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Identification and Characterization of Three DNA-binding Proteins on the Promoter of the Human *MDR*1 Gene in Drug-sensitive and -resistant Cells

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In multidrug-resistant cell lines (K562/ADM), *MDR*1 was amplified and transcriptionally activated. But the mechanism of *MDR*1 expression is unknown in K562/ADM cells. A 131 bp DNA fragment upstream from the major cap site of *MDR*1 contained CAAT-like and GC-box-like motifs and showed promoter activity in a CAT expression assay, resulting in a 3.5-fold enhancement of *MDR*1 promoter activity in K562/ADM as compared with that in K562 cells. In the CAT assay using deletion mutants we found that sequences containing the CAAT-like motif and the GC-box-like motif were required for *MDR*1 proximal promoter activity. To understand the details of *MDR*1 transcription in K562/ADM cells we analyzed the interaction between the proximal promoter and DNA-binding protein(s). We have identified three DNA-binding proteins on the *MDR*1 promoter from K562/ADM and parental K562 cells. The first binding protein to the *MDR*1 promoter was NF-R1, which recognized the sequences containing the CAAT-like motif and the GC-box-like motif as revealed by using a gel mobility shift assay. The second protein was NF-R2, which bound to the sequence containing the CAAT-like motif. The third protein, which bound to the sequence containing the GC-box-like motif was designated NF-R3. NF-R2 and NF-R3 in the resistant cells formed different bands in the gel mobility shift assays as compared with those in the sensitive cells, respectively. The observed difference might be related to the transcriptional activation of *MDR*1 in K562/ADM cells.

Key words: Multidrug resistance — *MDR*1 gene — CAT assay — Gel mobility shift assay

One of the major problems in cancer chemotherapy is multidrug resistance due to the expression of P-glycoprotein, which is an energy-dependent efflux pump and acts as a transporter of various structurally non-related compounds such as anthracyclines, actinomycin D, *Vinca* alkaloids, colchicine and epipodophyllotoxins.[1-3] P-Glycoprotein, which is encoded by the *MDR*1 gene,[4] is overexpressed in some multidrug-resistant cell lines.[5-8] It was found that some tissues and tumors innately overexpress the *MDR*1 gene.[9,10] In some renal tumors, transcriptional activation of a single *MDR*1 gene has been shown to cause multidrug resistance.[11] Transcriptional enhancement is now believed to be a common mechanism of resistance in patients.[11]

Mammalian gene expression is appropriately regulated by various *cis*-acting elements of the promoter region such as the CCAAT-box and the GC-box, which specifically interact with *trans*-acting regulatory factor.[12] In HeLa cells, at least four CCAAT-box-binding proteins have been detected; these are CTF,[13,14] CP1, CP2,[15,16] and NF-Y.[17,18] Differences in the ranges of sequence recognition by these proteins lead to specific interaction between these proteins and various CCAAT-box motifs. Thus, transcription of a specific gene could be regulated by such interaction. Moreover, the expression of several genes lacking the TATA-box in their promoter region, for example, the *ras* gene has been shown to require the GC-box-binding protein Sp1.[19,20]

The control mechanisms of transcription of the *MDR*1 gene still remain to be solved. The genomic structure of the 5′-flanking region of the *MDR*1 gene was determined, and the upstream region from the major transcription start site was confirmed to have promoter activity by CAT[4] assay in KB cells.[21] The *MDR*1 upstream region was reported to contain putative CAAT- and GC-boxes, but lacks the TATA-box, which is a common signal for eukaryotic mRNA transcription by RNA polymerase II.[21]

We have obtained adriamycin-resistant cells, K562/ADM, from human myelogenous leukemia K562 cells.[8] K562/ADM cells contain the amplified *MDR*1 gene.[22] In K562/ADM cells, we observed a 3.5-fold increase in *MDR*1 proximal promoter activity using CAT assay. CAT assay using deletion mutants revealed that the sequences containing the CAAT-like motif and the GC-box-like motif were required for *MDR*1 proximal promoter activity. We have identified three DNA-binding

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[4] The abbreviations used are: CAT, chloramphenicol acetyl transferase; ADM, adriamycin; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kb, kilobase; kDa, kilodalton.

proteins which might be required for the expression of the *MDR*1 gene in the multidrug-resistant cells.

MATERIALS AND METHODS

Cell culture An adriamycin-resistant subline (K562/ADM) was established from human myelogenous leukemia K562 cells in our laboratory,[8] and was cultured in suspension in RPMI 1640 medium (Nissui Co., Ltd.) supplemented with 5% fetal bovine serum and 100 μg/ml kanamycin.

