The Homeodomain Coordinates Nuclear Entry of the Lhx3 Neuroendocrine Transcription Factor and Association with the Nuclear Matrix*

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LIM homeodomain transcription factors regulate development in complex organisms. To characterize the molecular signals required for the nuclear localization of these proteins, we examined the Lhx3 factor. Lhx3 is essential for pituitary organogenesis and motor neuron specification. By using functional fluorescent derivatives, we demonstrate that Lhx3 is found in both the nucleoplasm and nuclear matrix. Three nuclear localization signals were mapped within the homeodomain, and one was located in the carboxyl terminus. The homeodomain also serves as the nuclear matrix targeting sequence. No individual signal is alone required for nuclear localization of Lhx3; the signals work in combinatorial fashion. Specific combinations of these signals transferred nuclear localization to cytoplasmic proteins. Mutation of nuclear localization signals within the homeodomain inhibited Lhx3 transcriptional function. By contrast, mutation of the carboxyl-terminal signal activated Lhx3, indicating that this region is critical to transcriptional activity and may be a target of regulatory pathways. The pattern of conservation of the nuclear localization and nuclear matrix targeting signals suggests that the LIM homeodomain factors use similar mechanisms for subcellular localization. Furthermore, upon nuclear entry, association of Lhx3 with the nuclear matrix may contribute to LIM homeodomain factor interaction with other classes of transcription factors.

A family of homeodomain transcription factors, initially named for three members (lin-11, Isl-1, and mec-3), shares a cysteine-rich domain containing two zinc-coordinated structures, referred to as the LIM domain (1, 2). The LIM domain has been shown to mediate protein-protein interactions between LIM and other proteins (1, 2). In addition to the LIM homeodomain (LIM-HD) proteins, the LIM domain superfamily includes factors lacking HDs, including kinases, GTPase-activating factors, and proteins that contribute to cellular architecture (1, 2).

LIM-HD proteins have been implicated in controlling developmental processes in many species, and aberrant activities of members of this class are associated with several human diseases (1, 2). In Caenorhabditis elegans, Mec-5 regulates mechanosensory cell differentiation (3), and in Drosophila, Apterous controls the fate of the wing imaginal disc (4). LIM homeobox genes such as Isl-1, Isl-2, and Lmx-1 are involved in the organization of motor neurons in the chick, zebrafish, and mouse (1, 2). LIM-HD factors in Xenopus are involved in neuroendocrine function and in induction by the Spemann organizer (1, 2). Gene knockout studies have established critical roles for LIM-HD factors in mammalian development. For example, embryos lacking the Lim1 gene do not develop head structures (5); mice deficient in Isl-1 lack motor neurons (6); and animals without Lhx4/Geh-4 die due to incomplete lung development (7). Mutations in the human LMX-1B gene are responsible for nail patella syndrome, a disease typified by maldevelopment of the fingernails, elbow joints, and knuckles (8).

To function as gene regulatory proteins, transcription factors must locate to the nucleus of the cell. The nuclear pore complex (NPC) mediates nuclear entry. Nuclear transport is a highly selective, ATP-dependent process that requires several components, including proteins that comprise the NPC (termed nucleoporins), and the presence of a nuclear localization signal (NLS) within the transported protein (9, 10). Although numerous types of NLSs have been reported, typically they are concise or multipartite sequences that contain regions of basic amino acids. These sequences can be located in all contexts within nuclear proteins, and proteins may utilize one or several NLS sequences (9–11). Multiple NLS sequences may function in combinatorial or redundant fashion (9, 10).

Within the nucleus, proteins may undergo additional trafficking, including targeting to the nuclear matrix. The nuclear matrix is operationally defined as the proteinaceous substructure that resists nuclease digestion and high salt extraction (12) and is composed of proteins such as the nuclear mitotic apparatus protein, NuMA (13–15). It has been suggested that transcriptionally active genes are associated with the nuclear complex; NLS, nuclear localization signal; EGFP, enhanced green fluorescent protein; GSU, a glycoprotein subunit; EMSA, electrophoretic mobility shift assays; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; PBS, phosphate-buffered saline; TSH, thyroid-stimulating hormone.

