Sequence-specific DNA Binding Activity of RNA Helicase A to the p16\(^{INK4a}\) Promoter*

Received for publication, May 24, 2000, and in revised form, October 4, 2000
Published, JBC Papers in Press, October 18, 2000, DOI 10.1074/jbc.M004481200

Sanna Myöhänen and Stephen B. Baylin‡
From the Johns Hopkins Oncology Center, Baltimore, Maryland 21231

*p16\(^{INK4a}\) is frequently altered in human cancer, often through epigenetically mediated transcriptional silencing. However, little is known about the transcriptional regulation of this gene. To learn more about such control, we initiated studies of proteins that bind to the promoter in cancer cells that do, and do not, express the gene. We identify RNA helicase A (RHA) as a protein that binds much better to the p16\(^{INK4a}\) promoter in the expressing cells. RHA has not previously been characterized to manifest sequence-specific DNA interaction but does so to the sequence 5' CGG ACC CGC TGC GC 3' in the p16\(^{INK4a}\) promoter. The Drosophila homologue to RHA, maleless (Mle), functions in the fly for 2-fold activation of male X-chromosome genes. In our experimental setting, RHA induces a similar modest up-regulation of male X-chromosome genes. In our experiments, RHA has not previously been characterized to manifest sequence-specific DNA interaction but does so to the sequence 5' CGG ACC CGC TGC GC 3' in the p16\(^{INK4a}\) promoter. The Drosophila homologue to RHA, maleless (Mle), functions in the fly for 2-fold activation of male X-chromosome genes. In our experimental setting, RHA induces a similar modest up-regulation of male X-chromosome genes.

Defects in the Rb-p16\(^{INK4a}\)-cyclin D pathway are one of the most commonly found abnormalities in a wide variety of cancer cells. Dysregulation of this pathway either through loss of Rb or p16\(^{INK4a}\) or amplification of cyclin D1 causes abnormal cell cycle regulation at the G1/S checkpoint (1). Whereas Rb is commonly mutated within the coding region (1), gene deletions and transcriptional silencing are preferred mechanisms for p16\(^{INK4a}\) loss in tumor cells (2). The transcriptional silencing is associated in most tumors with abnormal hypermethylation that extends over the proximal promoter and the 5' untranslated region of the gene both in primary tumors and in cell lines (2). Several studies have established that loss of p16\(^{INK4a}\) function is an early event in tumor progression (3, 4), and it has been postulated that loss of p16\(^{INK4a}\) expression is essential for tumor growth (5, 6).

Despite the widespread alterations of p16\(^{INK4a}\) in tumorigenesis, little is known about the molecular events that control its transcriptional expression and that might predispose tumor cells to abnormal transcriptional silencing of the gene. We have thus initiated a search for specific DNA-binding proteins whose function, if compromised, might contribute to events leading to such abnormal regulation of the p16\(^{INK4a}\) gene promoter, including hypermethylation of the region. In this paper, we describe a sequence-specific differential binding of human RNA helicase A (RHA)\(^1\) to the p16\(^{INK4a}\) promoter sequences in cells with unmethylated versus methylated endogenous p16\(^{INK4a}\) genes. RHA is a homologue of maleless (Mle), a dosage compensation gene in Drosophila (7). Mle, along with msl-1, msl-2, and msl-3, is needed for the hyperactivation of the single male X-chromosome in flies (8), and this complex colocalizes with hyperacetylated H4Ac16 on the X-chromosome and in some autosomal loci (9). In this regard, in our studies, RHA appears to have a potential modulatory role for p16\(^{INK4a}\) transcription.

EXPERIMENTAL PROCEDURES

Gel Shift Analysis—Nuclear proteins from culture lines were extracted according to Dignam et al. (10). Protein amounts were measured using the Bradford assay (Bio-Rad). All oligonucleotides were from Life Technologies, Inc. Binding reactions were performed in 1× binding buffer (50 m M KCl, 10 m M Tris-HCl, pH 7.9, 1 m M EDTA, 10% glycerol, 5 m M MgCl\(_2\), 1% Nonidet P-40, 1 m M dithiothreitol, 100 m g/ml pepstatin, 100 m g/ml leupeptin, 100 m g/ml aproptin, and 1 m M phenylmethylsulfonyl fluoride) for 15 min on ice in a volume of 20 m l. Each reaction contained, in addition, 1 m g of poly(dA)·poly(dT) (Amersham Pharmacia Biotech) as a nonspecific competitor. Nuclear extracts were preincubated with nonspecific and specific competitors for 10 m in ice before addition of labeled oligonucleotide. Gel shifts were run for 2 h in 1× low salt, tris-acetate EDTA (6.75 m M Tris-HCl, pH 7.9, 1 m M EDTA, 33 m M sodium acetate, pH 7.9) on a 5% nondenaturing polyacrylamide gel.

