Purification and characterization of NF-R1 that regulates the expression of the human multidrug resistance (MDR1) gene

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ABSTRACT
We have purified a protein, NF-R1, that specifically binds to two unrelated motifs—the ATTCA/GTC motif and the GC-box motif—on the MDR1 proximal promoter. Purified NF-R1 has been confirmed by southwestern blotting to be a 110-kDa protein. Methylation interference analysis revealed that the nucleotides were in close contact with purified NF-R1 on the ATTCA/GTC and GC-box motifs. The nucleotides were required for the binding of NF-R1, as seen by competition gel mobility shift assay using point mutated oligonucleotides. In a CAT expression assay using the corresponding point-mutated MDR1 promoter fused to a CAT gene, binding inhibition of NF-R1 to the promoter resulted in 2- to 3-fold increases of CAT activity, as compared to the intact promoter in Adriamycin-resistant K562 cells. Thus NF-R1 has a relation to the negative regulation of the MDR1 gene transcription.

INTRODUCTION
A problem of cancer chemotherapy is multidrug resistance that is caused by the expression of P-glycoprotein. The MDR1 gene codes P-glycoprotein (1), which is an energy-dependent efflux pump and acts as a transporter of such various structurally unrelated compounds as actinomycin D, Adriamycin, colchicine and Vinca alkaloids (2-4). Drug resistance correlates with levels of MDR1 RNA in cultured cells and some tumors (5-10). In some renal tumors (9) and neuroblastomas (10), high expression levels of a single MDR1 gene has been shown to cause multidrug resistance.

The regulatory mechanism of the MDR1 gene transcription remains to be studied, though the promoter region of the MDR1 gene has been isolated and sequenced (11). In some unrelated colon cancer specimens and adrenocortical carcinomas the MDR1 gene is transcribed from the downstream promoter (12), whereas in KB-V1 cells the MDR1 gene is known to have the upstream and downstream promoters (11). This downstream promoter has been confirmed to be functional, i.e., a 131-bp fragment of the MDR1 upstream region from major CAP site has the promoter activity in a CAT expression assay in Adriamycin-resistant K562 (K562/ADM) cells (13). The MDR1 downstream promoter lacks the TATAA box (14), which is a common signal for eukaryotic mRNA transcription by RNA polymerase II.

We have previously shown that deletion of two regions (−131 to −100 and −66 to −37 relative to the major CAP site) resulted in 5-fold and 2-fold respective decreases of promoter activity in a CAT expression assay and that a DNA binding protein, NF-R1, bound to these two regions (13). Moreover, additional DNA-binding proteins, NF-R2 and NF-R3, could bind to the sequences from −126 to −102 and −64 to −39, respectively (13).

In this paper we report the purification of NF-R1 from K562/ADM cells and show that NF-R1 recognized the ATTCA/GTC motif (−119 to −111) and GC-box motif (−52 to −41) on the MDR1 proximal promoter. The nucleotides that were in close contact with NF-R1 were determined. We show that the point mutation that inhibited the binding of NF-R1 to the MDR1 promoter caused 2- to 3-fold increases of promoter activity.

MATERIALS AND METHODS
Gel mobility shift assay
To prepare the MDR1 proximal promoter probe, p131MDR-CAT (13) was digested with BamHI and Sall followed by Klenow DNA polymerase I treatment using [α-32P]dCTP. The resulting labeled DNA was purified from the agarose gel after electrophoresis. This BamHI-Sall fragment was used as a probe. Binding reaction was performed as described previously (13). In reaction with purified NF-R1 no carrier DNA was used.

Purification of NF-R1
A flow chart for NF-R1 purification is shown in Fig. 1. Nuclear extract of K562/ADM cells was prepared according to the method of Dignam et al. (15). The extract was fractionated by a Sephacryl S-300 column chromatography. DNA-binding activity of NF-R1 was detected by a gel mobility shift assay (13). The resulting

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fraction containing NF-R1 was dialyzed against 50 mM KCl containing DNA column buffer [20 mM Tris-HCl (pH 8.0), 2.5 mM MgCl₂, 1 mM EDTA, 20% ethylene glycol, 0.5 mM PMSF] for 10 hours at 4°C. Extract from 5×10⁶ cells yielded approximately 20 mg of protein using BCA protein assay reagent (Pierce Chemical Company, Rockford IL). The fraction was loaded onto a specific DNA-sephrose column in the presence of 0.4 mg/ml of poly(dI-dC). The specific DNA-sephrose columns were prepared using the following three double-stranded oligonucleotides. The 25mer and 46mer oligonucleotides contained the ATTCAGTCA motif (Fig. 2), and the 26mer oligonucleotide contained the GC-box motif (Fig. 2).

