Mol. Cells, Vol. 10, No. 5, pp. 592–597

Communication

Molecules and Cells

A Physical Interaction of UvrD with Nucleotide Excision Repair Protein UvrB

Byungchan Ahn*
Department of Molecular Microbiology, University of Ulsan, Ulsan 680-749, Korea.

(Received on May 26, 2000)

The dual-incision nature of the reaction of UV-irradiated DNA catalyzed by the UvrABC complex potentially leads to excision of a damaged fragment. However, neither fragment release under nondenaturing conditions nor the UvrBC proteins are turned over. The addition of the UvrD protein to the incised DNA-UvrBC complex results in excision of the incised damaged strand and in the turnover of the UvrC protein. In an effort to better understand the involvement of UvrD in the excision step, immunoprecipitation was used to detect interacting proteins with UvrD in the DNA repair. In this communication, it is shown that UvrA and UvrB are precipitated with UvrD in solution but the UvrAB complex is not. In the incision complex, UvrB could be precipitated and the preincubation of UvrD with UvrB revealed an inhibitory effect on the turnover of the incision complex. These data imply that UvrB in the incision complex seems to recruit UvrD to the 3′ incised site of the incised strand by protein-protein interaction and to allow initiation of unwinding by UvrD from the resulting nick in a 3′ to 5′ direction.

Keywords: Helicase II; Immunoprecipitation; Nucleotide Excision Repair.

Introduction

The nucleotide excision repair (NER) in *Escherichia coli* is catalyzed by the multisubunit incision endonuclease UvrABC that initiates the excision of a large number of different DNA adducts including pyrimidine dimers (Grossman *et al.*, 1998; Sancar, 1996). The UvrA dimer forms the UvrAB complex, which is an active protein complex searching for DNA damage with its helicase activity (Oh and Grossman, 1989). Once the complex recognizes DNA damage, the UvrBC-DNA complex is formed by the interaction of a stable preincision UvrB-DNA complex with the UvrC protein (Orren and Sancar, 1989; 1990). It is believed that UvrB cleaves a phosphodiester bond four to five phosphates 3′ to the damage and that the eighth phosphate backbone 5′ to the damage is incised by UvrC (Lin and Sancar, 1992; Zou *et al.*, 1995). In the excision step of NER, it has been reported that the UvrD protein is involved in the turnover of UvrC and releases the incised DNA lesion, making repair synthesis possible by DNA polymerase and DNA ligase (Caron *et al.*, 1985; Husain *et al.*, 1985; Orren *et al.*, 1992). Although significant progress have been made in understanding the overall mechanism of *E. coli* NER, the molecular aspects of excision by UvrD remain unclear.

UvrD displays a 3′ to 5′ polarity in the DNA unwinding of blunt ended duplex DNA, nicked DNA, and a 3′-overhang containing duplex DNA *in vitro*, in that a 3′ single-stranded DNA flanking the duplex facilitates the initiation of unwinding (Maston, 1986; 1989; Runyon *et al.*, 1990). Optimum unwinding by the prebound UvrD-DNA complexes requires a 3′ single-stranded DNA tail of 36–40 nts (Ali *et al.*, 1999). When compared the length (12–13 nts) of the incised fragment to the requirement of the 3′ tail for UvrD, although there is a dramatic DNA structural change near the 3′ incision position (Visse *et al.*, 1994), the incised length may not be enough for UvrD to recognize a 3′ tail directly. However, a recent report revealed that UvrD had a potential interaction with MutL, a component of the methyl-directed mismatch repair pathway (Hall *et al.*, 1998), suggesting that protein-protein interactions may be responsible for directing the involvement of UvrD in DNA repair.

In an effort to understand how the UvrD protein recognizes the incised site for its helicase activity, immunoprecipitation was used to identify NER proteins

* To whom correspondence should be addressed.
Tel: 82-52-259-2359; Fax: 82-52-259-1694
E-mail: bcahn@uou.ulsan.ac.kr

Abbreviations: NER, nucleotide excision repair; 6xHIS, 6 histidines.

interacting with UvrD. UvrA and UvrB were precipitated with UvrD in solution and UvrB in the incision complex was precipitated as well. These results suggest that UvrB in the incision complex seems to recruit UvrD to the incised site and to allow initiation of unwinding by UvrD from the resulting nick.

