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ZNF143 ACTIVATES GENE EXPRESSION IN RESPONSE TO DNA DAMAGE AND BINDS TO CISPLATIN-MODIFIED DNA

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We have identified a cisplatin-inducible gene, the *mitochondrial ribosomal protein S11* (*MRP S11*) gene, by means of mRNA differential display. Functional analysis of the *MRP S11* promoter showed that a Staf binding site in the promoter is required for both basal promoter activity and cisplatin-inducible activity. We also found that Staf binding activity is significantly increased in nuclear extracts from cells treated with cisplatin. ZNF 143 and ZNF 76 are human homologues of the Xenopus transcriptional activator, Staf. *ZNF 143* expression is induced by cisplatin but *ZNF 76* expression is not. However, *ZNF 143* expression is not induced by transplatin, which is clinically ineffective. *ZNF143* is an inducible gene by other DNA damaging agents such as γ-irradiation, etoposide and adriamycin. ZNF 143 also binds preferentially to cisplatin-modified DNA. These results suggest that ZNF 143 participates in cellular responses to DNA damage.

Key words: *MRP S11;* ZNF143*; Staf; cisplatin; DNA damage*

Cisplatin is a widely used anticancer agent, and DNA damage is believed to be the main basis of the antitumor effect of cisplatin. Since cisplatin resistance is a major obstacle to the treatment of solid tumors, understanding the molecular basis of the DNA damage response and of cisplatin resistance is important for clinical protocols. Cisplatin resistance is thought to involve several mechanisms, including increased drug efflux, the presence of cellular thiols, increased nucleotide excision repair activity and decreased mismatch repair activity.[1–4] In addition, several mechanisms are thought to contribute to the DNA damage response associated with cisplatin resistance. To understand fully these molecular mechanisms, it is desirable to identify and characterize all the transcription factors that are activated by DNA damage. We have previously shown that transcription factors such as Y-box binding protein (YB-1) and the CCAAT-binding transcription factor 2 (CTF2) are overexpressed in human cancer cell lines resistant to cisplatin.[5,6] YB-1 has been shown to bind preferentially to cisplatin-modified DNA[5] and is activated by nuclear translocation and by transcriptional mechanisms. Oct1 participates in the cellular response to DNA damage,[7,8] and its induction involves posttranscriptional mechanisms. Both c-Myc and AP-1 are also activated and involved in cellular responses to cisplatin.[8] One cisplatin-inducible cDNA clone has been shown to encode the *vacuolar H^+-ATPase subunit c* (*ATP6L*),[9,10] and analysis of its expression revealed that both Sp1 and Oct1 are critical for its activation by anticancer agents.[11]

In order to identify novel mechanisms involved in the DNA damage response, we have used differential display to compare gene expression between control and cisplatin-treated cells. Here, we characterize one cisplatin-inducible gene, *MRP S11*, whose product, MRP S11, is a component of the ribosomal small subunit and binds to 12S rRNA.[12] MRP S11 appears to have RNA binding but not DNA binding activity. We have isolated the promoter region of *MRP S11* in order to identify its transcriptional activators. Functional analysis revealed a critical Staf binding site in the core of the promoter. The zinc finger transcription factor, Staf, originally identified in the frog, is involved in transcriptional regulation of snRNA type and mRNA promoters transcribed either by RNA polymerase II or III.[13] Two human homologues of Staf, ZNF 76 and ZNF 143, have been isolated.[14] Here we show that activation of the transcriptional activator ZNF 143 upregulates the transcription of *MRP S11*. We present evidence that *ZNF 143*, but not *ZNF 76*, is induced by DNA damage. We further demonstrate that ZNF 143 is one of the cellular proteins that specifically recognize cisplatin-damaged DNA.

MATERIAL AND METHODS

Cell culture, drugs, irradiation and antibody

The following cell lines were cultured in Eagle's minimal essential medium (Nissui Seiyaku, Tokyo, Japan): human epidermoid cancer KB cells[15] and human prostate cancer PC3 cells.[16] Cisplatin, transplatin, etoposide, adriamycin and 5-fluorouracil were purchased from Sigma Chemical (St. Louis, MO). Actinomycin-D was purchased from Nacalai Tesque (Kyoto, Japan). All drugs were added directly to the culture medium at the indicated concentrations and times. Irradiation was carried out with a ^{137}Cs γ-cell-40 Exactor (Nordion, Kanata, Ontario, Canada) radiation source at a dose rate of 0.938 Gy/min. An anti-SPH binding factor (SBF) antibody against ZNF 143 was kindly provided by Dr. G.R. Kunkel.[17] An antimethyl-CpG-binding protein 2 (MeCP2) antibody was purchase from Santa Cruz Biotechnology (Santa Cruz, CA).

Differential display

The differential display method was performed using the differential display kit from Takara Shuzo (Kyoto, Japan) as described previously.[9] In brief, reverse transcription was carried out using 9 anchored primers. Twenty-four primers of 10 mer were used in a PCR with the appropriate anchored primer. KB and cisplatin-treated KB cells were analyzed simultaneously. Gels were dried and autoradiographed for 1–2 days. DNA was eluted from gel by boiling and reamplified by PCR. cDNA fragments were then cloned into pGEM-Teasy (Promega, Madison WI) and sequenced with an automated sequencer 377 (PE Applied Biosystems, CA).

Northern blot analysis

Total RNA was isolated using Sepasol (Nacalai Tesque). RNA samples (20 μg/lane) were separated on a 1% formaldehyde-agarose gel and transferred to a Hybond N^+ filter (Amersham

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Biosciences, Piscataway, NJ) with 10 × SSC. *Eco*RI cDNA fragments of *MRP S11*, *ZNF 143* and *ZNF 76* and cDNA containing 316 bp of *GAPDH* coding sequence were labeled with random primer using the Megaprime DNA labeling kit (Amersham Biosciences). Prehybridization and hybridization were performed as described previously.[9]

Isolation of genomic clones of MRP S11 and DNA sequencing

MRP S11 cDNA was identified by mRNA differential display as a gene induced in cisplatin-treated KB cells.[9] MRP S11 genomic clones were isolated from a human placental genomic library in EMBL3 by screening with the cDNA. All positive phages were mapped with *Sal*I. Several genomic DNA fragments were also used as hybridization probes to confirm overlapping regions. Genomic DNA flanking first exon was subcloned into pUC18 and sequenced above.