ds 25mer
GGAATCAGCATTCAGTCAATCCGGGCC
TTAGTCGTAAGTCAGTTAGGCCCGG

Nuclear extract

Sephacryl S-300 column

NF-R1  NF-R2, NF-R3

DNA affinity column  X 2

purified NF-R1

Figure 1. Schematic representation of the purification procedure of NF-R1. Crude nuclear extract was fractionated by a S-300 chromatography. Active fractions were subjected to a specific DNA affinity column. To purify NF-R1 crude NF-R1 fractions were reapplied to the same column. Active fractions were determined by gel mobility shift assays using the MDR1 proximal promoter region as a probe.

XhoI

| -130 | -120 | -110 | -100 | -90 | -80 | -70 | -60 | -50 | -40 |
| CTCGAGGAAATCGCATCCAGGTCAATCCGGGCCGGAAGCAGTCAATCGTCATCTCTGGTTAGGAAGCTGAATGGCTGAGTTGGCAGAGACAGGGCGGGGGGCGGGCTGAG |

HaeII

AATCAGCATCCATCCGGGCCGGAAGCAGTCAATCGTCATCTCTGGTTAGGAAGCTGAATGGCTGAGTTGGCAGAGACAGGGCGGGGGGCGGGCTGAG 25mer
AATCAGCATCCATCCGGGCCGGAAGCAGTCAATCGTCATCTCTGGTTAGGAAGCTGAATGGCTGAGTTGGCAGAGACAGGGCGGGGGGCGGGCTGAG 26mer
AGGAACAGCGCAGCGGCGGAAGCTGAATGGCTGAGTTGGCAGAGACAGGGCGGGGGGCGGGCTGAG

SaeI

| -30 | -20 | -10 | +1 | +10 | +15 |
| CACAGGGCTCTCGCTCTCTCTCGCCCACAGGGAAAGCGCTAGCTGAGCTCATTCCGAGTGC |

unspecific 25mer  GGAAGCCTGAGGCTCATTCCGAGTGC

Figure 2. The MDR1 proximal promoter and location of oligonucleotides for experiments. A sequence of the MDR1 proximal promoter (−133 to +15 relative to the major transcription start site +1) is shown. 25mer (−126 to −102) and 46mer (−126 to −81) sequences containing the ATTCAGTCA motif, 26mer (−64 to −39) sequence containing the GC-box motif and nonspecific 25mer (−10 to +15) sequence are shown. The two motifs are underlined.
The labeled probes were treated with 1 μl of dimethyl sulfate for 1 min on ice in 50 μl of 50 mM cacodylic acid, pH 8.0, according to the methods of Maxam and Gilbert (17). The partially methylated oligonucleotide was used as a substrate in a gel mobility shift assay scaled up to 100 μl, which contained 10^6 cpm of oligonucleotide probe in addition to 40 ng of purified NF-R1. After incubation at 4°C for 30 min, the reaction mixture was loaded onto a 4% polyacrylamide gel and electrophoresed. After autoradiography at room temperature the free probe and bound complex were excised from the gel, and the gel was ground and extracted overnight at 37°C in buffer containing 500 mM sodium acetate, 1 mM DTT, 1 mM EDTA, 0.1% SDS, 10% methanol and 50 μg/ml of protease K. The extracted DNA was purified using an Elutip column (Schleicher & Schull, Dassel, Germany) and phenol extracted followed by piperidine cleavage. Samples were resuspended in formamide, loaded onto a 12% polyacrylamide-8M urea gel, and electrophoresed for 1 hour at 3000 V.

**Plasmid construction**

The MDR1 proximal promoter fragment (−131 to +9) was prepared by digestion of the genomic fragment of the MDR1 promoter region with TaqI followed by a repair reaction with Klenow DNA polymerase I. For site-directed mutagenesis this promoter fragment was cloned into Smal site of pUC8; the resulting plasmid was digested with EcoRI and BamHI and cloned between EcoRI and BamHI sites of amber mutant phage M13tv18 (Takarashuzo Co. Ltd. Tokyo, Japan). Phosphorylated and mutated anti-sense oligonucleotide (see Fig. 6) was used in the introduction of point mutation by a TAKARA site-directed mutagenesis system Mutan-G, based on the method of Kramer et al. (18). From several plaques, single-stranded DNA was recovered and sequenced to confirm incorporation of the mutation. In order to amplify the mutated promoter fragment (−131 to +9) containing the appropriate restriction sites on both ends, the resulting mutated phage was used as a template for PCR by using 5'-CGGATTCGAAATTCGATTCGCCAGGAAATCCAGC-3' (for CM3 through CM12 mutations) or 5'-CGGATTCGCCCAGG-3'.

