MRG15 Activates the B-myb Promoter through Formation of a Nuclear Complex with the Retinoblastoma Protein and the Novel Protein PAM14*

James K. Leung‡¶, Nathalie Berube‡, Susan Venable‡, Saira Ahmed‡, Nikolai Timchenko‡**, and Olivia M. Pereira-Smith‡

From the ¶Roy M. and Phyllis Gough Huffington Center on Aging, the ‡Departments of Molecular Virology and Microbiology, Molecular and Cell Biology, and Medicine, and the **Department of Pathology, Baylor College of Medicine, Houston, Texas 77030-3498

Received for publication, April 17, 2001, and in revised form, June 27, 2001
Published, JBC Papers in Press, August 10, 2001, DOI 10.1074/jbc.M103435200

The MORF4-Related Gene on chromosome 15 (MRG15) is a member of a novel family of genes originally identified in studies to reveal cell senescence-inducing factors. MRG15 contains several predicted protein motifs, including a nuclear localization signal, a helix-loop-helix region, a leucine zipper, and a chromodomain. These motifs are commonly associated with transcription factors, suggesting that MRG15 may likewise function as a transcriptional regulator. To examine the potential function(s) of MRG15, we sought to identify cellular factors associated with this MRG family member. In this regard, we have found that both the retinoblastoma tumor suppressor (Rb) and a novel nuclear protein PAM14 (Protein Associated with MRG, 14 kDa) specifically associate with MRG15. We have further demonstrated that these interactions require the helix-loop-helix and leucine zipper domains of MRG15. Interestingly, we have found all three proteins present in a multiprotein complex, suggesting that at least some of their functions may be interdependent. Although the functions of PAM14 have yet to be elucidated, Rb has several well characterized activities, including repression of E2F-activated promoters such as that of B-myb. Significantly, we have demonstrated that MRG15 blocks the Rb-induced repression of this promoter, leading to B-myb promoter activation. Collectively these results suggest that MRG15 regulates transcription through interactions with a cellular protein complex containing Rb and PAM14.

Replicative senescence, or the terminal loss of proliferative potential exhibited by normal cells in culture, is viewed as both a model for aging at the cellular level and as a tumor suppressor mechanism (1). In studies to identify cell senescence-related genes, we cloned MORF4 (MORtality Factor on chromosome 4), a novel gene that induces a senescent phenotype upon introduction into a subset of immortal human cell lines (2). It was subsequently shown that MORF4 is a member of a novel gene family whose protein products share several common structural motifs, including a nuclear localization signal, a helix-loop-helix region, and a leucine zipper. As these domains are frequently found in transcriptional regulators, we hypothesized that the MORF4 family members may function similarly to regulate transcription. Consistent with this possibility, we have previously established that members of this family are localized within the nucleus of cells (2).

Upon cloning each member of the MORF4 gene family, we found that in addition to MORF4, only two of the other family members were expressed, MRG15 and MRG (MORF4-Related Genes found on chromosomes 15 and X, respectively) (2). MRG15 is of particular interest because it is expressed in a wide variety of human tissue types and is highly conserved across multiple species, including flies (Drosophila melanogaster), worms (Caenorhabditis elegans), yeast (Schizosaccharomyces pombe and Saccharomyces cerevisiae) and plants (Arabidopsis thaliana) (3). This cross-species conservation suggests that MRG15 possesses an activity fundamentally important to one or more cellular processes.

Sequence alignment data demonstrate that MRG15 is nearly identical to MORF4 (96% amino acid similarity). Unlike MORF4, however, MRG15 fails to induce a senescent phenotype upon introduction into immortal cell lines, and MRG15 RNA levels decline with the onset of senescence (2). The most striking structural difference between MRG15 and MORF4 is that MRG15 contains an additional predicted domain at its amino terminus that codes for a chromodomain. The chromodomain is a motif identified in several proteins that function as negative or positive regulators of transcription, including proteins from D. melanogaster and S. cerevisiae such as the Msl-3, polycomb, HP1, and SWI/SNF proteins (4–7). These regulators do not appear to bind DNA directly but rather associate indirectly with specific sites on chromatin via interactions with transcriptional repressors or activators as well as with proteins that influence chromatin accessibility to such transcriptional regulators. The Msl-3 protein, for example, has been implicated in the regulation of dosage compensation in Drosophila by acting in a multimeric complex that binds to hundreds of specific sites on the male X.

MRG; GST, glutathione S-transferase; HA, hemagglutinin; GFP, green fluorescent protein; CHR, chromodomain; LUE, leucine zipper motif; HLH, helix-loop-helix; CMV, cytomegalovirus; CMV-MJ-Hel1, CMV-transformed human fibroblasts; EJ, bladder carcinoma cell line; PAGE, polyacrylamide gel electrophoresis.