Construction of MRP S11 promoter-luciferase reporters and expression plasmids

The promoter and the first exon region of *MRP S11* gene, between −1814 and 263, was amplified by PCR using the following primer pairs; 5′-AAGCTTCCAAATCTTGCGCCAGC-3′ and 5′-AAGCTTGAATTGACCCCAGTCAG-3′ and subcloned into the *Hind*III site of pGL3 basic vector (Promega). It is designated pMRP S11 Luc1. For deletion constructs, pMRP S11 Luc1 was digested with *Alw*NI (pMRP S11Luc2), *Acc*I (pMRP S11 Luc3), *Bgl*I (pMRP S11 Luc4) and *Bam*HI (pMRP S11 Luc5). The products were self-ligated after fill-in, or subcloned into the *Sma*I-*Hind*III site of the pGL3 basic vector. For expression plasmids, full-length cDNA fragments of human *ZNF 143* and *ZNF 76* were amplified by PCR with the primer pairs given below and then subcloned into pGEM Teasy (Promega), and *Eco*RI cDNA fragments of *ZNF 143* and *ZNF 76* were cloned into the pGEXT-6P vector (Amersham Biosciences), creating the constructs GST-ZNF 143 and GST-ZNF 76, respectively. For *ZNF 143*, the primers were 5′-ATGACAGAGTTTCCTGGAGG-3′ and 5′-TTAATCATCCAACCCTGGCG-3′, and for *ZNF 76*, they were 5′-ATGGAGAGCTTGGGCCTGCAC-3′ and 5′-TCAGCAGCCACTCTCCGACAC-3′. *Sp1* cDNA (encoding amino acids 30 to the C terminus) was kindly provided by Dr. Robert Tjian (University of California, Berkeley, CA) and it was cloned into pGEXT-4T vector (Amersham Biosciences). The resultant construct is GST-Sp1. GST-HMG1 and GST-TBP have been described previously.[5,18]

Transient transfection

Aliquots of 4×10^4 KB cells were seeded into 12-well tissue culture plates. On the following day, they were transfected with 0.4 μg luciferase reporter plasmid DNA using 2 μl of Superfect reagent (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. The β-galactosidase reporter gene, CH110 (Amersham Biosciences), was cotransfected as an internal control. After incubating for 3 hr, the cells were washed and further cultured at 37°C for 24 hr in fresh medium, then harvested. For cisplatin treatment, the cells were transferred to fresh medium with or without 2 μM cisplatin after 6-hr growth and harvested 12 hr later

Luciferase assay

Lysed cells were assayed for luciferase activity using the Picagene kit (Toyoinki, Tokyo, Japan); light intensity was measured for 2 sec with a luminometer (Luminescencer-JNRII AB-2300; ATTO, Tokyo, Japan). β-galactosidase was assayed according to the protocol of Promega.

GST fusion proteins

GST fusion proteins were induced by isopropyl-1-thio-β-D-galactopyranoside as described previously.[5] Briefly, *E. coli* cells were sonicated for 10 sec in binding buffer (50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 120 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride), and soluble fractions were obtained by centrifugation at 21,000*g* for 10 min at 4°C. GST fusion proteins were bound to 15 μl of glutathione-sepharose 4B in a 50% slurry in binding buffer for 4 hr at 4°C, washed 3 times with binding buffer and eluted with 50 mM Tris-HCl (pH 8.0) and 20 mM reduced glutathione according to the manufacturer's protocol (Amersham Biosciences).

Preparation of nuclear extracts

Nuclear extracts were prepared as described previously.[6] Briefly, 2×10^7 cells were collected in phosphate-buffered saline, resuspended in 2 ml of ice-cold 10 mM HEPES-KOH (pH 8.0), 10 mM KCl, 0.1 mM EGTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride and incubated on ice for 15 min. They were lysed with Nonidet P-40 at a final concentration of 0.5%, and the lysate was centrifuged at 500*g* for 10 min. The resulting nuclear pellet was resuspended in 300 μl of ice-cold 20 mM HEPES-KOH (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride and incubated for 30 min on ice with frequent gentle mixing. Following centrifugation at 21,000*g* for 5 min at 4°C to remove insoluble material, the supernatant (nuclear fraction) was stored at −80°C until use. Protein concentrations were determined by the method of Bradford.[19]

Western blotting

Nuclear extracts (50 μg of protein) of PC3 cells prepared with buffer C were separated on a 7.5% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) using a semidry blotter. Prestained protein marker (Nacalai Tesque) was used as a molecular weight standard. Immunoblot analysis was performed with an appropriate dilution of the antibody.

Electrophoretic mobility shift assay (EMSA)

For preparation of the ^{32}P-labeled oligonucleotide probe, the oligonucleotides were annealed with complementary strands. The double-stranded products were end-labeled with [γ-^{32}P] ATP (Amersham Biosciences) using polynucleotide kinase (Takara Suzo, Kyoto, Japan) and purified from the gel. Thereafter, half of each labeled probe was treated with 0.3 mM cisplatin or 0.3 mM transplatin at 37°C for 12 hr and purified by ethanol precipitation. Numbers of platinum atoms bound to the oligonucleotides were determined by atomic absorption spectroscopy (Polarized Zeeman atomic spectrophotometer Z-8200; Hitachi, Tokyo, Japan). The mean amounts of platinum bound to DNA were about 6.7 platinum atoms/oligonucleotide in cisplatin and about 3.1 platinum atoms/oligonucleotide in transplatin under our conditions. EMSAs with nuclear extract and purified GST fusion proteins were performed as described previously.[5,6] Briefly, 4 μg of nuclear extract protein was incubated for 30 min at 4°C in a final volume of 20 μl containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM EDTA, 1 mM dithiothreitol, BSA 0.1 mg/ml, 10% glycerol, Nonidet P-40 0.05%, 0.1 μg of poly(dI-dC) and 4 ng of ^{32}P-labeled oligonucleotide probe in the presence or absence of competitors. For supershift assays, preincubation was performed in the presence of 2 μl of anti-SBF antibody for 30 min at 4°C prior to the addition of radiolabeled probes. GST fusion proteins were incubated for 5 min at room temperature in a final volume of 10 μl containing 5% glycerol, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 20 μM $ZnCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol and 4 ng of ^{32}P-labeled oligonucleotide probe.[5] Products were analyzed on nondenaturing 4% polyacrylamide gels using a bioimaging analyzer (BAS 2000; Fuji Photo Film, Tokyo, Japan). The relative amounts of DNA-protein complex and total DNA were obtained by integrating the volumes of their respective band (DNA-protein complex) or bands (total DNA) by using the BAS 2000 (Fuji Photo Film). Kinetic analysis was performed as described previously.[20] For the measurement of the protein-DNA association constants by kinetic assays, the increase in the amount of complex was monitored at various times after the addition of 1–15 nM 3 GST fusion proteins (ZNF 143, TBP and HMG1) to ~

1 nM cisplatin-modified DNA probe. The raw data were fit to Equation 1:

$$1/[\text{protein}]_0 \ln([\text{probe}]_0/\{[\text{probe}]_0 - [\text{complex}]\}) = k_{on}t \quad \text{(Eq. 1)}$$

The left side of the equation was plotted against time in sec, and the k_{on} value for each probe was obtained from the slope of the plot. In the equation, $[\text{protein}]_0$ and $[\text{probe}]_0$ indicate the initial concentrations of GST fusion proteins and cisplatin-modified DNA probe, respectively, and [complex] is the concentration of complex at each time point.

To determine the dissociation rate constants (k_{off}), 1–15 nM GST fusion proteins and ~ 1 nM probe were mixed at 25°C for 30 min to reach the equilibrium state. Dissociation of the protein-DNA complexes was initiated by the addition of 200 ng of poly(dI-dC) at different time points to ensure that all dissociation reactions were complete at the same time. The decrease in the amount of complex was followed over a 1- or 2-hr time period. The data were fit to Equation 2 to obtain the first-order rate constant for the dissociation reaction:

$$\ln([\text{complex}]/[\text{complex}]_0) = -k_{off}t \quad \text{(Eq. 2)}$$

where [complex] indicates the concentration of complex at time t and $[\text{complex}]_0$ is the complex concentration under the initial binding conditions. The natural logarithm of [complex] divided by $[\text{complex}]_0$ was plotted *versus* time, and the negative slope of the fit provided the k_{off} value. Values of the dissociation constant, K_d, were calculated from these results ($K_d = k_{off}/k_{on}$).

