The Binding of UvrAB Proteins to Bubble and Loop Regions in Duplex DNA*

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Six proteins, UvrA (104 kDa), UvrB (76 kDa), UvrC (66 kDa), UvrD (70 kDa), polymerase I (103 kDa), and ligase (70 kDa), are necessary for DNA nucleotide excision repair in Escherichia coli (1, 2). The UvrABC endonuclease acts on diverse types of dissimilar DNA damages ranging from bulky lesions such as TT cyclobutane dimers, 2-aminofluorene-DNA adducts, aflatoxin B1 adducts (3), and psoralen adducts as well as non-bulky lesions such as thymine glycols and AP sites (4). The UvrABC endonuclease complex catalyzes the hydrolysis of the eighth phosphodiester bond 5' to the lesion (5, 6).

In the presence of ATP, UvrA binds to both damaged DNAs with a binding affinity (Kd) of 10^9 to 10^10 M^-1 and to undamaged DNAs with a Kd of 10^8 to 10^9 M^-1 (7, 8). ATP is not required, however, for specific binding of the UvrA to damaged sites. For example, ATPγS completely inhibits specific binding of UvrA to damaged sites, whereas nonspecific binding is enhanced. In the presence of ATP there is a little unwinding (ΔL = 0.5) of a supercoiled DNA, whereas there is about 100° or about 3 bp of unwinding per (UvrA) when ATP is substituted for ATPγS (9).

When damaged DNAs are included in an unwinding reaction, the UvrA protein induces enhanced unwinding of the DNA helix. A UvrA mutant with cysteine substitution for phenylalanine at 740 failed to bind to dsDNA and ssDNA (10), suggesting that the C-terminal zinc finger motif of UvrA possesses a DNA binding activity. From velocity sedimentation experiments, it was suggested that UvrA and UvrB subunits interact to form a complex in solution in a UvrA-to-UvrB stoichiometry of 2:1 (11). The ATPase resulting from interaction of UvrA and UvrB had a lower Vmax and Km of UvrA when compared to UvrA by itself (12). Several experimental facts suggest that UvrA and UvrB proteins form nucleoprotein complexes with undamaged DNA. In DNA unwinding experiments, the UvrAB complex induced a 150° to 220° unwinding of undamaged DNA per UvrA dimer in the presence of ATPγS, nearly twice that seen for UvrA alone (9). The UvrA protein in the presence of increasing amounts of UvrB induces a gradual increase in the unwinding of damaged DNA. The cryptic UvrB ATPase became activated by UvrA in the presence of dsDNA, ssDNA, or UV dsDNA (13, 14). Associated with the DNA unwinding activity and the ssDNA-stimulated ATPase activity of the UvrAB complex is a unique helicase-like activity (15). The UvrAB helicase acts unidirectionally (5' to 3') on short duplexes and D-looped DNA. This helicase activity requires nucleoside triphosphate-hydrolysis of either ATP or dATP (16). It also shows a strong preference for unwinding duplex DNA possessing a 5' ssDNA flanking region (17). The strand displacement activity of the UvrAB complex is inhibited by UV photoproducts. Like other DNA helix-tracking processes, the ATP-dependent action of the UvrAB complex simultaneously generates both transient positive and negative supercoils in relaxed covalently closed circular DNA templates (18).

Slow dissociation of the UvrA from single-stranded DNA and a marginal discrimination factor of UvrA between damaged and undamaged DNA molecules may contribute to the processivity of the helicase activity of the UvrAB complex which needs to translocate along the strand to which the complex binds (7, 19). In this model, the UvrAB complex binds initially to undamaged sites followed by unidirectional translocation along the DNA. This raises the question whether there are preferred binding sites in undamaged DNA.

Because of the effects on the RNAP-induced DNA structure around the transcription start site on DNA repair by UvrABC endonuclease, bubble and loop regions in duplex DNA were examined for binding sites of the UvrAB complex independent of RNAP. Here we report that the UvrAB complex binds to bubble and loop regions with an affinity similar to that for damaged DNA, and that preferential incision was observed in the strand complementary to the strand along which the UvrAB complex translocates.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from Life Technologies, Inc. or New England Biolabs and used in buffers suggested by the
supplied. T4 DNA ligase was obtained from New England Biolabs. T4 polynucleotide kinase was from Life Technologies, Inc. Sequenase version 2.0 and plasmid pT218U were purchased from U. S. Biochemical Corp. The Escherichia coli cDNA synthesis (ECL) kit was purchased from Amersham Corp. P1 nuclease, bacteriophage M13KO7 and plasmid pT218U were from Pharmacia Biotech Inc. E. coli strain DH5α (F−, end A1, his R7(k−, m−), sup E44, thi−1, lac−, rec A1, gyr A96, rel A1, Δ[argF− lac yaa])U169, Δ[80 lac ZM15]) was obtained from Life Technologies, Inc. The E. coli CJ 236 (dut 1, ung 1, thi 1, rel A1) (P15F・Cm−) used to generate ura−containing phage was from Dr. J. D. Roberts, National Institute of Environmental Health Sciences, Research Triangle Park, NC.

