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Purification of a novel UV-damaged-DNA binding protein highly specific for (6–4) photoproduct

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ABSTRACT

UV damage-specific binding proteins are considered to play important roles in early responses of cells irradiated with UV, including damage recognition in the DNA repair process. We have surveyed nuclear and cytoplasmic proteins which bind selectively to UV-irradiated DNA using an electrophoretic mobility shift assay. We detected four distinct binding activities with different mobilities in fractions separated from HeLa cells by heparin chromatography. Three of them were found in nuclear extracts and one in cytoplasmic extracts. We purified one of the binding factors from nuclear extracts to homogeneity, which was designated NF-10 (the 10th fraction of nuclear extract on heparin chromatography). It migrated as a 40 kDa polypeptide in SDS–PAGE, and bound to UV-irradiated double-stranded DNA but not to unirradiated DNA. The binding pattern of the NF-10 protein to DNA irradiated with UV corresponded to the induction kinetics of (6–4) photoproduct. Removal of (6–4) photoproducts from UV-irradiated DNA by (6–4) photoproduct-specific photolyase diminished the binding of NF-10 protein. These results suggest that the NF-10 protein binds to UV-damaged DNA through (6–4) photoproduct. Immunoblot analysis using a monoclonal antibody revealed that the NF-10 protein was expressed in cell lines from all complementation groups of xeroderma pigmentosum, indicating that the NF-10 protein is a novel UV-damaged-DNA binding protein.

INTRODUCTION

Ultraviolet light (UV) induces two main types of photoproduct in cellular DNA, cyclobutane pyrimidine dimer (CPD) and (6–4) photoproduct. Persistence of these lesions can interfere with essential processes such as transcription and DNA replication, possibly leading to cell death, mutation or neoplastic transformation (1,2). In human cells these lesions are known to be repaired by a nucleotide excision repair (NER) system (3). This system is universal in all organisms and plays an essential role in protecting DNA from various genotoxic agents in the environment.

Recently the functional repair reaction has been reconstituted *in vitro* with highly purified mammalian repair proteins (4,5). For the dual incision reaction XPA, TFIIH (XPB and XPD), XPC/HHR23B, XPF/ERCC1, XPG and replication protein A (RPA) were shown to be required as a minimal set. However, in these *in vitro* systems the efficiency of the excision reaction seems to be much less than that *in vivo*, although the predominant lesion repaired is (6–4) photoproducts in both systems (1,6,7). It is possible that some accessory proteins may stimulate the excision rate *in vivo*. XPE and ERCC7-11 proteins (8), the genes of which have not yet been cloned, may have such roles.

Among the multiple steps of NER, recognition of UV damage in DNA is thought to be a rate limiting step. Hence a recognition factor(s) could be one of the factors affecting the efficiency of NER. Numerous studies have been carried out to identify a UV-damaged-DNA binding protein by means of filter binding assay and electrophoretic mobility shift assay (EMSA) (9–12). The first UV-damaged-DNA binding protein was found using a filter binding assay by Feldberg and Grossman (9), who later partially purified it from human placenta (13). This same factor has been rediscovered using EMSA and purified from human placenta, HeLa cells and monkey cells (14–16) and designated UV-DDB. UV-DDB was found to be a complex of 125 and 41 kDa polypeptides and to have a high affinity for UV-damaged DNA, especially (6–4) photoproduct (17). It was also reported that UV-DDB activity was absent in some, but not all, nuclear extracts prepared from xeroderma pigmentosum complementation group E (XP-E) cell lines (10–12). However, XP-E cell lines lacking this binding activity (UV-DDB$^-$) showed only a mild defect in NER (1), suggesting that DDB cannot be the only factor required to achieve optimal NER.

In this paper we report the purification to homogeneity of an ~40 kDa protein from HeLa cells which specifically binds to UV-irradiated DNA. Partial characterization revealed that this binding activity was specific for (6–4) photoproduct-containing DNA and present in nuclear extracts from all XP complementation groups.