The sequences of the oligonucleotides used for the probes in EMSAs were as follows: MRP S11 oligo, 5′-GGTTCGTTCTCCCACAATGCACCGCGGCT-3′ and 3′-AAGCAAGAGGGTGTTACGTGGCGCCGAGG-5′; human U6 RNA (hU6) oligo, 5′-GCCTATTTCCCATGATTCCTTCATATTTTGC-3′ and 3′-GGATAAAGGGTACTAAGGAAGTATAAACGGG-5′; mtTFA oligo, 5′-GCAGATTTCCCATAGTGCCTCGCTAGTGGC-3′ and 3′-GTCTAAAGGGTATCACGGAGCGATCACCGG-5′; NF-κB oligo, 5′-TCGAAGGGGACTTTCCCAAGGGGACTTTCCCA-3′ and 3′-TCCCCTGAAAGGGTTCCCCTGAAAGGGTCTAG-5′; GC oligo, 5′-GGCCGGGGCGGGGCGATCGGGGCGGGGGC-3′ and 3′-GGCCCCGCCCCGCTAGCCCCGCCCCCGGG-5′. The Staf binding sites were underlined.

RESULTS

Isolation of MRP S11 cDNA by differential display

To isolate cisplatin-inducible genes, differential display was performed on total RNA from paired KB and cisplatin-treated KB cells. After cDNA amplification and subcloning, differentially expressed cDNAs were sequenced. One of the cDNAs was identical to that of *human mitochondrial ribosomal protein S11* (data not shown). To examine the expression of this cDNA, designated *MRP S11*, KB cells were treated with 15 μM cisplatin and the expression of *MRP S11* mRNA was determined at 5 time points. Cisplatin caused a time-dependent increase in *MRP S11* mRNA levels. Northern blot analysis showed that its level increased more than 10-fold by 12 hr after treatment with cisplatin (Fig. 1).

Characterization of promoter of MRP S11

To isolate a genomic clone encoding the 5′ region of the *MRP S11* gene, a human genome library was screened with the previously isolated cDNA clone. Two clones containing nonidentical inserts, EMBL-S11G1 and EMBL-S11G2, were characterized. The overlap of these clones is shown schematically in Figure 2(*a*), and the positions of exons were mapped by alignment of the cDNA with the published human sequence. The nucleotide sequence of the first exon and its 5′ flanking region is shown in Figure 2(*b*). Based on the published sequence, genomic fragments containing the first exon were amplified by PCR, using EMBL-S11G1 phage DNA as the template.

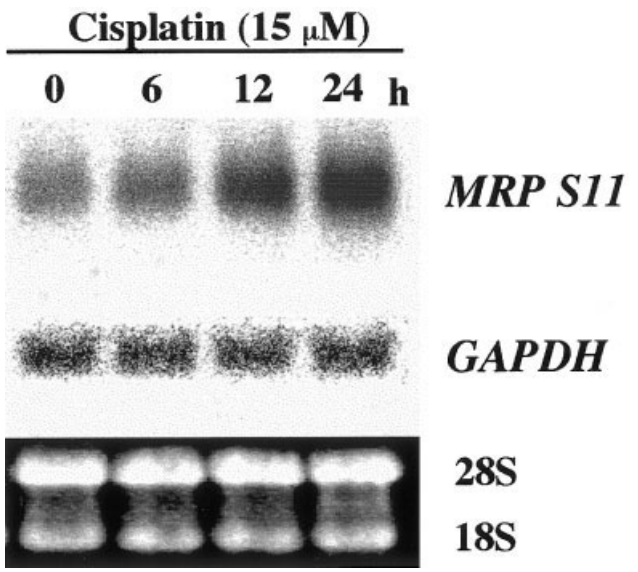
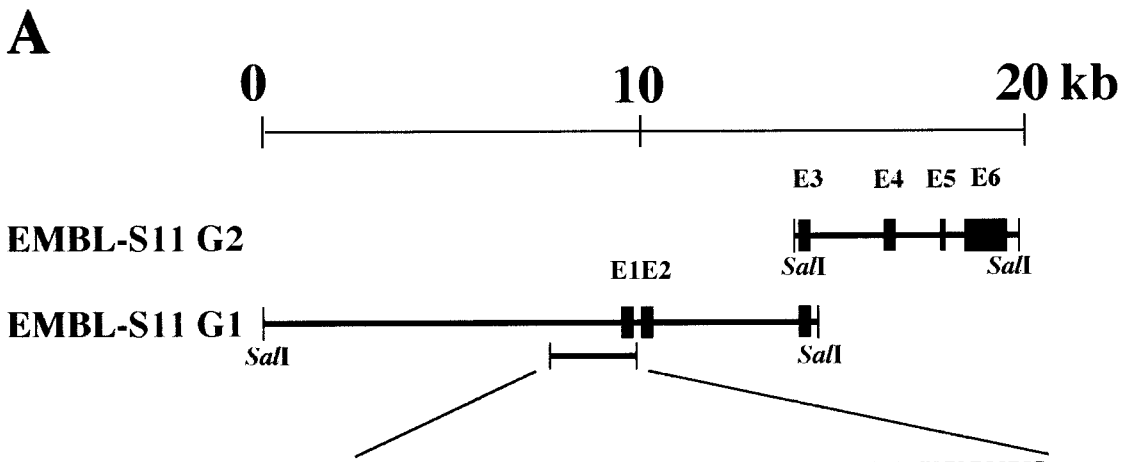


FIGURE 1 – Northern blot analysis of *MRP S11* expression in cisplatin-treated KB cells. KB cells were incubated with cisplatin (15 μM) for the indicated times, and *MRP S11* mRNA (1.1 kb) and *GAPDH* mRNA (1.4 kb) were assayed by Northern blotting. Twenty μg of total RNA was loaded per lane. Gel staining is also shown.

To determine whether the region upstream of the first exon has promoter activity, the available restriction sites were utilized to construct a series of deletion reporter constructs and these were tested by transient transfection in human cancer cell lines. The luciferase assays demonstrated that the region between −1874 and −386 might contain the negative regulatory elements. DNA extending only as far as −87 yielded full promoter activity, whereas the region to −40 had no activity (Fig. 3*a*). The region between −87 and −40 contains a binding site (22 bases in length) for the frog transcription factor, Staf, as shown in Figure 2(*b*). We next examined the effect of cisplatin on promoter activity and found that the luciferase activities of Luc 3 and 4 were increased about 4- to 5-fold by cisplatin treatment. However, the promoter activity of Luc 5 was not increased by cisplatin (Fig. 3*b*). These observations suggest that the Staf binding site is required for activation of the *MRP S11* promoter by cisplatin.

