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Functional Analysis of the Nuclear LIM Domain Interactor NLI

LINDA W. JURATA[1] AND GORDON N. GILL[2]*

Biomedical Sciences Graduate Program[1] and Department of Medicine,[2] University of California at San Diego, La Jolla, California 92093-0650

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LIM homeodomain and LIM-only (LMO) transcription factors contain two tandemly arranged Zn^{2+}-binding LIM domains capable of mediating protein-protein interactions. These factors have restricted patterns of expression, are found in invertebrates as well as vertebrates, and are required for cell type specification in a variety of developing tissues. A recently identified, widely expressed protein, NLI, binds with high affinity to the LIM domains of LIM homeodomain and LMO proteins in vitro and in vivo. In this study, a 38-amino-acid fragment of NLI was found to be sufficient for the association of NLI with nuclear LIM domains. In addition, NLI was shown to form high affinity homodimers through the amino-terminal 200 amino acids, but dimerization of NLI was not required for association with the LIM homeodomain protein Lmx1. Chemical cross-linking analysis revealed higher-order complexes containing multiple NLI molecules bound to Lmx1, indicating that dimerization of NLI does not interfere with LIM domain interactions. Additionally, NLI formed complexes with Lmx1 on the rat insulin I promoter and inhibited the LIM domain-dependent synergistic transcriptional activation by Lmx1 and the basic helix-loop-helix protein E47 from the rat insulin I minienhancer. These studies indicate that NLI contains at least two functionally independent domains and may serve as a negative regulator of synergistic transcriptional responses which require direct interaction via LIM domains. Thus, NLI may regulate the transcriptional activity of LIM homeodomain proteins by determining specific partner interactions.

Cell fate decisions made throughout development result in diverse, differentiated cell types capable of unique functions. One class of transcription factors known to influence the developmental programs of undifferentiated precursor cells consists of the LIM domain-containing transcription factors (for reviews, see references 13 and 20). Some nuclear LIM domain proteins contain a DNA binding homeodomain; others, known as LIM-only proteins (LMO), lack an obvious DNA binding element but associate with DNA binding factors (37, 51, 52). LIM transcription factors are required for head formation (44), neurogenesis (39, 55), pituitary development (46), erythropoiesis (54), pancreas development (2), and the determination of other cell types (9, 12, 14, 15). However, few gene targets and transcriptional mechanisms have been identified which account for the phenotypes resulting from targeted deletion of these genes.

The basic helix-loop-helix (bHLH) proteins constitute a second class of transcription factors involved in cellular differentiation (33). bHLH proteins are involved in muscle formation (36), B-cell development (6, 59), neurogenesis (7, 10, 22, 31), hematopoiesis (40, 42, 47), and myeloid differentiation (27), and they appear to function biologically by directing precursor cells to exit the cell cycle (38) and to activate cell-type-specific genes. In general, cell-type-restricted bHLH factors (B class) require heterodimerization with the widely expressed A class bHLH proteins, which include HEB, ITF2, and the products of the E2A gene, E12 and E47, to bind DNA and activate transcription (23, 24, 30, 34). During neurogenesis, expression of the bHLH proteins neuroD and NSCL-1 overlaps that of the LIM homeodomain proteins, which are found in postmitotic cells of the neural tube (7, 31, 50), suggesting that both classes of transcription factors may be coordinately involved in neuronal development. Functional convergence of the LIM and bHLH classes of transcription factors has been demonstrated by the association between LMO2 (Rbtn2, Ttg-2) and the bHLH protein Tal1 in hematopoietic (51, 52) and leukemic (29) cells and the synergistic activation of the rat insulin I promoter by the LIM homeodomain protein Lmx1 and Pan1/Pan2 (19), the hamster equivalents of E47/E12 (16, 35).

A glucose-responsive minienhancer region extending from −247 to −198 of the rat insulin I promoter (18) contains two closely spaced, functionally interdependent elements known as Far and FLAT (17, 19, 26). This minienhancer, in addition to a similar but more proximal element (−112 to −104), is required for high levels of insulin expression in pancreatic β cells (26). The Far element, extending from −239 to −230, contains a consensus E box (CANNTG) for bHLH binding (8, 34) and is capable of binding complexes containing E12 and E47 (16, 17, 35, 53). Although it is unknown which B-class bHLH factor is involved in binding to this element with E12/E47, it is unlikely that the E2A proteins function as homodimers at this site because functional homodimers appear to be restricted to B cells (45). The FLAT element (−222 to −208) contains multiple TAAT motifs and binds the LIM homeodomain protein Lmx1 (19). When cotransfected in a non-insulin-producing cell line, E47 and Lmx1 synergistically activate high levels of transcription from the Far-FLAT minienhancer (19).

A widely expressed, 43-kDa nuclear protein, NLI/Ldb1, was recently identified and demonstrated to bind directly to the LIM domains of all LIM class transcription factors (1, 25), suggesting a common mechanism for transcriptional regulation of these tissue-specific factors. But how NLI affects the function of LIM domain-containing proteins remains unknown. To gain a better understanding of how NLI may function in transcriptional processes, we have dissected the functional domains of NLI. In this study, we demonstrate that NLI can specifically associate with both endogenous and transfected LIM domain-containing transcription factors in vivo. A 38-residue carboxy-

* Corresponding author. Mailing address: Department of Medicine, University of California at San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0650. Phone: (619) 534-4310. Fax: (619) 534-8193. E-mail: ggill@ucsd.edu.

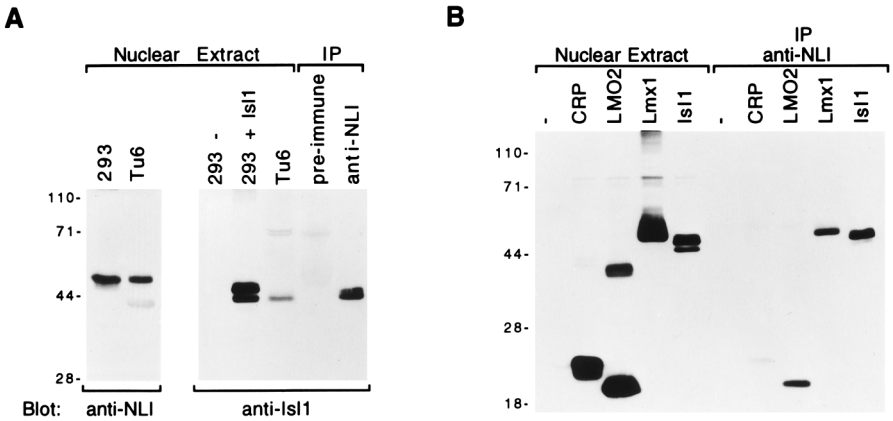

FIG. 1. In vivo association of endogenously expressed NLI with LIM domain transcription factors. (A) Nuclear extracts from 293 cells, 293 cells transfected with the Isl1 cDNA, and pancreatic tumor Tu6 cells were immunoblotted with anti-NLI and anti-Isl1 antibodies. The amount of Tu6 extract loaded represents 20% of the volume of extract used for immunoprecipitation. Tu6 nuclear extracts were immunoprecipitated (IP) with preimmune serum or anti-NLI antibodies and subsequently blotted with anti-Isl1 to visualize coprecipitating proteins. (B) Nuclear extracts from 293 cells transfected with empty expression plasmid (−), HA epitope-tagged CRP, LMO2, Lmx1, and Isl1 cDNAs were immunoprecipitated with anti-NLI antibodies and then immunoblotted with anti-HA monoclonal antibody. The nuclear extract showing expression of the four proteins represents 2.5% of the volume subjected to immunoprecipitation.

terminal region of NLI is sufficient for binding to LIM domain proteins in solution and on DNA, and the amino-terminal 200 amino acids are capable of mediating NLI homodimerization. Higher-order complexes containing multiple NLI molecules bound to Lmx1 are formed in solution, suggesting that complex formation may be involved in regulation. Using the Lmx1 and E47 transcriptional synergy on the rat insulin I promoter as a model system, we demonstrate that NLI interferes with LIM homeodomain/bHLH transcription from this promoter, suggesting that NLI may function, at least in part, by editing specific transcriptional responses.

