Transcriptional Synergy between LIM-Homeodomain Proteins and Basic Helix-Loop-Helix Proteins: the LIM2 Domain Determines Specificity

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LIM-homeodomain proteins direct cellular differentiation by activating transcription of cell-type-specific genes, but this activation requires cooperation with other nuclear factors. The LIM-homeodomain protein Lmx1 cooperates with the basic helix-loop-helix (bHLH) protein E47/Pan-1 to activate the insulin promoter in transfected fibroblasts. In this study, we show that two proteins originally called Lmx1 are the closely related products of two distinct vertebrate genes, Lmx1.1 and Lmx1.2. We have used yeast genetic systems to delineate the functional domains of the Lmx1 proteins and to characterize the physical interactions between Lmx1 proteins and E47/Pan-1 that produce synergistic transcriptional activation. The LIM domains of the Lmx1 proteins, and particularly the second LIM domain, mediate both specific physical interactions and transcriptional synergy with E47/Pan-1. The LIM domains of the LIM-homeodomain protein Isl-1, which cannot mediate transcriptional synergy with E47/Pan-1, do not interact with E47/Pan-1. In vitro studies demonstrate that the Lmx1.1 LIM2 domain interacts specifically with the bHLH domain of E47/Pan-1. These studies provide the basis for a model of the assembly of LIM-homeodomain-containing complexes on DNA elements that direct cell-type-restricted transcription in differentiated tissues.

Development of differentiated cells depends on the differential expression of transcriptional activators (23). Moreover, cooperative effects of several activators with restricted expression patterns determine the subset of genes turned on in a given cell type (4, 18, 25, 33). Determination of the molecular basis of cooperation between activators that leads to synergy in promoting the transcription of cell-type-restricted genes will help define the molecular basis for the existence of differentiated cells.

LIM-homeodomain (LIM-HD) proteins are a major class of transcriptional activators that cooperate with other activators to direct cellular differentiation. LIM-HD proteins contain a DNA-binding homeodomain and two N-terminal, zinc-binding LIM domains. The LIM-HD protein Lmx1.2 specifies a dorsal cell fate during vertebrate limb development (28, 32). Lim1 is a LIM-HD protein found in the organizer region of vertebrate embryos that is essential for formation of head structures (30). The mec-3 and lin-11 LIM-HD proteins are required for the asymmetric division of precursor cells in *Caenorhabditis elegans* (12, 39). Other LIM-HD proteins, including Isl-1, mec-3, and apterous, have been found to be crucial components of nervous system development in different species (22, 27, 39).

The LIM domains in LIM-HD proteins and several non-homeodomain-containing proteins are often found in pairs (and sometimes clusters of three) at the N or C termini of proteins, and they have been found to mediate both intramolecular and intermolecular interactions (6). LIM domains in the LIM-only protein cysteine-rich protein (CRP) appear to mediate both dimerization and the interaction of CRP with another LIM-only protein, zyxin (10, 29). Of particular relevance to synergy between E47/Pan-1 and Lmx1.1 is the observed in vivo interaction between LMO2, which is composed

entirely of LIM domains, and the class B basic helix-loop-helix (bHLH) proteins TAL1, LYL1, and LYL2 (37). LMO2 was found to have little or no interaction with the class A bHLH protein E47, E12, or HEB. The LMO2-TAL1 interaction is thought to be important for normal erythroid development (38) and participates in T-cell tumorigenesis (21). P-Lim (Lhx3; Xlim3 in *Xenopus*) is a LIM-HD protein required for specification of pituitary cell lineages (31) that synergizes with Pit-1 in transcription (3); the N-terminal LIM domains of P-Lim are required for this synergy, and they interact with the Pit-1 POU domain in vitro (3).

Lmx1.1, a LIM-HD protein originally identified in a pancreatic β-cell line, can synergistically cooperate with the bHLH protein E47/Pan-1 to activate the insulin gene promoter (16). These two proteins bind adjacent DNA elements in an insulin promoter minienhancer. Neither of these proteins alone will promote high-level transcription of insulin minienhancer-linked genes, but together they increase transcription 200- to 1,000-fold. The LIM domains of Lmx1.1 are required for synergy with E47/Pan-1 but may have a negative effect on Lmx1.1-dependent transcription in the absence of E47/Pan-1.

A clearly related *Lmx1* gene has been identified in the chicken, where it is expressed in subsets of differentiating cells in the developing neural tube (34) and the limb bud (28, 36). Although the role of the Lmx1 proteins in the differentiation of these tissues remains uncertain, we have chosen to examine the interaction between Lmx1.1 and E47/Pan-1 as an example of transcriptional synergy in differentiated tissues. In the studies described here, we dissected the molecular basis for cooperation between Lmx1.1 and E47/Pan-1 in regulating promoter activity.

MATERIALS AND METHODS

Cloning of Lmx1.2. The λgt11 HIT T15 M2.2.2 cDNA library used to obtain the Lmx1.2 cDNA has been described previously (15). Hybridization screening

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was performed with the human genomic Lmx-1-like fragment 94-43 (5). Sequence of the cDNA was obtained by automated sequencing with an ABI 321 DNA sequencer.

Plasmid construction. Plasmids pGBWZ and pGAWZ, used for expressing GAL4 DNA-binding domain (D:BD) and GAL4 activation domain (AD) fusion proteins, respectively, in *Saccharomyces cerevisiae* SFY 562, were modified from Clontech plasmids pGBT9 and pGAD424; the details of plasmid construction can be obtained upon request. The Mash-1 (Nj1:19) cDNA was the gracious gift of Richard Anderson (Dallas, Tex.).

Yeast transformation. *S. cerevisiae* SFY 562 was transformed by the electroporation method of Edman (8). Filter and liquid β-galactosidase assays were performed as described in the Clontech protocol, with a minor modification: after overnight culture in proper dropout medium, the transformed yeast cells were collected by centrifugation, resuspended in 100 μl of H$_2$O, frozen in liquid nitrogen, and thawed at room temperature for liquid β-galactosidase assay.

