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Evidence for a novel DNA damage binding protein in human cells

(cell cycle/nucleotide excision repair/UV radiation)

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ABSTRACT **We describe a novel DNA damage binding activity in nuclear extracts from a normal human fibroblast cell strain. This protein was identified using electrophoretic mobility shift assays of immunopurified UV-irradiated oligonucleotide substrates containing a single, site-specific cyclobutane pyrimidine dimer or a pyrimidine (6-4) pyrimidinone photoproduct. Compared with the (6-4) photoproduct, which displayed similar levels of binding in double and single-stranded substrates, the protein showed somewhat lower affinity for the cyclobutane dimer in a single-stranded oligonucleotide and negligible binding in double-stranded DNA. The specificity and magnitude of binding was similar in cells with normal excision repair (GM637) and repair-deficient cells from xeroderma pigmentosum groups A (XP12RO) and E (XP2RO). An apparent molecular mass of 66 kDa consisting of two subunits of ≈22 and ≈44 kDa was determined by Southwestern analysis. Cell cycle studies using centrifugal cell elutriation indicated that the binding activity was significantly greater in G1 phase compared with S phase in a human lymphoblast cell line. Gel supershift analysis using an anti-replication protein A antibody showed that the binding protein was not antigenically related to the human single-stranded binding protein. Taken together, these data suggest that this activity represents a novel DNA damage binding protein that, in addition to a putative role in excision repair, may also function in cell cycle or gene regulation.**

Nucleotide excision repair (NER) is the principal defense mechanism used by cells to mitigate the lethal, mutagenic, and carcinogenic effects of DNA damage caused by exposure to genotoxic agents, such as solar UV radiation. Many of the proteins involved in the NER pathway have been identified by complementation of the repair-deficient autosomal recessive disease, xeroderma pigmentosum (XP). Cloning of these genes in repair-deficient human and Chinese hamster ovary cells has facilitated the development of an *in vitro* repair assay and greatly increased our understanding of mammalian NER. Based on recent advances, a functional model has emerged that offers a plausible basic scheme for the sequence of protein–DNA and protein–protein interactions involved in the removal of bulky adducts from the genome (1). The DNA repair complex is produced by the sequential assembly of multiple gene products, some of which are subunits of the general transcription factor TFIIH, at the damage site. Although this model is comprehensive, there are at least 12 complementation groups associated with DNA damage tolerance in rodent cells, a few of which correspond to XP proteins but many of which do not, and their role in NER is not clear. Hence, it is reasonable to believe that additional, as yet unidentified proteins are involved in mammalian NER. By examining interactions of cell extracts with damaged DNA, a more complete understanding of NER can be obtained.

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Using this approach, Feldberg and Grossman (2) provided the first evidence for a human protein binding to damaged DNA [i.e., double-stranded, UV-irradiated DNA from which the cyclobutane pyrimidine dimers (CPDs) were removed by enzymatic photoreactivation]. Further characterization showed that the protein recognizes DNA damage produced by a broad range of physical and chemical agents including ionizing radiation (3) and various genotoxic chemicals (4, 5). Extracts from normal human fibroblasts and XP groups A, C, and D showed comparable activities. Subsequently, this protein was shown to be absent in the XPE cell line GM02415B, thus implicating it in NER (6). By using different approaches, it has become evident that the binding activity of the XPE factor is directed toward non-CPD damage, such as the pyrimidine (6-4) pyrimidinone photoproduct [(6-4)PD]. Using purified XPE factor and synthetic DNA substrates containing a single type of thymidine dimer, Reardon *et al.* (7) showed that the XPE factor binds the *trans, syn*-CPD, (6-4)PD, and Dewar pyrimidinone with high affinity, but it binds the *cis, syn*-CPD only slightly better than the undamaged substrate. Correlation between the binding affinity and the degree of helical distortion measured for each photoproduct [i.e., 44° for the (6-4)PD compared with 9° for the CPD (8, 9)] suggests that recognition of damaged DNA by the XPE factor may be dependent on the helical bend caused by the damage rather than the damage *per se*.

Work by Protić and coworkers (10) has shown that the binding activity is expressed constitutively in primate cells but is reduced to negligible levels immediately after UV irradiation. It is probable that the XPE protein is sequestered at DNA damage sites immediately after treatment and recovers to preirradiation levels as the photodamage is removed by excision repair. In addition, they observed a 2- to 6-fold increase in the binding activity in cultured primate cells by 48 h postirradiation (11). Induction of XPE protein in monkey kidney cells is observed following treatment with various DNA-damaging agents, including UV light, mitomycin C, and the polymerase inhibitor aphidicolin. Nuclear extracts from cells from some XPE patients are deficient in both the constitutive and inducible binding activities (12). Microinjection of purified XPE factor restores DNA repair synthesis to normal levels in XPE cell lines that lack this activity (i.e., XP2RO and XP82TO) but not in other XP cell lines that are not deficient in the XPE factor (13). These data support the notion that the XPE factor may function in a damage tolerance mechanisms such as NER.

