Preferential Binding of the Xeroderma Pigmentosum Group A Complementing Protein to Damaged DNA

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ABSTRACT: The xeroderma pigmentosum group A complementing protein (XPAC) is involved in an early step of nucleotide excision repair, the main process that removes UV damage and many chemical lesions from DNA. To explore the properties and function of XPAC, recombinant protein encoded by the human XPAC cDNA was expressed with an N-terminal polyhistidine tag in *Escherichia coli* and purified to homogeneity. The soluble fusion protein could correct the repair defect *in vitro* of XP-A cell extracts. XPAC protein bound to DNA with a preference for UV-irradiated over nonirradiated DNA, as determined by a gel electrophoresis mobility shift assay with a 258 base pair DNA fragment (the association constant was $\sim 3 \times 10^6$ M^{-1} for the fragment irradiated with 6 kJ/m^2 UV light). Removal of cyclobutane pyrimidine dimers from UV-irradiated DNA by enzymatic photoreactivation did not significantly reduce binding of XPAC to the irradiated fragment, indicating that binding was mostly due to (6–4) photoproducts, with a preference for a (6–4) photoproduct over an undamaged base pair up to 300-fold. Undamaged single-stranded DNA competed about 4-fold more effectively than undamaged double-stranded DNA for binding of XPAC to a UV-irradiated fragment. In addition, XPAC bound to DNA treated with the chemotherapeutic agent *cis*-diamminedichloroplatinum(II). The results suggest that XPAC functions as a key component in recognition of DNA damage during repair.

A defect in nucleotide excision repair, the main process that removes UV damage and many chemical lesions from DNA, can have severe consequences for an organism. This is clearly demonstrated by the human disorder xeroderma pigmentosum (XP),[1] a recessively inherited syndrome that encompasses seven repair-deficient complementation groups (XP-A to XP-G) and a variant group. The XP-A form is one of the most frequently encountered and is caused by inactivating mutations in the *XPAC* gene (Satokata et al., 1992a,b,c). The inability of an XP-A individual's cells to remove DNA damage results in a greatly elevated incidence of skin lesions, including tumors (Cleaver & Kraemer, 1989). The *XPAC* gene encodes the XPAC polypeptide of 273 amino acids (M_r 31,347) and contains a zinc finger motif that is necessary for the function of the gene product (Miura et al., 1991; Miyamoto et al., 1992).

The XPAC protein has an essential function in nucleotide excision repair, where it works in coordination with other repair proteins, including additional *XP* gene products. Nucleotide excision repair synthesis does not occur in cells where XPAC protein is absent or inactivated by mutation. The defect in repair can be traced to a failure of XP-A cells to incise DNA at damaged sites in an early stage of repair (Fornace et al., 1976; Erixon & Ahnström, 1979; Kaufmann & Briley, 1987; Squires & Johnson, 1988). Studies with cell extracts likewise show that XPAC protein is needed *in vitro* for incision of damaged DNA (Shivji et al., 1992). Functional XPAC is not necessary for viability of mammalian cells, and humans can survive and undergo early development normally even when the gene is completely inactivated. However, many individuals with XP-A acquire progressive neurological abnormalities during the first few decades of life. This has been hypothesised to occur by gradual accumulation of unrepaired lesions in metabolically active (but nondividing) neurons (Robbins, 1989). Indeed, a class of DNA lesions introduced by an oxidative mechanism is removed by nucleotide excision repair, and this removal is defective in XP-A cell extracts (Satoh et al., 1993).

We previously isolated small quantities of XPAC from calf thymus tissue on the basis of its ability to correct the repair deficiency of XP-A cell extracts *in vitro* (Robins et al., 1991). The purified protein had no apparent catalytic activity (such as nuclease activity). However, XPAC was found to bind DNA and had a preference for UV-irradiated DNA in a filter-binding assay (Robins et al., 1991), suggesting that XPAC plays a role in the recognition of DNA damage during repair. Other investigators, however, independently isolated XPAC and did not detect an increased affinity of the protein for UV-irradiated DNA (Eker et al., 1992; Sugano et al., 1991). In order to study the DNA binding further, we have now purified active recombinant XPAC protein from *Escherichia coli*. The DNA-binding properties of the protein were investigated using a gel mobility shift assay with damaged and undamaged double-stranded DNA fragments.

MATERIALS AND METHODS

Plasmids. XPAC cDNA (nucleotides −23 to +862) cloned into the *Eco*RI site of the expression vector pGEM7Zf (+) (Promega) was a kind gift of Dr. K. Tanaka (Tanaka et al., 1990). To facilitate subcloning into expression vectors, the cDNA was amplified by the polymerase chain reaction in a 100-μL mixture containing 10 ng of pGEM7Zf (+)/*XPAC* DNA, deoxynucleoside triphosphates (200 μM each), 20 pmol

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[1] Abbreviations: XP, xeroderma pigmentosum; XPAC, xeroderma pigmentosum group A complementing protein; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; T4 endo V; pyrimidine dimer-DNA glycosylase/endonuclease from phage T4.

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each of primers 5′-CCACATATGGCGGCGGC-CGACGGGGCTTT-3′ and 5′-ACTGTCGACTCA-CATTTTTTCGTATGT-3′, and 2.5 units of Pfu polymerase (Stratagene) in buffer 2 supplied by the manufacturer. Reactions were for 30 cycles of 1 min at 94 °C, 1.5 min at 56 °C, and 3 min at 72 °C. The underlined G in the second primer removed an *Nde*I site from the cDNA and changed codon 270 (Tyr) from TAT to TAC. The 838-bp product had an *Nde*I restriction site near one end and a *Sal*I site near the other; it was digested with these enzymes, ligated into *Nde*I- and *Sal* I-cleaved pT7.7 (Studier, 1991), and transformed into *E. coli* strain DH5α. The entire *XPAC* cDNA from a selected clone was sequenced by the dideoxy method; no errors due to the possible infidelity of DNA synthesis by Pfu polymerase were detected. The cDNA was excised from pT7.7 with *Nde*I and *Sal*I and cloned into the same sites of pCITE-2a (Novagen) in order to provide suitable restriction sites for introduction into pET-15b (Novagen). *XPAC* was excised from pCITE-2a with *Nde*I and *Xho*I and cloned into identical sites in pET-15b.