MATERIALS AND METHODS

Plasmid constructions. Glutathione *S*-transferase (GST) fusion constructs were generated by ligating restriction fragments or *Pfu* polymerase (Stratagene)-generated PCR products in frame into pGEX-KG or pGEX-KT (21). For in vitro transcription-translation reactions and transfections, PCR products were ligated into pcDNA3M containing a hemagglutinin epitope tag (56) or pcDNA3-FLAG. pcDNA3-FLAG was generated by ligating the annealed oligonucleotides 5′-GATCCTGTTGGTAAAATGGAAGACTACAAAGACGACGACGACAA AG-3′ and 5′-AATTCTTTGTCGTCGTCGTCTTTGTAGTCTTCCATTTTAC CAACAG-3′ to *Bam*HI-*Eco*RI-digested pcDNA3 (Invitrogen). Transfection constructs requiring a simian virus 40 T antigen nuclear localization signal sequence were generated by ligating the annealed oligonucleotides 5′-A ATTCGGTGCTCCTCCAAAAAAGAAGAGAAAGGTAGCTGGTG-3′ and 5′-AATTCAACAGCTACCTTTCTCTTCTTTTTTGGAGGAGCACCG-3′ into the *Eco*RI site of pcDNA3M. Intact reading frames were verified by sequencing the 5′ and internal fusion junctions of each construct. The rat insulin I minienhancer (−247 to −198) luciferase reporter construct was generated by annealing and ligating the oligonucleotides 5′-GATCCCTTCATCAGGCCATCTGGC CCCTTGTTAATAATCTAATTACCCTAGGTCTAA-3′ and 5′-GATCTTAG ACCTAGGGTAATTAGATTATTAACAAGGGGCCAGATGGCCTGATGA AGG-3′. After digestion with *Bam*HI and *Bgl*II, the purified fragment containing five minienhancer copies was ligated into a *Bam*HI site upstream of the minimal thymidine kinase promoter (−39 to +51) driving a luciferase reporter gene. The Isl1, Lmx1, and E47 cDNAs were generous gifts from M. Montminy, M. German, and C. Murre, respectively.

Protein purification and protein-protein interaction assays. Coimmunoprecipitations from transfected 293 and untransfected Tu6 cells were performed as described previously (25). Nuclear extracts were prepared by lysis of cells in hypotonic lysis buffer (10 mM Tris [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, protease inhibitors). The nuclei were washed once and then solubilized in 1 ml of RIPA buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 200 μg of ethidium bromide per ml to prevent DNA-dependent interactions [28]). The clarified supernatant was precleared with protein A-Sepharose and then immunoprecipitated with 5 μl of preimmune serum or anti-NLI polyclonal antibody and 15 μl of protein A-Sepharose. After being washed four or five times with RIPA buffer, the complexes were visualized by SDS-polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide) and immunoblotting.

GST fusion proteins were purified by standard procedures (48) after induction of pGEX-containing BL21DE3pLysS cultures with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at room temperature in 2× yeast tryptone (YT) medium. For LIM domain-containing proteins, 1 mM $ZnCl_2$ was included in the induction. Protein concentrations were determined by Bradford analysis.

Assays of the binding of in vitro-translated proteins to GST fusions were performed as described previously (25). Labeled proteins were transcribed from the T7 promoter of pcDNA3M and translated in the presence of [^{35}S]methionine and, for LIM domain-containing proteins, 1 mM $ZnCl_2$ (Promega TNT coupled reticulocyte lysate system). Labeled protein (10 μl) was incubated for 1 h at 4°C with 100 pmol of GST fusion bound to 12.5 μl of glutathione-agarose after preincubation of the GST fusion for 1 h in phosphate-buffered saline (PBS)–1% Triton X-100–25 mg of bovine serum albumin per ml–1 mM dithiothreitol (DTT). The beads were washed once with 25 mg of bovine serum albumin per ml in PBS and then three times with PBS before being analyzed by SDS-PAGE (12.5% polyacrylamide) followed by fluorography.

For double immunoprecipitation experiments, 25 μl of in vitro translation reaction mixture containing [^{35}S]methionine, 1 mM $ZnCl_2$, and 0.375 μg each of a pcDNA3M and a pcDNA3-FLAG construct was diluted with 225 μl of 25 mM Tris (pH 7.5)–300 mM NaCl–1% Triton X-100–protease inhibitors–200 μg of ethidium bromide per ml. The mixture was precleared with 25 μl of protein A-Sepharose (Sigma) for 1 h at 4°C and then immunoprecipitated with 2 μl of anti-FLAG antibody (Eastman Kodak) followed by 12.5 μl of protein A-Sepharose for 1 h each. The complexes were washed twice with dilution buffer and then once with 25 mM Tris (pH 7.5)–140 mM NaCl–200 μg of ethidium bromide per ml before being boiled in SDS lysis buffer (20 mM Tris [pH 7.5], 50 mM NaCl, 1% SDS, 1 mM DTT). The supernatant was diluted with 200 μl of RIPA and immunoprecipitated with 2.5 μl of anti-HA 12CA5 antibody (BAbCo) and protein A-Sepharose. After being washed three or four times, the proteins were analyzed by SDS-PAGE (12.5% polyacrylamide) and fluorography.

For cross-linking analysis, 25-μl translation reaction mixtures containing [^{35}S]methionine were diluted with 200 μl of 50 mM HEPES (pH 7.4)–150 mM NaCl–1% Triton X-100–10% glycerol–protease inhibitors. Following centrifugation at 14,000 × *g* for 30 min at 4°C, freshly prepared bis(succinimidyl) suberate (BS^3, Pierce) or buffer was added to a final concentration of 3 mM, the mixture was incubated for 30 min at room temperature, and the reaction was terminated for 15 min at room temperature with 250 mM glycine prior to SDS-PAGE (7.5% polyacrylamide) and fluorography.

EMSA. Annealed rat insulin I minienhancer oligonucleotides were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The electrophoretic mobility shift assay (EMSA) conditions were essentially as described previously (19). A total of 3 μl of unlabeled in vitro transcription/translation products were added to a 10-μl final reaction volume containing 20,000 cpm of probe (1 fmol), 10 mM HEPES (pH 7.7), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 3% Ficoll, 200 μg of poly(dI-dC)-poly(dI-dC) per ml, and 1% polyvinyl alcohol. The reaction mixtures were incubated at room temperature for 30 min and analyzed by nondenaturing polyacrylamide gel electrophoresis (5% polyacrylamide; 80:1 acrylamide/bisacrylamide ratio) in 0.5× Tris-borate-EDTA (TBE) at 4°C. All the proteins were generated from the pcDNA3M plasmid except for NLI (1-200fLID) and NLI (Δ200–300), which were generated from pcDNA3-FLAG.