Southwestern Analysis—Nuclear proteins (30 m g) were separated on a 7.5% denaturing polyacrylamide gel. Proteins were transferred to an Immobilon\(^\text{TM}\) (Millipore) membrane, which was treated with 6 m M guanidine HCl in binding buffer (10 m M Hepes, pH 7.9, 1 m M EDTA, 1 m M dithiothreitol, 50 m M KCl) for 5 m in. The membrane was then denatured by treatment with decreasing concentrations of guanidine-HCl (3, 1.5, and 0.75 m) in binding buffer and finally with binding buffer alone. Each step was for 5 m at room temperature. The membrane was blocked in 5% milk in binding buffer overnight at 4 °C and then incubated with 100,000 cpm of concatenated B4 oligonucleotide (see Table 1) in the binding buffer for 1 h at 4 °C and washed three times for 5 m in each with cold binding buffer.

DNA Affinity Purification and MALDI-MS Protein Identification—Concatenated B4 molecules (see Table 1) with a biotin label in one end were attached to streptavidin-coated magnetic beads (Dynal AB). Total nuclear protein fraction from cell line NCI-H69 (0.5 × 10\(^9\) cells) was

---

* This work was supported in part by a Public Health Service Grant (R01 CA43318) from the NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 410-955-8506; Fax: 410-614-9884; E-mail: sbaylin@jhmi.edu.

1 The abbreviations used are: RHA, human RNA helicase A; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; kb, kilobase(s); TSA, trichostatin A; TnT, transcription and translation.

This paper is available online at http://www.jbc.org
mixed with nonspecific competitor poly(A)·poly(T) (60 ng/ml) and oligo B4M1 (2 ng/ml; see Table 1) in 1× binding buffer with proteinase inhibitors. After 50 min of incubation on ice, the mixture was centrifuged for 10 min at 17,000 x g, and the supernatant was added to the streptavidin-coated magnetic beads. The binding reaction was incubated for 10 min at room temperature with rotation. Beads were washed two times with binding buffer and eluted with 0.4 M KCl in binding buffer. Eluted proteins were separated on a 7.5% SDS-PAGE gel and Coomassie stained. Selected proteins were excised and analyzed by MALDI-MS at the Keck Foundation (Yale University, New Haven, CT).

**Plasmid Constructs**—For transfection studies, the ATG site initiating translation of the p16\(^{\text{INK4a}}\) was mutated to a BamHI site with polymerase chain reaction mutagenesis using an oligonucleotide, 5′ AAA GGA TCC GCT GCC CGC CCG CT 3′. p16\(^{\text{INK4a}}\) promoter constructs of 0.9 kb (EcoRI-digested region) or 2.0 kb (HindIII-digested region) upstream of the ATG were cloned in pGL3 luciferase vector (Promega). Mutation in the RHA binding site described under “Results” was introduced by polymerase chain reaction mutagenesis with the oligonucleotide 5′ CTG GCT GTC CAT CAG AGG TGT GGG CGG ACC GTC TGC C 3′ (with the underlined base representing the site where C in the original sequence is mutated to A (mutant oligo B4M1; see Table I)). The polymerase chain reaction oligonucleotide included an endogenous BstEII site in the 5′ end, allowing the replacement of a BstEII-BamHI fragment in the wild-type constructs. All constructs were confirmed by sequencing. An expression plasmid containing the RHA cDNA in pcDNA3 was a gift from Dr. Che-Gun Lee.

**Transfections**—One day before transfections 2 × 10\(^6\) U1752 cells were plated and the next day transfected with 1 mg of wild-type or mutant p16\(^{\text{INK4a}}\) promoter-luciferase constructs and 8 μl of LipofectAMINE™ (Life Technologies, Inc.). In cotransfection experiments, 1 μg of luciferase construct was transfected along with 1 μg of RHA expression plasmid or with vector plasmid alone. Transfected cells were incubated for 4 h. NCI-H69 cells were transfected with 1 μg of plasmids and 2.5 μl of DMRIE transfection reagent (Life Technologies, Inc.) for 4 h. A renilla luciferase plasmid (Promega) was used to control for transfection efficiency, and luciferase activities were measured at 24 h using the Dual Luciferase kit (Promega). All transfections were done in triplicate, and results are shown from three independent experiments.

**TnT Reactions**—The RHA cDNA in the pSRSET plasmid was a kind gift from Dr. Che-Gun Lee. Deletion constructs AvnIII and XhoI were prepared from the full-length cDNA by removing amino acid regions 841–1270 and 693–1052, respectively, from the C-terminal end of the protein. 1 μg of plasmid DNA was transcribed and translated using the Quick Coupled Transcription/Translation system from Promega. 1 μg of the reaction was analyzed on SDS-PAGE gels or in a gel shift assay.

**Drug Treatments**—Trichostatin A (TSA) treatments were for indicated periods with 500 nm TSA (Wako). Control reactions were treated with EtOH. The caspase-3-specific inhibitor DEVD (final concentration 50 μM) was added 1 h before addition of TSA.

