Comparative Analysis of Binding of Human Damaged DNA-binding Protein (XPE) and *Escherichia coli* Damage Recognition Protein (UvrA) to the Major Ultraviolet Photoproducts: T[c,s]T, T[t,s]T, T[6-4]T, and T[Dewar]T*

(Received for publication, February 17, 1993, and in revised form, May 24, 1993)

Joyce T. Reardon‡, Anne F. Nichols‡, Scott Keeney§, Colin A. Smith, John-Stephen Taylor\*, Stuart Linn$, and Aziz Sancar‡

From the ‡Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599; the §Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720; and the ¶Department of Chemistry, Washington University, St. Louis, Missouri 63130

Human cells contain a protein that binds to UV-irradiated DNA with high affinity. This protein, the damaged DNA-binding protein (DDB), is absent from some *Escherichia coli* mutants; therefore, it has been suggested that it may be the damage recognition subunit of a human excision nuclease complex. However, the identity of the UV photoproduct bound by DDB and the role of this protein in nucleotide excision repair have been controversial. In this study, we used several synthetic DNA substrates, each of which contains one of the major UV photoproducts, and DDB purified to apparent homogeneity to quantify the specific binding of DDB to various photoproducts. For comparison, the binding of the same photoproducts by the *Escherichia coli* damage recognition protein UvrA, which is known to be a subunit of the *E. coli* excision nuclease, was also measured. UvrA and DDB each bound with high affinity to T[c,s]T, T[t,s]T, and T[Dewar]T, but only marginally discriminated between an undamaged oligomer and an oligomer with a T[c,s]T. In contrast to these similarities with regard to the binding to UV photoproducts, UvrA bound to another excision repair substrate, the psoralen-thymine monooadduct, with high specificity, whereas DDB was unable to distinguish between psoralen-adducted DNA and undamaged DNA. We conclude that DDB may play a special role in the repair of UV damage, but it cannot be the sole damage recognition subunit of human excision nuclease.

Recently, a number of mammalian proteins that bind with high affinity to damaged DNA have been described (Chu and Chang, 1988; Toney et al., 1988; Hirschfeld et al., 1990; Hasegawa et al., 1991; Pf and Lippard, 1992). Of these, the damaged DNA-binding protein (DDB) originally described by Chu and Chang (1988) is of special interest because it was reported to be lacking in xeroderma pigmentosum complementation group E (XP-E) cells (and named XPE-binding factor), raising the possibility that it may be the damage recognition subunit of a human excision nuclease complex. Furthermore, a monkey protein with similar properties was found to be inducible by UV light or mitomycin C, providing additional evidence that this protein might be a major factor in DNA repair (Hirschfeld et al., 1990).

More recent studies, however, have complicated the picture. The protein that was first thought to be specific for cyclobutane pyrimidine dimers (Pyr[c,s]Pyr) in UV-irradiated DNA (Chu and Chang, 1988), and perhaps a mammalian homolog of photolyase that had lost its cofactor during evolution (Patterson and Chu, 1989), was subsequently reported to not bind to Pyr[c,s]Pyr, but more likely to a (6-4) photoproduct (Pyr[6-4]Pyr) in UV-irradiated DNA (Hirschfeld et al., 1990; Treiber et al., 1992). In addition, when cell strains from 12 XP-E patients were tested, only three were missing DDB activity (Katoaka and Fujiwara, 1991; Keeney et al., 1992), raising the possibility that there may not be a causal relationship between the lack of this protein and the xeroderma pigmentosum syndrome.

Finally, the purification and characterization of DDB from human cells (Keeney et al., 1993) and its homolog from monkey cells (Abramic et al., 1991) revealed that DDB may be identical to the "damage-specific DNA-binding protein" first identified by Feldberg and Grossman (1976) in human placenta and later purified and extensively characterized by Feldberg and colleagues (Feldberg, 1980; Feldberg et al., 1982; Carew and Feldberg, 1985). These authors reported that the protein had broad specificity, binding to DNA damaged by UV light, ionizing radiation, NaHSO$_3$, OsO$_4$, and an enzymatic superoxide-generating system. Since xeroderma pigmentosum cells are not unusually sensitive to most of these agents, these findings also put into question the relation of the protein to XP-E and generalized excision repair.

