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LIM Factor Lhx3 Contributes to the Specification of Motor Neuron and Interneuron Identity through Cell-Type-Specific Protein-Protein Interactions

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Summary

LIM homeodomain codes regulate the development of many cell types, though it is poorly understood how these factors control gene expression in a cell-specific manner. Lhx3 is involved in the generation of two adjacent, but distinct, cell types for locomotion, motor neurons and V2 interneurons. Using in vivo function and protein interaction assays, we found that Lhx3 binds directly to the LIM cofactor NLI to trigger V2 interneuron differentiation. In motor neurons, however, Isl1 is available to compete for binding to NLI, displacing Lhx3 to a high-affinity binding site on the C-terminal region of Isl1 and thereby transforming Lhx3 from an interneuron-promoting factor to a motor neuron-promoting factor. This switching mechanism enables specific LIM complexes to form in each cell type and ensures that neuronal fates are tightly segregated.

Introduction

Combinatorial transcription codes are prevalent in the developing central nervous system (CNS) and are used to specify a large number of distinct cell types with a limited repertoire of factors. Many aspects of our understanding of CNS development have emerged through studies of the spinal cord where numerous transcription factors have been demonstrated to contribute to the specification of neuronal and glial cell identity (Jessell, 2000; Lee and Pfaff, 2001). Despite overwhelming evidence that transcription factors act in a combinatorial manner to regulate different gene outputs depending on their cellular context, the biochemical basis for these codes has remained elusive, as has an understanding of the mechanisms that control the context-dependent activities of factors.

V2 interneurons (INs) and motor neurons (MNs) are two cell types in the ventral spinal cord that participate in neuronal circuits for the coordination of locomotor activity but have markedly different axonal projections and neurotransmitter properties (Lee and Pfaff, 2001). The transcription codes involved in the formation of V2 INs and MNs are well defined (Kessaris et al., 2001; Marquardt and Pfaff, 2001). These neurons represent two classes of cells that develop adjacent to one another in the ventral spinal cord as a result of the graded inductive activity of Sonic hedgehog (Shh). The early steps involved in the generation of V2 INs and MNs depend on transcriptional repressors that lead to the formation of precise domains of progenitor cells for each class of neuron (Briscoe et al., 2000; Muhr et al., 2001). The V2 IN progenitor cells require Pax6, Nkx6.1, Nkx6.2, and Irx3, whereas MN progenitor cells express Pax6, Nkx6.1, Nkx6.2, Olig2, and MNR2 (Jessell, 2000; Marquardt and Pfaff, 2001). Since these progenitor factors act by repressing transcription, it is thought that other downstream effectors of cell fate become activated in the appropriate domains (Lee and Pfaff, 2001; Muhr et al., 2001).

The LIM homeodomain factors represent candidate effectors of cell identity in the spinal cord (Tsuchida et al., 1994), becoming expressed by V2 INs and MNs during their exit from the cell cycle. Both V2 INs and MNs express Lhx3 (Lim3) and the closely related factor Lhx4 (Sharma et al., 1998), but only MNs express Isl1 (Ericson et al., 1992). Thus, a simple LIM code involving Lhx3 in the presence or absence of Isl1 distinguishes these two cell types. Mice lacking Lhx3 (and the redundant factor Lhx4) fail to generate V2 INs and the proper types of MNs (Sharma et al., 1998), and mutation of the *Isl1* gene disrupts MN development altogether (Pfaff et al., 1996). As further evidence that Lhx3 and Isl1 control cell identity, ectopic expression of Lhx3 triggers V2 IN formation, whereas the combination of Lhx3 and Isl1 leads to the ectopic upregulation of a MN marker (Tanabe et al., 1998).

