Identification of a mammalian nuclear factor and human cDNA-encoded proteins that recognize DNA containing apurinic sites

(DNA-binding proteins/DNA repair/ λ gt11)

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ABSTRACT Damage to DNA can have lethal or mutagenic consequences for cells unless it is detected and repaired by cellular proteins. Repair depends on the ability of cellular factors to distinguish the damaged sites. Electrophoretic binding assays were used to identify a factor from the nuclei of mammalian cells that bound to DNA containing apurinic sites. A binding assay based on the use of β -galactosidase fusion proteins was subsequently used to isolate recombinant clones of human cDNAs that encoded apurinic DNA-binding proteins. Two distinct human cDNAs were identified that encoded proteins that bound apurinic DNA preferentially over undamaged, methylated, or UV-irradiated DNA. These approaches may offer a general method for the detection of proteins that recognize various types of DNA damage and for the cloning of genes encoding such proteins.

Cellular DNA is continually subjected to spontaneous and environmentally induced damage that, if left unrepaired, can result in cell death, mutation, or neoplastic transformation. Most DNA repair mechanisms, including base and nucleotide excision repair as well as direct reversal repair pathways, involve the participation of enzymes that recognize DNA structural alterations (1–3). An apurinic (AP) or apyrimidinic site is produced by the hydrolysis of the N-glycosylic bond that links a base to deoxyribose, and such sites are an important type of DNA damage. Spontaneous release of purines constitutes one of the most frequently occurring types of DNA damage under normal physiological conditions (4, 5). AP and apyrimidinic sites are also generated by various chemical or radiation-induced modifications of DNA bases or by the action of DNA repair glycosylases (1–3). They are mutagenic in bacterial and mammalian systems (6–9). Hence, an understanding of the proteins involved in the detection and repair of AP sites and of the genes encoding those proteins is important for comprehending the biological consequences of this type of damage.

We report here the use of an electrophoretic assay to detect factors in mammalian cells that bind to DNA containing AP sites. We also report the development of an approach for the cloning of cDNAs encoding proteins that bind preferentially to DNA containing AP sites.

MATERIALS AND METHODS

DNA Probes. Restriction fragments to be used in the binding assays with mammalian nuclear extracts were isolated from various plasmids. They were 3'-end-labeled using the Klenow fragment of DNA polymerase I and [α -

³²P]dNTPs. AP sites were then introduced into the probes. Radiolabeled DNA was methylated by incubation in 200 μ l of 50 mM sodium cacodylate/1 mM EDTA with 1 μ l of dimethyl sulfate (DMS) for 30 sec at room temperature (10, 11). Reactions were terminated by the addition of 50 μ l of 1.5 M NaCl/1.0 M 2-mercaptoethanol containing poly(dI-dC)·poly(dI-dC) (Pharmacia) at 100 μ g/ml. Samples were then ethanol-precipitated, resuspended in 10 mM Tris·HCl, pH 4.5/1 mM EDTA, and incubated at 50°C for 30 min. Undamaged probes were treated identically except that DMS was omitted and they were resuspended in 10 mM Tris·HCl, pH 8.0/1 mM EDTA.

Probes to be used for detection of AP DNA-binding fusion proteins encoded by recombinant bacteriophages were derived by nick-translation (12) of various plasmids to a specific activity of $1\text{--}2 \times 10^8$ dpm/ μ g, using [α -³²P]dCTP. AP and undamaged probes were then generated by the procedure described above for the probes used in the nuclear extract binding assays. Additional assays were performed with probes that were not treated with DMS but were incubated at pH 4.5 and with probes that were methylated but treated at pH 8.0 instead of 4.5. DNA probes that were irradiated with 254-nm light (2000 J/m²) were also tested.