**Western Blotting and Immunoprecipitations**—Western blotting was performed using standard protocols (ECL, Amersham Pharmacia Biotech). Antibodies and protein-A-agarose were purchased from Santa Cruz Biotechnology (Sp1 PEP2, Sp3 D-20, Sp4 V-20), except for topoisomerase, which was from TopoGEN, Inc. RHA antiserum was a kind gift from Dr. Che-Gun Lee. Immunoprecipitations with acetylated lysine antibodies were performed as described in the product manual (New England Biolabs) and washed five times with phosphate-buffered saline before loading on a 7.5% SDS-PAGE gel.

**RESULTS**

**Putative Sp1 Elements at the p16\(^{\text{INK4a}}\) Promoter Bind Sp1 and Sp3 in Vitro**—With respect to differences in normal and abnormal control of p16\(^{\text{INK4a}}\) transcription, we first chose to examine Sp1 binding activities for the p16\(^{\text{INK4a}}\) promoter. Such sequences have been proposed for a role in protection of CpG islands from methylation (11–13). We extracted nuclear proteins from several cell lines, representing tumors of different tissue types, and with or without p16\(^{\text{INK4a}}\) hypermethylation-associated transcriptional silencing, to establish whether any gross differences were detected in binding of Sp1 family proteins (14) to p16\(^{\text{INK4a}}\) sequences in gel shift assays. The double-stranded oligonucleotides used in the binding studies are described in Table I. Gel shift analysis with all three putative Sp1 elements from the proximal p16\(^{\text{INK4a}}\) regulatory region showed strong binding of three specific proteins (a, b, and c in Fig. 1, A and B). Using Sp1- and Sp3-specific antibodies in supershift analysis (data not shown), we identified two Sp3-specific shifted complexes (b and c) and one Sp1 specific shift (a) at each site (Fig. 1, A and B). We did not detect any quantitative differences in binding of these two proteins between different culture lines with or without methylation of the endogenous p16\(^{\text{INK4a}}\). Furthermore, all cell lines also had high levels of expression of both Sp1 and Sp3 by Western analysis (data not shown).

**Detection of a Protein with Decreased Binding Activity to p16\(^{\text{INK4a}}\) Regulatory Region in Cell Lines with an Epigenetically Silenced p16\(^{\text{INK4a}}\) Gene**—Although protein extracts from all cell lines had uncompromised binding of Sp1 and Sp3 factors to p16\(^{\text{INK4a}}\)-specific sequences, one of the oligonucleotides produced a distinctive pattern of shifted complexes (Fig. 1B). Oligonucleotide p16B, located at 76 to 48 nucleotides from the translation start site (Fig. 1C, position B), shifted a protein that was not recognized by Sp1, Sp3, or Sp4 antibodies in supershift analysis (data not shown), and there were marked differences between the relative binding activities for this complex for the different cell lines used in the study. The complex was markedly diminished in each cell line with a hypermethylated endogenous p16\(^{\text{INK4a}}\) gene (Fig. 1B, arrow). This decreased binding was especially evident by comparing the intensity of the novel band to that for the Sp3 band (Fig. 1B, c). These differences were even more clearly apparent in gel shifts in which we used an unlabeled Sp1 consensus oligonucleotide to compete for the shifted complexes due to Sp1 or Sp3 binding (Fig. 2A). Although some binding was detectable for the novel complex in every cell line, binding activity was strongest in the NCI-H69 cell line and other cell lines with an unmethylated and a highly expressed p16\(^{\text{INK4a}}\) gene and much weaker in cell lines with a hypermethylated, epigenetically silenced p16\(^{\text{INK4a}}\) gene (Fig. 2A).

**Characterization of the DNA Sequence for the p16\(^{\text{INK4a}}\)-binding Protein**—By testing a series of oligonucleotides surrounding the Sp1 consensus sequence (data not shown), we determined the minimal binding sequence, 5′ CGG ACC GCG TGC GCT G 3′ (sequence B4 in Table I), that was able to compete away the novel complex. This sequence overlaps with the 3′ end of the Sp1 site in oligonucleotide p16B (Table I) and extends downstream. We next designed a panel of mutant oligonucleotides to test the sequence specificity of the interaction (Table I). Interestingly, all the mutations inside the minimal sequence

---

**Table I**

<table>
<thead>
<tr>
<th>Oligonucleotides used in gel shift analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16A 5′</td>
</tr>
<tr>
<td>p16B 5′</td>
</tr>
<tr>
<td>p16C 5′</td>
</tr>
<tr>
<td>B4 5′</td>
</tr>
<tr>
<td>B4M1 5′</td>
</tr>
<tr>
<td>B4M2 5′</td>
</tr>
<tr>
<td>B4M3 5′</td>
</tr>
<tr>
<td>B4M4 5′</td>
</tr>
<tr>
<td>B4M5 5′</td>
</tr>
<tr>
<td>Sp1 5′</td>
</tr>
</tbody>
</table>