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matrix, and several proteins involved in gene regulation have recently been demonstrated to be targeted to the matrix. These include the pituitary transcription factor Pit-1 (16) and histone acetyltransferase (17). The nuclear matrix may provide a functional scaffold for chromatin and has been proposed to mediate the actions of both extranuclear and extracellular regulatory signals that result in altered gene expression (18).

Although putative domains necessary for nuclear localization of some non-LIM HD proteins have been described, the mechanisms that promote nuclear entry of HD and non-HD LIM proteins are not well understood. Sequences within the HD in the yeast repressor o2 (19), mammalian Tif-1 (20), mammalian Tst-1/Oct-6 (21), and plant KNOTTED-1 (22) non-LIM homeoproteins mediate nuclear localization. Some HD proteins also exhibit regulated nuclear localization. For example, the nuclear accumulation of the Drosophila Extradenticle HD factor is promoted by its association with Homothorax, another HD protein (23). In addition, some non-HD LIM proteins such as SLIMMER contain NLSs (24). Other types of LIM proteins interact with regulatory nuclear LIM interactor (NLI) proteins (also known as Lbd1/CLIM/Chip) via LIM domain-dependent associations (25–27). NLI factors mediate the dimerization and modulate the transcriptional activities of LIM-HD proteins (28–30). Furthermore, NLI has been demonstrated to facilitate the nuclear localization of the non-LH3 HD protein LMO4 (31).

In this study, to investigate the mechanism by which LIM-HD proteins enter the cell nucleus, the signals required for nuclear entry and nuclear matrix association of the Lhx3 LIM-HD molecule (also known as P-Lim/LIM-3) were characterized. In rodents, the Lhx3 gene is expressed in specific regions of the developing central nervous system and then becomes restricted to the cells of the developing pituitary and their derivatives in the adult gland (32–34). Analyses of mice with deleted Lhx3 genes have revealed that Lhx3, and the related Lhx4 protein, are critical for the early structural development of the anterior and intermediate pituitary lobes and for the subsequent differentiation of the hormone-secreting cell types that characterize these tissues (35, 36). Furthermore, transient expression of Lhx3 (and the coordinated actions of the Lhx4, Isl-1, and Isl-2 LIM-HD factors) within the nervous system during early embryogenesis is critical for the specification of motor axons (37). Importantly, the primary sequence of Lhx3 is highly conserved from Drosophila to humans (38–40). Lhx3 has two LIM domains, a central HD and a carboxyterminal conserved sequence known as the LIM-3/Lhx3-specific domain (40). Our laboratory and others (33, 39–41) have demonstrated that Lhx3 activates pituitary trophic hormone genes, acting either alone or in cooperation with other transcription factors, such as Pit-1.

We demonstrate that nuclear localization of Lhx3 is not dependent on the LIM domains but requires multiple sequences within and near the HD. Identified NLS-containing domains were linked to green fluorescent protein to confirm their function in nuclear localization. The NLSs lie within Lhx3 protein domains important for transcriptional activity. The identified nuclear localization sequences are conserved within the Lhx3 family of proteins and in LIM-HD proteins in general, suggesting that other LIM-HD proteins that serve as essential developmental transcription factors may use similar complex signals for nuclear translocation. In addition, we demonstrate that Lhx3 is associated with the nuclear matrix and that sequences within the HD mediate targeting to the matrix.

**Experimental Procedures**

Expression Vectors/Site-directed Mutagenesis—Expression vectors for EGFP-murine Lhx3 (EGFP-mLhx3) and EGFP-porcine Lhx3 (EGFP-pLhx3, pLhx3-EGFP) fusion proteins were generated by cloning complementary DNA sequences (cDNAs) into pEGFP-C1 or pEGFP-N1 (CLONTECH, Palo Alto, CA). Deletion mutants of EGFP-Lhx3 were generated by digestion with restriction enzymes and re-ligation. Site-directed mutagenesis was performed using QuikChange (Stratagene, La Jolla, CA). Sequences of mutagenic oligonucleotides are available upon request. DNAs encoding NLs were ligated to the EGFP cDNA in pEGFP-C1. The integrity of all plasmids was confirmed by DNA sequencing (Department of Biochemistry and Molecular Biology, Indiana University School of Medicine).