Overproduction and Purification of XPAC Protein. pET-15b/*XPAC* was transformed into the *E. coli* expression strain BL21 (DE3) pLysS (Studier, 1991) and maintained on media containing chloramphenicol and ampicillin (both at 25 μg/mL). One colony was inoculated into 200 mL of growth medium (2× TY containing 1% glycerol, 50 μg/mL ampicillin, and 25 μg/mL chloramphenicol) at 37 °C. At an OD_{600} of 0.6 the cells were collected by centrifugation, washed with phosphate-buffered saline (PBS), and resuspended in 2 L of fresh growth medium at 37 °C. At an OD_{600} of 0.64 the cells were collected, washed with PBS, resuspended in 2 L of fresh growth medium without glycerol, but with 1 mM isopropyl β-thiogalactopyranoside (IPTG) and 10 μM $ZnCl_2$, and incubated for a further 3 h at 28 °C. If cells were incubated at 37°C during induction, the majority of the recombinant XPAC was produced in an insoluble form. Some of this could be solubilized in guanidine hydrochloride and refolded by stepwise dialysis to give protein (46 μg/mL) of limited activity. Much better results were obtained by growing the cells at 28 °C, which gave a high yield of soluble XPAC. Cells were harvested, washed with PBS, and stored frozen at −80 °C.

Thawed cells were resuspended in 50 mL of buffer A (50 mM potassium phosphate, pH 8.0, 100 mM KCl, 0.01% Nonidet P 40, 1 mM EDTA, and 10% glycerol) containing 0.5 μM phenylmethanesulfonyl fluoride (PMSF) and 8 μg/mL aprotinin (both from Sigma) and disrupted by sonication on ice. Subsequent steps were performed at 4° C. The sonicate was centrifuged at 85000*g* in an SW28 rotor (Beckman) for 1 h. The supernatant (35 mL, 980 mg of protein) was resonicated briefly to shear any residual DNA and was loaded onto a 3-mL Ni^{2+} NTA–agarose (Qiagen) column in buffer A at a flow rate of 20 mL/h. The column was washed sequentially with buffer A containing 1, 20, 50, and 100 mM imidazole. XPAC protein was detected by Coomassie staining of 12% sodium dodecyl sulfate (SDS)–polyacrylamide minigels. The major peak of XPAC eluted in the 50 mM imidazole fraction (24 mL, 24 mg of protein). Aprotinin (8 μg/mL) and 1 mM dithiothreitol (DTT) were added to this fraction and loaded at a flow rate of 10 mL/h onto a 1.5-mL native DNA–cellulose column (Pharmacia) preequilibrated with buffer B (25 mM Hepes·KOH, pH 7.8, 10% glycerol, 1 mM EDTA, and 1 mM DTT) containing 0.1 M KCl. Bound protein was eluted with a 20-mL linear gradient of 100 mM to 1 M KCl in buffer B. XPAC eluted over a broad range with a peak at around 575 mM KCl. Fractions containing XPAC were pooled (8 mL, 3.2 mg of protein), adjusted to 1 mM potassium phosphate and 25 μg/mL aprotinin, and loaded on a 0.8-mL hydroxyapatite column preequilibrated with buffer C (25 mM Hepes·KOH, pH 7.8, 10% glycerol, 0.4 mM EDTA, 1 mM DTT, and 0.5 M KCl) containing 1 mM potassium phosphate at a flow rate of 10 mL/h. Bound protein was eluted with a 20-mL linear gradient of 1 to 200 mM phosphate in buffer C. XPAC eluted with a peak at 85 mM phosphate. Fractions containing pure protein (as judged by silver staining of gels) were pooled, and the protein concentration was determined with a Coomassie reagent kit (Pierce). The yield of homogeneous protein was 1.64 mg at 745 μg/mL.

In Vitro DNA Repair Assay. Reaction mixtures (50 μl) contained 250 ng each of UV-irradiated plasmid pBluescript KS^+ (Stratagene) and undamaged plasmid pHM14 (Rydberg et al., 1990) in buffer as described (Robins et al., 1991). Extracts were prepared as described previously (Jones et al., 1992). Reaction mixtures were incubated for 3 h at 30 °C. Plasmid DNA was purified from the reaction mixtures, linearized with *Bam*HI, and separated on a 1% agarose gel containing 0.5 μg/mL ethidium bromide. Repair synthesis was quantified by autoradiography and densitometry.

Substrates for DNA Binding. A double-stranded DNA probe was prepared by digesting the pBluescript-derived plasmid pHM14 with *Hin*dIII and *Pvu*II to give 4 fragments of 2511, 792, 258, and 162 bp, which were separated on a 2% agarose gel in buffer containing 40 mM Tris–acetate (pH 8.0), 1 mM EDTA, and no ethidium bromide. The 258-bp fragment (corresponding to nucleotides 715–972 of pBluescript) was purified by electroelution and radiolabeled by filling in the *Hin*dIII-generated 5′ overhang. Fragment (500 ng) in 250 μL of buffer containing 10 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 7.5 mM DTT, 20 μM each of TTP, dCTP, and dGTP, 200 μCi of [α-^{32}P]dATP (3000 Ci/mmol; Amersham) and 5 units *E. coli* DNA polymerase I large (Klenow) fragment (New England Biolabs) was incubated at 20 °C for 20 min. Cold dATP (20 μM) was added, and incubation continued for 5 min. Unincorporated label was removed on a NAP-5 column (Sephadex G25, Pharmacia) preequilibrated with 10 mM Tris·HCl (pH 8.0) and 1 mM EDTA (TE buffer). DNA was extracted with phenol/chloroform (1:1), ethanol precipitated, and dissolved in TE buffer. Radioactivity in the fragment was quantified by precipitating samples with trichloroacetic acid onto glass fiber filters and scintillation counting.

UV Irradiation and Photoreactivation. DNA was UV-irradiated at room temperature with a 254-nm (peak) source in TE buffer at a fluence rate of 2 W/m^2 as determined by a Latarjet dosimeter. For photoreactivation, 12.5 ng of DNA was in 50 μL of buffer containing 10 mM potassium phosphate, pH 7, 100 mM NaCl, 0.1% bovine serum albumin, and 5 mM DTT in the presence or absence of 44 ng of *Anacystis nidulans* photolyase (Eker et al., 1990), kindly provided by Dr. A. P. M. Eker. Samples were then irradiated at room temperature with blue light (Philips TLDK 30W/03 lamps, λ_{max} = 420 nm) for 30 min. After incubation with proteinase K (100 μg/mL) and SDS (1%) for 30 min at 37 °C and extraction with phenol/chloroform, DNA was recovered by ethanol precipitation. The efficacy of photoreactivation was determined by incubating 0.6 ng of DNA in a reaction mixture (10 μl) containing 50 mM Tris·HCl (pH 7.9), 1 mM EDTA, and 1 mM DTT, with or without 15 ng of T4 pyrimidine dimer DNA glycosylase (T4 endo V, a gift of R. S. Lloyd) at 37 °C for 45 min. The reaction was terminated by adding 16% formamide and 3.5 mM EDTA and heating at 68 °C prior

to loading on a denaturing 8% polyacrylamide gel. The sites of incision were located with a marker produced by dideoxy sequencing of pHM14 using the primer 5′-AGCTTATC-GATA-3′.