---

**Characterization of the DNA Sequence for the p16\(^{\text{INK4a}}\)-binding Protein**—By testing a series of oligonucleotides surrounding the Sp1 consensus sequence (data not shown), we determined the minimal binding sequence, 5′ CGG ACC GCG TGC GCT G 3′ (sequence B4 in Table I), that was able to compete away the novel complex. This sequence overlaps with the 3′ end of the Sp1 site in oligonucleotide p16B (Table I) and extends downstream. We next designed a panel of mutant oligonucleotides to test the sequence specificity of the interaction (Table I). Interestingly, all the mutations inside the minimal sequence
affected the binding, and most of them abolished it altogether (Fig. 2B). Importantly, mutation in the overlapping Sp1 consensus site, which abolished Sp1 binding, did not eliminate binding of the newly detected protein (data not shown). Using the information from these competition studies, we constructed a concatemerized B4 oligonucleotide. This labeled, concatemerized probe was used in a Southwestern analysis to determine the information from these competition studies, we constructed a concatemerized B4 oligonucleotide. This labeled, concatemerized probe was used in a Southwestern analysis to determine the size of the unknown DNA-binding protein. We did not detect any nuclear proteins binding to this probe from cell lines that had a transcriptionally active p16\textsuperscript{INK4a} gene (Fig. 2C), whereas a single 130-kDa protein band was detected from cells that had a transcriptionally active p16\textsuperscript{INK4a} gene (Fig. 2C).

DNA Affinity Purification and MALDI-MS Analysis Identified the Protein as RNA Helicase A—We used a concatemerized double-stranded wild-type sequence probe, B4 (Table I), labeled at one end with biotin, to purify the DNA binding activity. To increase the stringency we added an excess of an oligonucleotide containing a single base pair mutation, B4MI (Table I), and a nonspecific homopolymer poly(dA)-poly(dT) to the binding buffer. Nuclear proteins from NCI-H69 cells were preincubated in the presence of competitors, and specific proteins binding to the recognition sequence were separated using streptavidin-coated magnetic beads. After extensive washing, DNA binding activity was eluted using increasing salt concentrations. Presence of the binding activity at each step was detected by gel shift analysis (Fig. 3A). Approximately equal amounts of two major proteins (130 and 160 kDa) were purified in the binding fraction eluted with 0.4 M KCl as detected by Coomassie staining of an SDS gel (Fig. 3B). Analysis of these proteins by MALDI-MS identified both proteins as human RNA helicase A, a homologue of the Drosophila dosage compensation protein, maleless (Mle). Both of these molecular mass forms of human RHA have been recognized by other investigators although the determinants of the size differences are not known (15, 16).

**RHA binds to the p16\textsuperscript{INK4a} promoter**—To verify that RHA indeed was the protein that produced the original shifted complex, we tested binding of RHA proteins, produced from an RHA cDNA (Fig. 4D) in a rabbit reticulocyte TnT lysate system, to the original p16\textsuperscript{INK4a}-specific oligonucleotide, p16B (Table I) using gel shift analysis. The lysate contained some endogenous RHA that was detected by anti-RHA antiserum, but we were able to produce exogenous RHA in higher quantities (Fig. 4A, lane 2). As shown in Fig. 4B, lane 2, this produced a much stronger complex in the gel shift than for the TnT reaction using a control template (Fig. 4B, lane 1). To further show that RHA indeed can specifically bind to the p16\textsuperscript{INK4a} regulatory region, we transfected an RHA expression plasmid and a vector
control into cultured cells and detected binding activity by gel shift analysis. Expression of the exogenous RHA but not of the vector alone caused an increase in the specific complex detected by gel shift (Fig. 4C). This increase in binding can be seen in both cell lines with or without a methylated endogenous p16INK4a gene (data not shown), but the relative increase in the shift was larger in the cell line, NCI-H69 (Fig. 4C), that initially contained more endogenous binding and has an unmethylated endogenous gene.

The p16INK4a Sequence-specific Binding Domain of RHA Is Located in the N-terminal End of the Protein—To determine what domains of RHA are needed for its sequence-specific binding to the p16INK4a promoter region, we deleted, from the RHA cDNA, portions encoding for the C-terminal end of the protein, and XhoI/1, lacking the conserved helicase domains five and six (amino acids 683–1052), retained full binding activity. Thus, the binding activity resides in the N-terminal end, which contains the two double-stranded RHA binding domains (15).

Binding of RHA Modulates p16INK4a Transcription—We tested whether RHA can modulate activity of the p16INK4a promoter in transient transfection assays. p16INK4a promoter-luciferase constructs containing 0.9 or 2.0 kb of wild-type p16INK4a promoter sequence upstream from the ATG site (Fig. 1C) and with or without a mutation in the RHA binding site were transfected into U1752 cells. The basal expression of the mutant constructs was consistently 30% less than that for the wild-type constructs at 24 h after transfection (Fig. 5A). In addition, cotransfection studies with a plasmid expressing human RHA produced a 1.5-fold increase in luciferase activity of the wild-type construct at 24 h, whereas activity from the mutant construct did not show this increase (Fig. 5B).
gene, to detect any mutations that might contribute to lowered activity of RHA. However, no mutations were found (data not shown). In addition, no overall differences were seen in the total amounts of RHA protein between cell lines by Western analysis (see Fig. 6). The identification of the 130-kDa protein under the denaturing conditions of the Southwestern analysis suggested that RHA is able to bind to the p16<sup>INK4a</sup> region without a partner protein. It then seemed most likely, from all of the above data, that binding differences between cell lines might be a result of differences in posttranslational modification of the RHA.