Yeast two-hybrid analysis. The methods used for yeast two-hybrid analysis have been described elsewhere (11) and conform to the description in the Clontech protocol. Briefly, *S. cerevisiae* SFY 562 was transformed with DBD and AD plasmid constructs and plated on SD plates deficient in Leu and Trp. After 3 days at 30°C, single colonies were picked and grown in SD deficient in Leu and Trp overnight at 30°C. The yeast cells were assayed for β-galactosidase activity by the method of Miller (24). The two-hybrid results in this study are the means of dual determinations from 3 to 10 individual transformations.

For yeast genetic system analysis of the transcriptional effects of various segments of the Lmx1.2 protein, *S. cerevisiae* SFY 562 was transformed with DBD constructs and plated on SD-Trp. Individual colonies were assayed for β-galactosidase activity after growth overnight at 30°C in SD-Trp. The results reported are the means of dual determinations from 3 to 10 individual transformations.

Western blot analyses for GAL4 DBD fusion proteins. Colonies of yeast transformed with a given DBD construct and AD vector construct were grown overnight at 30°C in selective medium containing 2% glucose. The yeast cells were pelleted, washed with H$_2$O, resuspended in sodium dodecyl sulfate (SDS)-gel sample buffer, and boiled prior to fractionation on SDS–12% polyacrylamide gels. The proteins were electrophoretically transferred to polyvinylidene difluoride membranes and probed with an anti-DBD mouse monoclonal antibody (Santa Cruz Biotechnology), which was detected with anti-mouse-horseradish peroxidase (Sigma) and enhanced chemiluminescence reagents (Amersham).

Mammalian cell transfection. The Syrian hamster kidney fibroblast line BHK-21 (20) was maintained and transfected as described previously (16). Luciferase assays were performed with 100 μg of protein extract (7). Each data point represents the mean of at least three independent transfections ± standard error (SE).

Affinity resin assay. To test interactions between portions of the E47/Pan-1 protein and Lmx1.1, we in vitro translated and [³⁵S]methionine labeled the E47/Pan-1 proteins, using the Amersham coupled transcription-translation system. The concentrations of the individual segments were normalized by the amount of radioactivity incorporated as judged by autoradiography after SDS-polyacrylamide gel electrophoresis of the reaction product. The glutathione *S*-transferase (GST) fusion proteins were produced in *Escherichia coli* via the pPIG plasmid system. Similar quantities of the GST fusion proteins (approximately 1 μg as estimated by Coomassie blue staining of SDS-polyacrylamide gel-fractionated material) bound to 25 μl of glutathione-Sepharose beads in a total volume of 100 μl of interaction buffer (20 mM HEPES [pH 7.5], 100 mM NaCl, 1 mM EDTA, 4 mM MgCl$_2$, 1 mM dithiothreitol, 0.02% [vol/vol] Nonidet P-40, 10% [vol/vol] glycerol, 0.5% [wt/vol] nonfat dry milk, 50 μg of ethidium bromide per ml) (3). After incubation with in vitro-translated proteins for 20 min at 37°C, the beads were washed three times with interaction buffer without ethidium bromide and solubilized in Laemmli sample buffer for fractionation on SDS-polyacrylamide gels and autoradiography.

Nucleotide sequence accession number. The full nucleotide sequence of the Lmx1.2 cDNA clone used in this study is available under GenBank accession no. U61141.

RESULTS

Lmx1.1 and Lmx1.2 are closely related LIM-HD proteins. The existence of two distinct Lmx1-encoding genes was suggested by the sequence of a Lmx1-like genomic DNA fragment obtained by exon trapping (5). Using this fragment as a probe, we screened a hamster insulinoma cell line (HIT T15) cDNA library and purified a cDNA insert encoding a protein closely related to hamster Lmx1. We have therefore renamed the original cDNA Lmx1.1 and the new cDNA Lmx1.2. The human Lmx1.1 gene lies on chromosome 1 (13), and Lmx 1.2 lies on chromosome 9 (5, 19). Two chicken cDNAs (28, 36) previously called Lmx1 are most similar to Lmx1.2 (93% amino acid identity with hamster Lmx1.2, compared with 68% identity

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Lmx1.2     -PLRPGPAT LGVLLGSDCP HPAVCEGCQR PISDRFLMRV    38
MLDGLKMEEN FQSAIETSAS FSSLLGRAVS PKSVCEGCQR VISDRFLLRL    50

NESSWHEECL QCAACQQALT TSCYFRDRKL YCKQDYQQLF AAKCSGCMEK    88
NDSFWHEQCV QCASCKEPLE TTCFYRDKKL YCKYHYEKLF AVKCGGCFEA   100

IAPTEFVMRA LECVYHLGCF CCCVCERQLR KGDEFVLKEG QLLCKGDYEK   138
IAPNEFVMRA QKSVYHLSCF CCCVCERQLQ KGDEFVLKEG QLLCKGDYEK   150

EKDLLSSVSP DESDSVKSED EDGDMKPAKG QGSQSKGGGD DGKDPRRPKR   188
ERELLSLVSP AASDSGKSDD EESLCKSAHG AGKGTSED-- -GKDHKRPKR   197

PRTILTTQQR RAFKASFEVS SKPCRKVRET LAAETGLSVR VVQVWFQNQR   238
PRTILTTQQR RAFKASFEVS SKPCRKVRET LAAETGLSVR VVQVWFQNQR   247

AKMKKLARRH -QQQQEQQNSQ RLGQEVLSSR ----MEGMMASYTP LAPPQQQIVA
AKMKKLARRQ QQQQQDQQNTQ RLSSAQTNGG GTAGMEGIMNPYTA MPTPQQ-LLA

MEQSPCGSSD PFQQGLTPPQ MPGDHMNPYG NDSIFHDIDS -DTSLTSLSDC   338
IEQSVYNA-D PFRQGLTPPQ MPGDHMHPYG AEPLFHDLDS DDTSLSNLGDC   351

FLGSSDVGSL QARVGNPIDR LYSMQSSYFA S   369 Lmx1.2
FLATSEAGPL QSRVGNPIDH LYSMQNSYFT S   382 Lmx1.1

| LIM1 | | LIM2 | | HOMEO | |
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FIG. 1. Deduced amino acid sequence of Lmx1.2 (top) and comparison with that of Lmx1.1 (bottom). The LIM domains of each are boxed, and the homeodomains are indicated by a solid line between the sequences. The Lmx1.2 cDNA used in these studies is apparently missing the codons encoding the first 10 to 12 amino acid residues. A schematic diagram of the arrangement of these domains within the Lmx1 proteins is shown below the sequences.

with hamster Lmx1.1). Based on the chicken cDNA sequence, the hamster Lmx1.2 cDNA described here is missing the portion encoding 10 N-terminal amino acids.