CAT assay For electroporation, cells were washed once with phosphate-buffered saline, and resuspended at a density of 2×10^7 cells/ml. Supercoiled DNA (30 μg) was added to 0.8 ml of cell suspension, mixed gently, then held on ice for 10 min. The mixture was then transferred to an ice-cold Gene Pulser cuvette (0.8 cm, Bio-Rad) and subjected to a single pulse of 500 μF, 400 V from a Gene Pulser apparatus (Bio-Rad). The cuvette was incubated on ice for a further 10 min, then the cells were transferred to 50 ml of RPMI 1640 medium. Cells were harvested 24 h posttransfection and assayed for CAT activity according to Gorman *et al.*[23] In order to quantify the CAT activity, radioactive spots corresponding to acetylated chloramphenicols were taken from thin layer plates, and radioactivity was measured by using a liquid scintillation counter. CAT activities were normalized with respect to protein amounts.

Plasmid construction Plasmid p131MDR-CAT which contained the promoter (−131 to +9) was constructed as follows. A polymerase chain reaction (PCR) of genomic DNA of K562/ADM was carried out using 5′-CGGATCCCGAGGAATCAGCATTCAGTC-3′ and 5′-GGTCGACCGAATGAGCTCAGGCTTCCT-3′ as primers, the resulting fragment (−131 to +9) was digested with *Bam*HI and *Sal*I and cloned between the *Bam*HI and *Sal*I sites of p8-CAT (pUC8 containing the CAT gene in the *Hin*dIII site). To construct p99MDR-CAT a PCR product of K562/ADM genomic DNA using 5′- CGGATCCGAGCAGTCATCTGTGGTG-AG-3′ and 5′-GGTCGACCGAATGAGCTCAGGCT-TCCT-3′ as primers was digested with *Bam*HI and *Sal*I and cloned between the corresponding restriction enzyme sites of p8-CAT. To construct pΔ66-37MDR-CAT a synthesized DNA fragment having appropriate sticky ends (−36 to +9) was cloned to p8-CAT digested with *Bam*HI and *Sal*I (p36MDR-CAT). Next, a synthesized DNA fragment (−131 to −67) having appropriate sticky ends was cloned into the *Ava*I site of p36MDR-CAT. All constructs were sequenced to confirm that they had the correct sequence.

Isolation of nuclei and preparation of S-300 fractions Cells at the logarithmic growth phase were harvested from ten liters of culture and washed twice with phosphate-buffered saline. The cells were resuspended in 10 m*M* Tris-HCl, pH 7.4, 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, 1 m*M* DTT and 0.1 m*M* PMSF at a cell density of 4×10^7 cells/ml, and then homogenized with 20 strokes using a Dounce homogenizer. The cell homogenate was centrifuged at 1,000*g* for 10 min, and nuclei were recovered in pellet form. Nuclear proteins were extracted from the nuclei essentially according to the method of Dignam *et al.*[24] Briefly, nuclei were suspended in 20 ml of buffer containing 20 m*M* Tris-HCl, pH 7.9, 1 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 25% ethylene glycol, 0.5 m*M* DTT and 0.5 m*M* PMSF, and homogenized in a Dounce homogenizer. The homogenate was gently stirred for 30 min at 4°C, and then was centrifuged at 30,000 rpm for 30 min. The resulting supernatant was dialyzed overnight at 4°C against 0.2 *M* KCl containing TM buffer (50 m*M* Tris-HCl, pH 7.9, 12.5 m*M* $MgCl_2$, 1m*M* EDTA, 1 m*M* DTT and 20% ethylene glycol)[25] and the nuclear proteins (100 mg) were fractionated in a 200 ml Sephacryl S-300 column. The obtained fractions were stored at −70°C unitl analysis.

Gel mobility shift assay Binding reactions were carried out by incubating an end-labeled DNA probe (5,000 cpm) with 5 μl of protein fraction (1 mg/ml) and 0.3–3 μg of poly(dI-dC) in a final solution of 25 μl of buffer containing 10 m*M* Tris-HCl, pH 7.5, 50 m*M* KCl, 1 m*M* EDTA, 1 m*M* DTT and 5% ethylene glycol. After 30 min at 0°C, the reaction mixtures were loaded onto a 4% polyacrylamide gel. For competition experiments, 500-fold molar excess of oligonucleotides was added to the reaction mixture prior to the addition of the protein fraction.

Oligonucleotides for competition assays Double-stranded oligonucleotides were synthesized and hybridized. The sequences were as follows;

25mer containing the CAAT-like motif: (from −126 to −102)

GGAATCAGCATTCAGTCAATCCGGGCC
TTAGTCGTAAGTCAGTTAGGCCCGGCC

26mer containing the GC-box-like motif: (from −64 to −39)

GCAGGAACAGCGCCGGGGCGTGGGCTGA
TCCTTGTCGCGGCCCCGCACCCGACTCG

Unspecific 25mer: (from −10 to +15)