Materials and Methods

Strains, enzymes, DNA and nucleotides *E. coli* BL21(DE3)-Δ*uvrD* was provided kindly by Dr. S. W. Matson (University of North Carolina, Chapel Hill). *E. coli* K-12 was from KCTC (Korea). pET33b(+) was from Invitrogen. Pfu DNA polymerase was from Stratagene. All DNA modifying enzymes were from New England Biolabs. 6xHIS monoclonal antibody was from Clontech. The monoclonal antibodies of UvrA, UvrB, and UvrC were kindly provided by Dr. Lawrence Grossman (The Johns Hopkins University). UvrA, UvrB, and UvrC were purified as described previously (Kovalsky and Grossman, 1994; Yeung *et al*., 1986). Primers for PCR were from the Core facility of The Johns Hopkins University.

The cloning and purification of HIS-tagged UvrD The *uvrD* gene in *E. coli* K-12 genomic DNA was amplified by PCR with Pfu DNA polymerase and two primers containing a *Nco*I and *Xho*I site, respectively, which flank the gene. Amplified *uvrD* was cloned into the *Nco*I and *Xho*I site of pET33b(+) to create an in-frame translational fusion with 6 histidines. This construct was designated pETUvrD-HIS. After transformation, colonies were screened for those containing plasmids encoding the *uvrD* gene by both restriction and PCR assay. The cloned construct was identified finally by a full-length DNA sequencing of the *uvrD* gene.

To over-express this new recombinant UvrD, the BL21(DE3)Δ*uvrD* cells containing pETUvrD-HIS were grown at 30°C to 0.5 of A_{600}. IPTG (Isopropyl-β-D-thiogalactopyranoside) was then added to a final concentration of 0.4 mM for induction. UvrD was purified in one step through a Ni^{2+}-binding column according to the supplier's protocol (Novagen). Bound proteins were eluted from the column with elution buffer containing 200 mM imidazole. Fractions were analyzed by SDS-PAGE and the pooled fraction was dialyzed against UvrD storage buffer (20 mM Tris-HCl, pH 8.3, 200 mM NaCl, 50% Glycerol, 1 mM EDTA, 0.5 mM EGTA, 30 mM β-mercaptoethanol). The new recombinant, UvrD, was designated UvrD-HIS. The helicase activity of the purified UvrD-HIS protein was examined.

Immunoprecipitation UvrA (10 pmole), UvrB (12.8 pmole), and or UvrC (15 pmole) were incubated with UvrD protein (12 pmole) in 100 μl of reaction buffer (20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 85 mM KCl, 100 μg/ml BSA, 2 mM ATP, 5% glycerol) at 25°C for 20 min. One hundred μl of dilution buffer (TBS: 20 mM Tris-HCl, pH 7.6, 137 mM NaCl + 1% Triton X-100 + 1 mg/ml BSA) was added to the reaction mixture and then incubated with 10 μl of Mouse IgG-agarose (Sigma) to prevent subsequent coprecipitation of non-tagged proteins at 4°C for 1 h followed by centrifugation. The supernatant of the mixture was incubated with 2 μl of 6xHIS mAb at 4°C for 1 h and the protein-antibody complex was precipitated with 10 μl of Protein G-agarose at 4°C for 1 h. The precipitates were washed with the dilution buffer, TBS and 50 mM Tris-HCl (pH 6.8). The proteins in the precipitate were resolved by SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were immunoblotted with monoclonal antibodies to UvrA, UvrB, UvrC, and UvrD.

UV streak test A streak test was performed on LB-agar plates containing IPTG as described previously (Thaigalingam and Grossman, 1991). The plates were incubated at 30°C for overnight.

