MRGX is a novel transcription factor that is a member of the mortality factor 4 (MORF4)-related gene family. MRG15, a closely related family member, is in a complex with the retinoblastoma tumor suppressor protein Rb and activates the B-myb promoter, which is tightly controlled by Rb/E2F through the E2F binding site. In this study we investigated the effect of MRGX on the B-myb promoter. Interestingly, MRGX repressed the B-myb promoter in EJ cells (human bladder carcinoma cells), which have a functional Rb, but activated B-myb in HeLa cells (human cervical carcinoma cells), which express a lower amount of Rb. This repression and activation was dependent on the helix-loop-helix and leucine zipper regions of the MRGX protein but not the N-terminal region. MRGX interacts with Rb through the helix-loop-helix and leucine zipper regions. Using a treatment of trichostatin A, which is a potent inhibitor of histone deacetylase (HDAC), we determined that the repression of the B-myb promoter by MRGX in EJ cells was dependent on HDAC activity. We confirmed the association of MRGX with HDAC1 by immunoprecipitation/Western analysis and determined that MRGX complexes had HDAC activity. The data indicate that MRGX can repress or activate the B-myb promoter depending on the cell type studied, suggesting that there may be tissue-specific functions of this protein.
MRGX Acts as Positive and Negative Transcriptional Regulator

RESULTS

Effect of Wild-type and Various Mutant MRGX Proteins on the B-myb Promoter in HeLa and EJ Cells—Co-transfection of MRGX with the wild-type B-myb promoter-reporter construct into HeLa cells resulted in a dose-dependent increase of luciferase activity (about 3-fold) similar to what we had observed with MRG15 in our previous studies (Fig. 1A) (16). In contrast, increasing amounts of MRGX repressed the B-myb promoter (3–4-fold) in EJ cells (Fig. 1B) (significance is p < 0.05 by one-way analysis of variance). Analysis of luciferase activity following co-transfection with various mutant MRGX constructs (Fig. 2A) demonstrated that the HLH and LZ regions were necessary for both the derepression activity in HeLa cells (Fig. 2B) and the repression activity in EJ cells (Fig. 2C) and that the unique N-terminal domain of MRGX was not required for function in either cell type. These results were strikingly different from those obtained with MRG15, because the unique N-terminal CHR in MRG15 was essential for B-myb promoter activation (17). It was also unexpected that the same regions of the MRGX protein were involved in activating and repressing the promoter in the different cell types. The results indicate that MRGX can act as an activator or repressor of transcriptional activity depending on the expression of other proteins in the cell.

We had found previously that MRG15 can interact with Rb through the HLH and LZ regions. These regions are highly homologous in the MRG15 and MRGX proteins. We therefore tested whether MRGX can also interact with Rb through this common C-terminal region using GST pulldown analysis of EJ nuclear extract. Fig. 3A shows the various deletion mutants used. All fusion proteins were equally expressed in Escherichia coli, and the mutant protein production was confirmed by Western analysis using an anti-GST antibody (Fig. 3B). The interaction of MRGX with Rb also occurred through the HLH and LZ regions, and the unique N-terminal region was not required (Fig. 3C). Previously, we reported that MRG15 interacted with a novel 14-kDa protein, PAM14 (16). We investigated whether MRGX also interacts with PAM14 using GST pulldown analysis. As indicated in Fig. 3D, MRGX interacted with PAM14, and the HLH and LZ regions, but not the unique N-terminal region in MRG15, were important for this interaction similar to what we had observed with MRG15. These data indicate that Rb and PAM14 interact with the MRG proteins through a common region.