UvrA, UvrB, and UvrC Proteins—UvrA, UvrB, and UvrC proteins were purified as described previously (20).

DNA and Nucleotides—Oligonucleotides were synthesized using a MilliGen/Bioscribe 7500 automated synthesizer supplied by Scott D. Muddiman, National Institute of Environmental Health Sciences, Research Triangle Park, NC. The CJ 236 strain of E. coli, transformed with pT218U was used to generate ura− containing ssDNA (21). Cells from a minimal plate were grown at 37°C until 595 of 0.4 in 2YT medium supplemented with 0.25 mM thioglycollate. The standard method for phage preparation (21) was used.

Mutagenesis—Mutagenesis was followed as described by Kunkel et al. (40). Two primers, the ‘Bubble’ primer (5′-CGGCTCAGGAAGGATCTTGRGAACATACGAGC-3′) and the ‘Loop’ primer (5′-CGGCTCACAAAGGGAGATCTTGRGAACATACGAGC-3′) were designed (22) as canons in a plasmid containing the changed sequence was selected, and then circular ssDNA was prepared by M13KO7 phage infection. The isolated single-stranded DNA oligomers were annealed in light and annealed with complementary circular ssDNA molecules (pT218R). A DNA fragment (187 bp) containing the bubble or loop region was obtained by cutting with restriction enzymes (Asel and Acc) and labeled at the 3′ end by a filling-in reaction. The labeled fragment was further separated on a 5% polyacrylamide gel (10 × 8 × 0.1 cm) and electroeluted from the gel.

UV Irradiation—DNA molecules were placed on parafilm floating on ice water and irradiated by ultraviolet (UV) light from a germicidal lamp at 130 J/m². Single-stranded DNA molecules were irradiated to 400 J/m² - 1.5 kJ/m².

Nucleoprotein Complex Formation—The standard binding buffer contains 30 mM Heps-HCl (pH 7.6), 85 mM KCl, 10 mM MgCl2, 1 mM Na2EDTA, and 0.05% BSA. The buffer for is the binding buffer without DTT and BSA. The UvrA protein (various concentrations) was added to labeled DNAs in the binding buffer. After 10−15 min of incubation, unlabelled and unirradiated CTD was added to a final concentration of 100 μM (bp) for a 10−s challenge immediately prior to filtration or loading on 4.5% polyacrylamide gels (10 × 8 × 0.1 cm). In the brief challenge, only the rapidly dissociating complexes and free proteins are removed (7).

Nitrocellulose Filter Binding—Nitrocellulose membrane filters were soaked in the binding buffer prior to use. For experiments with DNA containing a bubble region, the filters were soaked in 0.4 M KOH for 20 min, then rinsed extensively with distilled water, and stored at 4°C in the filter binding buffer until used (7). The sample (20 μl) was filtered at a rate of 100 μl/s and the filters washed with 300 μl of the filter binding buffer. The filters were dried for 10 min under a heat lamp, and the radioactivities determined by scintillation counting.

Gel Mobility Shift Assay—Nucleoprotein complexes were resolved in 4.5-5% polyacrylamide gels (10 × 8 × 0.1 cm)/TBE (89 mM Tris, 89 mM borate, and 1 mM EDTA) at 70 volts and 15-20 mA at room temperature. For the detection of the UvrA−DNA complex, the gel and running buffer containing 10 mM MgCl2 and 1 mM ATP was pre-equilibrated for 60 min.

Binding Experiments—Apparent dissociation constants for UvrA or UvrB binding to DNA substrate can be determined by measuring the extent of complex formation as a function of protein concentration. The dissociation constant (Kd) was determined at 50% formation of nucleoprotein complexes. The rates of association of UvrA or UvrB to DNA substrates were measured by adding an aliquot of UvrA or UvrB to a solution of 32P-labeled DNA fragments in the binding buffer with ATP on ice. After a rapid mixing by a quick spin at 4°C, the reaction mixtures were incubated at 37°C. The reaction samples were challenged at varying times by the addition of 100 μM (bp) CTD for 10 s before being filtered through nitrocellulose filters or loading into polyacrylamide gels. The challenge removes UvrA nonspecifically bound to DNA. The rate of complex formation is given by the following equations (38)

\[
k_{d+} = \frac{1}{P_0} \ln \frac{P_0 - P_D}{P_0 - P_{D_0}}
\]

where t0.5 denotes time at which complex formation is 50% and P0 denotes the initial concentration UvrA and GC-bubble DNA substrates.