GGAAGCCTGAGCTCATTCGAGTAGC
CCTTCGGACTCGAGTAAGCTCATCG

Southwestern blotting S-300 fraction was mixed with SDS-PAGE buffer. The mixture finally contained 72 m*M* Tris-HCl, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerin, 4% ethylene glycol, 2.5 m*M* $MgCl_2$, 0.2 m*M* EDTA and 0.005% bromophenol blue. After incubation for 5 min at room temperature, the samples were sub-

jected to SDS-PAGE using a 4–20% gradient polyacrylamide gel at 4°C. The proteins were transferred electrophoretically to a nitrocellulose filter in a solution consisting of 25 m*M* Tris-HCl, pH 7.5 and 190 m*M* glycine. In order to detect specific binding, the nitrocellulose filter was covered with 5% skim milk (Yukijirushi Co., Ltd.) in 50 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 1 m*M* EDTA and 1 m*M* DTT,[26] and incubated for an hour at room temperature. Following two washes of the filter with TNE-50 buffer consisting of 10 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 1 m*M* EDTA and 1 m*M* DTT, the filter was incubated for one hour at room temperature in TNE-50 buffer containing 2×10^6 cpm of ^{32}P end-labeled DNA and 10 μg/ml of poly(dI-dC) as non-specific DNA. The filter was washed in three changes of TNE-50 buffer and then exposed to Kodak XAR-5 film.

RESULTS

***MDR*1 proximal promoter required CAAT-like motif and GC-box-like motif for transcription** We have cloned a genomic DNA containing *MDR*1 promoter from K562/ADM cells by using a polymerase chain reaction method. This DNA fragment proved to be the same as the corresponding genomic fragment in KB-V1 cells as revealed by dideoxy nucleotide sequencing (Fig. 1). The 131 bp upstream region from the major transcription start site contains the CAAT-like motif (AGTCAAT −115 to −109) and the GC-box-like motif (GGGGCGTGGG −52 to −43). This upstream region showed promoter activity in the CAT assay using p131MDR-CAT (Fig. 2). The CAT assay using p131MDR-CAT revealed that *MDR*1 proximal promoter activity in K562/ADM cells was enhanced 3.5-fold as compared with that in K562 cells (Fig. 2). To delimit the sequences essential for the expression of the *MDR*1 gene, we constructed deletion mutants of p131MDR-CAT and transfected these constructs into K562/ADM and K562 cells. In K562/ADM cells we observed that deletion to −99 caused a 5-fold decrease of activity, therefore, the sequence containing the CAAT-like motif included *cis*-acting element(s) for

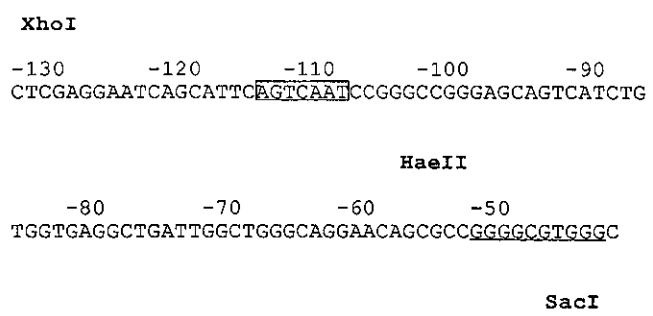

Fig. 1. The nucleotide sequence of the *MDR*1 gene proximal promoter region. The nucleotide sequence of the *MDR*1 gene promoter region of K562/ADM cells is shown. The major transcription start point is indicated by +1.[21] Several recognition sites of restriction enzymes are shown above the sequences. Numbers indicate the position relative to the major transcription start site. The CAAT-like motif is boxed, and the GC-box-like motif is underlined.