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**Figure 3.** Purification of NF-R1 by the specific affinity columns using the ATT-CAGTCA motif and GC-box motif oligonucleotides. A, NF-R1 was purified by the ATT-CAGTCA motif-specific affinity column. Silver staining SDS-polyacrylamide gels of fractions from affinity column are shown. a, c show marker proteins. b shows the first cycle eluted fraction. d, 0.2 M; e, 0.4 M; f, 0.6 M; g, 0.8 M; h, 1.0 M KCl second cycle eluted fraction. Numbers show the molecular weights in kilodaltons of the marker proteins. Arrow indicates the position of the NF-R1 protein. B, NF-R1 was purified by affinity columns using the 46mer and 26mer oligonucleotides. a, marker proteins; b, 1.0 M KCl eluted fraction of the 46mer column; c, 1.0 M KCl eluted fraction of the 26mer column. Arrow shows the position of NF-R1. C, Oligonucleotides containing the ATT-CAGTCA and the GC-box motifs were bound to both NF-R1 purified by the ATT-CAGTCA motif (lane a) and the GC-box motif (lane b) columns. The proteins were separated by electrophoresis on a denaturing SDS-PAGE. Southwestern blot analysis was carried out using 29mer (ATT-CAGTCA motif) and 30mer (GC-box motif) probes. The labeled probes were made with Klenow DNA polymerase I treatment of 5' protruding ends of 25mer and 26mer oligonucleotides with [α-32P]dCTP (see Materials and Methods).

**Figure 4.** Competition gel mobility shift assay of NF-R1. Assays were carried out by using 1 ng of BamHI-SalI fragment (−131 to +9; see Materials and Methods) as a probe and 5 ng of purified NF-R1. Control reaction was carried out as shown in lane a. Increasing quantities (b, 0.1 pmol; c, 0.5 pmol; d, 1.0 pmol) of the nonspecific 25mer (−10 to +15) and specific 46mer (−126 to −81), 26mer (−64 to −39) and 25mer (−126 to −102) oligonucleotides were added. Arrows indicate monomer and dimer complexes of NF-R1.
AATCAGCATTCAGTCA-3' (for GM mutations) and 5'-GGT-CGACCGAATGAGCTAGGCTCTTCT-3' as primers. The amplified fragment was digested with BamHI and SalI and cloned between BamHI and SalI sites of p8CAT (13). For CM1 and CM2 constructs, point mutation was directly introduced by the PCR method using mutated primer 5'-CGGATCCGAATTCC-CCGAGGCCTCAGC-3' (CM1) or 5'-CGGATCCGAATTCC-CCGAGGAATGTGC-3' (CM2) and 5'-GGTCGACCGAATGAGCTACGGCTCTTCT-3'. The amplified mutated promoter fragments were cloned into p8-CAT after digestion with BamHI and SalI. All constructs were sequenced.

**Transfection assay**

The procedure used for transfection was that of Ogura et al. (13). A transient transfection assay was carried out using an electroporation method with efficiency controls by cotransfection of a β-galactosidase-expressing construct pSV-β-galactosidase (Promega, Madison, WI). For a CAT assay, cells were collected 48 hours after transfection, and the assay was carried out according to the methods of Gorman et al. (19).

**RESULTS**

**Purification of NF-R1**

We previously reported that NF-R1 bound to sequences containing the ATTCAGTCA motif (25mer oligonucleotide; see Fig. 2) and the GC-box motif (26mer oligonucleotide) on the MDR1 proximal promoter (13). Thus, we have purified and analyzed NF-R1 in order to clarify the functional role of NF-R1 on the MDR1 proximal promoter.

