A DNA Binding Protein from Human Placenta Specific for Ultraviolet Damaged DNA†

Ross S. Feldberg*,‡ and Lawrence Grossman§

ABSTRACT: A DNA-binding protein specific for ultraviolet irradiated DNA has been purified extensively from human placenta. The binding preparation is free of exonuclease, polymerase, endonuclease, and *N*-glycosidase activity. The binding activity is salt dependent and is specific for double-stranded irradiated DNA. DNA from which the pyrimidine dimers have been monomerized by the action of photolyase (photoreactivating enzyme) remains an effective substrate for the binding protein, suggesting that the protein recognizes photoproducts other than pyrimidine dimers. This is supported by the finding that DNA irradiated under conditions which introduce only pyrimidine dimers is not a substrate for the binding protein. Examination of three of the xeroderma pigmentosum complementation groups has revealed no deficiency in this binding activity.

The irradiation of cells with ultraviolet (uv) light can result in mutation or cell death due to the introduction of a variety of photoproducts into the cellular DNA. Studies with bacterial cells have demonstrated the importance of processes involved in the recognition and removal of damaged bases from DNA. The best known process for DNA repair is the excision repair of uv-induced pyrimidine dimers. This process involves an initial endonucleolytic incision adjacent to the dimer, followed by exonucleolytic removal of the dimer and a number of adjacent nucleotides. The resulting cavity in the DNA is filled by a DNA polymerase and the sequence of repair completed by the action of polynucleotide ligase. The genetic and enzymatic evidence supporting this model has recently been reviewed (Grossman et al., 1975).

In mammalian cells, however, only indirect evidence has supported the existence of a similar process. Although DNA polymerase and ligase activities have been known for some time, only recently has an exonuclease with potential excision-repair properties been identified in human cells (Doniger and Grossman 1975; Doniger and Grossman, in preparation). No one has yet isolated a pyrimidine dimer-specific endonuclease (correndo II) from mammalian cells, although the presence of such an enzyme has been inferred from studies on the human skin disorder xeroderma pigmentosum (Robbins et al., 1974; Cleaver, 1974). This genetic disorder, characterized by an abnormal sensitivity to uv light, appears, on the basis of indirect biochemical evidence, to reflect a deficiency in the activity of a correctional endonuclease (Paterson et al., 1973; Wilkins, 1973).

Although it has been impossible to identify a pyrimidine dimer-specific endonuclease in mammalian cells, a variety of investigators have identified enzymes able to incise DNA with photodamage other than pyrimidine dimers. An endonuclease able to act at nonpyrimidine dimer damage in uv or γ-irradiated DNA has been isolated from human cells as well as from calf thymus (Bacchetti et al., 1972; Brent, 1973; Bacchetti and Benne, 1975), and an enzyme has been isolated from rat liver (van Lancker and Tomura, 1974) which will attack either uv or *N*-acetoxyaminofluorene-treated DNA. The biological significance of this nonpyrimidine dimer photoproduct has also been suggested by a report on the uv-stimulated repair replication in chick embryo fibroblasts (Paterson et al., 1974). Cells which have been allowed to monomerize pyrimidine dimers by in vivo photoreactivation showed no decrease in their level of repair replication, suggesting that photodamage other than pyrimidine dimers was being removed.

In this report, we have made use of a membrane filter binding assay to isolate from human cells a protein which specifically binds to uv-irradiated DNA (uv-DNA). The properties of this protein make it unique among the known DNA repair proteins and again strongly suggest the presence of nonpyrimidine dimer photodamage in DNA after irradiation at low doses of uv light.

Experimental Section

Materials

Chemicals and Cells. Human placentas from normal births were obtained from a local hospital within 4 h of delivery. Human fibroblasts from individuals with xeroderma pigmentosum were grown in culture and supplied through the generosity of Dr. J. H. Robbins. Whatman DEAE-cellulose (DE-52) was used without precycling. Bio-Gel P-100 polyacrylamide beads (Bio-Rad) were treated before use according to the manufacturer's instructions. Phosphocellulose powder (Whatman P-11) was precycled according to the method of Yoneda and Bollum (1965). Single-stranded, uv-irradiated DNA cellulose, prepared by the method of Litman (1968), was a generous gift of Dr. L. Hamilton.

Nucleic acids. Tritium-labeled *Escherichia coli* DNA, prepared according to the method of Mahler (1967), was a gift

† From the Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received December 16, 1975.* Publication No. 1078. This study was supported by American Cancer Society Grant No. NP-8F and ERDA Contract No. AT(11-1) 2814.

‡ R.S.F. was supported in part by a National Cancer Institute, National Institutes of Health, postdoctoral fellowship (F2CA00082A). Present address: Department of Biology, Tufts University, Medford, Mass. 02155.

§ Present address: Department of Biochemical and Biophysical Sciences, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Md., 21205.

[1] Abbreviations used are: uv-DNA; ultraviolet-irradiated DNA; DEAE, diethylaminoethyl; RFI, replicative form I; EDTA, (ethylenedinitrilo)-tetraacetic acid; SSC, standard saline citrate; AMP, adenosine 5′-monophosphate; PRE, photoreactivating enzyme.

TABLE I: Purification of a UV-DNA Specific Binding Protein from Human Placenta.

