MRG1 Binds to the LIM Domain of Lhx2 and May Function as a Coactivator to Stimulate Glycoprotein Hormone α-Subunit Gene Expression*

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Tissue-specific expression of the α-subunit gene of glycoprotein hormones involves an enhancer element designated the pituitary glycoprotein basal element, which interacts with the LIM homeodomain transcription factor, Lhx2. In the present studies we have explored the function of the LIM domain of Lhx2 in stimulating α-subunit transcription. When fused to the GAL4 DNA-binding domain, the LIM domain of Lhx2 was shown to contain a transcriptional activation domain. Furthermore, in the context of an α-subunit reporter gene in which a GAL4-binding site replaced the pituitary glycoprotein basal element, the LIM domain enhanced both basal and Ras-mediated transcription. In addition, a synergistic response to Ras activation was observed when the Lhx2 LIM domain and the transactivation domain of Elk1 are directed to a minimal reporter gene. A yeast two-hybrid screen identified the recently described melanocyte-specific gene-related gene 1 (MRG1) as an Lhx2 LIM-interacting protein. MRG1 was shown to bind Lhx2 in vitro, and a co-immunoprecipitation assay provided evidence that endogenous MRG1 forms a complex with Lhx2 in αT3-1 cells. Expression of MRG1 in αT3-1 cells enhanced α-subunit reporter gene activity. MRG1 was also shown to bind in vitro to the TATA-binding protein and the transcriptional coactivator, p300. These data suggest a model in which the Lhx2 LIM domain activates transcription through interaction with MRG1 leading to recruitment of p300/CBP and the TATA-binding protein.

Members of the LIM homeodomain family of transcription factors have been shown to contribute to the regulated expression of the α-subunit of the glycoprotein family of hormones (1). The ability of the pituitary to secrete the gonadotropin hormones, follicle-stimulating hormone, and luteinizing hormone is crucial for normal reproductive function. The synthesis and secretion of the gonadotropins involves an enhancer element designated the PGBE of the glycoprotein hormone α-subunit gene (1, 12). Initial studies demonstrated that LIM homeodomain factor-2 (Lhx2, also designated LH-2) can bind to a pituitary-specific enhancer element designated the PGBE of the mouse α-subunit gene (1). Because both the PGBE and a separate, structurally distinct DNA element designated the GNH-RE are required for GnRH responsiveness of the mouse glycoprotein hormone α-subunit promoter (12), the finding that Lhx2 binds to the PGBE implies that this LIM factor plays a role in transcriptional responses to GnRH. It has also been shown that a related LIM factor, Lhx3 (also designated pLIM or LM3) can also enhance α-subunit gene expression (13). Targeted disruption of the Lhx3 gene in mouse results in loss of pituitary organogenesis (14), demonstrating that LIM factors also play an important developmental role in the formation of the pituitary.

The specific role that the LIM domains play in transcriptional activation is somewhat unclear. The LIM domain, named for the genes of the first three members of the family, lin-11 (15), isl-1 (16), and mec-3 (17), is characterized by the presence of two zinc finger motifs that involve cysteine and histidine or aspartate residues that tetrahedrally coordinate a zinc atom (18, 19). There is evidence that some LIM domains can inhibit DNA binding of the associated homeodomain (20–22). This would suggest that the LIM domain may negatively regulate LIM factor activity. However, it is not clear that inhibition of DNA binding is a general phenomenon for LIM factors (23). Functional studies of Xlim-1 in Xenopus laevis have shown that deletion or mutation of the LIM domain of Xlim-1 results in the induction of secondary axis formation, whereas the wild type factor has no effect (24). This has been interpreted as evidence for a negative role for the LIM domain in regulating transcription. However, in the absence of more mechanistic information about Xlim-1 action, other interpretations are possible. In contrast to the view that LIM domains play a negative role in regulating DNA binding and transcription, some LIM factors have been shown to demonstrate synergistic transcriptional activation with other transcription factors (13, 25).

Recently, a putative co-activator was identified independently in several labs that binds to members of the LIM homeodomain protein family and nuclear LIM only proteins (24, 26, 27). This LIM-binding protein has been termed NLI (nuclear LIM interactor), LIM domain-binding factor and cofactor of LIM domain proteins. It has been suggested that NLI may have a positive effect on transcription by relieving the inhibitory effects of the LIM domain in the context of the full-length Xlim-1 in vivo (28). However, NLI has also been shown to inhibit the synergy between the LIM homeodomain factor Lmx-1 and the basic helix-loop-helix transcription factor, E47 (29). Thus, like the LIM domain itself, it is not yet clear
whether NLI plays a positive or negative role in mediating or regulating LIM factor function.