This paper is available on line at http://www.jbc.org

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
chromosome and induces hypertranscription likely through modification of chromatin structure (8). Interestingly MRG15 exhibits sequence similarity to the Drosophila Mal-3 protein over its entirety, suggesting that MRG15 may similarly affect gene expression in cells by associating with specific transcription factors in multimeric nuclear complexes.

The fact that the chromodomain and leucine zipper motifs have been implicated in protein-protein interactions suggested that MRG15 associates with one or more cellular factors. Therefore, identification of such interacting proteins is expected to provide critical insight into MRG15 function. We report here the identification of two MRG15-associated factors as a novel nuclear protein PAM14 (Protein Associated with MRG, 14 kDa) and the retinoblastoma tumor suppressor protein (Rb). Rb is known to repress the promoters of many genes, including those involved in cell cycle progression (9–12). We elected to study one paradigm of an Rb/E2F repressed promoter, the B-myb promoter (13–15), and have shown that MRG15 blocks Rb-mediated repression of this promoter. These results suggest that MRG15 forms a nuclear protein complex with PAM14 and Rb that may function to control transcription from Rb-regulated promoters.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Commercially available rabbit hemagglutinin (HA) polyclonal antibodies (Santa Cruz Biotechnology) and rabbit polyclonal Rb (C-15) antibodies (Santa Cruz Biotechnology) were used for immunoblot analysis. The polyclonal MRG15 antibody was generated by immunizing rabbits with a peptide containing the sequence from the 5′-chromodomain region of the protein (DEWVPESRVKL) and was used for immunoblot analysis. Commercially available HA monoclone antibodies (Roche Molecular Biochemicals) or Rb monoclone antibodies were used for immunoprecipitation assays. Commercially available anti-rabbit and anti-mouse secondary antibodies (Fierce) were also used. Plasmids—Wild-type and mutant B-myb promoter constructs in the pGL2 luciferase reporter (wt myb and mut myb) were generously provided by N. Dyson (13). The mutant B-myb promoter-reporter construct contains a mutation in the E2F binding site (deletion of nucleotides –208 to –206) that eliminates Rb binding and repression. To isolate the full-length PAM14 cDNA, an internal primer was synthesized and used for rapid amplification of cDNA ends with the AP1 primer (CLONTECH). The resulting amplicons were directly ligated into the PCRII vector using a TA cloning strategy (Invitrogen). The inserts corresponding to the 5′-end of the PAM14 cDNA were sequenced, and the following primers were used to amplify the open reading frame from a human heart library (CLONTECH): 5′-AGCTTCCACCATGCGGCCCCTGG-3′ and 5′-GCCCGATCCAGGGCCGCCCTGG-3′. The 424-base pair product was cloned into the HindIII and BamHI sites of the EGF-P11 (CLONTECH) and pcDNA3.1 (Invitrogen) vectors and the BamHI/EcoRI sites of the glutathione-S-transferase (GST) expression tag vector pGEX4T1 (Amersham Pharmacia Biotech) to generate PAM14:EGFP-N1, PAM14:pcDNA3.1, and PAM14:pGEX4T1, respectively. A HA tag was introduced at the carboxyl terminus of PAM14:pcDNA3.1 by polymerase chain reaction methods.

The MRG15 cDNA was introduced into the SalI/NcoI sites of pGEX4T1 (Amersham Pharmacia Biotech), the BamHI/SalI sites of the pGBT9 yeast two-hybrid bait vector, and the BamHI/XbaI sites of pcDNA3.1 to generate MRG15:GexP4T1, MRG15:pGBT9, and MRG15:pcDNA3.1, respectively. A HA tag was placed at the carboxyl terminus of MRG15 in pcDNA3.1 by polymerase chain reaction methods. MRG15-GST mutants lacking the chromodomain (CHR), helix-loop-helix (HLH), or leucine-zipper (LEU) domain were generated by inverse polymerase chain reaction of wild-type MRG15 and cloned into the PstI site of pGEX4T1 to generate –CHR: pGEX4T1, –HLH: pGEX4T1, and –LEU: pGEX4T1, respectively. Primer pairs used to create the respective constructs were: –CHR 5′-GGCTGGAGCTTCAATAATGGGACAAC-3′ and 3′-CHR 5′-GCCCTGACGAAAGAGAGGCCCATGGAGA-3′; –HLH 5′-AAAATCGAGGATCCATTGTTCTTCTTATTGACG-3′ and 3′-HLH 5′-AATCGCTGAACATCCCACTGAAAGAG-3′; and –LEU 5′-GCCCTGAGGAGCAGGATGTTGAGTG-3′ and 3′-LEU 5′-GCCCTGAGGACCATCGAGTATA-3′. All constructs were verified by sequencing and restriction enzyme analysis.

