RNA helicase A (RHA) is a member of the DEAD/H family of RNA helicases and unwinds duplex RNA and DNA. Recent studies have shown that RHA regulates the activity of gene promoters. However, little information is available about the in vivo relevance of RHA in the regulation of natural genes. We previously characterized a nuclear protein (MEF1) that binds to the proximal promoter of the multidrug resistance gene (MDR1) and up-regulates the promoter activity. In the present study, we isolated and identified RHA as a component of the MEF1 complex by using DNA-affinity chromatography and mass spectrometry. The antibody against RHA specifically disrupted the complex formation in electrophoretic mobility shift assay, confirming the identity of RHA. Western blotting showed that RHA in drug-resistant cells had a higher molecular weight than that in drug-sensitive cells. Similar results were obtained when FLAG-tagged RHA was overexpressed in these cells. This size difference probably reflects posttranslational modification(s) of RHA in drug-resistant cells. Chromatin immunoprecipitation revealed that RHA occupies the MDR1 promoter in vivo. Overexpression of RHA enhanced expression of the MDR1 promoter/reporter construct and endogenous P-glycoprotein (P-gp), the MDR1 gene product, and increased drug resistance of drug-resistant cells but not the drug-sensitive counterpart. Introduction of short interfering RNA targeting the RHA gene sequence selectively knocked-down RHA expression and concomitantly reduced P-gp level. Thus, our study demonstrates, for the first time, the involvement of RHA in up-regulation of the MDR1 gene. Interactions of RHA with other protein factors in the MEF1 complex bound to the promoter element may contribute to P-gp overexpression and multidrug resistance phenotype in drug-resistant cancer cells.

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RNA helicase A (RHA)*/nuclear DNA helicase II (referred to hereafter as RHA) is a member of the DEAD/H family of RNA helicases. Proteins of this family have seven conserved motifs in the helicase core and bind ATP (1). Aside from the central helicase core, RHA contains two copies of a double-stranded RNA-binding domain at its N terminus and an Arg-Gly-Oly (RGG) box at its C terminus that binds single-stranded nucleic acids (2). RHA unwinds both duplex RNA and DNA in an ATP-dependent fashion (3, 4).

Originally, RHA was identified as a human homologue of the Drosophila MLE protein (5). Recent experimental data reveal that RHA is a multifunctional protein involved in many nuclear events including transcriptional regulation. For example, RHA mediates interaction between the general coactivator CREB-binding protein (CBP)/p300 and RNA polymerase II (Pol II), which activates a chloramphenicol acetyltransferase reporter construct containing the cAMP-responsive element via CREB (6). RHA is also known to enhance the expression of a CREB-dependent gene by interaction with a methyl-CpG binding domain protein, MBD2a (7). Interestingly, the breast cancer-specific tumor suppressor protein (BRCA1) is recruited to Pol II by RHA (8), enabling BRCA1 to act as a transcriptional coactivator. In addition to functioning as a bridging factor between transcription factors, RHA is shown to bind directly to a DNA sequence in the promoter of the p16INK4a gene, leading to an increase in the promoter activity (9). Another recent study demonstrates an intriguing finding that RHA interacts with double-stranded (ds) DNA and topoisomerase IIα, indicating the involvement of RHA in the regulation of chromatin structure (10).