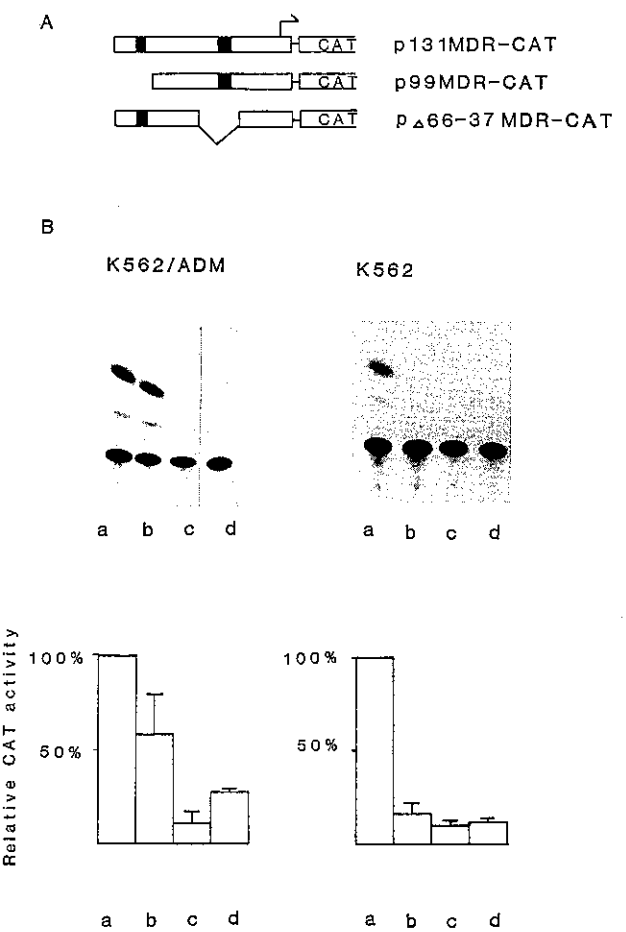

Fig. 2. Transcriptional activity of *MDR*1 promoter-deletion mutants in both K562/ADM and K562 cells. (A) Structure of pMDR-CAT constructs. The filled boxes represent the CAAT-like and GC-box-like motifs, respectively. An arrow showed the major transcription start point. (B) CAT activities in K562/ADM and K562 cells transfected with the following plasmids: lane a, pSV2CAT; lane b, p131MDR-CAT; lane c, p99MDR-CAT; lane d, pΔ66-37MDR-CAT. Relative CAT activity was expressed as percent of CAT activity of pSV2CAT in both cells. The graphs represent mean values from three independent experiments.

the expression of the *MDR*1 gene. To confirm the effect of the sequence containing the GC-box-like motif we constructed pΔ66-37MDR-CAT. Deletion of the sequence (−66 to −37) resulted in a 2-fold decrease in activity as compared with p131MDR-CAT. Thus we concluded that the sequences containing the CAAT-like motif and the GC-box-like motif were both required for efficient promoter activity. In K562 cells, CAT activity of the intact promoter was relatively low and deletion of both sequences caused a slight decrease of CAT activity.

MDR1 promoter interacts with DNA-binding proteins separated by gel filtration column chromatography To identify DNA-binding proteins from nuclear extract of K562/ADM which recognize specifically the *MDR*1 promoter, DNA-binding activities of the nuclear extract were investigated by using a gel mobility shift assay (Fig. 3). We used the 131 bp upstream region from the *Xho*I site (−132) to the *Sac*I site (−2) as a probe in the gel mobility shift assay. The nuclear extract from K562/ADM cells was fractionated by Sephacryl S-300 column chromatography. Two DNA-binding activities, shown as fractions A (lanes 6 and 7) and B (lane 9), could be discriminated in the gel mobility shift assay using the *Xho*I-*Sac*I probe. In lanes 4 and 5 faint bands were observed but this observation was not reproducible. Similar results were obtained in K562 cells (data not shown).

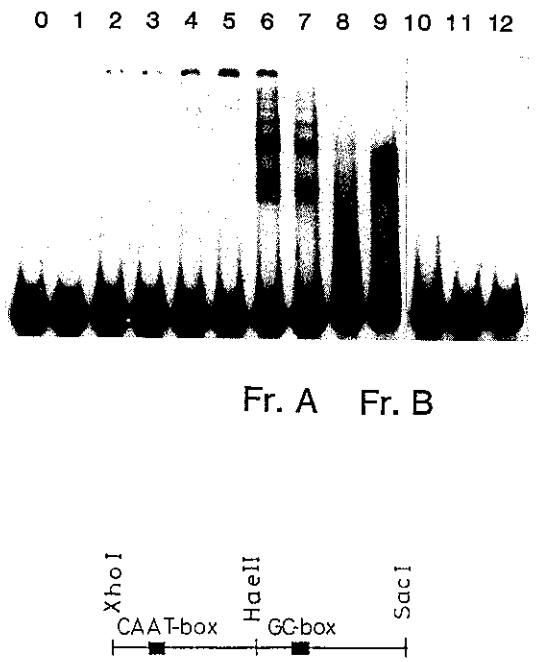

Fig. 3. Binding proteins to the *MDR*1 promoter detected by a gel mobility shift assay. Gel mobility shift assay of the nuclear extract fractionated by Sephacryl S-300 column chromatography. Plasmid pUM8 which contained a genomic DNA fragment of the *MDR*1 upstream region (*Pst*I-*Pst*I 1 kb) of K562/ADM was obtained by colony hybridization screening of *Pst*I-digested genomic DNA fragments cloned into pUC8. This plasmid pUM8 was digested with *Xho*I and end-labeled by Klenow DNA polymerase I treatment. Following *Sac*I digestion, the resulting *Xho*I-*Sac*I fragment was used in a gel mobility shift assay. Lane 0 is free probe without protein. In the following lanes, 5 μl of fractions from Sephacryl S-300 column chromatography were incubated with 5,000 cpm of the *Xho*I-*Sac*I probe and 2 μg of poly(dI-dC) for 30 min at 0°C according to "Materials and Methods" then subjected to 4% polyacrylamide gel electrophoresis. Lanes 6 and 7 were designated fraction A and lane 9 was designated fraction B.