The primary amino acid sequence of hamster Lmx1.2 is 68% identical and 85% similar to the hamster Lmx1.1 sequence and, like the Lmx1.1 sequence, displays a homeodomain, which is identical in sequence between the two proteins, and two LIM domains (Fig. 1). The 50-amino-acid-residue region immediately C terminal to the homeodomain of Lmx1.2 is glutamine-rich like the corresponding region of Lmx1.1. The remainder of the C-terminal regions of both proteins are acidic, as are the regions of both proteins between the second LIM (LIM2) domain and the homeodomains. The two Lmx1 proteins are much more closely related to each other than to any other LIM-HD proteins and therefore constitute a distinct class of LIM-HD protein.

The LIM domains of the Lmx1 proteins are highly conserved, with 61 and 83% identity between their LIM1 and LIM2 domains, respectively. The LIM1 and LIM2 domains of the *Drosophila* LIM-HD protein apterous are 56% identical and 39% identical, respectively, to the corresponding domains of Lmx1.2. A variety of other LIM-HD proteins have LIM domains exhibiting approximately this same level of identity with the LIM domains of the Lmx1 proteins, as does the LIM-only protein LMO1. The LIM-only protein CRP has very little sequence identity with the Lmx1 proteins outside those residues that define the characteristic structure of a LIM domain (26). In contrast to the sequence similarity between LIM2 domains in a variety of proteins, there is only 30% identity between the LIM1 and LIM2 domains of Lmx1.2, and the conserved residues are common to all LIM domains. Thus, there may be significant functional divergence between LIM1 and LIM2 domains in the LIM-HD proteins.

Lmx1.1 is composed of functionally discrete domains. To dissect the transcriptional functions of various segments of Lmx1.1 in yeast, we fused various portions of the Lmx1.1 cDNA to the coding sequence for residues 1 to 147 of the GAL4 DBD and determined the effects of these fusion pro-
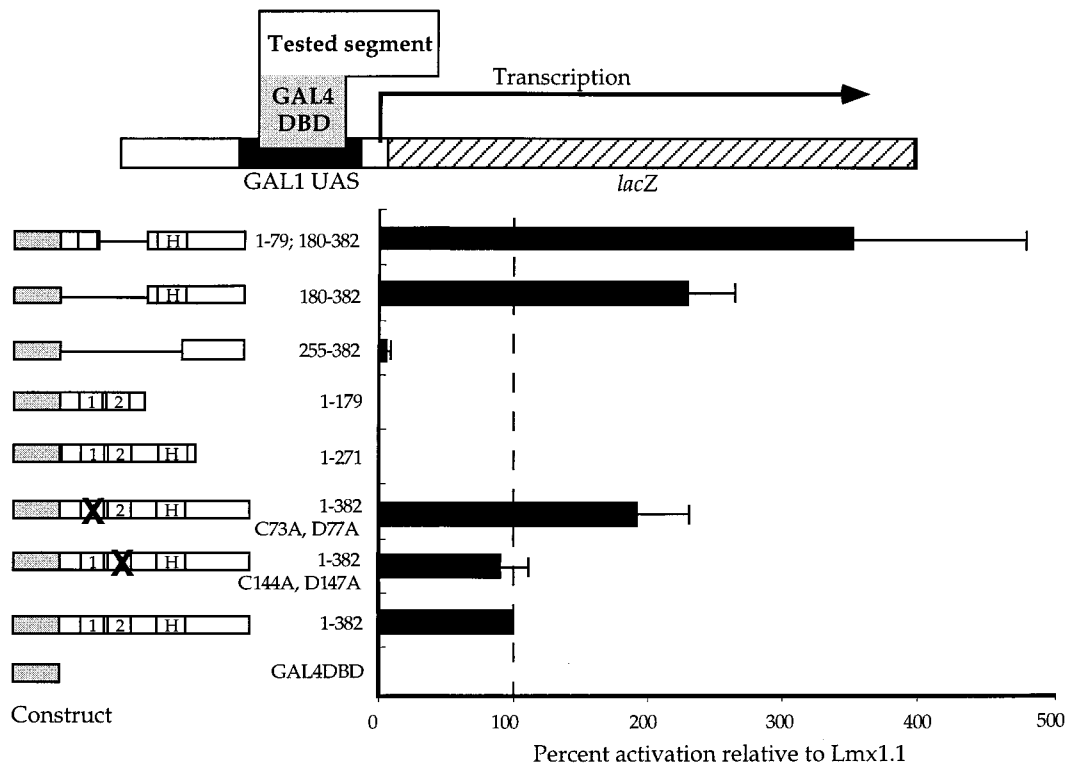
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FIG. 2. Localization of transcriptional activation domains in Lmx1.1 in a yeast genetic system. Various segments of Lmx1.1 were expressed as fusion proteins with the GAL4 DBD in strains bearing the GAL1 upstream activation sequence (UAS)-*lacZ* reporter gene as in the schematic diagram. β-Galactosidase activity was measured, and the value obtained for the DBD-Lmx1.1 fusion was set at 100%. The activity obtained with each of the other constructs is reported as a percentage of this activity. The results shown are the means of 5 to 10 independent transformations ± SE. 1, LIM1 domain; 2, LIM2 domain; H, homeodomain.

teins on transcription of a *lacZ* reporter gene linked to multiple upstream copies of the GAL4 DNA-binding site (Fig. 2). Full-length Lmx1.1 (amino acids 1 to 382) fused to the GAL4 DBD leads to high-level transcription of the *lacZ* reporter gene (198 Miller units). Deletion of the C-terminal 120 amino acids of Lmx1.1 (Lmx1.1 1-271) eliminates transcriptional activation. These 120 C-terminal residues (Lmx1.1 255-382) fused with DBD also activate transcription on their own, but since the β-galactosidase activity produced by this construct is 5% of that produced by the Lmx1.1-DBD fusion, more N-terminal sequences are required for full transcriptional activity. Similar experiments with Lmx1.2 indicate that its C-terminal domain is also a major general transcriptional activation domain; the DBD fusion protein of residues 1 to 369 of Lmx1.2 produces β-galactosidase activity 216% ± 23% of that observed with residues 1 to 382 of Lmx1.1, but the DBD fusion of residues 1 to 256 of Lmx1.2, representing C-terminal truncation 11 residues after the homeodomain, results in <1% of the activity of the DBD–Lmx1.2 1-369 fusion.

