Catalytic Sites for 3’ and 5’ Incision of *Escherichia coli* Nucleotide Excision Repair Are Both Located in UvrC*

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Nucleotide excision repair in *Escherichia coli* is a multistep process in which DNA damage is removed by incision of the DNA on both sides of the damage, followed by removal of the oligonucleotide containing the lesion. The two incision reactions take place in a complex of damaged DNA with UvrB and UvrC. It has been shown (Lin, J.-J., and Sancar, A. (1992) *J. Biol. Chem.* 267, 17688–17692) that the catalytic site for incision on the 5’ side of the damage is located in the UvrC protein. Here we show that the catalytic site for incision on the 3’ side is in this protein as well, because substitution R42A abolishes 3’ incision, whereas formation of the UvrBC-DNA complex and the 5’ incision reaction are unaffected. Arg42 is part of a region that is homologous to the catalytic domain of the homing endonuclease I-TevI. We propose that the UvrC protein consists of two functional parts, with the N-terminal half for the 3’ incision reaction and the C-terminal half containing all the determinants for the 5’ incision reaction.

Nucleotide excision repair in *Escherichia coli* is initiated by the binding of the UvrA-B complex to DNA containing a damage. Following this, UvrB is loaded onto the site of the damage, and the UvrA protein is released. The resulting UvrB-DNA preincision complex is bound by UvrC, leading to incision of the DNA at the fourth or fifth phosphodiester bond on the 3’ side of the damage. This 3’ incision is immediately followed by hydrolysis of the eighth phosphodiester bond at the 5’ side of the damage (for reviews see Refs. 1 and 2). Following 5’ incision, often further DNA cleavage is observed 7 nucleotides from the damage (for reviews see Refs. 1 and 2). Following this, UvrB is loaded onto the site of the damage, this 3’ incision, this residue is part of a region that is homologous to the catalytic domain of the homing endonuclease I-TevI, indicating that the catalytic site for 3’ incision during nucleotide excision repair is located in this homologous region.

**EXPERIMENTAL PROCEDURES**

**Protein Purifications**—Purification of the Uvra, UvrB (9), and UvrC (10) proteins have been described. UvrC(R42A) was expressed from plasmid pCA161, which was constructed by site-directed mutagenesis of pBl2 (11) using the oligonucleotide GACCTGAAAAACCGCTTCCAGCGTACTGTC. The UvrC(R42A) protein was overproduced in the ΔuvrC strain CS4927(12) and purified by the same procedure as the wild type UvrC protein. For the purification of UvrC (351–610) plasmid pCA137 was constructed in which the truncated UvrC protein fused to a His tag at its C terminus is expressed from the T7 promoter. The insert of pCA137 was synthesized by polymerase chain reaction using primers CAGTAACCATATGAGGGCGCATTATCTGAAA and AGCGTTATCTGTTCAATGACGAGGT using pBl12 as template. The polymerase chain reaction product was restricted with *Nde*I and *Bam*HI (sites are underlined in the primers), and the resulting fragment was inserted in PET11a (13). For overproduction of the truncated protein strain CS5434 was constructed by transferring the ΔuvrC gene mutation from CS4927 (12) into strain BL21(DE3) (13), by P1 transduction. CS5434 containing pCA137 was grown in LB until *A*~OD~ was 0.4 and 0.5 mM isopropyl-β-D-thiogalacto-pyranoside was added. After 2 h of induction the cells were collected. For the purification of the truncated UvrC protein the method described for wild type UvrC was adapted. After the phosphocellulose column, the proteins were loaded on a blue-Sepharose column (Amersham Pharmacia Biotech) in 0.1 M KPO4 (pH 7.5), 0.1 M KCl, 25% glycerol. The truncated UvrC protein was eluted with a 0.1–1.0 M KCl gradient in the same buffer. Samples containing UvrC were finally loaded on a Ni2⁺ column that was eluted with a 0–0.25 M imidazole gradient in 0.1 M KPO4 (pH 7.5), 0.5 M KCl, 25% glycerol.