Incision assay Incision was carried out as described previously (Ahn and Grossman, 1996). UvrA (1.0 pmole), UvrB (1.3 pmole), UvrC (1.5 pmole), and or UvrD (6 pmole) were incubated with 200–500 ng of UV-damaged plasmid DNA (320–350 J/m^2). The plasmid DNAs were then analyzed by agarose gels and detected by ethidium bromide staining.

UvrD Helicase assay To detect UvrD helicase activity, a blunt-ended duplex DNA substrate (121 bp) was prepared by *Bam*H I and *Pvu*II restriction enzyme digestions of pTZ18R followed by fill-in of T4 DNA polymerase and [α-^{32}P] dTTP. Purified UvrD proteins were incubated with the prepared DNA substrate in the standard reaction buffer (20 mM Tris-HCl: pH 7.6, 35 mM NaCl, 2.5 mM $MgCl_2$, 2 mM ATP, 4 mM β-mercaptoethanol) for 20 min at 37°C and the reactions were then terminated with stop solution (20 mM EDTA, 0.2% SDS). The DNA was analyzed by electrophoresis through a 9% polyacrylamide gel followed by autoradiography of X-ray film.

Results and Discussion

Biochemical characterization of HIS-tagged UvrD In order to identify proteins interacting with UvrD in solution using immunoprecipitation with monoclonal antibody against UvrD, UvrD was tagged with 6 histidines (6xHIS) at the C-terminal. To determine the ability of UvrD-HIS to function in UvrABC-mediated nucleotide excision repair, the complementation of this new recombinant UvrD to UV resistance was examined by a UV streak test. The plasmid pETUvrD-HIS was transformed into *E. coli* strain BL21(DE3)Δ*uvrD* which is UV sensitive (George *et al*., 1994). As shown in the UV streak test (Fig. 1A), UvrD-HIS, supplied on a plasmid, complemented the UV-sensitive phenotype of *E. coli* strain BL21(DE3)Δ*uvrD*, suggesting that the expressed UvrD-HIS protein retains its function in the DNA repair process. The UvrD-HIS protein was purified through a Ni^{2+}-HIS bind column with a single band purity (Fig. 1B). The purified UvrD-HIS protein could unwind blunt ended duplex DNA in a protein concentration-dependent manner (Fig. 1C). These results, consistent with those of previous observations (Matson, 1989; Runyon *et al*., 1990), indicate that the recombinant UvrD-HIS has an active helicase activity.