To help to understand the role of DDB in recognition and repair of UV damage, we have investigated the binding of DDB purified to apparent homogeneity (Keeney et al., 1993) to synthetic DNA substrates containing only one of the major UV photoproducts (T[c,s]T, T[t,s]T, T[6-4]T, and its Dewar isomer, T[Dewar]T) at a single location. For comparison, we also analyzed the binding to these substrates of the *Escherichia coli* UvrA protein, which is known to be the damage recognition subunit of the *E. coli* excision nuclease. We find that DDB and UvrA bind to T[6-4]T, T[Dewar]T, and T[c,s]T with high affinity ($K_D = 10^{-10}$ to $10^{-11}$ M), but to T[t,s]T with affinity only marginally above that for undamaged DNA. In contrast to similarities in substrate preference regarding UV photoproducts, UvrA and

21301
DDB behave very differently with another substrate known to be repaired by nucleotide excision, the psoralen-thymine monoadduct. UvrA bound to this substrate with high affinity, whereas DDB failed to bind to the psoralen-thymine adduct specifically. Thus, it appears that if DDB is involved in excision repair, it participates as a damage recognition protein for only a subset of DNA lesions repaired by the human excinuclease(s).

MATERIALS AND METHODS

Substrates

49-mers containing centrally located dithymidine photocoupled were prepared as described and were determined to be >95% pure (Taylor et al., 1987; Taylor and Brockie, 1988; Smith and Taylor, 1993). Approximately 5 pmol of each oligonucleotide were 5'-end-labeled with [γ-32P]ATP (7000 Ci/mmol; ICN) and T4 polynucleotide kinase (New England Biolabs, Inc.). Labeled oligomers were precipitated with ethanol, resuspended in annealing buffer (50 mM Tris-HCl, pH 8.0, and 100 mM NaCl), and annealed with 2-4-fold excess unlabeled complementary oligomer by heating at 75 °C for 10 min and cooling to 30 °C over a 2-3-h period. Double-stranded substrate was separated from nonhybridized single-stranded oligomers by electrophoresis on a 10% polyacrylamide gel and purified by electroelution. The purified DNA was stored either in annealing buffer or in standard binding buffer (47.5 mM Tris-HCl, pH 8.2, 55 mM KCl, 4.5 mM MgCl2, 100 μg/ml bovine serum albumin, and 10% (v/v) glycerol). When indicated, binding buffer contained 2 mM ATP. Labeled duplex without photoproduct was obtained by photoreactivation of Tc,sT-containing oligomer with E. coli DNA photolyase as follows. Oligomer at 2.5 nM was mixed with photolyase at 360-nm light. DNA was extracted once with phenol and twice with ether, precipitated with ethanol, resuspended in 1 pg/ml bovine serum albumin, and 10% (v/v) glycerol. When indicated, binding buffer contained 2 mM ATP. Labeled duplex without photoproduct was obtained by photoreactivation of Tc,sT-containing oligomer with E. coli DNA photolyase as follows. Oligomer at 2.5 nM was mixed with photolyase at 360-nm light. DNA was extracted once with phenol and twice with ether, precipitated with ethanol, resuspended in 1 x annealing buffer, and renatured as described above. The efficiency of photoreactivation was tested by digestion with MseI, which is inhibited by Tc,sT at its TTAA recognition site, nucleotides 21–24 of the 49-mer. Essentially all of the photoreactivated substrate was cut by this enzyme, whereas none of the cis,syn-substrate was digested (data not shown).

DNA-binding Proteins

E. coli photolyase (Sancar and Sancar, 1984) and the UvrA and UvrB subunits of (A/B) excinuclease (Thomas et al., 1985) were purified as described previously. The DDB heterodimer was purified from HeLa cells as described (Keeney et al., 1993). For binding experiments with DDB enriched for p124 relative to p41, the putative heterodimer was centrifuged through a 20–40% (v/v) glycerol gradient as described by Keeney et al. (1993), and DDB activity was located in the gradient fractions. As analyzed by SDS-polyacrylamide gel electrophoresis followed by double staining with colloidal Coomasie Blue followed by silver, the peak fractions contained approximately stoichiometric quantities of both subunits and were the source of the heterodimer used in binding assays. Fractions from the trailing edge of the peak of DDB activity were substantially enriched for p124 (Keeney et al., 1993) and were used for DNase I footprinting where indicated.

