

Transcriptional Activation of the Human HMG1 Gene in Cisplatin-resistant Human Cancer Cells¹

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ABSTRACT

The nonhistone chromosomal protein, high mobility group 1 (HMG1), which is ubiquitously expressed in higher eukaryotic cells, preferentially binds to cisplatin-modified DNA. The observation that HMG1 is overexpressed in cisplatin-resistant human cancer cells suggests that cisplatin resistance may be closely associated with HMG1. To decipher the mechanism of HMG1 overexpression in cisplatin-resistant cells, we isolated two overlapping genomic DNA clones containing the entire human HMG1 gene. These clones, which span ~15 kb of contiguous DNA, include 5 kb of the 5' flanking region as well as the entire coding sequence. We sequenced 1500 bp upstream of the first exon. The segment proximal to the transcription initiation site did not contain a TATA box but did possess an activating transcription factor site, an activator protein-2 site, one CCAAT box, and two CCAAT-binding transcription factor/nuclear factor-1 (CTF/NF-1) sites. HMG1 promoter activity was 3–10-fold higher in cisplatin-resistant KB-CP20 cells than in parental KB cells. An *in vivo* footprint experiment showed several differences of dimethyl sulfate modifications between KB and KB-CP20 cells in the area around the CTF/NF-1 sites. In addition, electrophoretic gel mobility shift assays showed that binding of a nuclear factor from cisplatin-resistant cells to the CTF/NF-1 site was significantly higher than the binding of the same factor from parental cells. Semiquantitative reverse transcription-PCR and Western blot analysis also showed that expression of CTF/NF-1 was 3–20-fold higher in the resistant cell line than in its parental counterpart. These findings suggest that, in cisplatin-resistant cells, the expression of HMG1 gene product is enhanced at the transcriptional level and that this probably occurs through the enhanced expression of the CCAAT binding factor, CTF/NF-1.

INTRODUCTION

Cisplatin is among the most widely used anticancer chemotherapeutic agents (1, 2). Frequently, tumor cells develop resistance to cisplatin, which may involve multiple mechanisms. One mechanism involves decreased net intracellular accumulation of cisplatin can limit drug effect. ATP-dependent active outward efflux of cisplatin through the plasma membrane is enhanced in some cisplatin-resistant cancer cell lines (3, 4). In another mechanism of resistance, intracellular pathways act to detoxify cisplatin; factors involved include glutathione *S*-transferase, glutathione (5), metallothionein (6), and thioredoxin (7). DNA repair is a third basis for resistance. Formation of cisplatin-DNA adducts is essential to cisplatin-induced cytotoxicity, and a defect in excision repair results in markedly enhanced sensitivity to cisplatin (8, 9). Numerous nuclear proteins preferentially bind to cisplatin-modified DNA, including linker histones H1 (10),

repair-related proteins (11), HMG1³ box-containing proteins (11), and transcription factors (12, 13). Because these proteins often are overexpressed in cisplatin-resistant cells (14), they may influence repair of cisplatin-DNA adducts. HMG1 is a nonhistone chromosomal protein that is abundant in eukaryotic cells (15). A member of the HMG family of proteins, HMG1 appears to be involved in DNA replication, transcription, and repair, as well as in other reactions involving binding to and recognition of DNA sequences (16–18).

In the present work, we demonstrated that HMG1 is overexpressed in three cisplatin-resistant cell lines. To better understand the mechanism of HMG1 overexpression in cisplatin-resistant cells, we isolated and characterized the HMG1 gene promoter. We compared the activity of this promoter in cisplatin-resistant cells and their drug-sensitive counterparts. Finally, we identified the specific factor that regulates HMG1 gene expression.

MATERIALS AND METHODS

Materials. [α -³²P]dCTP and [γ -³²P]ATP were obtained from Amersham Pharmacia Biotech. Restriction enzymes, Taq polymerase, and DNA-modifying enzymes were from Takara Shuzo (Kyoto, Japan). Synthetic oligonucleotides were obtained from Hokkaido System Science Co. Ltd. (Hokkaido, Japan). Sepasol for RNA extraction was from Nakarai Tesque, Inc. (Kyoto, Japan).

Cell Culture. All cell lines were cultured in either Eagle's minimal essential medium or RPMI 1640, both containing 10% fetal bovine serum, 0.292 mg/ml L-glutamine, 100 units/ml penicillin, and 100 μ g/ml kanamycin. KB/VJ300 and KB/VM-4 cells were obtained from human epidermoid cancer KB cells by sequential selection with increasing doses of vincristine or teniposide, respectively (19). KB and KB-CP4 cells were kindly provided by Dr. Akiyama (Kagoshima, Japan; Ref. 20); A2780, A2780-E80, KB, and KB-CP20 cells were donated by Dr. Fojo (National Cancer Institute, Bethesda, MD; Ref. 21); and PC3 and PC5 cells were kindly provided by Dr. Nakagawa (Ohita, Japan; Ref. 3).

Cloning and Sequencing. Starting with RNA from KB cells, human HMG1 cDNA was cloned by RT-PCR, using the primers 5'-AACAT-GGGCAAAGGAGATCC-3' and 5'-TACCAGGCAAGGTTAGTGGC-3'. The resulting cDNA was subsequently sequenced.