Fraction	Vol (ml)	Total Protein (mg)	Total Binding Act. (units × 10^{-4}) (a) Uv-DNA	(b) Unirrad.	Sp Act. (a − b)/mg
I, combined sonic supernatant	1000	10 000	200	200	---
II, DEAE-cellulose eluate	200	512	4.4	2.0	47
III, phosphocellulose eluate	63	8	1.3	0.3	1 250
IV, DNA-cellulose eluate	7	0.2	0.7	0.01	34 500
V, Bio-Gel P-100 eluate	19	---	0.5	---	---

of Dr. Inga Mahler. Tritium-labeled replicative form I ϕX-174 DNA (RFI) was prepared by a method which avoided the use of ethidium bromide–CsCl gradients. This procedure (Feldberg and Grossman, in preparation) involves lysis of infected cells with alkali, an RNase and heat treatment, Sevag extraction of protein, ethanol precipitation and Sephadex G-100 column chromatography. RFI DNA is isolated free of host DNA and nicked circles by alkali denaturation followed by reneutralization and nitrocellulose column chromatography.

Enzymes. The endonuclease from *Micrococcus luteus* specific for uv-irradiated DNA (correndonuclease II) employed in these experiments was a G-75 fraction (Riazuddin and Grossman, in preparation) containing 250 "BAP-units" (Kaplan and Grossman 1971) per ml and was provided by Dr. S. Riazuddin. Yeast photoreactivating enzyme, 80 000 transforming units/ml (Madden and Werbin 1974), was a generous gift of Dr. J. Madden. *E. coli* unwinding protein (Molineux et al., 1974) was a gift from Dr. V. McKay.

Methods

Ultraviolet Irradiation. ϕX-174 RFI DNA was irradiated in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 20 mM NaCl at 1–2 μg/ml in a 2-mm path-length quartz cuvette. A germicidal lamp emitting primarily at 254 nm was employed and the incident dose, measured using a Black Ray uv monitor (Ultra-Violet Products, Inc.), was 1.4 J m^{-2} s^{-1}. To introduce only pyrimidine dimer photoproducts into DNA (Rahn et al., 1973), irradiation at 360 nm in the presence of acetophenone and silver nitrate was employed. RFI DNA was diluted 1:2 in 10 mM sodium phosphate buffer (pH 7.0) containing 133 mM NaCl, 100 mM acetophenone, and 10 μM silver nitrate. Irradiation was carried out for 120 min in a 2-mm path-length quartz cuvette positioned 2 cm from two GE F15T8/BL black light fluorescent lamps. Control irradiations under the same conditions, but in the absence of acetophenone, were also carried out. Ultraviolet irradiation of tritium-labeled *E. coli* DNA was carried out in SSC at a concentration of 40 μg/ml using a germicidal lamp.

Enzyme Assays. (A) Binding Activity. The ability of the protein to bind radioactive DNA was measured by a filter binding assay similar to that of Riggs et al. (1970). The standard assay mixture contained 10 mM Tris-HCl (pH 8.1), 83 mM NaCl, 10–40 ng of RFI DNA, and an aliquot of protein in a total volume of 0.30 ml. Since ϕX-174 RFI DNA contains 11 000 nucleotides, each assay contained 3–12 × 10^{-15} mol of ϕX-174 double-stranded circles (sp act. 500 cpm/10^{-15} mol of circles). Incubation was generally carried out at 0 °C for 30 min after which the assay mixture was diluted with 2 ml of 0.30 M NaCl–0.03 M sodium citrate (2 × SSC), filtered immediately through Millipore HAWP membrane filters, and the filters washed with 2 ml of 2 × SSC. After drying under a heat lamp, the radioactivity retained on the filter was counted in Liquifluor (New England Nuclear Corp.). One unit of binding activity is defined as that amount of enzyme which retains at equilibrium 1 × 10^{-15} mol of RFI circles on a Millipore filter under the standard conditions of dilution and filtration. With uv-irradiated DNA, a unit of binding activity must be determined at a saturating dose of irradiation (>200 J/m^2 for RFI DNA).

(B) Endonuclease Activity. The endonuclease activity of the protein was determined by using the sensitive "nicking assay" described by Braun and Grossman (1974).

Miscellaneous. X-irradiation of the RFI DNA was carried out under N_2 in a 10 mM Tris-HCl–1 mM EDTA buffer (pH 8) containing 10^{-3} M L-histidine to minimize indirect effects. The biological infectivity of the X-irradiated DNA was tested by Dr. Inga Mahler using the spheroplast transfection assay described by Benzinger et al. (1971). Salt gradients were checked with a Radiometer conductivity meter. ϕX-174 RFI DNA was cross-linked using 4,5′,8-trimethylpsoralen (Paul Elder and Co.) as described by Cole (1971).

Results

Purification of Binding Protein

All operations were carried out at 4 °C. The buffers used were: buffer A, 30 mM Tris-HCl buffer (pH 8.0), 0.2 mM EDTA, and 1.0 mM 2-mercaptoethanol; buffer B is the same as buffer A but with the addition of 10% glycerol; buffer C, 50 mM potassium phosphate buffer (pH 7.5) containing 0.2 mM EDTA, 6 mM 2-mercaptoethanol, and 5% glycerol; buffer D, 200 mM potassium phosphate buffer (pH 7.5) containing 0.2 mM EDTA, 6 mM 2-mercaptoethanol, and 5% glycerol. The results of a typical purification are summarized in Table I.