The molecular basis for the activities of LIM factors has begun to emerge through identification of binding cofactors and structure/function analyses in *Drosophila*. The LIM domain coordinates zinc in a finger-like manner and functions as a protein-protein interaction module (Dawid et al., 1998). The best-characterized high-affinity binding partner for the LIM factors is the broadly-expressed nuclear LIM interactor NLI (Ldb1, CLIM2) or the ortholog Chip in *Drosophila* (Agulnick et al., 1996; Bach et al., 1997; Jurata et al., 1996; Morcillo et al., 1997), though other factors such as Ptx1 and E47 have also been reported to interact with LIM domains (Bach et al., 1997; Johnson et al., 1997). The N-terminal region of NLI is capable of self-dimerization, while the C-terminal region interacts indiscriminately with the LIM domains of all nuclear-class LIM factors (Jurata and Gill, 1997). Studies of Chip and the LIM homeodomain protein apterous have provided functional evidence that the relevant complex for wing patterning and neuronal development in *Drosophila* consists of a tetrameric arrangement of a Chip dimer bridging two apterous proteins (Milan and Cohen, 1999; van Meyel et al., 1999, 2000). Numerous cells coexpress multiple LIM factors during development (Hobert and Westphal, 2000), and in such cases it is expected that a combination of both homo- and heteromeric NLI:LIM complexes will be present (Jurata et al., 1998, 2000). It remains unresolved

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whether the multiple predicted complexes act additively to regulate cell identity or whether some mechanism exists to select for a particular LIM complex.

A paradox emerges when considering the actions of Lhx3 in the specification of both V2 INs and MNs. Namely, the same NLI:Lhx3-containing complexes predicted to exist in V2 INs are also likely to be present in MNs, illustrative of a general problem in reusing factors. In this scenario, MNs are predicted to express complexes active in V2 IN generation, and would therefore be expected to exhibit hybrid neuronal properties. This potential for crossregulation of V2 IN genes in MNs becomes unmasked in *HB9* mutants (Arber et al., 1999; Thaler et al., 1999), providing genetic evidence that HB9 is part of the fail-safe mechanism for ensuring that V2 IN and MN identity is properly segregated. To understand the mechanistic basis for the context-dependent activity of Lhx3 in these two neuronal types, we examined the biochemical underpinnings of the LIM code. Using misexpression of Isl1 and Lhx3 in the dorsal region of the chick embryonic spinal cord together with protein interaction studies, we determined that a 2NLI:2Lhx3 tetramer is involved in V2 IN generation, whereas 2NLI:2Isl1:2Lhx3 hexamers are found to drive MN differentiation. Assembly of the tetrameric and hexameric complexes involve two distinct types of protein interactions by Lhx3, which serves as the basis for converting it from an IN-factor to a MN-factor. The competitive interactions of Lhx3 lead to the formation of hexamers at the expense of tetramers in MNs, thereby serving as a switching mechanism for regulating its cell type-specific functions.

Results

The Combinatorial Actions of Isl1, Lhx3, and NLI Specify Neuronal Subtypes

In the ventral spinal cord, Lhx3 is expressed by V2 INs and MNs as they emerge from the ventricular zone (Sharma et al., 1998), while Isl1 is observed exclusively in postmitotic MNs (Figures 1A and 1D; Ericson et al., 1992). This initial LIM code involving Isl1 and Lhx3 has not been well appreciated, however, due to the rapid extinction of Lhx3 from MNs as they migrate laterally (Figure 1A). The function of LIM factors is thought to be dependent on their cofactor NLI. Correspondingly, we found NLI expression to be upregulated in postmitotic neurons (Figure 1B; Jurata et al., 1996). Thus, cells acquiring a V2 IN identity express Lhx3 and NLI, whereas those becoming MNs express Isl1 in addition to Lhx3 and NLI (Figure 1D).