**Construction of Damaged DNA Substrates**—The DNA sequence of substrate G1 is shown in Fig. 1. Substrate G2 is substrate G1 with a single-stranded nick at the 3’ incision position. The cholesterol lesion was synthesized as a phosphoramidite-protected nucleoside building block as described.2 Using automated oligonucleotide synthesis, this building block was directly introduced into DNA. For 5’ labeling 4 pmol of the cholesterol-containing oligo was incubated with 10 units of T4 polynucleotide kinase in 70 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 5 mM dithiothreitol, and 3 pmol of [γ-32P]ATP (7000 Ci/mmol, ICN). After incubation the reaction was quenched with 25% formamide and denatured DNA was fractionated by sucrose gradient centrifugation. The samples were dried and digested with Type I restriction enzymes. The resulting oligonucleotides were separated by denaturing PAGE. Autoradiography revealed the labeled products of the corresponding restriction reactions. In this paper we show that substitution R42A in UvrC abolishes 3’ incision. This residue is part of a region that is homologous to the catalytic domain of the homing endonuclease I-TevI, indicating that the catalytic site for 3’ incision during nucleotide excision repair is located in this homologous region.

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2 Y. Monaco, K. I. Van de Wetering, N. J. Meeuwennoord, H. A. Van den Elst, H. R. Stuijvenberg, R. Visse, G. F. Moolenaar, E. Verhoeven, N. Goosen, G. A. Van der Marel, and J. H. Van Boom, submitted for publication.
incubation at 37 °C for 45 min, the reaction was terminated by incubation at 80 °C for 10 min in the presence of 20 mM EDTA. G1 was constructed by hybridizing 4 pmol each of the 50-mer top strand and the 50-mer bottom strand in the presence of 50 mM NaCl and 1 mM EDTA. G2 was constructed by hybridizing the cholesterol-containing 31-mer, the adjacent 19-mer, and the 50-mer bottom strand. The substrates were purified from the nonincorporated nucleotides by G50 gel filtration in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl.

Incision Assay—The DNA substrates (40 fmol) were incubated with 2.5 nM UvrA, 100 nM UvrB, and 50 nM (mutant) UvrC in 20 μl of Uvr-endo buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 100 mM KCl, 0.1 μg/μl bovine serum albumin, and 1 mM ATP) as described (14). After the indicated times the reaction was terminated by adding 2 μl of 2 μg/ml glycogen followed by ethanol precipitation. The incision products were analyzed on a 15% acrylamide gel containing 7 M urea.

Gel Retardation Assay—The DNA substrates (40 fmol) were incubated with 2.5 nM UvrA, 100 nM UvrB, and 20 nM (mutant) UvrC in Uvr-endo buffer. The mixture was incubated at 37 °C, and the protein-DNA complexes were analyzed by loading the samples on a 3.5% native polyacrylamide gel.
RESULTS AND DISCUSSION

The N-terminal region of the UvrC protein contains a region with similarity to the GIY-YIG family of intron-encoded homing endonucleases (Ref. 15 and Fig. 2). It has been postulated that this region in the homing nucleases constitutes the catalytic domain, because amino acid substitutions of residues Arg27 or Glu75 in the I-TevI nuclease result in proteins that can bind a homing site substrate but no longer cleave this DNA (16, 17). To determine whether this region of UvrC has a similar function we mutated the conserved residue Arg42 by replacing it with Ala. The UvrC(R42A) mutant protein behaved identically to the wild type protein during the purification procedure, indicating that the overall physical properties of the protein are not affected by the mutation.

Incubation of the 3'-prenicked substrate G2 with UvrA, UvrB, and UvrC(R42A) resulted in a 5' incision as efficient as with the wild type UvrC (Fig. 3A, lanes 5 and 6). In contrast, the double-stranded substrate G1 was hardly incised with the mutant UvrC (Fig. 3A, lane 3). The very low amount of incision observed with UvrC(R42A) appeared to be due to uncoupled 5' incision (not shown). Low amounts of uncoupled 5' incision have been observed also on a BPDE-modified DNA substrate (18). Taken together our results demonstrate that UvrC(R42A) is defective in 3' but not in 5' incision. The additional 5' incision was also efficiently induced by the mutant UvrC. This damage-independent cleavage by UvrBC has been shown to use the same catalytic site of UvrC that is used for 5' incision (3). Gel retardation analysis showed that UvrC(R42A) formed UvrBC-DNA complexes comparable with the wild type protein (Fig. 3B, lanes 2 and 3). Taken together, the results strongly indicate that residue Arg42 of UvrC is part of the catalytic site for 3' incision, suggesting that the region that is homologous to the homing nucleases forms the catalytic domain.