**Cells, Cell Culture, and Transfections**—CMV-transformed human fibroblasts (CMV-MJ-Hel1), EJ bladder carcinoma-derived cells, HeLa cervical carcinoma cells, and Saos2 osteosarcoma cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Ham's minimum essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. All transfections were carried out using LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer's instructions. To generate stable clones of cells expressing HA-tagged PAM14, CMV-MJ-Hel1 and EJ cells were transfected with PAM-HA:pDNA3.1. Twenty-four hours post-transfection, 3,000—10,000 cells/80-mm dish cells were subcultured into Earle's minimum essential medium (Life Technologies, Inc.) supplemented with 1 mg/ml G418 (Life Technologies, Inc.) and incubated at 37° C in 5% CO2 incubator. The cells were subjected to X-gal staining to verify expression of the fusion proteins. The bacterial lysates were subsequently harvested and purified on Sepharose 4B glutathione (Amersham Pharmacia Biotech). For GST pull-down assays with cell lysates, EJ or Saos2 cells transiently transfected with the indicated plasmids were harvested in Nonidet P-40 lysis buffer (20 mg/ml Tris-HCl, pH 7.5, 200 mM NaCl) and incubated at 37° C. Coverslips with attached cells were rinsed briefly in a 0.5 µM/µl 4′,6-diamidino-phenylindole solution, and coverslips were affixed to microscope slides with mounting medium (VectorShield). Microscopy was performed using an Applied Precision DeltaVision microscope (Issaquah, WA) fitted with an Olympus IX70 microscope. Images were acquired via wide field sectioning using fluorescent light. The stacked images, usually 20—55 sections, were subjected to point spread function analysis for better image quality on Silicon Graphics software (SGI, Mountain View, CA). Magnification of all cells was maintained at 600×. PAM14-HA was seen as red, MRG15-GFP as green, 4′,6-diamidino-phenylindole nuclear staining as blue.

**Yeast Two-hybrid Analysis**—A yeast two-hybrid screen was performed using the Matchmaker two-hybrid system (CLONTECH). Yeast or murine fibroblast (3T3) DNA library derived from DM16 cells (a gift from J. Campisi) cloned into the activator plasmid pGAD424 were transformed into the PJ69-4A yeast strain (a gift from J. Campisi), which contains three different inducible reporter markers for adenine, histidine, and β-galactosidase synthesis. Plasmid DNA was isolated using DNA extraction buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA), 0.2 M of phenol/chloroform (1:1), and 0.3 g of l-proline. Plasmid DNA derived from each yeast colony was transformed into the DH5α Escherichia coli strain, and the DNA inserts were sequenced to determine the identity of the interacting clones. The prey plasmids identified in the screen were each retransformed with the MRG15 bait plasmid by the lithium acetate method (16) and selected on plates lacking tryptophan and leucine. These yeast were streaked on plates with adenine, tryptophan, and leucine, and expression of β-galactosidase was assayed as described above. Yeast colonies were selected on plates lacking histidine and adenine, and expression of β-galactosidase was assayed as described above. Yeast colonies were selected on plates lacking histidine and adenine, and expression of β-galactosidase was assayed as described above.
P-40, 0.1% SDS, 50 mM Tris, pH 8.0, solubilized in 2× sample buffer, run on an SDS-polyacrylamide protein gel, and either transferred to a nitrocellulose membrane (Bio-Rad) for immunoblot analysis or silver-stained using the Silver Stain Plus Kit (Bio-Rad) according to the manufacturer’s instructions. For GST pull-down assays with in vitro transcribed/translated products, in vitro transcription/translation was performed on the indicated plasmid constructs using the T7 Single Tube Protein Kit (Novagen). In vitro transcribed/translated protein products were then incubated with the GST proteins and glutathione-Sepharose beads in binding buffer (50 mM Tris, pH 8.0) for 2 h, then washed four times with Nonidet P-40 lysis buffer, run on a 12% polyacrylamide gel, dried, and developed by autoradiography.