Genomic HMG1 DNA clones were isolated by screening a human placenta library in EMBL3 with the HMG1 cDNA probe. Positive phages were mapped with *EcoRI* and *SalI*. Several restriction fragments around the first exon were subcloned into pUC18 and sequenced. All plasmids were sequenced from both ends using an ABI 373 automated sequencer. Primer extension analysis was performed as described, using the primers, 5'-CCTCAGCGAGGCACAGAGTCG-3', derived from the sequence of the first exon of the HMG1 gene (22).

Northern Blot Analysis. Total RNA from parental and cisplatin-resistant cells was isolated using Sepasol reagent. RNA samples (20 μ g/lane) were separated on a 1% formaldehyde-agarose gel and transferred to a Hybond N+ filter (Amersham Pharmacia Biotech) with 10 \times SSC. Prehybridization and hybridization were performed as described previously (19).

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³The abbreviations used are: HMG, high-mobility group; RT-PCR, reverse transcription-PCR; DMS, dimethyl sulfate; EMSA, electrophoretic mobility shift assay; CTF/NF-1, CCAAT-binding transcription factor/nuclear factor 1; ATF, activating transcription factor; AP, activator protein; nt, nucleotide.

Construction of Luciferase Reporter Plasmid. The Pica gene vectors PGV-P2 and PGV-B2 were purchased from Nippongene (Tokyo, Japan), and pCH110 (pSV- β -gal) was obtained from Amersham Pharmacia Biotech. Different deletions of the 5' regions of the HMG1 gene were ligated into the *Hind*III site of PGV-B2. Details of constructions are available upon request. Plasmid DNAs were isolated using Qiagen-tip columns (QIAGEN) according to the manufacturer's instructions.

DNA Transient Transfection and Luciferase Assay. Using 8 μ l of SuperFect (QIAGEN), we transfected DNA (2 μ g of plasmid) into KB and KB-CP20 cells when the cells were 60–80% confluent. After incubation for 8 h at 37°C, the cells were washed and incubated for another 36 h in culture medium. The cells were lysed in 200 μ l of 25 mM Tris phosphate (pH 7.5) containing 1% Triton X-100 and centrifuged at 14,000 \times *g* for 15 s. Luciferase activity in the resulting supernatants was assayed using a Picagene kit (Toyooki, Tokyo, Japan) as described previously (23). Light intensity was measured for 15 s with a luminometer (Dynatech ML 1500; JEOL, Japan).

All cells were cotransfected with pSV- β -gal as a control for transfection efficiency, and β -galactosidase activity was measured using a GAL-XE kit (Aurora, Costa Mesa, CA).

In Vivo Footprint Analysis. DNA was extracted from cells treated *in vivo* with DMS (24), and incubated with 1 M piperidine at 90°C for 30 min. As a control guanine ladder, genomic DNA from KB cells was treated *in vitro* with DMS and incubated with piperidine. Ligation-mediated PCR was performed as described (25, 26). The nucleotide sequences of the HMG1 upper strand primers were as follows:

Primer 1: 5'-GGAGCCAGACGCAGCC-3'

Primer 2: 5'-GCTCTGTAACATTACTCTCCAGCCAGCG-3'

Primer 3: 5'-GCTCTGTAACATTACTCTCCAGCCAGCGCGGCTC-3'

The HMG1 lower strand primers were as follows:

Primer 1': 5'-CTCTGCTTTTCCCGTAGCAGC-3'

Primer 2': 5'-GCTTCTGCCTCTGCCCTCTCCCG-3'

Primer 3': 5'-GCCTCTGCCTCTCCCGCTCAGCC-3'

Primers 1 and 1' were used for first-strand synthesis, whereas primers 2 and 2' were used for PCR amplification. Primers 3 and 3' were labeled at their 5' ends with [γ -³²P]ATP and used for detection of the ladder. Samples were analyzed on a 6% polyacrylamide sequencing gel.

Preparation of Nuclear Extracts and EMSA. Nuclear extracts were prepared as described (23). Briefly, 2 \times 10⁷ cells were collected with PBS; resuspended in 2 ml of ice-cold 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride; and incubated on ice for 15 min. The cells were lysed with dropping 0.6% NP-40, and the lysate was centrifuged at 400 \times *g* for 10 min. The resulting nuclear pellet was resuspended in 300 μ l of ice-cold 20 mM HEPES-KOH (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride; incubated for 15 min on ice with frequent gentle mixing; and centrifuged for 5 min at 4°C in a microcentrifuge to remove insoluble material. The resulting supernatant (nuclear extract) was stored at -70°C, and its protein concentration was determined (27).

EMSA were performed as described (13). Briefly, 6 μ g of nuclear extract protein was incubated for 30 min at room temperature in 20 μ l of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 8% glycerol, 1 mM DTT, 0.1 μ g of poly(dIdC), and 1 \times 10⁴ cpm of ³²P-labeled oligonucleotide probe (1 ng) in the absence or presence of various nucleotide competitors (data not shown). The reaction mixtures were applied to a nondenaturing 4% polyacrylamide gel and subjected to electrophoresis at 7 W for 1.5 h in 44.5 mM Tris-borate, 1 mM EDTA. The gels were subsequently exposed to X-ray film with intensifying screens. The sequence of oligonucleotides used for EMSAs is as follows.