Interestingly, deletion of the N-terminal portions of Lmx1.1 containing the LIM domains from such DBD fusions (Lmx1.1 1-79, 180-382, and Lmx1.1 180-382) increases the transcriptional activity of the remainder of the protein two- to threefold. Two point mutations in the LIM1 domain (C73A and D77A) presumed to disrupt the characteristic zinc finger structure of LIM domains also increase the transcriptional activity of the fusion protein nearly twofold. Similar point mutations in the LIM2 domain (C144A and D147A) have little effect on transcriptional activation. It should be noted that because none of these fusion proteins could be detected by Western blotting, we cannot rule out the possibility that these effects are due to

differences in protein stability; however, these results are comparable to those of our previous studies in mammalian cells in which deletion of the LIM domains of Lmx1.1 led to a four- to fivefold increase in transcription of a reporter gene linked to the insulin minienhancer (13).

The LIM domains of Lmx1 proteins mediate interactions with E47/Pan-1. We used a yeast two-hybrid system to characterize physical interactions between Lmx1 proteins and E47/Pan-1. Yeast cells were transformed with (i) a β-galactosidase reporter gene plasmid containing multiple upstream copies of the GAL1 upstream activation sequence, (ii) a plasmid directing expression of a GAL4 DBD alone or fused with portions of the Lmx1.1 protein containing the LIM domains in various arrangements, and (iii) a plasmid directing expression of the GAL4 transcriptional AD alone or fused with Pan-1, Lmx1.1, or Mash-1, another bHLH domain-containing transcription factor (17) (Fig. 3). In this assay, the GAL4 DBD did not interact with any of the AD fusion proteins, but the DBD fusion protein with the region of Lmx1.1 containing the LIM domains displayed an interaction with E47/Pan-1 that leads to β-galactosidase activity that is 10-fold higher than the background level of activity found in yeast expressing this LIM domain DBD construct and the AD domain alone (results from 10 individual transformations) (Fig. 3B and C). Mutational disruption of the LIM1 domain in such DBD constructs did not prevent interaction with E47/Pan-1, but similar disruption of LIM2 completely eliminated E47/Pan-1 interaction (Fig. 3C). Two-hybrid analysis of Lmx1.2 also indicates that the LIM domains of this protein mediate a similar interaction with E47/Pan-1 (data not shown).

We also observed an interaction between the Lmx1.1 LIM

FIG. 3. The LIM2 domain of Lmx1.1 mediates interaction between the LIM-HD protein and E47/Pan-1, but both are required for interaction with Lmx1.1. (A) Schematic representation of the yeast two-hybrid experiments. (B) Interaction between GAL4 DBD fusions of LIM domain-containing N-terminal 179-amino-acid segment of Lmx1.1 (and similar segments in which one LIM domain has been replaced by another copy of the other) and of Pan1, Mash-1, and Lmx1.1 proteins fused to the GAL4 AD. Vector refers to transformation with plasmid pGAWZ, expressing the GAL4 AD alone. The β-galactosidase activity for each double transformation (DBD construct plus AD construct) is shown (23). Values are the means ± SE of results of duplicate determinations from three independent transformations. (1, LIM1 domain; 2, LIM2 domain; H, homeodomain. (C) The N-terminal portion of Lmx1.1 containing the LIM domains or mutationally disrupted LIM domains were expressed as DBD fusion proteins. E47/Pan-1 and Lmx1.1 were expressed as AD fusions. The β-galactosidase activity shown for the double transformants is in Miller units. Values are the means ± SE of results of duplicate determinations from three independent transformations. (1, LIM1 domain; 2, LIM2 domain; H, homeodomain; X, mutational disruption of the marked domain. The Mash-1 AD construct used in these studies strongly interacted with an E47/Pan-1 DBD fusion (41).

domain-DBD fusion protein and intact Lmx1.1 fused to AD (Fig. 3B), suggesting that one or both LIM domains may interact intramolecularly within a given molecule or might mediate dimerization. Although LIM domains in other contexts have been shown to be dimerization domains, we did not ob-

serve interactions between Lmx1.1 LIM-DBD and Lmx1.1 LIM-AD constructs in yeast (data not shown). No interaction was detected between the Lmx1.1 LIM domains and Mash-1.