Extraction. Human placenta was obtained from a local hospital and washed clean of clotted blood with buffer B. The membraneous sheath and some of the larger pieces of connective tissue were removed and the soft tissue minced into small pieces, washed with buffer B, and dried by straining the liquid through cheesecloth. The damp tissue was weighed, resuspended in buffer B (approximately 2 ml/g of tissue), and homogenized in a Waring Blendor for 2 min. The placental tissue weight was generally 200–300 g. The homogenized tissue was centrifuged for 15 min at 10 000*g* in the Sorval GSA rotor. The pellet was resuspended in buffer B, homogenized a second time, and centrifuged as above. The two homogenization supernatant fluids were used as starting material for the preparation of a human correxonuclease (Doniger and Grossman, in preparation).

The pellet from the homogenization steps was resuspended in buffer A (2.5 ml/g of tissue) and sonically disrupted for six 1-min pulses using a Branson Sonifier. The extract was centrifuged at 10 000*g* for 15 min, the supernatant retained, and the pellet resuspended in buffer A and sonically disrupted as

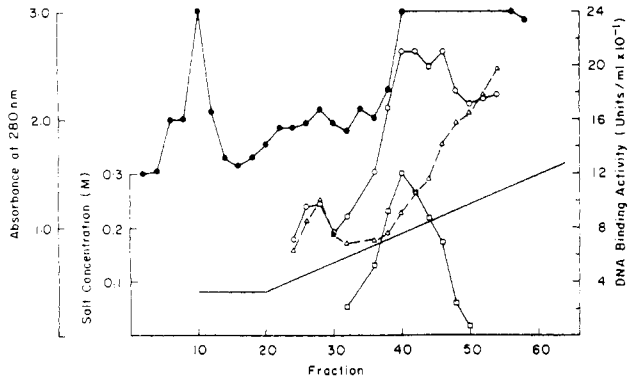

FIGURE 1: Chromatography of combined sonic supernatant fractions on DEAE-cellulose. Absorbance at 280 nm (●—●); DNA-binding activity with unirradiated DNA (△—△), and with DNA irradiated at 300 J/m^2 (○—○); uv specific activity (irradiated DNA binding minus unirradiated DNA binding) (□—□); salt concentration (—).

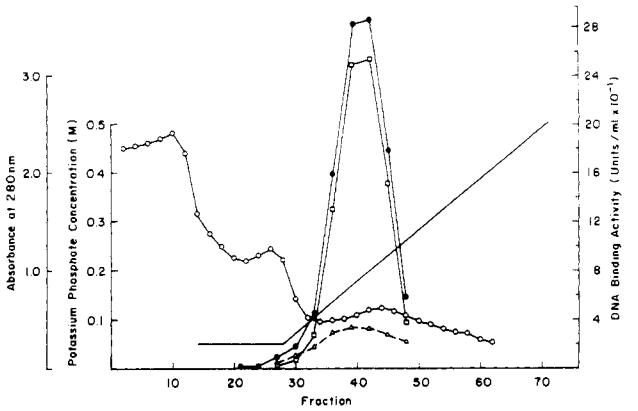

FIGURE 2: Chromatography of fraction II on phosphocellulose. Absorbance at 280 nm (○—○); DNA-binding activity with unirradiated DNA (△—△), and with DNA irradiated at 300 J/m^2 (●—●); uv-specific activity (□—□); salt concentration (—).

above. After centrifugation, the two sonic supernatant solutions were combined and used for the preparation of the damage-specific binding protein. The final volume was approximately 1200 ml (fraction I). At this stage, no uv-specific binding activity could be observed due to the high level of nonspecific binding.

DEAE-Cellulose. The combined sonic supernatant solutions were brought up to 10% in glycerol and applied to a column of Whatman DE-52 (12.6 cm^2 × 17 cm) equilibrated with buffer B. When the protein solution was adsorbed, the column was washed with buffer B containing 0.08 M KCl until a red protein band was washed off the column. Binding activity was eluted with a 700-ml linear gradient from 0.08 to 0.32 M KCl in buffer B. DNA binding activity showing specificity for uv-irradiated DNA eluted at a KCl concentration of 0.17M (Figure 1). Fractions containing uv-specific binding activity were pooled (fraction II) and applied directly to a phosphocellulose column.

Phosphocellulose. The pooled DE-52 fractions were applied to a column of Whatman P-11 (4.9 cm^2 × 15 cm) equilibrated in buffer C. After adsorption of the protein, the column was washed with one column of buffer C and then eluted with a 250-ml linear gradient from 50 mM to 500 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM EDTA, 6 mM 2-mercaptoethanol, and 5% glycerol. A peak of binding activity specific for uv-irradiated DNA eluted at 0.175 M potassium phosphate (Figure 2). The P-11 fractions containing activity were pooled (fraction III) and applied directly to a DNA-cellulose column.

DNA Cellulose. The combined P-11 fractions were applied to a uv-irradiated, single-stranded DNA cellulose column (1.8 cm^2 × 3.5 cm) equilibrated with buffer D containing 50 mM NaCl. This column was washed with 15 ml of equilibration buffer, with 25 ml of 200 mM NaCl-containing buffer D, and with 25 ml of 1.0 M NaCl-containing buffer D. Uv-specific DNA binding activity was eluted with the 1.0 M NaCl wash (fraction IV). At this point in the purification, the protein level was too low to be measured accurately by optical density measurements.