**Immunopopulation—Protein A/G BioMag beads (Polysciences)** were preincubated with 8 µg of HA or Rb monoclonal antibodies for 1 h at 4 °C. Nuclear extracts of Ej and CMV-MJ-HeL1 clones stably expressing PAM14-HA were prepared by resuspension of cells in buffer A (25 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 5 mM dithiothreitol) and higher salt extraction of proteins in the nuclear pellet in buffer B (25 mM Tris-HCl, pH 7.5, 0.42 M NaCl, 1.5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 25% sucrose). The lysate was then incubated with the antibody-bound beads for 3 h at 4 °C, washed four times in Nonidet P-40 lysis buffer, separated by SDS-PAGE, and immunoblotted with the indicated antibodies.

**Luciferase Assays—** EJ cells were transfected with the indicated expression plasmids and harvested with Reporter Lysis Buffer (Promega), and the luciferase activity was determined using the Luminescence Assay Kit (Promega) and a Monolight 2010 luminometer. The data was normalized to the amount of protein in the samples as determined by the Bradford assay (Bio-Rad). Luciferase assays were performed in triplicate to verify reproducibility, and protein assays were performed in duplicate. Statistical analyses were performed using the Student’s t test.

**RESULTS**

**Interaction of MRG15 with a Novel Nuclear Protein, PAM14—** MRG15 encodes several motifs implicated in mediating protein-protein interactions, suggesting that this protein associates with other cellular factors. Therefore, to identify potential MRG15-interacting proteins, we screened a human fibroblast cDNA library by yeast two-hybrid assay using full-length MRG15 as bait. A total of 2 × 10⁵ transformants were screened, yielding seven colonies that grew under selection. The strength of each interaction was assessed by β-galactosidase reporter activity upon co-transfection of MRG15 and the plasmids expressing each interacting protein into yeast (data not shown), and the strongest interacting protein, PAM14, was isolated for further analysis. As further verification of this interaction, yeast co-expressing PAM14 and MRG15 or the positive controls SV40 T antigen and p53 were competent for growth under adenine/tryptophan/leucine (−ATL) and histidine/adenine/tryptophan/leucine (−HATL) amino acid selection, whereas yeast co-expressing the SV40 T antigen and either PAM14 or MRG15 failed to grow under selection (Table I). Thus, MRG15 and PAM14 are not intrinsic trans-activating proteins. Interestingly, unlike many leucine zipper proteins, MRG15 was not found to homodimerize in this system (Table I).

Cloning and sequencing of the full-length PAM14 revealed it to be a 1205-nucleotide cDNA encoding a novel 14-kDa protein of 127 amino acids (GenBank™ accession number AF116272) (Fig. 1). Secondary structure analysis (Expsys) predicted this protein primarily consists of a coiled-coil helical structure throughout its length. To physically examine the MRG15-PAM14 interaction, we performed GST pull-down assays and found that the MRG15-GST fusion protein but not GST alone bound HA-tagged PAM14 (PAM14-HA) (Fig. 2A). Likewise, in the converse experiment, PAM14-GST but not GST alone bound HA-tagged MRG15 (MRG15-HA) (Fig. 2A). Furthermore, the interaction between these two cellular factors is direct as MRG15-GST retained the ability to bind in vitro transcribed and translated PAM14 (Fig. 2B).

**Table I. Interaction of PAM14 with MRG15 in yeast two-hybrid assays**

| Gal4 DBD | Gal4 TD | Growtha |
|---------|---------|---------|
| p53     | SV40 T  | +       |
| MRG15   | SV40 T  | +       |
| PAM14   | SV40 T  | +       |
| MRG15   | MRG15   | +       |
| MRG15   | PAM14   | +       |

a Growth on yeast plates lacking tryptophan and leucine (−TL) indicates the presence of both bait and prey plasmids in the yeast strain, whereas growth on plates also lacking adenine (−ATL) or adenine and histidine (−HATL) indicates interaction between the bait and prey proteins as this leads to activation of a Gal4 promoter and subsequent transcription of genes required for adenine and histidine synthesis.

**Fig. 1.** Amino acid sequence of the novel MRG15-associated protein PAM14. Underlined sequence denotes regions with predicted coiled-coil motifs.

For interaction with PAM14, we generated a panel of MRG15-GST deletion mutants lacking the predicted structural domains (Fig. 2C) and demonstrated that the wild-type and mutant GST fusion proteins were expressed to equivalent levels (Fig. 2D). In GST pull-down assays, the MRG15 mutant lacking the amino-terminal chromodomain (−CHR) bound PAM14 with similar affinity as wild-type MRG15 (Fig. 2E). By contrast, the MRG15 mutants lacking either the helix-loop-helix region (−HLH) or leucine zipper (−LEU) failed to bind PAM14 (Fig. 2E). Thus, these results suggest that the helix-loop-helix and leucine zipper domains but not the chromodomains of MRG15 are important for interaction with PAM14.