The number of AP sites present in the DNA probes employed in the various binding experiments was estimated using DNA nicking assays. Unlabeled, supercoiled plasmid DNA substrates were subjected to the depurination conditions described above and then incubated with an AP endonuclease, *Escherichia coli* endonuclease III (13), and electrophoresed in a 0.9% agarose gel (14). The conversion of depurinated, supercoiled DNA (form I) to nicked, relaxed circular DNA (form II) was quantified as described (14). DMS plus acid treatment introduced at least 1 AP site per 1000 base pairs (bp), whereas acid treatment alone resulted in at least 1 AP site per 1250 bp (data not shown). No AP sites were detected in the DMS-treated DNA that was maintained at pH 8.0 or in the untreated DNA. The level of damage in the UV-irradiated probes corresponded to approximately 1 cyclobutane pyrimidine dimer per 50 bp (15). In each experiment, the same batch of radiolabeled DNA was used and modifications, binding reactions, and autoradiography were performed in parallel.

Binding with Mammalian Nuclear Extracts. Extracts of mammalian cell nuclei were tested for the capacity to bind to probes that contained various types of damage. Nuclear extracts were prepared from various mammalian cell lines as described by Dignam *et al.* (16). For the binding reactions, extract containing 16 μ g of protein was diluted to 25 μ l with

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Abbreviations: AP, apurinic; DMS, dimethyl sulfate; IPTG, isopropyl β -D-thiogalactopyranoside; LTR, long terminal repeat.

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50 mM NaCl/10 mM Tris-HCl, pH 7.5/1 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol containing poly(dI-dC)poly(dI-dC) (4 μ g) and probe (10,000 dpm). The reaction mixtures were incubated for 20 min at room temperature (11, 17, 18). The entire reaction mixtures were electrophoresed in nondenaturing 5% polyacrylamide gels and then the gels were dried and autoradiographed. Reaction mixtures included EDTA because we found that no AP DNA-specific complex was detected when binding was assayed in the presence of Mg^{2+} (data not shown).

Isolation of Recombinant Clones. Cloning was performed using an amplified, oligo(dT)-primed, λ gt11 recombinant cDNA library (19) prepared from the human T-cell lymphoma line Jurkat (Clontech). Phage plaques were screened for the presence of β -galactosidase fusion proteins that bound to radiolabeled AP DNA, using the approach described by Vinson *et al.* (20) with slight modifications. Recombinant phage were adsorbed to *E. coli* Y1090, plated in medium containing 0.7% agarose, and grown at 37°C. After 4 hr, nitrocellulose filters that had been soaked in 10 mM isopropyl β -D-thiogalactopyranoside (IPTG) were placed on top of the cultures to induce fusion-protein expression, and the incubations were continued at 37°C overnight. Filters were then removed from the plates and dried at room temperature. All subsequent manipulations were performed with gentle shaking at 4°C in binding buffer consisting of 40 mM KCl, 25 mM Hepes KOH (pH 7.9), 3 mM $MgCl_2$, and 1 mM dithiothreitol. Filters were treated with 6 M guanidine hydrochloride in binding buffer (20). They were then treated with five successive 2-fold serial dilutions of guanidine hydrochloride in binding buffer and incubated for 30 min in 5% (wt/vol) instant nonfat dry milk (Carnation) in binding buffer (20). Binding was allowed to proceed for 2 hr with the appropriate probe ($1-2 \times 10^6$ dpm/ml).

Lysogens. Lysogens were generated by infection of *E. coli* Y1089 and extracts were prepared from IPTG-induced cultures as described (19). The λ gt11 chicken ovalbumin cDNA clone was obtained from Clontech. Twenty-five microliters of extract from each culture was electrophoresed in a NaDodSO₄/7.5% polyacrylamide gel and electroblotted to nitrocellulose (21, 22). Binding assays with DNA probes were performed as described for phage plaques in Fig. 4, except that double-stranded (10 μ g/ml) and single-stranded (5 μ g/ml) salmon sperm DNA were included in the reaction mixtures.