In addition to the XPE factor, other putative DNA damage binding activities have been identified. Chu and Chang (14) used electrophoretic mobility shift assay (EMSA) to identify a nuclear factor that recognizes DNA treated with UV light or cisplatin. Along similar lines, Chao *et al.* (15) identified two nuclear factors from a human cell line selected for cisplatin

Abbreviations: CPD, cyclobutane pyrimidine dimer; T◇T, thymidine CPD; (6-4)PD, pyrimidine (6-4) pyrimidinone photoproduct; T(6-4)C, thymidine-cytidine (6-4)PD; NER, nucleotide excision repair; XP, xeroderma pigmentosum; EMSA, electrophoretic mobility shift assay.

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resistance that recognize UV-irradiated DNA. Consistent with previous reports (16), competition experiments using UV-irradiated and cisplatin-modified DNA suggest that the UV binding activity is distinct from the cisplatin binding activity. Other studies have described a binding activity for DNA modified with the carcinogen *N*-acetoxy-2-acetylaminofluorene (17) that is distinct from the XPE binding activity (18). DNA damage binding proteins have also been reported in *Drosophila melanogaster*. Work by Todo and coworkers (19, 20) has shown that *D. melanogaster* embryo cells contain 30-kDa and 14-kDa proteins that recognize damaged DNA, and data from repair-deficient mutants suggest a possible involvement of these proteins in NER.

DNA damage binding proteins may be multifunctional, involved in excision repair as well as recombinational repair, postreplication repair, and gene regulation through their association with transcription factors. We have characterized a DNA damage binding protein, which appears to be distinct from the XPE factor and may function in DNA damage tolerance mechanisms in human cells. The singular pattern of cell cycle expression displayed by this binding activity (i.e., G1 > S) is intriguing and suggests that, in addition to its role in NER, this protein may also function in other metabolic pathways such as transcriptional regulation or DNA replication.

MATERIALS AND METHODS

Cell Culture. The normal human fibroblast cell strain GM637 was grown in α-MEM medium with 10% fetal bovine serum at 37°C. XPE strain GM02415B (XP2RO) was obtained from Coriell Institute (Camden, NJ) and grown in α-MEM supplemented with a 2× concentration of essential and nonessential amino acids and 20% fetal bovine serum. The lymphoblast cell line WI-L2/B5 was grown in RPMI 1640 medium containing 10% fetal bovine serum in spinner flasks.

Nuclear Extracts. The hypotonic shock method of cell lysis followed by salt extraction was performed according to the method of Dignam *et al.* (21). Cells were cultured in 150-mm dishes until they were 90% confluent. For some experiments the cells were washed in ice-cold phosphate-buffered saline (PBS) and irradiated with UVC light. Cells were then scraped off the plates into PBS, collected by low speed centrifugation, incubated in hypotonic salt solution on ice, and lysed with a few strokes of a Dounce homogenizer (type B). The ruptured cells were separated from the intact nuclei from which proteins were extracted in high salt buffer (i.e., 0.42 M NaCl) by passage through a 271/2-gauge syringe. Protease inhibitors including pepstatin, leupeptin, aprotinin, and phenylmethylsulfonyl fluoride were routinely used in the buffers. Extracted proteins were dialyzed at 4°C to remove salt, and protein concentrations were estimated using Coomassie blue reagent (Pierce). The binding activity of these extracts was stable for several months at −70°C.

Synthesis of UV-Damaged Substrates. The production of UV-damaged DNA substrates used in EMSA was carried out according to Ghosh *et al.* (22). Briefly, oligonucleotides (30 bp long) were designed such that a single dipyrimidine site was contained in a restriction cassette. Inhibition of restriction digestion could thus be used to test for the presence of photodamage at that site (23). The CPD and (6-4)PD oligonucleotides used in this study were ≈98% pure as determined by their refractivity to restriction digestion. Single-stranded oligomers were treated with 30 kJ/m^2 UVC light emitted by five narrow band germicidal lamps (Phillips Sterilamps G8T5, Phillips Electronic Instruments, Mahwah, NJ). The fluence was measured using a NS 254 probe (TD #11280) coupled to a model IL 1400A radiometer/photometer (International Light, Newburyport, MA). Antibodies raised against specific photodamage such as the CPD or the (6-4) photoproduct were used to immunoprecipitate oligomers containing one or the other photoproduct. Single-stranded oligonucleotides were used as such or were reannealed to the complementary strand and gel-purified to obtain pure double-stranded substrates. Each purified double-stranded oligomer was tested for photoproduct yield using the appropriate restriction enzyme.