In the present studies we sought to further define the function of the LIM domain of Lhx2 in the transcription of the glycoprotein α-subunit gene. We have shown the LIM domain of Lhx2 is sufficient to activate transcription when directed to the PGBe of the α-promoter. Furthermore, we have identified a LIM-interacting transcriptional activator, MRG1, that is capable of mediating enhanced transcription of the α-promoter.

MATERIALS AND METHODS

Reporter Genes and Expression Constructs—Luciferase reporter genes containing the −507 to −46 region of the mouse glycoprotein hormone α-subunit gene or the −507 to −205 region linked to a minimal promoter have been described previously (12, 30). The PGBe and GnRH-RE mutant α-subunit luciferase reporter genes were constructed by subcloning double-stranded oligonucleotides containing the GAL4-binding site, GGAAGACTCTCCCG, into the NotI restriction site of the previously described block PGBe and GnRH-RE mutant α-subunit promoter constructs (30). The 5× GAL4-binding site luciferase reporter and the GAL4-Elk1 expression constructs have been described previously (31). The mutant GALA-Elk1 S383A expression vector was constructed by oligonucleotide-directed mutagenesis using standard techniques to remove a major MAPK phosphorylation site (32). To prepare GAL4-LIM domain and GAL4-NLI expression constructs, the appropriate coding regions were isolated by polymerase chain amplification and subcloned into the pDS343 vector (gift of C. Ingraham) containing the GAL4 (1–147) DNA-binding domain downstream of the cytomegalovirus promoter (33). For construction of an MRG1 expression vector, the complete coding sequence for MRG1 was isolated by polymerase chain reaction amplification from αT3–1 cell cDNA using primers based on the known sequence (34), and the coding sequence was cloned into pcDNA3. The Lhx2 and MRG1 maltose-binding protein (MBP) fusion proteins were generated by subcloning appropriate fragments into the bacterial expression vector pMAL-c2 (New England Biolabs).

Cell Culture and Transfections—αT3–1 and NS20Y cells were maintained in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.). For transient transfection assays cells were plated in 6-well plates 24 h prior to transfection and then treated with a suspension of 5–10 μl of LipofectAMINE reagent (Life Technologies, Inc.) and 1–3 μg of total DNA in 1 ml of serum-free OPTI-MEMI (Life Technologies, Inc.) according to the manufacturer's recommendations. After a 12–14 h incubation, an equal volume of Dulbecco's modified Eagle's medium containing 20% fetal bovine serum was added to the transfected cells. 6 h later the cells were lysed and assayed for luciferase activity (35). To assess transfection efficiency, cells were transfected with a cytomegalovirus-β-galactosidase reporter gene (36), and β-galactosidase activity was determined and used to normalize luciferase light units.

 Yeast Two-hybrid Screen—To prepare a VP16-αT3–1 cDNA fusion library, duplex cDNA was prepared using RNA prepared from αT3–1 cells and reagents from Amersham Pharmacia Biotech following the manufacturer's recommendations. The cDNA termini were modified by ligation to an adapter containing a NotI restriction site, and then the cDNA was amplified by the polymerase chain reaction. The amplified cDNA was digested with NotI and subcloned into the NotI site downstream of the VP16 activation domain in the yeast two-hybrid library vector, VP16 (37). A modified bait vector, BTMYeA2, which allows expression of both a LexA fusion and a second protein, was used for the two-hybrid LIM domain of Lhx2 (residues 42–182) was isolated by the polymerase chain reaction and subcloned into EcoRI and BamHI sites, which are downstream of the LexA DNA-binding domain of BTMYeA. NLI was cloned downstream of the ADH promoter of the BTMYeA. The strategy of expression of both the LexA-Lhx2 LIM fusion protein and NLI was based on the possible identification of factors that interacted with the NLI complex. However, subsequent analysis of the factors that were isolated in the screen demonstrated that none of the LIM-binding factors were dependent on NLI for binding. The bait and cDNA library plasmids were transformed into the L40 yeast strain (MATα his32300 trp1-901 leu2-3, 112 ade2 lys2::(lexAop)5-His3 URA3::(lexAop)5-lacZ GAL4 gal80) (37) and selected for positive, actively cloning using histidine minus medium in the presence of 30 mg α-aminonitrile per liter. The yeast cells were collected, and nuclei were isolated and extracted as described previously (1). Nuclear extracts were incubated with M2 FLAG antibody immobilized on agarose beads (Kodak) overnight at 4 °C and subsequently washed five times in 1 ml each of 50 mM Tris, pH 7.8, 150 mM NaCl, and 0.5% Nonidet P-40 (Sigma). The immunoprecipitates were resolved on a denaturing 10% polyacrylamide gel, transferred differentially to a semi-dry transfer apparatus following the manufacturer's instructions (Bio-Rad). Lhx2 and Lhx3 were detected using polyclonal antibodies. The Lhx2 antibody was prepared by immunizing rabbits with a glutathione S-transferase fusion to a fragment of the coding sequence of mouse Lhx2. The antibody to mouse Lhx2 was a generous gift of Dr. S. Pafruit (38). MRG1 was detected using a polyclonal antibody to MRG1 at a dilution of 1:1000, a generous gift of T. Shioda. GAL4 fusion proteins were detected using monoclonal antibodies (Santa Cruz). Immunoblots were developed using horseradish peroxidase-conjugated secondary antibodies (Sigma), followed by detection with an enhanced chemiluminescence reagent (REN Life Science Products).