Band Mobility Shift Assay

The equilibrium binding constants and the dissociation rate coefficients for DDB and UvrA were determined by band mobility shift assays. The protein-DNA complexes were separated on 18% polyacrylamide gels (18 x 12 x 0.2 cm) containing acrylamide/methylenebisacrylamide at 30:1 in 50 mM Tris borate and 1 mM EDTA, pH 8.3, at a constant current of 30 mA. The gels were autoradiographed for visual inspection and quantitatively analyzed with an Ambis radioanalytic detection system. RESULTS

Binding of DDB and UvrA to Major UV Photoproducts—The substrates were 49-mer duplexes that contained a centrally located T-T photoproduct and were >95% pure. The sequence of the substrate (5′-TGCACGAATTAAGCAATTCGTAATCATGGTCATAGCT). The binding constants obtained from the slopes of the lines in Fig. 1, and several conclusions can be drawn. First, Tc,sT and Tc,sT are the best substrates, followed by Tc,DpW,t; the affinity for Tc,sT is equal to or marginally above that for T-T. Second, DDB binds to all high affinity substrates ~5 times more tightly than does UvrA. Third, ATP hydrolysis decreases the affinity of UvrA for all substrates tested ~5-fold.

The interactions of the two proteins with the substrates were also analyzed by kinetic experiments using the band mobility shift assay (Figs. 3 and 4 and Table 1). The off rate for UvrA is exceedingly slow in the absence of ATP and is somewhat stimulated by ATP hydrolysis. Thus, it appears that ATP lowers the
these higher order complexes are more readily observed with DDB. Perhaps more interestingly, DDB, which binds to T[6-4]T, mer. A, stranded DNA contaminant. T[DewarlT, and T[t,s]T with 5-fold higher affinity compared to UvrA (see Table I). The faint band below compared to UvrA is because DDB has 3-fold higher nonspecific binding also (Bertrand-Burggraf et al., 1991). The reason that only with DDB at high protein concentrations, are observed with UvrA concentration was 0.43 nM.

complexes. 

DNase Z Footprints FIG. 1. Binding of UvrA and DDB to T[6-4]T-containing oligomer. A, binding of UvrA in the absence and presence of ATP. The oligomer concentration was 0.28 nm, B, binding of DDB. The substrate concentration was 0.43 nm. F, free DNA; B and B1-B3, protein-DNA complexes. B1 is the specific complex; B2 and B3, which are seen here only with DDB at high protein concentrations, are observed with UvrA also (Bertrand-Burggraf et al., 1991) and result from nonspecific binding of a second and a third molecule of a binding protein to DNA that already is complexed with one molecule specifically. The reason that these higher order complexes are more readily observed with DDB compared to UvrA is because DDB has 3-fold higher nonspecific binding affinity than UvrA (see Table I). The faint band below F is single-stranded DNA contaminant.

equilibrium binding constant by stimulating dissociation. Perhaps more interestingly, DDB, which binds to T[6-4]T, T[Dewar]T, and T[t,s]T with 5-fold higher affinity compared to UvrA, also dissociates 4–17 times faster. For DDB, the association rate coefficients calculated from the equilibrium binding constant and the dissociation rate coefficient (ka = KA × koff) are in the range of 1–5 x 104 M⁻¹ s⁻¹, within the range of diffusion-controlled rate constants. In contrast, the rate constants for UvrA are in the range of 2–6 x 106 M⁻¹ s⁻¹ and are at least an order of magnitude slower than those for diffusion-controlled reactions. When ATP was present in the reaction mixture, the off rates of UvrA were a little faster; and thus, the calculated on rates were somewhat higher, but were still below the limit of diffusion-controlled reactions.

DNase I Footprints of DDB and UvrB—To characterize further the interaction of DDB with DNA, we conducted DNase I footprinting experiments (Fig. 5). For comparison, we used UvrB since the size of the UvrB footprint (~33 base pairs) combined with the end effects on DNase I cleavage precludes formation of a well defined UvrA footprint on these oligomers, whereas the smaller UvrB footprint (~20 base pairs) is easily discernible. (UvrB alone is not a DDB; it is delivered to the damage site by UvrA, which in turn dissociates from the UvrB-DNA complex (Orren and Sancar, 1989; Bertrand-Burggraf et al., 1991).) DNase I footprints with T[c,s]T are not included in Fig. 5 because the specific binding affinities of both proteins for T[c,s]T are not sufficiently different from the nonspecific binding to yield a footprint (data not shown).