EMSA. Approximately 2 ng of CPD or (6-4)PD oligonucleotide were end-labeled with [γ-^{32}P]ATP (sp. act. 3000 Ci/mol) and incubated with nuclear extract in a total volume of 20 μl, containing 0.5 μg of nonspecific competitor poly(dI-dC) per ml and binding buffer (20 mM Tris, pH 8.0/50 mM Hepes/0.2 mM KCl/2.5 mM EDTA/25 mM $MgCl_2$/5 mM DTT/60% glycerol). After 20 min at 23°C, the reaction products were resolved on a 5% native polyacrylamide gel, which was then dried and exposed to x-ray film. Quantitative analysis was performed using the IMAGEQUANT phosphorimaging system (Molecular Dynamics).

Competition Experiments. Homologous competition experiments were performed using an unlabeled oligomer containing the same lesion as the probe. The probe was incubated with increasing molar excess of competitor DNA before addition of nuclear extract. Heterologous competition experiments were performed using increasing molar excess of an unlabeled oligomer that had not been exposed to UVC light. The conditions for the binding reaction and electrophoresis were as mentioned above.

Southwestern Analysis. Nuclear extracts from GM637 cells were fractionated on 5% polyacrylamide gels in the presence or absence of 0.2 M DTT. Gels were electroblotted to BA85 nitrocellulose membrane (Schleicher and Schuell) overnight at 4°C. The membrane was blocked overnight at 4°C in a solution consisting of 2.5% (wt/vol) dried milk powder, 25 mM Hepes (pH 8.0), 1 mM DTT, 10% (vol/vol) glycerol, 50 mM NaCl, and 1 mM EDTA. The membrane was then probed for 4 h at room temperature with 2 × 10^6 cpm/ml end-labeled double-stranded oligomer containing a specific photoproduct. After three washes at room temperature with TNE-50 (10 mM Tris, pH 7.5/50 mM NaCl/1 mM EDTA/1 mM DTT) the blots were exposed to x-ray film (Amersham).

Counterflow Centrifugal Elutriation and Population Characterization. One liter of suspension culture of WI-L2/B5 lymphoblasts was harvested by centrifugation and resuspended in 10 ml of elutriant medium consisting of RPMI 1640 supplemented with 1% fetal bovine serum. The 10-ml suspension was gently expelled through a 25-gauge syringe and then loaded into a Beckman JE-6B rotor at an elutriant flow rate of 10 ml/min and rotor speed of 2200 rpm. After an initial flushing with 150 ml, cells were elutriated from the rotor in 50-ml fractions by increasing the flow rate by a factor of 1.1 between fractions. On average, 13 fractions were collected from each experiment. Aliquots of each elutriated fraction were counted in a Coulter counter, and the volume distribution of the cells was analyzed on a Coulter Channelizer. Approximately 2 × 10^6 cells were washed twice in Hepes buffer (20 mM Hepes, pH 7.0/137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4) and fixed in 65% ethanol. Fixed cells suspended in Hepes buffer were stained with propidium iodide (10 mg/ml) and treated with RNase A (100 mg/ml; Sigma). Cellular DNA content was analyzed by flow cytometry on a Coulter Epics Elite Flow Cytometer. Estimates of the proportion of cells in each phase of the cell cycle were made using the cell cycle program MULTICYCLE (Phoenix Flow Systems, San Diego). Nuclear extracts were made from each of the fractions as described above.

RESULTS

Substrate Affinity. Specificity of the binding activity was determined using 30-mers containing a single site-specific UV-induced (6-4)PD or CPD (Fig. 1). Because UV irradiation produces primarily CPDs at thymidine dinucleotides and

5' ACATGTGTACGAATATTAAAGGTAGACATG 3'	AATATT	SspI
5' ACATGTGTACGGATATCAAAGGTAGACATG 3'	GATATC	EcoRV

FIG. 1. Substrate sequences. Sequences of the oligonucleotides showing the specific dipyrimidine sites. Shown are 30-mers used in the immunoprecipitation procedure with dimeric damage (boldface type) located within the restriction enzyme recognition site (underlined).