In Vitro Binding Assays—MBF fusion proteins were expressed in E. coli and immobilized on amylase resin as described (39). In vitro binding reactions were performed in 10 μl HEPES, pH 7.4, 150 mM NaCl, and 0.1% Tween-20. Radiolabeled proteins for analysis of binding were generated by in vitro transcription using bacteriophage SP6 or T7 RNA polymerase (40) and translation in a reticulocyte lysate in the presence of [35S]methionine using reagents from Promega. FLAG epitope-tagged p300 was expressed and purified from baculovirus. After incubation of the binding reactions for 2 h at 4 °C, the resin was washed 5 × 1 ml in the binding buffer. Bound proteins were resolved on 10% polyacrylamide denaturing gels and visualized by autoradiography or by Western blotting using the anti-FLAG monoclonal antibody as described above.

RESULTS

Lhx2 and Lhx3 Are Both Present in the αT3–1 Gonadotrope-derived Cell Line, and Both LIM Factors Can Activate the α-Subunit Promoter—Previous studies from this laboratory have provided evidence that Lhx2 can bind to the PGBe of the α-subunit gene and stimulate α-subunit promoter activity (1). The Lhx2 cDNA was isolated from a library prepared from the gonadotrope derived, αT3–1 cell line (41), and this cell line was confirmed to contain Lhx2 mRNA (1). More recently it has been shown that αT3–1 cells also contain Lhx3 mRNA and that Lhx3 can also activate the α-subunit promoter (13). These separate studies raise the possibility that both Lhx2 and Lhx3 may contribute to activation of the α-subunit gene. To further explore this possibility we examined the expression of Lhx2 and Lhx3 in αT3–1 cells at the protein level. Immunoblots demonstrated that both Lhx2 and Lhx3 are indeed present in extracts of αT3–1 cells (Fig. 1A). The ability of these LIM factors to activate the α-subunit promoter in heterologous cells was then compared (Fig. 1B). Expression vectors for Lhx2 and Lhx3 were both able to stimulate expression of an α-subunit reporter gene. Interestingly, deletion of the LIM domain of Lhx2 eliminated activation of the α-subunit reporter gene. Although the role that the LIM domain plays in transcriptional activation has been somewhat controversial, these findings provide evidence that within this specific context, the LIM domain of Lhx2 is necessary for transcriptional activation. The findings that both Lhx2 and Lhx3 are present in αT3–1 cells and that both factors can activate the α-subunit promoter provide evidence that both Lhx2 and Lhx3 may play a role in α-subunit regulation in this cell line. In the present studies we have focused our attention on further characterization of Lhx2. However, it should be noted that both Lhx2 and Lhx3 appear to contribute to transcription of the α-subunit gene.

The LIM Domain of Lhx2 Contains a Transcriptional Activation Domain—The preceding experiments offer evidence 3-aminotriazole.

Immunoprecipitation and Immunoblot Analysis—For immunoprecipitation studies, αT3–1 cells were cultured in 150-mm2 plates and transfected using LipofectAMINE with either pcDNA3 or pcDNA3 directing the expression of FLAG epitope-tagged Lhx2 as described above.

48 h after transfection the cells were collected, and nuclei were isolated and extracted as described previously (1). Nuclear extracts were incubated with M2 FLAG antibody immobilized on agarose beads (Kodak) overnight at 4 °C and subsequently washed five times in 1 ml each of 50 mM Tris, pH 7.8, 150 mM NaCl, and 0.5% Nonidet P-40 (Sigma). The immunoprecipitates were resolved on a denaturing 10% polyacrylamide gel, transferred differentially to a semi-dry transfer apparatus following the manufacturer's instructions (Bio-Rad). Lhx2 and Lhx3 were detected using polyclonal antibodies. The Lhx2 antibody was prepared by immunizing rabbits with a glutathione S-transferase fusion to a fragment of the coding sequence of mouse Lhx2. The antibody to mouse Lhx2 was a generous gift of Dr. S. Pafruit (38). MRG1 was detected using a polyclonal antibody to MRG1 at a dilution of 1:1000, a generous gift of T. Shioda. GAL4 fusion proteins were detected using monoclonal antibodies (Santa Cruz). Immunoblots were developed using horseradish peroxidase-conjugated secondary antibodies (Sigma), followed by detection with an enhanced chemiluminescence reagent (REN Life Science Products).
that the LIM domain of Lhx2 may contain a transcriptional
activation domain. To directly evaluate the transcriptional ac-
tivation potential of the LIM domain of Lhx2, we constructed
expression vectors in which the yeast GAL4 DNA-binding
domain was fused to the LIM domain of Lhx2. The activity of the
GAL4-LIM fusion constructs was assessed by transfection of
αT3–1 cells. Transcription of the α-subunit gene in these cells is
stimulated by GnRH (12) through a pathway that involves
GnRH (12). Immunostaining with antibody to either Lhx2 or Lhx3 as indicated (A). To examine the ability of Lhx2 and Lhx3 to enhance α-subunit promoter activity, NS20Y cells were transfected with 0.2 µg of a reporter con-
struct containing the −507 to +46 region of the mouse α-subunit
promoter (B). The cells were also transfected with 0.2 µg of either an
empty expression vector or expression vector for Lhx2, Lhx3, or the LIM
deletion mutant of Lhx2 as indicated. A cytomegalovirus-β-galactosi-
dase reporter construct (0.5 µg) was transfected to assess differences in transfection efficiency. Data are reported as the relative luciferase
activity from three transfections (± S.E. normalized to β-galactosidase
activity).