The LIM2 domains of Lmx1.1 and Lmx1.2 are responsible for their interaction with E47/Pan-1. To discern whether
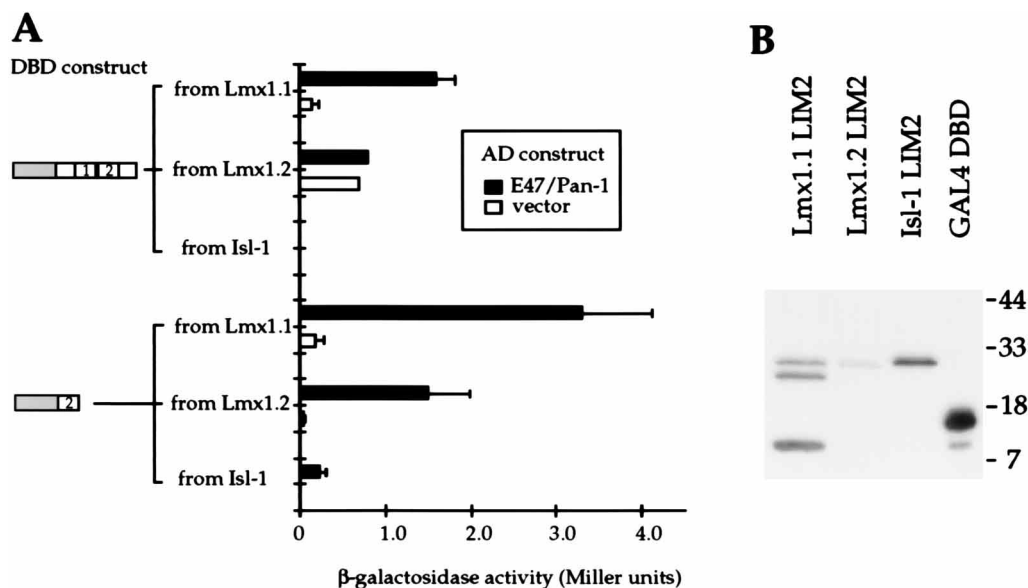
FIG. 4. The LIM domains and minimal LIM2 domains of Lmx1.1 and Lmx1.2, but not Isl-1, interact with E47/Pan-1. (A) The LIM domain-containing segments or the minimal LIM2 domains of Lmx1.1, Lmx1.2, and Isl-1 as DBD fusion proteins were tested for interaction with AD fusions of E47/Pan-1. The β-galactosidase activity shown for each double transformation (DBD construct plus AD construct) is given in Miller units. Values are the means ± SE of results of duplicate determinations from three independent transformations. The values obtained for the LIM domain-DBD fusion from Lmx1.2 are from a single transformation; subsequent transformations with this construct also displayed similar levels of β-galactosidase activity between yeast cotransformed with the AD vector or E47/Pan-1 AD constructs. (B) Anti-GAL4 DBD Western blots confirmed similar levels of expression of the Lmx1.1 LIM2-DBD and Isl-1 LIM2-DBD fusion proteins in doubly transformed yeast. Sizes are indicated in kilodaltons.

LIM1, LIM2, or both are required for interaction with E47/Pan-1, we tested DBD fusions with the N-terminal portion of Lmx1.1 in which one LIM domain was replaced with the other so that the fusion proteins contain either tandem LIM1 domains with no LIM2 domain or tandem LIM2 domains with no LIM1 domain (Fig. 3B). The tandem LIM2 domain-containing DBD construct greatly increased the β-galactosidase activity (approximately fourfold) in two-hybrid transformations with the E47/Pan-1–AD constructs relative to DBD constructs containing the normal LIM1-LIM2 arrangement of the LIM domains. This more than additive effect may represent an increase in affinity of the two-LIM2 protein for E47/Pan-1. In contrast, DBD fusion constructs containing tandem LIM1 domain segments did not display β-galactosidase activity above background levels.

In contrast to the LIM domains of Lmx1.1, the LIM domains of Isl-1 do not mediate an interaction with E47/Pan-1 (Fig. 4A). The LIM domains of Lmx1.2 have a high background transcriptional activation when expressed as DBD fusions, making it difficult to assess whether this construct interacts with E47/Pan-1. The minimal LIM2 domains of Lmx1.1 and Lmx1.2 are sufficient for interaction with E47/Pan-1, but the LIM2 domain of Isl-1 is not. Anti-GAL4 Western blots of yeast transformed with these constructs showed that the minimal LIM2-DBD fusions of Lmx1.1 and Isl-1 are expressed at similar levels, but that of Lmx1.2 is expressed at somewhat lower levels (Fig. 4B). Thus, the results of these two-hybrid studies, normalized for the amount of fusion protein found in such yeast transformants, indicate that the LIM2 domains of the Lmx1 proteins display similar affinities for E47/Pan-1, but the LIM2 domain of Isl-1 displays a much lower affinity for the bHLH protein. Although the β-galactosidase activity obtained with the single LIM2 (from Lmx1.1)-DBD fusion was higher than that obtained for the LIM1-LIM2 (from Lmx1.1)-DBD fusion, Western blots revealed that there was much more of

the former protein present in the yeast than the latter protein, which was undetectable.

The LIM2 domain of Lmx1.1 mediates transcriptional synergy with E47/Pan-1. The ability of the LIM2 domain to interact with E47/Pan-1 in yeast cells is paralleled by its ability to promote synergy between Lmx1.1 and E47/Pan-1 in stimulating transcription of a reporter gene linked to an insulin minienhancer in hamster (BHK-21) cells (Fig. 5). As reported previously, Lmx1.1 and E47/Pan-1 synergize strongly in this system (16). A version of Lmx1.1 with two LIM1 domains rather than a LIM1-LIM2 pair displays no synergy with E47/Pan-1, but a similar version of Lmx1.1 with two LIM2 domains displays enhanced synergy with E47/Pan-1 compared to intact Lmx1.1. This increased transcriptional synergy parallels the increased affinity of tandem LIM2 domains of Lmx1.1 for E47/Pan-1 detected in the two-hybrid system. The close correlation of the two-hybrid results with the mammalian cell transfection studies indicates that the physical interaction between the LIM2 domain of Lmx1.1 and E47/Pan-1 mediates the transcriptional synergy observed between these proteins.

In the same study, we found that Lmx1.2 is comparable to Lmx1.1 in its ability to synergize with E47/Pan-1. The cDNA of Lmx1.2 that we have used in these studies is missing the codons corresponding to what are presumably 10 to 12 N-terminal amino acids, but it appears that these residues are not required for transcriptional synergy.

