MRG1 Expression in Fibroblasts Is Regulated by Sp1/Sp3 and an Ets Transcription Factor*

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MRG1 (melanocyte-specific gene 1 (MSG1)-related gene), a ubiquitously expressed transcription factor that interacts with p300/CBP, TATA-binding protein and Lhx2, is the founding member of a new family of transcription factors. Initial characterization of this newly discovered transcription factor has underscored its potential involvement in many important cellular processes through transcriptional modulation. We previously demonstrated that MRG1 can be induced by various biological stimuli (Sun, H. B., Zhu, Y. X., Yin, T., Sledge, G., and Yang, Y. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13555–13560). As a first step in understanding its role in different biological processes, we investigated mechanisms that regulate transcription of the mouse MRG1 gene in fibroblasts. Transient transfection of Rat1 fibroblast cells with sequential 5′-deletions of mouse MRG1 promoter-luciferase fusion constructs indicated that the −104 to +121 region contains the full promoter activity. Deletion and site-directed mutations within this region revealed that the Ets-1 site at −97 to −94 and the Sp1 site at −51 to −46 are critical for MRG1 expression in fibroblasts. Gel mobility shift and supershift assays performed with Rat1 nuclear extracts identified nucleoprotein complexes binding to the Ets-1 site and the Sp1 site. In Drosophila SL2 cells, which lack the Sp and Ets family of transcription factors, expression of Sp1, Sp3, and Ets-1 or Elf-1 functionally stimulated MRG1 promoter activity in a synergistic manner. These results suggest that multiple transcription factors acting in synergy are responsible for MRG1 expression and the responsiveness of cells to different biological stimuli.

MRG1† and MSG1 are the members of a new family of transcription factors, which share a conserved C-terminal acidic domain (the CR2 domain) that accounts for their transcriptional activity. Through CR2 (1–4), MRG1 and MSG1 interact with the CH1 domain of the p300/cAMP response element-binding protein (CBP) complex, nuclear proteins that function as coactivators for basal transcription complexes (5–7). MRG1 also interacts with a LIM homeodomain transcription factor, Lhx2, to enhance Lhx2-dependent transcription possibly through recruitment of p300/CBP and TATA-binding proteins (8). The interaction of MRG1 with Lhx2 is dependent on the N-terminal region of MRG1, a region not conserved in MSG1. MSG1 interacts with Smad4 and enhances Smad-mediated transcription in a p300/CBP-dependent manner (4, 9). The Smad interaction domain resides in the N terminus of MSG1 and thus is unique to MSG1. Based upon these findings, it has been speculated that MRG1 family proteins bind p300/CBP through their conserved C-terminal regions (the CR2 region) while interacting with DNA-binding proteins through the unique N-terminal regions, thus regulating the p300/CBP-dependent transcriptional activation of different target genes (4).

Besides their structural differences, MRG1 and MSG1 show a distinct pattern of expression that may also determine their functional specificity (1–3, 10). MSG1 transcripts are predominantly expressed in cultured human and mouse epidermal melanocytes, whereas MRG1 transcripts are detected in all of the human and mouse cell lines and adult tissues examined (1–3, 10). Similarly, MRG1 and MSG1 expression profiles during early development are distinct from each other (2). MSG1 is predominantly expressed in a subset of mesoderm derivatives, whereas MRG1 transcripts are restricted to anterior visceral endoderm prior to gastrulation. Interestingly, MRG1 is expressed during heart development and its expression can be detected throughout embryogenesis from 8.5 days post-conception, which is consistent with the ubiquitous expression of this gene in adult tissues. These results suggested that MRG1 and MSG1 might play different roles during mouse embryogenesis.

MRG1 was cloned in our laboratory as part of an effort to isolate cytokine-inducible genes. Overexpression in Rat1 cells results in loss of cell contact inhibition, anchorage-independent growth in soft agar, and tumor formation in nude mice, suggesting that MRG1 is a transforming gene (10). Bhattacharya et al. demonstrated that MRG1 is up-regulated by hypoxia and dexamethasone (3), and represses HIF-1α-mediated transactivation through competitive interaction with the CH1 domain of p300/CBP. Elevated expression of MRG1 in hypoxic cells has been postulated to negatively regulate the cellular response to hypoxia, which is cytokine-regulated.