Other Modified Substrates. DNA (25 ng in 50 μL of TE buffer) was treated with *cis*-diamminedichloroplatinum(II) (cisplatin; Sigma) by adding an equal volume of the drug dissolved in water to give final cisplatin concentrations of 0, 30, 60, 120, 240, and 480 nM. Reaction mixtures were incubated at 37 °C for 16 h in the dark, reactions were terminated by adding NaCl to 500 mM, and DNA was recovered by ethanol precipitation. Cisplatin–DNA lesions were reversed by incubating 12.5 ng of adducted DNA with 0.2 M NaCN in 10 mM Tris·HCl (pH 8.0) and 0.5 mM EDTA at 37 °C for 16 h.

For modification with psoralens, 40 ng of DNA was incubated in 90 μL of TE buffer containing either ^{3}H-labeled 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen (220 μg/mL, 12 Ci/mM) or ^{3}H-labeled 5-methylisopsoralen (330 μg/mL, 6 Ci/mM), both from HRI Associates, Berkeley, CA. The mixture was illuminated with fluorescent UV-A lamps (λ_{max} = 365 nm), and the DNA was extracted with phenol/chloroform and ethanol-precipitated. The number of psoralen adducts per fragment was determined by scintillation counting.

A 90-mer double-stranded DNA substrate with the sequence 5′-ACGTTGTAAAACGACGGCCAGTGAATTCCC-GGGGATCCGTCGACCTGCAGCCAAGCTTGGCG-TAATCATGGTCATAGCTGTTTCCTGTGT-3′ on one strand was a gift of S. Griffin and P. Karran. The complementary strand of the control substrate contained C opposite the underlined position, and the mismatched substrate contained a T. Oligonucleotides were labeled as described (Branch et al., 1993).

DNA Binding Assay. In all cases, irradiated or chemically modified DNA was ethanol-precipitated and redissolved twice after treatment to remove impurities that could interfere with DNA-binding assays. DNA binding reaction mixtures (20 μL) contained 25 mM Hepes·KOH (pH 7.0), 0.2 ng of ^{32}P-labeled DNA, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 670 ng of bovine serum albumin (BSA), 10% glycerol, 1 mM DTT, 0.01% Nonidet P 40, and XPAC protein or BSA control as indicated. After 30 min of incubation at 30 °C, glutaraldehyde was added at a concentration of 0.25% for 5 min. The reaction mixtures were loaded immediately on vertical 1.5-mm 2% agarose gels, and electrophoresis took place for 2 h at 150 V in 40 mM Tris–acetate (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$, and 10% glycerol. Gels were fixed in 8% trichloroacetic acid for 30 min, dried, and subjected to autoradiography. Levels of free probe were determined by scintillation counting of excised bands. Duplicate samples were analyzed in all experiments, in addition to control reaction mixtures that contained equivalent amounts of additional BSA in place of XPAC protein. The level of free probe in a given reaction mixture containing XPAC was normalized against the corresponding BSA control sample.

Competition Experiments. As an indication of the binding constant to undamaged DNA, the extent of binding of XPAC protein to a labeled fragment containing specific sites was measured in the presence of an excess of unlabeled, undamaged DNA. The inverse of the apparent specific equilibrium constant, K_{app}^{-1}, is a linear function of the concentration of competitor, [N] (Mazur & Grossman, 1991): $K_{app}^{-1} = ([S]_0 - [AS])([A]_0 - [AS])/[AS] = 1/K_s + K_N[N]/K_s$, where $[S]_0$ and $[A]_0$ are the total concentrations of damaged sites and XPAC monomers, respectively, and [AS] is the detected concentration of XPAC–DNA complexes. Single-stranded or double-stranded M13mp18 DNA (25–200 ng) was added to 20-μL binding reaction mixtures containing 0.2 ng of UV-irradiated (6 kJ/m^2) 258-bp DNA fragment and 0.5 μg of XPAC. For the purpose of Figure 5, the effective concentration of damaged sites was calculated from the number of (6–4)photoproducts present per probe fragment [an average of 0.25 (6–4)photoproducts induced per 100 bp by each kJ/m^2].

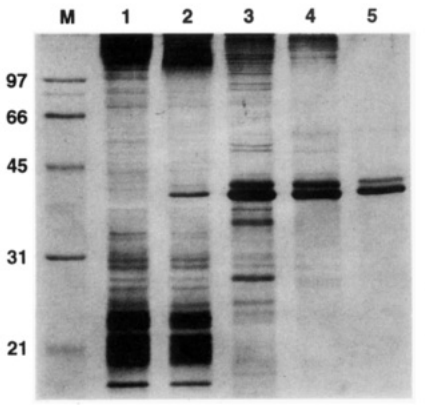


FIGURE 1: Purification of His-tagged XPAC fusion protein expressed in *E. coli*. Proteins were separated on a 12% SDS–polyacrylamide minigel (0.75-mm thickness) and silver-stained. Lane M, 0.5 μL Bio-Rad silver-stained markers; lane 1, uninduced bacterial whole-cell lysate (2.8 μg); lane 2, induced bacterial cell lysate supernatant fraction (2.8 μg); lane 3, pooled fractions after Ni^{2+} NTA–agarose chromatography (4 μg); lane 4, pooled fractions after DNA–cellulose chromatography (0.8 μg); lane 5, pooled fractions after hydroxyapatite chromatography (0.75 μg).

RESULTS

Overproduction and Purification of XPAC Protein. Initially, XPAC cDNA was cloned into the *Escherichia coli* vector pT7.7 to produce recombinant protein. Expression of the full-length protein was very poor, and extensive purification was required, but the polypeptide was able to complement the DNA repair defect of XP-A cell extracts *in vitro* (not shown). In contrast, a truncated form of XPAC lacking the amino-terminal 58 amino acids was synthesized from the same vector with a much higher yield, but in this case most of the product was insoluble. Resolubilized product had some complementing activity, consistent with information indicating that the N-terminal region of XPAC is partially dispensable for its main function. Transfection of mutant XPAC cDNA lacking the 5′ region of the gene confers considerable UV resistance on XP-A cells (Tanaka et al., 1990), and the N-terminal sequence of XPAC protein is very divergent between species (Shimamoto et al., 1991).