Lmx1.1 LIM domains interact with the bHLH region of E47/Pan-1 in vitro. To eliminate the unlikely possibility that the interaction that we observed between the LIM domains and E47/Pan-1 was mediated by one or more intermediary yeast proteins, we also tested for interactions between these proteins in vitro, using an affinity resin assay (29). In such an assay, in vitro-translated and ^{35}S-labeled E47/Pan-1 interacts with a GST-Lmx1.1 fusion protein bound to a solid support but not to similar amounts of either GST or GST fused with an
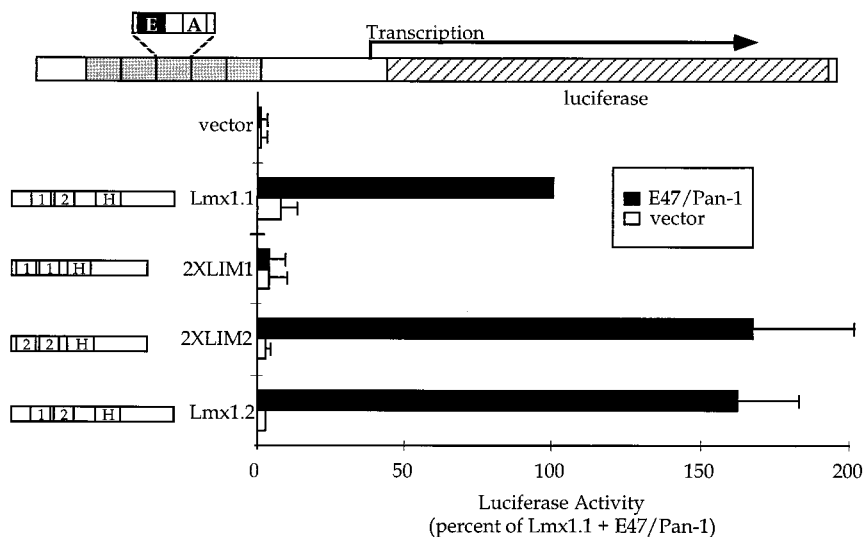
FIG. 5. Synergistic activation of the insulin minienhancer by Lmx1 proteins and E47/Pan-1 in BHK-21 cells and the effect of replacing LIM2 with LIM1 and LIM1 with LIM2. A reporter plasmid with the luciferase gene under the control of a minimal prolactin promoter and five copies of the insulin minienhancer (see schematic) was cotransfected with a plasmid expressing Lmx1 proteins with the normal arrangement of LIM1 and LIM2 domains or with constructs encoding versions of Lmx1.1 in which LIM1 is replaced by another copy of LIM2 (2XLIM2) and in which LIM2 is replaced by another copy of LIM1 (2XLIM1). Each was also cotransfected with an expression plasmid with an E47/Pan-1 cDNA insert (solid bars) or no cDNA insert (open bars). Luciferase activity of the wild-type Lmx1.1–Pan-1 cotransfection was set at 100%. Results are the means ± SE of three independent transfection experiments.

N-terminally truncated portion of the Lmx1.1 polypeptide lacking the LIM domains (Fig. 6A).

We also used the affinity resin assay to define the portion of E47/Pan-1 that interacts with Lmx1.1. We generated a number of deletion mutants of E47/Pan-1, produced them by in vitro translation, and examined the interaction of each of the E47/Pan-1 segments with a GST-Lmx1.1 fusion protein. The N-terminal 570 amino acids of E47/Pan-1 did not display an interaction with GST-Lmx1.1, but the C-terminal 137 amino acids, which contain the bHLH domain, interact with GST-Lmx1.1 as well as intact E47/Pan-1 did (Fig. 6A). Several other in vitro-translated C-terminal deletions of E47/Pan-1 representing amino acid residues 1 to 177, 1 to 324, and 1 to 485 showed no interaction with Lmx1.1 (Fig. 6B). N-terminal deletions representing amino acid residues 178 to 647 and 225 to 647 were both able to interact with Lmx1.1. in vitro as well as intact E47/Pan-1 (Fig. 6C). We were able use the affinity resin assay to narrow the portions of E47/Pan-1 required for interaction with GST-Lmx1.1 to amino acids 510 to 608 (Fig. 6B). This segment contains little more than the bHLH domain.

A fusion protein consisting of GST and the minimal LIM2 domain of Lmx1.1 interacts with E47/Pan-1 and its bHLH-containing region as efficiently as intact Lmx1.1 does in vitro (Fig. 7). In contrast, the LIM1 domain of Lmx1.1 interacts much less well with Pan-1 and its bHLH domain. Although it is possible that proteins present in the reticulocyte lysate used for translation could mediate these interactions, these in vitro results support the conclusion that the synergistic interactions of Lmx1.1 and Pan-1 are mediated, at least in part, by direct contact of the LIM2 and bHLH domains.

DISCUSSION

Our results indicate that the LIM domains of Lmx1 proteins physically interact with the bHLH domain of E47/Pan-1 to promote transcriptional synergy. The Lmx1.1 LIM2 domain is dominant over LIM1 in promoting both physical interaction and synergy, and the presence of two LIM domains greatly

strengthens these interactions. The LIM-bHLH interaction displays remarkable specificity, since Lmx1 proteins do not interact with the bHLH protein Mash-1, and E47/Pan-1 does not interact with the LIM-HD protein Isl-1. These studies provide a molecular basis for understanding one type of cooperation between transcriptional activators required for transcription of cell-type-restricted genes. LIM-bHLH interactions probably represent one class of specific interactions out of many that are required to precisely assemble a highly efficient cell-type-specific transcriptional complex.

LIM domains mediate functionally significant protein-protein interactions in other cell-type-specific transcriptional complexes that are distinct from the insulin minienhancer complex that we have described. The LIM domains of the LIM-only proteins LMO1 and LMO2 have been found to interact with the bHLH domains of the class B bHLH proteins TAL1, TAL2, and LYL1, but not with E47 or other class A bHLH proteins (37). These LMO proteins are non-DNA-binding transcription factors, and the interaction of LMO2 with TAL1 may play a role in erythroid differentiation (21, 35, 38). It is not yet known whether one or both of the LIM1 and LIM2 domains of LMO2 are responsible for this interaction, but it is worth noting that the LIM2 domain of Lmx1.1 is 46% identical with the LIM2 domain of LMO2. The sequence differences between these two LIM2 domains must determine the binding specificity of these domains. LMO2 is able to interact with TAL1 when the bHLH protein is heterodimerized with E47, indicating that the site of interaction is close to, but distinct from, the bHLH-bHLH interaction site. In another example, the LIM domains of the LIM-HD protein P-LIM interact with the POU-domain transcription factor Pit-1 in vitro (3). These LIM domains also mediate transcriptional synergy between P-Lim and Pit-1. Given these examples, LIM domains in transcription factor proteins should be viewed as presumptive interaction domains that allow for cooperation within transcriptional complexes.