The promoter region of human MRG1 has been reported but not analyzed in detail (11), and the transcription factors that regulate it have not been identified. We have now isolated the mouse MRG1 genomic sequence and analyzed the promoter activity by deletion mapping and transient expression in cells. The basal mouse MRG1 promoter maps to region to −104 to +121, which is highly conserved in the human and mouse genes. Deletion analysis in conjunction with site-directed mutagenesis shows that an Ets-1 site at −97 to −94 and an Sp1

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‡ The abbreviations used are: MRG1, MSG1-related gene; MSG1, melanocyte-specific gene 1; STAT3, signal transducers and activators of transcription 3; CBP, cAMP response element-binding protein; EMSA, electrophoretic mobility shift assay; SL2, Schneider’s Drosophila cell line 2; kb, kilobase(s); PCR, polymerase chain reaction; SIE, sis-interacting element; CMV, cytomegalovirus; MAPK, mitogen-activated protein kinase; GABP, GA-binding protein.
site at -51 to -46 are critical for MRG1 promoter activity. Electrophoretic mobility shift assays (EMSA) show that Sp1 and Sp3 bind the Sp1 element, whereas transcription factors binding to the Ets-1 element are yet to be identified. Co-transfection in insect cells that lack Sp and Ets factors demonstrates that Sp1, Sp3, Ets-1, or Elf-1 are important for MRG1 gene expression and therefore for the responses of cells to different biological stimuli.

**EXPERIMENTAL PROCEDURES**

*Cell Lines and Reagents—*NIH3T3, Rat1, and HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Schneider’s *Drosophila* cell line 2 (SL2) was maintained in Schneider’s *Drosophila* medium supplemented with 10% fetal bovine serum at room temperature with atmospheric CO2. Cell culture reagents were obtained from Life Technologies Inc. (Gaithersburg, MD). Antibodies to nuclear proteins Sp1, Sp3, and STAT3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

*Isolation of Mouse Genomic DNA Clone—*A 129/SvJ mouse liver genomic library in Lambda FIXII (Stratagene) was screened using the 2.1-kb MRG1 cDNA as the probe. The probe was random-labeled with [γ-32P]dCTP using the Prime-It kit (Stratagene). A 5-kb HindIII fragment from one of the positive clones was subcloned into the HindIII site of a cloning vector, pUC19, resulting in plasmid pUCHi. Sequence analysis showed that the fragment contains a 2.7-kb 5'-flanking region, the first and the second exons, the introns, and most of the third exon. To identify putative cis-regulatory elements, 1 kb of the mouse MRG1 gene 5'-flanking region was analyzed with the Mat Inspector program (12). The transcription start site was predicted by the neural network promoter prediction (NNPP) program (13).

*Plasmid Construction—*The 1036 to +121 region relative to the transcription start site was PCR-amplified using primers: P-1036, 5'-CCCAAGCTTCCCAACCCGTCAGGCAAGA; and P+121, 5'-GGGGGATCCGTTTCGAGGGCCAGGTCTT. The PCR product was digested with HindIII and Kpn1 and inserted into a promoterless luciferase reporter vector (PRL-CMV/AUG), resulting in plasmid pXP-104. The transcription start site was predicted by Mat Inspector and therefore for the responses of cells to different factors, 48 h after transfection, cell extracts were prepared, and luciferase activity was determined with Luciferase assay systems (Promega). The transcription start site was predicted by the neural network promoter prediction (NNPP) program (13). The transcription start site was predicted by the neural network promoter prediction (NNPP) program (13).

*Electrophoretic mobility shift assays (EMSA)—*These were performed as described previously (15). The medium was changed 24 h after transfection. After another 24 h, cell extracts were prepared and luciferase and β-galactosidase assays were performed as described above.

For transfection of SL2 cells, 1 day prior to transfection, cells were plated onto 6-well plates at 2 x 106 cells/well and transfected by the calcium phosphate method as described (16). Each well received 10 μg of DNA, including 5 μg of indicated luciferase reporter construct and varying amounts of expression plasmid such as pPacSp1 (17), pPacUSp3 (16), pPacUETS-1, or pPacUET-18 (18). Variable amounts of the expression plasmids were adjusted with the control plasmid pPacO. The medium was changed 24 h after addition of DNA, and the cells were harvested for luciferase assays 48 h after transfection. Luciferase values were normalized against total protein concentrations determined by protein assay (Bio-Rad).