Certain types of cancers, including some of the acute myelogenous leukemia, display intrinsic resistance to multiple chemotherapeutic drugs. Many other cancers acquire multidrug resistance (MDR) during chemotherapy. This has been a major clinical obstacle for successful treatment of cancer patients. MDR is frequently associated with overexpression of P-glycoprotein (P-gp), a Mr 170,000 ATP-dependent transmembrane protein encoded by the MDR1 gene. P-gp is capable of pumping a number of structurally unrelated compounds out of the cell by utilizing the energy of ATP hydrolysis (11–15), which results in a decrease in the intracellular accumulation of the compounds and resistance to drug cytotoxicity. P-gp is also expressed normally in epithelial cells of the liver, kidney, and small and large intestine and capillary endothelial cells in the brain, ovary, and testis, where it acts as a barrier to the uptake of xenobiotics and promotes their excretion (15). Introduction of exogenous MDR1 gene through the gene transfer approach (16–18) has established a direct association between P-gp expression and the MDR phenotype. In addition to its efflux pump activity, P-gp has been shown to have anti-apoptotic function via inhibition of drug-, Fas-, tumor necrosis factor-, or UV irradiation-mediated activation of caspase pathways (19–23). This work reinforces the importance of inhibition of MDR1 gene expression in circumventing MDR and eventually eliminating tumor cells.
Detailed mechanisms regulating P-gp overexpression are not well understood; however, studies on transcriptional regulation of the human MDR1 gene reveal a complex pattern. The promoter of MDR1 gene contains a number of cis-elements for binding of transcription factors, including SP1, EGR1, and NF-Y (24). Our laboratory has shown that nuclear factor kB/p65 and c-fos transcription factors bind to the CAAT sequence in the promoter region, exerting a negative regulatory effect on sensitive MCF-7 human breast cancer cells, but not on their resistant cell counterparts (25). We have also demonstrated that in a nuclear protein, the MDR1 promoter-enhancing factor (MEF1) binds a region from nucleotide −118 to −111, which results in up-regulation of the MDR1 gene (26). A 23-base region from nucleotide −123 to −100 including the sequence we described above is suggested to be important for the regulation of the MDR1 gene (24). This short region of the promoter bears several closely located or overlapping binding elements, which provide a potentially cooperative or competitive mechanism for transcription factor action. This organization of cis-elements may also provide an opportunity for construction of multiprotein complex containing DNA-binding factors and cofactors. Although many of the proteins bound to this region of the MDR1 promoter remain to be characterized, the cis-elements in the region have clearly been shown to functionally control the transcription activity of the MDR1 promoter (24). In the present study, we report that RHA is a component of a multiprotein complex that interacts with the −118/−111 sequence, designated as a CAAT-like motif (26), in the MDR1 promoter of a drug-resistant cell line derived from acute myelogenous leukemia cell line HL-60 that is drug sensitive. We demonstrate that RHA is involved in up-regulation of the MDR1 expression in drug-resistant but not drug-sensitive cells.

EXPERIMENTAL PROCEDURES

Cell Lines, Plasmids, Antibodies, and Oligonucleotides—The HL-60 human acute myelogenous leukemia cell line, its MDR1-mediated multidrug-resistant derivative HL-60/Vinc, and the derivative of the HL-60/Vinc cell line, HL-60/VCR, which expresses high levels of P-gp, have been described previously (26). To maintain the MDR phenotype, HL-60/VCR and HL-60/Vinc cells were cultured in medium containing 1 and 0.1 μg/ml vinristine (VCR), respectively.

The MDR1 promoter −196/-454 was amplified previously referred to as pGL3-B/wt-MDR1), was as described previously (26). The FLAG-tagged RHA expression vector, pCDNA3-FLAG-RHA, was kindly supplied by Dr. Chee-Gun Lee (University of Medicine and Dentistry of New Jersey, Newark, NJ).

Antibodies were obtained from the following sources: RHA rabbit polyclonal antibody was commercially raised by US Biological against amino acid residues 585–599 of RHA protein (GenBank accession number NP_001348); RHA rabbit polyclonal antibody was collected by centrifugation at 50,000 × g, and the pellet was dissolved in 2 ml of buffer C (50 mM Hepes, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) and dialyzed against buffer C (500-mmol buffer) at 4°C. After electrophoretic separation, bands were visualized by silver staining with a Silver Stain Plus kit (Bio-Rad), excised, and in-gel-digested with trypsin. Protein identification was performed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) at the Indiana University School of Medicine Proteomics Core Facility, Pittsburgh, PA.

Western Blotting—Cells were harvested and lysed in Nonidet P-40 buffer containing protease inhibitor mixture (Sigma). Equal microgram amounts of protein were subjected to SDS-7.5% PAGE and then transferred onto Immobilon polyvinylidene difluoride membrane (Millipore). Membranes were incubated with the indicated antibodies. Secondary antibodies were either anti-RHA monoclonal or anti-(full-length)-RHA at room temperature followed by a 15-min treatment with 125 mM glycine to quench horseradish peroxidase (Amersham Biosciences). Immunoreactive protein bands were visualized by using the ECL kit (Pierce).