MATERIALS AND METHODS

Cell strains and culture

HeLa cells were obtained from Dr H. Yasuda (Kanazawa University). The normal (WI38VA13) and XP-A (XP2OSSV and XP12ROSV) cell lines were from Dr K. Tanaka (Institute for Molecular and Cellular Biology, Osaka University). XP-C (XP4PASV), XP-D (XP6BESV), XP-F (XP2YOSV) and XP-G

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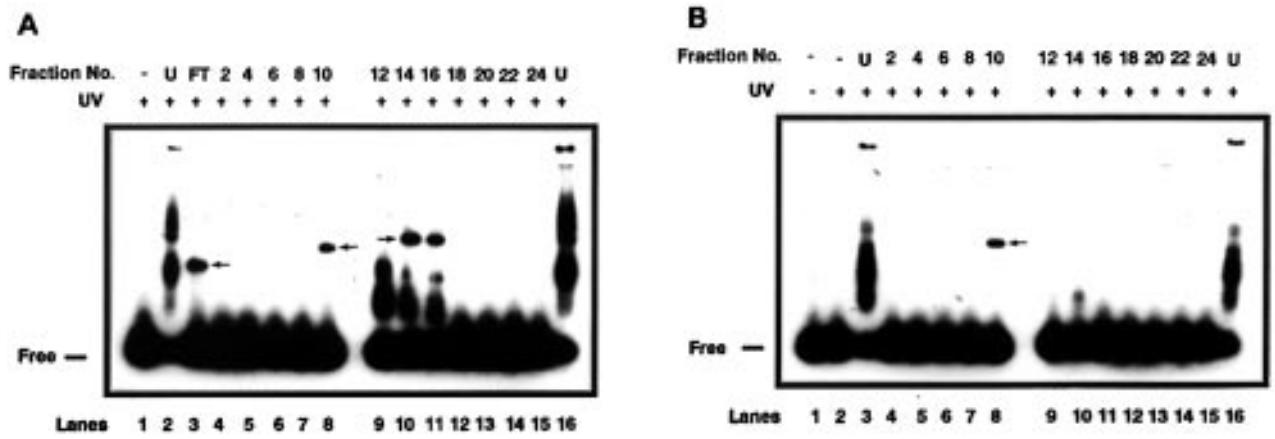

Figure 1. Identification of UV-damaged-DNA binding proteins in fractionated nuclear and cytoplasmic extracts. Nuclear (**A**) or cytoplasmic (**B**) extracts were loaded on a HiTrap heparin column and fractionated with a linear salt gradient of 0.1–1.0 M KCl. One microliter of each fraction was tested for binding activity to UV-irradiated (10 kJ/m^2) oligonucleotide probe using an EMSA. U, unfractionated nuclear or cytoplasmic extract; FT, flow-through fraction. The bands we designated are indicated by arrows.

(XP3BRSV) cell lines were from Dr T. Yagi (Kyoto University). Lymphoblastoid cell lines, normal (GM01953A), XP-B (GM02-252A), XP-E (GM02450E) and the XP variant (GM01646A) were purchased from the Human Genetic Mutant Cell Repository (Camden, NJ). HeLa cells were grown in RPMI 1640 medium with 5% calf serum and antibiotics. SV40-transformed cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. Lymphoblastoid cell lines were grown in RPMI 1640 medium with 15% FBS and antibiotics.

Electrophoretic mobility shift assay (EMSA)

A *Hin*dIII cassette (46/50 bp, 5′-GTACATATTGTCGTTAGAA-CGCGTAATACGACTCACTATAGGGAGA-3′/5′-AGCTTCT-CCCTATAGTGAGTCGTATTACGCGTTCTAACGACAATA-TGTAC-3′; Takara Shuzo Co.) was used as an oligonucleotide probe for EMSA. One hundred nanograms of the oligonucleotide were end-labeled using polynucleotide kinase and [γ-^{32}P]ATP. After removal of kinase and unincorporated [γ-^{32}P]ATP, the DNA was irradiated with 10 kJ/m^2 UV from a germicidal lamp. A typical reaction mixture (10 µl) contained 1 ng ^{32}P-labeled probe, 2 µg poly(dI·dC)·poly(dI·dC) and 1µl fractionated cell extracts in binding buffer [50 mM Tris–HCl, pH 8.0, 60 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 0.5 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA)]. After incubation at 30°C for 15 min 2.5 µl 5× loading dye (250 mM Tris–HCl, pH 8.0, 50% glycerol, 0.25% sodium azide, 0.25 mg/ml bromophenol blue) was added and 1 µl of the reaction mixture was resolved at 4°C on 8–25% gradient polyacrylamide gels using the Phast system (Pharmacia LKB Biotechnology). After electrophoresis the gels were wrapped and exposed to X-ray film.