*Nuclear Extracts and EMSA—*Nuclear extracts from Rat1 cells were prepared as described (19). Synthesized double-stranded oligonucleotides were end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. Approximately 0.2 ng of the labeled oligonucleotide (20,000 cpm) was added to 10 μg of nuclear extracts in a final volume of 20 μl containing 1 μg of poly(dI-dC), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl2, 4% glycerol, and 1 mM dithiothreitol and incubated for 15 min at room temperature. Subsequently, the DNA-protein complexes were separated from the free probe by electrophoresis through a 5% non-denaturing polyacrylamide gel. Gels were dried and subjected to autoradiography in the presence of intensifying screens (DuPont) at -80°C. For supershift analysis, 1 μl of antibody was incubated with nuclear extracts at 4°C for 1 h in binding buffer, followed by an additional incubation for 30 min at room temperature with labeled oligonucleotides. For competition analysis, unlabeled DNA was incubated with nuclear extracts at 4°C for 20 min before the addition of the labeled probe.

The oligonucleotides used as EMSA probes were annealed prior to labeling. The sequences of the unique strands of the oligonucleotides were used as follows: MRG1 Sp1 (−56/−42), 5'-TAAAAGCTTGCGGCTCGCCCTCTTC; MRG1 Ets1 (−101/−85), 5'-GATTGCCTGGCAGCTCTT. The transcription start site was predicted by the neural network promoter prediction (NNPP) program (13). The transcription start site was predicted by the neural network promoter prediction (NNPP) program (13). The transcription start site was predicted according to the neural network promoter prediction (NNPP) program (13). The transcription start site was predicted according to the neural network promoter prediction (NNPP) program (13). The transcription start site was predicted according to the neural network promoter prediction (NNPP) program (13). The transcription start site was predicted according to the neural network promoter prediction (NNPP) program (13). The transcription start site was predicted according to the neural network promoter prediction (NNPP) program (13). The transcription start site was predicted according to the neural network promoter prediction (NNPP) program (13).

**RESULTS**

*Isolation and Characterization of Mouse MRG1 Genomic Sequence—*To understand the molecular basis for the ubiquitous expression of MRG1, we analyzed its promoter activity. A 5-kb HindIII fragment from a mouse MRG1 genomic clone, which contains 2.7 kb upstream sequence from the transcription start site and all of the coding regions, was subcloned into the pUC19 vector. Similar to the human MRG1 gene (11), the mouse gene consists of three exons, separated by two small introns (data not shown). The transcription start site was predicted according to the neural network promoter prediction (NNPP) program (13). The transcription start site was predicted according to the neural network promoter prediction (NNPP) program (13).

Potential regulatory elements have been identified through the Mat Inspector program (12). These include C/EBP sites at −998 and −758, Sp1 sites at −748, −698, −648, −348, −178, and −51; AP4 site at 718; AP2 site at −218; Ets binding sites at −278, −97, and −44; MZF-1 site at −65; and an E-box at −148 (Fig. 1A) (the corresponding GenBank® accession number for mouse MRG1 gene is AF295547). Extensive identity exists between mouse and human proximal promoter regions (Fig. 1B), introns and exons (data not shown), indicating that the MRG1 gene is well conserved during evolution. As shown in Fig. 1B, there is an overall 80% identity between mouse and human MRG1 genes in the proximal promoter region between −190 and −121. Sequences further upstream are more diverse, suggesting −190 to −121 may play an important role in MRG1 expression.

*Identification of Transcription Elements Responsible for MRG1 Expression—*To assess the promoter activity of the MRG1 5'-flanking region, deletion mutants were generated using the firefly luciferase reporter vector, pXP2 (14). Each
Characterization of MRG1 Promoter

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**Characterization of MRG1 Promoter**

| Characteristic | Mouse MRG1 Gene | Rat1 Cell Transfection | HepG2 Cell Transfection | NIH3T3 Cell Transfection |
|----------------|-----------------|------------------------|-------------------------|-------------------------|
| Promoter      | -166 to +2750   | -166 to +2750          | -166 to +2750           | -166 to +2750           |
| Activity      | -104 to -44     | -104 to -44            | -104 to -44             | -104 to -44             |
| Ets-1 Element | TTCGCCC         | TTCGCCC                | TTCGCCC                 | TTCGCCC                 |
| Sp1 Element   | GGGCGCC         | GGGCGCC                | GGGCGCC                 | GGGCGCC                 |
| MZF-1 Element | GGGGCC          | GGGGCC                 | GGGGCC                  | GGGGCC                  |
| STAT3 Element | GGGATC          | GGGATC                 | GGGATC                  | GGGATC                  |
| Promoter Activity | -104% to +121% | -104% to +121%        | -104% to +121%         | -104% to +121%         |