![Immunoblot](image1)

**Fig. 1.** Lhx2 and Lhx3 are present in αT3–1 cells, and both LIM
factors can enhance α-subunit reporter gene activity in heter-
ologous cells. Nuclear extracts from αT3–1 cells were resolved by
denaturing gel electrophoresis and transferred to a membrane before
immunostaining with antibody to either Lhx2 or Lhx3 as indicated (A).

To determine whether transcription-stimulating activity of the Lhx2 LIM domain was dependent on the intact structure of the LIM domain, cysteine residues 52 and 55, which are involved in zinc binding (18, 19), were mutated to alanine. This mutation, which presumably disrupts the structure of the LIM domain, was found to substantially decrease
reporter gene activity. All of the GAL4 fusion proteins were expressed at comparable levels (Fig. 2C).

![Diagram](image2)

**Fig. 2.** The LIM domain of Lhx2 contains a transcriptional activation domain. αT3–1 cells were transfected with either 0.2 µg of
a reporter construct containing five copies of a GAL4-binding site up-
stream of the E1b TATA box luciferase reporter (A and B) or an α-sub-
unit reporter gene containing the −507 to −205 region of the mouse
glycoprotein hormone α-subunit gene placed upstream of a minimal
TATA box linked to luciferase (D and E) or a mutant α-subunit reporter
gene in which the PGBE was replaced with a GAL4-binding site (D, F,
and G). The cells were also transfected with 0.2 µg of either an empty
expression vector control or an expression vector for constitutively active Ras and 0.2 µg of an expression vector for the GAL4 DNA-
binding domain alone (GAL4) or a GAL4 DNA-binding domain fusion
with muscle LIM protein (GAL4-MLP), with Lhx2 or Lhx3 LIM do-

teins (GAL4-Lhx2 LIM or GAL4-Lhx3 LIM) or an Lhx2 LIM domain
mutant in which cysteine residues 52 and 55, which interact with a zinc
atom, were replaced with alanine (GAL4-LIM Mut). The cells were also
transfected with 0.5 µg of a cytomegalovirus-β-galactosidase vector to
assess differences in transfection efficiency. Data are reported as the
relative luciferase activity from three transfections (± S.E. normalized to
β-galactosidase activity. The relative expression of the GAL4 fusion
proteins was assessed by immunoblot analysis of nuclear extracts from
αT3–1 cells, which were transfected with the indicated constructs (C).

We also assessed the ability of the LIM domains of Lhx2 to activate transcription in the context of the α-subunit promoter. For these studies the wild type PGBE element of α-subunit
promoter was replaced with one copy of a GAL4-binding site (Fig. 2D). This allowed GAL4-LIM fusion proteins to be di-
rected to the mutated PGBE in transient transfection studies. As described previously (31), expression of the wild type α-sub-
unit reporter gene was induced severalfold by activated Ras (Fig. 2E). Consistent with previous studies (1), replacement of the
PGBE with a GAL4-binding site reduced basal expression of the α-subunit reporter gene and also reduced the ability of
activated Ras to stimulate reporter gene expression in the absence of the GAL4-Lhx2 LIM domain construct (data not shown). Neither the GAL4 DNA-binding domain alone nor the
GAL4-MLP fusion were able to activate basal expression or support a Ras response (Fig. 2F). In contrast, the GAL4-Lhx2
LIM fusion construct increased basal reporter gene activity and
FIG. 3. Expression of the LIM domain of Lhx2 inhibits the ability of Ras to stimulate α-subunit reporter gene activity in a concentration-dependent manner. αT3–1 cells were transfected with 0.2 μg of either the 507 to −205 wild type α-subunit reporter gene (A) or a thymidine kinase luciferase reporter (B) and either 0.2 μg of an empty expression vector control or vector for constitutively active Ras and the indicated amount of a GAL4-Lhx2-LIM domain expression vector. Data are reported as relative luciferase activity from three transfections ± S.E. corrected for transfection efficiency.