To dissect the functional basis of the Lhx3/Isl1 LIM codes for V2 IN and MN differentiation, in ovo electroporation of chick embryos was used to introduce combinations of the LIM genes into ectopic locations on one side of the neural tube (plus sign in figures). We focused our analysis on dorsal spinal cord cells as they endogenously express NLI and have no exposure to Shh or ventral progenitor factors (i.e., Nkx 6.1/6.2, Olig2, MNR2). Newly generated V2 INs and MNs were identified by their selective expression of the transcription factors Chx10 in V2 INs and Isl2 or HB9 in MNs (Figures 1C and 1D; Ericson et al., 1992; Tanabe et al., 1998; Tsuchida et al., 1994). Ectopic expression of Lhx3 alone in dorsal spinal cord regions induced Chx10$^+$ V2 IN differentiation without activating Isl2 (Figures 1E–1G; Tanabe et al., 1998). Inclusion of Isl1 with Lhx3 inhibited the formation of V2 INs and instead, Isl2$^+$ MNs were generated (Figures 1H–1J). These data differ slightly from previous observations using viral coinfection, which suggested that Isl1 and Lhx3 triggered incomplete MN generation (Tanabe et al., 1998). To determine whether Isl1 and Lhx3 produced bona fide ectopic MNs, we examined further markers of these cells. In addition to Isl2, the transcription factor HB9, axonal adhesion protein SC1, and neurotransmitter synthesis enzyme choline acetyltransferase were found to be expressed by the ectopic cells (see Supplemental Figures S1 at http://www.cell.com/cgi/content/full/110/2/237/DC1), indicating that Isl1 and Lhx3 properly specify MNs. The difference from the previous report probably reflects the more efficient coexpression of proteins using in ovo electroporation. In summary, coexpression of Isl1 with Lhx3 has two clear effects: it blocks the V2-inducing activity of Lhx3, and it promotes MN differentiation.

Biochemical and genetic evidence indicate that LIM factor activity is dependent on the function of the LIM bridging molecule NLI. Given the expression of NLI in developing V2 INs and MNs, we assessed its involvement in spinal cord development using dominant-negative constructs analogous to their *Drosophila* counterparts. Specifically, we used either the dimerization domain (DD)-containing N-terminal region or the LIM-interaction domain (LID)-containing C-terminal region (Milan and Cohen, 1999; van Meyel et al., 1999). First, we compared the induction of ectopic neurons by Lhx3 and Isl1 in the presence or absence of dominant-negative NLI constructs. V2 IN generation by Lhx3 was completely abolished in the presence of the DD or LID (Figures 2A and 2B; data not shown). Likewise, MN induction by Isl1 and Lhx3 together was disrupted by either dominant-negative construct (Figures 2C and 2D; data not shown). These dominant-negative effects are very efficient, presumably because electroporated cells receiving the LIM constructs have a high incidence of cotransfection by the dominant-negative DNAs, which are then coexpressed with similar timing and at similar levels.

The previous data established the participation of NLI in the induction of ectopic cells by LIM factors. Thus we predicted that the formation of the endogenous V2 INs and MNs would also depend on NLI function. To test this hypothesis, a variety of dominant-negative constructs were used including the DD region of NLI, the C-terminal region of Isl1 (see below), and the LIM-only factor LMO4. LMO4 acts as a dominant negative by interacting with the LID region of NLI, thereby competing for binding between NLI and the LIM homeodomain factors (Milan et al., 1998). Though the overall efficiency of these dominant-negative constructs is limited by the timing and number of cells that are electroporated, each significantly reduced the quantity of V2 INs and MNs in the ventral spinal cord without altering the pattern of upstream progenitor factors Nkx6.1 and Olig2 (Figures 2E–2H; data not shown). We found that V2 INs were inhibited more effectively than MNs (Figures 2E–2H), suggesting that the slight lag in IN generation relative to MN generation might provide more time for high levels