In Fig. 2A a schematic representation of the UvrC protein is
shown. All identified residues of the catalytic site for the 5’ incision reaction are located in the C-terminal half of the protein. This second half of UvrC also contains the DNA-binding domain that is homologous to ERCC1 and that has been shown to be important for 5’ incision (10). The proposed catalytic domain for the 3’ incision is in the N-terminal half together with the coiled-coil domain that interacts with UvrB. This domain has been shown to be essential for 3’ incision. The positions of the different domains suggest that UvrC might consist of two functional halves, one for each incision event. To test this, we tried to overproduce and purify the two halves of the protein separately. Attempts to purify the N-terminal part of UvrC, either spanning residues 1–243 or residues 1–350 were unsuccessful, because both truncated proteins formed insoluble aggregates in the cell (not shown). The C-terminal part, from residues 351 to 610, however, was successfully purified. As expected, because both the catalytic site and the UvrB-binding domain for 3’ incision are absent, substrate G1 was not incised at all by UvrC (351–610) (Fig. 4A, lane 2). Substrate G2, however, was efficiently incised at the 5’ incision position by the C-terminal half of the protein UvrC (Fig. 4B, lanes 6–8). The truncated UvrC protein did not induce the additional 5’ incision. This is in agreement with the observation that this damage-independent incision event requires the interaction between the coiled-coil domains of UvrB and UvrC (3) because in UvrC (351–610) this interaction domain is lacking (Fig. 2A).

The efficiency of the 5’ incision induced by the truncated UvrC protein was somewhat lower compared with that of wild type UvrC (Fig. 4B). This might be due to the presence of the His tag fused to the C-terminal end of the mutant protein. On the other hand it cannot be excluded that the N-terminal half of UvrC contributes to the 5’ incision reaction, e.g., by stabilizing the conformation of the C-terminal half of the protein or by stabilizing the UvrBC-DNA complex. The efficient incision by the UvrC (351–610) mutant (50% in 10 min), however, demonstrates that all important determinants for 5’ incision are located in the C-terminal half of the protein.

In the past it was shown that a fusion of the maltose-binding protein (MBP)9 with part of the UvrC gene containing the C-terminal 314 amino acids (Fig. 2A) is capable of complementing for UV sensitivity in vivo (19). The same protein was shown to be able to induce 3’ incision in vitro, albeit at only about 1% of wild type UvrC activity. These observations are contradictory to the results presented here, because the MBP fusion protein is not only lacking the catalytic domain for 3’ incision, as identified in this paper, but also the coiled-coil domain for interaction with UvrB. The only way to explain the results reported for the MBP fusion protein is that both the in vivo studies and the protein overexpression for the in vitro studies were done in an E. coli strain that expresses a partially active UvrC protein from the chromosome. The E. coli strain used in the MBP fusion studies contained uvrC279::Tn10, which has a Tn10 insertion in the 3’ half of uvrC. As a result this strain might produce a truncated UvrC protein that still has all the domains for 3’ incision and that might therefore still incise damaged DNA at the 3’ side. The lack of the C-terminal part prevents subsequent 5’ incision, and hence the strain is deficient for repair. The introduced MBP fusion protein, however, which like UvrC (351–610) is expected to be fully active in 5’ incision, can complete the repair reaction, and as a result UV resistance is restored. The observed incision in vitro could then be explained by a co-purification of the chromosome-encoded Uvr fragment with the MBP fusion protein.

In the eukaryotic nucleotide excision repair system, the 3’ and 5’ incisions are made by different proteins (20, 21). In this paper we show that the two incisions in the E. coli system are induced by the same protein but that for each incision event distinct protein domains are used. This suggests that also in the ancestral bacterial repair system the two incisions were induced by two different proteins and that most likely during evolution these two proteins have fused into one.

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