Chromatin Immunoprecipitation (ChIP) Assay—HL-60/Vinc cells (2 × 10^6 cells in 1.2 ml of normal growth medium) were seeded in a 12-well plate. Twenty-four h later, the cells were cross-linked with 1% (v/v) formaldehyde (final concentration), 1% (v/v) EDTA for 10 min at room temperature followed by a 15-min treatment with 125 mM glycine to quench cross-linking. The soluble chromatin was prepared by using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). In brief, the cross-linked cells were lysed in SDS lysis buffer supplemented with protease inhibitor mixture (Sigma) and sonicated (six times; 10 s each; 40% of ultrasonic power) using a Misonix sonicator equipped with a cup horn that was pre-cooled with filling with ice water. The sonication resulted in DNA fragment sizes of 0.2–1.3 kb as analyzed on 6% polyacrylamide gels. Part (~1%) of the whole cell lysate was taken as input chromatin for PCR analyses. The rest was precleared
with salmon sperm DNA/protein A-agarose beads. The supernatant was incubated overnight at 4°C with anti-RHA or anti-full-length RHA and anti-vector was used as negative control. The chromatin-antibody complex was collected through incubation with the agarose beads for 1 h at 4°C and centrifugation. The beads were washed and eluted with buffers as specified by the manufacturer (Upstate Biotechnology). The eluted chromatin and the input chromatin were incubated at 65°C for 4 h to reverse the cross-links, followed by proteinase K digestion at 45°C for 1 h. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The purified DNA fragments were used as templates for PCR amplification to analyze the protein occupancy of the MDR1 promoter.

The PCR primers spanning the −198/+43 (241 bp) region in the promoter of the MDR1 gene (GenBank™ accession number L07624) were as follows: 5′-TCAGAGGGTGACGGGA-3′ (forward) and 5′-GGGCTCTGCTTCTTTAGA-3′ (reverse). PCR conditions were 1 cycle of 5 min at 94°C for initial denaturation, 30 cycles of 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C; and a final extension for 7 min at 72°C. PCR products were separated by 8% PAGE and visualized with ethidium bromide staining. For siRNA transfection, 10^4 cells were mixed with 4 μg of pRL-SV40 Vector (Promega), and 2 μg of expression vector pcDNA3-FLAG-RHA or empty vector pcDNA3 in 1 ml of serum-free medium in a 6-well plate using FuGENE6 (Roche Applied Science). The pRL-SV40 Vector contains the Renilla luciferase gene driven by the early SV40 enhancer/promoter, used as an internal control for transfection efficiency, and the promoter-less pGL3-Basic vector was used as a negative control. The pcDNA3 vector was used to keep the amount of DNA constant in each transfection mixture. The ratio of FuGENE6 to DNA was 3:1. The cell/DNA mixture was incubated at 37°C for 7 h and then supplemented with 4 ml of normal growth medium. The cells were cultured for an additional 40 h and harvested. Cell lysates were prepared, and the luciferase activity was measured with a dual luciferase reporter assay system (Promega) in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Transfections were performed in triplicate three times. Firefly luciferase light units were normalized against Renilla luciferase activities as expressed in the method.

Introduction of RHA Expression Vector or siRNA into Cells by Electroporation—HL-60/Vinc or HL-60 cells (2 × 10^5) were transiently transfected with 2 μg of pcDNA3-1uc, 0.03 μg of pRL-SV40 Vector (Promega), and 2 μg of expression vector pcDNA3-FLAG-RHA or empty vector pcDNA3 in 1 ml of serum-free medium in a 6-well plate using FuGENE6 (Roche Applied Science). The pRL-SV40 Vector contains the Renilla luciferase gene driven by the early SV40 enhancer/promoter, used as an internal control for transfection efficiency, and the promoter-less pGL3-Basic vector was used as a negative control. The pcDNA3 vector was used to keep the amount of DNA constant in each transfection mixture. The ratio of FuGENE6 to DNA was 3:1. The cell/DNA mixture was incubated at 37°C for 7 h and then supplemented with 4 ml of normal growth medium. The cells were cultured for an additional 40 h and harvested. Cell lysates were prepared, and the luciferase activity was measured with a dual luciferase reporter assay system (Promega) in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Transfections were performed in triplicate three times. Firefly luciferase light units were normalized against Renilla luciferase activities as expressed in the method.