Expression of ZNF 143 is cisplatin-inducible

Two human zinc finger transcription factors, ZNF 76 and ZNF 143, are highly homologous to the frog Staf activator.[14] To investigate whether cisplatin induction of MRP S11 involves an interaction with either of these factors, we first examined Staf binding activity in a nuclear extract of KB and PC3 cells. Binding was detected when the nuclear extract was incubated with labeled MRP S11 oligo and was markedly reduced by the presence of homologous unlabeled DNA (Fig. 4*a*). Similar results were obtained with an unlabeled oligonucleotide corresponding to the Staf binding site of the *human U6 RNA* gene and *mitochondrial transcription factor A* (*mtTFA*) gene. An unlabeled oligonucleotide corresponding to the NF-κB binding motif did not compete. The level of the Staf DNA-protein complex was significantly higher in extracts of cisplatin-treated cells when assayed with either of the 2 labeled probes (Fig. 4*b*). We further noted that the band retarded by these 2 probes was supershifted by the addition of anti-SPH binding factor (SBF) antibody against ZNF 143, but not by rabbit IgG (Fig. 4*c*). We found 2 retarded bands, C1 and C2. The C1 indicates the positions of the supershifted bands. The C2 is shown as the retarded probe at the origin of the gel, which was described previously.[17] Similar results were obtained with nuclear extracts of PC3 cells (data not shown).



B

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      CAATCTGGGA AAATTCAAAG GGCAAAAAAC TACCTTAATC TGGCAAAATT
-1632 AAAAGGAAAA AAAAAACAGG AAGAATAAAA CAGAGACATG GCACACTTGG
      CAAGCTCAGA AGTTTATCAC CCAACATAAC CAGATACACA GTCAAAACAC
      CATGCAGAAA GGAAATGGAG GGAAAAAAAG GTCCTCTGAA CTTTGGGCTT
      GAGTTACATT TGCCAAAAAT GTTACAATGT CACTGGGGGT CAAGAGTGCT
-1432 ACAAAAACAG AGAGCTGGGT TGGGCAATGC CTGCTGACTC ACCACAATCC
      AGTCATGTCA GTCTTTGATC AGAATCTCAC TCACCAAGGA CTTTCCTGTT
      CAGGGACTTT CAAGAGGTTG TTCCTTCTTC CTAGAATGTT TTTCCTCACA
      TTCTTTCTTC TGGGAGAGTC CTACTCATCT TTCTGATTTC AATTTAAATA
-1232 TGACTGTCTC AGAGAGACAT TTCTCATAAA GAGAAAAACC TGTATTCAGA
      AAGTCTGAGG CATTCCTTGA CACTCACCAT AAAATCAAAA TGTGATGTAT
      TTGTTAAACA CTCTATAGTG CCTTGTGTTT TCCTTAACAA CACTTATCAG
      GGTCTGCATT TAGTTGTATG ATTATTTGGT TACTGACTGA TCCCCTCACT
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      ATGAACATCT GTAGGTTGAG TAACAAATAT TTGTCAAGCG AGCGAGTAAA
      TGAATAAATC AAGCAAAAAG CACAGAAGAG AAGTTTATAG TTCTCAACAG
 -832 ACCAAGACAA AAACATCTCG GATTCTGAGG AAACATATCA CCCTTCAAAC
      ACTTTCTGTA GCGACCTCAC ACCTTCACAG CCACTACACT GTTTTTGCCC
      ATGCTTCCTC ACTTGCCCCT CACACAGATA GCCCCATGAG ATAAAACAGA
      GTAGGCTTAT CATAGGCATC TGACAGATGT GCCCACCGCC TCTTGTATTT
 -632 CCTTCTTAAC ATTCATCACA TCACAAAGAC TTTCTCCCTC CAGACTTGTA
      AGCTCCTAGT GACACGGACC GATTCTTGTT CACCACAGGA TCCCTATTGT
      GTTGCATGAA CAAGAAGTAG AGTATCTCCA AAGAGTTGTC TAAGGTGACA
      ATAAACACGG CCAATGAAAA GAAACCACTT TTTGTTGAAC ATCTATGTAC
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      TTAAGTTCAG AGAGGTGAAA TTACTCGCTC AAAGTCACGC AGAATAAACG
      TCACTCTGAA CCCTGCATCT CAGTCCCACC TGCTGCAGTA GAGACGCCAT
      CTCTTCCTGC AATGGGGTCA ACGGCTTGGA GACTACAGGT GGCCGCTGCA
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      CCGCCATCTT TCGTTCTCCC ACAATGCACC GCGGCTCCCA GGGATCCCTC
  -32 AGCCGCGGGA GCGGCAAACT AGGGGAAAGC TCAGTCCCGG AACTGCTAGA
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FIGURE 2 – Restriction map and sequence of MRP S11. (*a*) Schematic exon map in 2 overlapping MRP S11 genomic clones, EMBL-S11 G1 and EMBL-S11 G2. The black boxes denote positions of identified exons E1–E6. (*b*) Nucleotide sequence of the 5′ upstream region, the first exon and part of the first intron of the *MRP S11* gene. Nucleotides are numbered relative to the transcription initiation site. The first exon is boxed and the ATG initiation site is in boldface. The Staf binding site is in boldface and underlined.

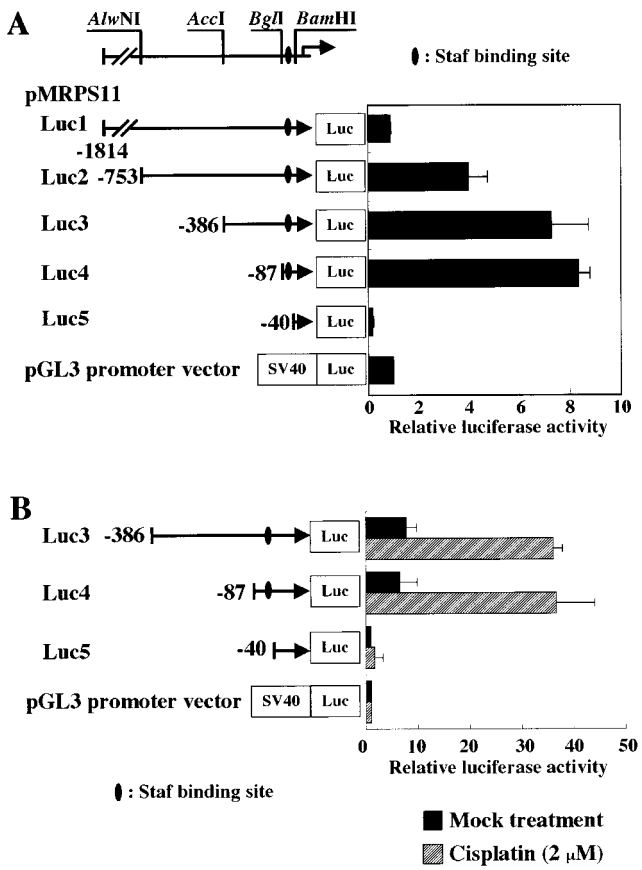

FIGURE 3 – Functional analysis of the MRP S11 promoter in KB cells. (*a*) Schematic representation of the MRP S11-luciferase reporter plasmids and relative promoter activity of the 5′ flanking region of the *MRP S11* gene. Deletion constructs of the 5′ flanking region of the *MRP S11* were subcloned into pGL3 basic vector upstream of the luciferase reporter gene. All reporter constructs were transiently transfected into KB cells, together with a β-galactosidase reporter as an internal control. The pGL3 promoter vector containing an SV40 promoter was used for normalization of luciferase activity, and the promoter activity of the pGL3 promoter vector was set at 1. Relevant restriction enzyme sites are also shown: *Alw*NI, *Acc*I, *Bgl*I, *Bam*HI. (*b*) Transcriptional activity of luciferase reporters with or without cisplatin treatment. All reporter constructs were transiently transfected into KB cells as described in text. After transfection for 9 hr, the cells were incubated for 12 hr in fresh medium with or without cisplatin (2 μM). The promoter activity of the pGL3 promoter vector was set at 1. The Staf binding site is shown as the black circular box. Error bars indicate SD.