Preparation of nuclear and cytoplasmic extracts

Nuclear and cytoplasmic extracts were prepared according to the method of Dignam *et al.* (18). All steps were performed at 4°C or on ice. In brief, late log phase cultures were harvested and washed with phosphate-buffered saline. The cell pellet was resuspended in ice-cold buffer A [10 mM HEPES–NaOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and allowed to stand on ice for 10 min. The cells were homogenized by 10 strokes in a Dounce homogenizer, the nuclei were collected by centrifugation at 1000 *g* for 10 min and the supernatant was saved for cytoplasmic extract preparation (see below). The nuclear pellet was subjected to an additional centrifugation at 25 000 *g* for 20 min and suspended in buffer B (20 mM HEPES–NaOH, pH 7.9, 25% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT). After homogenization and gentle stirring for 30 min particulate material was removed by centrifugation and the supernatant was dialyzed against buffer C (20 mM HEPES–NaOH, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF).

Cytoplasmic extract was prepared by gently mixing the above supernatant with 0.11 vol, buffer B followed by centrifugation and dialysis against buffer C.

Purification of NF-10 protein

Nuclear extracts prepared from a total of 60 l HeLa cell culture were loaded onto a heparin column (80 ml) equilibrated with 400 ml buffer D (20 mM HEPES–NaOH, pH 7.9, 10% glycerol, 100 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT) and the column washed with 400 ml of the same buffer. Proteins were eluted with 400 ml of a linear KCl gradient (0.1–1 M) in buffer D at a flow rate of 1 ml/min. Fractions showing the binding activities were pooled, dialyzed against buffer E (20 ml Tris–HCl, pH 8.0, 10% glycerol, 0.5 mM DTT) and applied to an anion exchange Fractogel EMD TMAE-650 (S) column (10 ml). After extensive washing proteins were eluted with 100 ml of a linear NaCl gradient (0–0.9 M) in buffer E at a flow rate of 1 ml/min. Active fractions were dialyzed against buffer F (50 mM HEPES–NaOH, pH 7.9, 0.2 mM EDTA, 10% glycerol, 0.5 mM DTT) and applied to a FPLC MonoS HR5/5 column (1 ml). The column was washed with 15 ml of the same buffer and proteins were eluted with 20 ml of a linear NaCl gradient (0–0.5 M) in buffer F at a flow rate of 1 ml/min. The active fractions were combined and stored in aliquots at –80°C after addition of glycerol to 20%.

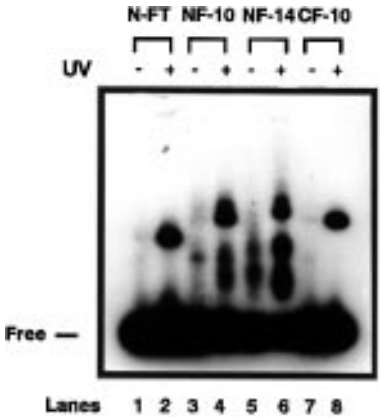

Figure 2. UV-dependent DNA binding of the heparin-fractionated fractions. ^{32}P-Labeled DNA probe was irradiated with (+) or without (–) 10 kJ/m^2 254 nm UV and assayed with N-FT (lanes 1 and 2), NF-10 (lanes 3 and 4), NF-14 (lanes 5 and 6) and CF-10 (lanes 7 and 8).