In Vitro Cell Culture, Transfection, and Luciferase Assays—Cell culture, transfection, and luciferase assays were performed as described (39). The porcine αGSU promoter and rat prolactin enhancer/promoter have been described (39). C3H1T1/2, COS-1, αTSH, 293, and 293T cells were transfected using CalPhos (CLONTECH). A6 and UMR 106-01 cells were transfected using LipofectAMINE (Life Technologies, Inc.).

In Situ Nuclear Matrix Extraction—UMR 106-01 or 293 cells were plated at 4 × 10⁴ cells/10 cm² in chamber slide flasks (Nalge Nunc, Naperville, IL) 24 h prior to transfection. Cells were transfected using 1.5 μg of plasmid DNA. After 48 h, cells were fixed or subjected to sequential extraction, stained, and mounted for immunofluorescence. Soluble cytoskeletal and nuclear proteins were sequentially extracted from cells as described (14, 42). Cells then were fixed in 3.7% formaldehyde and processed for immunodetection of nuclear antigens and chromatin staining as described below.

Confoocal and Fluorescence Microscopy—Confocal microscopy was performed on live or fixed cells as described (40). Phase contrast and fluorescent digital images were captured using a Zeiss Axiovert TV light microscope (Thornwood, NY) with a phase contrast ×100 (NA 1.3) oil-immersion lens. Fluorescent images of triple-labeled cells were obtained using narrow band pass rhodamine, fluorescein, and DAPI filters and a CCD camera (Photometrics, Inc., Tucson, AZ).

Generation of Lhx3 Polyclonal Antiserum—A glutathione S-transferase-mLhx3 expression vector was constructed by cloning the mLhx3a cDNA into pGEX-KT as described (39). Fusion protein was expressed and affinity purified as described (39). Purified protein was sonicated in PBS/Freund’s adjuvant (1:1) and used to immunize rabbits. Preimmune and immune serum was collected and used in DNA binding assays at 1:20–1:200.

Indirect Immunofluorescence—293 cells were plated on chamber slides. 48 h after transfection, cells were fixed in 4% paraformaldehyde, extracted in 0.1% Triton X-100/PBS, and blocked in 2% bovine serum albumin/PBS. Mouse anti-Myc monoclonal antibody 9E10 ascites (Developmental Studies Hybridoma Bank, University of Iowa) was used at 1:20–1:40. Mouse anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) was used at 1:1000. Cells were visualized by confocal microscopy as described above. UMR 106-01 cell nuclear matrix filaments were detected with an anti-NUma antibody as described (43). Removal of nuclear DNA was verified by staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) as described (44).

Cell Extracts and Electrophoretic Mobility Shift Analysis—Whole-cell extracts were prepared as described (45). Electrophoretic mobility shift assays (EMSA) were performed as described (39). Total protein was determined by the Bradford method (Bio-Rad). Antibodies or control preimmune serum was added before the addition of probe to confirm the specificity of interactions.

Western Analysis—Western assays were performed as described (39). An anti-EGFP polyclonal antibody conjugated to hors eradish peroxidase (CLONTECH) was used at 1:300.