Because Staf binding activity increased in nuclear extracts of cisplatin-treated cells, we investigated whether the expression of *ZNF 76* and/or *ZNF 143* is cisplatin-inducible. Interestingly, Northern blot analysis showed that the level of *ZNF 143* mRNA increased when cells were treated with cisplatin, whereas that of *ZNF 76* was reduced (Fig. 5*a*). To determine whether ZNF 143 protein levels also increased, we performed Western blotting. Consistent with the Northern blot analysis, cisplatin treatment increased the cellular level of ZNF 143 protein 3- to 5-fold after 24 hr. MeCP2 expression was almost constant during cisplatin treatment (Fig. 5*b*).

We next examined the induction of *ZNF 143* mRNA in KB cell treated with different chemotherapeutic agents, including γ-irradiation. We first assayed the cytotoxic effects of these drugs and used 5- to 6-fold drug concentrations and the irradiation dose of IC_{50}. Both doses and exposure times were enough to induce the cellular stress for DNA damage or growth arrest. As shown in Figure 6(*a*), most of the DNA damaging agents were shown to induce the expression of *ZNF 143*. In contrast actinomycin-D and 5-fluorouracil did not induce the expression of *ZNF 143*. Further, *ZNF 143* mRNA expression was not induced by the treatment with transplatin, which is clinically ineffective (Fig. 6*b*).

Preferential binding of ZNF 143 to cisplatin-modified DNA

To investigate the ability of ZNF 143 to interact with cisplatin-modified DNA *in vitro*, full-length *ZNF 143* cDNA was fused to GST, and the fusion protein was expressed in bacteria. To determine whether GST fusion proteins are useful in analyzing DNA binding activity, it was first necessary to purify the protein. As a positive control, *HMG1* and *TBP* cDNA were fused to GST, as each of these proteins is known to bind cisplatin-modified DNA. The purified fusion products in each case migrated as mixtures of full-length and faster migrating proteins when analyzed by SDS-PAGE (Fig. 7*a*). The faster migrating proteins were immunoreactive with anti-GST antibody (data not shown), indicating that they were proteolytic products. We used the purified fusion proteins to assess DNA binding activity with an oligonucleotide probe, GC oligo. GST-ZNF143 was unable to bind well to transplatin-modified and unmodified GC oligo, but it bound to cisplatin-modified GC oligo (Fig. 7*b*). Both HMG1 and TBP failed to bind to any of the unmodified oligonucleotides, but did preferentially bind to the cisplatin-modified oligonucleotide (Fig. 7*b*). We calculated the DNA binding affinity by kinetic analysis.[20] To determine the association rate constants for GST fusion proteins, we performed EMSA and analyzed the data. The bimolecular binding reactions reach the equilibrium state within 10 min for the GST fusion proteins (data not shown). The raw data collected within 200 sec were fit to Equation 1 (Fig. 8*a*). The association rate constants (k_{on} values) for cisplatin-modified GC oligo were 1.9×10^5/MS, 1.8×10^5/MS and 2.1×10^6/MS of ZNF143, TBP and HMG1, respectively (Table I). The k_{on} value of ZNF143 for cisplatin-modified GC oligo was almost similar to that of TBP. On the other hand, the k_{on} value of HMG1 for cisplatin-modified GC oligo was about 10-fold higher than that of ZNF143. After dissociation reaction, decreased amounts of the complex were detected (data not shown). The dissociation reaction data were fit to Equation 2 (Fig. 8*b*). Based on these results, we calculated dissociation constants K_d (k_{off}/k_{on}) for each protein (Table I). For K_d values of ZNF 143 and TBP, ZNF 143 has about a 1.5-fold higher binding affinity than TBP, because ZNF 143 possesses a lower off rate. The K_d values for HMG1 and TBP are consistent with the values reported previously.[4,20] As the GC oligo contains 2 Sp1 binding sites, we also examined Sp1 binding to it as a control. As expected, GST-Sp1 recognized the GC oligo well, but not the cisplatin-modified oligo (Fig. 7*b*).

DISCUSSION

To gain insight into how cancer cells respond to DNA damage, we have identified and characterized novel cisplatin-inducible

FIGURE 4 – Binding of nuclear proteins to the MRP S11 Staf binding site analyzed by EMSA. (*a*) Competition assays for MRP S11 oligo binding. Oligonucleotide sequences are shown in text. Ten-, 25- and 50-fold molar excesses of unlabeled oligonucleotides were incubated with nuclear extracts of KB cells (left) or PC3 cells (right) and labeled MRP S11 oligo. (*b*) Analysis of Staf binding proteins in nuclear extracts of cells treated with cisplatin. Nuclear extracts of KB cells (left) or PC3 cells (right) incubated with cisplatin (15 μM) for the indicated times, and control extracts were incubated with MRP S11 oligo or mtTFA oligo for 30 min at 4°C. (*c*) Analysis of Staf binding proteins in nuclear extracts by supershift assay. Nuclear extracts from KB cells treated with cisplatin (15 μM) for 24 hr were preincubated with 2 μl of anti-SBF antibody for 30 min at 4°C and labeled MRP S11 oligo was added. The arrowhead C1 indicates the positions of the supershifted bands. The arrowhead C2 is shown as the retarded probe at the origin of the gel, which was described previously.[17] The arrow indicates the principal retarded band and F denotes free probe.