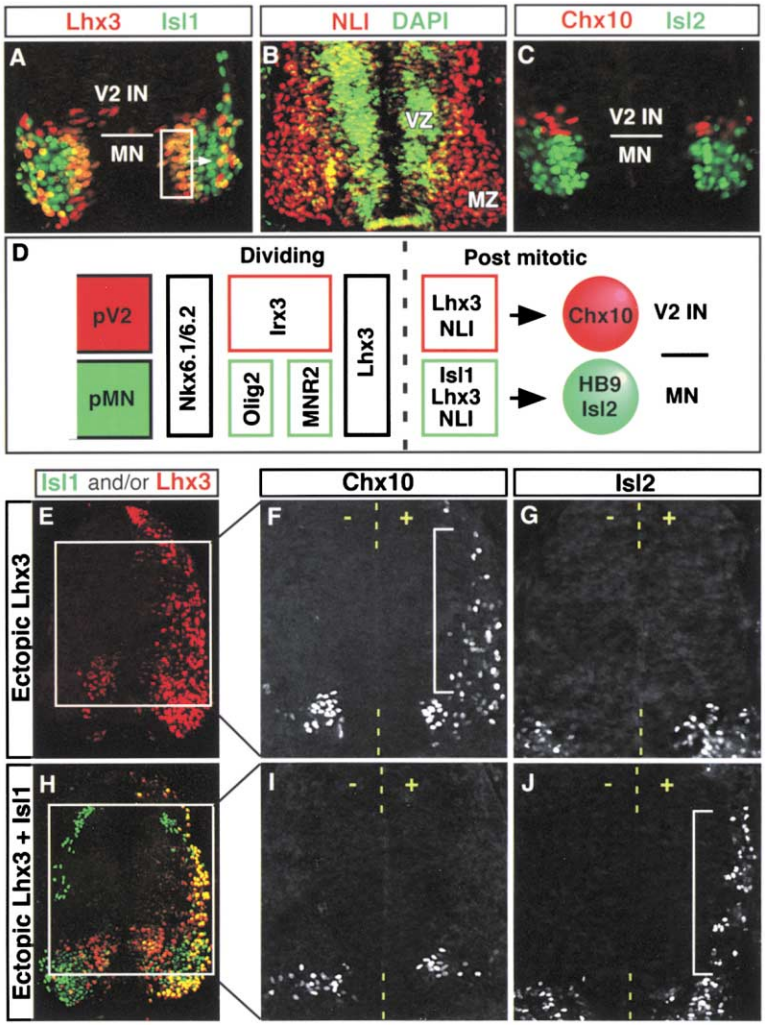

Figure 1. Lhx3, Isl1, and NLI Form a Transcriptional Code for the Specification of Ventral Spinal Cord Neurons

(A–C) Immunohistochemical analysis of HH stage 24 chick ventral neural tube.

(A) V2 INs express Lhx3 but not Isl1. MNs initially express both genes as they exit the cell cycle (white rectangle). Upon further differentiation and lateral migration (arrow), MNs continue to express Isl1, while only a subset maintains Lhx3.

(B) All spinal neurons express NLI with higher levels in the postmitotic mantle zone (MZ) than in the proliferative ventricular zone (VZ). DAPI nuclear stain is pseudocolored green for clarity.

(C) V2 INs and MNs are clearly delineated by the postmitotic markers Chx10 and Isl2, respectively.

(D) Illustration showing the expression of transcription factors in the progenitor cells (pV2 and pMN) and their relationship to the LIM factors and late markers for V2 INs and MNs.

(E–J) Cell fate analysis on serial sections from HH stage 24 chicks electroporated (plus side) with constructs listed on left. Coelectroporation resulted in >80% of cells expressing both constructs. White boxes in (E) and (H) highlight the region of focus in serial images.

(E–G) Misexpression of Lhx3 in the dorsal neural tube generates ectopic V2 INs (32 ± 10 dorsal Chx10$^+$ cells/section) with no change in Isl2$^+$ MN number.