Bio-Gel P-100. The pooled DNA cellulose fractions were desalted by column chromatography on a Bio-Gel P-100 column (4.9 cm^2 × 21 cm) equilibrated with buffer B. The uv specific DNA binding activity eluted in the void volumn (fraction V), indicating a molecular weight in excess of 100 000.

Polyacrylamide Gel Electrophoresis. The extremely low level of protein present in fractions IV and V made analysis of the purity of the preparation difficult. Electrophoresis of 35 units of binding activity revealed two extremely faint Coomassie brilliant blue staining bands, one immediately behind the bromophenol blue dye front and the other near the top of the gel. Attempts to slice unstained gels into fractions and assay directly for binding activity were unsuccessful. When unstained gels were immersed in 5 μg/ml of uv-irradiated *E. coli* DNA for several minutes, washed with 2 × SSC, and dipped in 11 μg/ml of ethidium bromide, two fluorescent bands of bound DNA were observed, one immediately behind the dye front, and the other near the top of the gel, corresponding to the faint protein bands observed.

Properties of the DNA-Binding Protein

Stability. The uv-DNA specific binding activity has been stored in both high salt (as fraction IV) and at low ionic strength (as fraction V) over several months at 0 °C with only a slight loss in activity. Binding activity is totally lost, however, upon dialysis or gel filtration on Sephadex or Agarose columns. It has been possible to recover low, but variable, amounts of activity from Sephadex columns by elution with high salt (0.5 M ammonium sulfate). This behavior probably reflects an interaction between the binding protein and carbohydrate. DNA binding activity is stable at 37 °C, but activity is lost at 50 and 60 °C with a half-life of 70 and 11 min, respectively. Incubation of the protein with 50 μg of RNase A (Worthington, preheated at 85 °C, 15 min) for 30 min at 37 °C gave no decrease in binding activity, whereas incubation with 20 μg of trypsin under the same conditions resulted in a total loss of binding activity.

Other Enzymatic Activities. Neither DNA polymerase nor exonuclease activity could be detected in fraction IV or V, using *E. coli* DNA as a substrate. Using the φX-174 nicking assay, less than 3% of the DNA was converted to a denaturable form after incubation with five binding units of fraction V protein. No nicking activity was detected in the presence of $MgCl_2$ (2 mM), NaCl (50 mM), $CaCl_2$ (2 mM), ATP (3 mM), cyclic AMP (0.3 mM), or 2-mercaptoethanol (2 mM). Incubation of the irradiated DNA with five binding units of *M. luteus* uv-endonuclease resulted in 100% of the DNA being converted to a denaturable form. From these results, it was concluded that the uv DNA specific binding protein contained no endonuclease activity.

The discovery in *E. coli* of an *N*-glycosidase that releases free uracil from DNA containing deaminated cytosine

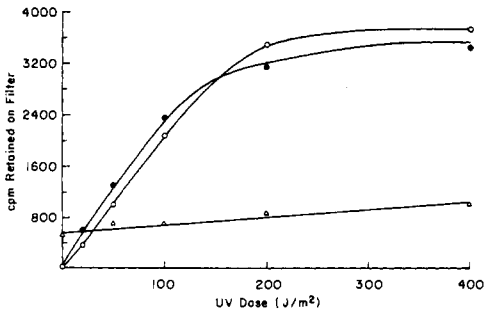

FIGURE 3: Dose-response for binding to ϕX-174 RFI DNA. A total of 7×10^{-15} mol (7500 cpm) of RFI circles was used in each assay. Incubation with 3.5 units of placental fraction uv was carried out for 30 min at 0 °C. Placental fraction IV (●—●); *M. luteus* correndonuclease II (○—○); *E. coli* unwinding protein (△—△).

(Lindahl 1974) suggested to us that the binding protein might be functioning in a similar manner with respect to some DNA photoproduct. Under the conditions of the nicking assay (denaturation with 0.10 M sodium phosphate, pH 12.1, 0.30 M NaCl, 25 mM EDTA for 5 min at room temperature), strand breaks would not have been introduced in the apurinic (or apyrimidinic) DNA. *N*-Glycosidase activity was assayed by making use of Lindahl and Andersson's observation (1972) that chain breaks are introduced adjacent to apurinic (or apyrimidinic) sugars by incubation of the DNA at high pH and elevated temperatures in the presence of a primary amine. After incubation of irradiated RFI DNA with fraction IV in the presence of 2 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 50 mM NaCl for 30 min at 37 °C, the assay mixture was adjusted to pH 12.0 with a solution containing 0.10 M sodium phosphate, 0.30 M NaCl, 25 mM EDTA, and 50 mM L-lysine. This denaturation mixture was incubated at 40 °C for 3 h before reneutralization and filtration through nitrocellulose filters. No significant increase in the amount of DNA retained on the nitrocellulose filters was observed, suggesting that the binding protein does not contain *N*-glycosidase activity.