(6-4)PDs at dipyrimidine sites where cytidine is located 3′ to thymidine (24), oligonucleotides containing either a thymidine CPD (T◇T) or a thymidine-cytidine (6-4)PD [T(6-4)C] were selected and immunopurified for these studies. The single-stranded damaged oligomers were annealed to the undamaged complementary strand and gel-purified, and the yield was determined by measuring loss of restriction enzyme digestion. About 0.1 pmol of each of the double-stranded, end-labeled substrates were incubated with increasing concentrations of nuclear extract from the human fibroblast cell line GM637, and the products were resolved on 5% polyacrylamide gels. Fig. 2 shows gel profiles for binding of nuclear extract to oligonucleotides containing either the T◇T or T(6-4)C. Incubation of both substrates with 2.5, 5.0, and 10 μg of extract produced a major retarded band (B1) and a band of slower mobility (B2). With the addition of 10 μg of extract, the greater binding affinity for the T(6-4)C substrate was evident. The appearance of an additional band at higher extract concentrations may result from nonspecific binding or interaction of multimers of the protein binding with the damaged DNA probe (see below). Binding to undamaged DNA was evident but significantly reduced compared with the damaged substrates (data not shown). Proteinase K and RNase digestions confirmed that the retarded complex is produced by protein–DNA interaction (data not shown).

Binding Specificity. Substrate specificity was confirmed by homologous and heterologous competition experiments using the double-stranded T(6-4)C probe and 40- to 800-fold molar excess of unlabeled double-stranded T(6-4)C and undamaged oligonucleotides as competitors. Fig. 3*A* shows the results of a homologous competition where a 40-fold molar excess of unlabeled oligomer reduced the binding of band B1 by ≈70%. At higher concentrations of competitor, some reduction in band B2 is also evident. Competition with a 100-fold excess of unlabeled double-stranded CPD oligomer reduced the binding

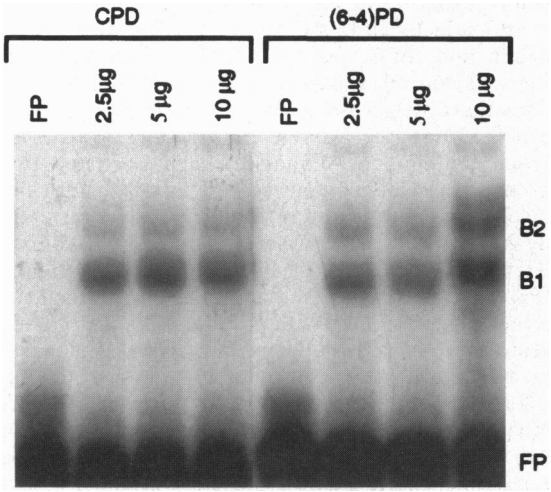

FIG. 2. Substrate affinity. Binding of a nuclear factor from GM637 cells to DNA substrates containing either a single cyclobutane pyrimidine dimer or a (6-4) photoproduct. Substrates were UV-irradiated and immunopurified with antibodies specific for either the CPD or the (6-4)PD and used in EMSAs. End-labeled substrates were incubated without extract or with 2.5, 5.0, or 10.0 μg of extract at room temperature and resolved on a 5% native polyacrylamide gel in high ionic electrophoresis buffer. FP, free probe.

of band B1 by ≈50% (data not shown). The same range of excess unlabeled oligomer containing no damage was used as the competitor in heterologous competition experiments as shown in Fig. 3*B*. Neither band B1 nor B2 competed for binding to the undamaged competitor. These results suggest that bands B1 and B2 result from specific interaction between the damaged substrates and the nuclear factor and that the slow moving band B2 may be a multimer of the faster migrating band B1.

We examined the ability of nuclear extracts to bind photodamage in single- and double-stranded DNA substrates. Double-stranded substrates were prepared (see *Materials and Methods*) and tested for their sensitivity to digestion by the restriction enzymes *Ssp*I, for T◇T, and *Eco*RV, for T(6-4)C. Samples containing the double-stranded substrates were electrophoresed for ≈8 min at 100 V before application of the single-stranded substrates. Whereas protein binding was comparable for single- and double-stranded T(6-4)C substrates, it was significantly greater for the T◇T in single- as opposed to double-stranded DNA (Fig. 4).