**Results**

Fluorescent Derivatives of Lhx3 Are Biologically Active—Fluorescent labeling has proved a powerful tool for the analysis of the properties of nuclear proteins (e.g., Refs. 46 and 47). To provide reagents to determine the subcellular location of Lhx3, we constructed expression vectors for enhanced Aequorea green fluorescent protein-Lhx3 fusion proteins (EGFP-Lhx3 or Lhx3-EGFP). To test the transcriptional activities of the Lhx3 fusion proteins, expression vectors were transiently transfected into human embryonic 293 cells with pituitary hormone promoter luciferase reporter genes. In these experiments, both Lhx3-EGFP and EGFP-Lhx3 activated the αGSU promoter in similar fashion to Lhx3 controls (Fig. 1A). EGFP alone was inactive. Similarly, Lhx3-EGFP and EGFP-Lhx3 fusion proteins cooper-
Small proteins such as EGFP (27 kDa) are able to diffuse passively through the NPC into the nucleus (48). By contrast, Lhx3-EGFP and EGFP-Lhx3 proteins were localized to the nucleus of transfected 293 cells (Fig. 2B and data not shown) and mouse αTSH pituitary thyrotrope cells (Fig. 2C). Similar results were obtained following the introduction of EGFP-Lhx3 into Xenopus laevis A6 kidney cells, mouse C3H10T1/2 fibroblast cells, monkey COS-1 cells, and rat UMR 106–1 osteosarcoma cells (data not shown). To confirm that nuclear localization of Lhx3 was not dependent upon association with EGFP, an expression vector for Lhx3 fused to a Myc epitope (39) was transiently transfected into 293 cells, and indirect immunofluorescence was observed using anti-Myc monoclonal antibodies. The Myc-Lhx3 molecule also was located entirely in the nucleus of transfected cells (Fig. 2D).

Signals Within and Near the HD Are Required for Nuclear Localization—To map the sequences responsible for subcellular localization of Lhx3, sequential deletion mutants of murine and porcine EGFP-Lhx3 were created (Fig. 3A). Fluorescence was observed by confocal microscopy after transfection of 293 cells. Deletion of the conserved Lhx3/LIM3-specific domain, for which a function has yet to be described, had no effect on nuclear localization (Fig. 3). However, removal of the HD caused detection of EGFP-Lhx3 within the cytoplasm (Fig. 3). These observations indicated that the LIM domains were not sufficient for nuclear localization and suggested that the nuclear localization signals lay within the HD and perhaps the carboxyl region of the molecule outside of the Lhx3/LIM3-specific domain. Interestingly, a molecule lacking residues downstream of amino acid 186 displayed similar fluorescence intensity in the nucleus and cytoplasm, whereas deletion of residues after position 188 resulted in predominantly nuclear detection (Fig. 3), suggesting that a sequence important to nuclear localization is located within this region of the HD.

Inspection of the Lhx3 HD and carboxyl-terminal amino acid sequence identified four regions that displayed similarity to reported nuclear localization signals (B1–B4, Fig. 4A). To investigate the importance of these candidate signals, the basic amino acids in each were mutated to alanines by site-directed mutagenesis, and intracellular localization was monitored by confocal microscopy (Fig. 4A). In the case of region B2, the proline and histidine residues also were mutated to alanines. Mutation of any one of the four regions did not affect nuclear localization (Fig. 4A), indicating that not one is required for nuclear localization. To test whether signals could act in tandem, mutations in all combinations were made either of two, three, or all four regions (Fig. 4A). Mutation of two basic regions of Lhx3 resulted in predominantly nuclear localization with some cytoplasmic fluorescence, except when one of the two mutations included B2; in these cases, localization remained nuclear (Fig. 4A). Experiments testing mutations of greater than two basic regions of Lhx3 indicated that the four signals act in additive fashion to mediate nuclear localization. Mutation of the three HD basic regions or of all four basic signals resulted in exclusion of the fluorescent Lhx3 derivatives from the nucleus (Fig. 4A). To control for the production of EGFP-Lhx3 fusion proteins of the predicted molecular masses, mutant proteins (and all other derivatives described in this study) were examined by Western analyses using an anti-EGFP antibody (Fig. 4B, and data not shown). All fusion proteins were of the predicted size; the Lhx3mutB123 protein, however, appeared to be less stable, and breakdown products often were seen (Fig. 4B).

The three NLSs in the HD (B1, B2, and B3) are conserved within all reported Lhx3/LIM3 proteins, and B4 is conserved within the vertebrate members of the family (Fig. 5A). Alignment...
ment of sequences of representative LIM-HD factors revealed that the basic nature of the B1 and B3 regions is similar in all members of the group. B2 is less conserved, and B4 is only found within Lhx3 and the closely related Lhx4 protein. A basic region, however, also is found downstream of the HD in the Lin-11 protein (Fig. 5).