Photolyase treatment

The photolyases for CPD and for (6–4) photoproduct (6,19) were treated as follows. An aliquot of 12 ng labeled probe irradiated with 10 kJ/m^2 UV was mixed with 5 µl photolyase in 50 µl binding buffer for EMSA and spotted onto parafilm. The samples were covered with a 100 mm dish containing water to prevent any increase in temperature and exposed to photoreactivating light (three FL10EX fluorescent lamps; Panasonic) for 30 min. The DNA samples were extracted with phenol/chloroform, precipitated with ethanol and dissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).

Preparation of monoclonal antibody against NF-10 protein

The purified NF-10 protein (MonoS fraction, 20 µg) was injected i.p. four times into BALB/c mice. Three days after the final booster injection of 50 µg NF-10 into the tail vain the spleen was excised from the mouse and cell fusion with myeloma cells (P3X63-Ag8.653) was performed as previously described (20). Culture supernatants from microtiter wells containing hybridomas were screened using an enzyme-linked immunosorbent assay (ELISA) and cells in the promising wells were cloned twice by limiting dilution. Western blotting was performed using a standard procedure and immunoblots were detected with an Enhanced Chemiluminescence kit (Amersham) according to the manufacturer's specifications.

RESULTS

Identification of binding activities to UV-irradiated DNA in fractionated nuclear and cytoplasmic extracts

We utilized an EMSA to identify UV damage-specific binding proteins. Under our assay conditions we could not detect clear bands with crude nuclear and cytoplasmic extracts (Fig. 1A, lanes 2 and 16, Fig. 1B lanes 3 and 16), probably due to non-specific DNA binding proteins. We fractionated nuclear and cytoplasmic extracts by heparin chromatography and tested each fraction for binding activity using an EMSA. We could detect four distinct bands (indicated by arrows) in different fractions (Fig. 1) and designated each fraction N-FT (flow-through fraction from nuclear extract), NF-10 (10th fraction from nuclear extract), NF-14 (14th fraction from nuclear extract) and CF-10 (10th fraction from cytoplasmic extract). These DNA binding activities were found to be specific for UV-irradiated DNA, but not for unirradiated DNA (Fig. 2), and to be dependent on fluence of UV (data not shown).