**Promoter Activity of Serial Deletion Constructs of the Mouse MRG1 Gene**

Rat1 cells were transiently cotransfected with 1.0 \( \mu \)g of sequentially deleted reporter constructs and 0.25 \( \mu \)g of pCMV\( \beta \) construct. Luciferase results were normalized by \( \beta \)-galactosidase activity. The experiments were done in triplicates. Data are the mean + S.D. \( P \) represents the promoterless luciferase vector pXP2. In A, promoter activities of eight deletion constructs are shown. The promoter activity of pXP-2700/+121 represents 100, and activities of other constructs are compared with that of pXP-2700/+121. In B, promoter activities of another three deletion constructs and a site-directed mutant are shown. The promoter activity of pXP-104/+121 represents 100.

**Identification of Transcription Factors Binding to Sp1 or Ets-1 Elements in MRG1 Promoter**

-To determine the biochemical composition of protein complexes binding to the proximal Sp1 or Ets-1 element of the MRG1 promoter, nuclear extracts from Rat1 fibroblast cells were analyzed by EMSA using a labeled Sp1 or Ets-1 oligonucleotide probe. As illustrated in Fig. 4A, three DNA-protein complexes (A, B, and C with arrows) were detected using labeled Sp1 probe (lane 2). These DNA-protein complexes were specific, because they disappeared in the presence of excess amounts of unlabeled Sp1 competitors (lanes 3 and 4), and inclusion of an excess amount of unlabeled mutated Sp1 oligonucleotide did not affect the formation of these complexes (lane 5). Because members of the Sp1 multigene family share the same binding consensus sequence (GC or GT box) (21), we performed supershift experiments to determine the identity of the three complexes. As shown in Fig. 4B, anti-Sp1 antibody inhibited complex A formation (lane 2), whereas complexes B and C were supershifted in the presence of anti-Sp3 antibody (lane 3). In the presence of both antibodies, complexes B and C were supershifted and the intensity of complex A decreased (lane 4). Anti-STAT3 was included as a negative control and was found not to affect the level of pXP-44/+121 (Fig. 2B). It should also be noted that deletion of the region between –92 and –57 also resulted in a significant decrease in the promoter activity. This region contains an MZF-1 binding site (GGGA sequence at –66 to –62). These results demonstrated that the Ets-1 site, Sp1 site, and/or Sp1 motif CCGCCC were important for the mouse MRG1 promoter activity.

**Contribution of Ets-1 and Sp1 Sites to the Proximal Promoter Activity of Mouse MRG1 Gene**

To further define the specific elements in the proximal –104 region that contribute to the proximal promoter activity, we generated a series of MRG1 promoter constructs with mutations in Ets-1 and Sp1 elements from pXP-104/+121. Ets-1 motif TTCC was mutated to TTAA, whereas Sp1 motif GGCGCC was mutated to TTGGCC. Upon transfection into Rat1 cells, constructs containing a specific mutation of Sp1 or Ets-1 element resulted in about 90 and 50% decreases in the promoter activity, respectively (Fig. 3). The results were similar in the NIH3T3 fibroblast cell line. In HepG2, disruption of the Sp1 site decreased 75% of the promoter activity. In all cases, mutations of both sites resulted in a 90–98% loss of the proximal promoter activity (Fig. 3), demonstrating that both Ets-1 and Sp1 elements are important for the MRG1 proximal promoter activity.
mobility of these complexes (lane 5). The gel shift pattern was similar when performed using nuclear extracts from NIH3T3 cells (data not shown). These results indicated that Sp1 is a component of complex A and complexes B and C contain Sp3, a typical gel shift pattern as previously described for the Sp1 site (21).