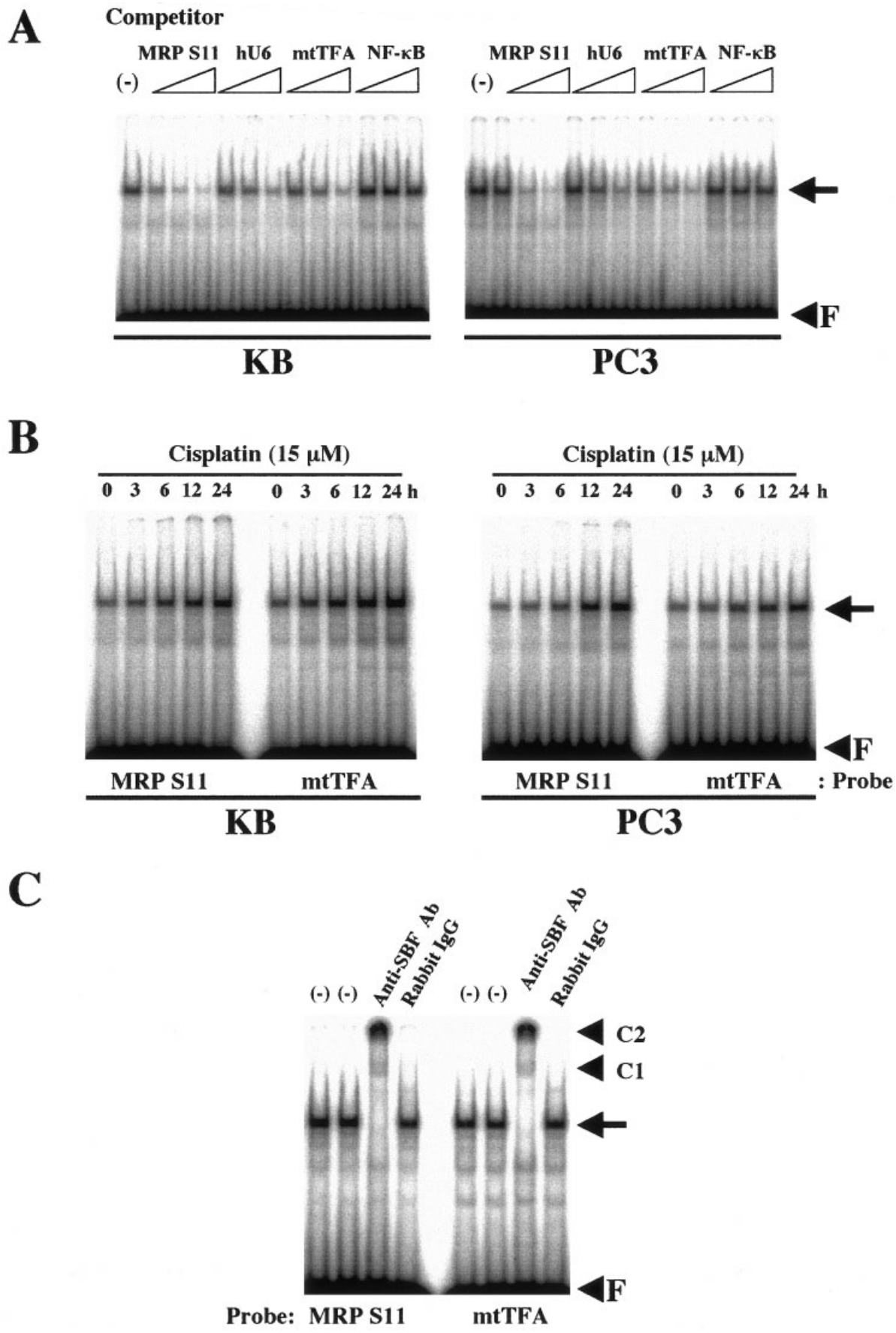
A
Competitor
MRP S11 hU6 mtTFA NF-κB
(-)
MRP S11 hU6 mtTFA NF-κB
(-)
F
KB
PC3
B
Cisplatin (15 μM)
0 3 6 12 24 0 3 6 12 24 h
Cisplatin (15 μM)
0 3 6 12 24 0 3 6 12 24 h
F
MRP S11 mtTFA
MRP S11 mtTFA : Probe
KB
PC3
C
(-) (-) Anti-SBF Ab Rabbit IgG
(-) (-) Anti-SBF Ab Rabbit IgG
C2
C1
F
Probe: MRP S11 mtTFA

FIGURE 4.

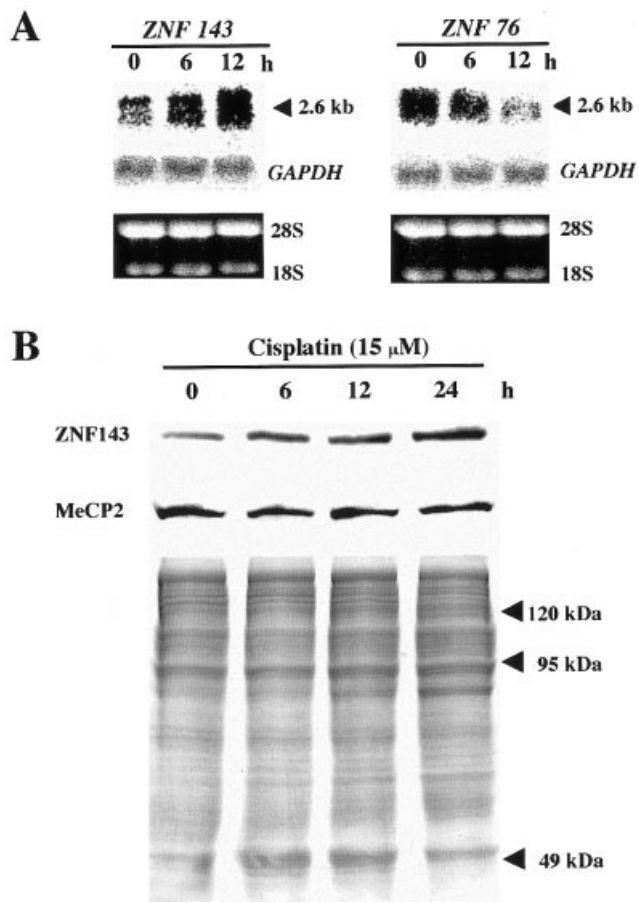

FIGURE 5 – Induction of *ZNF 143* and *ZNF 76* in KB cells treated with cisplatin. (*a*) Northern blot analysis of *ZNF 143*, *ZNF 76* and *GAPDH* mRNA in cells treated with cisplatin. KB cells were incubated with or without cisplatin (15 μM) for the indicated times, and total RNAs were assayed by Northern blotting. Total RNAs were assayed by Northern blotting. Twenty μg of total RNA was loaded per lane. Gel staining is also shown (each bottom panel). (*b*) Induction of ZNF 143 protein in PC3 cells was treated with cisplatin (15 μM) for the indicated times. Cells were harvested and nuclear extracts were prepared as described in text. Fifty μg of the nuclear extracts were loaded on a 7.5% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. Immunoblot analysis was performed with antiserum to ZNF 143 (95 kDa) and antibody to MeCP2 (76 kDa). Gel staining with Coomassie Brilliant Blue (R-250) is also shown (bottom).