Because RHA has been shown to interact with the transcriptional coactivator cyclic AMP response element-binding protein (17), which has acetylase activity, one possible modification of RHA is acetylation. To evaluate the role of acetylation in the RHA binding to the p16<sup>INK4a</sup> promoter, we first treated U1752 and NCI-H69 cells with TSA, a specific histone deacetylase inhibitor (18), for 18 h and analyzed DNA binding by gel shift assay. As shown in Fig. 6A, TSA treatment of U1752 cells did not affect the DNA binding activity of RHA to a p16<sup>INK4a</sup>-specific oligonucleotide (lanes 1 and 2). However, in NCI-H69 cells, TSA treatment caused a marked decrease in the sequence-specific DNA binding of RHA (Fig. 6A, lanes 3 and 4). A complete time course indicated that this decrease became apparent 12 h after TSA administration (data not shown). Interestingly, immunoblot analysis of total proteins with RHA-specific antibodies revealed a difference in isoforms of RHA between control-treated and TSA-treated NCI-H69 cells (Fig. 6B, lanes 3 and 4), which also appeared only after 12 h of TSA treatment (data not shown). This indicated to us that TSA treatment caused a shift in either posttranslational modification or degradation of the protein in NCI-H69 cells; this was not seen in U1752 cells (Fig. 6B, lanes 1 and 2).

We next analyzed control- and TSA-treated samples by immunoprecipitations with acetylated lysine antibodies. Immunoreactive RHA could be immunoprecipitated from both NCI-H69 and U1752 cells (Fig. 6C, lanes 1 and 3) with acetylated lysine antibodies. However, the amount of RHA complexed with acetylated lysine antibodies was considerably less in U1752 cells (a cell line with reduced RHA binding activity to the p16<sup>INK4a</sup>-specific sequence) than in NCI-H69 cells. Yet, both cell lines contain the same steady state amount of total RHA (Fig. 6B, lanes 1 and 3). In NCI-H69 cells the smaller isoform of RHA produced by TSA treatment was brought down less efficiently with acetylated lysine antibodies than was the full-length form (Fig. 6C, lane 4). In U1752 cells no change was seen in RHA complexed with acetylated lysine antibodies after TSA treatment (Fig. 6C, lane 2).

It was interesting that TSA induced a change in the form of RHA associated with the decreased binding in NCI-H69 cells. In addition to its direct inhibition of histone deacetylases, TSA has been shown recently to have later, and indirect, effects on apoptosis associated with induction of caspase activity (19). In the NCI-H69 cells, the new form of RHA seen after TSA treatment caused a shift in either posttranslational modification or degradation of the protein in NCI-H69 cells; this was not seen in U1752 cells (Fig. 6B, lanes 1 and 2).

We next analyzed control- and TSA-treated samples by immunoprecipitations with acetylated lysine antibodies. Immunoreactive RHA could be immunoprecipitated from both NCI-H69 and U1752 cells (Fig. 6C, lanes 1 and 3) with acetylated lysine antibodies. However, the amount of RHA complexed with acetylated lysine antibodies was considerably less in U1752 cells (a cell line with reduced RHA binding activity to the p16<sup>INK4a</sup>-specific sequence) than in NCI-H69 cells. Yet, both cell lines contain the same steady state amount of total RHA (Fig. 6B, lanes 1 and 3). In NCI-H69 cells the smaller isoform of RHA produced by TSA treatment was brought down less efficiently with acetylated lysine antibodies than was the full-length form (Fig. 6C, lane 4). In U1752 cells no change was seen in RHA complexed with acetylated lysine antibodies after TSA treatment (Fig. 6C, lane 2).

It was interesting that TSA induced a change in the form of RHA associated with the decreased binding in NCI-H69 cells. In addition to its direct inhibition of histone deacetylases, TSA has been shown recently to have later, and indirect, effects on apoptosis associated with induction of caspase activity (19). In the NCI-H69 cells, the new form of RHA seen after TSA treatment was estimated to be 145 kDa, 15 kDa smaller than the full-length (160 kDa) RHA. Analysis of the RHA amino acid structure revealed the sequence EEVD, a putative caspase-3 cleavage site, in the N-terminal portion of the molecule between the two previously identified double-stranded RNA binding domains (Fig. 4D). Cleavage at this site would cut a 15-kDa fragment off the N-terminal portion of the RHA molecule. To test whether TSA might be inducing such cleavage, we incubated NCI-H69 cells with the caspase-3 specific inhibitor DEVD before addition of TSA. This inhibitor substantially reduced formation of the 145-kDa TSA-specific form of RHA and also substantially reversed the TSA-induced decrement in RHA.