Sucrose gradient analyses of the nuclear extracts of EJ cells had demonstrated that MRG15 was present in at least two complexes, MRG15-associated factor 1 and MRG15-associated factor 2 (17). MRG15-associated factor 1 involved MRG15, Rb, and PAM14, and MRG15-associated factor 2 contained MRG15 and the histone acetyltransferase hMOF (17, 21). When MRGX was analyzed on these gradients, it was found to be present in multiple fractions but not those involving hMOF (data not shown). In view of these results we determined whether an HDAC activity was associated with the repressive activity of MRGX and performed co-transfections of MRGX into EJ cells with the B-myb promoter-reporter construct. We treated the cells with trichostatin A, a specific inhibitor of HDAC, 24 h later and observed loss of the B-myb promoter repression by MRGX following the treatment (Fig. 4A). We confirmed that MRGX interacts with HDAC1 by immunoprecipitation/Western analysis, because Rb-HDAC1 complexes have been implicated in repression of E2F-activated promoters (22–24), and MRGX interacts with Rb. HA-tagged MRGX co-precipitated with HDAC1 in an immunoprecipitation using anti-HA antibody. Immunoprecipitation with an unrelated anti-HIS antibody served as a control (Fig. 4B). HDAC activity in anti-HA

Plasmid Constructs—The B-myb promoter-reporter construct was a gift from N. Dyson (Massachusetts General Hospital). To generate a MRGX expression vector for use in mammalian cells, the MRGX cDNA was amplified from cDNA derived from human neonatal foreskin fi-broblasts (HCO2A strain derived in the Smith laboratory) using primers that incorporate EcoRV and XhoI at the 5′ and 3′ ends, respectively, and introduced into this into the EcoRV/XhoI sites of pcDNA3.1 (Invitrogen).

Large Deletion Mutants of MRGX—MRGX deletion mutants were cloned into the EcoRV/XhoI sites of pGEX4T1 to generate MRGX-DEL1: pGEX4T1-MRGX-DEL1; MRGX-DEL2:pGEX4T1-MRGX-DEL2:pGEX4T1; and MRGX-DEL: pGEX4T1, respectively. Primer pairs used to create the respective constructs were: DEL1, 5′-TTT GAT ATC TAT GCA GAA GAC TCC TGG AAA-3′; DEL1, 5′-TTT CTC GAG TAC CAG GCA TAT TTT AG-3′; and DEL2, 5′-TTT CTC GAG TAC CAG GCA TAT TTT AG-3′, respectively.

Expression—HEK293 cells were plated 2 × 10^5 cells in 35-mm tissue culture dishes and transfected the next day using LipofectAMINE (Invitrogen).

SDS-PAGE and Western Analysis—Transformed B-myb promoter-reporter construct was cotransfected with wild-type or mutant B-myb promoter-reporter construct into HeLa cells. After 48 h, the lysates were prepared by scraping the dishes with ice-cold SDS-PAGE sample buffer (150 mM Tris/HCl, pH 6.8, 2.5% SDS, 20% glycerol, 0.01% bromphenol blue), separated by SDS-PAGE, and transferred to a nitrocellulose membrane (Bio-Rad) for immunoblot analysis.

Immunoprecipitation and HDAC Activity—EJ cells were transfected with a C-terminal HA-tagged MRGX construct (pcDNA 3.1 MRGXHA). After 48 h, the cells were lysed in the lysis buffer (50 mM Tris, pH 7.5, 120 mM NaCl, 1.5 mM MgCl_2, 0.4% Nonidet P-40), supplemented with a protease inhibitor mixture (Calbiochem), and kept on ice for 30 min, and the lysates were prepared by centrifugation at 14,000 × g for 10 min. The precipitates were applied to 10% PAGE, and proteins were transferred to nitrocellulose membrane followed by immunoblot detection. HDAC activities in the precipitates were measured using a HDAC assay kit (Upstate Biotechnology).