The dominant role for LIM2 in promoting interactions between Lmx1.1 and the bHLH domain of E47/Pan-1 leaves
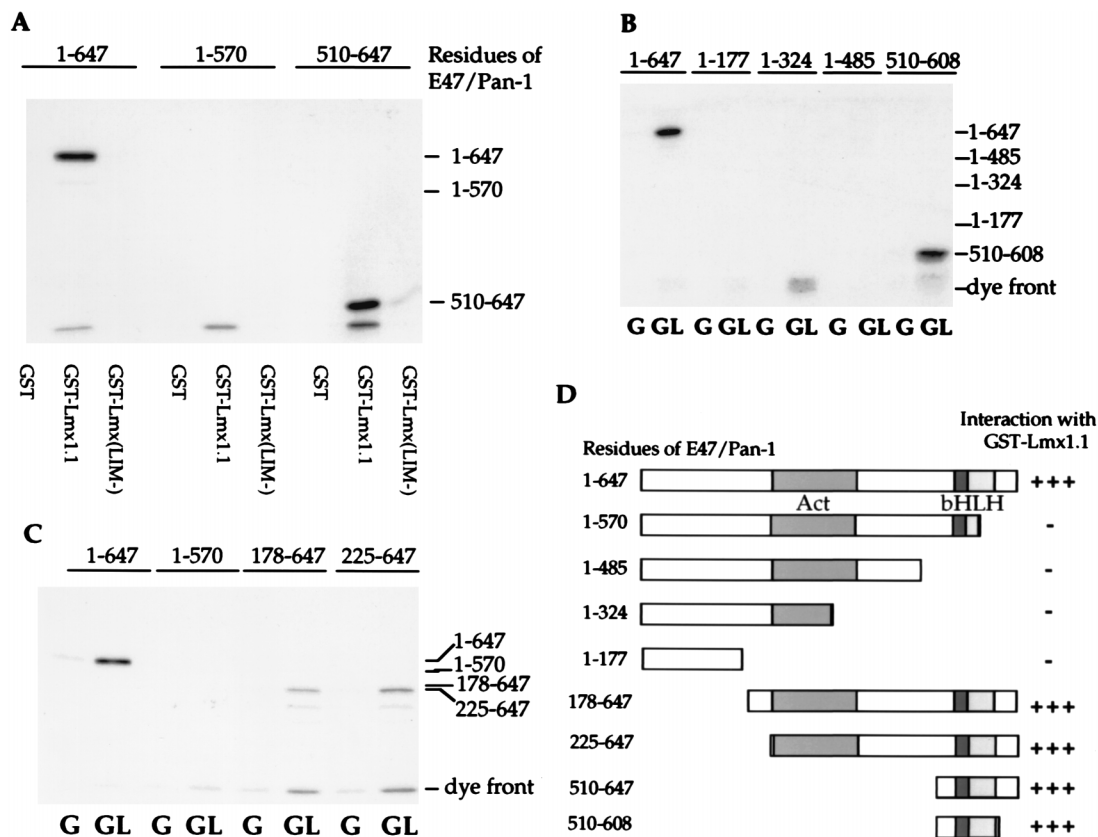
FIG. 6. Lmx1.1–Pan-1 interactions in vitro require the Lmx1.1 LIM and E47/Pan-1 bHLH domains. (A) GST alone or GST fusion proteins (GST-Lmx1.1 and GST-Lmx1.1 lacking the LIM domains) bound to beads (approximately 1 μg of protein each) were exposed to [³⁵S]methionine-labeled, in vitro-translated intact E47/Pan-1 (residues 1 to 647) and two deletions, Pan 1-560 and Pan 510-647. Similar amounts of radioactive protein were used for all incubations. The beads were washed and solubilized in SDS-polyacrylamide gel sample mix prior to electrophoresis and autoradiography. (B and C) Labeled in vitro translation products representing the residues of E47/Pan-1 indicated were incubated with GST-glutathione-Sepharose (G) or GST-Lmx1.1-glutathione-Sepharose (GL), washed, and analyzed as for panel A. The electrophoretic mobility of each in vitro-translated product is indicated. (D) Summary of affinity resin interactions between GST-Lmx1.1 and deletion mutants of E47/Pan-1. The shaded areas represent (from the N terminus) the general activation (Act) domain of E47/Pan-1 and the basic and helix-loop-helix domains (bHLH). −, background levels of interaction; +++, interaction similar to that shown for E47/Pan-1 in panels A to C.

open the question of the function of LIM1. Arber and Caroni (1) also found a dominant role for LIM2 of the LIM-only protein muscle LIM protein (MLP) in promoting association of this protein with the actin cyctoskeleton; additional LIM domains, including the LIM1 domain of MLP, potentiated the binding, but different potentiating LIM domains resulted in an apparent altered specificity of binding. Thus the Lmx1.1 LIM1 domain, which is clearly potentiating, may contribute to the specificity of the interaction as well. Alternatively, the LIM1 domain may specify interaction with other, unidentified target proteins.

LIM domains also exist in proteins that appear to have no association with transcriptional complexes, and in certain cases these proteins appear to mediate specific protein-protein interactions that allow assembly of protein complexes that function outside the nucleus. A number of studies have indicated that LIM domains in the LIM-only proteins CRP, CRP2, and CRP3 have the capacity both for self-association and for interaction with other proteins (1, 10). We found no direct evidence for LIM-LIM association in Lmx1.1-derived LIM domains in the two-hybrid or in vitro systems. However, the LIM domains of Lmx1.1 do appear to mediate intramolecular interactions, and it remains for us to determine which portions of Lmx1.1 participate.