The rate of dissociation of UvrA−DNA or UvrB−DNA complexes was measured by the addition of CTD. UvrA or UvrB plus UvrA were incubated with 32P-labeled GC-bubble oligomers in the binding buffer at 37°C for 15 min. The dissociation was initiated by adding CTD at 37°C to a final concentration of 100 μM (bp). The amount of labeled DNA bound as a function time was determined by the nitrocellulose filter binding assay. Dissociation kinetic data were analyzed by the following equations (38)

\[
k_{d-} = \ln \frac{P_D}{P_{D_0}}
\]

where P0 represents the extent of protein-DNA complex at zero times and PD denotes the extent of remaining protein-DNA complex at various times.

Western Blotting—Proteins were incubated with GC-bubble oligomers at 37°C for 15 min, and protein-DNA complexes were resolved on a 4.5% polyacrylamide gel. After electrophoresis, the gel was soaked in 1 x chamber buffer (25 mM Tris, 192 mM glycerine, pH 8.3) for 30 min to equilibrate. The proteins in the gel were transferred onto a nitrocellulose membrane using the Hoefer protocol. A monoclonal antibody against UvrA and UvrB (22) were used in this blotting. Protein bands were detected by the ECL kit.

KmNO4 Reactions on DNA Substrates Containing a Bubble or Loop Region—End-labeled DNA substrates containing bubble or loop regions were incubated with UvrA and UvrB proteins in the presence of 45 ng of unlabeled plasmid DNA in the binding buffer for 10 min at 37°C. After incubation, the reactions were mixed with 1 μl of 100 mM KmNO4 and incubated for 2 min at 37°C. The reactions were terminated by the addition of 2 μl of 8 M (β-mercaptoethanol and placed on ice, followed by the addition of 23 μl of stop solution (4 x NH4OAC and 35 mM EDTA) (23). After a phenol/chloroform extraction, each sample was precipitated with six volumes of 100% ethanol and 20 μg of glycogen and then treated with 1 x-piperidine as described by Maxam and Gilbert (25). Sequencing reactions were performed as described by Maxam and Gilbert with the end-labeled DNAs. After drying in vacuo, all samples were resuspended in formamide loading buffer (85% formamide, 1 x TBE, 0.01% xylene cyanol, and 0.01% bromphenyl blue) and electrophoresed through a 5% sequencing gel (40 x 38 x 0.04 cm) in 1 x TBE. The gel was dried and exposed to Kodak XAR-5 film using a DuPont Lightning Plus intensifying screen or to the Phosphor imaging plate. The extent of KmNO4 reactivity was quantified by use of a Fuji BioPhosphorImager.

P1 Nuclease Reaction—The reaction mixture, as prepared in the KmNO4 reaction, was incubated with 1 μl of P1 nuclease (1 units/μl) for 30 s at 37°C. The reaction was terminated according to the procedure in the KmNO4 reaction. Precipitated DNAs were resuspended in the...
formamide loading buffer and electrophoresed through a 5% sequencing gel.

Incision by UvrABC Endonuclease or Micrococcus luteus UV-endonuclease (5, 26)—Each reaction mixture was conducted in a volume of 10 μl. The final reaction compositions were 30 mM Tris, 85 mM KCl, 10 mM MgCl₂, 1 mM DTT, 100 μg/ml BSA, and 2 mM ATP. After incubating the DNA substrates with 7.5 nM UvrA and 34 nM UvrB protein at 37 °C for 10 min, incision was initiated by the addition of 30 nM UvrC protein and incubated for 5 min. The reactions were terminated by the addition of 5 μl of the formamide loading buffer and heated immediately at 90 °C for 3 min. The samples were loaded onto a 5–6% sequencing gel. The M. luteus UV-endonuclease reaction was carried out using the same conditions as that in the UvrABC incision reaction. The extent of incision was quantified by use of a Fuji PhosphorImager.

RESULTS

UvrA Binding to GC-bubble Oligomer—To investigate the binding of UvrA to undamaged DNAs, a synthetic DNA oligomer containing a 12-base pair DNA mismatch and oligomers with various G+C levels (Fig. 1A) were used in a gel mobility shift assay. Fig. 1B shows the gel mobility shift assay monitoring UvrA binding to the DNA substrates in the presence of ATP. A stable nucleoprotein complex was formed only with GC-bubble oligomers, having been challenged with a high concentration of unlabeled DNA prior to being loaded on a 4.5% native polyacrylamide gel. UvrA seems to bind to this substrate as a dimer, as already described (7). Duplex DNA oligomers (GC and AT) and the fragment (GN) of a similar length showed less than 5% of complex formation over the same ranges of UvrA concentration. In Fig. 1C, the binding of UvrA to the GC-bubble oligomer is presented as a function of UvrA concentration. The equilibrium dissociation constant (Kd) for the UvrA is 5 × 10⁻⁹ M. The binding constant for the GC-bubble oligomer can be compared to values for damaged sites (Table I): 1 × 10⁸ M⁻¹ for the psoralen adduct thymine-4'-hydroxymethyl-4,5,8-trimethyl (8); 4.2 × 10⁸ M⁻¹ for the trans,syn-dimer (27); 2 × 10⁶ M⁻¹ for UV-damaged DNA (7). The affinity of UvrA binding to the GC-bubble oligomer is similar to that for damaged DNA.