Nc: 5'-GGCTTTGGATTGAAGCCAATATGAGG-3' and 3'-CGAAACCTAACTTCGGTTATACTCCG-5'

N1: 5'-CCGTGAGTTGGCGGTAGCCAATAGGAGC-3' and 3'-GCACTCAACCGCCATCGGTTATCTCCG-5'

N2: 5'-GGACAATGGGAGGTATCGGGCCAATGAGC-3' and 3'-CTGTACCCTCCATAGCCCGTTACTCGC-5'

ATF: 5'-CAATCCTTGATGACGTGTCC-3' and 3'-TTAGGAACACTGACACAGGG-5'

The anti-CTF1 antibody for supershift assay was kindly provided by Dr. Tanese, New York Medical Center (28).

Western Blotting. Nuclear extract containing 100 μ g of protein was separated on 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Millipore) using a semidry blotter. Prestained protein marker (New England Biolabs) was used as a molecular weight standard. The membrane was immunoblotted with antiserum to CTF/NF-1 used in the super shift assay.

RT-PCR. Total RNA was isolated from KB and KB-CP20 cells using Sepasol reagent, and first-strand cDNA was synthesized by reverse transcription of 1 μ g of RNA in 20 μ l, using 5 μ M random primers and mouse mammary tumor virus reverse transcriptase (Life Technologies, Inc.).

PCR amplification was performed in a 10- μ l volume containing various amount of cDNA together with 10 \times PCR buffer, 250 μ M each deoxynucleotide triphosphate, Ex Taq polymerase, and 1 μ M each primer.

The human CTF/NF-1 primers were 5'-GCTCACGCACGAAGTAGGCCA-3' and 5'-GGCGAGGGAAGAAGACCTTTGCTATCCC-3'. The human β_2 -microglobulin primers were 5'-ACCCCACTGAAAAAGATGA-3' and 5'-ATCTCAAACCTCCATGATG-3'.

Amplification was performed for a predetermined optimal number of cycles. PCR products were separated by electrophoresis on 2% agarose gels, which were stained with ethidium bromide. To quantify the amount of DNA, each gel was analyzed on a FLA2500 densitometer (Fuji, Japan).

RESULTS

Expression of HMG1 mRNA. Human HMG1 cDNA that had been isolated by RT-PCR and sequenced was used as a hybridization probe in Northern blotting. When we analyzed the levels of HMG1 mRNA in three independent cisplatin-resistant cell lines, KB-CP4, A2780E80 and KB-CP20, we found that the levels in these resistant cells were 3–9-fold higher than in their respective parental cells (Fig. 1).

Cloning of HMG1 Genomic Clones. We also used the cloned HMG1 cDNA to screen a human placental genomic DNA library. Finally, we isolated two overlapping genomic clones, EMBL-H1G1 and -H1G2, that together formed a functional HMG1 gene (data not shown).

Sequence Analysis of the Promoter Region. A 4.7-kb *Hind*III fragment of EMBL-H1G2 was subcloned into pUC18. This fragment, designated pH1-5, was mapped (data not shown). The nucleotide sequences of the first exon and first intron as well as that of the 5' flanking region of the gene were determined (Fig. 2).

We carried out primer extension to precisely define the start site for

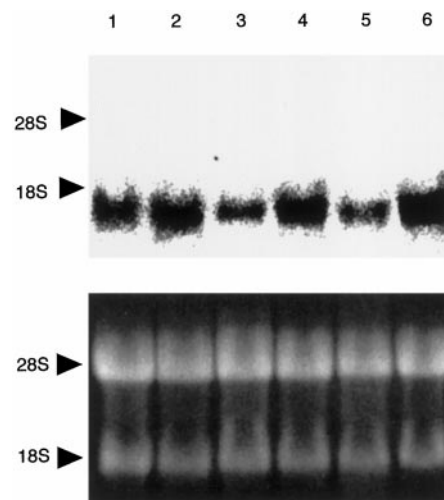


Fig. 1. Expression of HMG1 mRNA in parental and cisplatin-resistant cells. RNA from each cancer cell line was separated on a 1% formaldehyde-agarose gel, transferred to a Hybond N+ membrane, and hybridized with an HMG1 cDNA probe (22). Gel staining is also shown (bottom panel). Lane 1, KB; Lane 2, KB-CP4; Lane 3, A2780; Lane 4, A2780-E80; Lane 5, KB; Lane 6, KB-CP20.