High expression of soluble XPAC was achieved by producing a fusion protein with an N-terminal leader sequence of 20 amino acids including a hexahistidine oligopeptide (His-tag). The latter motif allows purification of the product on a column containing immobilized nickel ions, and the observations noted above suggested that the additional amino acids would have little effect on the activity of the protein. *XPAC* cDNA was cloned into the vector pET-15b under control of a regulated T7 RNA polymerase promoter and propagated in the *E. coli* strain BL21 (DE3) pLysS. Addition of IPTG to the growth medium results in synthesis of T7 RNA polymerase and production of XPAC protein. When the culture was incubated at 28 °C, ~50% of the protein was soluble, and it was purified to apparent homogeneity in three steps (Figure 1). Both bands of the characteristic protein doublet in the final product cross-

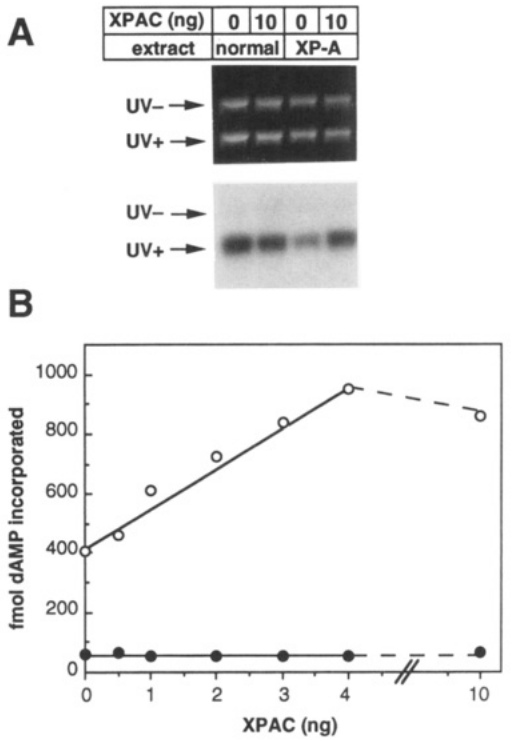


FIGURE 2: Complementation of XP-A whole-cell extracts by purified recombinant XPAC protein. (A) His-tagged XPAC protein was added as indicated to whole-cell extract protein (125 μg) from repair-proficient normal 1BR.3N cells or XP12RO (XP-A) cells and incubated for 3 h at 30 °C in repair synthesis buffer that contained irradiated (UV+) and nonirradiated (UV–) plasmid DNA and [α-^{32}P]-dATP. DNA was isolated, linearized, resolved by electrophoresis on an ethidium bromide-stained agarose gel (upper panel), dried, and subjected to autoradiography (lower panel). (B) Quantification of repair synthesis in UV-irradiated DNA (○) and nonirradiated DNA (●).

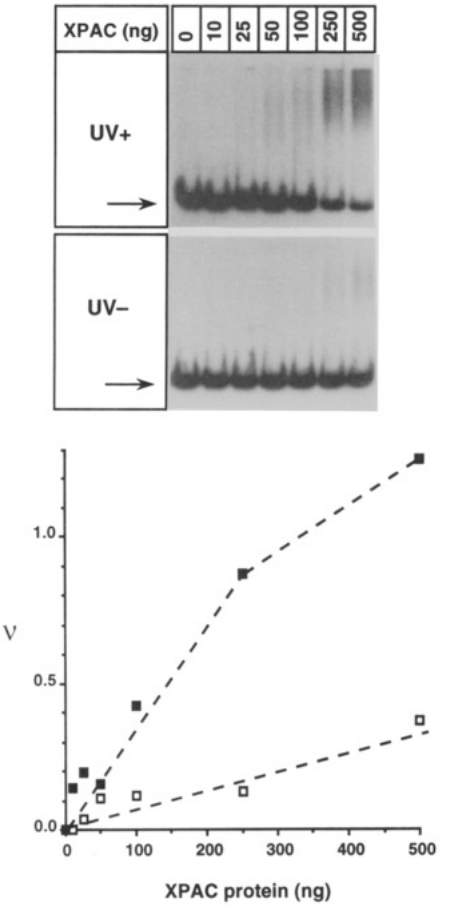


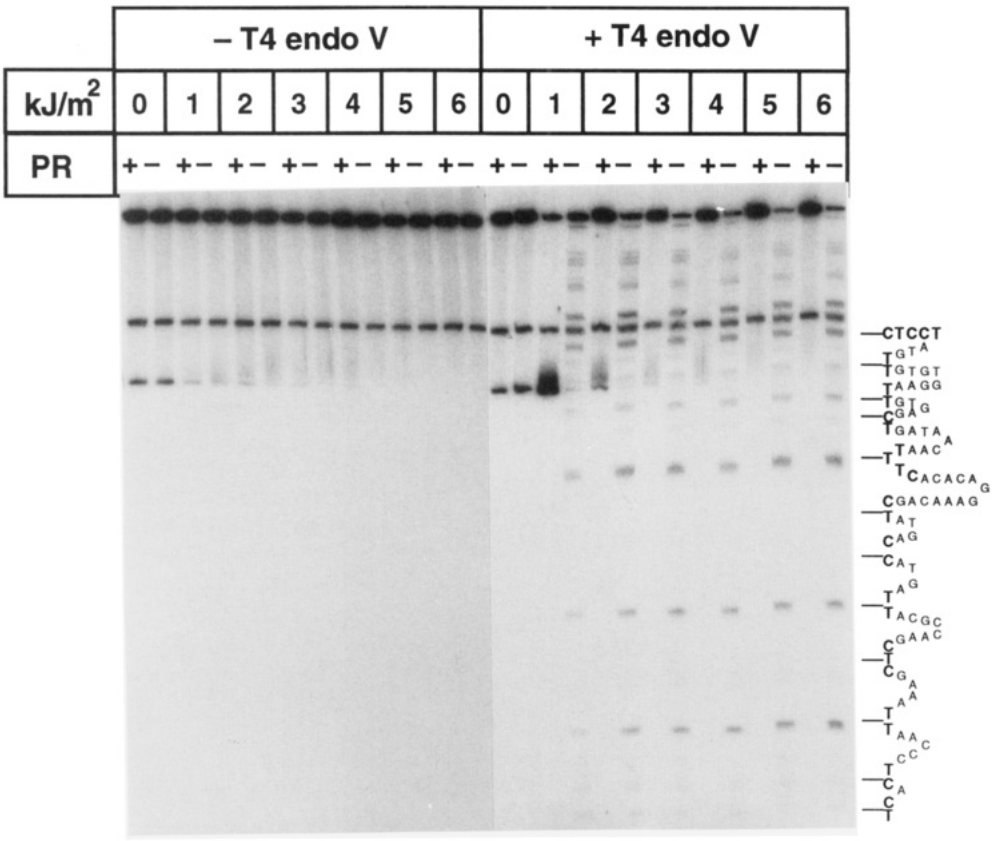
FIGURE 3: Titration of XPAC UV-damaged DNA binding activity. (A, top) A ^{32}P-labeled 258-bp double-stranded DNA probe (0.2 ng), UV-irradiated (6 kJ/m^2, UV+) or unirradiated (UV–), was incubated with recombinant XPAC protein for 30 min at 30 °C and separated on vertical 2% agarose gels. Both free probe (indicated by the arrow) and slower migrating complexes are visible. (B, bottom) Quantification of binding of XPAC to UV-irradiated DNA (■) and nonirradiated DNA (□). Bands representing free unbound probe (shown in panel A) were excised, quantified by scintillation counting, and normalized against a parallel experiment performed with bovine serum albumin in place of XPAC. The parameter ν (negative natural logarithm of the fraction of unbound probe) is plotted against the amount of protein in each reaction mixture; each point is an average of two experiments.