The Formation of UvrAB-GC-bubble Oligomer Complex—The stimulation of the ATPase of the UvrAB complex in the presence of dsDNA or ssDNA suggests that the UvrAB complex is able to bind productively to these DNA molecules (12). To enable demonstration of the potential helicase activity, the UvrAB complex needs to bind to single-stranded regions in a helicase substrate. However, no UvrAB-DNA (undamaged) complex has been detected directly because the UvrA dissociates very quickly from undamaged DNA.

Fig. 2A shows a gel mobility shift assay monitoring the formation of the UvrAB-DNA complex with the GC-bubble oligomer. To stabilize nucleoprotein complexes during electrophoresis, 1 mM ATP and 10 mM MgCl₂ were included in a gel and buffer. A constant amount of UvrB was incubated with various G+C levels (Fig. 1A) were used in a gel mobility shift assay. Indicated amounts of UvrA (1, 5, 10, 25, and 50 nM) were incubated at 37°C for 15 min with terminally labeled oligomers (1.7 nM) in 10 μl reaction mixtures. Samples were loaded on to a 5–10% (w/v) vertical polyacrylamide gel, and run it at 75 V for 50 min. The gel was then autoradiographed. Lanes 1–5 show DNA binding with the GN substrate (panel A), and lanes 6–10 with GC-bubble. Lanes 1 and 6, 1 nM; lanes 2 and 7, 5 nM UvrA; lanes 3 and 8, 10 nM UvrA; lanes 4 and 9, 25 nM UvrA; lanes 5 and 10, 50 nM UvrA. F., free DNA; B, UvrA-DNA complex. C, quantitative analysis of UvrA binding. The extent of UvrA-DNA complex formation from gel mobility shifts was quantitated using the PhosphorImager and are presented as a function of concentrations.

FIG. 1. Binding of UvrA to DNA. A, sequences of DNA substrates for the binding of UvrA or UvrAB. AT, 76.7% A+T sequence from the AT rich sequence of the λ ori region; GN, 55% A+T sequence from pT218R plasmid by cutting with restriction enzymes; GC, 65-bp oligomer, and 60% of G+C sequence; GC-bubble, 63-bp oligomer containing 12-base pair DNA mismatch in the middle of DNA fragment. B, gel mobility shift assay. Indicated amounts of UvrA (1, 5, 10, 25, and 50 nM) were incubated at 37 °C for 15 min with terminally labeled oligomers (1.7 nM) in 10 μl reaction mixtures. Samples were loaded on to a 4.5–5% (w/v) vertical polyacrylamide gel, and run it at 75 V for 50 min. The gel was then autoradiographed. Lanes 1–5 show DNA binding with the GN substrate (panel A), and lanes 6–10 with GC-bubble. Lanes 1 and 6, 1 nM; lanes 2 and 7, 5 nM UvrA; lanes 3 and 8, 10 nM UvrA; lanes 4 and 9, 25 nM UvrA; lanes 5 and 10, 50 nM UvrA. F., free DNA; B, UvrA-DNA complex. C, quantitative analysis of UvrA binding. The extent of UvrA-DNA complex formation from gel mobility shifts was quantitated using the PhosphorImager and are presented as a function of concentrations.
in lane 3 even when challenged by CTD. This band is in the same position as that of the DNA shift band in Fig. 2A (lanes 1–4), indicating that the protein band in the Western blot is associated with the GC-bubble oligomer. In the presence of UvrB protein, a new shifted band was observed but the UvrA-DNA band disappeared (Fig. 2B, lanes 4 and 5). The position of the new shifted band matched to that in Fig. 2A (lanes 5 to 8).

UvrB alone is not able to bind to any type of DNA (28). In lane 7, free UvrB was detected but no UvrB-CTD was detected. When the UvrB was incubated with UvrA and the GC-bubble oligomer, a shifted band was detected (Fig. 2B, lanes 8 and 9), which in the same position as that in lane 7, free UvrB was detected but no UvrB-CTD was detected.

When the UvrB was incubated with UvrA and the GC-bubble oligomer, a shifted band was detected (Fig. 2B, lanes 8 and 9), which in the same position as that in lane 7, free UvrB was detected but no UvrB-CTD was detected. Therefore, the shifted DNA contains both the UvrA and UvrB protein, indicating that the UvrAB complex is able to form a stable nucleoprotein complex with the GC-bubble oligomer. This detection of the UvrAB-DNA (undamaged) complex is the first directly monitoring the intermediate of the preincision complex.