Fig. 5 reveals several interesting features. First, even though the molecular masses of UvrB and DDB are 78 and 165 kDa, respectively, the two proteins yield footprints of comparable sizes on both the damaged (Fig. 5, A–C) and the undamaged (Fig. 5D) strands of duplex containing various photoproducts. Second, the presence of T[6-4]T or its Dewar isomer, but not T[t,s]T, results in a very strong hyperversensitive site immediately 3' to the photolesions. This indicates that in contrast to T[c,s]T' or T[t,s]T, T[6-4]T' or its Dewar isomer introduces a severe kink in DNA as such sites are hypersensitive to DNase I digestion (Suck et al., 1988). Third, the binding of UvrB creates a hyperversensitive site 11 nucleotides 5' to the photodimer, whereas binding of DDB makes the sixth phosphodiester bond 5' to the dimer hypersensitive to DNase I. Both proteins appear therefore to kink the DNA, but they kink it at different distances from the lesion and kink it to different degrees. Fourth, the footprint obtained with the putative DDB heterodimer is different from that of DDB enriched for the 124-kDa subunit: the binding by the latter form causes hypersensitivity at the sixth phosphodiester bond 3' to the photopro-
**TABLE I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_A$</th>
<th>$k_{on}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(6-4) photoproduct</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UvrA</td>
<td>$2.4 \times 10^9$</td>
<td>$2.2 \times 10^{-4}$</td>
</tr>
<tr>
<td>UvrA (+ATP)</td>
<td>$3.7 \times 10^8$</td>
<td>$9.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>DDB</td>
<td>$1.6 \times 10^{10}$</td>
<td>$8.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Dewar photoproduct</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UvrA</td>
<td>$1.0 \times 10^9$</td>
<td>$1.7 \times 10^{-4}$</td>
</tr>
<tr>
<td>UvrA (+ATP)</td>
<td>ND</td>
<td>$2.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>DDB</td>
<td>$4.7 \times 10^9$</td>
<td>$2.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>trans,syn-Dimer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UvrA</td>
<td>$2.7 \times 10^9$</td>
<td>$2.4 \times 10^{-1}$</td>
</tr>
<tr>
<td>UvrA (+ATP)</td>
<td>$4.2 \times 10^8$</td>
<td>$1.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>DDB</td>
<td>$1.3 \times 10^{10}$</td>
<td>$3.7 \times 10^{-3}$</td>
</tr>
<tr>
<td>cis,syn-Dimer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UvrA</td>
<td>$2.6 \times 10^8$</td>
<td>$2.8 \times 10^{-2}$</td>
</tr>
<tr>
<td>UvrA (+ATP)</td>
<td>$1.2 \times 10^8$</td>
<td>ND</td>
</tr>
<tr>
<td>DDB</td>
<td>$1.7 \times 10^9$</td>
<td>$&gt;4.6 \times 10^{-2}$</td>
</tr>
<tr>
<td>PR cis,syn-dimer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UvrA</td>
<td>$1.6 \times 10^8$</td>
<td>ND</td>
</tr>
<tr>
<td>DDB</td>
<td>$5.5 \times 10^8$</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not determined; PR, photoreactivated.

duct, whereas in the heterodimer footprint, this site is protected (Fig. 5C). Thus, while the presence of stoichiometric amounts of the p41 subunit changes the character of the footprint, it does not change the size. Nonetheless, this change in binding character is the first indication that p41, which copurifies with p124, is a functional subunit of DDB. The footprints of UvrB and DDB are shown schematically in Fig. 6. The two are quite similar, but there are differences with regard to the length of the protected regions and the locations of the hypersensitive sites.