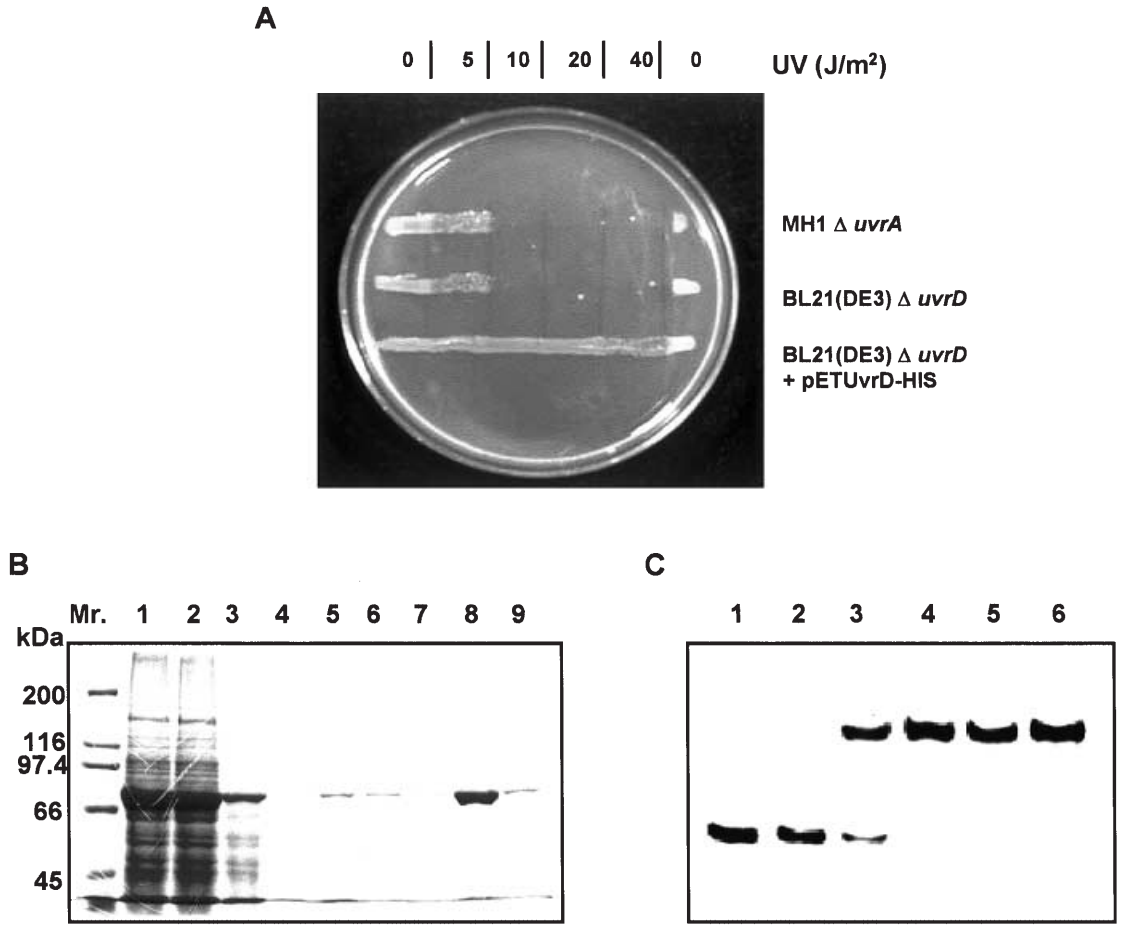

Fig. 1. Biochemical activity of histidine-tagged UvrD protein. **A.** UV streak test. The constructed pETUvrD-HIS plasmid was transformed into the BL21(DE3)Δ*uvrD* strain. MH1, Δ*uvrA*; *uvrA* deletion mutant. BL21(DE3), Δ*uvrD*; *uvrD* deletion mutant. **B.** The purification of UvrD-HIS through the Ni^{2+}-HIS bound resin. Lane 1, molecular weight standard; lane 2, cell extract; lane 3, soluble fraction; lane 4, flow through; lane 5, washing with binding buffer; lanes 6–8, washing with washing buffer; lanes 9 and 10, elution fractions. **C.** The helicase activity of UvrD-HIS. Lane 1, no protein; lane 2, 10 nM, lane 3, 30 nM; lane 4, 100 nM; lane 5, 200 nM; lane 6, heat denatured substrate.

A physical interaction of UvrD with nucleotide excision repair proteins UvrA and UvrB To detect any interaction of UvrD protein with UvrA, UvrB, or UvrC in solution, each purified protein was incubated in the reaction buffer. Proteins which bind nonspecifically to the solid-phase matrix were removed by preadsorption of the incubated protein mixture using Mouse IgG-agarose followed by centrifugation. The supernatant was incubated with anti-6xHIS monoclonal antibody exclusively specific for the 6xHIS tag. The complex of antibody, UvrD-His, and Uvr proteins was precipitated with Protein G-agarose which interacts with the antibody. The precipitated sample was washed to remove proteins that are not tightly bound to the matrix. The precipitate was analyzed by SDS-PAGE and then the proteins were detected using the antibodies specific for UvrA, UvrB, and UvrC, respectively (Kovalsky and Grossman, 1994). No nonspecific precipitation was detected in the absence of UvrD (data not shown). With the incubation with UvrD, UvrA, and UvrB were precipitated but UvrC was not (Fig. 2A, lanes A, B, and C). The two thick bands were heavy chains (H) and light chains (L) of IgG, respectively. When UvrA and UvrB were pre-incubated, no protein was precipitated with UvrD (lane AB). However, UvrB was still precipitated even with pre-incubation of UvrB and UvrC (lane BC). These results suggest that UvrD interacts with UvrA or UvrB in the context of the immunoprecipitation system, that the interacting sites of UvrD for UvrA and UvrB may overlap, and that there may be a differential affinity of UvrB for UvrA and UvrD.