-1337 AACCTTAGT TATGATGCT TATAAGCAT ATAAAAATC AAATAACAC GATGTAGTG ATTTATCCA AATTGTACT
 GTTACTTTG GAGGAATTGG TTGTGGATT TAGAAGTGA CAATAACCA TTAGAGAGT AAAAAAAGG GAAGTCCTGT
 ATAACTGAAT AGGGGCTGG TCGTCTCAG CTAAGAGGA TAGATAGAT TTTCATAAG GACTGTATA ACTGTAGAGT
 CTTCAAGACT GCCCACTACA GCCAGACAT TTCTCTACA GACGTAAGTA TGTCTATTA TACCAGCCAG CTTGTGGAGT
 TAGAGAACC ACAGCCTGTG GATTTGGGCA CATCACTCT CTGCTGCAT CTAATCCTT GGTGAACAT ATACTACAA
 CTCCTGTAAT CTTAGAGCAG TTACATGGAT GCACTAAGT GTGTGATGG AGGGAAGAT AGCATCACAA GAATTCGTTT
 GATTGTACA GTAATCCACA CCAATCGAAA TAAATAGCC AAACAGTTA GAATGTGCT TCTAATGAAT TTGCTAATC
 TACCAGGGCC TCACAACAT TTCTCTCAG AGTACCCTAT ATACAACCA GTGGCTGTC TTAAATTACA TGGAAATTT
 ATTTGATTC CAGGAAGTTA AGACAGTCT TTGTATTTA ATAAAAGTT CTAGACATG TGTGAACTC GATCAACCA
 AGAAAAAGG TGAACCTGAA TGCTGTCTA CTACTCAAG ATTTCAAGT CTACAGGAG CTGACGTGAC GGCCTATAG
 CCCAAAAAG GGGTATTTA AACCCGGCT CCGGGTGTG GGGTGGTCT TTTGCTGAG GAGTTGAGA CACTTGTGGA
 AAAGTCAGC CTTTTCGCT CCGCCGGCC CTGCGGTGTG GGGTGGTCT GGGTAAACA CATGCACACA TACACCATAG
 AGCTCTGTT TTCCCTGAG AGCTCTGCT TCTGCTCTG CTCTCCCGC CTGACCTCT TTGCCCGGCA TAGACACACA
 TTCAAGTTG CCGCTGTTT CAATCCTTGA TGACTGTCT CCGGAGAC CCAATAGCAA ACGGGCTCTG GTCAGACAA
 -57 TTGAGTGTAT CGGCAATG AGCAGCCC GTGATTTGCA GATACCAAT AGGAGCCG CTGGCTGAG AGTAATGTTA
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CGCCGGGCAA GTGAGAGCC GACGGGCACT GGCGACTCT GTCCCTGCT GAGCTGAGT CTGGGCGAG GTGGGCGG
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 +2184 ACATGGGCAA AGGAGATCT AAGAAGCCA GAGGCAAAAT GTATCATAT GCAITTTTTG TGCAAACTTG TCCGGAGGAG
CATAAGAAGA AGCACCCAGA TGCTCAGTC AACCTCTCAG AGTTTCTAA GAACTGCTCA GAGAGGTGGA AGTgagagc

Fig. 2. Nucleotide sequence of the 5' upstream region, first exon, first intron, and second exon of the HMG1 gene. Nucleotides are numbered relative to the transcription initiation site, which was determined by primer extension. The first and second exons are boxed. The intron sequence is in lowercase letters. The sequences that serve as the recognition sites for the CTF/NF-1 transcription factor are underlined and boxed. The ATF and AP-2 sites and the CCAAT boxes are underlined.

transcription. The cDNA products extending from the primer were analyzed by electrophoresis. We found that transcription was initiated at one major site (data not shown). Sequence comparison with the cDNA also indicated that the first exon was 157 bp in length and that

the second exon started 14 bp upstream from the initiation codon. Although no TATA or TATAA sequences were detected in the 5' upstream region, one was found in the ATF site, one in the AP-2 site, and three CCAAT boxes were found in the proximal promoter region, all of which may be binding sites for transcription factors. The sequence of the proximal two CCAAT boxes resembled that of the CTF/NF-1 binding site.

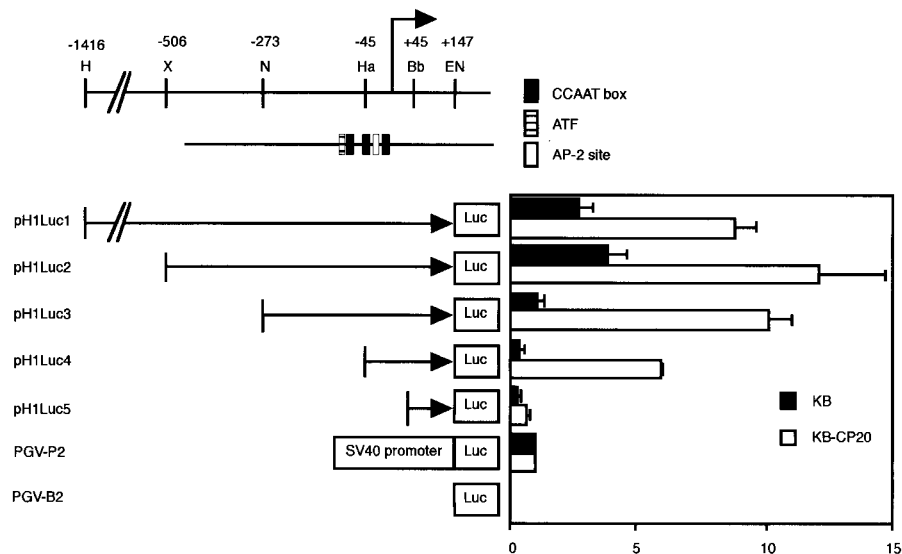
Up-Regulation of HMG1 Gene Expression in Cisplatin-resistant Cells. To determine whether the region upstream of the putative transcriptional start sites can activate transcription, available restriction sites were used to construct a series of deletion plasmids (Fig. 3). The basal transcriptional activity of the HMG1 gene promoter was measured in KB cells transiently transfected with various luciferase reporter plasmids. We observed maximal luciferase activity with pH1Luc2. In addition, the promoter activity of pH1Luc3 was almost as high as that of PGV-P2, indicating that the region between nt -273 and -45 is essential for basal promoter activity (Fig. 3).