110 kDa DNA binding protein, NF-R1 could recognize sequences containing the CAAT-like motif and the GC-box-like motif Fraction A formed two major bands and a faint band in the gel mobility shift assay using the *Xho*I-*Sac*I probe containing the CAAT-like and GC-box-like motifs (Fig. 4A). The formation of these DNA-protein complexes was subject to competition by 500-fold molar excess of 25mer (−126 to −102) or 26mer (−64 to −39) oligonucleotide including the CAAT-like motif and the GC-box-like motif, respectively (Fig. 4A). An unspecific 25mer oligonucleotide (−10 to +15) could not inhibit the formation of these complexes. These data suggested that this DNA binding protein designated NF-R1, recognized both sequences, i.e., the CAAT-like motif and the GC-box-like motif. NF-R1 showed similar affinity to both sequences in competition gel shift assays (data not shown). The two bands observed in the gel mobility shift assay seem to correspond to monomer and dimer of DNA-protein complex, respectively. In fact, in the gel mobility shift assay using a *Xho*I-*Hae*II probe (−132 to −58), a major single band was observed, while in the experiment using a *Hae*II-*Taq*I probe (−58 to +7) a single band was detected (Fig. 4B). The formation of these bands was subject to competition by the specific 25mer and 26mer oligonucleotides in the case of both probes (data not shown). Moreover NF-R1 might have affinity for an additional recognition site on the *Xho*I-*Hae*II region, since a faint band having corresponding mobility was observed, as shown in Fig. 4B.

We next characterized NF-R1 in Southwestern blotting using double-stranded oligonucleotide probes including the CAAT-like motif and the GC-box-like motif with fraction A from K562/ADM motif and the GC-box-like motif with fraction A from K562/ADM and K562 cells. Each oligonucleotide bound to a 110 kDa protein in fraction A obtained from K562/ADM cells (Fig. 4C). This 110 kDa protein was also detected in fraction A from K562 cells by each oligonucleotide. Moreover each detected band was subject to competition by the cold 25mer and 26mer oligonucleotides (data not shown). These data support the conclusion that NF-R1 is the 110 kDa protein which recognizes the sequences containing