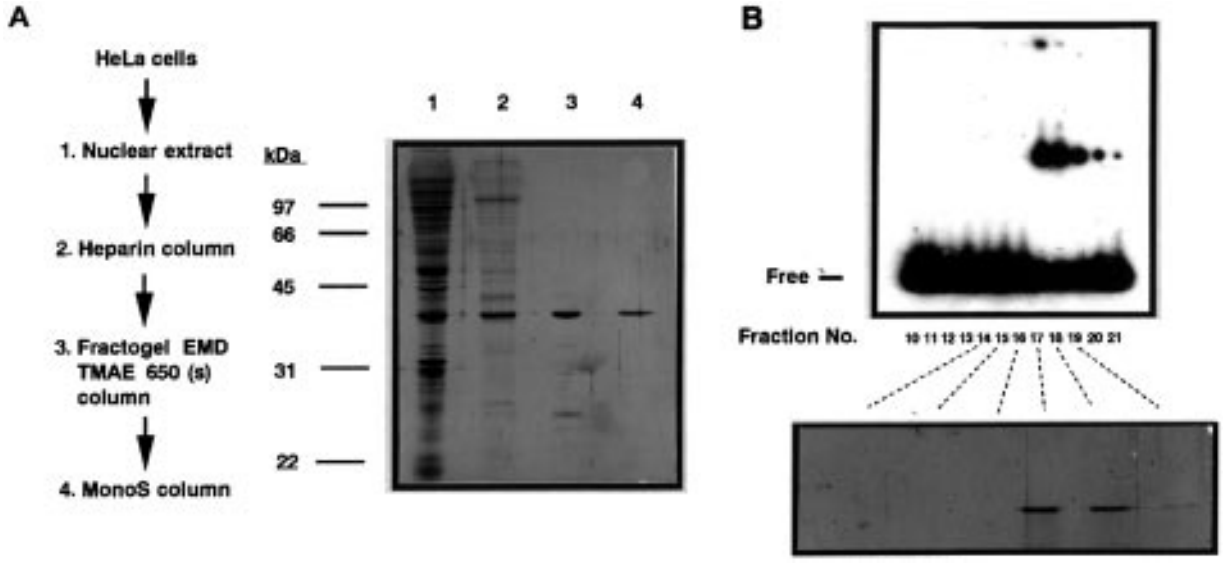

Figure 3. Purification of NF-10 protein from HeLa cells. (**A**) Silver stained SDS–polyacrylamide gel showing NF-10 protein at various stages of purification. An outline of the purification procedure is shown on the left. Samples from each purification step (indicated by numbers) were analyzed by SDS–PAGE (12.5% polyacrylamide) and silver staining. The marker proteins used were phospholipase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (22 kDa). (**B**) The correlation of UV-damaged-DNA binding activity with 40 kDa polypeptide. Fractions 14–19 (indicated) from the MonoS column were tested for binding to UV-damaged DNA (10 kJ/m^2) by an EMSA (top) or for the presence of NF-10 by SDS–PAGE followed by silver staining (bottom).

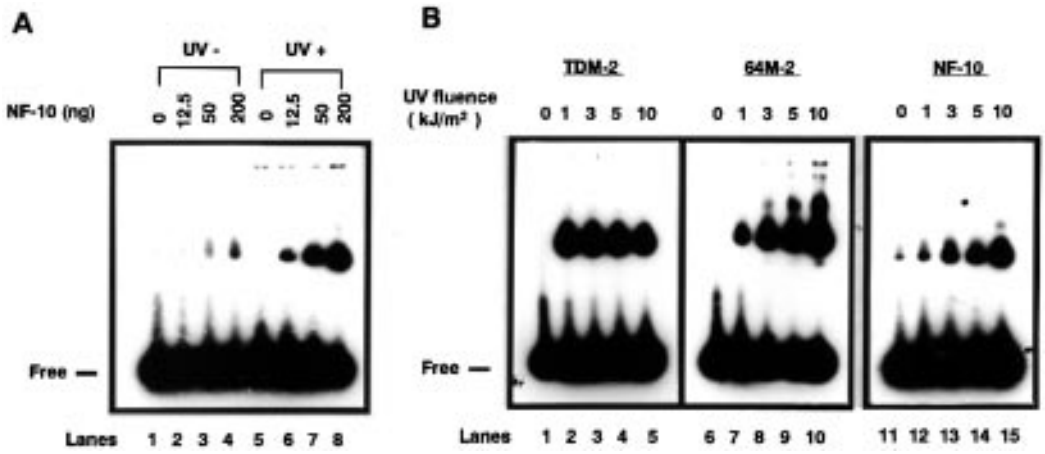

Figure 4. Characterization of the NF-10 protein. (**A**) Titration of NF-10 binding to unirradiated and UV-irradiated DNA. ^{32}P-Labeled DNA was irradiated with (lanes 5–8) or without (lanes 1–4) 10 kJ/m^2 254 nm UV and assayed with the indicated amount of purified NF-10 protein. (**B**) UV fluence dependence of NF-10 binding to DNA. ^{32}P-Labeled DNA probe was irradiated with 0, 1, 3, 5 or 10 kJ/m^2 254 nm UV and assayed with UV damage-specific monoclonal antibodies (lanes 1–10) or 200 ng purified NF-10 protein (lanes 11–15). TDM-2 (lanes 1–5) and 64M-2 (lanes 6–10) antibodies were used to determine the induction kinetics of CPD and (6–4) photoproduct respectively.

Purification of NF-10 protein from nuclear extracts

Among these fractions we tried to purify the NF-10 protein further by means of conventional chromatography and FPLC. Active fractions eluted from the final purification step, a MonoS column, contained a single polypeptide with an apparent molecular mass of ~40 kDa on SDS–PAGE (Fig. 3A, lane 4). Furthermore, the binding activity to UV-damaged DNA in the each fraction correlated well with the intensity of the 40 kDa band on a silver stained SDS gel (Fig. 3B), indicating that this 40 kDa polypeptide is the NF-10 protein with UV-damaged-DNA binding activity.