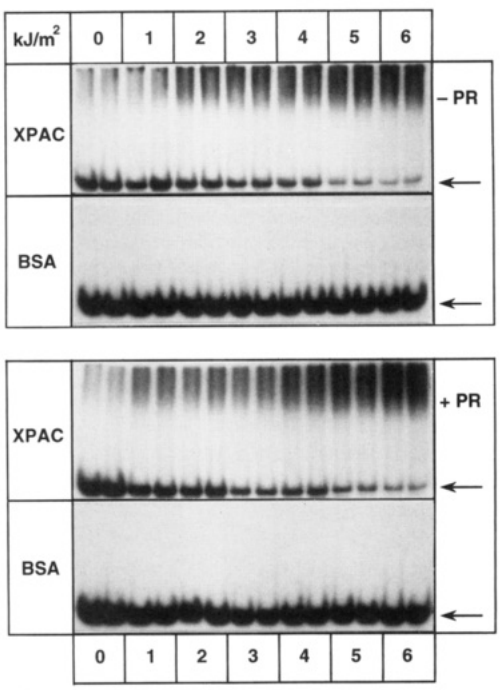
reacted with antisera raised against peptides derived from the cDNA sequence (Robins et al., 1991). In our experience, the proportion of the lower band of the XPAC doublet is correlated with the complementing activity of the preparation.

Purified XPAC fusion protein complemented the DNA repair activity of XP-A cell extract (Figure 2) and had a specific activity of ~350 units/μg, about 30-fold lower than the value quoted for XPAC isolated from calf thymus tissue (Robins et al., 1991). One unit of complementing activity is the amount needed to double the repair signal obtained with XP-A extract alone, and estimates of specific activity obtained with this definition of a complementing unit depend strongly on the background synthesis produced in UV-irradiated DNA by the particular XP-A cell extract used (this background synthesis does not represent true nucleotide excision repair). Part of the difference in specific activity between the calf thymus and *E. coli* preparations may arise because of this complication. It is also possible that the protein produced in *E. coli* might lack a posttranslational modification that affects activity, or that the presence of the N-terminal tag quantitatively affects the complementing activity, despite the considerations listed earlier. Further, recombinant XPAC was identified visually by staining protein from column fractions, and it is conceivable that not all of the pooled fractions were equally active.

XPAC has a Stokes radius of 33 Å as determined by gel filtration chromatography, and the recombinant protein had an S value of 2.2 as determined by velocity sedimentation in a glycerol gradient (not shown). These hydrodynamic values were used to derive a molecular mass of 30 kDa for the protein (with a frictional ratio of 1.6), using the method of Siegel and Monty (1966) and assuming a partial specific volume of 0.725 cm^3/g. This is close to the molecular mass of 31 kDa predicted from the *XPAC* cDNA sequence and indicates that XPAC exists in solution as a monomer.

Preferential Binding to UV-Irradiated DNA. Once it was established that recombinant XPAC protein could complement repair-defective XP-A extracts, the DNA-binding characteristics of the protein were investigated. A previous preliminary study using a nitrocellulose filter-binding assay indicated that XPAC from calf thymus had a preferential affinity for UV-irradiated double-stranded DNA (Robins et al., 1991). In the present experiments we used a gel mobility shift assay to examine the binding of XPAC to a 258-bp DNA fragment labeled at one end with ^{32}P. Figure 3A shows an example in which the DNA fragment was irradiated with UV (6 kJ/m^2, top) or nonirradiated (bottom) and incubated with increasing amounts of XPAC before the mixture was subjected to electrophoresis in order to detect DNA–protein complexes. XPAC bound to irradiated DNA (and less avidly to undamaged DNA), as shown by a reduction in the amount of free probe with increasing amounts of XPAC in the reaction mixture and the appearance of slower migrating complexes.





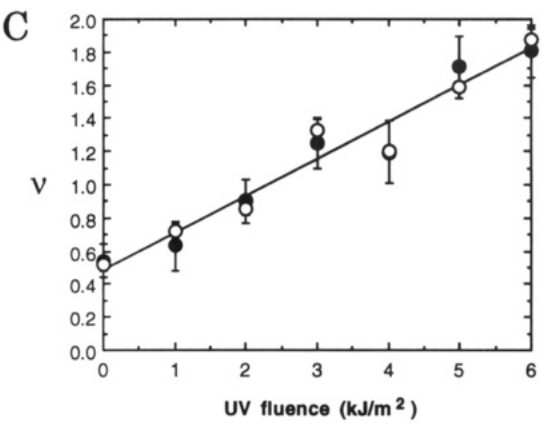


FIGURE 4: Effect of photoreactivation of UV-irradiated DNA on XPAC binding. (A) T4 endo V digestion of photoreactivated DNA substrates. The double-stranded 258-bp DNA fragment used in the XPAC-binding experiments was irradiated with the indicated UV fluence, illuminated under blue light in the presence (PR+) or absence (PR–) of *A. nidulans* photolyase, and then incubated with or without T4 endo V. The 3′-labeled products were resolved on 8% polyacrylamide denaturing gels. The major, slowest migrating band is the labeled strand of the probe; a few minor bands of unknown origin did not interfere with the assay. At the right, the partial sequence of the ^{32}P-labeled strand of the probe is indicated (nucleotides 773–865, numbering from pBluescript). (B) Gel mobility shift analysis of XPAC binding to UV irradiated DNA after treatment with photolyase. DNA was incubated with 500 ng of XPAC or BSA in a gel mobility shift assay. Upper panels, No photoreactivation of DNA; lower panels, photoreactivated DNA. Duplicate assays were loaded at each UV fluence. (C) Quantification of photoreactivation experiments. The mean value of ν from three experiments is plotted against the UV fluence. Points represent DNA which had been incubated with (○) or without photolyase (●). Bars represent the standard error of the mean.

Parallel control reaction mixtures with BSA in place of XPAC showed no complexes (see Figure 4B). Binding of XPAC to DNA gave a diffuse mobility shift, even at low protein concentration. This pattern was observed with a variety of different buffer systems, gel compositions, and running temperatures and apparently reflects partial dissociation of

complexes during electrophoresis. Brief treatment with glutaraldehyde allowed the complexes to be most reproducibly detected.

For quantification, the reduction in the amount of free probe upon binding was measured by scintillation counting of bands excised from the dried gel. Counting of entire excised lanes established that all of the probe added to the reaction was present in the gel, either as unbound DNA or in complexes. The negative logarithm of the fraction of unbound probe in a reaction (ν) is proportional to the average number of XPAC molecules bound per fragment and allows quantification of the binding (Figure 3B). The reciprocal of the concentration at which 1/2 of the probe is bound ($\nu = 0.69$) gives an estimate of the equilibrium association constant. For the UV-irradiated probe K_{UV} is about 3×10^6 M^{-1}, a value in agreement with a Scatchard plot of the data. For nonirradiated probe, the constant is about 6×10^5 M^{-1}. Below, we discuss the interpretation of these values with respect to the affinity of XPAC for damaged *vs* nondamaged sites.