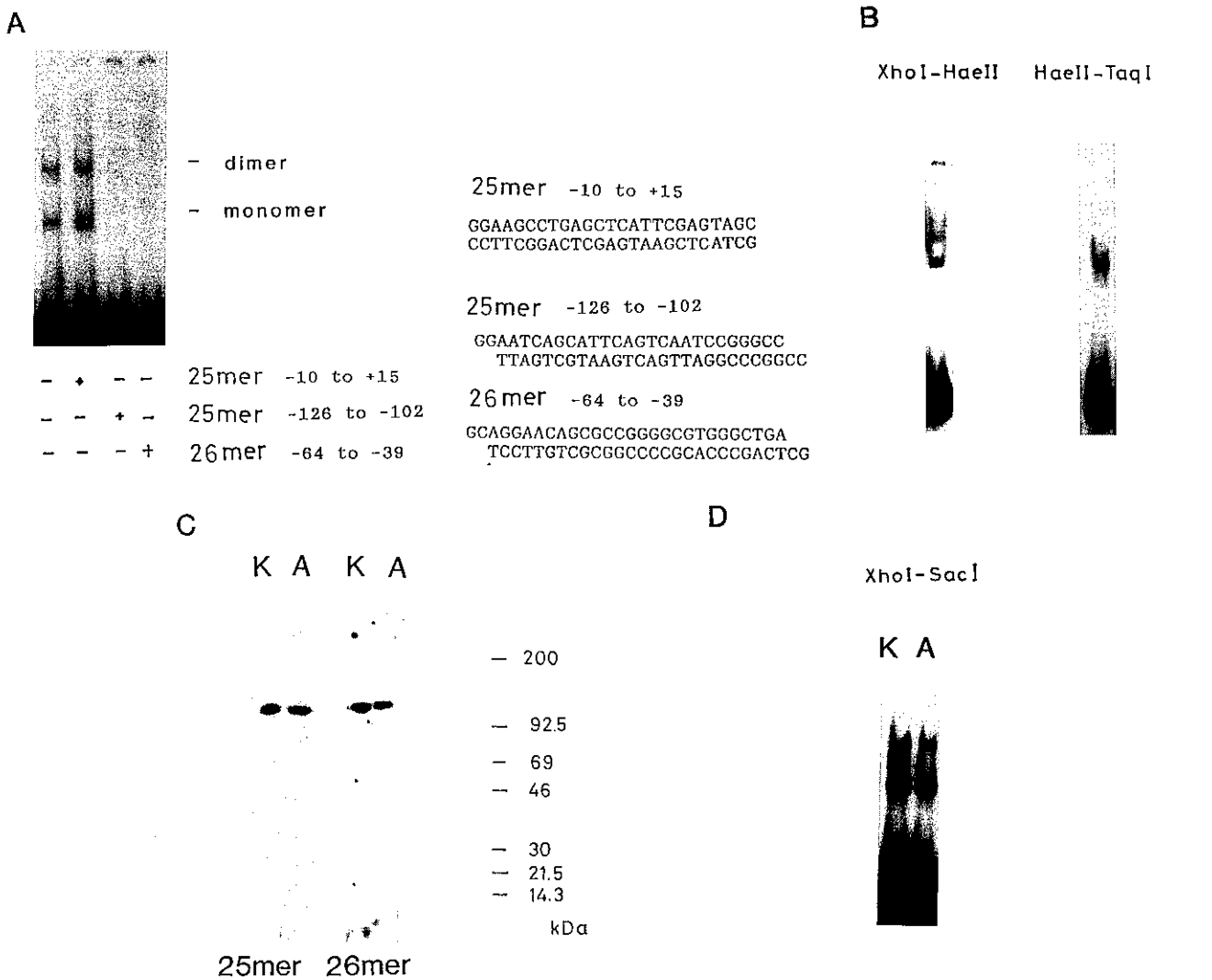

Fig. 4. Gel mobility shift assay and Southwestern blotting of NF-R1. (A) Gel mobility shift assay using the *Xho*I-*Sac*I probe was carried out as described in the legend to Fig. 3. "−" refers to no addition of the indicated conpetitors, and "+" indicates the addition of 500-fold molar excess of the indicated competitors. The sequences are shown in the right of the panel. 25mer (−126 to −102) and 26mer (−64 to −39) oligonucleotides are specific, but another 25mer oligonucleotide (−10 to +15) is unspecific. (B) Gel mobility shift assays of fraction A were carried out using a *Xho*I-*Hae*II probe containing the CAAT-like motif and a *Hae*II-*Taq*I probe containing the GC-box-like motif. The *Xho*I(*Taq*I)-*Taq*I (−131 to +9) fragment was prepared by digestion of pUM8 with *Taq*I and labeled by Klenow DNA polymerase I treatment. This *Xho*I(*Taq*I)-*Taq*I fragment was digested by *Hae*II; the resulting 77 bp *Xho*I(*Taq*I)-*Hae*II and 63 bp *Hae*II-*Taq*I fragments were used for a gel mobility shift assay. (C) The double-stranded 25mer oligonucleotide containing the CAAT-like motif and 26mer containing the GC-box-like motif were repaired with Klenow DNA polymerase I using [α-^{32}P]dCTP, and the resulting 29mer and 30mer oligonucleotides were used as probes. Southwestern blottings using the 29mer and 30mer double-stranded oligonucleotides were performed with fraction A as described in "Materials and Methods." K and A indicate fraction A obtained from K562 and K562/ADM cells. Each lane contained approximately 3 μg of protein. The numbers along the autoradiogram indicate molecular weights of the size markers. (D) NF-R1 was detected in each fraction A from from K562 and K562/ADM cells by a gel mobility shift assay. In this experiment the *Xho*I-*Sac*I probe was used.

the CAAT-like motif and the GC-box-like motif. A ubiquitous GC-box-binding protein Sp1 is known to have a molecular weight of 105–95 kDa.[25] NF-R1 has a different molecular weight and different DNA-binding properties in relation to the CAAT-like motif as compared with Sp1.

To confirm that NF-R1 in K562/ADM cells is the same as that in K562 cells, we compared both proteins in a gel mobility shift assay. Fig. 4D shows that the same shifted bands were observed, and therefore, NF-R1 has the same characteristics in K562/ADM and K562 cells.

NF-R2 in K562/ADM and K562 cells showed different DNA binding properties The DNA-protein complex band detected in the gel mobility shift assay of fraction B using the *Xho*I-*Sac*I probe was not subject to competition by oligonucleotide bearing the CAAT-like motif. When the 25mer oligonucleotide containing the CAAT-like motif was added in a gel mobility shift assay, a complex which might be formed by another protein remained as a faster mobility band (data not shown). Therefore the complex band detected by using the *Xho*I-*Sac*I probe was thought to be a mixture of complexes formed by different proteins. In order to characterize the first binding protein, NF-R2 included in fraction B, a gel mobility shift