Comparison of the Gel Mobility Shift and the Nitrocellulose Filter Binding Assays—From the DNA binding experiments involving UvrA with the GC-bubble oligomer, a filter efficiency of 0.7 was obtained. In detecting the UvrAB-DNA complex, a 30% formation of UvrAB-DNA complexes was measured from the gel mobility shift assay (Fig. 2C) and a 75% complex obtained from the filter binding (Fig. 2D). The low level of forma-
tion in the gel may be due to the lack of stability of the UvrAB-DNA complex during electrophoresis.

Equilibrium Properties of UvrAB-DNA Complexes—The binding of UvrAB to the GC-bubble oligomer was quantitated by nitrocellulose filter binding. The filter binding was carried out using varying UvrA concentrations ranging from 0 to 80 nM in the presence of a 2-fold molar excess of UvrB (Fig. 2D). The apparent equilibrium dissociation constant ($K_d$) was determined as the amount of UvrA protein existing as a monomer that resulted in 50% of the protein being bound to the DNA. The apparent equilibrium dissociation constant ($K_d = 5 \times 10^{-9}$ M) for UvrAB to the GC-bubble oligomer is remarkably similar to that of UvrA (Table I). In addition, this constant is similar to that measured with UvrA for AP-DNA and UvrAB for BA-DNA (29).

Association Kinetics—The rate of association of UvrA with the GC-bubble oligomer was determined by measuring the extent of formation of UvrA-DNA complex at various times. A value for the association rate constant was then determined (see "Experimental Procedures"). The experimentally determined association rate constant for UvrA binding to the GC-bubble oligomer was $1.1 \times 10^9$ M$^{-1}$ s$^{-1}$ (Table I), which is greater than that estimated for single-stranded DNA (7). The association rate constant for UvrAB-DNA complex formation was also obtained from filter binding experiments. The experimentally determined association rate constant for the UvrAB complex binding to the GC-bubble oligomer was $1.4 \times 10^9$ M$^{-1}$ s$^{-1}$ (Table I), which is similar to that for UvrA alone.

Dissociation Kinetics—The time course for the dissociation process of UvrA from the GC-bubble oligomer revealed a half-life of 80 min for the UvrA-DNA complex, corresponding to a dissociation rate of $k_d = 1.4 \times 10^{-9}$ s$^{-1}$. This rate constant is less than that (3.7 × 10$^{-3}$ s$^{-1}$) of UV-damaged sites (7) and other photoproducts (trans, syn-dimer; $1.1 \times 10^{-3}$ s$^{-1}$; Dewar photoproduct; $2.1 \times 10^{-3}$ s$^{-1}$) (27) (Table I). The experimentally determined dissociation rate constant ($k_d$) of the UvrAB complex is $1.5 \times 10^{-3}$ s$^{-1}$, corresponds to a half-life of 7.5 min. The UvrAB complex remained on the GC-bubble oligomer for less time than UvrA but longer than UvrA on damaged sites (7) (Table I). The results show that the presence of UvrB affects the complex stability or the dissociation rate, which may be due to the translocation of the UvrAB complex.

The Construction of DNA Substrates Containing a Bubble or Loop Regions—The binding of UvrAB complex to the GC-bubble oligomer implies that a single-stranded region in duplex DNA may provide a preferred binding site for the UvrAB complex. This binding is consistent with the requirement for a single-stranded region for UvrAB helicase activity. Whether a bubble or loop region can serve as a start site for the translocation of the UvrAB complex and for repair by the UvrABC endonuclease is fundamental. Thus, a longer DNA substrate (187 bp) containing a bubble or loop region was constructed as described under "Experimental Procedures."

P1 Digestion and KMnO$_4$ Modification—To confirm the sequence and nature of the single-stranded region in a bubble and loop region, P1 nuclease and KMnO$_4$ were used and the reactive sites were mapped (Fig. 3) P1 nuclease is specific for single-stranded region and digests a phosphodiester bond at pA-N residue (32). As shown in Fig. 3, most of the P1 sites are cleaved. Thymines in single-stranded DNA regions are specifically modified by treatment with KMnO$_4$, resulting in breakage of the phosphodiester bond by piperidine. The results from the treatment of both P1 nuclease and KMnO$_4$ are consistent with the sequences in the bubble and loop region (Fig. 3), suggesting that the DNA fragment has a single-stranded region as expected in the construction of the bubble and loop region.