In other LIM-HD proteins, LIM domains have been suggested to be negative regulatory domains. The LIM domains of

Xlim-1 have a negative effect on the capacity of this protein to promote muscle differentiation in *Xenopus* embryos (32). Given the sequence similarity between the LIM domains of all of the LIM-HD proteins, it is tempting to speculate that they
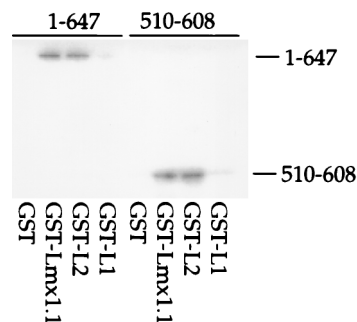

FIG. 7. LIM2 but not LIM1 interacts with E47/Pan-1 and its bHLH region. GST alone or GST fusion proteins (GST-Lmx1.1, GST-LIM2, and GST-LIM1) bound to beads (approximately 1 μg of protein each) were exposed to [³⁵S]methionine-labeled, in vitro-translated intact E47/Pan-1 (residues 1 to 647) or the segment of E47/Pan-1 between residues 510 and 608 encompassing the bHLH region. Similar amounts of radioactive protein were used for all incubations. The beads were washed and solubilized in SDS-polyacrylamide gel sample mix prior to electrophoresis and autoradiography. The electrophoretic mobility of the in vitro-translated products is indicated on the right.
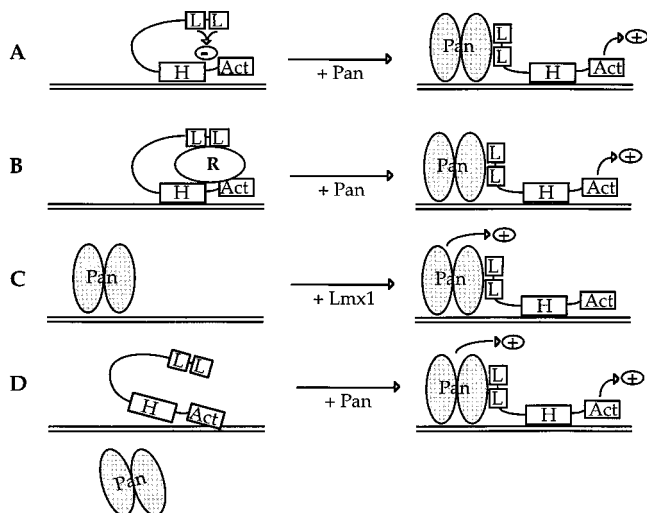
FIG. 8. Models for the synergistic activation of insulin minienhancer (see text for description). H, homeodomain; L, LIM domain; Act, activation domain; Pan, E47/Pan-1; E, E box; A, A element; −, negative transcriptional regulation; +, positive transcriptional regulation.

play analogous regulatory roles. There do appear to be some differences in the behavior of these domains, however. For example, the LIM domains of Isl-1 have been reported to profoundly affect the DNA-binding properties of this LIM-HD protein (2), but the DNA binding of Lmx1.1 is affected subtly, if at all, by the presence or absence of LIM domains (19a). In addition, our observation that the LIM domains inhibit transcriptional activation when bound to the GAL4 DBD demonstrate that inhibition of homeodomain-DNA binding alone cannot explain the inhibitory effect of the LIM domains.

The LIM domains of Lmx1.1 have a negative effect on transcription that is somewhat analogous to the properties of LIM domains in Xlim-1. LIM domains in Lmx1.1 have a negative effect on transcription in the absence of E47/Pan-1. Our previous studies using transfected fibroblasts found that deletion of the LIM domains of Lmx1.1 greatly increased the ability of the remainder of the protein to increase transcription of genes linked to the insulin minienhancer in the absence of E47/Pan-1 (16). Our yeast genetic analysis of the transcriptional activation properties of Lmx1.1-DBD fusion proteins also point to either a masking effect of the Lmx1.1 LIM domains on Lmx1.1 general transcriptional activation domains or a negative effect of the LIM domains on protein stability.

Taken together, these LIM deletion-mutation studies suggest that LIM domains have the capacity to mask certain functions of LIM-HD proteins; the extent or precise effect of this masking may vary in individual proteins. The apparent dual role of LIM domains in LIM-HD proteins as intramolecular inhibitors and intermolecular activators suggests that synergy may involve an allosteric mechanism in which LIM domains inhibit transcriptional activation by the LIM-HD protein until it is juxtaposed with a cooperating protein with which the LIM domains preferentially interact (Fig. 8, model A).

Several alternative models to explain synergy can be proposed. The LIM domains may bind a repressor that is released when the LIM domains interact with E47/Pan-1 (Fig. 8, model B), although a repressor would need to be expressed in both yeast and mammalian cells in order to explain our data. Alternatively, interaction with the LIM domains may induce changes in E47/Pan-1 that expose new transcriptional activa-

tion regions (Fig. 8, model C). Finally, interaction between Lmx1 proteins and E47/Pan-1 may increase the DNA-binding affinity of the complex (Fig. 8, model D). More than one of these models may prove to be correct, meaning that LIM-bHLH interactions may contribute to synergy on several levels.

The capacity of Lmx1.1 and E47/Pan-1 to bind to adjacent segments of the insulin promoter and interact to elevate transcription synergistically defines a specific mechanism that may be important in β cells, but given the complexity of the insulin promoter, it is likely that multiple complexes containing a diversity of proteins cooperate to regulate the insulin gene in vivo. Indeed, the β-cell homeodomain-containing protein PDX-1 (pancreatic duodenal homeobox protein 1) has been found to bind A-element DNA and synergistically promote transcription with E47 (25). Although PDX-1 does not contain LIM domains, our studies suggest that it will be worthwhile to examine PDX-1 and other β-cell homeodomain-containing proteins for their ability to specifically interact with bHLH proteins. Further illustrating potential for complexity of the insulin transcription complex, the POU-HD transcription factor hepatocyte nuclear factor 1α (HNF-1α) has recently been shown to be important for insulin production in humans (40). Like Lmx1.1 and PDX-1, HNF-1α binds one of the A sites in the insulin minienhancer and stimulates insulin transcription in transfected fibroblasts (9, 14). It is currently unknown whether HNF-1α synergizes with E47/Pan-1.

Given the demonstrated importance of LIM-HD proteins and bHLH proteins as participants in cell-type-specific transcriptional complexes, it is tempting to speculate about the generality of bHLH LIM-HD interactions in these complexes. The regulation of each cell-type-specific gene is likely to result from precise assembly of unique sets of cell-type-restricted transcriptional activators. We have identified domains in two of these activators that allow for their specific cooperation in transcriptional activation.

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