Removal of Cyclobutane Pyrimidine Dimers from UV-Irradiated DNA. The two major types of lesions introduced into DNA by UV irradiation at 254 nm are cyclobutane pyrimidine dimers and (6–4) photoproducts, formed in a ratio of about 3:1 (Mitchell, 1988). Mammalian cells remove both photoproducts by XPAC-dependent nucleotide excision repair, and they remove (6–4) photoproducts 5–10-fold faster than cyclobutane dimers (Mitchell, 1988). Experiments *in vitro* also show that most repair of UV-irradiated DNA by normal cell extracts takes place at (6–4)photoproducts (Wood, 1989; Sibghat-Ullah et al., 1989; Szymkowski et al., 1993).

In order to determine the relative contribution of the different photoproducts to the preferential binding of XPAC to UV-irradiated DNA, we made use of enzymatic photoreactivation to selectively repair the cyclobutane pyrimidine dimers. The DNA probe fragment was UV-irradiated and then incubated with photolyase from *A. nidulans* in the presence of blue photoreactivating light. Control samples were illuminated under the same conditions in the absence of photolyase. To confirm that photoreactivation took place, DNA was treated with T4 pyrimidine dimer-DNA glycosylase (T4 endo V), which cleaves the phosphodiester bond at sites of cyclobutane pyrimidine dimers. Separation on a denaturing polyacrylamide gel revealed the cleavage products (Figure 4A, right). The fraction of DNA cleaved at each fluence was quantified and showed that an average of ~2 cyclobutane pyrimidine dimers were introduced per fragment by 1 kJ/m^2 irradiation. After photoreactivation, sites susceptible to cutting with T4 endo V disappeared, and the DNA was restored to the same pattern as without T4 endo V (compare left and right sides of Figure 4A, lanes +PR).

Photoreactivated and non-photoreactivated substrates were used in DNA-binding assays with a constant amount of XPAC protein (0.5 μg). An autoradiograph of a gel from a representative experiment is shown in Figure 4B, and quantification of data from three separate experiments is given in Figure 4C. Strikingly, complete removal of cyclobutane pyrimidine dimers by photoreactivation had no significant effect on this binding (within the detection limits of the assay). Thus, XPAC has a marked preference for non-cyclobutane dimer lesions in irradiated DNA, very likely for (6–4) photoproducts. These abundant photoproducts are known to be well-recognized by the XPAC-dependent nucleotide excision repair system and are not reversed by the photolyase utilized here. We further note that the formation of DNA–XPAC complexes increased in proportion to the UV fluence,

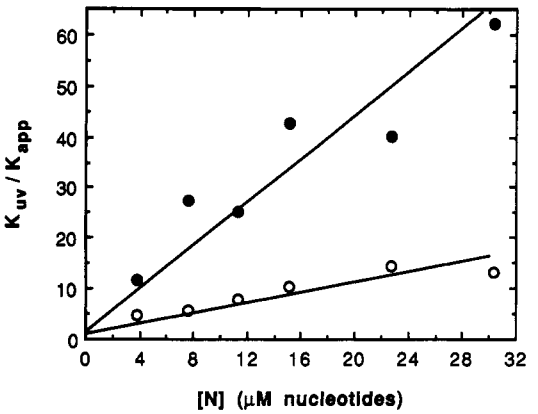


FIGURE 5: Effect of addition of competitor DNA to binding reactions. Circular single-stranded (●) or double-stranded M13 DNA (○) was added at the indicated nucleotide concentrations [N] to binding reaction mixtures containing 0.2 ng of UV-irradiated probe (6 kJ/m^2) and 0.5 μg of XPAC, and the data was analyzed as described in the text, with $K_{UV} = 3 \times 10^6$ M^{-1}.

even in the range from 2 to 6 kJ/m^2 where cyclobutane pyrimidine dimer formation and reversal has reached photochemical equilibrium. The yield of (6–4) photoproducts continues to increase with UV fluence above 2 kJ/m^2 (Lippke et al., 1981).

Binding to Double-Stranded vs Single-Stranded DNA. Our previous filter-binding study indicated that XPAC could also bind to single-stranded DNA (Robins et al., 1991). Eker et al. (1992) further showed that XPAC could bind single-stranded DNA following transfer of the protein to a membrane and renaturation. To quantitatively compare the binding of XPAC to single-stranded and double-stranded DNA, we performed competition assays. Binding reaction mixtures including 0.2 ng of UV-irradiated DNA and 0.5 μg of XPAC were supplemented with increasing concentrations of single-stranded M13mp18 viral DNA or double-stranded M13mp18 replicative form DNA. Single-stranded DNA was a better competitor than double-stranded DNA, as judged from a plot of competitor concentration *vs* the ratio of K_{UV} to the apparent equilibrium association constant (Figure 5). The ratio of the slopes of the two curves is 4.4, and thus the data indicate that XPAC binds undamaged circular single-stranded DNA ~4-fold more avidly (on a molar nucleotide basis) than undamaged circular double-stranded DNA. In experiments with a nitrocellulose filter-binding method, fewer complexes of XPAC with single-stranded DNA were detected than with double-stranded DNA (Robins et al., 1991). It seems likely that the efficiency of retaining single-stranded DNA–XPAC complexes on filters is significantly lower than the retention efficiency for double-stranded DNA–XPAC complexes, as has been noted for UvrA–DNA complexes (Mazur & Grossman, 1991). The competition experiments may therefore reflect more accurately the relative affinity for undamaged double-stranded *vs* single-stranded DNA.

Binding of XPAC to Other Types of Damaged DNA. Cells from xeroderma pigmentosum group A are defective in repairing all DNA damage processed by nucleotide excision repair. As a first step in determining whether XPAC protein has an affinity for DNA lesions caused by agents other than UV light, DNA was modified with the compound *cis*-diamminedichloroplatinum(II) (cisplatin). DNA adducts formed by treatment with this chemotherapeutic drug *in vitro* include 1,2-intrastrand d(GpG) cross-links (~65% of total lesions) and d(ApG) cross-links (~25%) as well as 1,3-intrastrand d(GpNpG) cross-links, interstrand cross-links, and