Discussion

Upon binding of the UvrAB complex to the site of damage, a stable pre-incision UvrB-DNA complex is formed resulting in the release of UvrA. Then, UvrC

interacts with UvrB in the UvrB-DNA intermediate and is believed to trigger an endonuclease activity of UvrB and UvrC. The complex of UvrBC with incised DNA is called an incision complex. In order to examine whether UvrD interacts with UvrB in the preincision and the incision complex, the UvrA, UvrB, and/or UvrC proteins were incubated with UV-irradiated DNA and then UvrD was added to the reaction followed by immunoprecipitation (Fig. 2B). The extent of precipitated UvrB was greater in the presence of UvrABC with UV-damaged DNA than in the presence of UvrAB and UV-damaged DNA and was similar to that of free UvrB in solution. Some precipitation of UvrA was detected as well, probably due to free UvrA or UvrA dissociated after the formation of the precision complex. However, no protein was precipitated in the absence of UV-damaged DNA (Fig. 2B, lane ABC). These results indicate that the interaction of UvrD with UvrD seems to be stronger in the incision complex than in the preincision complex and that the interaction may be dependent on UV-damaged DNA when all three UvrABC proteins are present.

To examine the functional importance of the interaction of UvrD with UvrB and UvrA in excision repair, the incision of DNA damage by UvrABC was investigated (Fig. 3A). The UV-damaged supercoiled DNA was incised partially (Fig. 3A, lane ABC) but most of the damaged supercoiled DNA was incised in the presence of UvrD and a linear form of DNA was