Molecular Weight Determination. Southwestern blotting was used to determine the approximate molecular weight of the protein. Double-stranded T(6-4)C and T◇T oligonucleotides were used as probes. Because polyacrylamide gel electrophoresis in the presence of a disulfide reducing agent such as DTT denatures proteins into their constituent subunits, we used both nonreducing and reducing conditions for protein fractionation. Under nonreducing conditions, two proteins of ≈66 kDa and ≈100 kDa were observed with both probes (Fig. 5, lanes 1 and 2). Binding to the T◇T probe was weaker than to the T(6-4)C probe (a trend also seen in EMSA). Under reducing conditions (i.e., with the addition of DTT), two bands of ≈22 kDa and ≈44 kDa were observed when probed with the T◇T substrate (lane 3). Similar results were observed using the (6-4)PD probe (data not shown). In three independent Southwestern blots using reducing conditions, the 100-kDa band was lost, and only a smear remained above the 44-kDa band. The presence (denaturing) or absence (native) of SDS during electrophoresis did not alter the sizes of the bands observed. These results suggest that the 66-kDa protein has two subunits of ≈22 kDa and ≈44 kDa.

Response of Binding Activity to UV Irradiation. To test for the effects of UV irradiation on binding activity, GM637 cells were treated with 30 J/m^2 UVC light, and nuclear extracts were made immediately after irradiation (0 h) or after different time intervals. This UVC dose induces ≈120 CPDs and ≈28 (6-4)PDs per megabase of cellular DNA. A nonirradiated sample was used as control. Cell extracts (10 μg) incubated with double-stranded T(6-4)C or a nonirradiated control showed no change in binding activity up to 6 h postirradiation (data not shown). This result is different from that obtained for XPE factor and suggests that this novel DNA damage binding activity may be present in considerably greater amounts in the cell or that other cellular (repair) proteins may be competing for binding to DNA damage.

Expression of DNA Damage Binding Activity During the Cell Cycle. Counterflow centrifugal elutriation separates cells in liquid suspension into small cells elutriating first and larger cells emerging at later times. Because we were unable to perform these experiments with the fibroblast cell line GM637, we used a B lymphoblast cell line derived from human spleen, WI-L2/B5, for the cell cycle experiments. DNA distribution was determined on propidium iodide-stained cells by cytofluorometry to evaluate the cell cycle phase represented by each fraction. Multicycle estimates of cell cycle characteristics for a typical fractionation of WI-L2/B5 cells are shown in Fig. 6*A*. It can be seen that the cells in early fractions had a predominantly G1 DNA content (fractions 4 and 5), while progressively larger cells represented later phases of the cell cycle. By fraction 15, most of the cells were in the G2 phase. We determined the DNA damage-specific binding activity in ex-

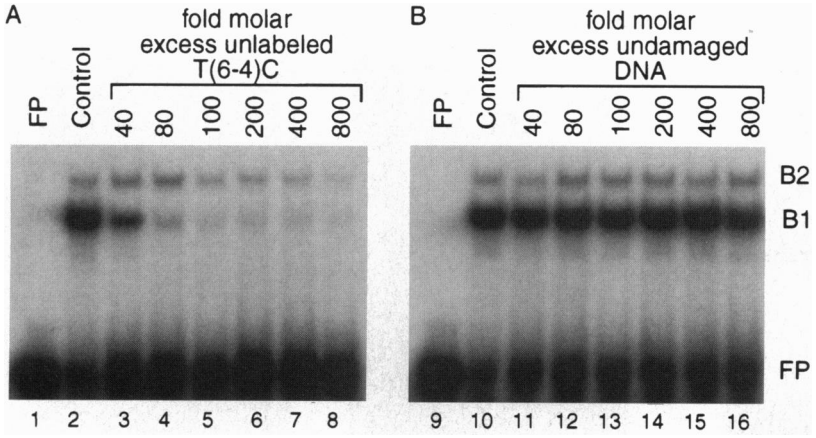

FIG. 3. Substrate specificity. End-labeled double-stranded T(6-4)C oligonucleotide was incubated with excess unlabeled competitor (with or without damage) and 10 μg of nuclear extract and resolved on 5% polyacrylamide gels. (*A*) Homologous competition with 40- to 800-fold molar excess unlabeled double-stranded T(6-4)C oligonucleotide. Lane 1 contains no extract, lane 2 contains no competitor, and lanes 3–8 show the reaction with 40- to 800-fold molar excess T(6-4)C oligonucleotide. (*B*) Heterologous competition using the same concentrations of undamaged competitor as in *A*. Lane 9 contains no extract, lane 10 contains no competitor, and lanes 11–16 show the reaction with 40- to 800-fold molar excess of unlabeled competitor.

tracts made from WI-L2/B5 cells fractionated into different phases of the cell cycle using double-stranded T(6-4)C as substrate. In Fig. 6*B*, a typical EMSA is shown, and it is clear that significantly more activity is observed early in the cell cycle compared with later stages (note increase in band B1 and decrease in free probe). The data in Fig. 6*C* represent four gel shift assays from two separate elutriation experiments, and it is clear that fractions 4 and 5, which include primarily G1-phase cells, display a two-fold greater binding activity than subsequent S-phase fractions. From these studies it is evident that the DNA damage binding activity we have been investigating shows a definitive cell cycle binding pattern.