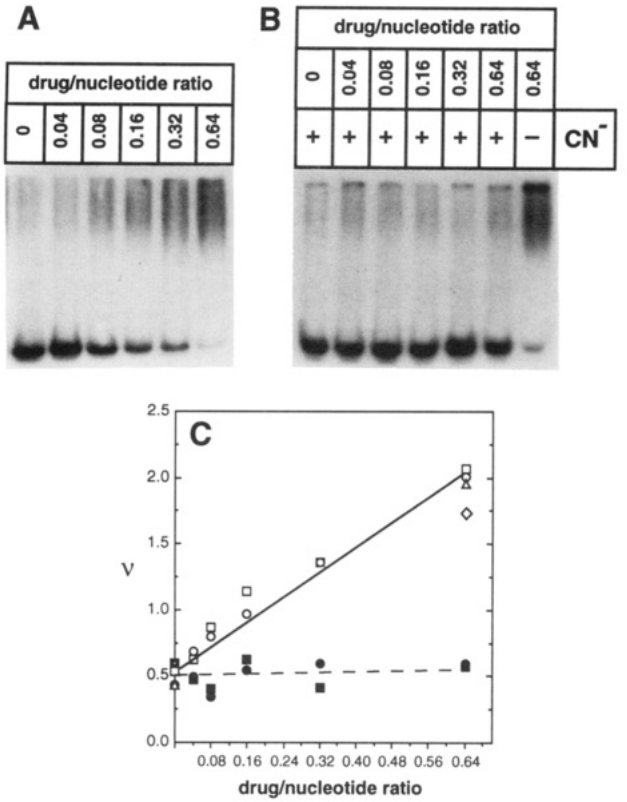


FIGURE 6: Binding of XPAC to DNA probe treated with *cis*-diamminedichloroplatinum(II). (A) ^{32}P-Labeled DNA probe was treated with cisplatin at the molar drug/nucleotide ratios indicated and incubated with 500 ng of XPAC in DNA-binding assays. (B) Cisplatin-damaged DNA probe from (A) was treated with sodium cyanide before incubation with 500 ng of XPAC in DNA-binding assays; the sample in the lane at the far right was mock-treated. (C) The negative natural logarithm of the fraction of unbound probe, ν, plotted *vs* the drug/nucleotide ratio. All points are the mean of duplicate assays: (□, ○) binding to platinated DNA (two experiments); (■, ●) binding to cyanide-treated platinated DNA; (◇, △) samples that were mock-treated during the cyanide incubation.

monoadducts. These lesions are acted on by nucleotide excision repair and are not removed by XP-A cells or XP-A cell extracts (Fraval et al., 1978; Hansson & Wood, 1989).

As shown in Figure 6A, XPAC bound to DNA treated with cisplatin with a higher affinity than to undamaged DNA, and the binding increased with greater cisplatin modification of the fragment. To confirm that the increased binding was due to platinum adducts formed during the incubation with cisplatin, the adducted DNA was treated with NaCN, which removes most cisplatin lesions from DNA (Lemaire et al., 1991). This reduced the XPAC binding to the level obtained with undamaged DNA (Figure 6B,C). In separate control experiments we confirmed directly that our NaCN treatment protocol removed platinum lesions from DNA, by monitoring the disappearance of a 1,2-intrastrand d(GpG) cross-link (Szymkowski et al., 1992) and a 1,3-intrastrand d(GpTpG) cross-link (unpublished data) from duplex DNA circles.

In contrast, we did not detect increased affinity of XPAC for probe containing moderate levels of photoactivated psoralen adducts. No effect on binding was seen after 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen modification to give ~3 interstrand cross-links per fragment or 5-methylisopsoralen modification to give ~6 monoadducts per fragment (data not shown). These lesions are substrates for nucleotide excision repair, but are much less well-recognized *in vitro* than other lesions (Hansson et al., 1989). Further, no additional binding of XPAC protein was detected to a 90-mer double-stranded substrate containing a single G·T mismatch (data not shown). Irradiation of the mismatched substrate with 4 kJ/m^2 UV did increase the binding, as anticipated. Human cells and extracts repair mismatched base pairs by a process distinct from nucleotide excision repair, and so the lack of affinity of XPAC for the mismatch is not unexpected.

DISCUSSION

Binding of XPAC to Damaged DNA. We have shown here that soluble recombinant XPAC protein produced in *E. coli* with an N-terminal polyhistidine tag can correct the defect of nucleotide excision repair-deficient XP-A cell extracts. The recombinant XPAC protein has a greater affinity for damaged double-stranded than for undamaged DNA. Increased binding was observed for DNA that had been UV-irradiated or treated with cisplatin. Undamaged single-stranded DNA was a better competitor for this binding than was undamaged double-stranded DNA. No significant increased binding was detected for a DNA fragment containing moderate levels of psoralen modification or a single G·T mismatch.

Photoreactivation experiments showed that the detected increase in binding to UV-irradiated DNA was not due to the most abundant type of lesion, cyclobutane pyrimidine dimers, but to another lesion, apparently (6–4) photoproducts. The data allow an estimation of the affinity of XPAC protein for a (6–4) photoproduct, relative to undamaged DNA. The estimated association constants from the binding data in Figure 3B are ~3×10^6 M^{-1} for the fragment irradiated with 6 kJ m^{-2} and ~6×10^5 M^{-1} for the nonirradiated fragment, about a 5-fold difference. However, the probe irradiated at this fluence contains only ~4 (6–4) photoproducts and ~250 undamaged base pairs. The affinity of XPAC for a damaged *vs* an undamaged site could thus be as high as 5(250/4), or about 300-fold. Another estimate can be made from the data in Figure 4C. With nonirradiated DNA, the parameter ν (proportional to the average number of XPAC molecules bound per fragment) is 0.5. The value of ν is increased by an additional 0.5 upon irradiation of the fragment with 2.2 kJ/m^2, a fluence that produces ~1.5 (6–4) photoproducts per 258-bp fragment. This suggests that XPAC has ~200-fold greater affinity for a (6–4) photoproduct over an undamaged site. We do not, however, have information on the binding site sizes for XPAC in damaged and undamaged DNA, nor do we know whether the complexes are qualitatively similar in the two cases. It is therefore not currently possible to give a more precise estimate of the relative affinities.

The lesions responsible for the increased affinity of XPAC to cisplatin-damaged DNA are unknown, but judging from the cyanide reversibility, they are probably a form of intrastrand DNA cross-link (Lemaire et al., 1991). Since different cisplatin–DNA lesions are formed with very different yields and appear to be reversed with different efficiencies, it is difficult to directly compare the relative binding of XPAC to (6–4)photoproducts *vs* specific cisplatin–DNA adducts.

What features of DNA modification cause the increased binding by XPAC? The greater affinity of XPAC for single-stranded over double-stranded DNA suggests the conservative hypothesis that XPAC recognizes the single-stranded character produced in DNA by lesions that most distort the double helix. The single-stranded character produced by some DNA damage is well-recognized. For example, S1 nuclease can incise heavily UV-irradiated (Heflich et al., 1979) or cisplatin-treated DNA (Butour et al., 1990), and some single-stranded DNA-binding proteins also have an affinity for cisplatin-treated duplex DNA (Toulmé et al., 1983; Clugston et al.,

1992). In contrast, psoralen adducts can produce significantly less of a structural change in the helix (Haran & Crothers, 1988), and a G·T mismatch is a base-paired structure without single-stranded character (Kalnik et al., 1988); both of these modifications are poorly bound by XPAC. In competition experiments like those shown in Figure 5, UV irradiation of single-stranded DNA did not change its efficiency as a competitor, consistent with the idea that XPAC recognizes single-stranded structure rather than the precise nature of DNA lesions.