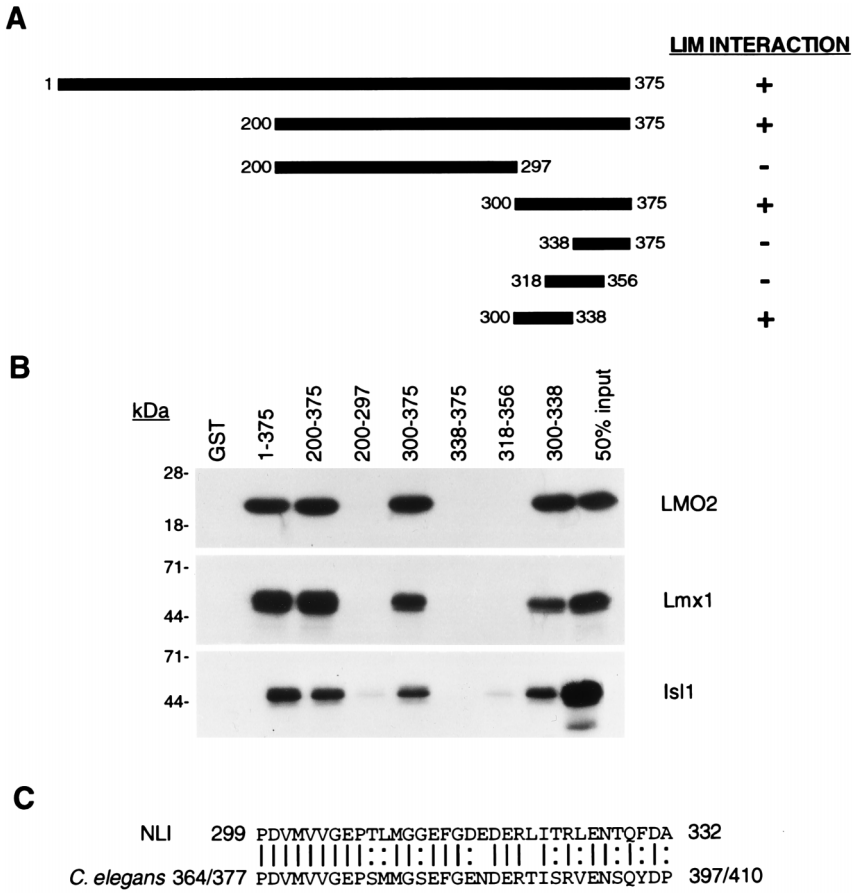

FIG. 2. Identification of the LID of NLI. (A) Representation of GST-NLI fusion constructs used for mapping experiments and summary of the results shown in panel B. Numbering indicates the amino acids of NLI included in each GST fusion construct. (B) GST fusions shown in panel A were incubated with the in vitro-translated, [^{35}S]methionine-labeled LIM proteins, and 50% of the in vitro-translated protein used in the binding reaction was loaded separately for estimation of binding affinity. Binding was visualized by SDS-PAGE and fluorography. (C) Homology of the LID to the predicted proteins encoded by alternatively spliced mRNAs derived from the *C. elegans* cosmid F58A3. Solid lines indicate amino acid identity, and colons represent similarity. Numbering reflects amino acid position, and for the *C. elegans* protein, dual numbering reflects differential amino acid positions resulting from alternative 5′ splicing of two mRNAs.

Lmx1 and its derivatives were translated in the presence of 1 mM $ZnCl_2$. Reticulocyte lysate containing pcDNA3M was used to equalize total protein. After translation, the proteins were diluted with an equivalent volume of PBS and centrifuged at 14,000 × *g* for 5 min at 4°C before being used in the EMSA. The GST fusions that eluted from the glutathione-agarose with 20 mM free glutathione were quantitated by Bradford analysis and diluted in PBS.

Cell lines, transfections, and enzymatic assays. Human kidney 293 EBNA cells (Invitrogen) were maintained in Dulbecco's modified Eagle's medium containing 10% enriched calf serum, and Tu6 pancreatic tumor cells (a gift from M. Montminy) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. For coimmunoprecipitation experiments, 293 cells in 100-mm dishes were transfected with 20 μg of cesium chloride-purified DNA by calcium phosphate precipitation (11). For luciferase assays, cells in 60-mm dishes were transfected with 10 μg of DNA. For each datum point, we used 0.5 μg of E47, 1.5 μg of Lmx1-containing pcDNA3M plasmids (cytomegalovirus [CMV] promoter), and 2.75 μg of luciferase reporter construct. A CMV–β-galactosidase plasmid (0.25 μg) was cotransfected for normalization of transfection efficiency, and empty pcDNA3M was used to equalize the total DNA concentrations. Cytoplasmic extracts for enzymatic assays were prepared by rapid freeze-thaw cycles in 0.1 M KH_2PO_4/K_2HPO_4 (pH 7.8)–1 mM DTT. The extracts were analyzed with a Monolight 2001 luminometer for luciferase activity after the addition of 100 μl of 1 mM luciferin (Analytical Luminescence Laboratory) to 300 μl of 0.1 M KPO_4 (pH 7.8)–5 mM ATP–15 mM $MgSO_4$. β-Galactosidase assays were performed in 50 mM Tris (pH 7.5)–100 mM NaCl–10 mM $MgCl_2$–0.75 mg of *o*-nitrophenyl β-D-galactopyranoside per ml, and the results were analyzed by measuring the optical density at 420 nm after stopping the reaction with an equivalent volume of 1 M Na_2CO_3.

Antibodies. The anti-NLI antibody used here has been described previously (25) and was diluted 1:5,000 for immunoblotting. The 39.4D5 monoclonal antibody was diluted 1:250 for anti-Isl1 immunoblotting. 39.4D5 was developed by Thomas M. Jessell and was obtained from the Developmental Studies Hybridoma Bank (Johns Hopkins University School of Medicine, Baltimore, Md.). The 12CA5 monoclonal anti-HA antibody (BAbCo) was used at 1:10,000 for immunoblotting.

RESULTS

NLI forms a complex with the endogenously expressed LIM homeodomain protein Isl1 in Tu6 cells and with transfected LIM domain transcription factors in 293 cells. We have previously shown that LIM domain transcription factors coprecipitate with NLI when human kidney 293 cells are cotransfected with NLI and LIM domain-expressing plasmids (25). However, it is unknown whether endogenously expressed NLI, which is found at modest levels in a wide variety of cell lines, associates with endogenous LIM domain proteins in vivo. Immunoblotting of nuclear extracts from the Isl1-expressing Tu6 pancreatic tumor cell line (32) revealed that this cell line also expresses NLI, which migrates identically to endogenously expressed NLI from 293 cells (Fig. 1A). Anti-Isl1 monoclonal antibodies recognized a nuclear protein from Tu6 cells which comigrated with Isl1 protein expressed exogenously in 293 cells, strongly suggesting that Tu6 cells express Isl1 and not an immunorelated protein. Finally, Isl1 from Tu6 nuclei was quantitatively precipitated by anti-NLI antibodies, indicating that most or all of the Isl1 in these cells is complexed with NLI.

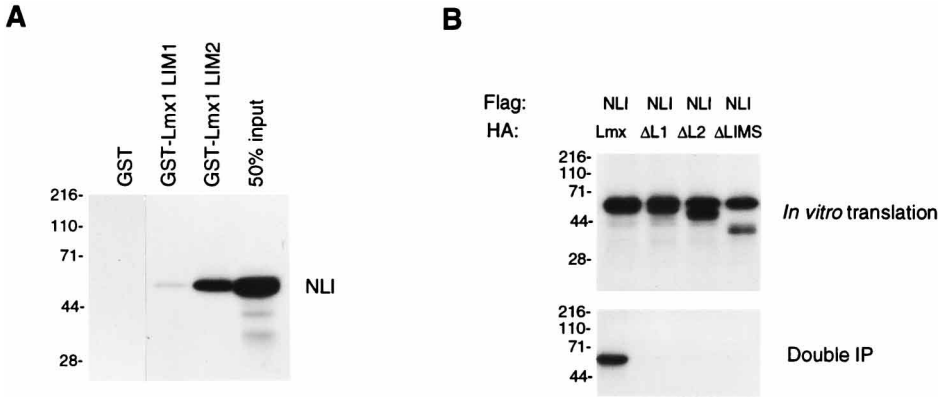

FIG. 3. Lmx1 LIM domain requirements for NLI interaction. (A) [^{35}S]methionine-labeled NLI was incubated with GST fusions of Lmx1 LIM domains as described for Fig. 2. (B) Double immunoprecipitation (IP) assay in which [^{35}S]methionine-labeled FLAG and HA epitope-tagged proteins were cotranslated in vitro. Complexes were first immunoprecipitated with anti-FLAG monoclonal antibodies, after which coprecipitating proteins were captured by anti-HA monoclonal antibodies followed by SDS-PAGE and fluorography. The upper panel shows 5% of the translated proteins used for the binding experiment, and the lower panel shows the results of the double immunoprecipitation. Lmx refers to full-length Lmx1, ΔL1 refers to a LIM1 deletion of Lmx1, ΔL2 refers to a LIM2 deletion of Lmx1, and ΔLIMS refers to a deletion of both LIM domains of Lmx1. Note that the molecular masses of Lmx1 and NLI are similar (382 and 375 amino acids, respectively) resulting in comigration of in vitro labeled proteins.