RESULTS

RHA Is a Component of MEF1 Complex That Interacts with the CAAT-like Motif—Our laboratory has previously identified a novel cis-acting element, CAAT-like, in the proximal promoter of the MDR1 gene and characterized a novel protein, MEF1, present in the multidrug-resistant HL-60/VCR cells (but not in the drug-sensitive HL-60 cells) that specifically binds to the CAAT-like sequence. Increased luciferase expression was observed by transient transfection of the wild-type MDR1 promoter/luciferase reporter construct, but not the construct with a deletion in the CAAT sequence. The CAAT-like motif behaves like an enhancer, as when it was cloned upstream of the SV40 promoter in the pG3-Luciferase Vector, a 7-fold increase in luciferase activity was also observed (26), which is consistent with the notion that enhancers increase transcription in a manner that is independent of their orientation and distance relative to the transcription start site (27). To help define the mechanism of MEF1 action, we sought to identify the proteins that interact with the CAAT-like region and/or MEF1 in HL-60/VCR nuclear extracts. We used a DNA-affinity chromatography procedure similar to that used previously to isolate MEF1 (26), except that bound proteins were eluted with SDS sample loading buffer, which allowed us to obtain eight major bands of polypeptides bound to the ds CAAT-like oligonucleotide as visualized by silver staining (Fig. 1, inset).

All the bands were subjected to peptide mass fingerprinting by MALDI-TOF MS. The resulting peptide masses were searched against predicted tryptic peptide masses for all the proteins contained within the National Center for Biotechnology Information protein database using the ProFound online search engine (prowl.rockefeller.edu). Identification was considered positive if the top-ranked candidate had a Z score of 2.0 or above and a probability score of 1.0 and the next protein candidate had a significantly lower probability (28). Based on these criteria, five of the eight proteins are positively identified (data not shown). One of the positively identified proteins (indicated by an arrowhead in the inset of Fig. 1) was analyzed in this study, and the rest are under investigation. Fig. 1 shows the MALDI-TOF spectrum of this protein. One hundred monoisotopic masses derived from Fig. 1 were submitted to ProFound. Twenty-one of the top 100 submitted peptide masses matched human RHA (Z score = 2.33) within 50 p.p.m. with 20% coverage. The probability (1.0) of an RHA peptide mass greater than 2.33 is the probability of lower than 10^-24 higher than the next candidate.

To confirm that RHA is present in the MEF1 complex interacting with the CAAT-like oligonucleotide, we performed EMSA by incubating nuclear extracts of HL-60/VCR cells with the radiolabeled CAAT-like oligonucleotide probe. A DNA-protein complex was detected (Fig. 2, lane 2). The complex was competed by the cold CAAT-like oligonucleotide (lane 3), but not by the mutant (deleted in the CAAT sequence) (lane 4) or unrelated oligonucleotides (lane 5), suggesting that the complex is specifically interacting with the CAAT-like element of the MDR1 gene. The polyclonal antibody against full-length RHA almost completely disrupted the formation of the DNA-protein complex, whereas it had no effect on the nonspecific (NS) band (Fig. 2, lane 6). Normal rabbit serum was used as a negative control, which could not disrupt the complex formation. These results demonstrate that RHA is a component of the MEF1 complex. To determine whether RHA binds directly to the CAAT-like element, we used the in vitro-translated RHA instead of nuclear protein extracts in EMSA, which showed no specific DNA-protein complex formed (results not shown), indicating that RHA may bind indirectly to the element.