Nuclear extract of K562/ADM was fractionated on a Sephacryl S-300 column, and the fraction containing NF-R1 was detected by a gel mobility shift assay. Other nuclear factors, NF-R2 and NF-R3, were separated in the gel filtration step (Fig. 1). We purified NF-R1 by using an ATTCAGTCA motif-specific DNA affinity column (polymerized 46mer oligonucleotide; see Materials and Methods and Fig. 2) and a GC-box motif-specific affinity column (polymerized 26mer oligonucleotide) from the NF-R1-containing fraction. We used the DNA affinity column with the 46mer oligonucleotide for the purification because NF-R1 had relatively low affinity to DNA-sepharose column with the 25mer oligonucleotide containing the ATTCAGTCA motif. Active fractions of the first cycle of each DNA affinity column were reapplied to the same affinity column. A 110-kDa protein was eluted between 0.4 M and 1.0 M KCl during the second cycle of the ATTCAGTCA motif-specific column (Fig. 3A). In a 1.0 M KCl fraction during the second cycle of the GC-box motif-specific column, NF-R1 was also eluted as the 110-kDa protein (Fig. 3B). NF-R1 was at least 70% pure as judged by a densitometric analysis. Both 110-kDa proteins purified by using the ATTCAGTCA and GC-box motif-specific columns were confirmed to be NF-R1 on southwestern blot analyses using 29mer and 30mer probes. The probes were end-labeled 25mer and 26mer double-stranded oligonucleotides, respectively (Fig. 3C; see Materials and Methods). A nonspecific 25mer probe (data not shown) could not bind to the 110-kDa protein. These results show that NF-R1 was purified by both the ATTCAGTCA and the GC-box motif-specific affinity columns.

To further support this conclusion NF-R1 purified by the ATTCAGTCA motif-specific affinity column was applied to the GC-box motif-specific column and eluted with 1.0 M KCl containing DNA column buffer. As expected, NF-R1 was bound and eluted (data not shown). Both NF-R1 purified by the ATTCAGTCA and GC-box motif affinity columns formed two DNA-protein complex bands in a gel mobility shift assay using the MDR1 proximal promoter probe. A competition gel mobility shift assay using NF-R1 purified by the ATTCAGTCA motif-specific column revealed that NF-R1 truly recognized two sequences containing the ATTCAGTCA motif and the GC-box motif. Fig. 4 shows that formation of two complex bands was competed by
both 1.0 pmol of the 46mer containing the ATTCACTGA motif and the 26mer containing the GC-box motif oligonucleotides, whereas that of the complex bands was not competed using an equal molar amount of nonspecific 25mer oligonucleotide to 46mer and 26mer oligonucleotides. In the assay using the 26mer oligonucleotide we observed a slight decrease of the total amount of radioactivity but the reason is not clear at present. The binding of NF-R1 was competed by the similar amount of the 25mer oligonucleotide containing the ATTCACTGA motif to the 46mer oligonucleotide. Thus relative low affinity of the column made with the 25mer oligonucleotide to NF-R1 might be derived from artificial polymerization of the oligonucleotide. The competition experiments using NF-R1 purified by the GC-box motif-specific column showed similar results (data not shown). Accordingly we used NF-R1 purified with the 46mer column in further experiments.

Contact sites of NF-R1 on the two sequences

To determine correct binding sites of NF-R1 on the MDR1 proximal promoter, we performed methylation interference experiments using purified NF-R1. Fig. 5 shows the nucleotides required for NF-R1 binding to the ATTCACTGA motif. Two guanine and five adenine residues in the 5'-AGCATCATTCA-3' sequence were protected on the top strand, whereas a single guanine residue and two adenine residues were protected on the bottom strand. NF-R1 could not bind to the GC-rich region flanked to the ATTCACTGA motif. Next, nucleotides required for NF-R1 binding to the GC-box motif were determined (Fig. 5). Five guanine and two adenine residues on the bottom strand of the 5'-CGCCGGGGCGTGGGCTGCT-3' sequence were protected; no guanine and adenine residues were protected on the top strand of the GC-box motif. Above results showed strand specificity of NF-R1 for the binding to DNA.

Lack of NF-R1 binding to the MDR1 promoter caused 2- to 3-fold increases of MDR1 promoter activity

To confirm the sequences required for NF-R1 binding, we carried out a competition gel mobility shift assay using mutated oligonucleotides (Fig. 6). Oligonucleotides containing two base substitutions corresponding to the NF-R1-binding region and its flanking sequences were synthesized and used as competitors. CM1 mutation slightly inhibited the binding of NF-R1, whereas CM2 mutation did not affect the binding. Sequence AA, which was located in 5' end of wild-type 25mer, was not required for NF-R1 binding according to the result of methylation interference experiment. However, substitution of these nucleotides might change the conformation of DNA so that NF-R1 binding was slightly inhibited. All substitution in 5'-GCATCATTCA-3' inhibited NF-R1 binding, as expected. The substitution of the GC-rich region flanked to the NF-R1 binding site did not affect the binding of NF-R1.