Substrate Specificity. When native, unirradiated RFI DNA was incubated with fraction V, less than 2% of the DNA was retained on a Millipore filter under the conditions of the binding assay. Irradiation of the DNA with 254-nm light resulted in a protein-dependent retention of DNA on the filters. The amount of radioactivity bound to the filter increased linearly with dose up to approximately 100 J/m^2, leveling off at higher exposures. Figure 3 shows a comparison of the dose-response curves for binding for the placental uv-DNA specific binding protein, *M. luteus* correndonuclease II, and the *E. coli* unwinding protein. In Figure 3, the maximum level of DNA retained on the filter was 50% of the input DNA. With a higher level of binding protein, it was possible to retain 100% of the DNA on the filter at exposures of 150 J/m^2 and greater (data not shown). From experiments carried out with a saturating level of binding protein, a half-maximal dose was calculated to be 50 J/m^2. However, quantitative arguments cannot be derived from these data, since the binding assay is a reflection of two separate phenomena: (a) the binding of the protein to photodamage on the DNA, and (b) the efficiency with which the protein-DNA complex is retained on the filter. It is possible that both of these parameters may vary with dose, making it impossible to derive conclusions about (a) without some knowledge of (b). Thus, it is of interest that the binding of the *M. luteus* correndonuclease II shows a dose-response curve very similar to the placental binding protein, whereas a dose-response curve for the endonuclease based on its nicking activity shows a saturation at exposures of around 15 J/m^2. It is clear from Figure 3, however, that the placental binding protein is recognizing some photodamage introduced into the DNA at low uv doses and not simply a gross distortion in the DNA as would be recognized by the *E. coli* unwinding protein.

TABLE II: The Effect of Photoreactivation on Binding Activity.[a]

Incubation 1	360-nm Light	Incubation 2	mol of RFI Circles Retained ($\times 10^{15}$)
PRE	−	No addition	6.8
PRE	+	No addition	0.2
Buffer	+	5.2 units of fraction V	6.6
PRE	+	5.2 units of fraction V	5.0
Buffer	+	0.25 BAP unit of *M. luteus*	6.3
PRE	+	0.25 BAP units of correndonuclease II	0.5

[a] ϕX-174 RFI DNA (21×10^{-15} mol/assay) irradiated with 100 J/m^2 of 254-nm light was preincubated for 30 min at 37 °C under two GE black light fluorescent bulbs at a distance of 15 cm with either buffer B or with yeast photoreactivating enzyme at a final concentration of 16 transforming units/ml. Incubation 2 was carried out in the dark and the samples were treated for 30 min at 0 °C and then treated as in the normal binding assay.

To determine if the placental binding protein was specifically recognizing pyrimidine dimers, the ability of the protein to bind to irradiated DNA which had been pretreated with yeast photolyase was examined. As a measure of the pyrimidine dimer content of the DNA, its ability to act as a binding substrate for the yeast photolyase and for the *M. luteus* correndonuclease II was also measured. These data are shown in Table II and indicate that, under conditions in which both photolyase and correndonuclease II binding are reduced by over 90%, the placental binding protein still shows a high level of binding.

The unexpected finding that the placental binding protein recognized irradiated DNA which had been pretreated with photoreactivating enzyme led us to further examine the photoproduct specificity of this protein. RFI circles were irradiated under conditions designed to introduce only pyrimidine dimers into the DNA (Rahn et al., 1973). The ability of this DNA to act as a binding substrate is indicated in Table III. These data reveal that DNA irradiated under these conditions, although an excellent substrate for photoreactivating enzyme binding, is not bound to the placental binding protein.

A variety of other DNA substrates have been tested for interaction with the placental binding protein. Unirradiated RFI DNA which had been nicked with pancreatic DNase I was not active as a binding substrate, nor was DNA into which cross-links had been introduced by 4,5′,8-trimethylpsoralen. Irradiated DNA pretreated with either pancreatic DNase I or with *M. luteus* correndonuclease II was not affected as a substrate for the placental binding protein. X-irradiated RFI DNA (200 000-rad dose), which was approximately 50% infective as measured by a spheroplast transfection assay, was retained on Millipore filters by placental binding protein fraction IV to the extent of 15% of the input DNA. It thus appears that the binding protein may recognize x-ray damage in DNA as well as uv photodamage.

The ability of single-stranded DNA to act as a binding substrate for both the placental binding protein and the *M. luteus* uv endonuclease was examined using tritium-labeled

TABLE III: Comparison of Binding to 254-nm Irradiated DNA and to DNA Irradiated at 360 nm with Acetophenone and Silver Nitrate.[a]

Protein	No irrad.	% of Input DNA Retained on Filter: 254 nm	360 nm + $AgNO_3$	360 nm + $AgNO_3$ + acetophenone
None	1	1	1	1
Fraction V	2	52	3	3
Yeast PRE	3	26	12	31

[a] ϕX-174 RFI DNA was irradiated, either at 254 nm (12×10^{-15} mol/assay) or at 360 nm (10×10^{-15} mol/assay), as described under Methods. The ability of these DNA samples to act as binding substrates was tested with 8 units of placental binding protein and with 24 units of photoreactivating enzyme (PRE) under the standard assay conditions.