The doublet appearing in both Isl1-transfected 293 cells and Tu6 cells most probably includes differentially phosphorylated forms of Isl1. Transfected LMO2 (Rbtn2, Ttg-2) and the LIM homeodomain proteins Lmx1 and Isl1 were likewise found to be associated with endogenously expressed NLI in 293 cells (Fig. 1B). The cytoskeleton-associated LIM protein CRP, previously shown not to interact with NLI (25), did not coprecipitate in this experiment.

A carboxy-terminal 38-amino-acid region of NLI mediates LIM domain binding. To define the functional domains of NLI, the LIM interaction domain (LID) was mapped by an in vitro assay in which GST fusions of NLI fragments were incubated with [^{35}S]methionine-labeled LIM domain proteins. A small region encompassing amino acids 300 to 338 of NLI was sufficient for high-affinity binding to LMO2, Lmx1, and Isl1 (Fig. 2A and B). Further subdivision of this domain resulted in loss of binding (data not shown). The ability of all three divergent LIM domain proteins to interact with the same small domain of NLI suggests that NLI associates with all LIM domain-containing transcription factors via the LID. No obvious structural motifs could be identified in the LID region, with the exception of a hydrophobic patch (VMVV) near the amino terminus and an acidic patch (DEDE) at the beginning of a predicted α-helix. Of interest, amino acids 299 to 332, which lie within the identified LID, are highly homologous to the *Caenorhabditis elegans* cosmid F58A3 and related mRNAs, containing 32 of 34 similar or identical residues (Fig. 2C). In addition, amino acids 46 to 68 and 80 to 110 showed significant homology to the *C. elegans* clones, implying functional conservation of these three domains. We have previously shown that there is an interaction between the *C. elegans* LIM homeodomain protein mec-3 and NLI, consistent with the presence of an NLI-related protein in *C. elegans* (25). These results suggest that a small carboxy-terminal domain of approximately 38 amino acids mediates the association of NLI with LIM domain-containing transcription factors and has been selectively maintained throughout evolution.

NLI preferentially interacts with LIM2 of Lmx1 but requires both LIM domains for high-affinity binding. Using GST-LIM domain fusions and [^{35}S]methionine-labeled NLI, we previously showed that NLI preferentially associates with LIM1 of LMO2 and Isl1 but LIM2 of mec-3 (25). By convention, the more amino-terminal of the two LIM domains present in all LIM homeodomain and LMO proteins is designated LIM1 and the carboxy-terminal LIM domain is designated LIM2. Using the same assay, we found that NLI interacted strongly with Lmx1 LIM2 and much more weakly with LIM1 (Fig. 3A). Similar results were obtained from the reverse experiment in which [^{35}S]methionine-labeled LIM domain deletions of Lmx1 were incubated with GST-NLI (data not shown). However, under conditions of a double-immunoprecipitation experiment in which Lmx1 and NLI were present in equimolar amounts, both LIM domains were found to be required for NLI interaction (Fig. 3B). In this experiment, Flag-tagged NLI and hemagglutinin (HA)-tagged Lmx1 proteins were cotranslated in vitro in the presence of [^{35}S]methionine. Complexes were first immunoprecipitated with anti-FLAG antibodies, after which coprecipitating proteins were captured with anti-HA antibodies for analysis. In this manner, the binding requirements of the comigrating NLI and Lmx1 could be investigated. Deletion of either LIM1 or LIM2 abolished the interaction of Lmx1 with NLI (Fig. 3B). Thus, under conditions in which a binding partner is present in great excess, as in the GST fusion experiments, a single LIM domain was sufficient for NLI interaction, but under double immunoprecipitation conditions, in which the partners are found at equimolar concentrations, two LIM domains were absolutely required.

The amino-terminal 200 amino acids of NLI mediate NLI homodimerization, but the dimerization state of NLI does not affect its interaction with Lmx1. Because many transcription factors form homodimers in addition to heterodimers and because dimerization is often involved in the regulation of transcriptional activity, the possibility of NLI self-association was investigated. To determine if NLI was capable of forming homodimers, the same double-immunoprecipitation approach described for Fig. 3B was used. HA epitope-tagged NLI coimmunoprecipitated with FLAG-tagged full-length NLI, NLI(1–200), and NLI(Δ200–300), indicating that NLI is capable of forming homodimers through a dimerization domain which lies within the amino-terminal 200 amino acids (Fig. 4A). This dimerization region could not be further subdivided, indicating that either a large surface or nonadjacent elements are required for dimerization. Chou-Fasman secondary-structure algorithms predict a long α-helical segment extending from

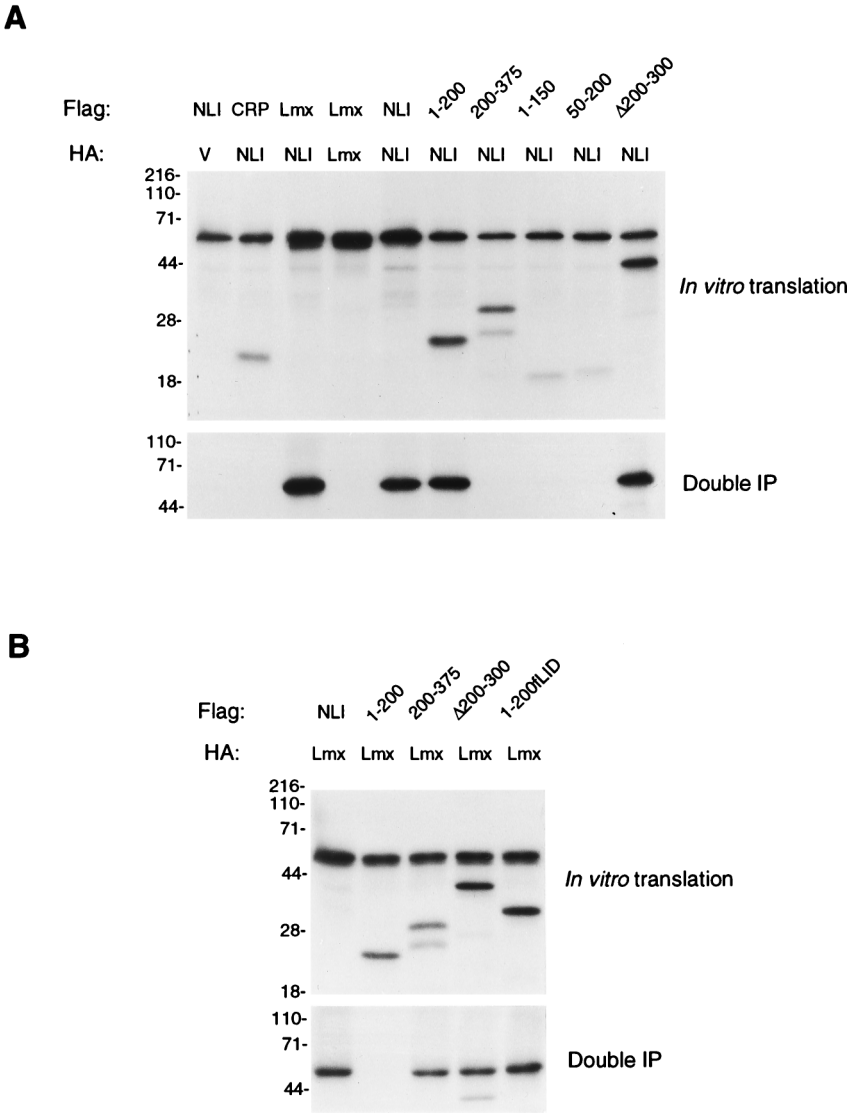

FIG. 4. NLI homodimerization requirements and the effects of homodimerization on interaction with Lmx1. (A) Double immunoprecipitations (IP) similar to those in Fig. 3B, in which complexes were precipitated by anti-FLAG antibodies, followed by precipitation with anti-HA antibodies. The upper panel reflects 5% of the proteins used for the binding assay for estimation of translation efficiency, and the lower panel shows the results of the double immunoprecipitation. Numbers in the FLAG-tagged row indicate the amino acid numbers of NLI fragments, and Δ200–300 represents a deletion of NLI amino acids 200 to 300. V refers to the HA vector pcDNA3M. (B) Double immunoprecipitation assay with numbering indicating NLI amino acid fragments included in each FLAG construct. 1–200fLID represents a construct containing the amino-terminal 200 amino acids of NLI (dimerization domain) fused to the LIM interaction domain (LID, residues 300 to 338) of NLI.