The potential interaction of the Ets-1 element with nuclear proteins was also evaluated by EMSA (Fig. 4C). Transcription factors in the Ets family recognize the sequence (A/G)(T/A)(G/C) with a similar structure in the DNA binding domain. The TCC sequence is absolutely essential for Ets family factors in the Ets-1, Ets-2, PU.1, Erg-1, Fli-1, and Elk-1) did not change the gel shift pattern (data not shown). The exact identity of proteins in each of the DNA binding sites was determined by surrounding nucleotides (22, 23). The −104/+121 region contains two Ets core consensus sequences: one at −97 to −94 (most closely matched Ets-1 recognition sequence) and the other one at −45 to −42. With MRG1 Ets-1 element (−101/−85) as a probe, three complexes were formed (lane 1) and could be competed by unlabelled −101/−85 oligonucleotide (lane 2), suggesting they are specific. Conversely, unlabelled oligonucleotide bearing a mutated Ets-1 element (TTCG mutated to TTAA) was unable to compete for binding (lane 3), indicating the core TTCG motif is critical for the formation of DNA-protein complexes. However, the unlabelled sre-interacting element (SIE) oligonucleotide (20), containing a core TTCG motif, could not compete for the complex formation (lane 6), suggesting that flanking sequences are also important for the formation of DNA-protein complexes. Interestingly, the other TTCG-containing oligonucleotide from the MRG1 promoter (−56 to −34) could compete for the formation of complex A but not complexes B and C. Mutations of the TTCG motif in region −56 to −34 did not significantly affect the promoter activity in Rat1 cells (data not shown). Most importantly, the addition of a 100-fold excess of unlabelled Ets/PEA3 binding oligonucleotide (lane 5) inhibited the formation of all three complexes almost as effectively as did MRG1−101/−85 oligonucleotide. These results strongly suggested that −101/−85 contains an Ets-1 binding site. However, addition of monoclonal antibodies directed against various members of Ets factor family (Ets-1, Ets-2, PU.1, Erg-1, Fli-1, and Elk-1) did not change the gel shift pattern (data not shown).

Fig. 3. Effect of site-specific mutations on mouse MRG1 proximal promoter activity. Site-specific mutations were performed on pXP-104/+121 (WT). Rat1 or NIH3T3 cells were cotransfected with 1.0 μg of MRG1 promoter construct and 0.25 μg of pCMVβ. HepG2 cells were cotransfected with 0.6 μg of MRG1 promoter construct and 0.2 μg of pCMVβ. Luciferase activity was normalized against β-galactosidase activity. The activity of the WT construct represents 100, and activities of mutated constructs were compared with the activity of WT. The solid circles represent a wild type sequence, whereas the circles with a cross represent a mutated sequence. Experiments were performed in triplicates. Shown are mean ± S.D.

To directly determine whether Sp1- and Ets-related Proteins—

Cooperative Activation of Mouse MRG1 Promoter by Sp1- and Ets-related Proteins—To directly determine whether Sp1- and Ets-related proteins could functionally modulate MRG1 promoter activity, Drosophila SL2 cells, which are deficient in functional Sp1, Ets-1 or Elf-1 alone could increase luciferase activity of pXP-104/+121, with Ets-1 being more potent than Elf-1 (Fig. 5D). They had no effect on the Ets-1 transactivation on pXP-104mEts-1, suggesting the transactivation by Ets-1 or Elf-1 is dependent on the integrity of the Ets-1 element. Consistent with these results, six copies of the −101/−85 region have been shown to enhance the activity of a heterologous TK promoter in various cell lines and to mediate the Ets-1 or Elf-1 transactivation in SL2 cells (data not shown). Finally, to test the possible functional interplay between the Sp1- and Ets-related proteins, we performed cotransfection experiments using pXP-104/+121 or pXP-104mEts-1, together with plasmds expressing different transcription factors. As shown in Fig. 5D, pPacUSp1 (1 μg) stimulated promoter activity by about 11-fold, whereas coexpression of Sp1 with Ets-1 stimulated pXP-104/+121 by about 35-fold. Cotransfection of Elf-1 with Sp1 also resulted in a cooperative functional interaction on MRG1 promoter. This synergy was dependent upon the integrity of the −101/−85 region and was not observed on mutant promoter construct pXP-104mEts-1. These results demonstrated synergistic activation of the MRG1 promoter by Sp1 and Ets transcription factors.