Also supported a Ras response that is similar in magnitude to that obtained with the wild type α-subunit construct (Fig. 2, compare E and F). The ability of the GAL4-Lhx2 LIM construct to permit a Ras response with the α-subunit reporter is in contrast to failure of this same construct to support a Ras response with the simple 5× GAL4 reporter (Fig. 2B). This observation suggests that within the context of the α-subunit gene, the Lhx2 LIM domain cooperates with other factors to enhance the response to Ras and activation of MAPK. The activity of the GAL4-Lhx2 LIM construct was dependent on an intact LIM structure as mutation of crucial cysteine residues within the one zinc finger greatly reduced both basal and Ras-stimulated reporter gene activity. We also compared the ability of the LIM domain of Lhx2 and Lhx3 to activate the modified α-subunit reporter gene (Fig. 2G). Expression of GAL4-LIM domain fusions of both Lhx2 and Lhx3 allowed Ras-induced activation of the α-subunit reporter gene (Fig. 2G).

Because both cytoplasmic and nuclear LIM domains have been shown to function as protein-protein interaction domains (46, 47), it seems likely that the LIM domain of Lhx2 functions as a transcriptional activation domain through recruiting other factors to the α-subunit gene. As an initial characterization of this possibility, we sought to determine whether the Lhx2 LIM domain could inhibit α-subunit expression (Fig. 3). For these studies, the wild type α-subunit reporter gene was co-transfected with the GAL4-LIM constructs. Because the wild type α-subunit reporter gene does not contain GAL4-binding sites, we anticipated that any effects of the GAL4 fusion proteins would be indirect through sequestration of specific proteins. Transfection of GAL4-Lhx2 LIM domain vector substantially inhibited Ras-induced expression of the α-subunit reporter in a concentration-dependent manner (Fig. 3A). Comparable effects were seen for the GAL4-Lhx2 LIM construct but not the Lhx2 LIM mutant (data not shown). This effect was specific for the α-subunit reporter because the GAL4-Lhx2 LIM construct had little or no effect on the thymidine kinase promoter (Fig. 3B). Similarly, an expression vector for GAL4-MLP had no effect on α-subunit gene expression (data not shown), again indicating the specificity of this effect.

To further define the structure-function relationship for the Lhx2 LIM domain, the effects of GAL4 fusion constructs containing the individual LIM domains, designated LIM1 and LIM2, were also tested (Fig. 4B). The second LIM domain (LIM2) was sufficient to permit a response to activated Ras, although the total activity is considerably reduced as compared with the activity of the intact LIM domain. Both LIM domains were expressed at comparable levels (Fig. 4C). This result is consistent with reports suggesting that one LIM domain may be sufficient for interaction with a binding partner, whereas the other LIM domain may function to potentiate binding (25, 44).

Direction of GAL4-NLI to the α-Subunit Gene Does Not Mimic the Effects of the GAL4-Lhx2 LIM Domain—Recent studies have identified NLI as a putative co-activator that binds to the LIM domain of both LIM homeodomain transcription factors and LIM-only nuclear factors (24, 26, 28, 29, 48, 49). Although it is clear that NLI binds to a group of nuclear LIM factors, the functional role of NLI has not yet been fully explored. It is possible that LIM-dependent recruitment of NLI to a promoter directly leads to transcriptional activation. To test this possibility a GAL4-NLI fusion vector was co-transfected with the mutant α-subunit reporter in which the PBGE was replaced with a GAL4-binding site (Fig. 5A). GAL4-NLI had little effect on basal reporter gene activity and did not support a Ras response (Fig. 5B). GAL4-NLI also did not increase the activity of the simple 5× GAL4 reporter gene (data not shown), consistent with the observation that NLI does not function as a transcriptional activator in yeast (28). Thus, direction of NLI to the PBGE of the α-subunit gene is not sufficient to activate transcription. This finding implies that the function of the LIM domain is not limited to the recruitment of NLI to the promoter. Of course, it remains possible that binding of NLI contributes to the transcriptional activity of the LIM domain.