*We next sought to determine the subcellular localization of PAM14 by examining cells expressing GFP-tagged PAM14 using fluorescence microscopy. Interestingly GFP-PAM14 localized to the nucleus of human cells but was excluded from the nucleoli (Fig. 3A), an expression pattern similar to that previously observed for GFP-tagged MRG15. In accordance with these results, we observed co-localization of PAM14 and MRG15 in HeLa cell nuclei by confocal microscopy (Fig. 3B).*

**MRG15 Interacts with Rb—** Although we predict the interaction between MRG15 and PAM14 to be significant, the lack of knowledge regarding PAM14 function made it difficult to gain insight into MRG15 function based on its association with this novel protein. We therefore attempted to identify additional MRG15-associated proteins with more established cellular functions. To this end, we performed GST pull-down assays of EJ cell nuclear extracts using MRG15-GST to identify MRG15-associated cellular factors. MRG15-GST-bound proteins were resolved by SDS-PAGE and visualized by silver staining, and bands unique to the MRG15-GST pull-down assay not detected in pull-down assays with GST alone were catalogued. As an additional control, the MRG15-GST protein not incubated with lysate was examined simultaneously to identify contaminating bacterial protein bands and MRG15-GST breakdown products. Pull-down assays were performed multiple times under conditions of increasing stringency, and in each of these experiments we consistently observed a MRG15-associated protein with an
approximate molecular mass of 100 kDa (p100) (Fig. 4A). To identify this protein, we obtained antibodies to several known nuclear proteins of the appropriate molecular weight, including Rb (17), p107 (18), and p130 (18) and tested them in immuno-
blots of MRG15-GST pull-down reactions. Using this screening approach, we succeeded in identifying Rb as a second MRG15-
associated cellular factor (Fig. 4B). To confirm the identity of Rb as a MRG15-associated protein, we performed similar GST

**Fig. 2. MRG15 and PAM14 interact in vitro.** A, wild-type MRG15-GST interacts with PAM14-HA. Nuclear extracts of EJ cells transfected with 4 μg of PAM14-HA were subjected to GST pull-down assays with the indicated GST fusion protein and then immunoblotted with HA antibodies. B, schematic of wild-type MRG15, as well as MRG15 deletion mutants lacking either the chromodomain (CHROMO) (–CHR), the helix-loop-helix domain (–HLH), or the leucine zipper domain (–LEU). aa, amino acids; NLS, nuclear localization signal. C, the wild-type and mutant MRG15-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 location of each fusion protein. D, the MRG15 helix-loop-helix and leucine zipper domains are required for interaction with PAM14. GST pull-down assays were performed as described in A using the indicated wild-type or mutant MRG15-GST fusion proteins.
represents the same field stained for 4,6-diamidino-2-phenylindole (DAPI) to visualize nuclei, and examined by confocal microscopy. Both GFP and HA antibodies were used to co-localize with MRG15 in the nuclei of cells. CMV-MJ-HeI1 cells stably expressing PAM14-HA were transfected with MRG15-GFP. Cells were incubated with both GFP and HA antibodies, 4,6-diamidino-2-phenylindole (DAPI) to stain nuclei, and examined by confocal microscopy. Each of the four panels represents the same field stained for 4,6-diamidino-2-phenylindole (DAPI) and merged images (right panel).

Pull-down experiments using lysates from Saos2 cells, which are known to lack functional Rb (Fig. 4B). As expected, we failed to detect the 100-kDa MRG15-associated band in these experiments, further suggesting that this MRG15-interacting protein is Rb.

To determine the region(s) of the MRG15 protein required for the Rb interaction, we utilized the MRG15-GST deletion mutants described above (see Fig. 2C) in GST pull-down assays. Similar to our results with PAM14, the MRG15 deletion mutant lacking the amino-terminal chromodomain bound Rb with similar strength as wild-type MRG15, whereas the MRG15 mutants lacking the helix-loop-helix or leucine zipper domains failed to bind Rb (Fig. 4C). Thus, the helix-loop-helix and leucine zipper regions are important both for the interaction of MRG15 with PAM14 as well as with Rb.

MRG15, PAM14, and Rb Are All Present in a Multiprotein Complex—Our observations that similar MRG15 domains are important for binding both PAM14 and Rb might suggest that these interactions are mutually exclusive. Therefore, to determine whether all three proteins co-exist in a multiprotein complex or whether MRG15 binds only one of the cellular factors at a time, we performed immunoprecipitations from EJ and CMV-MJ-HeI1 cell lines stably expressing PAM14-HA. Upon immunoprecipitation of PAM14-HA from nuclear extracts of these cells, we observed co-immunoprecipitation of both MRG15 and Rb, suggesting that all three proteins were present in a multiprotein complex (Fig. 5, A and B). Similar results were obtained when nuclear extracts from EJ and CMV-MJ-HeI1 clones were immunoprecipitated with an Rb antibody (Fig. 5, A and B), further suggesting that MRG15 is able to simultaneously complex with PAM14 and Rb.