RESULTS

Binding of Mammalian Nuclear Proteins to AP DNA. Initially, we tested whether proteins that bind to DNA containing AP sites (AP DNA) could be detected in mammalian nuclear extracts by electrophoretic mobility-shift assays (23, 24). Nuclear proteins were incubated with a radiolabeled 72-bp restriction fragment and resolved by electrophoresis. Multiple binding activities were detected when the 72-bp fragment, which comprised the enhancer sequences of the murine retrovirus SL3-3 (25), was incubated with nuclear proteins from mouse or human cells (Fig. 1). Murine retroviral enhancers are known to bind to several proteins (17, 18, 26). In each experiment, a variable amount of DNA was retained at the origin of each lane. The nature of this material is unknown. When the DNA was treated successively with DMS and acidic pH, a procedure that introduces AP lesions (4, 5, 27), the amounts of the complexes formed were lower than those seen with undamaged DNA. However, the introduction of AP sites also resulted in the appearance of a new complex (Fig. 1). Presumably, this complex was generated by the binding of a protein that recognized the AP sites in the probe.

FIG. 1. Binding of AP (+) and undamaged (-) DNAs with mammalian nuclear extracts. Extracts from four murine cell lines (T, T-cell line L691-6; E, erythroleukemia line DS-19; M ϕ , macrophage line J774; and B, B-cell line J558) and from the human erythroleukemia line K562 were tested as described (17, 18). Arrows show the position of the AP DNA complex. F indicates the position of free DNA.

Three approaches were used to test whether the binding was specific for AP DNA rather than for methylated DNA or for a specific sequence within the 72-bp probe. One was to compare the binding of DNA probes that either contained or did not contain AP sites. The others were to test different DNA sequences as probes and to test binding in the presence of unlabeled competitor DNA that contained AP sites. Incubation of DNA at increasingly acidic pH and at higher temperatures leads to the generation of increased numbers of

FIG. 2. Specificity of the binding activity in mammalian nuclear extracts for AP DNA. Arrows show the positions of the AP DNA complexes. The nature of the radioactive material at the origins of the lanes is unknown and its levels were variable. (A) Methylated probes were prepared as described in *Materials and Methods*, except that following ethanol precipitation they were resuspended at the pH indicated and incubated for 30 min at the temperature (T, °C) indicated. (B) (Upper) Binding of heparin-agarose-fractionated J558 nuclear extract to a 30-bp restriction fragment with or without AP sites. Crude nuclear extract was loaded onto a heparin-agarose column (Bio-Rad) in 0.1 M KCl/20 mM Hepes KOH, pH 7.9/0.2 mM EDTA/0.5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/20% glycerol. Fractions were eluted in the same buffer containing the concentration of KCl indicated and were tested for binding with an AP or undamaged (-) probe. (Lower) The fraction that was eluted from heparin-agarose at 0.2 M KCl was tested with four different restriction fragments of the sizes indicated. These were derived by *Bst*NI and *Eco*RV digestion of the SL3-3 LTR plasmid (25). Probes tested were either undamaged (-) or contained AP sites. For this experiment, 20,000 dpm of AP DNA or 10,000 dpm of undamaged DNA was used per reaction.

FIG. 3. Binding of an AP probe to heparin-agarose-fractionated J558 extract in the presence of excess unlabeled AP or undamaged DNA. The fraction eluted at 0.2 M KCl was tested for binding to an undamaged (UND) or AP probe (10,000 dpm, ≈ 0.1 ng). Binding to the AP probe was tested with no competitor DNA (-), with 2.5 μ g of competitor DNA that contained AP sites, or with 2.5 μ g of undamaged competitor. The competitor DNAs were derived by *Hae* III digestion of a 5-kbp plasmid of the SL3-3 LTR in pBR322 (17). AP sites were introduced into the competitor DNA by DMS methylation followed by pH 4.5 treatment at 50°C as described for the radiolabeled probes.

AP sites (4, 5, 27). A 42-bp fragment from the SL3-3 long terminal repeat (LTR) was methylated using DMS and concentrated by ethanol precipitation at pH 8.0. Aliquots were resuspended in buffers of pH 4.5 or pH 7.0 and incubated at 50°C or 37°C, respectively, for 30 min and then tested in binding assays. The AP-specific complex was detected with the probe that was incubated at pH 4.5 at 50°C but not with the methylated probe incubated at pH 7.0 at 37°C (Fig. 2A). Thus, binding was correlated with the treatment that introduced AP sites into the probe.