Constructions of Small Deletion/Point Mutants—To make the HLH small deletion and LZ point mutation in human MRGX, we used pcDNA3.1 (+) hMRGX as a template for PCR mutagenesis. Primers used for PCR were: 5′-TTT GAT ATC TAT GCA GAA GAC TCC TGG AAA-3′ and 5′-TTT CTC GAG TAC CAG GCA TAT TTT AG-3′, respectively. After PCR amplification, fragments were digested by BamHI and XbaI and ligated into pcDNA3.1 (+). The HLH mutant MRGX has a 5-amino acid deletion at position 136 (total of 288 amino acids). The LZ mutant MRGX has a leucine (3.1) point mutation at position 352.

Effect of placing a 3′ splice site within the 5′ UTR of the E2F-activated promoters (22, 23) and the characteristic HDAC enzyme activity of MRGX were measured using the luciferase assay kit (Upstate Biotechnology).

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and anti-HDAC1 immunoprecipitates was determined and was 5–10-fold greater than control in the EJ cell lysate (Fig. 4C).

This HDAC1 activity was inhibited by about 80% by the addition of 250 mM sodium butyrate, an inhibitor of HDACs (data not shown). These results indicate that MRGX is present in a complex with Rb and HDAC, primarily HDAC1, and represses the B-myb promoter via the E2F binding site on this promoter.

DISCUSSION

The identification of MORF4 (1) as an inhibitor of proliferation in immortal human cell lines assigned to complementation group B for indefinite division (4, 6) has led to the identification of a novel, very interesting family of genes. MRG15 and MRGX, the only two other family members that are expressed, have now been found to be involved in transcriptional regulation. New data presented here demonstrate that MRGX can stimulate or repress a gene promoter depending on the context of the cell type, irrespective of the expression of MRG15. This supports many previous reports in the literature (25, 26) that indicate the dynamic nature of nucleoprotein complexes in cells. Thus, depending on other proteins available in the cells and the composition of nucleoprotein complexes, transcriptional activity will vary. A recent report by Yamagoe et al. (27) highlights this best using fluorescence resonance energy transfer analysis. They have demonstrated that p300/CBP-associated factor, a HAT, and HDAC1 are in close proximity in HeLa cells, that HATs are integrated into a large multiprotein HDAC complex, and that the coordinated activity of the two enzymes determines the expression of genes controlled by E2F. Further, YY1 and Sp1 interact with both HATs and HDACs and acquire an activator or repressor that is dependent on the promoter context and other factors. In the case of MRGX overexpression, the activity of the HDAC complex is dominant over the HAT complex present in EJ cells. Because the N-terminal region of MRGX was not necessary for repression of the B-myb promoter, the link to a HDAC-Rb-E2F complex is strengthened, as Rb binding requires the LZ and HLH domains of the MRGX.

The results have led us to propose the following model. EJ cells, which have a functional Rb, require the interaction of MRG15 with a histone acetyltransferase, such as hMOF, to modify chromatin around the B-myb promoter and permit access to the Rb/E2F site to allow for activation of the promoter. Because MRGX lacks a CHR, which we have shown is neces-

FIG. 1. Action of MRGX on the B-myb promoter in HeLa and EJ cells. A, MRGX activates the B-myb promoter in HeLa cells. HeLa cells were transfected with 0.5 μg of a luciferase-expressing plasmid under the control of the B-myb promoter either alone or together with 0.25–1.0 μg of a plasmid expressing wild-type MRGX. Cells were lysed 24 h post-transfection, the amount of luciferase activity in each lysate was determined using a luminometer, and luciferase activity was normalized to the amount of protein in each sample. The results represent three independent experiments. B, MRGX represses the B-myb promoter in EJ cells. EJ cells were transfected as described above, and luciferase activity was determined. The results represent three independent experiments.
necessary to recruit hMOF, it is unable to perform this function in EJ cells and instead binds to the HDAC-Rb-E2F complex that has been described as present at E2F sites (28, 29) and represses the B-myb promoter. HeLa cells express the papilloma virus proteins E6 and E7, and Rb is inactivated and degraded by E7 in these cells. These lower Rb levels may result in