The Binding of the UvrAB Complex to Bubble and Loop Regions—The direct binding of the UvrAB complex to bubble or loop regions was monitored by P1 and KMnO$_4$ protection. Labelled DNA substrates (187 bp) containing the bubble or loop region were incubated with UvrAB complexes for 10 min at 37 °C and treated with P1 nuclease or KMnO$_4$ as described. The reactivity of P1 was reduced by UvrAB proteins (Fig. 4, A and B, lanes 3 and 8) by 50% at the bubble region in both strands. A similar level of reduction was detected in the loop region, suggesting that the UvrAB complex binds to these regions. In addition, the reactivity of KMnO$_4$ in the bubble and loop region was reduced to 50% in the presence of the UvrAB complex (Fig. 4, A and B, lanes 5 and 10). DNase I footprints revealed the unique protection at the bubble region (Fig. 4C), reflecting the binding of the UvrAB complex to a bubble or loop region.

Preferential Binding of the UvrAB Complex to Damaged Sites on DNA Containing Bubbles or Loops—To see the effect of the bubble and loop regions on damage recognition of the UvrAB complex, M. luteus UV-endonuclease protection was performed with the DNA substrates containing a bubble or loop region where the top strand of the DNA substrate was labeled and irradiated by UV light. M. luteus UV-endonuclease is specific for pyrimidine photodimers (26). The binding of the UvrAB complex to pyrimidine photodimers is monitored by its effect on the extent of the M. luteus UV-endonuclease cleavage. These data are shown in Fig. 5. No protection was observed with duplex DNA, even though the UvrAB complex was incubated with the substrate before adding the M. luteus UV-endonuclease. However, the cleavage by M. luteus UV-endonuclease was reduced in the DNA substrate containing a bubble or loop. The protection against the nicking by the M. luteus UV-endonuclease was specifically localized downstream (leftward from bubble or loop region, in Fig. 7, A and B) of bubble or loop in a 3' to 5' direction. These results imply that DNA damage recognition of the UvrAB complex occurs directionally in the DNA substrate containing a bubble or loop and that preferential binding to the bubble or loop region increases UvrAB complex recognition.

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**Fig. 3.** Single-stranded DNA region in DNA substrates containing bubble and loop regions. Purified labeled DNA fragments (187 bp) were treated with KMnO$_4$ or P1 nuclease and analyzed on a 6% sequencing gel. After electrophoresis, the gel was dried and autoradiographed. Summary of reactive sites of P1 nuclease and KMnO$_4$. Positions were determined by Maxam-Gilbert sequencing for G+A.
Incision of UV-damaged Sites by the UvrABC Endonuclease—Incision of UV-damaged DNA substrates by the UvrABC endonuclease is shown in Fig. 6, A and B. Each DNA substrate was incubated with the UvrAB proteins for 10 min at 37 °C, and incision was initiated by the addition of UvrC proteins. The reactions were further incubated for 5 min, and then 5 μl of sequencing loading solutions were added to the reactions, followed by heating at 90 °C for 4 min to stop incision. In Fig. 6A, the top strand labeled at its 3' end was irradiated by UV. Lane 1 shows the damaged sites of duplex DNA cleaved by the M. luteus UV-endonuclease. The differential level of incision may due to the extent of photo-product formation. Lane 2 shows the incision of duplex DNA by the UvrABC endonuclease. In lane 3, the bubble substrate was incubated with UvrA and B and incised with UvrC. The damaged sites downstream (leftward from bubble or loop region in Fig. 7, A and B) of the top strand in a 3' to 5' direction were more efficiently incised (4–5-fold, marked with arrows) when compared to lane 2. The extent of incision was gradually decreased at the 3' end, consistent with the extent of M. luteus UV-endonuclease protection (Fig. 5). Lane 4 shows the level of incision of damaged sites in loop substrate. The extent (2–4-fold) and pattern are almost similar to those in lane 3. It seems that the UvrAB complex recognizes the damaged site on the strand complementary to the strand to which it is bound and along which the UvrAB complex translocates. In other words, the strand along which the UvrAB complex translocates is the 5' to 3' strand and the damaged site recognized and incised by the UvrABC is in the complementary strand. However, this kind of directional incision is not observed in M. luteus UV-endonuclease catalyzed incision. 2 When the bottom strands (Fig. 6B) of the DNA substrates were incised by the UvrABC endonuclease, the pattern and the extent (4–6-fold) of incision were the same as shown in the top strand.

DISCUSSION

The ability of UvrA or the UvrABC complex to bind preferentially to bubble or loop regions in duplex DNA is presented. The fact that preferential and directional incision was observed in a 3' to 5' direction from the bubble or loop regions strongly supports the involvement of translocation of the UvrABC complex in NER. From previous studies (39), it was shown that the RNAP can generate start sites in promoter regions for the initiation of repair. The synthetic loop and bubbles of themselves are sufficient for this recognition. The findings in this study reveal the important aspects of damage recognition by the UvrABC complex: 1) bubble or loop regions can act as start
sites to which UvrAB or UvrA bind with the same affinity as that to damaged sites; 2) the UvrAB complex translocates along 5' to 3' strand which is consistent with the directionality of the UvrAB helicase; and 3) during translocation, damaged sites on the complementary strand are preferentially recognized by the UvrAB complex and incised by UvrABC endonuclease complex.