Binding Activity of the Protein in the XPA and XPE Cell Lines. Nuclear extracts were prepared from XP groups A (XP12RO) and E (XP2RO) cells, and binding activity for damaged DNA was determined. In Fig. 7, it is evident that nuclear extracts from XPA and XPE fibroblasts bound the double-stranded T(6-4)C substrate with affinities comparable with that seen in the normal fibroblasts.

DISCUSSION

We have characterized a DNA damage binding protein that appears to be distinguishable from the XPE factor and shows a distinct pattern of cell cycle expression. EMSA has been routinely used to identify and characterize DNA binding proteins in general and DNA damage binding proteins (i.e., putative repair proteins) in particular. By using this approach, the XPE factor was originally purified from human placenta (2) and more recently characterized in cell extracts and purifed protein from cultured cells (6, 10). The substrates used in these earlier studies were predominantly UV-irradiated DNA fragments in which formation of a specific photoproduct was verified by enzymatic and or chemical digestion. More recently, synthetic oligonucleotides into which defined types of dimeric UV damage had been inserted have been used to characterize the binding properties of purified binding protein (7). In the current study, we designed short oligonucleotides containing a single dipyrimidine combination (*i.e.*, T-T or T-C) within a restriction cassette such that the induction of UV lesions could be confirmed by the inability of a restriction enzyme to cleave the recognition site. Oligonucleotides containing single, site-specific UV photoproducts were immunoprecipitated using purified rabbit immunoglobulin that had been shown to recognize and bind a specific type of UV photodamage. These substrates were then used in EMSA with nuclear extracts from the normal human fibroblast cell line GM637.

EMSA has identified a nuclear factor in normal human fibroblasts that binds both the T◇T and T(6-4)C in single-stranded DNA, with a higher affinity for the latter. Although the protein binds T(6-4)C with similar affinity in single- and double-stranded DNA, it recognizes T◇T predominantly in single-stranded DNA. Homologous and heterologous competition experiments serve to further verify this binding specificity and indicate that both bands B1 and B2 are associated with damage recognition protein(s). The data suggest that the slower-migrating band B2 may be an aggregate involving

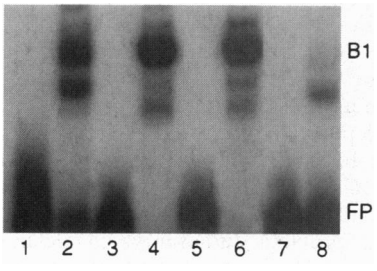

FIG. 4. Binding of the nuclear factor to single- and double-stranded damaged substrates. Single-stranded substrates [i.e., T(6-4)C and T◇T] were either annealed to the undamaged complementary strand or not and used as probes in binding reactions with 15 μg of nuclear extract. Lanes 1 and 2 show the double-stranded T(6-4)C probe alone or with nuclear extract; lanes 3 and 4 show single-stranded T(6-4)C substrate with and without nuclear extract; lanes 5 and 6 show the single-stranded T◇T probe alone or with nuclear extract; and lanes 7 and 8 show double-stranded T◇T substrate with and without nuclear extract.

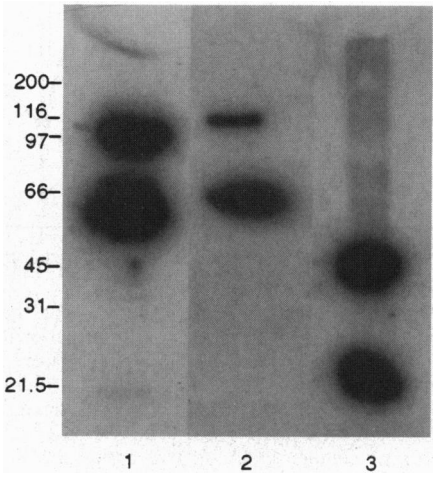

FIG. 5. Molecular weight determination using Southwestern analysis. Nuclear extract from GM637 cells was fractionated on a 10% polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot was blocked as described and probed with 2×10^6 cpm/ml end-labeled double-stranded oligonucleotide. Lane 1 shows the double-stranded T(6-4)C probe; lane 2 shows the double-stranded T◇T probe; and lane 3 shows the double-stranded T(6-4)C probe when DTT was present during electrophoresis. Molecular weight markers are on the left.