**Binding to Psoralen-adducted DNA**—One important property of a nucleotide excision nuclease, both in pro- and eukaryotes, is its wide substrate range (Sancar and Sancar, 1988; Huang et al., 1992; Svoboda et al., 1993a). Thus, the damage binding subunit of the excision nuclease is expected to bind preferentially to all lesions that are excised by the nuclease. Even though DDB does not discriminate so well between undamaged DNA and DNA containing T[c,s]T, the evidence presented so far is consistent with DDB being the damage recognition subunit of the human excision nuclease. We wished to test this premise further by measuring the binding of DDB to another bulky adduct known to be repaired by the human excision nuclease, the psoralen-thymine monoadduct (Svoboda et al., 1993a). Fig. 7 shows that while UvrA binds with high affinity to psoralen-adducted DNA, DDB does not discriminate between psoralen-adducted and undamaged DNAs (compare to Fig. 1). Thus, while UvrA and DDB behave very similarly with regard to their affinities for UV-damaged DNA, they differ drastically in their binding to a psoralen-thymine monoadduct, which is repaired efficiently by both *E. coli* (Van Houten et al., 1988) and human (Wood et al., 1988; Sibghat-Ullah et al., 1989; Reardon et al., 1991) excision nucleases.

**Binding to Photoproducts in Single-stranded DNA**—The apparent specificity of DDB for UV photoproducts raises the possibility that this protein may not be so general a damage recognition protein as the UvrA protein of *E. coli*, which recognizes backbone distortions rather than unique structural features of certain lesions. Instead, it was conceivable that DDB, like DNA photolyase, recognizes one lesion or a class of lesions specifically and not the overall deformity in the backbone of the DNA duplex. If such were the case, DDB should bind to the photoproducts in single-stranded DNA with nearly equal affinity as does the *E. coli* photolyase to T[c,s]T (Sancar et al., 1985; Hu-
Fig. 4. First-order rate plots of dissociation kinetics of UvrA and DDB complexes with T\((6-4)\)T, T\([\text{Dewar}]\)T (DW), and T\([ts]\)T (TS). The data points were obtained from experiments conducted as described for Fig. 3. With the exception of the 30-min point for DDB-T\([ts]\)T, the data points are the averages of two to four experiments. The error bars indicate standard deviations, and where no error bar is shown, the standard deviation was <2% and is hidden by the symbol. ○, -ATP; □, +ATP.

**DISCUSSION**

DDB was first identified in human placenta by Feldberg and colleagues (Feldberg and Grossman, 1976; Feldberg et al., 1982) as a protein that bound with high affinity to DNA damaged by UV, ionizing radiation, sodium bisulfite, or N-methyl-N-nitrosourea. The protein was rediscovered by Chu and Chang (1988), who conducted band mobility shift assays with nuclear extracts from normal and xeroderma pigmentosum cell lines. These authors also reported that two XP-E cell lines tested were both missing DDB activity, whereas xeroderma pigmentosum cell lines from all other complementation groups and normal cells contained the binding protein. In addition, it appeared that this protein also bound to cisplatin-damaged DNA with high affinity (Chu and Chang, 1990; Chao et al., 1991). These observations constituted a reasonable case for DDB being the sole damage recognition subunit of a human excision nuclease that removes all bulky adducts from DNA. However, several subsequent studies of this protein cast serious doubts on this simple hypothesis. Hirschfeld et al. (1990) found that the simian homolog of DDB bound with similar affinity to UV-irradiated and photo-reactivated DNA and that the protein did not bind to a defined substrate containing a single T\([ts]\)T, and this finding has been recently confirmed with extracts from human cells (Treiber et al., 1992). It was therefore concluded that the major binding determinant in UV-irradiated DNA was the (6-4) photoproduc, which is not subject to photoreversal by DNA photolyase. Since all xeroderma pigmentosum cell lines, including XP-E, are defective in the removal of Pyr\([c,s]\)Pyr, these results raised the possibility that the lack of DDB in XP-E cell lines observed by Chu and Chang may be incidental. In fact, this possibility gained support when it was discovered that of 12 XP-E cell strains tested for activity, only three were defective in DDB (Kataoka and Fujiiwara, 1991; Keeney et al., 1992). Could the binding of DDB to damaged DNA be a “side reaction” of a protein not related to DNA repair (Feldberg et al., 1982)? The availability of purified protein (Abramic et al., 1991; Keeney et al., 1993) has made it possible to address this question directly.