Different Isoforms of RHA Exist in Drug-sensitive and -Resistant Cells—Because RHA was isolated from the highly drug-resistant HL-60/VCR cell line, we asked whether it exists in HL-60/Vinc cells that express moderate resistance (26), as well as in the drug-sensitive HL-60 cells that do not express P-gp. We performed Western blotting analysis of cell lysates from the
three cell lines mentioned above using the antibody against the peptide derived from RHA sequence. RHA bands were detected in all the cell lines, with the major bands from the two drug-resistant cell lines showing slower mobility than that from HL-60 drug-sensitive cells (Fig. 3), revealing different molecular weights of RHA present in the drug-resistant and -sensitive cells. To rule out the possibility that this size difference is due to a rearrangement or a large insertion in the RHA gene of drug-resistant cells that occurs during the process of drug selection, we transiently transfected HL-60/Vinc and HL-60 cells with pcDNA3-FLAG-RHA expression plasmids containing the FLAG coding sequence and the full-length RHA coding sequence. The cells were subsequently used for Western blot analysis with anti-FLAG monoclonal antibody. As shown in Fig. 3B, the FLAG-tagged full-length RHA also displays the two isoforms: one isoform of Mr ~150,000 in the drug-resistant HL-60/Vinc cells (left lane), and one of Mr ~127,000 in the drug-sensitive parental HL-60 cells (right lane). These results suggest that RHA proteins in the drug-resistant cells are probably modified posttranslationally.

**In Vivo Association of RHA with the MDR1 Promoter**—After association of RHA with the CAAT-like region of the MDR1 gene was established in vitro by the DNA-affinity chromatography and EMSA, we investigated the in vivo relevance of RHA with the MDR1 promoter using the ChIP assay. This assay determines the protein occupancy of a specific DNA sequence.
Immunoprecipitation of DNA using either the polyclonal anti-serum generated against the RHA peptide or preimmune serum was performed on formaldehyde-cross-linked extracts from HL60/Vinc cells. After reversal of cross-links and proteinase K digestion, a fragment covering nucleotides −198 to +43 (241 bp) of the MDR1 promoter was amplified by PCR. With total chromatin (input) as a template, the DNA fragment of expected molecular weight was observed (Fig. 4A, top panel). A strong DNA band was obtained from the chromatin precipitated with anti-RHA, whereas the chromatin precipitated with preimmune serum (negative control) generated only a very weak band (Fig. 4A, bottom panel).

To confirm this result, another polyclonal antibody against full-length RHA, an unrelated monoclonal anti-Bax, and a normal rabbit serum were used. Similarly, the MDR1 promoter DNA was precipitated with the anti-RHA serum, whereas very little or no DNA was obtained with the rabbit serum or anti-Bax (Fig. 4B). The weak signal obtained from the precipitates by preimmune or normal rabbit serum was considered as a background due to nonspecific immunoprecipitation. We conclude that RHA can interact with the MDR1 gene in vivo.

RHA Enhances the Transcriptional Activity of the MDR1 Promoter and Increases Drug Resistance in Drug-resistant Cells—We next performed a functional study in HL-60/Vinc cells to determine the effect of RHA on the MDR1 gene. The luciferase reporter gene construct containing the MDR1 promoter (pMDR1-luc) was transiently cotransfected with the RHA expression vectors (pcDNA3-FLAG-RHA) or the empty vector (pcDNA3). Luciferase activities were measured with a luminometer. As shown in Fig. 5, a 3-fold increase in the transcriptional activity of the reporter gene was obtained by cotransfection with pcDNA3-FLAG-RHA compared with the activity from the cotransfection with pcDNA3. The promoter-less pGL3-Basic that was cotransfected with pcDNA3-FLAG-RHA showed a background activity and was not enhanced by the RHA expression vector (data not shown), indicating that the increased activity by RHA occurs through the MDR1 promoter. However, this enhancement of RHA on the MDR1 promoter in drug-resistant HL-60/Vinc cells was not observed in the drug-sensitive HL-60 cells, and the luciferase activity in HL-60 cells is much lower than that in HL-60/Vinc cells (Fig. 5). These results indicate that HL-60 cells probably lack a factor(s) that is necessary for RHA to enhance the MDR1 gene activity. The factor(s) is probably responsible for formation of the RHA isoforms (Fig. 3) and the slowly moving form might be a functional one.