The promoter activity of these constructs was then compared in KB and cisplatin-resistant KB-CP20 cells to identify the promoter sequences responsible for high-level expression. We found that promoter activity in resistant cells was higher than that in KB cells, using all of the deletion constructs. In resistant cells, significant promoter activity was observed after transfection with pH1Luc1, pH1Luc2, pH1Luc3, and pH1Luc4, but not with pH1Luc5. These findings thus suggest that the -45 region upstream of the transcription initiation site is responsible for the up-regulation of HMG1 expression in cisplatin-resistant cells.

In Vivo Footprint Analysis of HMG1 Promoter. As shown above, the present sequence analysis revealed three CCAAT boxes and AP-2 and ATF sites in the promoter region of the HMG1 gene, all of which may be binding sites for transcription factors. Protein-DNA interactions in the proximal promoter region may be detected by *in vivo* DMS footprint experiments. When we assayed DMS modifications of the HMG1 gene promoter in KB and KB-CP20 cells, several structural alterations were observed (Fig. 4). The most distal ATF binding site was clearly protected in both parental and cisplatin-resistant cells (Fig. 4A). Protection of the ATF site was also observed in the lower strand (Fig. 4B) as well as concomitant hypersensitive signals (guanines at nt -99, -98, and -97) in the vicinity of the ATF site. These observations strongly suggest that this ATF binding site constitutively binds ATF or related factor(s).

In contrast, the AP-2 site showed hypersensitive signals, but not

Fig. 3. Schematic representation of reporter plasmids and relative promoter activity of the 5' flanking region of the HMG1 gene. Deletion constructs of the 5' flanking region of HMG1 were subcloned into Basic Vector 2 (PGV-B2) upstream of the luciferase reporter gene. Relevant restriction enzyme sites are also shown: H, HindIII; X, XhoI; N, NotI; Ha, HaeIII; Bb, BbvI; EN, EcoNI. All reporter constructs were transiently transfected into both KB and KB-CP20 cells, along with the β -galactosidase reporter gene (pSV- β -gal) as an internal control. Promoter Vector 2 (PGV-P2) reporter gene containing an SV40 promoter was also transfected to normalize luciferase activity. Bars, SD.



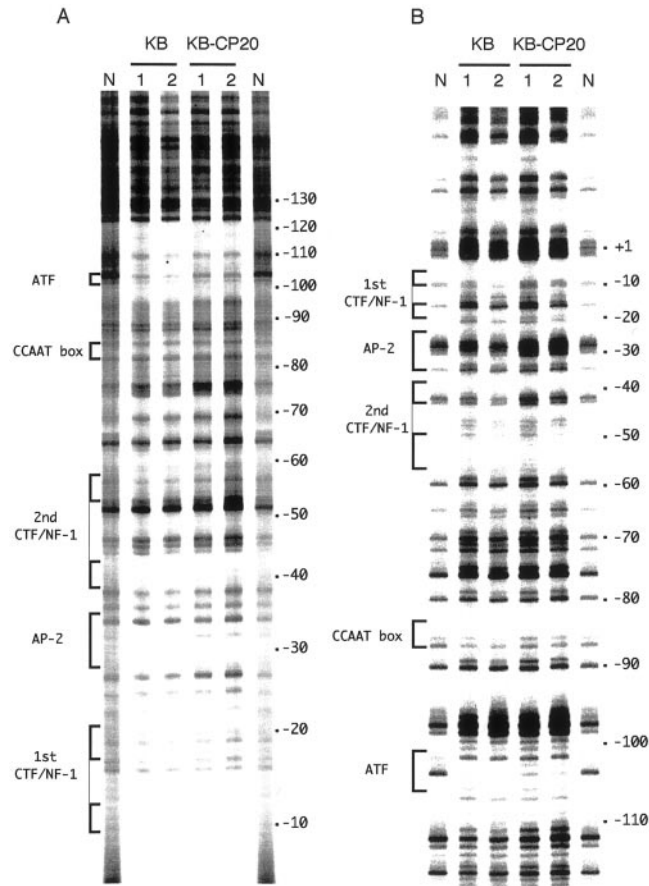


Fig. 4. *In vivo* footprint analysis of the HMG1 gene promoter. *Panel A* corresponds to the upper (nontranscribed) strand, whereas *panel B* corresponds to the lower (transcribed) strand. Potential *cis*-acting DNA elements are shown at the left of each panel, and the positions relative to the transcription initiation site are shown to the right. Lane *N* contains naked DNA, purified from untreated KB cells and treated with DMS *in vitro*. Lanes 1 and 2 contain DNA from nuclei treated *in vivo* with 0.05 and 0.1% DMS, respectively.

protection, in both the upper and lower strands, suggesting that this site on the HMG1 gene does not function as an AP-2-binding element. In addition, a higher intensity of hypersensitive signals in cisplatin-resistant cells than in parental cells was observed.