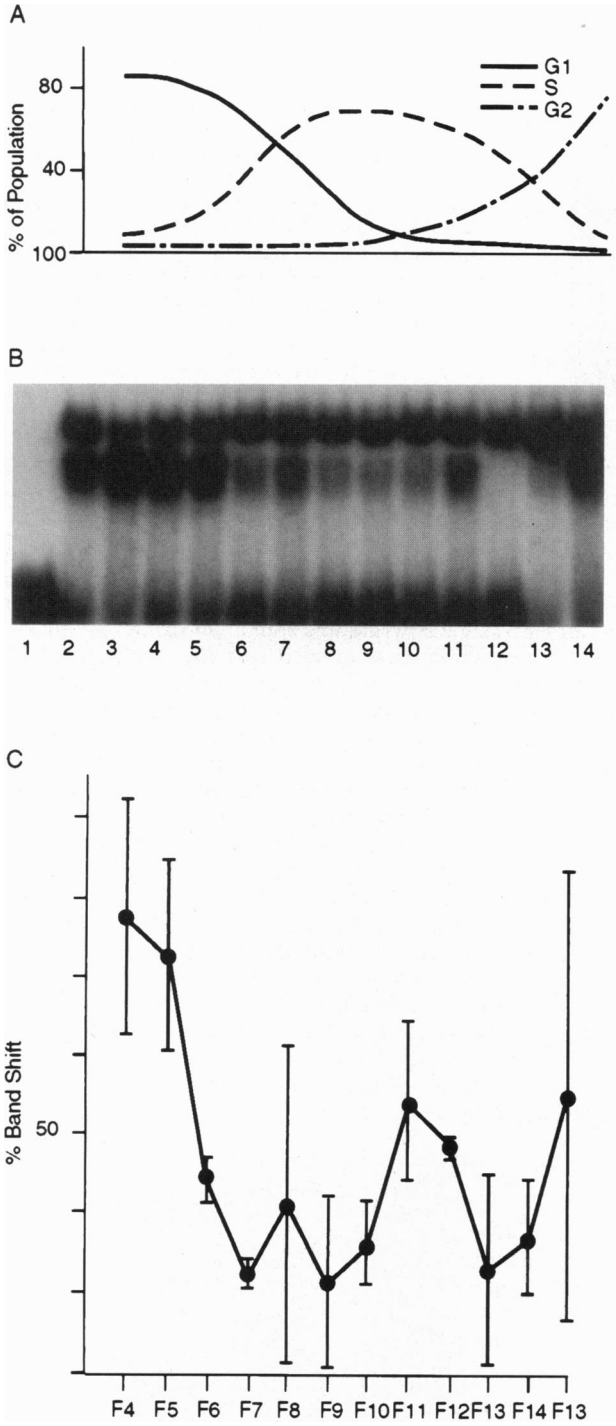

FIG. 6. Modulation of the binding activity during the cell cycle. Counterflow centrifugal elutriation was used to fractionate cells into different phases of the cell cycle. Nuclear extracts were made from each fraction and tested for the ability to bind a double-stranded (6-4)PD substrate. (*A*) Graphical representation of the distribution of cells in the G1, S, and G2 phase of the cell cycle based on propidium iodide staining of cellular DNA. (*B*) A typical EMSA using the different fractions is shown. Lane 1 contains no extract; lane 2 shows activity of the total asynchronous cell population before fractionation; and lanes 3–14 show cell cycle fractions 4–15. (*C*) Statistical representation of the data from four EMSAs from two separate elutriation experiments. Percentage band shift (*y* axis) was calculated from the ratio of bound to free as determined by phosphorimaging analysis. The *x* axis represents the fraction numbers from cell elutriation.

multiple molecules of the binding protein associated with band B1 or a separate DNA damage binding protein of higher