**Fig. 2. Nuclear localization of the Lhx3 protein.** Cultured cells were transiently transfected with expression vectors for the indicated proteins and observed by krypton-argon laser-scanning confocal microscopy. Upper panels (A–C) show direct fluorescent images (scale bar, 10 μm); lower panels (E–H) show phase contrast images of the same fields. Note that only a subset of cells is transfected. EGFP is localized to both cytoplasmic and nuclear compartments of 293 cells in control transfections (A). Lhx3-EGFP and EGFP-Lhx3 are restricted to the nuclei of 293 cells (B) and mouse aTSH pituitary thyrotrope cells (C). By using indirect immunofluorescence with an anti-Myc antibody, Myc epitope-tagged Lhx3 is detected in the nuclei of transfected 293 cells (D).

**Fig. 3. Nuclear localization of Lhx3 requires HD and carboxyl-terminal domain sequences.** 293 cells were transiently transfected with the indicated EGFP-Lhx3 deletion constructs and examined by confocal microscopy as described in Fig. 2. The nuclear location of each protein is given in A. Murine Lhx3 (mLhx3) and porcine Lhx3 (pLhx3) were analyzed. LSD denotes the Lhx3/LIM-3-specific domain (40, 49). N, nucleus; C, cytoplasm. Representative photomicrographs are shown in B.

**Fig. 4. Mutations in the HD Impair the Ability of Lhx3 to Transactivate Target Genes.**—Three of the four identified NLSs (B1, B2, and B3) of Lhx3 lie within the DNA binding HD; the 4th, B4, is located in the carboxyl terminus adjacent to the HD. The function of this region has not been defined, but reports have speculated that the carboxyl terminus contains both transactivation domains and sequences important for regulation of...
Lhx3 activity (32–34, 39, 40). To test directly the contributions of the four signals to Lhx3 DNA binding and gene activation function, each site was mutated in the native Lhx3 cDNA, and expression vectors were constructed. Co-transfection assays were performed to assess Lhx3 activation of both the αGSU and PRL promoters. Mutation of any of the HD NLSs abolished the ability of Lhx3 to activate the αGSU promoter (Fig. 7A).

Similar results were observed in assays of the ability of these molecules to cooperate with Pit-1 in activation of PRL (data not shown). Surprisingly, mutation of B4 increased Lhx3 function 2–3-fold (Fig. 7A), suggesting that this region may mediate the actions of repressive signaling pathways. In parallel controls to monitor the DNA binding activities of the mutant Lhx3 molecules, extracts from transfected cells were used in EMSA experiments. As expected, mutation of the HD abolished Lhx3 binding to a consensus DNA recognition site (Fig. 7B). The DNA binding activity of the Lhx3 B4 mutant was comparable to that observed for wild-type controls (Fig. 7B), indicating that this mutation does not alter DNA binding function.

Lhx3 Associates with the Nuclear Matrix—Following demonstration of the nuclear localization of Lhx3, we determined whether the Lhx3 molecule was associated with the nuclear matrix. Following demonstration of the nuclear localization of Lhx3, we determined whether the Lhx3 molecule was associated with the nuclear matrix. Following demonstration of the nuclear localization of Lhx3, we determined whether the Lhx3 molecule was associated with the nuclear matrix.
EGFP-Lhx3 was transiently transfected into cultured cells, and 48 h after transfection, cells were treated by sequential extraction with detergent, deoxyribonuclease, ammonium sulfate, and 2 M sodium chloride to remove soluble nuclear components. Significant EGFP-Lhx3 fluorescence remained following all extractions (Fig. 8, D and H), whereas detergent treatment removed detectable fluorescence from control cells transfected with EGFP alone (Fig. 8B). In similar experiments with EGFP-Lhx3-transfected UMR 106-1 cells, the nuclear matrix protein NuMA was co-visualized by indirect immunofluorescence as a control. After extraction, EGFP-Lhx3 was retained in cells displaying NuMa fluorescence (Fig. 8, H and J), confirming that Lhx3 is associated with the nuclear matrix.