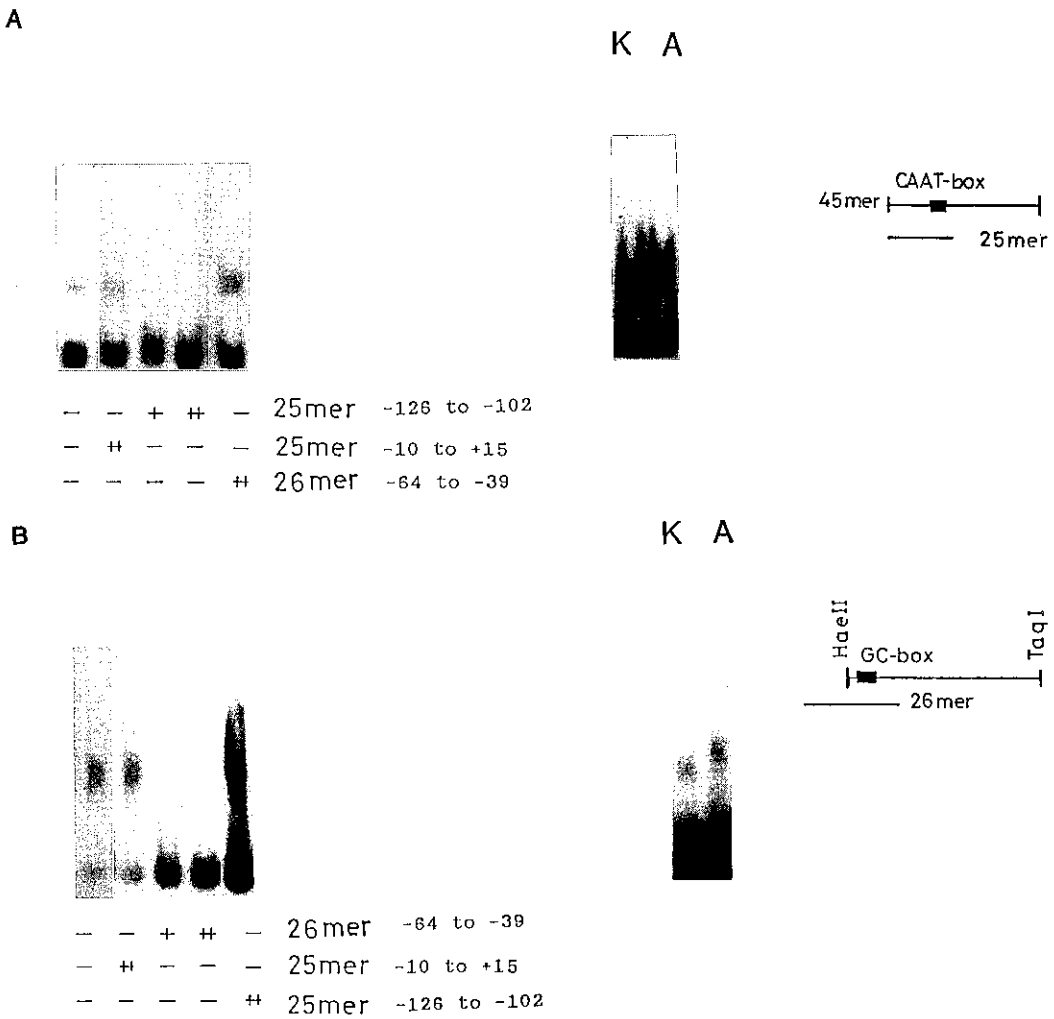

Fig. 5. NF-R2 and NF-R3 detected by gel mobility shift assays. K and A indicate fraction B obtained from K562 and K562/ADM, respectively. "+" and "++" indicate the addition of 250-fold and 500-fold molar excess of the indicated competitors, respectively. "−" refers to the absence of the indicated competitors. (A) Specific binding of NF-R2 to the sequence containing the CAAT-like motif. Gel mobility shift assay of NF-R2 with a 45mer probe containing the CAAT-like motif was carried out as described in "Materials and Methods" except that 0.5 μg of poly(dI-dC) was used per assay. The double-stranded 45mer probe (−126 to−81) was chemically synthesized and hybridized. The sequences of the competitors are shown in Fig. 4A. (B) Specific binding of NF-R3 to the sequence containing the GC-box-like motif. Gel mobility shift assay was carried out by using the *Hae*II-*Taq*I probe (the preparation of the *Hae*II-*Taq*I probe is described in the legend to Fig. 4B) with 0.5 μg of poly(dI-dC). The sequences of the competitors are given in Fig. 4A.

assay was performed by using a 45mer double-stranded oligonucleotide (−126 to −81). The formation of the complex with the 45mer oligonucleotide in fraction B from K562/ADM cells was subject to competition by a 500-fold molar excess of the cold 25mer oligonucleotide bearing the CAAT-like motif. Addition of the unspecific 25mer oligonucleotide (−10 to +15) and the 26mer oligonucleotide containing the GC-box-like motif (−64 to −39) could not influence the formation of the DNA-protein complex (Fig. 5A). Thus, the binding of NF-R2 was confirmed to be specific for the 25mer sequence. Similar results were obtained in K562 cells; NF-R2-like protein was also present in K562 cells as revealed by competition assay (data not shown). We compared the characters of NF-R2s between K562/ADM and K562 cells in the gel mobility shift assay since NF-R2 was not detected by using Southwestern blotting. NF-R2s in K562/ADM and K562 cells formed different bands (Fig. 5A).