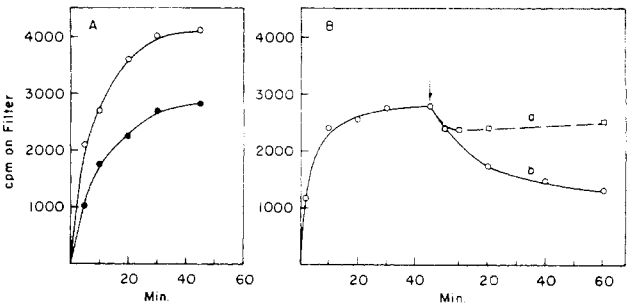

FIGURE 4: Time course of association and dissociation of placental fraction V. (A) Standard binding assay with 10×10^{-15} mol of RFI circles/assay. At various times, samples were diluted with 2 ml 2 × SSC, filtered through Millipore filters, and washed with 2 ml of 2 × SSC. Fraction V, 5.2 units (●—●), and 10.4 units (○—○). (B) At 45 min the assay mixtures were diluted with 1.2 ml of either (a) 10 mM Tris-HCl, 80 mM NaCl, pH 8 (□—□), or with (b) 0.30 M NaCl, 0.03 M sodium citrate (○—○). After dilution, the samples were incubated at 25 °C for varying lengths of time. Immediately before filtration, 0.5 ml of 8 × SSC was added to the (a) samples and 0.5 ml of 2 × SSC was added to the (b) samples to maintain the same set of filtration conditions.

TABLE IV: Native and Denatured *E. coli* DNA as a Binding Substrate.[a]

Protein	% of Input DNA Retained on Filter: Native DNA Irrad.	Native DNA Nonirrad.	Denatured DNA Irrad.	Denatured DNA Nonirrad.
None	27	28	28	22
Fraction V	75	34	33	23
Correndo II	60	21	22	16
Unwinding protein	53	58	100	95

[a] Tritium-labeled *E. coli* DNA (40 μg/ml; 1.25×10^4 cpm/μg) was irradiated to a total dose of 850 J/m^2. The irradiated and nonirradiated DNA samples were denatured by incubation in a boiling water bath for 10 min followed by quick cooling. Each assay contained 0.32 g of DNA (5000 cpm). Binding assays were diluted with 2 ml of 0.5 × SSC and washed with 2 ml of 0.5 × SSC. The high background levels of binding are a characteristic of *E. coli* DNA.

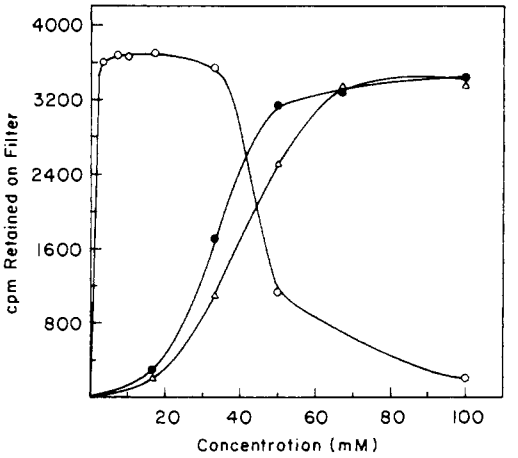

FIGURE 5: Uv-specific DNA binding activity as a function of ionic strength. The assay mixture contained 13.2×10^{-15} mol of RFI circles and nine units of fraction V. NaCl (●—●); KCl (△—△); $MgCl_2$ (○—○).

E. coli DNA. These data (Table IV) indicate that the binding protein is largely specific for double-stranded irradiated DNA. The minimal (5% above background) binding shown with unirradiated DNA and with single-stranded irradiated DNA was not seen when ϕX-174 or λDNA was employed as a substrate.

One photoproduct introduced into DNA at low uv doses that might be involved in binding protein recognition is the cytosine hydrate, in which a molecule of water is introduced across the 5,6 double bond of cytosine. The work of Johns and his coworkers (Johns et al., 1965; Deboer et al., 1970), however, has suggested that this photolesion is not very stable and will spontaneously dehydrate or deaminate to generate cytosine or a uracil hydrate. The relative instability of this photoproduct was employed in testing its involvement in binding. RFI DNA irradiated at 100 J/m^2 was incubated at 60 °C for 60 min to eliminate all or a part of the hydrates present. Since this level of exposure is on the linear portion of the dose–response curve, any decrease in photodamage content should be reflected in a decrease in the amount of DNA retained on the filter. When the heated irradiated DNA was tested as a substrate it was bound to the same extent as unheated irradiated DNA, leading us to conclude that hydrates were not the photoproduct being recognized.

Time Course of Association and Dissociation. As shown in Figure 4A, both the rate and the extent of binding are dependent on the protein level. Figure 4B shows the rate of dissociation after a 5× dilution in either assay buffer (10 mM Tris-HCl, pH 8.1, 80 mM NaCl) or in a high-salt buffer (2 × SSC). These data indicate that the binding is reversible and that dissociation is favored at higher salt concentrations. It is possible to quantitatively recover the bound DNA by denaturation of the protein on the filter with a 1% solution of sodium lauryl sulfate.

Salt Requirement. The human placental binding activity is markedly salt dependent (Figure 5). For NaCl and KCl, the dependency appears to be sigmoidal, and full binding is not reached until a concentration of 60 mM. With $MgCl_2$, full stimulation of binding occurs at a concentration of 3 mM ($\Gamma/2 = 9$) and inhibition is found at concentrations greater than 30 mM ($\Gamma/2 = 90$). These results suggest an ion-specific effect rather than simply an ionic-strength effect.