**Fig. 3. Affinity purification of the protein.** Nuclear proteins from the NCI-H69 cell line were incubated (see “Experimental Procedures”) in buffer containing a biotin-labeled, concatemerized B4 oligonucleotide for the wild-type RHA binding sequence with a mutant RHA binding site as competitor (oligonucleotide B4M1; see Table 1). Bound protein was isolated with streptavidin-coated magnetic beads. A, specific DNA binding activity of purification fractions as monitored by gel shift assays with B4 as the labeled probe (the arrow marks the position of the specific complex). Lanes are as follows: 1, initial incubation mixture on ice; 2, supernatant following centrifugation of the mixture before addition of beads; 3, unbound supernatant fraction following incubation with beads; 4 and 5, supernatants from first and second washes, respectively, of beads with binding buffer; 6, supernatant from elution of beads with 0.4 M KCl (note elution of the specific binding protein); 7, supernatant from final elution with 1 M KCl. B, proteins eluted in washes from A as detected by SDS-PAGE and Coomassie staining. Lanes are as follows: 1 and 2, washes 1 and 2, respectively, with binding buffer; 3, elution with 0.4 M KCl. Arrows indicate the 160- and 130-kDa proteins that were analyzed by MALDI-MS.

**Differences in the Sequence-specific Binding Activity of RHA Are Associated with Posttranslational Modifications**—We next wanted to identify factors that might affect the sequence-specific binding of RHA and be determinants of differences in this activity between the cell lines studied. We first sequenced RHA coding regions from the cell line U1752, which has a low sequence-specific binding activity and a methylated p16<sup>INK4a</sup> region, to detect any mutations that might contribute to lowered activity of RHA. However, no mutations were found (data not shown). In addition, no overall differences were seen in the total amounts of RHA protein between cell lines by Western analysis (see Fig. 6). The identification of the 130-kDa protein under the denaturing conditions of the Southwestern analysis suggested that RHA is able to bind to the p16<sup>INK4a</sup> region without a partner protein. It then seemed most likely, from all of the above data, that binding differences between cell lines might be a result of differences in posttranslational modification of the RHA.

Because RHA has been shown to interact with the transcriptional coactivator cyclic AMP response element-binding protein (17), which has acetylase activity, one possible modification of RHA is acetylation. To evaluate the role of acetylation in the RHA binding to the p16<sup>INK4a</sup> promoter, we first treated U1752 and NCI-H69 cells with TSA, a specific histone deacetylase inhibitor (18), for 18 h and analyzed DNA binding by gel shift assay. As shown in Fig. 6A, TSA treatment of U1752 cells did not affect the DNA binding activity of RHA to a p16<sup>INK4a</sup>-specific oligonucleotide (lanes 1 and 2). However, in NCI-H69 cells, TSA treatment caused a marked decrease in the sequence-specific DNA binding of RHA (Fig. 6A, lanes 3 and 4). A complete time course indicated that this decrease became apparent 12 h after TSA administration (data not shown). Interestingly, immunoblot analysis of total proteins with RHA-specific antibodies revealed a difference in isoforms of RHA between control-treated and TSA-treated NCI-H69 cells (Fig. 6B, lanes 3 and 4), which also appeared only after 12 h of TSA treatment (data not shown). This indicated to us that TSA treatment caused a shift in either posttranslational modification or degradation of the protein in NCI-H69 cells; this was not seen in U1752 cells (Fig. 6B, lanes 1 and 2).

We next analyzed control- and TSA-treated samples by immunoprecipitations with acetylated lysine antibodies. Immunoreactive RHA could be immunoprecipitated from both NCI-H69 and U1752 cells (Fig. 6C, lanes 1 and 3) with acetylated lysine antibodies. However, the amount of RHA complexed with acetylated lysine antibodies was considerably less in U1752 cells (a cell line with reduced RHA binding activity to the p16<sup>INK4a</sup>-specific sequence) than in NCI-H69 cells. Yet, both cell lines contain the same steady state amount of total RHA (Fig. 6B, lanes 1 and 3). In NCI-H69 cells the smaller isoform of RHA produced by TSA treatment was brought down less efficiently with acetylated lysine antibodies than was the full-length form (Fig. 6C, lane 4). In U1752 cells no change was seen in RHA complexed with acetylated lysine antibodies after TSA treatment (Fig. 6C, lane 2).