FIG. 2. Small mutations in the HLH and LZ but not the N-terminal region of MRGX abolish B-myb promoter activation in HeLa cells and repression in EJ cells. A, schematic diagram of MRGX mutants, which were used in transfection experiments. MRGX DEL1 has a deletion in the N-terminal region, which is a novel region specific to MRGX. MRGX HLH5d has a deletion of five conserved amino acids in the HLH region. MRGX LA has one leucine to alanine mutation in the LZ domain. NLS, nuclear localization signal; MSL-3, male-specific lethal-3. B, wild-type (WT) and mutant MRGX were co-transfected with the B-myb promoter-reporter plasmid into HeLa cells. After 24 h, luciferase activities were measured. C, wild-type and mutant MRGX were co-transfected with the B-myb promoter-reporter plasmid into EJ cells. After 24 h, luciferase activities were measured. At least two independent experiments performed in triplicate were done for the transfections described above.
FIG. 3. MRGX interacts with Rb, and the LZ and HLH domains are necessary for the interaction between MRGX and Rb in vitro. A, schematic diagram of wild type and mutant MRGX proteins. A schematic diagram of wild-type MRGX, as well as MRGX deletion mutants lacking the MRGX-specific N-terminal domain (DEL1), the N-terminal region including part of the HLH domain (DEL2), a large region at the N terminus (DEL3), or the LZ domain (DEL Leu). NLS, nuclear localization signal; PKC, protein kinase C; MSL-3, male-specific lethal-3. B, the wild type and mutants of MRGX GST fusion proteins are expressed to equivalent levels. Equivalent aliquots of bacterial lysates expressing each GST fusion protein were separated by SDS-PAGE, transferred to a membrane, and immunoblotted with GST antibodies. Asterisks indicate the location of each fusion protein. C, bead-immobilized GST-tagged proteins were incubated with EJ nuclear lysates for 3 h, then washed four times in radiimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris-HCl, pH 8.0), solubilized in 2× sample buffer, run on an SDS-PAGE protein gel, and transferred to a nitrocellulose membrane for immunoblot analysis. The membrane was probed with anti-Rb antibody. D, GST pulldown assays were performed as indicated above using nuclear lysates from PAM14-HA-expressing EJ cells and subsequently immunoblotted with HA antibodies.
Fig. 4. Association of MRGX with HDAC in EJ cells. A, loss of MRGX repression of the B-myb promoter following trichostatin A (TSA) treatment. MRGX and B-myb promoter-reporter plasmids were co-transfected into EJ cells. After 18 h, the histone deacetylase inhibitor, trichostatin A, was added to the cultures at 1 μM, and the cells were incubated for 24 h. Cell lysates were prepared, and luciferase activities were measured. B, MRGX co-immunoprecipitates with HDAC1. A HA-tagged MRGX-expressing plasmid was transfected into EJ cells. After 48 h, total cell lysates were prepared and subjected to immunoprecipitation (IP) with rabbit anti-HA, anti-HDAC1 (positive control), or anti-HIS antibodies (negative control). Western analysis was done using an anti-HDAC1 antibody. C, HDAC activity is present in MRGX complexes. HDAC activity in the immunoprecipitates used in B was measured using a HDAC assay kit (Upstate Biotechnology).
lowered repression at the various E2F responsive promoters, and overexpression of increasing amounts of MRGX may be able to disrupt the complex on the B-myb promoter and activate this promoter rather than repress it in HeLa cells (30, 31). We have performed the majority of our studies with immortal cells, which are easier to transfec and analyze, to obtain information that can now be applied to normal young and senescent human cells. Determining the composition and number of nucleoprotein complexes present in normal cells should aid greatly in our understanding of normal cell aging processes versus immortalization and dysregulated growth control and of the molecular mechanisms involved in cell cycle regulation.

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MRGX Is a Novel Transcriptional Regulator That Exhibits Activation or Repression of the B-myb Promoter in a Cell Type-dependent Manner

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