The Homeodomain Targets Lhx3 to the Nuclear Matrix—To map the amino acid sequences required for association of Lhx3 with the nuclear matrix, we determined whether EGFP fusion proteins containing specific regions of the Lhx3 molecule remained with the matrix following extraction. Deletions of the amino and carboxyl termini of the molecule demonstrated that the LIM domains and the Lhx3/LIM3-specific domain, respectively, are not required for matrix association (Fig. 9). By contrast, deletion of the carboxyl-half of the HD, or the entire HD, prevented matrix targeting (Fig. 9). To confirm the importance of the HD in matrix association, fusion proteins containing the HD region, the HD alone, or portions of the HD were tested. These experiments indicated that the HD could alone confer nuclear matrix targeting (Fig. 9). Whereas a peptide containing the B1 and B2 regions of the HD was not associated with the matrix, a peptide containing regions B3 and B4 was strongly retained with the matrix following extraction (Fig. 9). Individual and combined site-directed mutagenesis of each of these sequences in the context of the intact molecule confirmed that the HD sequences, but not the B4 sequence, are required for nuclear matrix association (Fig. 9). Together, these data demonstrate that the HD mediates the targeting of Lhx3 to the nuclear matrix and that the B3 sequence is central to this function.

DISCUSSION

In this study, we demonstrate that the Lhx3 LIM-HD protein has four NLS sequences: three within the HD and a fourth in the carboxyl terminus. Mutation of single NLS sequences does not prevent nuclear localization, indicating redundancy of individual NLS sequences. However, mutation of two or more specific signals prevents nuclear-specific accumulation of Lhx3, demonstrating that the signals act in combinatorial fashion. Our experiments reveal that the LIM domains of Lhx3 are not required for its nuclear localization or association with the nuclear matrix. Interactions with nuclear LIM-interacting proteins are, therefore, not essential for nuclear accumulation. However, interactions of the LIM domains with cofactors such as the NLI proteins (reviewed in Ref. 50) and MRG1 (41), and with transcription factors such as Pit-1 (33, 39) have been shown to regulate the activity of LIM-HD factors such as Lhx3. Furthermore, the LIM domains also provide a transcriptional activation function to the Lhx3 molecule (41).

The nature of the three Lhx3 HD NLS sequences is conserved within the LIM-HD protein family. The B2 NLS signal
is not as strongly conserved as the basic B1 and B3 signals, but similar sequences are found at this position in many members of the family (Fig. 5). These observations suggest that sequences related to the identified Lhx3 NLS motifs may serve as NLSs within other LIM-HD proteins. The B4 NLS is conserved in the Lhx3/LIM3 class of LIM-HD factors and the Lhx4 LIM-HD protein but is not positionally conserved in other LIM-HD proteins examined. This implies that the B4 signal may confer activities that are specific to this subclass of LIM-HD factors. Two of the Lhx3 NLS sequences (B1 and B3) are similar to signals within the HDs of other HD proteins. The B1 NLS is defined by amino acids Lys\(^{161}\)–Arg\(^{164}\) and is similar in location and content to basic regions that act as NLSs in the TTF-1 and Tst-1 homeoproteins (20, 21). The B3 signal located at Arg\(^{211}\)–Lys\(^{221}\) lies within the predicted DNA recognition helix of the Lhx3 HD. This helix of the TTF-1 and PDX-1 factors has been shown to contain NLS activities (20, 51), but the sequences identified by these studies are not identical to the defined Lhx3 B3 NLS. It also is possible that the B3 sequence functions together with the B4 signal as an extended bipartite NLS. The B2 NLS located at Pro\(^{185}\)–Glu\(^{191}\) is the weakest NLS of the four identified signals; mutation of this signal in tandem with mutation of one other NLS does not prevent nuclear accumulation of Lhx3 (Fig. 4). However, the observation that Lhx3 mutated at all four NLSs is exclusively cytoplasmic, whereas Lhx3 mutated at B1, B3, and B4 still partially enters the nucleus, demonstrates that the contribution of B2 is significant. This NLS sequence is similar to the human c-MYC oncoprotein NLS (52). This region of an HD has not previously been characterized as an NLS and, therefore, defines a new type of HD NLS sequence.