(H–J) Coelectroporation of Lhx3 and Isl1 promotes MN formation in the dorsal spinal cord visualized by Isl2 expression (15 ± 8 cells/section) without generating V2 INs. Dorsal Isl1$^+$ cells (green) seen on both sides of the spinal cord in (H) represent the endogenous D2 IN population. Each image is representative of 20 embryos.

of the dominant-negative proteins to accumulate prior to LIM function. These findings establish a role for NLI in the specification of V2 INs and MNs.

2NLI:2Lhx3 Tetramers Trigger V2 IN Differentiation

NLI mediates the formation of tetrameric LIM complexes via an N-terminal self-dimerization domain (DD) and a C-terminal LIM interaction domain (LID) (Jurata et al., 2000). Structure/function-based rescue studies in the fly have shown that tetrameric complexes, composed of two molecules of Chip and two molecules of the LIM homeodomain protein apterous, direct axon pathfinding of a subset of INs as well as aspects of wing development (Milan and Cohen, 1999; van Meyel et al., 1999, 2000). Similarly, the biologically relevant V2 IN complex might be the 2NLI:2Lhx3 tetramer (Figure 3A). The predicted structure of the V2 IN complex is consistent with the involvement of NLI based on dominant-negative constructs that interfere with self-dimerization (DD) or NLI:LIM interactions (LID or LMOs) (see Figure 2).

We characterized the precise identity of the Lhx3-containing complexes involved in V2 IN specification using in vivo structure/function tests. As expected, the generation of V2 INs by ectopic Lhx3 required the N-terminal LIM domains of Lhx3, presumably for interactions with NLI (Figure 3B). Similarly, mutation of a critical asparagine residue in the homeodomain region of the C terminus of Lhx3 eliminated its activity, indicating a requirement for DNA binding (Figure 3C). A chimeric molecule, in which the LIM domains of Lhx3 were replaced by the LIM domains of another LIM homeodomain factor Lhx1 (L1-Lhx3), was equally effective in inducing V2 IN programs (Figure 3D). These data suggest that in V2 IN differentiation, the sole function of the Lhx3 LIM domains is to bind NLI. To test this, a chimeric protein was constructed by directly fusing the DD of NLI to the homeodomain of Lhx3 (DD-Lhx3), mimicking the putative 2NLI:2Lhx3 tetramer while bypassing the need for NLI:LIM interactions. Expression of this chimeric molecule fully recapitulated the activity of ectopic Lhx3 (with endogenous NLI) in V2 IN generation (Figure 3E).

The biochemical activity of the chimeric molecules was tested in vitro by performing sequential coimmunoprecipitations of in vitro translated epitope-tagged proteins (Jurata et al., 1998). As expected, the L1-Lhx3 chimera and the native Lhx3 dimerized only in the presence of NLI (Figure 3F, lanes 1–4). In contrast, the DD-