Characterization of the purified NF-10 protein

Using the purified NF-10 protein we tried to characterize the binding activity. As shown in Figure 4A, the binding activity to UV-irradiated DNA (lanes 5–8) was much stronger than to unirradiated DNA (lanes 1–4), indicating that the binding activity of NF-10 has a preference for damaged DNA.

In order to specify photoproducts recognized by NF-10, we determined UV fluence dependence of the binding, because two kinds of major UV photoproduct, CPD and (6–4) photoproduct, show different induction patterns (1). As measured with damage-specific antibodies, the induction of CPD reached a maximum at ~1 kJ/m^2 (Fig. 4B, lanes 1–5), while that of (6–4) photoproduct increased linearly up to 10 kJ/m^2 (Fig. 4B, lanes 6–10). The binding pattern of NF-10 protein is similar to that of 64M-2 antibody (Fig. 4B, lanes 11–15), raising the possibility that NF-10 protein binds to (6–4) photoproduct in UV-irradiated DNA.

To verify this possibility we employed two kinds of enzymatic photoreactivation to selectively remove either CPDs or (6–4) photoproducts from an UV-irradiated oligonucleotide probe. As shown in Figure 5A, treatment of UV-damaged DNA with *Escherichia coli* photolyase diminished the binding of TDM-2 antibody, but not that of 64M-2 antibody, indicating specific photoreactivation of CPDs. On the other hand, photoreactivation by (6–4) photolyase specifically suppressed the binding of 64M-2 antibody without affecting TDM-2 binding (Fig. 5B). Binding of the

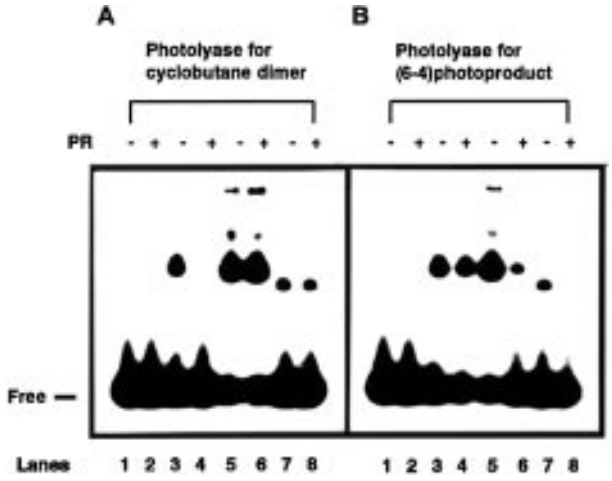

Figure 5. Effects of photoreactivation on NF-10 binding to UV-damaged DNA. ^{32}P-Labeled DNA irradiated with 10 kJ/m^2 254 nm UV was incubated with (PR+) or without (PR–) *E.coli* photolyase specific for CPD (**A**) or *Drosophilla* (6–4) photolyase specific for (6–4) photoproduct (**B**) under photoreactivating light. These DNA probes were assayed with buffer (lanes 1 and 2), TDM-2 antibody (lanes 3 and 4), 64M-2 antibody (lanes 5 and 6) and 200 ng purified NF-10 protein (lanes 7 and 8) for binding.

NF-10 protein decreased after photoreactivation of (6–4) photoproducts (Fig. 5B), but not after that of CPDs (Fig. 5A), leading us to conclude that this NF-10 protein recognizes (6–4) photoproduct in DNA or (6–4) photoproduct-related DNA structure.

Establishment and characterization of a monoclonal antibody raised against NF-10 protein

We have produced a monoclonal antibody directed against the purified NF-10 protein as described in Materials and Methods. The anti-NF-10 antibody reacted with purified NF-10 protein in an ELISA (data not shown) and with the 40 kDa protein in nuclear extract in Western blot analysis (Fig. 6A). This antibody could also