To examine whether RHA increases the activity of endogenous MDR1 gene, we transfected HL-60/Vinc cells with pcDNA3-FLAG-RHA or pcDNA3 empty vector. In a time course experiment, expression of RHA reached the highest level at 24 h after transfection as revealed by reverse transcription-PCR (Fig. 6A). We then performed Western blotting with antibodies against FLAG or P-gp using the HL-60/Vinc cell lysates prepared 24 h after transfection. Expression of FLAG-RHA fusion protein was verified (Fig. 6B, left panel). With the same cell lysate preparations, a much stronger signal of P-gp expression was detected in the pcDNA3-FLAG-RHA-transfected cell lysate than in the empty vector-transfected cell lysate (Fig. 6B, right panel). Thus, RHA can up-regulate both luciferase reporter gene driven by the MDR1 promoter and the endogenous MDR1 gene.

Because RHA can elevate the levels of P-gp expression, and drug resistance can be induced by P-gp overexpression, we tested the effect of RHA overexpression on the degree of drug resistance in HL-60/Vinc and HL-60 cells by MTT assay. We first established cell lines that stably express RHA. The expression of FLAG-RHA fusion protein in HL-60/Vinc and HL-60 cells was confirmed by Western blotting using anti-FLAG monoclonal antibody (Fig. 6C, left panel). It is noteworthy that the two isoforms of RHA were also demonstrated in these stably transfected cells (indicated by the arrowheads in Fig. 4B), immunoblots from cells that were transiently transfected with pcDNA3-FLAG-RHA expression vectors were probed with antibodies against RHA or β-actin (loading control).
6C). As in the transiently transfected HL-60/Vinc cells, elevated P-gp levels were also detected in the stably transfected HL60/Vinc cells (Fig. 6C, right panel). However, RHA overexpression in stably transfected HL-60 cells could not induce P-gp expression. The stably transfected cells were then incubated with the indicated concentrations of VCR for 72 h and analyzed by the MTT method. As shown in Fig. 6D, RHA transfection increased the mean survival of HL-60/Vinc cells by ~17% at 1 μM and ~63% at 5 μM, compared with the untransfected counterpart. At a concentration as high as 15 μM VCR, ~78% of the transfected cells still survived. However, introduction of RHA into HL-60 cells basically had no effect. This result reveals that RHA increases the resistance of HL-60/Vinc cells but not the drug-sensitive parental HL-60 cells to drug cytotoxicity.

**Transient Silencing of RHA Expression by RNA Interference Leads to Reduction of P-gp Level**—Because overexpression of
RHA increases the P-gp level in the drug-resistant cells, we speculated that inhibition of RHA expression would inhibit P-gp expression. To test this speculation, we utilized the RNA interference technology to posttranscriptionally silence the RHA gene. Introduction of chemically synthesized 21-nucleotide RNA duplexes (known as short interfering RNA or siRNA) targeting the RHA gene sequence led to a marked decrease in endogenous RHA (Fig. 7, left panel) and P-gp (Fig. 7, right panel) levels compared with the levels in nonspecific siRNA-transfected cells. The data further demonstrated that RHA does play a critical role in regulation of the MDR1 gene.

**DISCUSSION**

We have isolated and identified a multiprotein complex that interacts with the CAAT-like sequence of the MDR1 proximal promoter in the nuclear extracts of multidrug-resistant cancer cells derived from acute myelogenous leukemia cell line HL-60 by using DNA-affinity chromatography and mass spectrometry. One of the proteins is RHA. RHA has been shown to possess ATPase and helicase activity (3, 4, 29). ATPases/helicases are essential for preinitiation and initiation of transcription to alter chromatin structure and to unwind the two strands of promoter DNA. It has also been demonstrated that RHA interacts directly with Pol II through its 50-amino acid transactivation domain located between amino acid residues 331 and 380 (30) and also interacts with other protein factors such as CBP (6) and BRCA1 (8), linking them to the basal transcription machinery containing Pol II. Notably, CBP is also found to associate with numerous transcriptional regulators through its multiple independent protein-interacting domains (31). These interactions might result in synergism between the transcriptional factors when bound to the same promoter in cis to facilitate the stable assembly of basal transcription factors into complexes, leading to an increase in Pol II transcription initiation rates. These features of the RHA molecule (i.e. ATPase/helicase activity and interaction with Pol II, CBP, and other protein factors) suggest that RHA could be an important transcriptional regulator. However, very few endogenous genes whose promoter activity is regulated by RHA have been identified (7, 9). Identification of natural targets for RHA transcriptional regulation would be critical to understand the biological and pathological roles of RHA. In this report, we describe a novel natural gene, the MDR1 gene, targeted by RHA. We show that exogenous RHA enhances expression of both the luciferase reporter gene driven by the MDR1 promoter and the endogenous P-gp, the protein product of the MDR1 gene. ChIP assay revealed that endogenous RHA occupies the MDR1 promoter. These data indicate that RHA is responsible for the overexpression of endogenous P-gp in the drug-resistant cancer cells.