Since nucleotide excision repair was originally defined in terms of removal of Pyr\([c,s]\)Pyr from DNA in both pro- and eukaryotes (see Friedberg (1985)), any protein suspected of being involved in excision repair of bulky adducts is expected to participate in Pyr\([c,s]\)Pyr removal as well. This *sine qua non* has been the source of some confusion in the DNA repair field because Pyr\([c,s]\)Pyr is not a good substrate for either the prokaryotic (Sancar and Rupp, 1983; Svoboda et al., 1993b) or the eukaryotic (Mitchell et al., 1985; Sibghat-Ullah et al., 1989; Wood, 1989) excision nucleases. Therefore, in our attempt to establish whether DDB is involved in general nucleotide excision repair of bulky adducts, we used the *E. coli* damage recognition subunit UvrA as a reference protein since it is known that UvrA is absolutely required for removal of Pyr\([c,s]\)Pyr (Boyce and Howard-Flanders, 1964). We found that both UvrA and DDB discriminate only modestly between Pyr\([c,s]\)Pyr-containing DNA and undamaged DNA while binding to 6-4 and...
Binding of XPE-DDP and UvrA to UV Photoproducts

**FIG. 5. DNase I footprints of UvrB and DDB on 49-base pair duplexes containing single UV photoproducts.** Substrates (0.6 nM) terminally labeled either in the damaged (A-C) or undamaged (D) strand were digested with DNase I in the absence of binding protein or in the presence of UvrA and UvrB or of DDB at the indicated concentrations. Following DNase I digestion, the DNA was precipitated with ethanol, resuspended in formamide/dye mixture, and resolved on 10% polyacrylamide sequencing gels. In A and B, lanes 1 and 2 contain Maxam-Gilbert sequencing reactions for G and G + A, respectively. In C, p124 + p41 and p124 indicate the putative DDB heterodimer and DDB enriched for the large subunit, respectively. In D, lane 6 contains the G + A Maxam-Gilbert sequencing reaction, **, hypersensitive site in the UvrB or DDB footprints; ***, hypersensitive site seen in the footprint of the p124-enriched form of DDB, but not in the footprint of the putative heterodimer. T, T[S]; DW, T[Devar].

<table>
<thead>
<tr>
<th></th>
<th>T, S</th>
<th>DW</th>
<th>6-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>UvrA (4 nM)</td>
<td>+ + - - - - -</td>
<td>+ + - - - - -</td>
<td>+ + - - - - -</td>
</tr>
<tr>
<td>UvrB (65 nM)</td>
<td>- - + + + + +</td>
<td>- - + + + + +</td>
<td>- - + + + + +</td>
</tr>
</tbody>
</table>

Lane 1 2 3 4 5 6 7 8

Other minor UV photoproducts with much higher affinity. In fact, this is a reflection of Pyr[c,s]Pyr being a relatively poor substrate in both E. coli and human cells; and therefore, the results are consistent with DDB being the damage recognition protein for the human excision nuclease.

However, there is one important difference between UvrA and DDB with regard to their affinities for damaged DNA. UvrA binds with high affinity to the psoralen-thymine monoaduct, whereas DDB has no measurable affinity for this lesion. It is conceivable that DDB discriminates between psoralen-adducted DNA and undamaged DNA at a level below our detection limit and that this discrimination is amplified by other subunits of the human excision nuclease. It is equally possible that in human cells there exists more than one "damage recognition" protein perhaps with overlapping but not identical specificity. One such candidate is the XP-A gene product (XPAC), which was recently shown by filter binding assays to bind to UV-irradiated DNA with high affinity (Robins et al., 1991). It must be noted, however, that Eker et al. (1992) were unable to detect damage-specific binding of XPAC using the Southwestern assay. Clearly, more work is needed to clarify the roles of various xeroderma pigmentosum proteins in damage recognition. Multiple damage recognition proteins with a hierarchy of roles in the assembly of the subunits involved in excision may also explain why XP-E cells are only moderately defective in Pyr[c,s]Pyr removal in contrast with XP-A cells, which appear to be completely defective (Friedberg, 1985).