To confirm that complex formation was not due to a sequence-specific binding protein, assays were performed with four different restriction fragments. First, nuclear extract from a murine B-cell line was fractionated on heparin-agarose because this treatment was found to reduce the level of binding of factors that did not recognize AP DNA specif-

ically. The fraction that was eluted at 0.2 M KCl contained most of the AP DNA-specific binding activity (Fig. 2B Upper). Binding assays performed with this fraction and any one of four probes of different DNA sequence into which AP sites had been introduced resulted in the formation of the complex (Fig. 2B Lower). Thus, we conclude that it was not formed by a sequence-specific binding protein but was due to the treatment that introduced AP sites into the probe.

Binding to an AP probe was also tested in the presence of excess unlabeled DNA that was either undamaged or treated to introduce AP sites (Fig. 3). The competitor containing AP sites inhibited binding to levels that were undetectable, while the same amount of undamaged competitor inhibited binding only slightly. Based on all these experiments, we conclude that the complex was formed by a cellular factor that recognized the AP sites.

Cloning of Human cDNAs Encoding AP DNA-Binding Proteins. Since AP DNA-binding activity could be detected in extracts of mammalian cells, we next wished to determine whether a clone of human cDNA encoding an AP DNA-binding protein could be obtained. We employed an approach similar to those that have been used to obtain clones of mammalian sequence-specific DNA-binding proteins (20, 28–32). The method utilized the binding of an AP DNA probe to the *lacZ*-cDNA-encoded fusion protein of recombinant λ gt11 phage (19) to detect clones that expressed the AP DNA-binding domain of a human protein. An amplified recombinant phage library derived from the human T-cell line Jurkat was screened with a DNA probe containing AP sites. Fusion-protein expression in phage plaques was induced with IPTG as described (19). Fusion proteins were adsorbed to nitrocellulose and subjected to denaturation in 6 M guanidine hydrochloride and renaturation as described (20). The immobilized proteins were then incubated with the AP DNA probe for 2 hr and recombinant phage that expressed AP DNA-binding proteins were detected by autoradiography.

Six AP DNA-binding clones were obtained in a screen of 7.5×10^4 recombinant phage. The specificity of binding of each of the six was tested by comparing in parallel assays the binding of the AP DNA probe with binding to undamaged

FIG. 4. Binding of protein from plaques of cloned λ gt11 recombinants to various DNA probes. (A) Binding of each of the six clones to AP (top row) and undamaged (CON, bottom row) DNAs. (B) Binding of clone 3 to probes containing the types of damage indicated (AP, DMS plus pH 4.5; CON, undamaged; DMS, DMS plus pH 8; pH 4.5, unmethylated plus pH 4.5; UV, UV-irradiated). A randomly selected clone (Neg. Con.) that does not bind to DNA is also shown. For the experiments shown here, 25–100 plaque-forming units of cloned recombinant phage were plated in 0.5 ml of 0.7% agarose in LB medium.

FIG. 5. Southern blot of *Eco*RI-digested DNA from each of the six recombinant phage clones hybridized to 32 P-labeled, plasmid-subcloned cDNA inserts of clones 3 and 11. The approximate amounts of DNA used from each of the clones were as follows: clone 3, 0.2 μ g; 4, 0.2 μ g; 6, 0.1 μ g; 7, 0.2 μ g; 10, 0.1 μ g; and 11, 0.4 μ g.

DNA. Each of the clones demonstrated a marked preference for AP DNA compared to undamaged DNA (Fig. 4A). Probes that contained other types of damage were also tested, including DNA that was methylated but maintained at pH 8.0 to prevent depurination, DNA that was irradiated with 254-nm light (2000 J/m²), and DNA that was not methylated but was depurinated by treatment at acidic pH. Fig. 4B shows that the extent of binding of the fusion protein from clone 3 was greatest with the probes that contained AP sites. The methylated and UV-irradiated probes did not bind more efficiently than the undamaged probe, indicating that the fusion protein did not recognize at least the major types of adducts produced by these treatments but was specific for AP DNA. Equivalent results were obtained with each of the other five clones (data not shown).