MRG15 Relieves Rb-mediated Transcriptional Repression—We next wanted to examine the functional significance of the MRG15-Rb interaction. Rb has well established roles in proliferation and differentiation through its ability to regulate gene transcription (19). However, Rb is not a classical transcription factor in that it does not possess sequence-specific DNA binding activities. Rather, Rb is recruited to promoters through interactions with transcription factors, such as those of the E2F family. In the case of E2F, Rb functions as a potent negative regulator of transcription in that it complexes with E2F family members to block transcription from E2F-responsive promoters (17). The association of Rb with MRG15 led us to hypothesize that MRG15 might influence the transcriptional regulatory activities of Rb. We therefore examined the effect of MRG15 on the well characterized E2F-responsive B-myb promoter. During G1 growth arrest, transcription of the B-myb gene is repressed upon sequestration of E2F into inactive complexes by Rb (13–15). To examine the effect of MRG15 on Rb-mediated repression of the B-myb promoter, EJ cells were co-transfected with a luciferase reporter construct driven by the B-myb promoter and increasing amounts of a MRG15 plasmid. We observed a statistically significant increase (3–5-fold, p < 0.01) in luciferase activity in a dose-responsive manner in...
the presence of 0.5 and 1.0 μg of MRG15 (Fig. 6A). This effect was specific to wild-type MRG15 as a MRG15 deletion mutant lacking the leucine zipper motif failed to stimulate B-myb promoter activity (Fig. 6A). To verify that the observed increase in B-myb promoter activity was due to a counteraction of Rb-mediated repression rather than a general MRG15 transcriptional activation function (i.e. providing nonspecific access to histone acetylases) we performed similar luciferase reporter assays with a mutant B-myb reporter construct lacking E2F binding sites. Although this mutant has higher basal B-myb promoter activity, this activity was not further increased upon introduction of MRG15 (Fig. 6B, p > 0.04), suggesting that MRG15 is not a general transcriptional activator. In addition, we failed to detect activation of the B-myb promoter by MRG15 in Rb−/− Saos2 cells (Fig. 6C), further suggesting that the activation of the B-myb promoter by MRG15 is mediated through an inhibition of transcriptional repression by Rb. As expected, the levels of wild-type and mutant B-myb promoter activity in Saos2 cells are similar (Fig. 6C), presumably due to a lack of Rb-mediated repression of the wild-type B-myb promoter. Collectively, these observations suggest that the interaction of MRG15 with Rb can result in the inhibition of Rb-mediated B-myb transcriptional repression.

**DISCUSSION**

The recent discovery of the cell senescence-inducing protein MORF4 has led to the subsequent identification of several MORF4-related genes, although gene expression has only been verified for two other members of this family, MRG15 and MRGX. Interestingly, although both MRG15 and MRGX exhibit a high degree of sequence similarity to MORF4, neither of these genes exhibit the senescence-inducing activity characterized for MORF4. However, the possibility remains that these MRG family members function in related aspects of cellular proliferation. Consistent with this notion is the observation that members of this family possess several putative protein interaction motifs commonly found in proteins with roles in transcriptional regulation. MRG15 represents a particularly interesting member of this family, both because it is widely expressed, even during embryonic development, and because it is conserved across multiple species including vertebrates, insects, yeast, and plants (2). Therefore, to gain functional insight into MRG15, we examined interactions of this protein with additional cellular factors. We report here the identification of two MRG15-associated cellular proteins, the retinoblastoma tumor suppressor Rb and the novel protein PAM14. Significantly we demonstrate that all three proteins exist in a multiprotein complex in cells and, furthermore, that MRG15 can relieve Rb-mediated transcriptional repression. From these data, we propose a model whereby the interaction of MRG15 with Rb and possibly PAM14 negatively regulates Rb-induced E2F-responsive promoter repression and, as a result, may facilitate cell cycle progression.