Activity in Xeroderma Pigmentosum Fibroblasts. Although no catalytic activity has yet been discovered associated with the uv-DNA specific binding protein, the biological role of the

protein could potentially be clarified if it could be shown that an absence of the protein results in cellular uv sensitivity. Since we cannot directly eliminate the protein from cells, we are limited to examining cells from individuals with an abnormal sensitivity to uv light. In this study, fibroblasts derived from patients with the autosomal recessive disease xeroderma pigmentosum were examined for their level of binding activity. The data for a normal cell line and for three of the five complementation groups known for this disease are shown in Table V. Although the group A cells showed a somewhat lower level of binding activity than the other cell lines, because we do not know what the range of binding activity will be in normal cells, it is impossible to assign significance to these quantitative differences. It is clear that the binding activity is not totally missing in any of the xeroderma lines examined, and thus the biological significance of this activity is still unresolved.

TABLE V: Binding Activity in Fibroblast Extracts.[b]

Cell Line		Description	Total cpm Bound/mg of extract
LaCol	CRL 1119	Normal fibroblast	2900
JayTim[a]	XP12BE	Xeroderma, Group A	2000
JayAr[a]	XP2BE	Xeroderma, Group C	2750
TeGer[a]	XPKABE	Xeroderma, Group D	3000

[a] Cells provided by Dr. J. Robbins, NIH. [b] Cells grown until nearly confluent were harvested by detaching the cells with 0.04% EDTA in phosphate-buffered saline and centrifuging at 1000g for 10 min. Fibroblasts (approximately 0.1 g wet wt) were suspended in 3 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM EDTA and 5 mM 2-mercaptoethanol and broken by sonic disruption. Cell debris was pelleted by centrifugation at 10 000*g* for 15 min and the protein concentration determined by measuring the optical density at 280 and 260 nm. The sonic supernatant was applied to a P-11 column (0.78 cm^2 × 11 cm) and eluted with a 50-ml linear gradient from 50 mM to 500 mM potassium phosphate, pH 7.5, containing 0.2 mM EDTA, 5 mM 2-mercaptoethanol, and 5% glycerol. The peak of uv-DNA specific binding activity eluted at 0.17 M potassium phosphate and was measured in the standard binding assay.

Discussion

A DNA-binding protein specific for uv or x-irradiated DNA has been purified from human placental tissue. Although the low level of protein present in the highly purified fractions has made it impossible to estimate the final purity, the protein is free of contaminating DNA polymerase, exonuclease, endonuclease, *N*-glycosidase, and nonspecific DNA binding activity. Binding occurs rapidly, even at 0 °C, and, unlike many DNA specific enzymes, requires a high concentration of monovalent cations. The inactivation of the binding activity by heat or trypsin is consistent with it being a protein. The purification steps employed were designed to avoid dialysis, a procedure which resulted in the total loss of binding activity. Analysis of the dialysate fluid revealed no binding activity, making it unlikely that the protein had migrated through the pores of the dialysis tubing. The loss of activity upon dialysis as well as the tendency of the protein to adsorb tightly to Sephadex gels suggests that carbohydrate may be a primary binding site for the protein.

Most surprising was the finding that the binding activity, although specific for uv-irradiated DNA, was not directed toward pyrimidine dimers. Pretreatment of irradiated DNA with sufficient photoreactivating enzyme to eliminate practically all binding to the *M. luteus* correndonuclease II resulted in only a 10–20% decrease in the binding of placental fraction V protein. This suggests that pyrimidine dimers are not the primary substrate for the binding protein. The 10–20% decrease in binding may reveal some minor affinity for pyrimidine dimers or it may show some more nonspecific effect of photoreactivating enzyme on the ability of the DNA to act as a substrate in the assay. In addition, irradiation of the DNA under conditions which supposedly introduce only pyrimidine dimers into the DNA (Table III) did not convert the DNA into a substrate for the binding protein. These two results suggested that the protein was recognizing some form of damage in the DNA other than pyrimidine dimers. A variety of other possible uv- induced lesions such as single-strand nicks or single-strand gaps were also eliminated as substrates for the binding protein.

The study of DNA repair has focused primarily on the fate of the pyrimidine dimer, the most stable and easily measured photoproduct. However, it is now becoming apparent that other photoproducts may also be introduced into DNA at physiological doses of uv light. Indirect evidence for the presence of nondimer photodamage has been presented by the work of Paterson et al. (1974). These workers measured repair replication in primary chick cells which had been incubated either under photoreactivating conditions or in the dark following uv irradiation. Using the *M. luteus* correndonuclease II as a probe for dimers, they found that, under conditions in which essentially all of the pyrimidine dimers had been photoreactivated, the cells still demonstrated repair replication. These results suggest the presence of some nonpyrimidine dimer photodamage introduced at low uv doses and subject to some form of excision repair.