The AP-2 site is located between two proximal CCAAT boxes (Fig. 4). Among the three CCAAT boxes detected in the HMG1 promoter region, two resembled the CTF/NF-1 binding site, TGGA/C(N)₅GCCAA. Moreover, a family of binding factors has been shown to bind to this palindromic element. Although no clear protection in the factor contact sites at either end of this consensus sequence was observed, a significant number of hypersensitive signals in the spacer region were detected, *e.g.*, at guanines -16 and -17 in the most proximal CTF/NF-1 site, and at guanines -45, -46, and -51 in the second CTF/NF-1 site (Fig. 4A). Similarly, the adenines at -50 and -48 and the guanine at -47 showed hypersensitive signals in the lower strand (Fig. 4B). Because hypersensitive sites in the vicinity of the consensus sequence were routinely observed (factor contact nucleotides), the presence of hypersensitive signals in the spacer regions of the CTF/NF-1 sites suggests that these CTF/NF-1 sites may be occupied by binding factors. Hypersensitive signals in the AP-2 site situated between two CTF/NF-1 binding sites could also be interpreted as evidence of the binding of factors to the CTF/NF-1 sites. With respect to the distal CCAAT box, slightly hypersensitive nucleotides were detected in both strands (guanines -88 and -81 in the upper strand, and guanines -76 and -90 in the lower strand), suggesting that this distal CCAAT box is occupied by a type of CCAAT binding factor, at least *in vivo*.

Characterization of Nuclear Factor Binding to CCAAT Boxes. To gain insight into the transcription factors that may regulate HMG1 gene expression, EMSAs were performed using nuclear extracts prepared from various drug-resistant cell lines. We designed four oligonucleotides probes as described in "Materials and Methods." The N1, N2, and ATF probes were based on the HMG1 promoter sequence, whereas Nc contained the CTF/NF-1-binding consensus sequence. We found that although the ATF probe formed multiple complexes, it formed equal numbers of complexes with nuclear extracts from parental and drug-resistant cells (data not shown). Probes N1, N2, and Nc each formed a single smear complex, suggesting that the protein that binds both N1 and N2 is CTF/NF-1. Alterations of the DNA-binding activities toward Nc, N1, and N2 were not observed in cell lines resistant to teniposide (KB-VM4) or vincristine (KB-VJ300; Fig. 5, *left*). However, we observed enhanced DNA-binding activities in all cisplatin-resistant cell lines compared with parental cells. Cross-competition experiments showed that a 20-fold molar excess of unlabeled N1 and N2 could disrupt interaction with either N1 or N2 (data not shown).

To determine whether CTF/NF-1 can bind N1 and N2, we performed supershift assays. As expected, in the supershift assay using Nc, N1, and N2 oligonucleotides as probes, anti-CTF/NF-1 antibody was able to supershift with similar mobility, but anti-c-myc antibody could not (Fig. 6). These findings suggest that the nuclear factors binding to probes N1 and N2 are identical.

We next investigated whether the increased binding activity in cisplatin-resistant cells was attributable to a simple quantitative alteration of CTF/NF-1. We analyzed the nuclear content of CTF/NF-1 protein in various cisplatin-resistant cells in comparison with sensitive counterparts. Western blot analysis demonstrated that the nuclear content of CTF/NF-1, which migrated at 60 kDa, was much higher in cisplatin-resistant variants than in their parental counterparts (Fig. 7A). This is consistent with the findings obtained from EMSAs. Primers were then designed to quantify the steady-state levels of CTF/NF-1 mRNA, and the levels of endogenous CTF/NF-1 mRNA expression were assayed using RT-PCR. The level of CTF/NF-1 mRNA, normalized relative to that of β_2 -microglobulin mRNA, was found to be 3-fold higher in KB-CP20 cells than in KB cells (Fig. 7B).

DISCUSSION

The HMG proteins are a family of chromatin proteins. Among these proteins, HMG1 and HMG2 are structurally and functionally similar, and

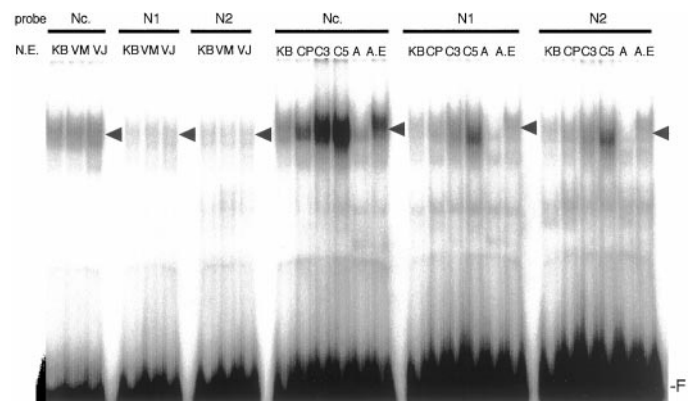


Fig. 5. Up-regulation of CCAAT box-binding factor activity in cisplatin-resistant cells. Nuclear factors were obtained from cell lines resistant to various drugs. The arrowheads indicate specifically retarded bands. F, free probes. Cell lines assayed were as follows: KB, drug-sensitive parental cells; VM, teniposide-resistant KB-VM4 cells; VJ, vincristine-resistant KB-VJ300 cells; C3, drug-sensitive PC3 prostatic cancer cells; C5, cisplatin-resistant PC5 cells; A, drug-sensitive A2780 ovarian cancer cells; A.E., cisplatin-resistant A2780-E80 cells.