It was interesting that TSA induced a change in the form of RHA associated with the decreased binding in NCI-H69 cells. In addition to its direct inhibition of histone deacetylases, TSA has been shown recently to have later, and indirect, effects on apoptosis associated with induction of caspase activity (19). In the NCI-H69 cells, the new form of RHA seen after TSA treatment was estimated to be 145 kDa, 15 kDa smaller than the full-length (160 kDa) RHA. Analysis of the RHA amino acid structure revealed the sequence EEVD, a putative caspase-3 cleavage site, in the N-terminal portion of the molecule between the two previously identified double-stranded RNA binding domains (Fig. 4D). Cleavage at this site would cut a 15-kDa fragment off the N-terminal portion of the RHA molecule. To test whether TSA might be inducing such cleavage, we incubated NCI-H69 cells with the caspase-3 specific inhibitor DEVD before addition of TSA. This inhibitor substantially reduced formation of the 145-kDa TSA-specific form of RHA and also substantially reversed the TSA-induced decrement in RHA.
binding to the p16\(^{\text{INK4a}}\) promoter (data not shown). These findings further localize the region of the protein responsible for the binding to the far N terminus of the molecule.

**DISCUSSION**

In the present study we have defined a sequence, 5'-CGG ACC GCG TGC GC-3', in the context of the p16\(^{\text{INK4a}}\) promoter region, that specifically binds RNA helicase A. We show that this binding activity of RHA is decreased in tumor cell lines with an epigenetically silenced p16\(^{\text{INK4a}}\) gene. This decrement appears to be associated with decreased amounts of RHA that immunoprecipitate with acetylated lysine antibodies. In an experimental setting RHA can induce a modest up-regulation of the p16\(^{\text{INK4a}}\) promoter, and this modulatory activity is dependent upon the sequence-specific interaction of RHA with the promoter. Finally, we show that RHA is a substrate for caspase-3 and that sequence-specific binding of RHA to the p16\(^{\text{INK4a}}\) promoter is sensitive to caspase cleavage.

**Transcriptional Activity of RHA**—The transcriptional modulatory activity of RHA in our study may best be considered in the context of defined functions of Mle, the RHA homologue in Drosophila (20). In the male fly, this protein functions, together with MSL proteins, to ensure 2-fold increase in transcription of the single male X-chromosome. Although Mle is only transiently or loosely associated with the complex, it may be the essential factor in the process by recruiting the other proteins (20).

The precise manner in which RHA modulates transcription is not known. RHA is a member of the DEAH-box DNA/RNA helicases (15), and it has previously been shown to have unwinding activity toward both RNA and DNA, suggesting a role in DNA replication and transcription (21). The sequence-specific binding for RHA that we demonstrate is the first described for this protein. This binding activity appears to reside within the region containing the two previously characterized, nonsequence-specific, double-stranded RNA binding domains at the far N-terminal end of the molecule. A glycine-rich RGG domain in the C-terminal end has been suggested to have a role in single-stranded DNA/RNA binding. Neither the double-stranded RNA binding domains nor the RGG domain is needed for the unwinding activity (15).

The transcriptional role of RHA in mammals has not yet been well characterized. In mice, knockout of the gene coding for this protein was recently shown to be embryonically lethal (22). However, the only characterized potential functions of human RHA, to date, are nuclear export and stability of retroviral RHAs (23, 24). Our studies now indicate a role for this helicase in transcriptional competence of specific genes. All of the properties mentioned above for the protein could be important in this process, and the actual degree of transcriptional
modulation in our experimental setting may tell only a small part of the story. The helicase activity of the protein and the ability to target other proteins to a transcriptional complex could facilitate the transcription of genes such as \( p16^{\text{INK4a}} \). For example, it has been shown that RHA has a role in cAMP-dependent transcriptional activation through direct interaction with the transcriptional coactivator cyclic AMP response element-binding protein (17) and that RHA can directly interact with RNA PolII (17, 25).

Posttranslational Modulation of RHA Binding and Differences in the Process between Tumor Cell Types—The differences that we have found between tumor cell lines in the sequence-specific DNA binding activity of RHA appear to be mediated by steady state differences in signal transduction involving the acetylation status of the protein or proteins complexed with it. From our experiments it seems probable that acetylation helps regulate the sequence-specific DNA binding of RHA. The amounts of RHA brought down with anti-acetylated lysine antibodies were much higher from NCI-H69 cells, which have a high sequence-specific DNA binding activity of RHA, than from U1752 cells, which have much lower binding activity. Coactivator cyclic AMP response element-binding protein, with which RHA can complex, has a histone acetyltransferase activity, but it has also been shown to modify nonhistone proteins such as p53 (26). Acetylation of specific lysine residues in the p53 protein has been shown to be important in induction of the sequence-specific binding activity (26).

Colocalization of Msl complexes in the fly with H4Ac16 indicates that X-chromosome dosage compensation is closely linked to chromatin structure and histone acetylation. Mle, the homolog of RHA, also colocalizes with H4Ac16 in distinct regions on autosomes (9). It is not surprising then that, in higher eukaryotes, RHA has functions in transcriptional activation associated with the status of protein acetylation.