The Lhx2 LIM Domain Functionally Cooperates with an Ets Transactivation Domain—The preceding experiments provide evidence that binding of Lhx2 to the PBGE site contributes to basal and Ras-stimulated transcription of the α-subunit gene. Previous studies have demonstrated that a different DNA element, the GnRH-RE, also contributes to GnRH-stimulated and presumably Ras-stimulated activation of the α-subunit gene (12). The endogenous factor that binds to the GnRH-RE has not been determined but the element does contain a core binding site for the Ets family of transcription factors. A role for an Ets factor in mediating responses to GnRH and Ras/ MAPK activation is consistent with the known ability of several Ets factors to be phosphorylated and activated by the mitogen-activated protein kinase (32, 50, 51). Therefore, we sought to determine whether binding of an Ets transcription factor at the GnRH-RE site is capable of supporting Ras-stimulated transcription. For
FIG. 5. Directed binding of the nuclear LIM-interacting protein to the PGBE of the α-subunit gene does not support a transcriptional response to activated Ras. αT-1 cells were transfected with 0.2 μg of the wild type α-subunit (A) or the PGBE to GAL4 mutant α-subunit (B) luciferase reporter genes and 0.2 μg of either an empty expression vector control or a vector for constitutively active Ras and 0.2 μg of an expression vector for the GAL4 DNA-binding domain (GAL4) or fusion of the GAL4 DNA-binding domain with the Lhx2 LIM domain (GAL4-Lhx2-LIM) or the nuclear LIM-interacting protein coding sequence (GAL4-NLI) as indicated. Data are reported as the relative luciferase activity from three transfections ± S.E. corrected for transfection efficiency. The relative expression of the GAL4 fusion proteins was assessed by immunoblot analysis of nuclear extracts from αT-1 cells that were transfected with the indicated constructs (C).

FIG. 6. The Elk1 carboxyl-terminal transcriptional activation domain can contribute to the Ras responsiveness of an α-subunit reporter gene and synergize with the LIM domain of Lhx2 to mediate Ras responsiveness. αT-1 cells were transfected with 0.2 μg of either the wild type α-subunit reporter gene (A and B) or the GnRH-RE to GAL4 site mutant α-subunit reporter (A and C) or a simple reporter gene containing three copies of a composite LexA–GAL4-binding site upstream of minimal promoter linked to luciferase (D and E). The cells were also transfected with 0.2 μg of either an empty expression vector control or a vector for constitutively active Ras and with 0.2 μg of an expression vector for the GAL4 DNA-binding domain (GAL4) or the LexA DNA-binding domain (LEX) or fusion of the GAL4 DNA-binding domain with the Elk1 carboxyl-terminal transcriptional activation domain (GAL4-Elk) or the Elk1 activation domain in which a crucial phosphorylation site at serine 383 has been mutated to alanine (GAL4-Elk-mut) or a fusion of the LexA DNA-binding domain with the Lhx2 LIM domain (LEX-LIM) as indicated. Data are reported as the relative luciferase activity from three transfections ± S.E. corrected for transfection efficiency.

these studies an α-subunit reporter gene was constructed in which the GnRH-RE sequence was replaced with a GAL4-binding site in the context of a wild type PGBE sequence (Fig. 6A). GAL4 fusion genes were constructed with the transactivation domains of Elk1 or a mutant Elk1 in which a crucial MAPK phosphorylation site at serine 383 was mutated to alanine (32). As expected (12), replacement of the GnRH-RE with a GAL4-binding site eliminated the ability of the α-subunit gene reporter to respond to activated Ras (Fig. 6C). Transfection of a GAL4-Elk1 fusion vector was able to restore Ras-activated reporter gene activity, whereas the MAPK phosphorylation mutant, Elk1-S383A, was unable to restore the Ras response. Similar results were obtained for GAL4 fusions containing the transactivation domains of two other MAPK-responsive Ets factors, Ets1 (50, 52) and Net (53) (data not shown). Of course this experiment does not identify the endogenous GnRH-RE-binding factor. However, the findings demonstrate that when directed to a site corresponding to the GnRH-RE, Ets factors are capable of contributing to Ras responsiveness of the α-subunit gene, presumably through a mechanism involving functional cooperation with a LIM factor binding to the PGBE.