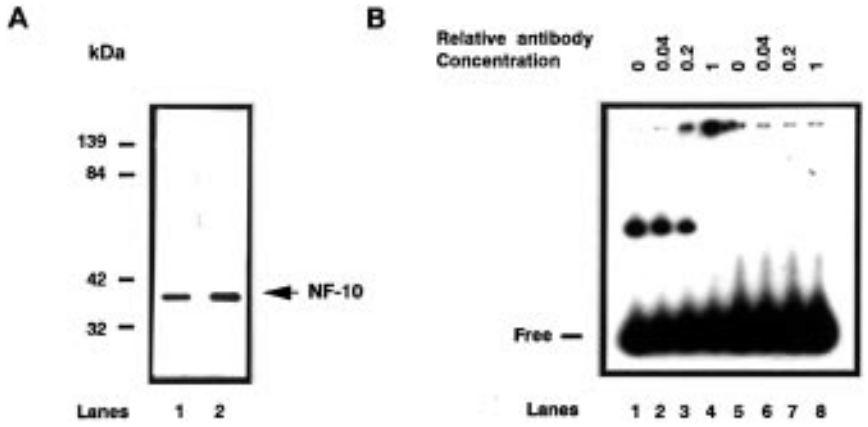

Figure 6. Characterization of the anti-NF-10 monoclonal antibody. (**A**) Immunoblot analysis of nuclear (lane 1) and cytoplasmic (lane 2) extract prepared from HeLa cells using anti-NF-10 antibody. One microgram of each extract was used and the immunoreactive band was developed by the enhanced chemiluminescence (ECL) system. (**B**) Effect of anti-NF-10 antibody on binding of purified NF-10 protein to UV-damaged DNA. Anti-NF-10 antibody was preincubated with (lanes 1–4) or without (lanes 5–8) 200 ng purified NF-10 protein on ice for 15 min and assayed with a UV-irradiated (10 kJ/m^2) DNA probe.

react with a 40 kDa protein in cytoplasmic extract. The 40 kDa band in Western blots was also detected in the CF-10 fraction, but not in the other heparin fractions from cytoplasmic extract (data not shown). These results suggest that NF-10 and CF-10 are identical. We tested the effect of the antibody on NF-10 binding to UV-damaged DNA (Fig. 6B). When purified NF-10 protein was preincubated with the anti-NF-10 antibody before addition to the binding reaction the band of UV-damaged DNA probe complexed with NF-10 shifted to the origin (lanes 1–4). In the absence of the NF-10 protein addition of the antibody did not change the mobility of the UV-irradiated probe (lanes 5–8), indicating the anti-NF-10 antibody does not interact with the oligonucleotide probe. Taking these results together, we conclude that this antibody is specific for the NF-10 protein.

Detection of the NF-10 protein in various XP cell lines

We surveyed the presence of NF-10 protein in various XP cells by immunoblot analysis to examine the relationships between NF-10 and XP gene products. As shown in Figure 7, we detected NF-10 protein in all nuclear extracts prepared from SV40 transformed fibroblasts and lymphoblastoid cells belonging to different XP complementation groups, XP-A to XP-G and the variant, as well as normal cells. This result indicates that NF-10 is a novel UV-damaged-DNA binding protein.

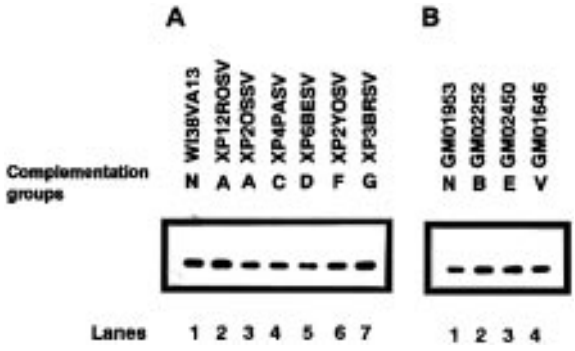

Figure 7. Immunoblot analysis of NF-10 protein in various XP cell lines. Nuclear extracts were prepared from SV40-immortalized (**A**) and lymphoblastoid (**B**) cell lines. Normal (N), WI38VA13 and GM01953; XP-A, XP12ROSV and XP2OSSV; XP-B, GM02252; XP-C, XP4PASV; XP-D, XP6BESV; XP-E, GM02450; XP-F, XP2YOSV; XP-G, XP3BRSV; XP variant, GM01646. Immunoblotting was performed as described in Figure 6.