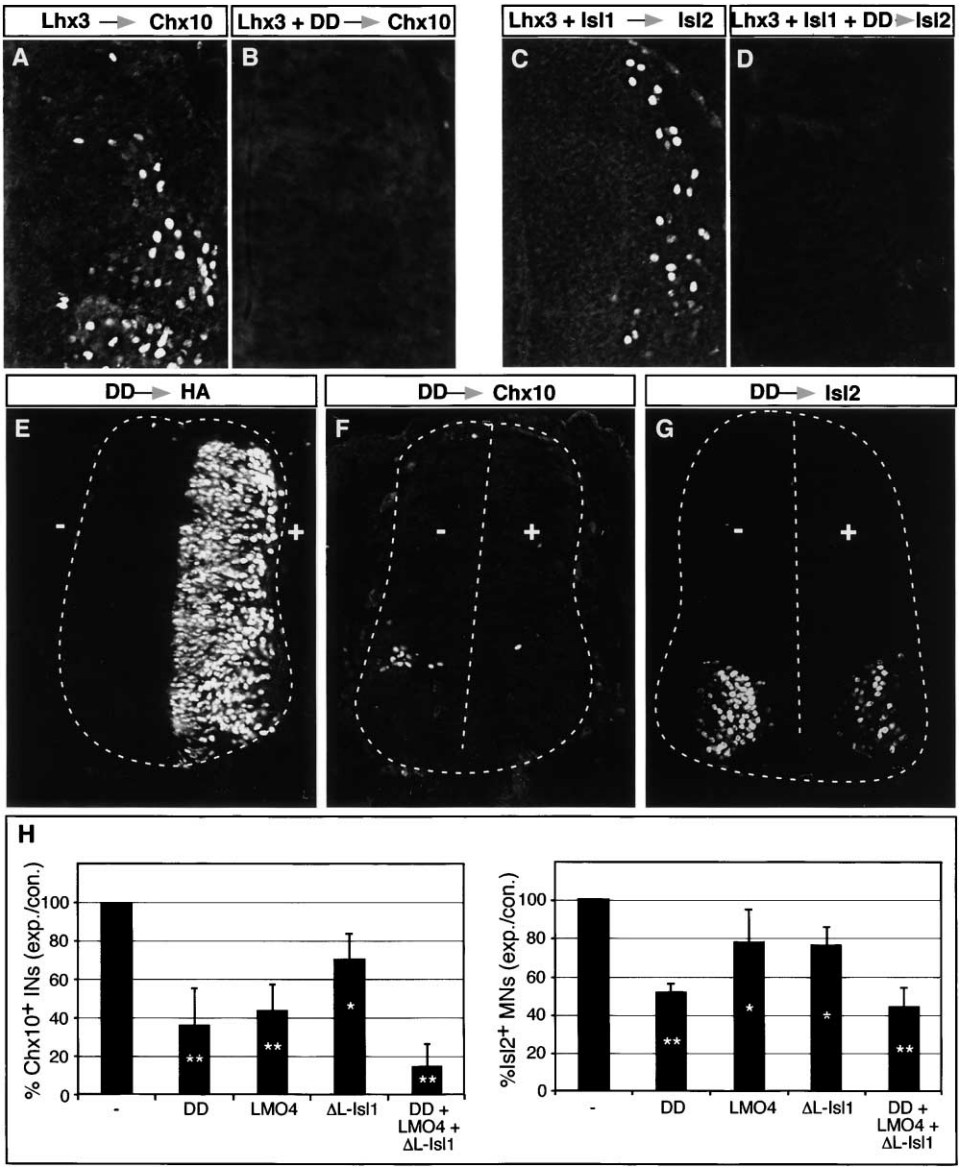

Figure 2. NLI Is Necessary for IN and MN Differentiation

Immunocytochemical analysis of cell differentiation in HH stage 24 chick embryos following DNA electroporation.

(A) Lhx3 expression in the dorsal neural tube triggers ectopic Chx10$^+$ IN differentiation.

(B) Coelectroporation of the dominant-negative dimerization domain (DD) of NLI with Lhx3 inhibits the formation of ectopic V2 INs.

(C) Isl1 and Lhx3 trigger ectopic Isl2$^+$ MN differentiation, (D) which is inhibited by the DD of NLI.

(E) Electroporation (+) of HA-tagged DD.

(F) DD inhibits endogenous V2 IN generation on the electroporated (+) side of the neural tube.

(G) DD inhibits the normal generation of MNs.

(H) Quantitative analysis of endogenous IN (Chx10$^+$) and MN (Isl2$^+$) formation using dominant-negative constructs alone and in combination. Percents were calculated by comparing the number of cells on the electroporated experimental side (exp.) to the nonelectroporated control side (con.). DD is expected to antagonize dimerization of NLI, the LIM-only factor LMO4 is predicted to compete with LIM homeodomain factors for binding to NLI, and ΔL-Isl1 should compete for Isl1 interactions with Lhx3 (see below). At least five sections from three embryos were analyzed in each case. Double asterisk indicates $p \leq 0.003$, and single asterisk indicates $p < 0.03$ using the one-tailed t test.