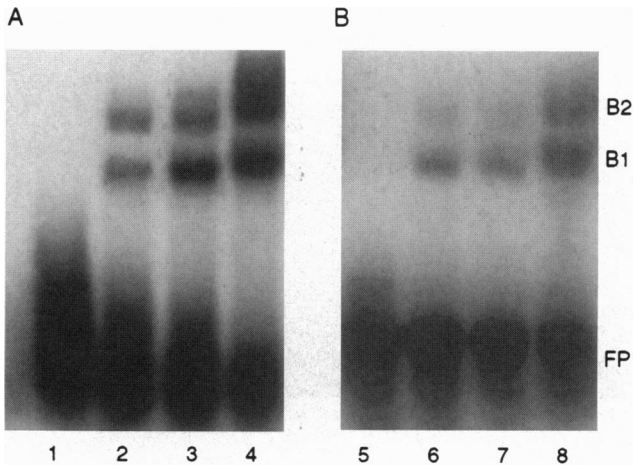

FIG. 7. Binding activity of nuclear extracts from XP groups A and E. Double-stranded T(6-4)C substrates were incubated with nuclear extracts made from XP groups A (XP12RO) and E (XP2RO) and subjected to electrophoresis. (*A*) Lane 1 contains no protein, and lanes 2–4 contain 5, 10, and 20 μg of XP12RO nuclear extract, respectively. (*B*) Lane 5 contains no extract, and lanes 6–8 contain 2.5, 5, and 10 μg of nuclear extract from XP2RO, respectively.

molecular weight. The latter interpretation is supported by Southwestern analysis, which shows a DNA damage binding protein of ≈100 kDa in addition to the 66-kDa protein associated with band B1. Although the molecular weight and binding specificity of the 100-kDa protein is similar to XPE factor, EMSA of XPE nuclear extracts indicate otherwise. Of particular interest is the observation that, in contrast to band B1, the activity associated with band B2 shows no cell cycle dependence.

Because this binding pattern is distinct from that observed for XPE factor, we compared the binding of our extracts with purified XPE protein (a gift of M. Protić) using immunopurified substrates. In these experiments (data not shown), XPE factor bound the T(6-4)C in double-stranded but not single-stranded DNA and did not recognize the CPD in either single- or double-stranded substrates. Additional data that further support the uniqueness of this DNA damage binding activity are as follows: (*i*) Southwestern analyses indicate that this protein is considerably smaller (i.e., half the size) than the XPE factor; (*ii*) unlike XPE factor, which shows decreased binding immediately following UV irradiation of cells and subsequent restoration to control levels (correlating with the kinetics of (6-4)PD excision), UV irradiation has no effect on the level of binding activity; and (*iii*) binding activity is not significantly different in XPE cell lines that are deficient in XPE factor compared with other XP cell lines and normal human fibroblasts. The difference between the results presented here and those from previous studies is not readily apparent but may arise from the unique extraction and analysis conditions used. During the preparation of nuclear extracts, we routinely use a cocktail of protease inhibitors in all of our buffers and, unlike most published reports, lyse the nuclei in high salt (0.42 M NaCl) buffer. In addition, and perhaps more importantly, is the fact that, unlike previous studies in which nuclear extracts were reacted with substrates in which the frequency and location of the binding site(s) (i.e., photoproduct) were not controlled (nor known), our extracts were reacted with a purified substrate containing a single, site-specific CPD or (6-4)PD. Although one other study had used similar substrates (7), purified XPE protein, rather than nuclear extracts, was tested.

Because comparable binding activities were observed in extracts from normal and repair-deficient XPA and XPE cells, the role of this protein in NER, as we understand it, is uncertain. By screening other repair-deficient human and

rodent mutant cell strains, the relationship between this activity and NER may be clarified. The most intriguing result from our studies on human DNA damage binding activity concerns its differential expression during the cell cycle. Data from counterflow centrifugal elutriation experiments indicate that this activity is modulated in a cell cycle-dependent manner with two-fold greater binding observed during G1 compared with S phase. This result suggested that the observed activity may in fact be associated with the human single-strand binding protein and is sequestered during S phase at replication forks and unavailable for binding in nuclear extracts. Gel supershift experiments with anti-replication protein A antibody (a gift of R. Wood, Clane Hall Laboratories, U.K.) showed that the binding activity was not antigenically related to human single-strand binding protein (data not shown).

The XPB and XPD gene products are essential repair proteins, yet function primarily as subunits of the general transcription factor TFIIH. It has been shown that some transcription factors can recognize and bind damaged DNA (25). Smith and MacLeod (26) have recently shown that the binding of Sp1 protein to the G-C box promoter is enhanced by the presence of a benzo[a]pyrene diolepoxide-I adduct within the promoter. Benzo[a]pyrene diolepoxide-I adducts outside the promoter region are also nonspecifically targeted by Sp1. The two-fold increase in binding activity during G1 phase suggests that this protein may have cellular responsibilities other than (or in addition to) DNA repair and may function as a transcription or replication factor. In addition to their participation in NER and gene regulation through their activity as transcription factors, DNA damage binding proteins may also function as structural elements in chromatin or the nuclear matrix (27). Hence, elucidation of the functional role of the novel DNA damage binding protein reported here will require further studies.

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