The binding constants of UvrA were determined by filter binding and gel mobility shift assays. The UvrA proteins bind to GC-bubble oligomers with a $K_d = 5 \times 10^{-9}$ M, indicating that UvrA may also prefer single-stranded regions in duplex DNA. The dissociation of UvrA from the bubble substrate is slower than from damaged DNA, but faster than from ssDNA (7).

The UvrAB complex is able to bind to a GC-bubble oligomer with the same affinity as that of UvrA (Table I). This observation substantiates the requirement of single-stranded regions for helicase activity. With such specificity, the UvrAB complex is able to bind to the single-stranded region in duplex DNA as well as to a damaged site. From DNase I footprinting experiments, it is shown that the UvrAB complex binds to a bubble region very effectively in the presence of GTP (Fig. 4C). From the P1 nuclease and KMnO$_4$ modification data, the UvrAB complex binds to both strands in the bubble or loop region (Fig. 4B). The binding constant (29) of UvrAB to BA-DNA is the same as that for its binding to the GC-bubble oligomer (Table I). The association rate constant of UvrAB to the GC-bubble oligomer is $1.4 \times 10^6$ M$^{-1}$ s$^{-1}$. This constant is the same order of magnitude as UvrA binding to damaged sites. The dissociation rate constant of the UvrAB complex from the GC-bubble oligomer is slower than that of UvrA from damaged sites, but faster than that of UvrA from the GC-bubble oligomer. This difference may be due to the translocation of UvrAB from the bubble region, resulting in running off DNA. However, the dissociation rate constant of UvrAB from damaged sites has not been determined. The rate $(6 \times 10^4$ M$^{-1}$ s$^{-1}$) of loading of UvrB to damaged sites in DNA (33) is most likely dependent on several earlier steps, including 1) unwinding DNA by UvrAB, 2) the recognition of damaged site of the UvrAB complex, and 3) release of UvrB to the damaged site. If there is competition
between unwinding and dissociation at the damaged site, the unwinding efficiency may influence the loading of UvrB. It is possible that the premelted DNA regions provide the UvrAB complex with a preferred binding site and that the formation of a productive complex is facilitated. The binding of the UvrAB complex to the promoter region in open complexes (39) is consistent with its binding to the bubble or loop substrate. Hence, the formation of UvrAB-DNA complexes with the bubble or loop region in undamaged sites may be an obligate intermediate in precission. Since scanning is suggested to explain the formation of the precission complex (19), such bubble or loop regions may provide start sites for scanning of DNA damage. Recently Daube and von Hippel (34) and Aiyar et al. (35) have developed a bubble template as a model system for the study of transcription elongation and initiation.

Because UvrAB helicase cannot displace a long oligomer (16), it has been thought that the unwinding activity may be involved in prepriming the precission step, i.e. localized unwinding DNA at damaged or at start sites. This model has been explained by a diffusional random binding of the UvrAB complex. However, it seems that the UvrAB complex may be able to rewind DNA behind itself during the translocation to maintain DNA integrity. During DNA replication, however, the syntheses of daughter strands needs complete separation of parental duplex DNA with the support of other proteins such as helicase, SSB, polymerase, etc. (36). In the supercolliding experiment of the UvrAB complex (18) and in this study, the coordinated unwinding of the duplex DNA during translocation in repair and transcription is strongly suggested (37).

Three different DNA substrates were used in following incision by the UvrABC endonuclease. The extent of its incision of duplex DNA is much less efficient when compared to the extent of digestion by the M. luteus UV-endonuclease because of the lack of directionality by this single reacting enzyme. With duplex DNA containing a bubble or loop region preferential incision is confined to downstream regions (leftward from bubble or loop region, Fig. 7, A and B) in a 3' to 5' direction. To explain the above incision results, the UvrABC complex has to sense those damaged sites on the strand complementary to strand along which the UvrAB complex translocates. The UvrAB complex indeed recognizes damaged sites on the 3' to 5' strand of DNA substrates containing the bubble or loop region preferentially and unidirectionally (Fig. 6).

The incision efficiency of damaged DNA is variable depending on the species of damage and concentration of UvrA in vitro. The formation of UvrB-DNA (at damaged sites) is observed at limiting concentrations of UvrA (29, 33). At higher concentrations of UvrA, the formation of UvrB-DNA is inhibited. If the UvrAB complex translocates from an undamaged site to a damaged one accompanied by unwinding, the efficiency of incision may not be influenced by UvrA concentration, but rather it can be affected by the binding constant of the UvrAB complex to the undamaged or preferred binding sites. The recognition of the damaged site may depend on the ability of a DNA adduct to block the translocation of the UvrAB complex. For example, although the UvrAB complex binds to the bubble or loop regions with an affinity similar to that of damaged sites, neither incision at the region nor UvrB-DNA complex was observed.