Lhx3 chimera self-associated independently of the bridging molecule NLI (Figure 3F, lane 5). Thus, biochemical and biological data demonstrate that 2NLI:2Lhx3 tetramers establish the identity of V2 INs.

Tetrameric Complexes Are Insufficient to Trigger MN Development

Differentiating MNs express the "code" Lhx3, Isl1, and NLI, and therefore present a more complicated biochemical scenario than *Drosophila* Chip$^+$/apterous$^+$ cells or NLI$^+$/Lhx3$^+$ V2 INs. Of the numerous complexes expected to form in cells with multiple LIM factors, the simplest would be three types of tetramers: 2NLI:2Lhx3, 2NLI:2Isl1, and the hetero combination 2NLI:Lhx3:Isl1. In order to assess the function of these complexes, we tested dimerizing analogs of Isl1 and Lhx3 created by fusing the DD of NLI to the C-terminal homeodomain-containing region of the LIM factors. Protein interaction

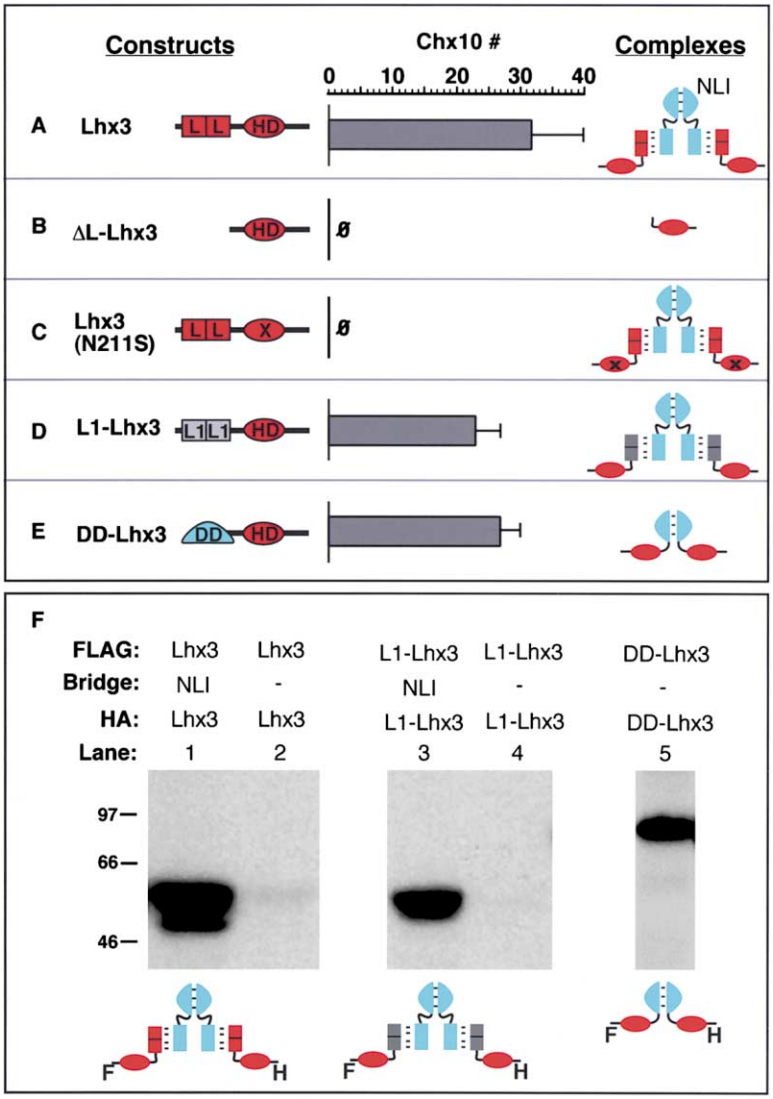

Figure 3. Lhx3 Forms Tetrameric Complexes with NLI to Specify V2 IN Identity

(A–E) Quantitative analysis of V2 IN differentiation in HH stage 24 chick embryos following electroporation. Mutant constructs are: ΔL-Lhx3, Lhx3 without LIM domains; Lhx3(N211S), Lhx3 with a missense mutation that disrupts DNA binding; L1-Lhx3, Lhx3 with the LIM domains of Lhx1; and DD-Lhx3, a fusion of the DD of NLI with the C-terminal end of Lhx3. Ectopic V2 IN generation was monitored by quantifying Chx10$^+$ cells dorsal to the endogenous V2 IN population (average number of ectopic Chx10$^+$ cells/section ± standard deviation) from ten sections in at least three embryos in each case. The list of complexes presents only major predicted structures. In several cases endogenous NLI (blue) mediates complex formation (A, C, and D).

(F) In vitro protein interaction assays using FLAG (F) and HA (H) epitope tags for immune precipitation. Complexes were assembled and then isolated with FLAG antibody. Coprecipitating HA-proteins were identified by immunopurification and electrophoresis. Molecular weight scales are indicated at left in kilodaltons. Neither Lhx3 nor L1-Lhx3 can self-associate unless full-length NLI is present as a bridging molecule, but DD-Lhx3 can self-associate.