The human nucleotide excision repair system removes (6–4) photoproducts from UV-irradiated DNA much more rapidly and efficiently than cyclobutane pyrimidine dimers. The preference of XPAC for binding to DNA containing (6–4)photoproducts may be an important factor contributing to this difference in repair. Even so, it seems surprising that significant binding of XPAC to cyclobutane pyrimidine dimers was not detected in the current experiments. It is clear that removal of dimers from mammalian DNA depends on the function of XPAC, since dimer removal is abolished in XP-A cells. Perhaps binding of XPAC to cyclobutane pyrimidine dimers is too weak to be detected by the present band-shift analysis. It will probably be worthwhile to make a further and more direct comparison of binding to the two photoproducts, using DNA fragments containing single pyrimidine dimers and (6–4) photoproducts (Szymkowski et al., 1993).

The poor binding of XPAC to cyclobutane dimers may be a limiting factor for repair of these photolesions in cellular DNA. This is suggested by studies of UV-resistant revertants of XP-A cells isolated in culture. One such XP-A revertant (designated XP129) has ~30% of the normal amount of XPAC (Jones et al., 1992). XP129 cells repair (6–4) photoproducts normally but have a nearly undetectable removal of cyclobutane pyrimidine dimers from the genome as a whole (Cleaver et al., 1987; Lommel & Hanawalt, 1993). Although the XPAC protein in XP129 has one amino acid difference from the wild type (McDowell et al., 1993), the result suggests that a modest reduction in XPAC levels has a larger effect on cyclobutane pyrimidine dimer repair than on (6–4) photoproduct repair. Another example is provided by the study of Ishizaki et al. (1992), in which it was found that transfer of a single chromosome 9 to XP-A cells (thus providing one active copy of *XPAC*) conferred a substantial ability to repair (6–4) photoproducts on the cells, but little ability to remove cyclobutane dimers from the bulk of the genome.

To compensate for inefficient recognition of dimers by the core nucleotide excision repair system, mammalian cells utilize alternative mechanisms for dealing with these lesions. Dimer repair is increased in active genes by utilizing the stalling of RNA polymerase at a lesion in the transcribed strand as a method to enhance recognition of the photoproduct (Mellon et al., 1986); consequently, even XP129 cells remove cyclobutane dimers from the transcribed strand of an active gene (Lommel & Hanawalt, 1993). Further, replicative DNA synthesis can often bypass dimers without repairing them, and without an immediately deleterious effect (Spivak & Hanawalt, 1992; Carty et al., 1993).

Comparison with Other DNA Damage Binding Proteins. (A) Comparison with E. coli UvrA Protein. UvrA is the DNA-binding subunit of the *E. coli* nucleotide excision repair enzyme, and it has a higher affinity for single-stranded DNA and damaged duplex DNA than for undamaged duplex DNA (Kacinski et al., 1981; Seeberg & Steinum, 1982). This feature provokes comparison with the XPAC protein. There is no significant overall sequence similarity between the two proteins, although both UvrA and XPAC have zinc finger motifs that are essential for function. Unlike the monomeric 31-kDa XPAC, the 104-kDa UvrA protein hydrolyzes ATP, forms a homodimer in solution, and has multiple domains that are required for damage discrimination. Truncating or altering the C-terminal domain of UvrA abolishes damage-specific binding (Claassen & Grossman, 1991; Wang & Grossman, 1993), but more than this domain is needed for damage recognition since a 35-kDa C-terminal fragment does not retain damage-discrimination properties (Myles & Sancar, 1991). Damage-specific binding of UvrA alone does not require the ATP-binding activity of UvrA (Myles et al., 1991) or the presence of ATP (Mazur & Grossman, 1991). The discrimination of UvrA for sites of DNA damage is about 1000-fold (Mazur & Grossman, 1991), apparently greater than that of XPAC. The preference of nucleotide excision repair for (6–4) photoproducts over cyclobutane dimers is also found in *E. coli*, where the UvrABC enzyme has a ~9-fold higher rate of incision at (6–4) photoproducts than at dimers (Svoboda et al., 1993).

(B) Comparison with RAD14 Protein. The *RAD14* gene is the *Saccharomyces cerevisiae* homolog of *XPAC* (Bankmann et al., 1992). Recently it was reported that recombinant RAD14 protein has affinity for UV-irradiated DNA (Guzder et al., 1993). As we have observed with XPAC, removal of cyclobutane pyrimidine dimers from the probe by photoreactivation had no effect on the binding of RAD14 to UV-damaged DNA, implying that (6–4) photoproducts were the major lesions recognized. In contrast to our findings with XPAC, no binding of RAD14 to nonirradiated duplex DNA was detected. It seems likely that RAD14 will have an affinity for other lesions removed by nucleotide excision repair.

(C) Comparison with XP-E Protein. Many research groups have used gel retardation assays to identify an activity in crude mammalian cell extracts that can bind to a UV-irradiated oligonucleotide. These studies generally agree that the major activity includes a polypeptide of ~125 kDa (distinct from XPAC) with a high affinity for (6–4) photoproducts in UV-irradiated DNA (Abramic et al., 1991; Treiber et al., 1992; Hwang & Chu, 1993). This protein appears to be identical to the ~120-kDa polypeptide originally purified from human placenta by Feldberg and Grossman (1976) as an activity that bound to UV-irradiated DNA in a filter-retention assay. Significantly, this binding activity is completely absent in extracts from some cell lines derived from XP-E patients (Chu & Chang, 1988; Hirschfeld et al., 1990; Kataoka & Fujiwara, 1991; Keeney et al., 1992), and it now appears very likely that defects in the protein are indeed responsible for XP-E. The discrimination of the XP-E protein for UV photoproducts over undamaged nucleotides might be up to ~10^5-fold (Hwang & Chu, 1993), much higher than the relative affinity of XPAC protein. This large difference indicates why the XP-E protein is by far the major activity detected in crude cell extracts in the presence of undamaged competitor DNA and why XPAC was not detected in extracts by this method.

Conclusion. The present study of the DNA-binding properties of XPAC has provided evidence that the protein functions in the repair process as a key factor in the recognition of different types of DNA damage during repair. XPAC is, however, only one of the components necessary to carry out the complex reaction of nucleotide excision repair in human cells. It must cooperate or interact with other repair proteins in ways that remain to be defined, so that lesions can be precisely located and repair events initiated. XPAC is the

only XP or ERCC protein produced so far as an active recombinant polypeptide. Synthesis of the protein by overexpression from its gene by recombinant methods has yielded sufficient material to allow us to begin an examination of its biochemical features. A similar approach for other *XP* and *ERCC* gene products should be a productive route toward an understanding of their mechanism of action.

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