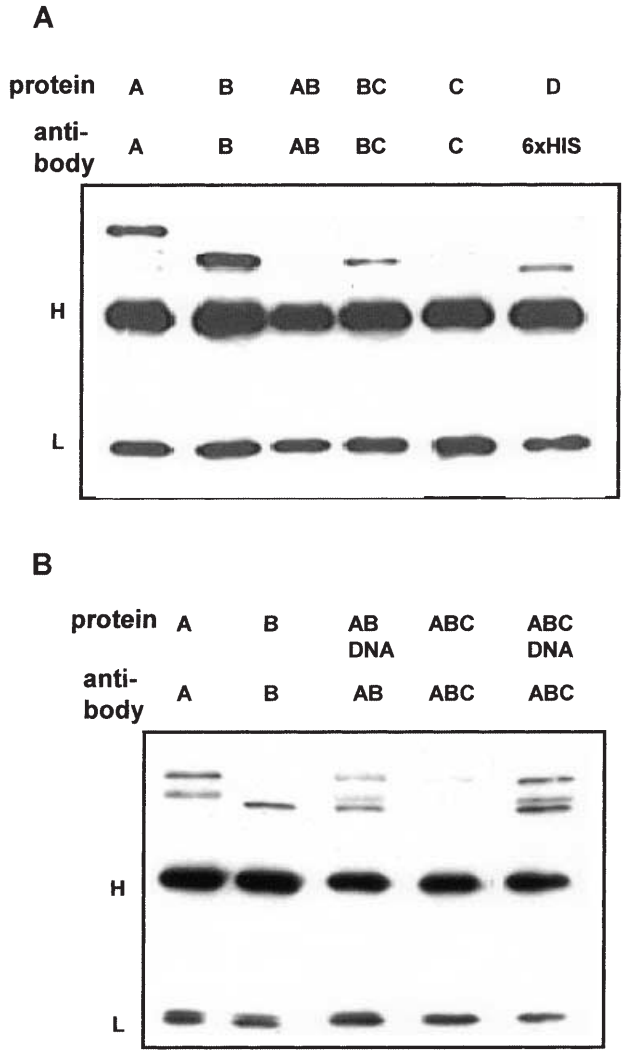

Fig. 2. Immunoprecipitation. **A.** Immunoprecipitation with UvrD. UvrA (10 pmole), UvrB (12.8 pmole), and/or UvrC (15 pmole) was incubated with UvrD (12 pmole) in 100 μl of reaction buffer at 25°C for 20 min. The reaction mixture was incubated with 10 μl of Mouse IgG-agarose to prevent subsequent coprecipitation of non-tagged proteins. The supernatant of the mixture was incubated with 2 μl of 6xHIS mAb at 4°C for 1 h and the protein-antibody complex formed was precipitated with 10 μl of Protein G-agarose at 4°C for 1 h. The precipitates were washed with the dilution buffer. The proteins in the precipitate were resolved by SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were immunoblotted with monoclonal antibodies to UvrA, UvrB, UvrC, and UvrD. **B.** Immunoprecipitation of UvrD in the presence of UV-damaged DNA (300 ng).

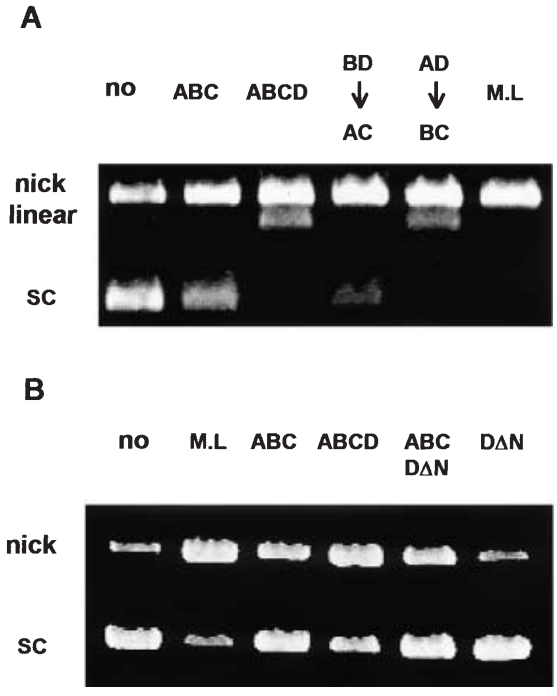

Fig. 3. Effect of the interaction of UvrD and UvrB on incision. **A.** The effect of UvrD on incision. UV-damaged supercoiled DNA (200 ng) was incubated with UvrABC (lane ABC) or UvrABCD (lane ABCD). UvrD was incubated with UvrB (lane BD) or UvrA (lane AD) before addition of other proteins and UV-damaged DNA. The reactions were analyzed on an agarose gel. M.L: *Micrococcus luteus* UV-endonuclease. **B.** The effect of UvrDΔN101 on incision. UV-damaged DNA (500 ng) was incubated and then a half of the reaction was loaded onto an agarose gel. Lane DΔN: only UvrDΔN101 was incubated with DNA.

detected (Fig. 3A, lane ABCD). This increased extent of incision and the appearance of a linear form of DNA are due to the turnover of UvrC by UvrD, in agreement with earlier reports (Caron *et al*., 1985; Husain *et al*., 1985; Orren *et al*., 1992). However, when UvrD was incubated with UvrB prior to the addition of UvrA, UvrC, and UV-damaged DNA, the linear form of DNA was not detected (Fig. 3A, lane BD $\rightarrow$ AC), whereas the pre-incubation of UvrA and UvrD produced the linear form of DNA (Fig. 3A, lane AD $\rightarrow$ BC). These results suggest that the interaction of UvrD with UvrB prior to formation of the UvrAB complex and the incision complex could affect NER incision due to the competitive interaction of UvrB with UvrA and UvrD. Thus, in order for the interaction of UvrD and UvrB to be functional in the NER process, the interaction should occur at the excision step.