In the present study, RHA was isolated from the nuclear extracts of the drug-resistant variant of the HL-60 cell line by DNA-affinity chromatography using ds oligonucleotides containing the CAAT-like sequence. EMSA experiments using the oligonucleotide as a probe showed that deletion of the CAAT sequence almost completely abolished the formation of the specific DNA-protein complex. The anti-RHA polyclonal antibody specifically disrupted complex formation. These data suggest that RHA exerts the regulatory effect through the CAAT-like element in the proximal promoter of the MDR1 gene. Our result that the in vitro-translated RHA could not form a complex with the CAAT-like oligonucleotide in EMSA (data not shown) may suggest that RHA does not directly bind DNA and that it is recruited to the element via a specific DNA-binding protein (i.e. an activator) such as MEF1 that we have identified (26) and functions as a coactivator. It is also likely that posttranslational modification(s) of RHA is required for its interaction with DNA (see below). Alternatively, other cofactors are required for its stable binding to the DNA sequence. Indeed, several other nuclear proteins are copurified with RHA in the DNA-affinity chromatography using the CAAT-like oligonucleotide coupled to CNBr-activated Sepharose 4B. These proteins might enhance DNA binding of RHA, although further evidence is necessary.

In a Western blotting analysis, RHA was detected in both drug-sensitive and -resistant cells; however, the molecular weight of RHA in drug-resistant cells is higher than that in the drug-sensitive ones in the SDS-PAGE. This difference in molecular size was also revealed by immunoblotting the exogenously introduced FLAG-tagged RHA in transiently or stably transfected cells. These results suggest that some posttranslational modification(s) of RHA occurs in drug-resistant cells. Protein phosphorylation and acetylation are two types of key regulatory mechanisms modifying the activity of transcription factors (32, 33). A recent report showed that DNA-dependent protein kinase phosphorlates recombinant RHA in an RNA-dependent manner (34). It was also reported that CBP interacts with RHA (6). CBP possesses intrinsic histone acetyltransferase activity (35, 36), which enables it to mediate transcriptional activation by remodeling chromatin, and it can also acetylate certain transcription factors, leading to transcriptional activation of specific genes (31). Another report demonstrated that RHA is immunoprecipitated with acetylated lysine antibodies and that decreased amounts of the immunoprecipitated RHA are related to reduced binding of RHA to the p16INK4a promoter (9). Two or more posttranslational modifications may be integrated in the same regulatory event for a particular transcription factor. For example, activation of sequence-specific DNA binding by p53 after DNA damage depends on both phosphorylation and acetylation status of p53 (37). Whether RHA in drug-resistant cells is modified by acetylation, phosphorylation, and/or other covalently modifying types is under investigation.

As described above, RHA interacts with many protein factors, including CBP (6), MBD2a (7), BRCA1 (8), topoisomerase IIα (10), SMN complex (38), and HAP95 (39), involved in diverse nuclear events. These findings imply that the functional specificity of RHA may be dependent on its associating factors. The proteins copurified with RHA in this report seem to differ from those mentioned above, although the identity of the copurified proteins that were determined here by MALDI-TOF MS analysis needs to be confirmed by other methods such as Western blot and gel supershift analyses. The copurified proteins may directly or indirectly associate with RHA. Elucidating the roles played by these proteins will help to understand the molecular mechanism of regulation of the MDR1 gene by RHA and by the MEF1 transcription factor complex.

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