Hybridization analysis was used to determine whether all six clones were derived from the same gene (Fig. 5). The results showed that five of the cDNAs cross-hybridized and thus were derived from the same gene or perhaps from closely related genes. Since three of the clones (nos. 4, 6, and 10) yielded the same size insert, they may have been derived by amplification of the same parent in the original library. The other isolate (clone 3) was derived from a distinct gene.

To confirm that the fusion protein was responsible for the binding activity, lysogens were prepared from clones 3 and 11 as described (19). Fusion-protein expression was induced in lysogen cultures and total protein extracts were prepared. These were fractionated by electrophoresis in NaDodSO₄/polyacrylamide gels and transferred by electroblotting to nitrocellulose (21, 22). The immobilized proteins were then subjected to denaturation in 6 M guanidine hydrochloride and renaturation (20). They were then tested for binding to radiolabeled AP and undamaged DNAs (Fig. 6). The fusion

FIG. 6. Nitrocellulose blot of lysogen extracts tested for binding to AP and undamaged (UNDAM) DNA probes. Extracts were prepared from clones 3 and 11 and from a λ gt11 chicken ovalbumin cDNA clone (O). Arrows denote the positions of the fusion proteins.

proteins of clones 3 and 11 bound well to the AP probe and only weakly to the undamaged probe. No equivalent binding activity was observed for proteins prepared from a λ gt11 chicken ovalbumin recombinant. We conclude that the portions of the fusion proteins encoded by the human cDNAs are responsible for the AP DNA-binding activity.

DISCUSSION

An AP DNA-specific complex was detected when nuclear extracts from human or murine cells were analyzed by the electrophoretic assay. Although a single AP DNA complex was detected in these experiments, it is unclear whether it was formed by the binding of a single factor or of multiple factors. Nuclear extracts from all the different cell lines gave complexes of the same mobility. Thus, it is possible that the same factor was present in all of them. We term it AP DNA-binding factor (ABF). It is also possible that there are additional ABFs that are present at levels too low to measure by this approach or that do not bind stably enough to be detected. Additional analyses will be required to determine whether ABF is identical to any of the previously characterized, chromatographically distinct forms of human AP endonucleases (1–3, 33–39).

The isolation of two distinct human cDNAs whose fusion proteins showed a preference for binding to AP DNA is consistent with the possibility that human cells contain multiple proteins that recognize AP sites in DNA (1–3, 33–39). Additional studies will be required to determine what role the AP DNA-binding proteins encoded by the cloned genes might play in the repair of AP sites. We do not yet know whether either of the cloned human cDNAs encodes the ABF that was detected in nuclei of human and murine cells. They may encode one of the AP endonucleases that have been isolated from various types of human cells, including lymphoblasts (1–3, 33–39). Regardless, the fact that these clones encode domains of proteins that exhibit preferential binding to AP DNA makes them good candidates for genes involved in DNA repair in human cells.

These results demonstrate (i) the feasibility of using binding assays to detect proteins that recognize AP DNA and (ii) that cells may contain multiple species of such factors. This approach is likely to be of general use for the identification of proteins that recognize various types of damage in DNA. Chu and Chang (40) used electrophoretic mobility-shift assays to detect at least one nuclear factor that bound to DNA damaged by UV irradiation or by the antitumor drug cisplatin. We did not detect formation of a complex of the mobility of ABF when a UV-irradiated (2000 J/m², 254 nm) DNA probe was tested with nuclear extract from mouse L691-6 lymphocytes (data not shown). Therefore, we conclude that ABF is not identical to the UV DNA-binding factor. Glazer *et al.* (41) reported the use of DNA-protein binding assays to detect factors from HeLa cells whose binding activity was stimulated by treating the cells with UV light. Recently, Toney *et al.* (42) reported the isolation of a cDNA encoding a protein that bound preferentially to DNA modified with cisplatin. Thus, these assays appear likely to be of general use for the detection of mammalian factors that might be involved in DNA repair.

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