DISCUSSION

MRG1 represents the founding member of a new family of transcription factors that may be involved in many physiological processes through its interactions with p300/CBP, TATA binding proteins and certain specific DNA-binding transcription factors (1–4, 8–10). Sequence alignment of mouse and human (11) MRG1 genes showed extensive identity in proximal promoter regions (Fig. 1B), exons and introns, implicating high conservation of MRG1 during evolution. Sequences upstream
Characterization of MRG1 Promoter

Fig. 5. Sp1- and Ets-related proteins activate mouse MRG1 promoter activity in Drosophila SL2 cells. A, Sp1 or Sp3 alone activates MRG1 promoter. SL2 cells were cotransfected with 5.0 μg of pXP-104/mSp1 with varying amounts (0.25–5.0 μg) of expression plasmids for Sp1 and Sp3. Cells were incubated with calcium phosphate-precipitated DNA for 24 h, then changed to fresh medium for another 24 h. The luciferase value was normalized against protein concentration. Fold induction represents the -fold increase in the luciferase activity against vector pPacO-cotransfected control. Data shown are representative of three experiments. B, Sp3 enhances Sp1-mediated transactivation of the MRG1 promoter. SL2 cells were transfected with pXP-104/mSp1 with 1.0 μg of pPacSp3 and varying amounts of pPacUSp3 (0–4.0 μg). Data are the mean ± S.D. from three experiments. C, Sp1/Sp3 activation of the MRG1 promoter is through the Sp1 site. SL2 cells were transfected with pXP-104/mSp1 or pXP-104mSp1 with 1.0 μg of expression plasmids for Sp1, Sp3, or Sp1+Sp3. Data shown are representative of three experiments. D, Sp1- and Ets-related proteins act in synergy in activation of MRG1. 5 μg of pXP-104/mSp1 or pXP-104mEts1 construct was cotransfected into SL2 cells with 1 μg of expression plasmids for Sp1, Ets-1, Sp1+Ets-1, Elf-1, and Elf-1+Sp1. Luciferase assays were performed as described. All the experiments were performed in triplicates. Data shown are mean ± S.D.

MRG1 Ets-1 oligonucleotides (−104/−51): 5′-GTATGATTCCGGGTATCCT-3′
MRG1 mEts-1 oligonucleotides (−104/−51): 5′-GATGTGATGAGATTCG-3′
MRG1 56/34 oligonucleotides: ETS/PEA3 oligonucleotides: SIE oligonucleotide:

Fig. 4. EMSA analysis of nuclear proteins bound to the mouse MRG1 Sp1 binding site. A, gel mobility shift assay for Sp1 site. 10 μg of Rat1 nuclear extract was incubated with Sp1 (−56/−42) probe alone (lane 2), or in the presence of unlabeled Sp1 (lane 3, 50-fold excess; lane 4, 100-fold excess) or mutated Sp1 (lane 5, 100-fold excess) oligonucleotide as competitors. Lane 1 shows the mobility of Sp1 probe alone without nuclear extract. Three Sp1-specific DNA-protein complexes are indicated as A, B, and C. B, gel mobility supershift assay for the Sp1 site. Mouse MRG1 Sp1 probe was incubated with 10 μg of Rat1 nuclear extract either alone (lane 1) or in the presence of anti-Sp1 (lane 2), anti-Sp3 (lane 3), anti-Sp1+anti-Sp3 (lane 4) or anti-STAT3 (lane 5). C, gel mobility shift assay for the Ets-1 site. Gel mobility shift assay was performed using Ets-1 (−101/−85) probe and Rat1 nuclear extract (15 μg) (lane 1). Competition analysis was performed in the presence of 100-fold excess of unlabeled Ets-1 (−101/−85) (lane 2), mEts-1 (−101/−85) (lane 3), MRG1 −56/−34 (lane 4), ETS/PEA3 consensus sequence (lane 5), or SIE oligonucleotide (lane 6). The sequences of the oligonucleotides used are shown at the bottom of the figure. Three Ets1-specific DNA-protein complexes are indicated as A, B, and C.

of −190 are more divergent, suggesting the proximal promoter region might play an important role in MRG1 gene regulation. Consistent with this inference, we mapped the basic promoter region of mouse MRG1 to −104/+121. We further identified two elements, Sp1 element (−51 to −46) and Ets binding site (−97 to −94) within this region to be essential for the promoter activity.