To further investigate the functional cooperation of Lhx2 and Elk1, a simple reporter gene was constructed that contained a multimer of the binding site for the bacterial repressor LexA adjacent to a GAL4-binding site. This construct permits analysis of the combined effect of the Elk-1 and LIM domain fusion proteins on a single promoter (Fig. 6D). The GAL4 and LexA DNA-binding domains alone have little or no transcriptional activity when co-transfected with this reporter. When the GAL4-Elk1 or LexA-Lhx2 LIM fusion constructs were transfected separately, there was little if any increase in either basal or Ras-stimulated activity. It should be noted that when GAL4-Elk1 is tested on a reporter gene containing five copies of a GAL4-binding site, the reporter gene is Ras/MAPK responsive (32). It is not clear whether the lack of Ras responsiveness with the GAL4-Elk1 construct and this reporter is due to the decreased number of binding sites or the different arrangement of the binding sites. Nonetheless, when both GAL4-Elk1 and LexA-Lhx2 LIM were expressed, a substantial synergistic response was observed. This activation was not observed when the GAL4-Elk1-S383A MAPK phosphorylation mutant was co-expressed with LexA-Lhx2 LIM. Thus when the Lhx2 LIM domain and the activation domain of Elk1 are directed to this reporter gene, the response mimics that of the wild type α-subunit reporter. Again, these findings are consistent with a model in which transcriptional responses of the α-subunit gene to Ras/MAPK activation involves the functional cooperation of Lhx2 and Elk1 or another Ets transcription factor.
Identification of a Factor That Interacts with the Lhx2 LIM Domain—To identify proteins that may be involved in mediating transcriptional responses to the Lhx2 LIM domain, the yeast two-hybrid assay (37, 54–56) was used to screen for Lhx2 LIM domain-interacting proteins. Approximately 12 million clones representing the αT3–1 fusion cDNA library were screened for factors that can interact with the LIM domain of Lhx2. Among the positive, LIM-interacting factors, NLI was detected several times, consistent with previous reports (24, 26, 27). The detection of NLI served as a positive control for the quality of the αT3–1 cDNA fusion library and suggested that appropriate conditions were used for the screen. In addition to NLI, a VP16 fusion cDNA corresponding to MRG1 amino acids 1–145 (34) was isolated from the screen. MRG1 is widely expressed in both adult tissues and in the later stages of the developing embryo (57). MRG1 and the closely related melanocyte-specific gene (MSG1) are 24- and 27-kDa nuclear proteins, respectively, that have a highly conserved transcriptional activation domain. Thus, the immunoprecipitation experiments provide evidence that Lhx2 and MRG1 can associate in vivo. Because it is possible that the association of Lhx2 and MRG1 could have occurred after disruption of the cells, we also tested for in vivo interaction using a mammalian two-hybrid assay. GAL4 fusions with the Lhx2-LIM domain or the Lhx3-LIM domain were activated by the VP16 fusion protein containing MRG amino acids 1–145 (Fig. 8B). A GAL4 fusion with MLP or the POU transcription factor, Pit1, were not activated by the VP16-MRG1. Thus, both co-immunoprecipitation studies and the mammalian two-hybrid assay provide evidence for a selective in vivo interaction of MRG1 and the LIM domains of Lhx2 and Lhx3.

MRG1 May Function as a Co-activator to Stimulate α-Subunit Glycoprotein Gene Expression—Transfection of αT3–1 cells with an expression vector for MRG1 was found to substantially activate the wild type α-subunit reporter construct (Fig. 9A). Importantly, this finding suggests that MRG1 can interact with endogenous factors to enhance α-subunit expression. Mutation of the PGBE to a GAL4-binding site disrupted the ability of MRG to stimulate the reporter gene, and MRG responsiveness was restored by transfection of either a GAL4-Lhx2 or -Lhx3 LIM domain expression vector (Fig. 9B). Thus the ability of MRG to activate the α-subunit reporter gene was dependent on the presence of the LIM domain at the PGBE site. MRG1 also failed to activate a TK-luciferase construct (Fig. 9C), providing additional evidence that the response is specific.

To begin to understand the mechanisms by which MRG1 functions as a potent transcriptional activator, we tested the ability of MRG1 to bind to the Ets-1 transcription factor, the TATA-binding protein and the widely utilized co-activator, CBP/p300 (Fig. 10). We found that immobilized MRG1 bound both TATA-binding protein and p300 but not Ets-1. The ability of MRG1 to bind to CBP/p300 is consistent with a report that appeared while this manuscript was in preparation demonstrating that an alternatively spliced isoform of MRG1, designated p35MRG1, binds CBP/p300 both in vitro and in vivo. (58).

**DISCUSSION**

These studies provide evidence that the LIM domain of Lhx2 can function as a transcriptional activation domain and en-
LIM Domain and Transcriptional Activation

FIG. 9. An MRG1 expression vector stimulates expression of an α-subunit reporter gene. The wild type α-subunit luciferase reporter gene (A) or the PBGE to GAL4 site mutant α-subunit reporter gene (B) or a reporter gene containing the herpes simplex virus thymidine kinase reporter (C) were transfected into αT3-1 cells with either 2.0 µg of an empty expression vector control or a vector directing expression of full-length MRG1. The cells also received 0.2 µg of an expression vector for GAL4 DNA-binding domain (GAL4) or a fusion of the GAL4 DNA-binding domain with the Lhx2 LIM domain (GAL4-Lhx2 LIM) or the Lhx3 LIM domain (GAL4-Lhx3 LIM) as indicated. Data are reported as the relative luciferase activity from three transfections ± S.E. correct for transfection efficiency.