NF-R3 has different properties in K562/ADM and in K562 cells To characterize another DNA binding protein in fraction B, a gel mobility shift assay of fraction B from K562/ADM cells was carried out by using the probe containing the GC-box-like motif (*Hae*II-*Taq*I). As a result, a second binding protein in fraction B, NF-R3, was detected. In the gel mobility shift assay using the *Hae*II-*Taq*I probe the binding of the NF-R3 in K562/ADM cells was subject to competition by the cold 26mer double-stranded oligonucleotide containing the GC-box-like motif as shown in Fig. 5B. In addition, formation of the DNA-protein complex was not subject to competition by the unspecific 25mer oligonucleotide (−10 to +15) or the 25mer oligonucleotide containing the CAAT-like motif (−126 to −81). Thus, this shifted band was confirmed to be formed by NF-R3, which is a putative GC-box-binding protein. In K562 cells we also detected NF-R3-like protein as revealed in a competition assay (data not shown). We analyzed the NF-R3s in K562/ADM and K562 cells using a gel mobility shift assay since NF-R3 could not be detected by using Southwestern blotting. The NF-R3s in the K562/ADM and K562 cells were confirmed to differ from each other because each NF-R3 formed a different complex in the gel mobility shift assay (Fig. 5B).

DISCUSSION

We showed a 3.5-fold enhancement of *MDR*1 proximal promoter activity in K562/ADM cells by CAT expression assay and established that sequences containing the CAAT-like motif and the GC-box-like motif were required for efficient *MDR*1 promoter activity as revealed by a deletion study. Kohno *et al.* have found 3-fold enhancement of *MDR*1 promoter activity in multidrug-resistant VJ-300 cells.[27] Moreover, we pointed out the possibility that the more upstream region from nucleotide −131 might play a role in transcriptional enhancement of the *MDR*1 gene in K562/ADM cells. Next we analyzed the interplay of several DNA-binding proteins on the *MDR*1 promoter region in both K562/ADM and K562 cells. NF-R1 is present in both cells. NF-R2 and NF-R3 in K562/ADM cells were different from the corresponding proteins in K562 cells. In the gel mobility shift assay, NF-R2 and NF-R3 in K562/ADM cells formed different shifted bands as compared with the corresponding proteins in K562 cells. It is of interest to know whether the detected differences of NF-R2 and NF-R3 between K562/ADM and K562 cells are significant in relation to the transcriptional activation of the *MDR*1 gene in K562/ADM cells. Recently it was reported that activation of mammalian transcription factor CREB (cAMP response element binding protein) required its phosphorylation by cAMP dependent protein kinase.[28] Therefore we speculated that multiple phosphorylation of NF-R2 and NF-R3 might cause mobility changes and enhancement of transcription of the *MDR*1 gene by the proximal promoter.

NF-R1 bound to the two sequences on the *MDR*1 proximal promoter. Several proteins that specifically bound to two motifs of unrelated sequences have been reported, such as HAP1,[29] TEF1,[30] and Oct-2.[31] The single DNA-binding domain of each protein is thought to recognize different sequence motifs based on the competition experiment using a gel mobility shift assay. Competition assay and Southwestern blotting suggested that NF-R1 could bind to the sequences containing the CAAT-like motif and the GC-box-like motif.

Several CCAAT-box-binding proteins for other genes have already been identified and well characterized, for example CTF[13, 14, 32, 33] and C/EBP.[34–36] Those studies revealed that multiple CCAAT-binding factors could be involved in the regulation of a number of diverse promoters. NF-R2 bound to the sequence containing the CAAT-like motif, and therefore, we speculated that NF-R2 might be a putative CAAT-box-binding protein which recognized the GTCAAT sequence on the *MDR*1 proximal promoter, even though it is slightly different from the typical CAAT-box motif, CCAAT.

Proteins that bind to GC-rich sequences on various promoters have been reported, such as Sp1,[19, 20] AP2,[37] ETF[38] and GCF.[39] Sp1 is a ubiquitous transcription factor that binds to the GC-box, of which the concensus sequence is $\begin{matrix}TA\\GG\end{matrix}GGCG\begin{matrix}TAAT\\GGGC\end{matrix}$,[25] and has a molecular weight of 105–95 kDa. The gene encoding Sp1 has been reported to belong to a family of related genes as shown by Southern blotting using an Sp1 cDNA probe harboring no zinc finger motif in HeLa cells.[40] NF-R3 might be

a putative GC-box-binding protein, and so NF-R3 might be related to Sp1 or other GC-box-binding proteins. To characterize NF-R3 in detail, purification of the protein will be required. Ueda *et al.* referred to another GC-box-like sequence (−69 to −61).[21] We have not found a factor bound to that sequence, but our results do not exclude the possibility that there might be other factors bound to the MDR1 proximal promoter in K562/ADM cells.

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