Sp1 is a well-characterized sequence-specific DNA-binding protein that is important for transcription of many cellular and viral genes that contain GC boxes in their promoters (17, 21). Three Sp1-related transcription factors (Sp2, Sp3, and Sp4) have been cloned. Sp2 does not recognize the same sequence as Sp1, and Sp4 expression is restricted to the brain. Sp3, on the other hand, is ubiquitously expressed and recognizes the same sequence as Sp1. Although Sp1 appears to be almost exclusively an activating transcription factor, Sp3 contains a transcriptional repression domain and can act as a transcriptional activator or repressor, depending on the promoter and cell type studied (16, 24). We have shown that Sp1 is part of complex A, whereas complexes B and C contain Sp3 (Fig. 4). In addition, we have demonstrated that both Sp1 and Sp3 transactivate MRG1 promoter in Drosophila SL2 cells. Interestingly, our data also suggested that Sp1 and Sp3 function in a cooperative manner for MRG1 promoter activity (Fig. 5). Sp1 has been shown to associate directly with members of the basal transcription machinery such as TFIIID components and has been viewed as a constitutive transcriptional activator that acts as a basal factor for TATA-less promoters. Ubiquitous and consti-
tutive expression Sp1/Sp3 may account for high basal expression of MRG1 in various cell types and tissues. In addition, despite its general role in transcription of housekeeping genes, Sp1 has been demonstrated to be involved in induced transcription of various genes responding to different biological stimuli (25–29). While Sp1 and Sp3 are ubiquitous nuclear factors, differences in expression level during different stages of development (30, 31) or in various cell types (31) along with specific post-translational modifications (32) are responsible for altering gene transcription in a development-specific and cell-specific manner (33). The correlation of Sp1 and MRG1 expression during development and responses to biological stimuli need to be further investigated.

The common structural feature of Ets family transcription factor is the presence of a unique DNA binding domain that recognizes the core GGAAT motif in a 10-bp DNA sequence (22, 23). Different Ets proteins exhibit low selectivity in binding site preference, in common with many other transcription factor families, including the homeodomain proteins. The residues flanking the GGA motif dictate whether a particular Ets domain will bind the site. This extending family contains proteins involved in cell growth, differentiation, and transformation. We have identified Ets-1 site at −101/−85 and its binding proteins to be essential for MRG1 expression. These binding proteins appear to be relatively ubiquitous, because the Ets-1 element served as an enhancer in HepG2, Rat1, NIH3T3, and HeLa cell lines (data not shown). However, we failed in our initial attempts to identify specific Ets factors involved in complex formation through gel supershift assays. This is consistent with difficulties encountered by others in the identification of specific Ets family members (18), in part due to the contribution of autoinhibitory binding domains present in each of the Ets factors (34–36). Nevertheless, two of the Ets family members, Ets-1 and Elf-1, which have very different expression patterns among tissues and cell lines, could activate transcription through the MRG1 Ets-1 element in SL2 cells (Fig. 5D). These data therefore strongly support the positive role of the MRG1 Ets-1 element in MRG1 expression. Given the fact that most cell types express multiple Ets proteins and these proteins can interact with similar or identical sequences, the existence of this functional Ets-1 site in MRG1 promoter may also account for the ubiquitous nature of MRG1 expression. Furthermore, several Ets family transcription factors have been shown to be nuclear targets of the Ras-Raf-MAPK signaling cascade (37). In many instances, specific phosphorylation of Ets proteins greatly enhances their ability to activate transcription and subsequent cross-talk of ubiquitous signaling cascades. Therefore, the transient up-regulation of MRG1 we reported previously (10) might in part be contributed by the Ras-Raf-MAPK signaling pathway. Further experiments are required to clarify this point.

It is well established that Ets factors bind DNA as monomers (except for GABPβ-gGABPβ complex) but can also associate with other transcription factors bound to their cognate motifs in the vicinity of the Ets binding site (22, 23). For example, Ets-1 and Sp1 interact to activate synergistically the human T-cell lymphotropic virus long terminal repeats (38). In addition, Sp1 activity can be modulated by factors that recognize DNA elements flanking or overlapping a GC box (39, 40). In the context of the mouse MRG1 promoter, the Ets-1 element is located 40 nucleotides upstream of the Sp1 element, which provides a close proximity for an Ets-related factor and Sp1 to interact. In this regard, it is interesting to find that indeed Sp1 and Ets factors synergistically activate the MRG1 proximal promoter in SL2 cells (Fig. 5D).

In summary, we cloned and functionally characterized mouse MRG1 promoter and identified two essential elements in the proximal promoter region. We showed that Sp1, Sp3, and an Ets-related transcription factor act synergistically to activate the mouse MRG1 promoter. The identification of these cis-elements and associated transcription factors provide the first explanation for the ubiquitous expression of MRG1 in cells and the possible mechanism of its up-regulated expression by different biological stimuli.

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