As to the GC-box motif, GM1 and 2 mutations did not inhibit NF-R1 binding. The results seemed to be reasonable, because NF-R1 could bind to -52 to -2 region of the MDR1 promoter.
GM3 mutation caused a decrease of binding affinity to NF-R1, whereas the guanine residues in 5'-CGGGGC-3' were not contact sites of NF-R1 according to the methylation interference experiment. This effect might be based on the conformational change of DNA. GM4 through GM7 mutations inhibited the binding of NF-R1.

These point mutations were introduced to the MDR1 proximal promoter fused to a CAT gene, and the constructs were transfected into K562/ADM cells in order to assess the effect of the inhibition of NF-R1 binding in vivo. As a result, all constructs with inhibitory effects to the binding showed 2- to 3-fold increases of promoter activity, though the mutations CM3 and CM6 showed relatively slight effect in vivo (Fig. 6A). The mutations, such as CM2, CM7, CM5, CM9, CM10, GM1 and GM2, which did not inhibit the binding of NF-R1 did not enhance promoter activity. These results suggest that NF-R1 relates to the negative regulation of the MDR1 gene expression.

**DISCUSSION**

We have purified and characterized NF-R1 that binds to the MDR1 proximal promoter region. We observed similar promoter activity of the more upstream region (−1568 to +9) as compared to the proximal promoter, therefore, the proximal promoter was thought to be essential for MDR1 expression (data not shown). Kohno et al. also reported that −1568 to +121 region of the MDR1 gene showed similar promoter activity of −255 to +121 region in natural resistant CV-1 cell (20).

We have shown that NF-R1 binds to two divergent sequences, the ATTCAGTCA and GC-box motifs on the MDR1 proximal promoter. It is interesting that NF-R1 recognizes not the GC-rich region flanked to the ATTCAGTCA motif but the GC-box motif. Several proteins bound to GC-rich region were reported, but these factors did not recognize all GC-rich elements. For example, well characterized factor, Sp1, did not interact with some of the GC-rich elements of EGF receptor gene promoter, which were recognized by GCF (21).

Specific recognition of the different sequences by the same protein has been reported in other promoters of eukaryotic genes: HAPI (22), C/EBP (23), COUP (24), TEF1 (25), Oct2 (26) and T Saf (27). In these cases a single DNA-binding domain of the protein is responsible for recognizing divergent sequences. A putative DNA-binding domain of NF-R1 might recognize both the ATTCAGTCA and GC-box motifs since the binding of NF-R1 to the ATTCAGTCA motif was competed not only by the 25mer sequence containing the ATTCAGTCA motif but also by the 26mer sequence containing the GC-box motif and vice versa. Thus, the ATTCAGTCA and GC-box motifs, which have no sequence homology, might create a similar binding site on the DNA in a particular conformation.

Methylation interference experiments revealed that NF-R1 mainly bound to one strand of the duplex DNA on both the ATTCA GTCA and GC-box motifs. This strand specificity has been observed in the case of ubiquitous transcription factors Sp1 (28) and TFIIIA (29). The sequences containing the ATTCAGTCA motif and the GC-box motif could also be recognized by promoter binding proteins NF-R2 and NF-R3, respectively. Thus strand specificity of NF-R1 might be related to the possible additional protein binding to the similar region of the DNA.

Results of the competition assay and the functional assay showed that CM2 mutation did not influence NF-R1 binding and promoter activity, whereas the mutated adenine residue of CM2 was a part of contact residues according to the result of the methylation interference assay. The reason of this discrepancy is not clear, but one of our speculation is that the adenine residue at −122 was not important for NF-R1 binding. In other mutations, however, the results of CAT expression assays were generally consistent with the results of NF-R1 binding assays except of the CM2 mutation.

Previous work has shown that deletion of the sequences from −131 to −100 and −66 to −37 resulted in 5-fold and 2-fold decreases of promoter activity, respectively (13). Nevertheless, we observed that point mutations within these two regions, which inhibited the binding of NF-R1, caused 2- to 3-fold increases of promoter activity. NF-R2 and NF-R3 could respectively bind to the CM and GM series oligonucleotides, which lacked the binding of NF-R1 (30). Thus we speculated that the effect of the putative positive factors NF-R2 and NF-R3 might overcome the negative effect of NF-R1. At this moment, however, it is difficult to conclude whether NF-R1 is truly a negative transcription factor by using a CAT assay system, because NF-R1/NF-R2 and NF-R1/NF-R3 bind respectively to similar DNA sequences. It has been assumed that negative cis-acting elements or trans-acting factors might play significant roles in appropriate gene expression. The interplay of NF-R1 and NF-R2 and/or NF-R3 need to be clarified for the better understanding of the MDR1 expression.

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