Since it has been reported that the N- and C-termini of UvrD are essential for the interaction with MutL (Hall *et al*., 1998), we constructed a mutant lacking the N-terminal 101 amino acid residues that failed to complement the loss of UvrD in excision repair (data not shown). This mutated gene product (UvrDΔN) was purified through a Ni^{2+}-HIS bind column (data not shown) and the purified protein was added to the UvrABC incision assay (Fig. 3B). As shown in the figure, the increased incision was not detected (lane ABCDΔN) when compared to lane ABCD, indicating that the N-terminus of UvrD would be required for its activity or turnover of the incision complex. We are currently exploring the role of the N-terminus of UvrD in excision repair, the investigation of the critical residues for the interaction of UvrB and UvrD, and the interaction of UvrD with UvrB in the incision complex using a DNA substrate containing a single adduct.

The results presented here propose that UvrB in the incision complex seems to recruit UvrD to the 3′ incised site of the incised strand by protein-protein interaction and to allow initiation of unwinding by UvrD from the resulting nick in a 3′ to 5′ direction. Therefore, this directional unwinding turns over UvrC and excises the incised fragment, but UvrB remains on the opposite strand.

Although *E. coli* contains at least 11 DNA helicases, the specific interactions of some helicases with other proteins have been reported in biological processes. For example, DnaB replication helicase interacts with DnaC in replication initiation (Wahle *et al*., 1989) and with τ subunit of *E. coli* replicative polymerase III in elongation (Kim *et al*., 1996). UvrD is the only helicase for nucleotide excision repair and methyl-directed mismatch repair processes. This UvrD interacts with MutL, a component of the methyl-directed mismatch repair pathway (Hall *et al*., 1998). Thus, this kind of specific protein-protein interaction provides the specificity of the helicase capable of functioning in biological processes. Furthermore, the discovery of a physical interaction between UvrD and UvrB may lead to advances in our understanding of nucleotide excision repair in eukaryotic systems where the TFIIH complex is composed of two kinds of helicases similar to UvrAB and UvrD, respectively (Drapkin *et al*., 1994; Wang *et al*., 1994).

Acknowledgments We thank Dr. L. Grossman (The Johns Hopkins University, USA) for the antibodies for UvrA, UvrB, and UvrC. We also thank for Dr. S. W. Matson (University of North Carolina, Chapel Hill) for kindly providing strains. This work was supported by GE (97-019-D0024) grant and KRF (97-002-D0024) to B. Ahn.

References

Ahn, B. and Grossman, L. (1996) The binding of UvrAB proteins to bubble and loop regions in duplex DNA. *J. Biol. Chem.* **271**, 21462–21470.

Ali, J. A., Maluf, N. K., and Lohman, T. M. (1999) An oligomeric form of *E. coli* UvrD is required for optimal helicase activity. *J. Mol. Biol.* **293**, 815–834.

Caron, P. R., Kushner, S. R., and Grossman, L. (1985) Involvement of helicase II (uvrD gene product) and DNA polymerase I in excision mediated by the uvrABC protein complex. *Proc. Natl. Acad. Sci. USA* **82**, 4925–4929.

Drapkin, R., Reardon, J. T., Ansari, A., Huang, J. C., Zawel, L., Ahn, K., Sancar, A., and Reinberg, D. (1994) Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. *Nature* **368**, 769–772.

George, J. W., Brosh, R. M., Jr., and Matson, S. W. (1994) A dominant negative allele of the *Escherichia coli uvrD* gene encoding DNA helicase II. A biochemical and genetic characterization. *J. Mol. Biol.* **235**, 424–435.

Grossman, L., Lin, C., and Ahn, B. (1998) DNA Damage and Repair, Vol. I, pp. 11–27, Humana Press, Totowa, NJ, USA.

Hall, M. C., Jordan, J. R., and Matson, S. W. (1998) Evidence for a physical interaction between the *Escherichia coli* methyl-directed mismatch repair proteins MutL and UvrD. *EMBO J.* **17**, 1535–1541.

Husain, I., Van Houten, B., Thomas, D. C., Abdel-Monem, M., and Sancar, A. (1985) Effect of DNA polymerase I and DNA helicase II on the turnover rate of UvrABC excision nuclease. *Proc. Natl. Acad. Sci. USA* **82**, 6774–6778.