FIG. 10. MRG1 can bind to the TATA-binding protein and p300 in vitro. MBP or an MBP-MRG1 fusion protein was immobilized on amyllose resin and incubated with radiolabeled Lhx2 (A), radiolabeled Ets-1 (B), radiolabeled TATA-binding protein (C), or FLAG epitope-tagged p300 (D). After washing the resin, the bound proteins were analyzed by denaturing gel electrophoresis and detected by autoradiography (A–C) or immunostaining (D). For comparison, 15% of the input was also analyzed (Input).

hance both basal and MAPK pathway-stimulated transcription of the α-subunit gene. Interestingly, the LIM domain is a zinc finger structure that forms a highly ordered structure (19, 60). Although there are only a few examples where the structures of transcriptional activation domains have been determined, in at least some cases the activation domain may not be highly structured. For instance the transcriptional activation domain likely involves the ability of this structure to recruit transcription factors or co-activators leading to synergistic activation. We have also demonstrated the ability of the LIM domain to synergize with members of the Ets family of transcription factors or co-activators leading to synergistic activation. We have demonstrated the ability of the LIM domain to synergize with members of the family of transcription factors and co-activators leading to synergistic activation. We have demonstrated that deletion of the LIM domain results in the formation of a secondary axis (24). Similarly, deletion of the LIM domain of Lhx3 enhances transcriptional activation by this factor in heterologous cells (27). In contrast, the present study as well as recent findings from another laboratory (65) have demonstrated that deletion of the LIM domain can decrease transcription-stimulating activity of LIM homeodomain factors. Although the Xenopus studies described above are consistent with a possible negative modulatory role for the LIM domain, other interpretations are possible. If the LIM domain is the major transcriptional activation domain, then deletion of the LIM domain could create an inactive transcription factor. Displacement of a wild type, active LIM homeodomain factor by the inactive LIM-deleted factor would then inhibit transcription. Deletion of the LIM domain of Xlim-1 may then cause formation of a secondary axis through inhibition of a LIM factor-dependent target gene. However, it has been demonstrated that Xlim-1 contains a carboxyl-terminal transcriptional activation domain (28) distinct from the LIM domain, and therefore it is not clear that deletion of the LIM domain would create a dominant negative form of Xlim-1. In any case, the present studies provide evidence that the LIM domain of Lhx2 can function as a transcriptional activation domain, and this function should be considered in evaluating the activity of LIM homeodomain deletion mutants.

The ability of the LIM domain to function as a transcriptional activation domain likely involves the ability of this structure to recruit transcription factors or co-activators. Several laboratories have identified NLI as a LIM-binding, putative co-activator (19, 24, 26, 27). As with the functional properties of the LIM domain itself, the role of NLI has not been clearly established. The results of several studies suggest that NLI acts to stimulate transcription (27, 28). In contrast, NLI has been shown to inhibit the synergy between Lmx-1 and E47 (29). Moreover, recent genetic studies of apterous in Drosophila...
suggest that the relative stoichiometry of LIM transcription factors and Chip, the Drosophila ortholog of NLI (66), is critical for proper function (47, 67, 68). Although the present studies do not resolve this issue, the results provide evidence that recruitment of NLI is not sufficient for transcriptional activation. Forced recruitment of GAL4-NLI to a mutant α-subunit reporter in which the PGBE was replaced with a GAL4 site was not sufficient for transcriptional activation. If NLI functions as a co-activator, its mechanism of action is probably substantially different than the well studied co-activator, CBP, which recruits MRG1 to the TATA-binding protein and the TATA-binding protein leading to transcriptional activation. Our findings are consistent with a model in which Lhx2 MRG1 is able to bind to the TATA-binding protein and p300/CBP, depending on the nature of specific interacting factors.

MRG1 and its isoforms may function as a co-activator modulator, capable of mediating p300/CBP recruitment and transcriptional activation in some circumstances or blocking the recruitment of p300/CBP, depending on the nature of specific interactions with individual transcription factors.

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MRG1 has a number of properties that are consistent with a possible function as a co-activator for Lhx2. MRG1 binds directly to the LIM domain of Lhx2 as determined by an in vitro binding assay. A co-immunoprecipitation assay provided evidence that endogenous MRG1 interacts with Lhx2. Importantly, MRG1 expression enhanced α-subunit reporter gene activity in a LIM domain-dependent manner. We found that MRG1 is able to bind to the TATA-binding protein and p300/CBP. Our findings are consistent with a model in which Lhx2 recruits MRG1 to the α-subunit promoter, which enhances recruitment of p300/CBP and the TATA-binding protein leading to transcriptional activation. Our findings have similarities and important differences with a report that appeared while this manuscript was in preparation (59). Similar to our findings, it was reported that an alternatively spliced isoform of MRG1, which is termed p35srj, binds p300. However, rather than leading to transcriptional activation, p35srj/MRG1 was able to compete with the hypoxia-inducible factor, HIF-1, for binding to p300 leading to a reduction in HIF-1 activity. Thus, MRG1 and its isoforms may function as a co-activator modulator, capable of mediating p300/CBP recruitment and transcriptional activation in some circumstances or blocking the recruitment of p300/CBP, depending on the nature of specific interactions with individual transcription factors.

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