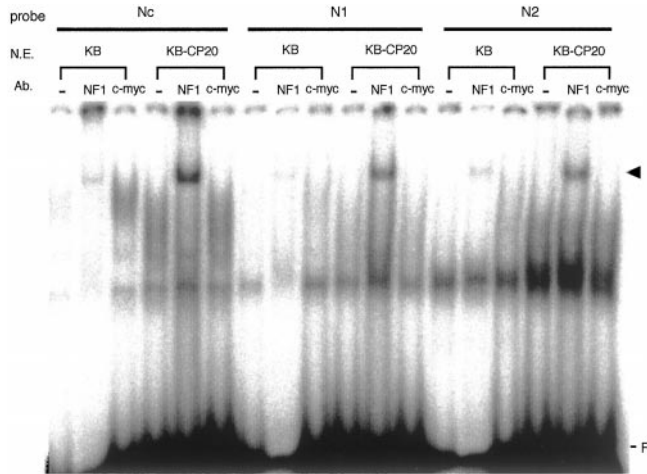


Fig. 6. Analysis of CCAAT-binding proteins by super shift assay. Nuclear extracts (N.E.) were preincubated with 2 μ l of anti-CTF or anti-c-myc antibody (Ab) for 30 min at 4°C followed by incubation with probes. The position of the super shifted band is indicated by the arrowhead. F, free probes.

both bind preferentially to cisplatin-modified DNA. As shown in Fig. 1, we demonstrated that HMG1 gene expression is up-regulated in all cisplatin-resistant cell lines. Identification of the promoter and transcription factors involved is essential for understanding the molecular mechanism of HMG1 up-regulation by cisplatin.

In the present study, the isolation and characterization of the human HMG1 gene were demonstrated. Although the genomic cloning of HMG1 was reported previously (29), the structure of the promoter was not shown. The overlapping clones, which cover the entire HMG1 gene, also contain the 5' flanking region of this gene (data not shown).

The 5' upstream sequence of the HMG1 gene was determined (Fig. 2). HMG1 gene was found to have three CCAAT boxes, but the sequences of these CCAAT boxes were different.

Transient transfection of reporter constructs showed that the promoter activity of the HMG1 gene is 3–10-fold higher in cisplatin-resistant cells than in KB cells. This indicates that HMG1 gene expression is transcriptionally up-regulated in resistant cells. The present study showed that the proximal region, from nucleotide –273 to nucleotide –45 upstream from the transcription initiation site, functions as a core promoter in KB cells. This finding also suggests that the most proximal CCAAT box is necessary not only for the manifestation of core promoter activity, but also for up-regulation of expression in cisplatin resistant cells.

The three CCAAT boxes in the KB parental cell line showed slight protection from DMS methylation. In contrast, the KB-CP20 cisplatin-resistant cells exhibited much less protection. However, hypersensitive sites near these CCAAT boxes, at nt –99 to –96, –31 to –28, and –2 to +4, were constitutively observed, and the extent of the hypersensitivity was higher in cisplatin-resistant cells than in parental cells. Moreover, concomitant protection at nt –104 was also constitutively observed within the ATF binding site. Taking the EMSA results and the overexpression of CTF/NF-1 (Figs. 6 and 7) into account, the findings suggest that these CCAAT boxes could be key elements in up-regulation of the HMG1 gene, whereas the surrounding region (nt –104 to the transcription initiation site) may act as part of the highly ordered regulatory complex (30).

It is important to characterize the nuclear factors that might mediate the transcriptional activation of the HMG1 gene in cisplatin-resistant cells. In the present EMSA experiments, the sequences of the two proximal CCAAT boxes were similar to the consensus sequence for

CTF/NF-1, but that of the distal CCAAT box was not (28, 31). The binding activity of two oligonucleotides (N1 and N2), was 3–8-fold higher when nuclear extracts from three cisplatin-resistant cells were tested than when extracts from parental cells were assayed. In contrast, the binding activity of a third oligonucleotide (ATF) to nuclear factors from KB-CP20 and KB cells were similar (data not shown). We also found that this enhanced CTF/NF-1 site binding activity is specific for cisplatin-resistant cells because the binding activity of nuclear extracts from vincristine- or teniposide-resistant cells were similar to the activities of extracts from parental cells (Fig. 5). A CTF/NF-1 dimer has been found to bind to viral and cellular promoters recognizing TGGA/C(N)₅GCCAA sequences, primarily through the two half-palindromes (32, 33).