Kim, S., Dallmann, H. G., McHenry, C. S., and Marians, K. J. (1996) Cocupling of a replicative polymerase and helicase: a tau-DnaB interaction mediates rapid replication fork movement. *Cell* **84**, 643–650.

Kovalsky, O. I. and Grossman, L. (1994) The use of monoclonal antibodies for studying intermediates in DNA repair by the *Escherichia coli* Uvr(A)BC endonuclease. *J. Biol. Chem.* **269**, 27421–27426.

Lin, J. J. and Sancar, A. (1992) Active site of (A)BC excinuclease. I. Evidence for 5′ incision by UvrC through a catalytic site involving Asp399, Asp438, Asp466, and His538 residues. *J. Biol. Chem.* **267**, 17688–17692.

Matson, S. W. (1986) *Escherichia coli* helicase II (*uvrD* gene product) translocates unidirectionally in a 3′ to 5′ direction. *J. Biol. Chem.* **261**, 10169–10175.

Matson, S. W. (1989) *Escherichia coli* DNA helicase II (*uvrD* gene product) catalyzes the unwinding of DNA RNA hybrids *in vitro*. *Proc. Natl. Acad. Sci. USA* **86**, 4430–4434.

Oh, E. Y. and Grossman, L. (1989) Characterization of the helicase activity of the *Escherichia coli* UvrAB protein complex. *J. Biol. Chem.* **264**, 1336–1343.

Orren, D. K. and Sancar, A. (1989) The (A)BC excinuclease of *Escherichia coli* has only the UvrB and UvrC subunits in the incision complex. *Proc. Natl. Acad. Sci. USA* **86**, 5237–5241.

Orren, D. K. and Sancar, A. (1990) Formation and enzymatic properties of the UvrB DNA complex. *J. Biol. Chem.* **265**, 15796–15803.

Orren, D. K., Selby, C. P., Hearst, J. E., and Sancar, A. (1992) Post-incision steps of nucleotide excision repair in *Escherichia coli*. Disassembly of the UvrBC-DNA complex by helicase II and DNA polymerase I. *J. Biol. Chem.* **267**, 780–788.

Runyon, G. T., Bear, D. G., and Lohman, T. M. (1990) *Escherichia coli* helicase II (UvrD) protein initiates DNA unwinding at nicks and blunt ends. *Proc. Natl. Acad. Sci. USA* **87**, 6383–6387.

Sancar, A. (1996) DNA excision. *Annu. Rev. Biochem.* **65**, 43–81.

Thiagalingam, S. and Grossman, L. (1991) Both ATPase sites of *Escherichia coli* UvrA have functional roles in nucleotide excision repair. *J. Biol. Chem.* **266**, 11395–11403.

Visse, R., King, A., Moolenaar, G. F., Goosen, N., and van de Putte, P. (1994) Protein-DNA interactions and alterations in the DNA structure upon UvrB- DNA preincision complex formation during nucleotide excision repair in *Escherichia coli*. *Biochemistry* **33**, 9881–9888.

Wahle, E., Lasken, R. S., and Kornberg, A. (1989) The dnaB-dnaC replication protein complex of *Escherichia coli*. I. Formation and properties. *J. Biol. Chem.* **264**, 2463–2468.

Wang, Z., Svejstrup, J. Q., Feaver, W. J., Wu, X., Kornberg, R. D., and Friedberg, E. C. (1994) Transcription factor b (TFIIH) is required during nucleotide-excision repair in yeast. *Nature* **368**, 74–76.

Yeung, A. T., Mattes, W. B., and Grossman, L. (1986) Protein complexes formed during the incision reaction catalyzed by the *Escherichia coli* UvrABC endonuclease. *Nucleic Acids Res.* **14**, 2567–2582.

Zou, Y., Liu, T. M., Geacintov, N. E., and Van Houten, B. (1995) Interaction of the UvrABC nuclease system with a DNA duplex containing a single stereoisomer of dG-(+)- or dG-(−)-anti-BPDE. *Biochemistry* **34**, 13582–13593.