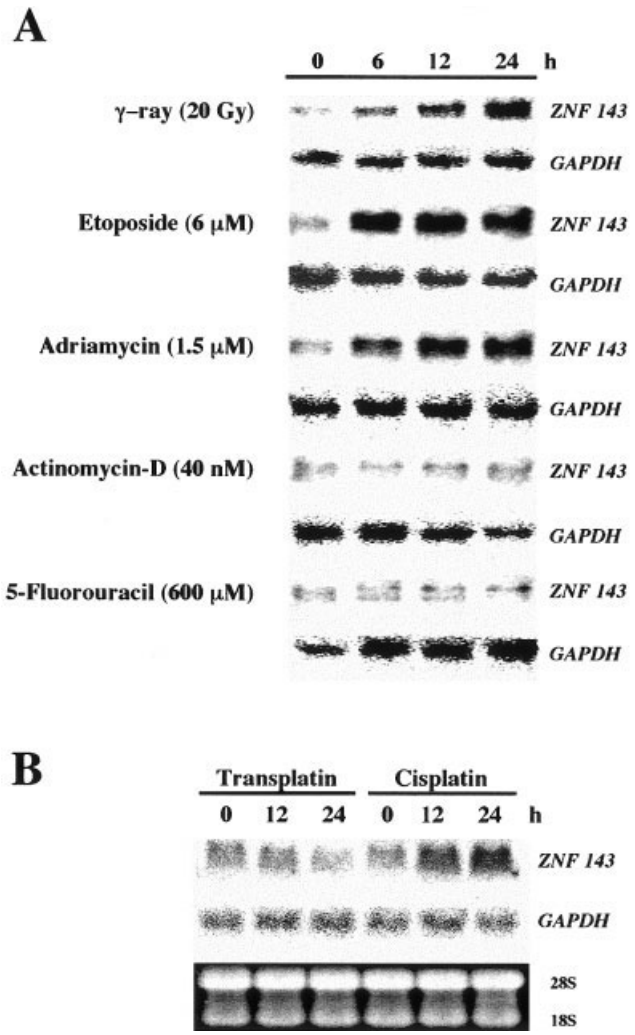

FIGURE 6 – Induction of *ZNF 143* mRNA in KB cell treated with different chemotherapeutic agents including γ-irradiation. (*a*) Northern blot analysis of *ZNF 143* mRNA (1.1 kb) in KB cells treated with γ-irradiation, etoposide, adriamycin, actinomycin-D and 5-fluorouracil. KB cells were irradiated using doses of 20 Gy of γ-rays and incubated for the indicated times. KB cells were incubated with those anticancer drugs at the indicated concentration for the indicated times. Total RNAs were assayed by Northern blotting. Twenty μg of total RNA was loaded per lane. Northern blot analysis of *GAPDH* mRNA (1.4 kb) is also shown (each bottom panel). (*b*) Northern blot analysis of *ZNF 143* mRNA (2.6 kb) in KB cells treated with transplatin (150 μM) and cisplatin (15 μM). Northern blot analysis of *GAPDH* mRNA (1.4 kb) and gel staining are also shown (bottom panel).

genes. This allowed us to study the cellular components involved in DNA damage signaling and in cellular sensitivity to DNA damaging agents. The *MRP S11* gene was isolated by differential display as a cisplatin-inducible gene. We found that cisplatin treatment of human cancer cells stimulated *MRP S11* mRNA expression, as well as nuclear Staf binding activity. Although there is no evidence that MRP S11 is directly involved in DNA damage, mitochondria are important cellular targets for DNA damage. The cooperative actions of several mitochondrial proteins may be involved in the DNA damage response pathway in mitochondria, and *mtTFA* has also been found to be cisplatin-inducible (data not shown). It is a member of the family of HMG proteins and can recognize both cisplatin-modified and oxidatively damaged DNA.[21] We have shown that YB-1 recognizes the inverted CCAAT box as well as cisplatin-modified DNA.[5] YB-1 is a major core protein of messenger ribonucleoprotein particles,[22] and its overexpression in mammalian cells inhibits protein synthesis.[23] It has been shown to bind specifically to RNA containing 8-oxoguanine,[24] suggesting that it may function to sequester oxidatively damaged mRNA molecules from the translational machinery. These findings suggest that MRP S11 may bind to cisplatin-damaged RNA as well as to ribosomal RNA. The persistence of damaged mRNA in the cell would be hazardous and might induce apoptosis. It has been shown that the apoptosis-related protein, DAP3, is a component of the small subunit of the mitoribosome.[25] This suggests that the mitoribosome, with MRP S11 as a constituent, may be involved in apoptosis. While the relation between mitochondria and DNA damage is not entirely clear, we speculate that MRP S11 may possess novel functions in DNA damage signaling.

We also found that ZNF 143 is a transcriptional activator of *MRP S11* expression. Staf is involved in transcriptional activation of snRNA-type genes, some of which are transcribed by RNA

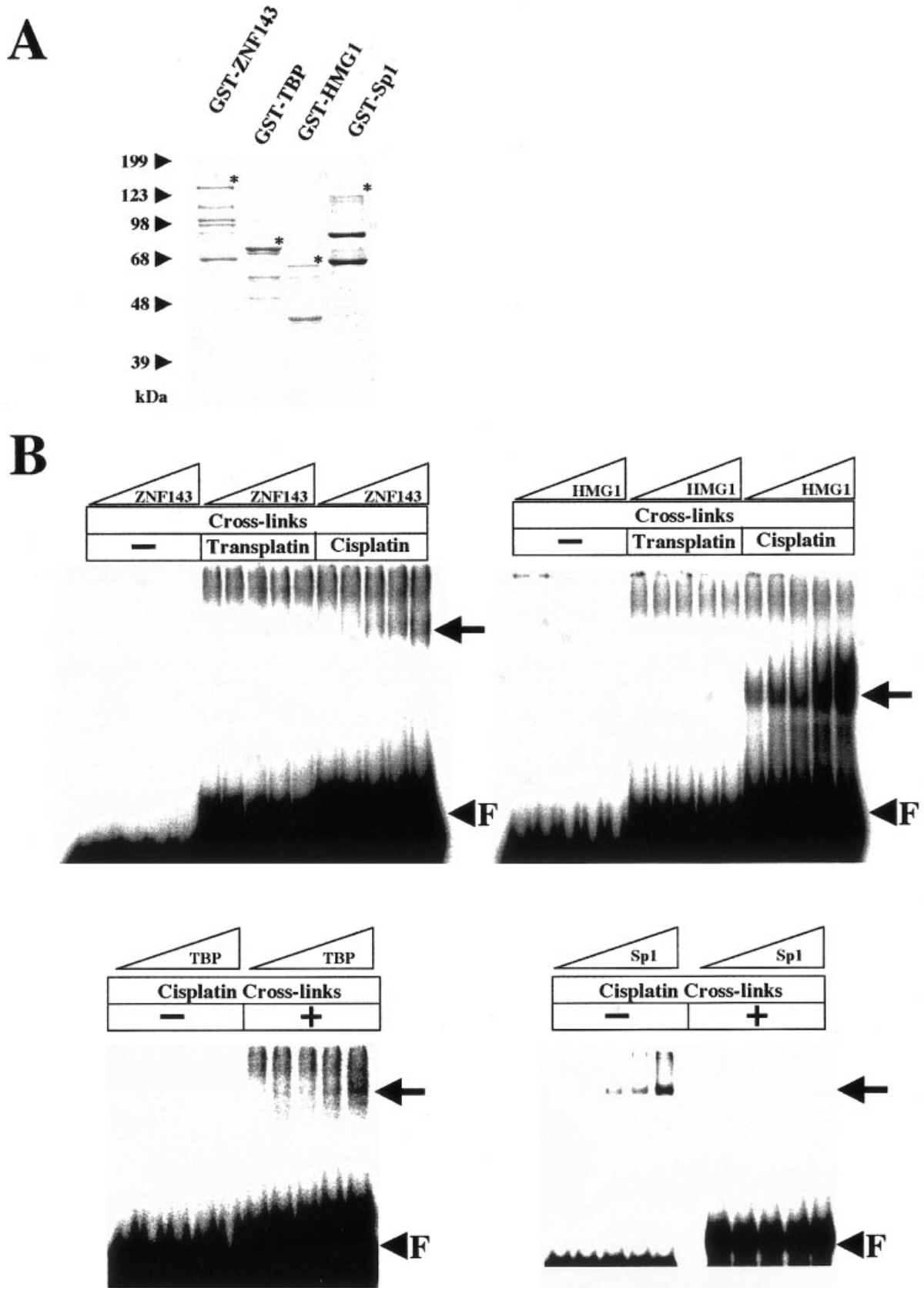

Figure 7 – EMSA using GST fusion proteins. (*a*) Purified GST fusion proteins were separated by SDS-PAGE (10%) and stained with Coomassie Brilliant Blue (R-250). Asterisks indicate the positions of full-length GST fusion proteins. (*b*) The drug-modified DNA binding activity of GST-ZNF 143, GST-HMG1, GST-TBP and GST-Sp1. Binding of purified GST fusion proteins to DNA oligo was analyzed by EMSA using the ^{32}P-labeled GC oligo with cisplatin or transplatin or without crosslinks (−). The amounts of GST fusion proteins were 6, 12, 25, 50 and 100 ng except for GST-HMG1. The amounts of GST-HMG1 were 0.6, 1.2, 2.5, 5 and 10 ng. The arrow indicates DNA-protein complexes and F denotes free probe.