amino acids 142 to 192, which contains a contiguous stretch of six hydrophobic residues (Met and Ile) spaced seven amino acids apart, reminiscent of a hydrophobic zipper. This region, and/or the 46 to 110 region encompassing the domains conserved in *C. elegans*, could be involved in NLI homodimerization. The NLI-NLI interaction was of comparable affinity to the Lmx1-NLI interaction, which has been determined by several different methods of analysis to be very high. HA-NLI did not coprecipitate with FLAG-CRP as expected. In addition, HA-Lmx1 did not coprecipitate with FLAG-Lmx, indicating that Lmx1 does not self-associate in solution.

NLI dimerization could either be required for or prevent the interaction with LIM domains. However, NLI(200–375), which is incapable of homodimerization (Fig. 4A) but contains the LID, bound Lmx1 with similar affinity to full-length NLI in the double-immunoprecipitation assay (Fig. 4B). Likewise, fusion of the dimerization domain to LID-containing fragments (Δ200–300 and 1–200fLID), did not alter high-affinity binding to Lmx1 (Fig. 4B).

Because the double-immunoprecipitation assay could not distinguish between dimers and higher-order complexes, in vitro cross-linking was used to investigate the stoichiometry of the Lmx1-NLI complexes. Treatment of in vitro-translated [^{35}S]NLI with the cross-linking agent BS^3 indicated the presence of NLI dimers, while treatment of Lmx1 alone showed no indication of dimerization (Fig. 5A), in agreement with the double-immunoprecipitation assay results. NLI, Lmx1, and cross-linked species run anomolously high on SDS-PAGE, with monomers displaying apparent molecular masses of approximately 60 kDa. Cross-linking of NLI cotranslated with Lmx1 resulted in a ~120-kDa band, which most probably consisted of comigrating NLI homodimers and NLI-Lmx1 het-

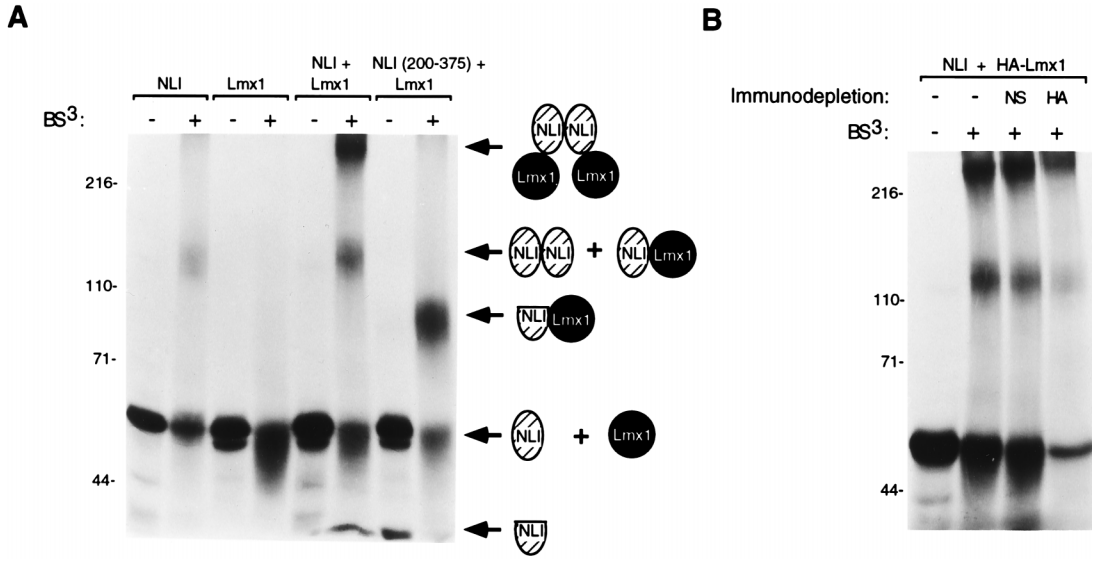

FIG. 5. Higher-order complexes containing NLI and Lmx1. (A) [^{35}S]methionine-labeled proteins cotranslated from pcDNA3M were treated (+) or not treated (−) with the cross-linking agent BS^3, followed by SDS-PAGE and fluorography. The likely constituents of the cross-linked species are depicted on the right. (B) Proteins were cotranslated as in panel A, except that NLI was produced from pcDNA3L (no tag). The complexes were immunodepleted with a nonspecific monoclonal antibody (NS) or an anti-HA monoclonal antibody (HA) prior to cross-linking.

erodimers, as well as a higher-molecular-weight complex. The high-molecular-weight complex generated by cross-linking probably represents 2 NLI-2 Lmx1, running well above the 216-kDa molecular mass marker. Formation of the large complex was dependent on NLI homodimerization, because cross-linking of truncated NLI lacking the dimerization domain (200 to 375) with Lmx1 resulted in only a single complex which migrated faster than NLI homodimers (Fig. 5A). The presence of Lmx1 in both the ~120- and ~240-kDa complexes was confirmed by specific immunodepletion of both complexes by an anti-HA antibody that recognized only Lmx1 (Fig. 5B). These results indicate that while NLI is able to homodimerize through the amino-terminal 200 amino acids, dimerization is not a prerequisite for binding to Lmx1 and does not interfere with binding to Lmx1. Higher-order complexes composed of two NLI molecules with two Lmx1 molecules indicated that NLI homodimerization and NLI-Lmx1 heterodimerization are not mutually exclusive events.

Requirements for complex formation between NLI and Lmx1 on DNA. To investigate whether the interactions defined in solution also applied to DNA-bound LIM homeodomain proteins, we analyzed the effects of NLI on Lmx1 binding to the rat insulin I minienhancer element by EMSA. Lmx1 has previously been shown to bind specifically to a TAAT site within this DNA element and to synergize with the bHLH protein E47, which binds to an adjacent E box, to generate high levels of transcription (19). Unlabeled, in vitro-translated NLI did not bind to the DNA probe (Fig. 6, lane 2), consistent with the lack of a known DNA binding domain. Specificity for HA-tagged Lmx1 binding to the probe was demonstrated by competition with cold probe and by supershifting with the anti-HA antibody (lanes 3 to 5). Increasing amounts of NLI progressively supershifted the Lmx1-DNA complex, indicating that NLI is capable of associating with DNA-bound Lmx1 (lanes 6 to 8). However, at higher NLI concentrations, inhibition of Lmx1 DNA binding was noticeable, as judged by the reduced intensity of the total signal compared to Lmx1 alone or Lmx1 supershifted with the anti-HA antibody (compare lane 8 with lanes 3 and 5). Addition of anti-NLI polyclonal antibodies to the Lmx1-NLI-DNA complex returned Lmx1 binding to its original state (lane 9). The anti-NLI antibodies strongly recognize the LID region of NLI (data not shown), compatible with interference with NLI and Lmx1 binding at the high antibody concentrations used in the EMSA. In vitro-translated E47 did not yield a protein-DNA complex under these EMSA conditions, and addition of E47 did not alter the complexes observed with Lmx1 alone or with Lmx1 plus NLI (data not shown). These results indicate that the affinities of E47 for this DNA element and for Lmx1 are significantly lower than those of Lmx1 and NLI, despite the strong transcriptional synergy observed in vivo (19).