The related proteins Rb, p107, and p130 are all members of the pocket protein family of transcriptional repressors named for their pocket-like protein interaction domain that typically binds LXXCXE motifs of target proteins. We found that MRG15 specifically interacts with Rb but fails to bind either p107 or p130. This observation, coupled with the fact that MRG15 lacks the canonical LXXCXE motif, suggests that the interaction between MRG15 and Rb does not occur through the pocket domain common to this family of proteins. The fact that PAM14 is predicted to possess extensive helical structure and that several domains of Rb are similarly predicted to form helices, suggests that MRG15 may target helical domains of these proteins. Both the helix-loop-helix and leucine zipper domains of MRG15, which are important for PAM14 and Rb binding, are similarly predicted to have a helical structure (3). It is of note that many transcription factors, such as the BZip proteins, likewise contain coiled-coil domains that mediate specific interaction with their partners (20).

Although Rb is represented in the human fibroblast cDNA library analyzed in the yeast two-hybrid screen, we failed to detect its interaction with MRG15 in this system. One possible explanation is that more transformants need to be analyzed to detect the Rb association in this assay. Alternatively the interaction between MRG15 and Rb may be mediated through another protein(s) not present in the library. Furthermore, MRG15 and/or Rb may require specific post-translational modifications, which may not occur in yeast, to promote their interaction. In this regard, both MRG15 and Rb are known to contain multiple phosphorylation sites that may regulate specific protein interactions. In particular, Rb is phosphorylated and thereby inactivated by multiple cyclin-dependent kinases during cell cycle progression (21). One possibility is that Rb phosphorylation may promote its interaction with MRG15, perhaps as an additional negative regulator of Rb function during the cell cycle.

Our observation that MRG15 de-represses the E2F-responsive B-myb promoter suggests that MRG15 may function to antagonize the transcriptional repressive functions of Rb. Although the specific E2F binding site(s) on Rb have yet to be elucidated, the fact that E2F lacks the LXXCXE motif suggests that the interaction does not involve the Rb pocket domain (22). As MRG15 likewise lacks the LXXCXE motif, one possibility is that it shares a similar Rb binding site with E2F. In this scenario, the interaction of MRG15 with Rb could be envisioned to displace E2F from Rb, thereby facilitating activation of E2F-responsive promoters.

2 J. Campisi, personal communication.
With regard to possible mechanisms of MRG15 function, the fact that this protein was not a self-activator in the yeast two-hybrid assay but was able to activate the B-myb promoter may suggest that the transcriptional activation functions of MRG15 are not direct but rather mediated through interactions with specific cellular factors. This idea is further substantiated by both the failure of MRG15 to activate either the wild-type B-myb (wt myb) promoter or a mutant B-myb (mut myb) promoter lacking the E2F-responsive sites either alone or together with 1.0 µg of a plasmid expressing MRG15, and luciferase activity in each transfection was determined as described in A. The data are compiled from three independent experiments, each performed in triplicate. B, MRG15 fails to activate a mutant B-myb promoter lacking E2F-responsive sites. EJ cells were transfected with 0.5 µg of a luciferase-expressing plasmid under the control of either the wild-type B-myb (wt myb) promoter or a mutant B-myb (mut myb) promoter lacking the E2F-responsive sites either alone or together with 0.25–1.0 µg of a plasmid expressing MRG15, and luciferase activity in each transfection was determined as described in A. The data are from one representative experiment, which was performed in triplicate.

With regard to possible mechanisms of MRG15 function, the fact that this protein was not a self-activator in the yeast two-hybrid assay but was able to activate the B-myb promoter may suggest that the transcriptional activation functions of MRG15 are not direct but rather mediated through interactions with specific cellular factors. This idea is further substantiated by both the failure of MRG15 to activate either a mutant B-myb promoter lacking E2F binding sites and by the inability of the MRG15 leucine zipper deletion mutant, which is impaired for Rb and PAM14 binding, to de-repress the wild-type B-myb promoter in Rb−/− Saos2 cells. Saos2 cells were transfected with 0.5 µg of a luciferase-expressing plasmid under the control of either the wild-type B-myb promoter or a mutant B-myb promoter lacking the E2F-responsive sites either alone or together with 0.25–1.0 µg of a plasmid expressing wild-type MRG15. Luciferase activity in each transfection was determined as described in A. The data are from one representative experiment, which was performed in triplicate.