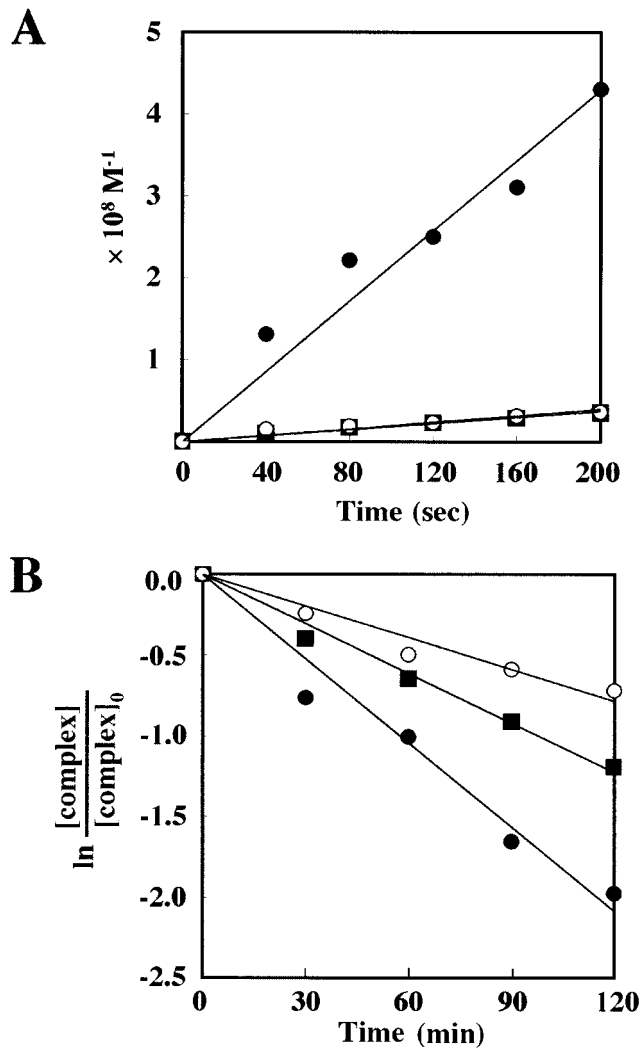

FIGURE 8 – Determination of kinetic parameters for GST fusion proteins binding to cisplatin-modified DNA. (*a*) The raw data collected within 200 sec were fit to Equation 1 to determine k_{on} values for ZNF 143 (open circles), TBP (closed squares) and HMG1 (closed circles). Lines were plotted according to Equation 1. (*b*) The natural logarithm of the complex concentration divided by the initial concentration at t = 0 is plotted *versus* time. To determine k_{off} values for ZNF 143 (open circles), TBP (closed squares) and HMG1 (closed circles), lines were plotted according to Equation 2.

polymerase II and others by RNA polymerase III.[13] Recently, 2 human Staf homologues, ZNF 143 and ZNF 76, have been cloned.[14] ZNF 143 and ZNF 76 are 84% and 64% identical to Xenopus Staf, respectively.[14] These 2 proteins bind the same DNA motif with similar affinity[13] but their expression in response to cisplatin treatment is very different. As shown in Figure 5(*a*), expression of *ZNF 143* is upregulated while *ZNF 76* is downregulated by cisplatin treatment. We also found that most of the DNA damaging agents were shown to induce the expression of *ZNF 143*. In contrast, actinomycin-D and 5-fluorouracil that did not produce

TABLE I – KINETIC PARAMETERS FOR ZNF 143, TBP AND HMG1 BINDING TO CISPLATIN-MODIFIED DNA

Protein	k_{on}, M^{-1} S^{-1}	k_{off}, S^{-1}	K_d, nM
ZNF 143	$1.9 \pm 0.1 \times 10^5$	$1.1 \pm 0.1 \times 10^{-4}$	0.59
TBP	$1.8 \pm 0.1 \times 10^5$	$1.6 \pm 0.2 \times 10^{-4}$	0.86
HMG1	$2.1 \pm 0.2 \times 10^6$	$2.7 \pm 0.3 \times 10^{-4}$	0.13

Values indicate the average and SD of at least 3 experiments.

DNA strand breaks did not induce the expression of *ZNF 143*. Taken together, these results indicated that the zinc finger-type transcription factor *ZNF 143* is induced after cell exposure to DNA damaging agents (Fig. 6). The upregulation of ZNF 143 protein by cisplatin may be directly involved in the cisplatin-inducible expression of *MRP S11*. *mtTFA* is also upregulated by cisplatin treatment (data not shown), and we have found that a Staf binding site is also located in the proximal promoter region of the *mtTFA* gene. In the light of these findings, it was of interest to examine whether ZNF 143 protein could recognize cisplatin-modified DNA.

Several cellular proteins that preferentially recognize cisplatin-modified DNA have been characterized.[2,5,26–28] Notable among them are the HMG proteins. We have recently shown that the Y-box binding protein, YB-1, also binds strongly to cisplatin-modified DNA.[5] This is the first evidence that a sequence-specific transcription factor can recognize cisplatin-modified DNA. As shown in Figure 7, we prepared GC oligo, which contains a series of cisplatin crosslinking sites. ZNF 143 protein recognized cisplatin-modified GC oligo. Cisplatin crosslinking near the TATA box has been shown to enhance interaction of TBP with the TATA box.[29] This implies that ZNF 143 may recognize the specific pattern of cisplatin crosslinking in DNA in a sequence-independent manner.

Tumor suppressors are involved in cellular responses to DNA damage; they accumulate in the nucleus after DNA damage and regulate the cell cycle and gene expression. The induction of ZNF 143 does not require the tumor suppressor p53, because PC3 cells with disrupted p53 still exhibited a significant induction of ZNF 143 (Fig. 4*b*). We have previously shown that tumor suppressor proteins interact directly with YB-1[30] and HMG1[31] and regulate the expression of their target genes and recognition of the DNA damage. Both YB-1 and HMG1 proteins are upregulated in cisplatin-resistant cells, suggesting that cisplatin-induced apoptosis may be controlled by the levels of proteins that recognize DNA damage as well as of tumor suppressor proteins. It will be of interest to examine whether ZNF 143 interacts with tumor suppressor proteins.

In conclusion, we believe that the *MRP S11* and *ZNF 143* genes provide novel insights into the mechanism of cisplatin genotoxicity. Although mitochondria are extensively involved in responses to DNA damage, little is known about how cisplatin-DNA crosslinks contribute to the sequence of events that leads to cell death. Furthermore, the novel finding that ZNF 143 is one of the group of proteins that recognize cisplatin-modified DNA suggests that the mechanism of cisplatin genotoxicity may be complex and multifactorial. It will be important to determine whether elevated expression of ZNF 143 induces drug resistance, since proteins of the nucleotide excision repair complex can recognize and remove cisplatin adducts from genomic and mitochondrial DNA.[1–4,32–34] We are currently investigating the interaction of ZNF 143 with repair proteins.

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