To evaluate the LIM domain and NLI requirements for complex formation on DNA, various constructs were analyzed by EMSA. As in the double-immunoprecipitation assay (Fig. 3B), both LIM domains were required for an effect of NLI on Lmx1-DNA complexes. Deletion of one or both LIM domains of Lmx1 prevented NLI supershifts (Fig. 7A, lanes 4, 6, and 8). Of interest, deletion of LIM domains did not significantly alter the affinity of Lmx1 DNA binding (lanes 1, 3, 5, and 7). This is in contrast to reported negative effects of LIM domains on DNA binding and transcription by some LIM homeodomain proteins (5, 43, 49, 58).

The LID-containing region of NLI was necessary to mediate NLI effects in the EMSA (Fig. 7B, lanes 3 to 5), while the dimerization domain was not required (lane 6). Although NLI(200–375) did not dramatically alter the migration of Lmx1 (lane 6), the presence of the NLI polypeptide in the complex was confirmed both by supershifting with an anti-epitope tag antibody and by disrupting the complex with the anti-NLI antibody (data not shown). The significance of the greater abundance of the Lmx1-NLI(200–375) complex compared to the Lmx1–full-length NLI complex is unclear (lane 6 versus lane 2) but could reflect an involvement of the dimerization domain in the partial inhibition of Lmx1 DNA binding by full-length NLI. However, it is more likely that the differential intensities may simply result from a more concentrated signal of the slightly shifted (200–375)-containing complex compared to the more diffuse signal from the slowly migrating, full-length

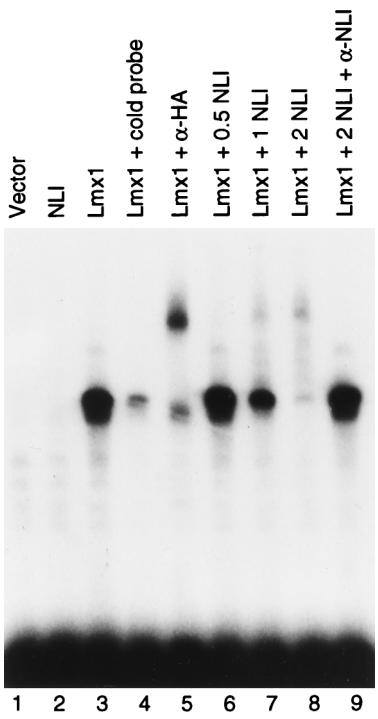

FIG. 6. Lmx1 and NLI complex formation on the rat insulin I minienhancer element as shown by EMSA with unlabeled proteins generated by in vitro translation to analyze binding to a [^{32}P]labeled DNA probe (−247 to −198 of the rat insulin I promoter). Vector refers to a pcDNA3M-containing translation reaction which was also used to equalize the total protein in each sample. Unlabeled probe used for competition was added at a 1,000-fold molar excess to the labeled probe. A 1-μl volume of Lmx1 was used in each lane, and the indicated volume (microliters) of NLI was included. α-HA refers to the 12CA5 monoclonal antibody, and α-NLI refers to anti-NLI polyclonal antibodies.

NLI-containing complex. Fusions of the dimerization domain to LID-containing fragments were capable of supershifting Lmx1 (lanes 7 and 8), albeit less completely than full-length NLI or NLI(200–375). Two supershifted complexes were observed for each of the constructs containing the dimerization domain, consistent with the cross-linking results shown in Fig. 5, which demonstrated the existence of both heterodimeric and higher-order complexes containing NLI multimers. The LID or the LID-containing carboxy-terminal 75 amino acids fused to GST were likewise capable of supershifting the complex (lanes 9 and 10), reconfirming that the LID defines a discrete domain sufficient for LIM domain binding. GST alone had no effect on Lmx1 DNA binding (data not shown). These experiments demonstrate that NLI is capable of associating with DNA-bound Lmx1, where it would be in a position to affect transcriptional processes. Thus, the requirements for the interaction of NLI with Lmx1 on DNA are the same as those for interaction in solution; i.e., both LIM domains of Lmx1, as well as the LID of NLI, are necessary to form NLI-Lmx1-DNA complexes. In addition, the ability of NLI to dimerize was not required for the association of NLI with DNA-bound Lmx1.

NLI negatively regulates the Lmx1/E47 synergistic activation from the rat insulin I minienhancer element. To address the functional consequences of the binding of NLI to Lmx1, we tested the effects of expression of NLI, Lmx1, and E47 on a luciferase reporter construct driven by five tandem copies of the rat insulin I minienhancer upstream of a minimal herpes simplex virus thymidine kinase promoter in 293 cells. None of these proteins significantly altered the activity from the reporter plasmid lacking the minienhancer (data not shown). Consistent with the inability of NLI to bind to the minienhancer in the absence of Lmx1 in vitro, reporter gene activity downstream of the minienhancer was unaffected by transfection of the maximal level of NLI used in the experiment (Fig. 8A). Surprisingly, transfection of increasing amounts of NLI had no effect on the ~8-fold activation by Lmx1 alone, nor did the expression of exogenous NLI affect transcription resulting from E47 alone (~16-fold).

As expected, the effects of Lmx1 plus E47 were strongly synergistic over those of either alone (Fig. 8B), in agreement with results of previous studies with BHK cells (19). Because of the phenotypic synergy observed between NLI and the LIM homeodomain protein Xlim-1 in injected *Xenopus* embryos, we expected a positive effect of NLI on Lmx1- or Lmx1/E47-mediated transcription. However, the introduction of increasing amounts of NLI progressively reduced the synergistic effects of Lmx1 plus E47 by 75%, in a dose-dependent manner. β-Galactosidase activity, used as an internal control to correct for transfection efficiency, showed no reduction in cells transfected with NLI, and Lmx1 and E47 levels were unaffected by NLI overexpression (Fig. 8B). Thus, NLI-mediated transcriptional inhibition did not result from a general inhibition of the CMV promoter. The flattening of the inhibitory NLI curve correlated with levels of NLI expression in the transfected cells as assayed by immunoblotting, in which the initial 10-fold induction of NLI expression observed from 0 to 1 NLI transfected was followed by a less than twofold increase from 1 to 2 and from 2 to 3 NLI transfected (Fig. 8C). The profile of NLI expression therefore suggests that complete inhibition of Lmx1/E47 synergy was not observed due to limitations in NLI expression, which barely exceeded the expression level of transfected Lmx1 (Fig. 8B). Deletion of the LID prevented NLI inhibition of Lmx1/E47 synergy, indicating that a specific association between Lmx1 and NLI through the LID is required for this effect (Fig. 8B). NLI(1–200) similarly had no effect on Lmx1/E47 synergy because it lacks the LID. However, NLI(200–375), which includes the LID but is incapable of dimerization, decreased Lmx1/E47 transcription levels by 86%, clearly indicating that NLI-mediated inhibition is independent of the homodimerization domain.

Reporter gene assays thus showed an NLI-mediated inhibition of Lmx1/E47 transcriptional synergy from the rat insulin I minienhancer element, without effects on basal levels of Lmx1 or E47 activity. This inhibition was specific for Lmx1/E47 synergy because NLI had no effect on transcription from the reporter construct alone, Lmx1 alone, E47 alone, or the expression levels of either of these proteins or the internal control β-galactosidase. Furthermore, inhibition was absolutely dependent on the LID of NLI and hence on the LID-LIM interaction between NLI and Lmx1, but it did not require homodimerization of NLI.