DISCUSSION

We have assayed UV-damaged-DNA binding activity in nuclear and cytoplasmic extracts from HeLa cells using an EMSA. We detected four shifted bands with different mobilities in fractions from heparin chromatography. These assumed binding factors were found to preferentially bind to UV-irradiated DNA compared with unirradiated DNA. Among these factors we have purified an ~40 kDa protein from the NF-10 fraction to homogeneity. The quantitative Western blotting data using an anti-NF-10 antibody revealed that the degree of purification of the 40 kDa band in the final fraction was ~400-fold, indicating that NF-10 is an abundant protein (data not shown). The photoreactivation experiments suggest that NF-10 protein bound to (6–4) photoproducts, but not to CPDs. Furthermore, the immunoblot analysis shows that this protein is expressed in all XP cell lines tested so far (A to G and the variant).

Several research groups have detected binding activity to UV-damaged DNA in mammalian crude extracts using an EMSA (10–12). Extensive purification revealed that the main UV-DDB activity was attributed to a complex of 125 and 41 kDa proteins with a high affinity for (6–4) photoproduct (14–16). Chu and Chang reported that this binding activity was absent in cells from two related XP-E patients, XP2RO (GM02415) and XP3RO (GM02450) (10). In this study, however, NF-10 binding activity was detected in fractionated nuclear extract from XP3RO (GM02450) cells (data not shown). In addition, our purified protein was detected in this cell strain by Western analysis (Fig. 7). It should be noted that the 125 kDa subunit could not be detected in our final fraction by silver staining (Fig. 3). Considering these data together, we conclude that NF-10 protein is different from UV-DDB, although we cannot exclude the remote possibility that

NF-10 is identical to the small subunit (41 kDa) of UV-DDB until the sequence of the NF-10 protein or gene is determined.

XPA protein has a calculated molecular weight of 31 kDa and migrates as an ~40 kDa protein (21,22). It has been also shown that XPA has a significant preference for binding to damaged DNA and has a higher affinity for (6–4) photoproducts than for CPDs (23). Although NF-10 protein appears to be similar to XPA protein, it is improbable that they are identical, for the following reasons. First, NF-10 protein did not show the doublet band on a SDS gel (Fig. 3) which is characteristic of XPA protein (22,23). Second, NF-10 protein was detected in XP-A cell lines (Fig. 7A) lacking XPA proteins (24). Third, anti-NF-10 antibody did not react with recombinant XPA protein and anti-XPA antibody (a generous gift from Dr K. Tanaka) did not react with the purified NF-10 protein (data not shown). Thus we can conclude that NF-10 is not XPA.

Here we have found a third UV-damaged-DNA binding protein with high affinity for (6–4) photoproduct in humans, distinct from XPA and probably also from UV-DDB. This raises the question of why human cells have so many (6–4) photoproduct binding proteins. The presence of these proteins may explain the much higher frequency of removal of (6–4) photoproducts than of CPDs *in vivo* (1,6). Reconstitution of the NER reaction *in vitro* has revealed that XPA protein is indispensable for dual incision of damaged DNA (4), consistent with the complete lack of damage removal in XP-A cells *in vivo* (1). Although XPA is thought to be involved in the process of damage recognition, the binding constant for UV-damaged DNA does not seem to be high enough (3×10^6/M) (23). It was recently reported that RPA could stimulate damage-specific DNA binding by XPA protein (25). Other UV-damaged-DNA binding proteins may have a role in enhancing the efficiency of damage recognition in NER. An alternative possibility is that some UV-damaged-DNA binding proteins may be involved in the cellular response after UV irradiation, rather than in the repair process *per se*. Functional analysis of NF-10 protein is under way to clarify the biological role of this protein.

The repair of CPDs is also important for cells, since CPD is the predominant lesion induced by UV. There is a possibility that another DNA damage binding factor(s) having a high affinity for CPD may be present in cells. Preliminary experiment showed that the binding activity of the crude NF-14 fraction appeared to be specific for CPD, not for (6–4) photoproduct. Purification and characterization of this protein may give us helpful information on the recognition and repair of CPDs.

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