The ability of MRG15 to increase B-myb promoter activation has implications for a potential role of MRG15 in cell cycle progression. The B-myb protein is an established transcriptional activator believed to promote cell cycle progression through stimulation of multiple effectors of cell growth, including Cyclin D1 and cdc2 (9, 23). Furthermore, overexpression of B-myb has been demonstrated to promote bypass of p53-induced G1 arrest (10), suggesting that B-myb also induces cell growth through inhibition of growth inhibitory molecules. B-myb expression correlates with cell cycle progression in that it is repressed during G0 and early G1 phases but stimulated in late G1 and S phases. Significantly this mRNA expression pattern is similar to that of MRG15; we have observed an approximately 2-fold increase in MRG15 mRNA levels at 4–8 h post-serum stimulation of normal quiescent fibroblasts, and these elevated mRNA levels persist up to 28 h poststimulation.
Young fibroblast cells 4–8 h poststimulation are in the early to mid-G1 phase of the cell cycle and enter S phase at 16 h postexposure to serum with maximal DNA synthesis at 24 h poststimulation. Thus, like B-myb, MRG15 expression correlates with cell cycle progression, and it therefore represents a putative growth-stimulatory factor. It is tempting to speculate that such potential proliferative functions of MRG15 are due to its ability to antagonize one or more activities of Rb, resulting in enhanced B-myb expression. Interestingly the PAM14 sequence has been deposited in the data base as PGR1 (GenBankTM accession number AF116272), a protein induced in quiescent lymphocytes following stimulation to enter the cell cycle.3 Thus, the ability to interact with PAM14 may also play an important role in the putative growth-stimulatory activities of MRG15.

The facts that MRG15 is expressed in a wide variety of tissues and is conserved across a large number of species suggest an essential function for this cellular factor. Our data showing that MRG15 forms multiprotein complexes within the nucleus that may antagonize Rb function further support this contention. Future studies aimed at examining the effect of MRG15 on additional Rb/E2F-repressed promoters and identifying additional cellular factors present within MRG15 complexes are therefore expected to provide critical insight into the role of this protein in the cell cycle and possibly other processes.

Acknowledgment—We thank N. Dyson for the B-myb promoter-reporter constructs.

REFERENCES

1. Smith, J. R., and Pereira-Smith, O. M. (1996) *Science* **273**, 63–67
2. Bertram, M. J., and Pereira-Smith, O. M. (2001) *Gene (Amst.)* **266**, 111–121
3. Bone, J. R., and Kuroda, M. I. (1996) *Genetics* **144**, 705–713
4. Kennison, J. A. (1995) *Annu. Rev. Genet.* **29**, 289–303
5. Koomin, E. V., Zhou, S., and Lacchese, J. C. (1995) *Nucleic Acids Res.* **23**, 4229–4233
6. Jones, D. O., Cowell, I. G., and Singh, P. B. (2000) *Bioessays* **22**, 124–137
7. Lorentz, A., Ostermann, K., Fleck, O., and Schmidt, H. (1994) *Gene (Amst.)* **143**, 139–143
8. Bashaw, G. J., and Baker, B. S. (1996) *Curr. Opin. Genet. Dev.* **6**, 496–501
9. Sala, A., and Calabretta, B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10415–10419
10. Lin, D., Fiscella, M., O’Connor, P. M., Jackman, J., Chen, M., Luo, L. L., Sala, A., Travali, S., Appella, E., and Mercer, W. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 10079–10083
11. Arzura, M., Introna, M., Passerini, F., Mantovani, A., and Golay, J. (1992) *Blood* **79**, 2708–2716
12. Raschella, G., Negroni, A., Sala, A., Pucci, S., Romeo, A., and Calabretta, B. (1999) *J. Biol. Chem.* **274**, 8540–8545
13. Lam, E. W., and Watson, R. J. (1993) *EMBO J.* **12**, 2705–2713
14. Hurford, R. K., Jr., Cobrinik, D., Lee, M. H., and Dyson, N. (1997) *Genes Dev.* **11**, 1447–1463
15. He, S., Cook, B. L., Deverman, B. E., Weihe, U., Zhang, F., Prachand, V., Zheng, J., and Weintraub, S. J. (2000) *Mol. Cell. Biol.* **20**, 363–371
16. Schiestl, R. H., and Gietz, R. D. (1989) *Curr. Genet.* **16**, 339–346
17. Harbour, J. W., and Dean, D. C. (2000) *Genes Dev.* **14**, 2395–2409
18. Clasen, M., and Dyson, N. (2001) *Exp. Cell Res.* **264**, 135–147
19. Zheng, L., and Lee, W. H. (2001) *Exp. Cell Res.* **264**, 2–18
20. Micklethyer, C., and Chmielewski, J. (1999) *Curr. Opin. Chem. Biol.* **3**, 724–729
21. Sherr, C. J. (1996) *Science* **274**, 1672–1677
22. Dahiya, A., Gavin, M. R., Luo, R. X., and Dean, D. C. (2000) *Mol. Cell. Biol.* **20**, 6799–6805
23. Sala, A., and Watson, R. (1999) *J. Cell. Physiol.* **176**, 245–250

---

3 J. K. Leung, N. Berube, S. Venable, S. Ahmed, N. Timchenko, and O. M. Pereira-Smith, unpublished data.