DISCUSSION

While the requirements for LIM homeodomain and LMO proteins for developmental processes are well documented, little is known about the mechanisms through which they affect transcription or the gene targets they regulate. Although Isl1 expression in the developing neural tube is necessary for the formation of motor neurons and interneurons (39), neuron-specific gene targets and cofactors for Isl1 have yet to be described. In addition, little is understood about the components of higher-order transcription complexes involving LIM domain-containing transcription factors on target promoters. However, two notable examples of synergistic transcriptional

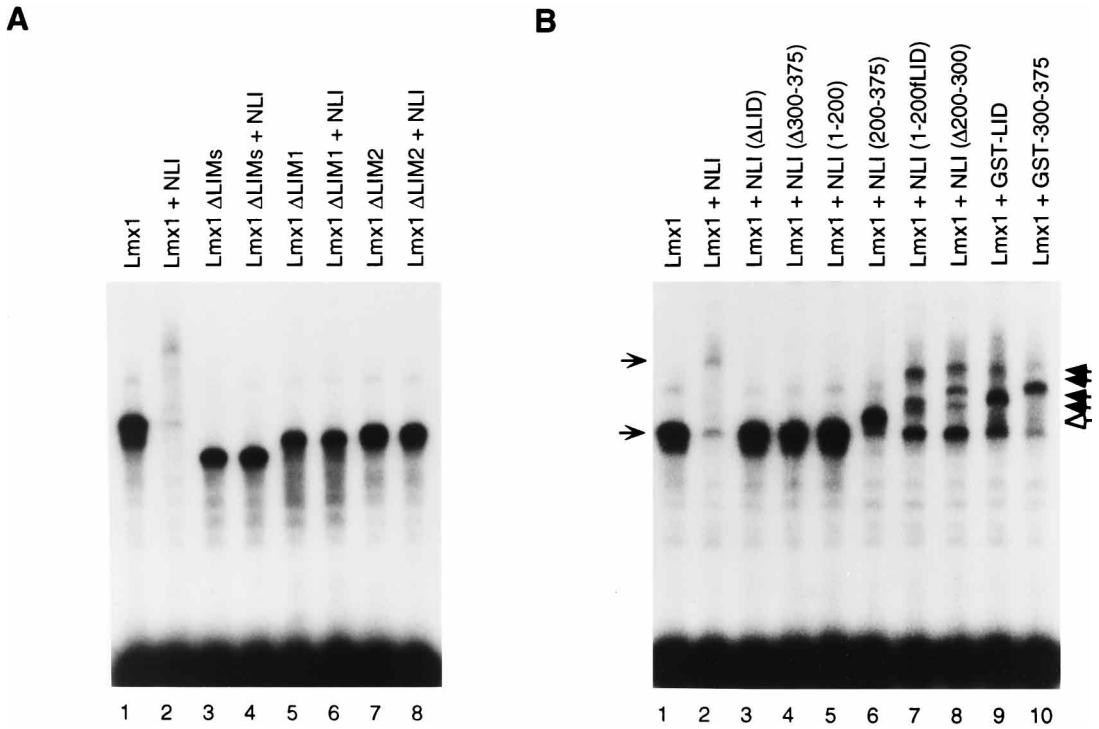

FIG. 7. Lmx1 LIM domain requirements and NLI requirements for complex formation on the rat insulin I minienhancer element. (A) EMSA showing the effects of NLI on the binding of various derivatives of Lmx1 to the minienhancer. Lmx1 proteins (1 μl) and NLI (2 μl) were used in the binding reactions. ΔLIMs refers to a deletion of both LIM domains of Lmx1, ΔLIM1 refers to deletion of LIM1, and ΔLIM2 refers to deletion of LIM2. (B) EMSA showing the effects of various NLI derivatives on Lmx1 DNA binding. ΔLID represents a deletion of the LIM interaction domain (300 to 338) of NLI, Δ300–375 reflects a C-terminal truncation, 1–200fLID represents a fusion of amino acids 1 to 200 with the LID, and Δ200–300 refers to a deletion of amino acids 200 to 300. GST fusion proteins were used at approximately 10-fold molar excess with respect to Lmx1, as evaluated by quantitative Western blotting of in vitro-translated proteins. The open arrow indicates the supershifted complex containing NLI (200 to 375) in lane 6; the black arrows indicate supershifted complexes for the constructs containing the dimerization domain in lanes 7 and 8. The small arrows on the left indicate Lmx1 alone and Lmx1 supershifted with NLI.

responses involving LIM homeodomain proteins have been described. In one case, the pituitary- and neuron-specific LIM protein Lhx3 (mLim-3, P-lim) was shown to synergize with the pituitary-specific POU homeodomain protein Pit-1 on the α-GSU, prolactin, and TSH-β promoters (5). Direct interaction between these factors via the LIM domains of Lhx3 was demonstrated, and the LIM domains were required for transcriptional synergy. Remarkable synergy between the LIM homeodomain protein Lmx1 and the ubiquitously expressed bHLH protein E47 on the rat insulin I promoter has also been demonstrated (19). While these factors independently occupy nearby sites on the DNA, they have recently been shown to physically interact with one another through the second LIM domain of Lmx1, and both LIM domains are required for synergy (24a, 19). It was therefore important to consider the functional consequences of the high-affinity nuclear LIM domain binding protein NLI on this synergistic relationship. In the present study, two functional domains of NLI were identified: a LIM-interacting domain and a homodimerization domain (Fig. 9A). In addition, we found that association of NLI with the LIM homeodomain protein Lmx1 inhibited the strong transcriptional synergy of Lmx1 and E47 on the rat insulin I minienhancer.

In vivo interactions of NLI with LIM domain transcription factors. NLI has been shown to form high-affinity complexes with LIM homeodomain and LMO proteins in vitro and in cotransfection experiments in vivo (1, 25). In this study, we show that nearly all of the endogenously expressed Isl1 in the Tu6 cell line is complexed with endogenous NLI. Similarly, endogenous NLI in 293 cells was found to associate with transfected LMO2, Lmx1, and Isl1. Because NLI is expressed at moderate levels throughout embryonic and adult tissues (1, 25) and in all cell lines tested (25) (see above), it is likely that endogenously expressed LIM domain transcription factors are commonly found in a complex with NLI. It remains possible, however, that NLI-LIM domain interactions are regulated by the expression levels of these proteins or competing proteins or by phosphorylation events.

Protein-protein interaction domains of NLI. The only precise LIM domain target requirements that have been described thus far are for the cytoplasmic protein Enigma, which contains three carboxy-terminal LIM domains (20, 57). Tyrosine-containing tight turns were found to be required for the interaction of Enigma LIM2 with the Ret tyrosine kinase and the interaction of Enigma LIM3 with the insulin receptor tyrosine kinase (56, 57). The present study shows that NLI contains a discrete 38-amino-acid carboxy-terminal LID region, which mediates the interaction with LIM domain-containing transcription factors. However, no tyrosine residues are found within the LID of NLI, and so a non-tyrosine-based recognition motif must be responsible for the interaction. The LID was absolutely required for interaction with Lmx1 in solution and on DNA, as well as for the functional effects of NLI on Lmx1-mediated transcription. The near identity of this region between the murine NLI and an NLI-related *C. elegans* protein argues for an evolutionarily conserved dependence of NLI function on the LID.