The results presented in this paper may also help in defining the quaternary structure of DDB. Feldberg et al. (1982) reported a native molecular weight of 120,000 by gel electropho-
resis and a maximum molecular weight of 400,000 by gel permeation chromatography for the protein from human placenta. Abramic et al. (1991) reported a monomer molecular weight of 126,000 for the protein purified from CV-1 monkey cells. They estimated a molecular mass of 210 kDa by gel permeation and suggested that the binding protein is a homodimer. Keeney et al. (1993) find stoichiometric amounts of a 124- and a 41-kDa protein in their purest fractions, which led them to propose that DDB is a heterodimer. However, DDB containing predominantly the 124-kDa subunit, when tested by gel mobility shift assay, exhibited the same binding properties as fractions containing both subunits; and thus, it was not possible to tell unambiguously whether the 41-kDa protein was a fortuitous contaminant or a true subunit of the binding protein. The footprinting experiments in Fig. 5C reveal an effect of the 41-kDa subunit on the footprint of the 124-kDa protein, providing strong evidence that the two proteins do interact and that DDB is a heterodimer of these two subunits.

Finally, this work has revealed some interesting aspects of the UvrA-DNA interactions. (i) The affinity of this protein for all substrates tested is higher in the absence of ATP, in contrast to an earlier report that ATP stimulated DNA binding (Seeberg and Steinum, 1982). It appears that ATP hydrolysis stimulates dissociation from DNA, which explains why the protein becomes nearly irreversibly bound to DNA nonspecifically in the presence of ATPyS (Seeberg and Steinum, 1982). (ii) Without specifying a particular lesion, it has been generally accepted that UvrA dissociates very rapidly (t1/2 < 5 s) from UV-irradiated DNA (Yeung et al., 1986). In contrast, we find this not to be a general property of UvrA-DNA complexes. The UvrA-DNA (T[c,s]T) complex is in fact very unstable, with koff = 2.8 x 10^-2 s^-1 (t1/2 = 25 s) in the absence of ATP. However, the complexes formed with other UV photoproducts are extremely stable, with half-lives of ~50 min for UvrA complexes with T[6-4]T, T[Dewar]T, and T[t,s]T. Again, in the presence of ATP, the dissociation of UvrA is more rapid by a factor of 4-12. (iii) UvrA associates with the substrate at an ~100-fold slower rate than in a diffusion-controlled reaction. Such slow association rates usually imply significant conformational change upon binding (see Wolfenden and Frick (1987)). (iv) Both kinetic and thermodynamic experiments reveal a 100-fold higher affinity of UvrA for the (6-4)-photoproduct compared to T[c,s]T (Sancar and Rupp, 1983) and the more recent excision rate estimates of ~10-fold more rapid excision of T[6-4]T than T[c,s]T (Svoboda et al., 1993a). The discrepancy between the binding affinity of UvrA and the excision rate can be accounted for at the step of UvrB loading and by the stabilities of the UvrB-DNA complexes, which are independent of the stability of the UvrA-DNA complexes since they involve different proteins.

Acknowledgments—We thank Ann Fischer for assistance in culturing human cells and Dr. Tom Brody for help in preparing DDB.

REFERENCES


FIG. 6. Diagram of DNase I footprints of UvrB and DDB. The diagram is based on data in Fig. 5. The adducted thymines are underlined. The boxes above and below the sequence indicate the areas of protection from DNase I digestion of the top and bottom strands, respectively. Arrows indicate DNase I-hypersensitive sites. The 3'-hypersensitive site for DDB disappears when stoichiometric amounts of p41 are present. Note that the footprint on the damaged strand is for DNAs containing T[c,s]T, T[6-4]T, and T[Dewar]T; the footprint for the undamaged strand is for a T[6-4]T-containing duplex.

Fig. 7. Binding of UvrA and DDB to 138-mer containing psoralen monoadduct. About 0.1 nM DNA was incubated with either of the binding proteins at 30 °C for 30 min, and the bound fraction was determined by mobility shift assays on 5% polyacrylamide gels. A, autoradiographs of the gels. F, free; B1 and B2, bound DNA bands. The diffuse complex at the highest concentration of DDB was not considered to be a uniform species. B, plot of the binding data shown in A. (a), UvrA; (C), DDB.

Fig. 8. Binding of DDB to single-stranded DNA with or without UV photoproducts. DNA at 0.3 nM was incubated with DDB in 15 μl of binding buffer for 30 min at 30 °C, and the bound fraction was determined by band mobility shifts on 5% polyacrylamide gels. (a), undamaged single-stranded 49-mer; (a), single-stranded 49-mer with T[Dewar]T; (O), double-stranded 49-mer with T[Dewar]T.
Feldberg, R. S. (1980) Nucleic Acids Res. 8, 1133-1143