show that the UvrC protein of B. subtilis can substitute for E. coli UvrC protein both in vivo and in vitro.
These findings have several implications.

First, the fact that the B. subtilis protein with 38% homology to E. coli UvrC complements the E. coli excision nuclease provides the first evidence of an excision nuclease in another species which repairs DNA damage by exactly the same mechanism as the E. coli (A)BC excinuclease. By the same reasoning, it is likely that the M. luteus, uvrA, and uvrB homologues (Shiota and Nakayama, 1988, 1989) also encode subunits of an enzyme that functions in the same manner as the E. coli (A)BC excinuclease.

Second, of the two patches of homology found between E. coli UvrB and UvrC proteins (Arikan et al., 1986; Backendorf et al., 1986) the one extending from position 349 to 354 of UvrC (new numbering) and 352 to 357 of UvrB appears to be nonessential for functional integrity of either E. coli protein. This homology has not been conserved in M. luteus UvrB (Shiota and Nakayama, 1988) nor in B. subtilis UvrC which can substitute for E. coli UvrC protein.

Third, it was found that the carboxyl-terminal 60 residues of UvrC are 34% homologous to the carboxyl terminus of the human ERCC-1 gene (Doolittle et al., 1986) which corrects the UV repair deficiency of a rodent UV-sensitive mutant cell line (van Duin et al., 1988). Surprisingly, the residues conserved between ERCC-1 and E. coli UvrC protein are not conserved in the B. subtilis UvrC homologue and therefore cannot be primary importance for the UvrC function.

Fourth, the sequence identity between the E. coli and B. subtilis UvrC proteins is only 36%, yet the B. subtilis protein complements the E. coli excision nuclease suggesting that the residues important for function must be within those identical in the two proteins. Considering the reaction mechanism of E. coli (A)BC excinuclease (Orren and Sancar, 1989), these residues are likely to be important for interacting with DNA or UvrB protein. It must be pointed out, however, that the incision kinetics with B. subtilis UvrC are reproducibly slower than with E. coli UvrC, most likely reflecting the suboptimal interactions between E. coli UvrB and B. subtilis UvrC, due to sequence divergence.

Finally, the results presented here along with two previous reports suggest that the E. coli UvrC protein is 610 amino acids long (M, = 68,510). Sancar et al. (1984) reported that the amino-terminal sequence of UvrC protein was blocked and suggested that the protein was 588 amino acids long because a plasmid with a deletion extending up to 20 nucleotides preceding the putative ATG codon in wrc (position 67-69) complemented wrc-, mutations. Moolessen et al. (1987) found that deletion of a GTG triplet 66 bp 5′ to this ATG coupled with a frameshift in an ATG triplet upstream in the vector resulted in a construct which was unable to complement wrc- in a qualitative assay and suggested that wrc actually initiated with the GTG codon 66 bp 5′ to the ATG codon proposed by Sancar et al. (1984). Chen et al. (1989) found the sequence homology between the B. subtilis and E. coli uvrC genes extended up to the GTG codon and proposed that both proteins initiated at this codon. Our amino-terminal sequencing confirms that prediction for the B. subtilis protein and very strongly suggests that the E. coli protein extends that far as well. If that is the case, the data of Sancar et al. (1984) indicate that the first 22 amino acids of E. coli UvrC protein are not essential for function.

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Reconstitution of nucleotide excision nuclease with UvrA and UvrB proteins from Escherichia coli and UvrC protein from Bacillus subtilis.

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