In summary, bubble or loop regions can confer start sites for the translocation of the UvrAB complex in the absence of RNAP. This synthetic region contributes to increased complementary incision by the translocation mechanism of the UvrAB complex. Preferential UvrAB binding and its directional translocation predetermine where incision is to occur.

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REFERENCES

1. Caron, P. R., Kushner, S. R., and Grossman, L. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 4925–4929
2. Hussein, I., van Houten, B., Thomas, D. C., Abdel, M. M., and Sancar, A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 6774–6778
3. Olekowksi, C. A., Mayernek, J. A., Lim, S. E., Groopman, J., Grossman, L., Wigan, G. N., and Yeung, A. T. (1993) J. Biol. Chem. 268, 7990–8002
4. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, ASM Press, Washington, DC
5. Yeung, A. T., Mattes, W. B., Oh, E. Y., and Grossman, L. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 6157–6161
6. Sancar, A., and Rupp, W. D. (1983) Cell 33, 249–250
7. Mazur, S. J., and Grossman, L. (1991) Biochemistry 30, 4422–4433
8. van Houten, B., Camper, H., Sancar, A., and Hearst, J. E. (1987) J. Biol. Chem. 262, 13180–13187
9. Oh, E. Y., and Grossman, L. (1986) Nucleic Acids Res. 14, 8557–8571
10. Wang, J., Mueller, K. L., and Grossman, L. (1994) J. Biol. Chem. 269, 10771–10775
11. Orren, D. K., and Sancar, A. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 5237–5241
12. Oh, E. Y., Claassen, H., Thilagam, S., Mazur, S., and Grossman, L. (1989) Nucleic Acids Res. 17, 4145–4159
13. Caron, P. R., and Grossman, L. (1988) Nucleic Acids Res. 16, 10991–10992
14. Sawy, T. W., and Grossman, L. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 6577–6581
15. Oh, E. Y., and Grossman, L. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 3638–3642
16. Oh, E. Y., and Grossman, L. (1989) J. Biol. Chem. 264, 1336–1343
17. Oh, E. Y. (1987) Mechanism of DNA Repair. Ph.D. thesis, Johns Hopkins University, Baltimore, MD
18. Koo, H. S., van Houten, B., Thomas, D. C., Abdel, M. M., and Sancar, A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 1212–1216
19. Grossman, L., and Thilagam, S. (1993) J. Biol. Chem. 268, 16871–16874
20. Yeung, A. T., Mattes, W. B., Oh, E. Y., Yaakunin, G. H., and Grossman, L. (1986a) Nucleic Acids Res. 14, 8535–8556
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Kovalsky, O. I., and Grossman, L. (1994) J. Biol. Chem. 269, 27421–27426
23. Sasse-Dwight, S., and Gralla, J. D. (1989) J. Biol. Chem. 264, 8074–8081
24. Lee, D. N., and Landick, R. (1992) J. Mol. Biol. 228, 759–777
25. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499–560
26. Grafstrom, R. H., Park, L., and Grossman, L. (1982) J. Biol. Chem. 257, 21469
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27. Reardon, J. T., Nichols, A. F., Keeney, S., Smith, C. A., Taylor, J.-S., Linn, S., and Sancar, A. (1993) J. Biol. Chem. 268, 21301–21308
28. Yeung, A. T., Mattes, W. B., and Grossman, L. (1986b) Nucleic Acids Res. 14, 2567–2582
29. Snowden, A., and Van Houten, B. (1991) J. Mol. Biol. 220, 19–33
30. Hayatsu, H., and Ukita, T. (1967) Biochem. Biophys. Res. Commun. 29, 556–561
31. Rubin, C. M., and Schmid, C. W. (1980) Nucleic Acids Res. 8, 4613–4619
32. Linn, S. M., and Roberts, R. J. (1985) Nucleases, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
33. Orren, D. K., and Sancar, A. (1990) J. Biol. Chem. 265, 15796–15803
34. Daube, S. S., and von Hippel, P. H. (1992) Science 258, 1320–1324
35. Aiyar, S. E., Helmann, J. D., and deHaseth, P. L. (1994) J. Biol. Chem. 269, 13179–13184
36. Kornberg, A., and Baker, T. A. (1992) DNA Replication, New York, W. H. Freeman & Co.
37. Tsao, Y. P., Wu, H. Y., and Liu, L. F. (1989) Cell 56, 111–118
38. Riggs, A. D., Bourgeois, S., and Cohn, M. (1970) J. Mol. Biol. 53, 401–407
39. Ahn, B., and Grossman, L. (1996) J. Biol. Chem. 271, 21453–21461
40. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