Another piece of indirect evidence for uv-induced nonpyrimidine dimer photodamage has been the finding of a uv, γ-specific endonuclease in mammalian cells. This enzyme has been extensively purified from calf thymus by Bacchetti and Benne (1975) who have confirmed that the enzyme is not directed toward pyrimidine dimers. It is possible that the placental binding protein and the uv γ-endonuclease are directed toward the same photoproduct. However, the two proteins differ significantly in their dose–response curves, with the endonuclease demonstrating a linear response up to exposures of 450 J/m^2 while the binding protein shows saturation with DNA irradiated at 150 J/m^2 or greater. This difference may reflect a real difference in the photoproducts recognized by the two proteins, or it may simply reflect a difference intrinsic to the assays employed. If a single photoproduct on an RFI circle is all that is needed to interact with a molecule of binding protein and retain the entire circle on a filter, then the binding assay is essentially a "one-hit" assay and is much more sensitive than the endonuclease assay. This difference in sensitivity could account for the difference in the dose–response curves. It may be possible to determine if the two proteins are directed toward a common photoproduct by measuring the effect of saturating levels of binding protein on the activity of the uv γ-endonuclease. In this regard, it is of interest that the binding protein did not inhibit the action of the *M. luteus* correndonuclease II on irradiated DNA, nor did pretreatment of irradiated DNA with the *M. luteus* correndonuclease II affect binding protein activity.

It is still difficult to predict the precise nature of the unidentified photodamage. Sugar phosphates do not absorb light above 220 nm. Although it has generally been accepted that the pyrimidine bases are much more sensitive to uv light than are the purines, recent reports (Salomon and Elad 1974; Iv-

anchenko et al., 1975) have suggested that under certain conditions the photoefficiency of purine conversions can be nearly that of pyrimidines. Another candidate for the photoproduct recognized is the pyrimidine adduct reported by Varghese and Wang (1967). It may now indeed prove possible to use the placental binding protein as a tool for the isolation and identification of this unknown lesion.

The biological significance of the placental binding protein and of the photodamage it recognizes remains to be determined. The activity has been found in the three complementation groups of xeroderma pigmentosum examined, suggesting that it does not play a role in the etiology of this disease. However, the two remaining complementation groups, as well as cells from patients with De Sanctis-Cacchione syndrome, have yet to be examined. It should be possible to gain some information on the biological role of the photodamage by using the binding protein as a probe for this lesion with DNA isolated from cells at various times after irradiation in vivo. Such information should tell us whether the unknown photodamage is being eliminated from the DNA.

Acknowledgments

We thank Dr. A. Braun, Dr. J. Doniger, Dr. L. Hamilton, Dr. J. Laval, Dr. I. Mahler, and Dr. S. Riazuddin for their encouragement during the course of this work.

References

Bacchetti, S., and Benne, R. (1975), *Biochim. Biophys. Acta 390,* 285.

Bacchetti, S., van der Plas, A., and Veldhuisen, G. (1972), *Biochem. Biophys. Res. Commun. 48,* 662.

Benzinger, R., Kleber, I., and Huskey, R. (1971), *J. Virol. 7,* 645.

Braun, A., and Grossman, L. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1838.

Brent, T. P. (1973), *Biophys. J. 13,* 399.

Cleaver, J. H. (1974), *Adv. Radiat. Biol. 9,* 1.

Cole, R. S. (1971), *Biochim. Biophys. Acta 254,* 30.

Deboer, G., Klinghoffer, O., and Johns, H. E. (1970), *Biochim. Biophys. Acta 213,* 253.

Doniger, J., and Grossman, L. (1975), *Biophys. Soc. Abstr. 15,* 297a.

Grossman, L., Braun, A., Feldberg, R., and Mahler, I. (1975), *Annu. Rev. Biochem. 44,* 19.

Ivanchenko, V. A., Titschenko, A. I., Budowsky, E. I. Simukova, N. A. and Wulfson, N. S. (1975), *Nucleic Acids Res. 2,* 1365.

Johns, H. E., LeBlanc, J. C., and Freeman, K. B. (1965), *J. Mol. Biol. 13,* 849.

Kaplan, J. C., and Grossman, L. (1971), *Methods Enzymol. 21,* 240.

Lindahl, T. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3649.

Lindahl, T., and Andersson, A. (1972), *Biochemistry 11,* 3618.

Litman, R. M. (1968), *J. Biol. Chem. 243,* 6222.

Madden, J. J., and Werbin, H. (1974), *Biochemistry 13,* 2149.

Mahler, I. (1967), *Methods Enzymol. 12A,* 693.

Molineux, I. J., Friedman, S., and Gefter, M. L. (1974), *J. Biol. Chem. 249,* 6090.

Paterson, M. C., Lohman, P. H. M., DeWeerd-Kastelein, E. A., and Westerveld, A., (1974), *Biophys. J. 14,* 454.

Paterson, M. C., Lohman, P. H. M., and Sluyter, M. L. (1973), *Mutat. Res. 19,* 245.

Rahn, R. O., Setlow, J. K., and Landry, L. C. (1973), *Photochem. Photobiol. 18,* 67.

Riggs, A. D., Suzuki, H., and Bourgeois, S. (1970), *J. Mol. Biol. 48,* 67.

Robbins, J. H., Kraemer, K. H., Lutzner, M. A., Festoff, B. W., and Coon, H. G. (1974), *Ann. Intern. Med. 80,* 221.

Salomon, I., and Elad, D., (1974), *Biochem. Biophys. Res. Commun. 58,* 890.

van Lancker, J. L., and Tomura, T. (1974), *Biochim. Biophys. Acta 353,* 99.

Varghese, A. J. (1972), *Photophysiology 7,* 207.

Wilkins, R. J. (1973), *Int. J. Radiat. Biol. 24,* 609.

Yoneda, M., and Bollum, F. J. (1965), *J. Biol. Chem. 240,* 3385.