A larger, 200-amino-acid, amino-terminal domain was found

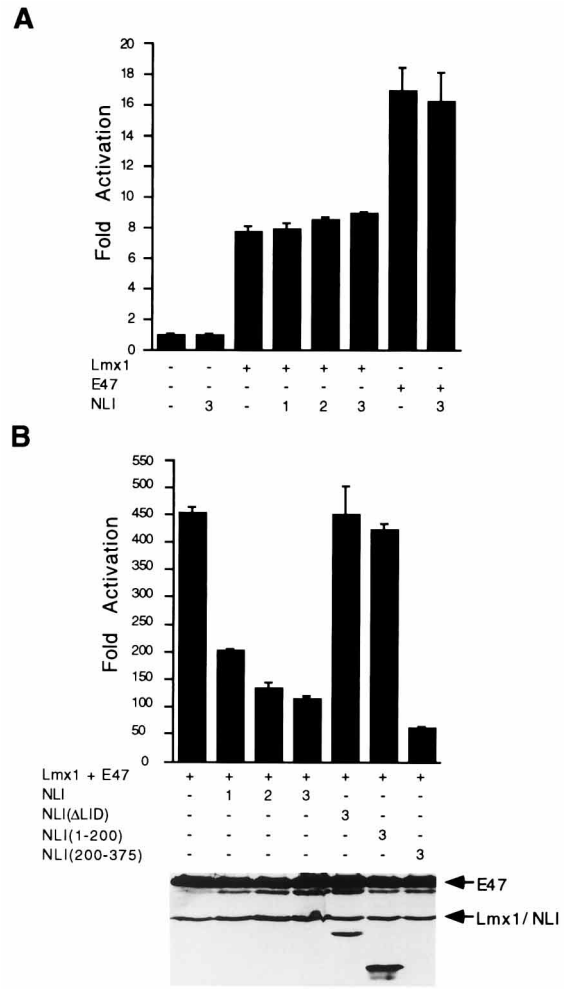

FIG. 8. Effects of NLI on Lmx1 and Lmx1/E47 transcriptional activation from the rat insulin I minienhancer. 293 cells were cotransfected with a luciferase reporter containing five copies of the rat insulin I minienhancer and CMV-driven expression plasmids (pcDNA3M) expressing Lmx1, E47, and NLI. The amount of NLI or NLI derivatives indicated represents the ratio of the amount of NLI-expressing plasmid relative to the amount of Lmx1 expressing plasmid (micrograms) included in the transfection. Reported values indicate the mean fold activation over the luciferase reporter transfected with pcDNA3M alone ± standard error of the mean for three independent samples. Protein expression was verified by immunoblotting nuclear extracts from assayed cells with the anti-HA monoclonal antibody, and the results shown are representative of at least three similar experiments. (A) Luciferase assay showing the effects of NLI on Lmx1-mediated and E47-mediated transcription. (B) The effects of NLI and NLI derivatives on Lmx1/E47 transcriptional synergy. For NLI(1–200) in which the putative nuclear localization signal sequence was deleted, a simian virus 40 T-antigen nuclear localization signal sequence was added to the amino terminus. (C) Densitometry scanning of an anti-NLI immunoblot from an identical transfection to that shown in panel B, showing the endogenous and transfected expression levels of NLI.

to serve as an interface for NLI homodimerization. However, because homodimerization of NLI exerted no influence over interactions with Lmx1 or effects of NLI in transcription assays, it is unclear what functional role NLI self-association plays. Our present studies indicate that NLI monomers and dimers are equally capable of interacting with Lmx1 and that tetrameric complexes of 2NLI-2Lmx1 which are dependent on the NLI dimerization domain are capable of forming. Because Lmx1 does not homodimerize in solution, it is probable that one function of NLI is to generate LIM transcription factor homodimers. LIM-LIM interactions between LIM-containing transcription factor proteins have been observed in vivo but not demonstrated in vitro (3), suggesting that endogenously expressed NLI may participate in the interaction.

It is also possible that homodimerization of NLI mediates the heterodimerization between different LIM domain transcription factors. It has been suggested that a LIM homeodomain combinatorial code defines the identity of specific motor neuron populations (50). Alternative combinatorial expression of *Isl1*, *Isl2*, *Lhx1* (*Lim1*), and *Lhx3* (*Lim3*) correlates with the identity and subsequent innervation targets of motor columns, suggesting that synergy of these proteins on specific gene targets may occur. NLI may play a role in assembling such complexes on target promoters.

NLI effects on Lmx1-dependent transcription from the rat insulin I minienhancer. The present studies also demonstrate that NLI negatively regulates transcriptional synergy by Lmx1 and E47 on the rat insulin I minienhancer, requiring the LID but not the homodimerization domain of NLI. Because NLI

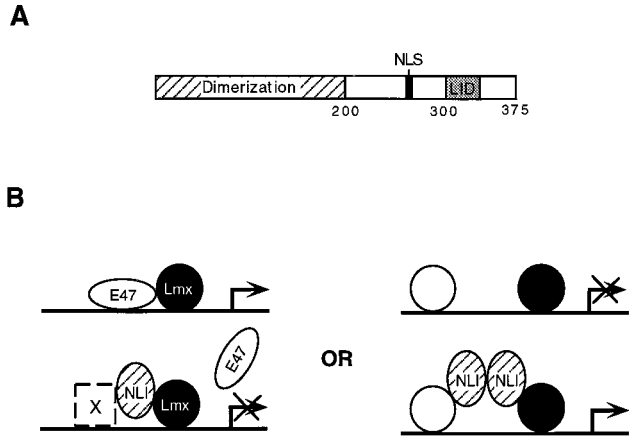

FIG. 9. (A) Schematic representation of the functional domains of NLI. NLS represents the putative nuclear localization signal sequence. (B) Proposed models for NLI function in transcription. A possible mechanism for NLI-mediated repression of transcription is depicted on the left. A hypothetical protein (X) may or may not be required for NLI-mediated inhibition of Lmx1/E47 transcription from the rat insulin I minienhancer. NLI may also function as a dimer in this context, but the ability to dimerize does not alter its activity. A possible mechanism for NLI-mediated transcriptional activation is illustrated on the right. In this context, NLI may function as an adapter protein to mediate the interaction between two LIM domain transcription factors via NLI homodimerization or between a LIM and a non-LIM factor. Dimerization of NLI may affect the involvement of NLI in positive transcriptional events involving a LIM and a non-LIM factor.

did not affect Lmx1-stimulated transcription alone, it is unlikely that the pronounced inhibition of the synergistic responses to Lmx1 plus E47 resulted from the reduced binding of Lmx1 to DNA seen with high concentrations of NLI in EMSA. In addition, the modest levels of NLI generated in the transfection experiments are probably well below the concentrations used in the EMSA. It remains possible, however, that under certain circumstances, high levels of NLI expression inhibit Lmx1 transcription by interfering with DNA binding.

It has recently been shown that Lmx1 and E47 directly contact one another with low affinity through LIM2 of Lmx1 and that Lmx1/E47 synergy is dependent on the presence of both LIM domains but, most importantly, LIM2 (24a). Intriguingly, NLI preferentially binds to LIM2 of Lmx1, but both LIM domains are required for high-affinity interaction. A similar situation has been described for other LIM domain-containing proteins, in which a single LIM domain mediates target specificity while a second, nonspecific LIM domain is additionally required for the interaction (3). Because NLI binds with high affinity to the LIM domains of Lmx1, it is likely that NLI displaces the lower-affinity interaction between Lmx1 and E47, thereby disrupting the Lmx1/E47 transcriptional synergy. It remains possible that NLI contacts an as yet unidentified protein, which displaces E47 from Lmx1 or the DNA (Fig. 9B, left). Although NLI inhibition of Lmx1/E47-mediated insulin expression in pancreatic cells is unlikely, NLI may function to edit the activity of this promoter in non-insulin-producing cells which also express Lmx1 and E47. For example, Lmx1 and the ubiquitous E47 are expressed in the developing notochord, neural tube, and limb bud of the embryo (41). Insulin production in these environments might therefore be abrogated by the high levels of NLI expressed in these regions during early development. Thus, while NLI edits an inappropriate Lmx1/E47-dependent transcriptional event, it may simultaneously serve to enhance Lmx1 homodimeric or heterodimeric transcription at another promoter within the same cell.

Positive roles for NLI in development and transcription have also been demonstrated. It has been reported that NLI synergizes with the LIM homeodomain protein Xlim-1 in the formation of ectopic muscle tissue and partial secondary axes when coinjected ventrally into *Xenopus* embryos (1). In addition, a protein closely related to NLI has recently been shown to mediate synergy between Lhx3 and the bicoid class homeodomain protein P-OTX on the α-*GSU* promoter, suggesting that NLI activates transcription under such circumstances by mediating interactions between synergizing elements (4) (Fig. 9B, right). Finally, our previous studies colocalized high levels of NLI protein with the onset of Isl1 expression in developing motor neurons (25). Because Isl1 is required for motor neuron formation (39), it is unlikely that NLI negatively regulates Isl1 activity at this stage of neuronal development.

NLI is thus postulated to be a versatile adapter protein which can both facilitate and prevent the interaction of LIM homeodomain and LMO proteins with other transcription factors; the resultant response can be to either enhance or repress transcription, depending on the promoter context. Identification of promoters involved in the developmental effects of LIM domain transcription factors will provide a valuable means for exploring the function of NLI in transcriptional processes during development.

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