The identified Lhx3 nuclear localization signals lie within important functional domains of the protein. The HD is a multifunctional protein structure that can mediate DNA binding, protein-protein interaction, and other activities (53). Our experiments demonstrate that the Lhx3 HD is required for DNA binding, nuclear localization, nuclear matrix association, and transcriptional activity. Individual mutation of the three HD NLSs rendered Lhx3 transcriptionally inactive and unable to bind to target DNA sequences. The fourth Lhx3 NLS lies within the carboxyl-terminal domain, and mutation of this motif led to an increased transcriptional response but no change in DNA binding activity. This suggests that this region of Lhx3 may be the target of repressive regulatory signals and that mutation of the B4 NLS disrupts these signaling pathways. Indeed, there are several consensus sites for post-translational modification of the molecule by phosphorylation in the carboxyl terminus adjacent to the B4 NLS. Alternately, this NLS could lie within a region important for interaction with transcriptional co-activator/co-repressor molecules, such as CREB-binding protein. These models are not mutually exclusive; a recent report demonstrated that selective interactions of Pit-1 with co-activator molecules mediate its transcriptional response to intracellular signals such as cAMP or growth factors (54).

In our experiments, EGFP molecules fused to pairs of the identified Lhx3 HD NLSs (B1 + B2 and B3 + B4) were observed to concentrate within the nucleoli of transfected cells (Fig. 6 and data not shown). Some homeoproteins do display nucleolar localization (55). However, the Lhx3 holoprotein displayed a diffuse nuclear localization and generally was excluded from the nucleoli. These observations suggest that the nucleolar localization conferred by the B1 + B2 and B3 + B4
peptides either is masked in the context of the intact protein or that the isolated peptides can adopt a structure that mimics a nuclear retention signal. Consistent with the latter hypothesis, pairs of basic sequences with structural similarity to the Lhx3 NLS sequences have been shown to function as nuclear retention signals in proteins such as fibuloblast growth factor 3 (56).

This study also demonstrates that Lhx3 is associated with the nuclear matrix and that the HD mediates this association. The conserved B3 region is the most important of the HD basic sequences for this activity, and a small peptide containing this region can alone confer nuclear matrix targeting. The B3 region contains a short sequence of 10 amino acids containing mostly basic residues. In the context of the Lhx3 holoprotein, all mutations of HD sequences abrogated nuclear matrix targeting; this is likely due to disruption of the HD structure. The loss of DNA binding in these mutants (Fig. 7) is consistent with this hypothesis.

The nuclear matrix is an insoluble, filamentous structure that has been proposed to form a scaffold for active chromatin and to mediate the actions of regulatory pathways that modulate transcription factor function (13–18). Few other homeodomain proteins have been demonstrated to be associated with the nuclear matrix, and the sequences that mediate such localization are largely uncharacterized. The SATB1 protein has an atypical HD that functions with another domain to allow interaction with DNA nuclear matrix attachment regions (57). Interestingly, Pit-1, a HD factor that cooperates with Lhx3 in synergistic activation of the PRL, TSHβ, and Pit-1 pituitary-specific genes (33, 39, 40) also is a nuclear matrix-associated protein (16). The interaction of these two transcription factors with the nuclear matrix may contribute to the transcriptional activation of genes in the pituitary. Pit-1 has a bipartite DNA binding domain known as the POU domain, which consists of a POU-specific domain and a POU-type homeodomain (reviewed in Ref. 58). Within Pit-1, the POU-specific domain forms the nuclear matrix-targeting signal (16). Like the HD of Lhx3, the Pit-1 POU-specific domain adopts a helical structure and contains basic sequences at each end of the domain (58). Another POU protein, Oct-1, also is present within the nuclear matrix fraction, and this interaction has been proposed to play a role in regulation of the TSHβ gene (59), but the sequences that confer matrix association of Oct-1 are unknown.

To our knowledge, this report provides the first description of the association of a LIM-HD factor with the nuclear matrix and the first characterization of the protein sequences required for the nuclear entry and nuclear matrix targeting of a LIM-HD protein. Future experiments will be required to determine the significance of the nuclear matrix in Lhx3 function and the role of the complex Lhx3 nuclear localization signals in its activities.

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