It remains to be determined how RHA binding and its modulation by translational modification mediate the biological roles of the \( p16^{\text{INK4a}} \) gene. \( p16^{\text{INK4a}} \), by controlling the functional status of Rb in the cyclin D-Rb pathway, plays a critical role for cell cycling. Interestingly, each of the tumor cell lines that we found to have increased binding activity of RHA have a mutant Rb gene and a marked increase in \( p16^{\text{INK4a}} \) expression, probably in response to the loss of Rb function in cyclin D-Rb pathway check point control (1). RHA could play a role in this increased expression. Furthermore, in normal cells, \( p16^{\text{INK4a}} \) expression is up-regulated in cells entering the quiescent state of senescence (27). Interestingly, we have shown that RHA is a substrate for caspase activity. Perhaps, caspase-mediated abrogation of RHA binding to the promoter is involved with an alternative signal for decreasing \( p16^{\text{INK4a}} \) expression in cells programmed for apoptosis or increasing expression of the gene in arrested cells entering senescence. This area may be a rich one for further exploration.

Does RHA Binding Play a Role in Aberrant Transcriptional Silencing and Methylation of the \( p16^{\text{INK4a}} \) Promoter?—We originally began the current study looking for factors that might render the \( p16^{\text{INK4a}} \) promoter region sensitive to aberrant hypermethylation during tumorigenesis. Indeed, we detected the novel RHA interaction partly because binding of this protein to the \( p16^{\text{INK4a}} \) promoter is reduced in tumor cells of different types that have an endogenously hypermethylated and epigenetically silenced \( p16^{\text{INK4a}} \) gene. How might the RHA interaction influence the methylation events? Interestingly, the binding site for RHA at the \( p16^{\text{INK4a}} \) promoter is located just downstream from, and partially overlaps with, a classical Sp1 consensus sequence. Several studies on the APR7 gene have suggested that Sp1 elements can protect CpG islands from methylation (11–13). However, it is now clear that the Sp1 protein alone is not enough to keep CpG islands free from methylation because Sp1-knockout mice do not hypermethylate CpG islands (28), and this site alone cannot protect transgene CpG islands from methylation (29).

The close overlap of the RHA binding site with an Sp1 site might suggest interaction between RHA and Sp1 in this region. Both transcription and DNA methylation could be influenced by such interaction. Recently, another helicase-like transcription factor was shown to interact with Sp1 and Sp3, and it was suggested that this protein, HLTF, might provide chromatin opening to facilitate transcriptional activation by Sp family members (30). We have shown in this study that RHA binding can also positively modulate transcriptional activity of \( p16^{\text{INK4a}} \) constructs in transient assays. Perhaps RHA makes the \( p16^{\text{INK4a}} \) promoter more accessible to transcriptional activators like Sp1 and thus helps render it protected from CpG island methylation.

We have made some initial attempts to see whether binding of RHA is important to keep \( p16^{\text{INK4a}} \) sequences free of methylation. We stably introduced the wild-type and mutant \( p16^{\text{INK4a}} \) promoter-luciferase constructs used in the present studies into DuPro and Du145 cells in which the endogenous \( p16^{\text{INK4a}} \) gene is methylated and unmethylated, respectively (31). We followed six independent wild-type and mutant clones in both cell lines for over 20 passages but observed no methylation in any of our clones (data not shown). In these experiments loss of RHA binding did not then increase the probability of methylation at the exogenous \( p16^{\text{INK4a}} \) promoter.

Whereas these results could rule out a role for RHA in \( p16^{\text{INK4a}} \) promoter methylation patterns in cancer, our results may not address this issue for a number of reasons. First, the
chromosomal context of the endogenous gene may be essential for any role of RHA. Second, the initial hypermethylation of p16\(^{INK4a}\) and loss of expression during tumorigenesis may require a selective pressure closely linked to a functional Rb gene. In cancers of multiple types, p16\(^{INK4a}\) is only altered, including promoter hypermethylation, when a wild-type Rb gene is present (1).

Active Rb has been shown by some workers to produce negative transcriptional pressure on the p16\(^{INK4a}\) promoter (32). This potential role for active Rb is also evident from recent studies where transcriptional silencing and hypermethylation of p16\(^{INK4a}\) provides an early step toward cellular immortalization by allowing cells to escape mortality checkpoint "M0" (6, 33). Importantly, this hypermethylation only occurs if the Rb gene and/or protein is present in a functional state (33). Both of the cell lines into which we introduced our constructs and our plasmids and for RHA antiserum. We thank Erica Seiguer, Jessa Jones, and Jennifer Hackett for technical assistance and Dr. Margaret Johns, Dr. Beth Cameron, and Dr. James Herman for their advice.

Acknowledgments—We thank Tammy Means for help in preparation of the manuscript. We thank Dr. Che-Gun Lee for RHA expression plasmids and for RHA antiserum. We thank Erica Seiguer, Jessa Jones, and Jennifer Hackett for technical assistance and Dr. Margaret Johns, Dr. Beth Cameron, and Dr. James Herman for their advice.

REFERENCES
26. Sakaguchi, K., Herrera, J. E., Suhr, S., Miki, T., Bustin, M., Vassilev, A.,
RNA Helicase A Binding to the p16^{INK4a} Promoter