The two proximal CCAAT boxes in the HMG1 promoter are not completely identical to those of the CTF/NF-1 consensus binding sites. The canonical spacer length of the CTF/NF-1 consensus sequence has been found to be 5–6 nt, and this spacer length has been reported to be an important determinant of binding affinity (28, 34, 35). We found, however, that the spacer lengths of the first and second CTF/NF-1 sites are 4 and 8 nt, respectively. Furthermore, these two CTF/NF-1 binding sites (N1 and N2) were shown to have a lower binding potential than the consensus CTF/NF-1 oligonucleotides (Nc). These divergences of spacer length might therefore account for the weak protection within factor contact sites. In addition, expression of the CTF/NF-1 protein was significantly increased 3–20-fold in cisplatin-resistant cells (Fig. 7A). Although expression of CTF/NF-1 mRNA was not detected by Northern blotting, CTF/NF-1 mRNA was 3-fold higher in cisplatin-resistant KB-CP20 cells than in parental KB cells by semiquantitative RT-PCR (Fig. 7B). Using the CTF/NF-1 primers, we observed two RT-PCR bands of 857 and 785 bp. Human CTF/NF-1 has been shown to encode multiple mRNA species for the alternative coding regions CTF1, CTF2, and CTF3 (36, 37), and differences in both forms and the amount of CTF/NF-1 binding activity among cell lines have been observed (38). This suggests that the present 857 and 785-bp products were derived from CTF2 and CTF3 mRNA, respectively. Although the intensities of the two bands are similar in KB cells, the intensity of the upper band is increased in

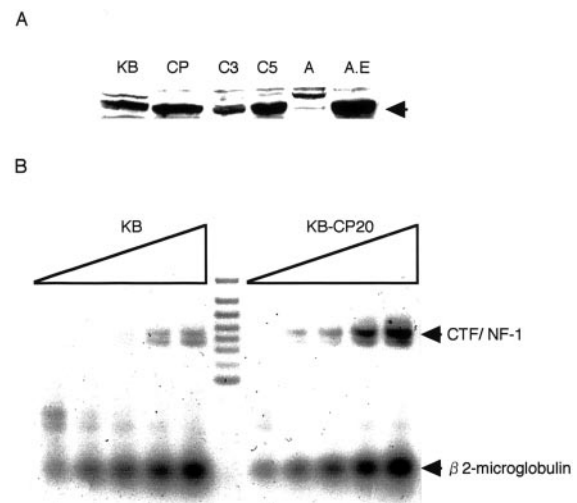


Fig. 7. Overexpression of CTF/NF-1 in cisplatin-resistant cells. A, nuclear extract (100 μ g) from each cell line was resolved on 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and then incubated with rabbit antibody against CTF/NF-1 and horseradish peroxidase-conjugated antirabbit IgG. The arrowhead indicates the 60-kDa CTF/NF-1 protein. B, total RNA was prepared from both KB and KB-CP20 cells, and expression of CTF/NF-1 and β_2 -microglobulin mRNA was assayed by RT-PCR. The amount of cDNA per lane was (right to left) 6.3, 12.5, 25, 50, and 100 ng. Amplification products were electrophoresed in 2% agarose gels, which were stained with ethidium bromide. Cell lines are as described in the legend for Fig. 5.

cisplatin-resistant cells, suggesting that CTF2 is the major CCAAT-binding species in the latter. We were unable, however, to detect the 1010-bp PCR product, which is derived from CTF1 mRNA, in either KB or KB-CP20 cells. Furthermore, it is of interest to examine whether other CTF/NF-1 target genes are overexpressed in cisplatin-resistant cells. Cisplatin-resistant cells were routinely maintained in medium containing cisplatin. Regulation of the forms of CTF/NF-1 has been shown to be sensitive to changes in cellular environment (38). However, CTF/NF-1 activity, CTF expression, and HMG1 expression were not induced by acute exposure of cells to cisplatin (data not shown). These findings suggest that CTF2 overexpression in cisplatin-resistant cells may be attributable to chronic exposure of the cells to cisplatin.

Chromatin remodeling, transcription factor binding, and transcription were inhibited by brief exposure of cells to cisplatin (39). Chronic exposure of cells to cisplatin may affect gene expression through chromatin remodeling activity and the cellular levels of transcription factors. We previously have shown that KB-CP4 cells overexpressed a transcription factor, YB-1, with the extent of YB-1 expression correlating with cellular sensitivity to the cytotoxic effect of cisplatin (40).

Interestingly, it has been shown that CTF/NF-1 can serve as an initiator of DNA replication (36), suggesting that overexpression of CTF/NF-1 may facilitate DNA replication and repair and may play a role in both gene expression and maintenance of genomic stability (41). Several different mechanisms for resistance to cisplatin have been demonstrated, including increased activity of DNA repair, increased levels of intracellular thiols, and reduced accumulation of cisplatin. In the same cisplatin-resistant cells used in this study, accumulation of cisplatin has been found to be decreased (4). Alterations of certain proteins have been demonstrated in KB-CP20 cells (20). Considering these findings together with our present results, cisplatin resistance appears to result from many different mechanisms. To clarify whether our observation is relevant to cisplatin resistance, isolation of stable transfectants that show overexpression of either the CTF/NF-1 or the HMG1 gene is under way in our laboratory.

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