assays revealed the ability of the chimeric factors DD-Lhx3 and DD-Isl1 to self-associate (Figure 4A, lanes 1 and 2). Unexpectedly, this combination failed to generate ectopic MNs (Figure 4B). Both chimeras were functionally active, however, as DD-Lhx3 triggered V2 IN formation (Figures 3E and 4B), and the combination of DD-Isl1 and wild-type Lhx3 promoted MN differentiation (see Figure 5B). Thus, the tetramer analogs formed by DD-Isl1 and DD-Lhx3 are insufficient for MN generation.

LIM Domains of Both Lhx3 and Isl1 Are Required for MN Development

The inability of tetrameric complexes to trigger MN differentiation prompted us to consider alternative complexes involving NLI and the LIM factors. Previous studies have shown that the LIM domains of Lhx3, but no other LIM factor, have the ability to bind with high affinity directly to the C-terminal region of Isl1, even in the absence of NLI (Figure 4A, lanes 3–5; Jurata et al., 1998). Moreover, dLim3 and dIslet are coexpressed in a subtype of fly MN, and direct binding between dLim3 and dIslet has also been observed (van Meyel et al., 1999). Although the interaction between Lhx3 and Isl1 is of high affinity and evolutionarily conserved, its function remains unknown.

To more precisely characterize the relationship between NLI, Isl1, and Lhx3, we performed a series of experiments using deletion constructs to isolate particular binding partners. First, we deleted the LIM domains of Lhx3 to eliminate its ability to bind either NLI or Isl1 (Figures 3F and 4A) and found that ΔL-Lhx3 and wild-type Isl1 were unable to promote MN differentiation (Figure 4C). Next, we removed the LIM domains of Isl1, which allows binding to Lhx3 (Figure 4A, lane 4) but not NLI (Figure 4A, lanes 6 and 7). ΔL-Isl1 and wild-type Lhx3 also failed to generate MNs (Figure 4D), establishing a requirement for the Isl1 LIM domains. Finally, we replaced the LIM domains of Lhx3 with those of Lhx1. This exchange allows the variant of Lhx3 (L1-Lhx3) to maintain binding with NLI (see Figure 3F) but excludes interactions with Isl1 (Figure 4A, lane 5). Although L1-Lhx3 was active in V2 IN specification, it failed to trigger MN differentiation with Isl1 (Figure 4E). In summary, LIM domains from different nuclear LIM factors can efficiently substitute for those in Lhx3 when promoting V2 IN development, presumably because they all mediate

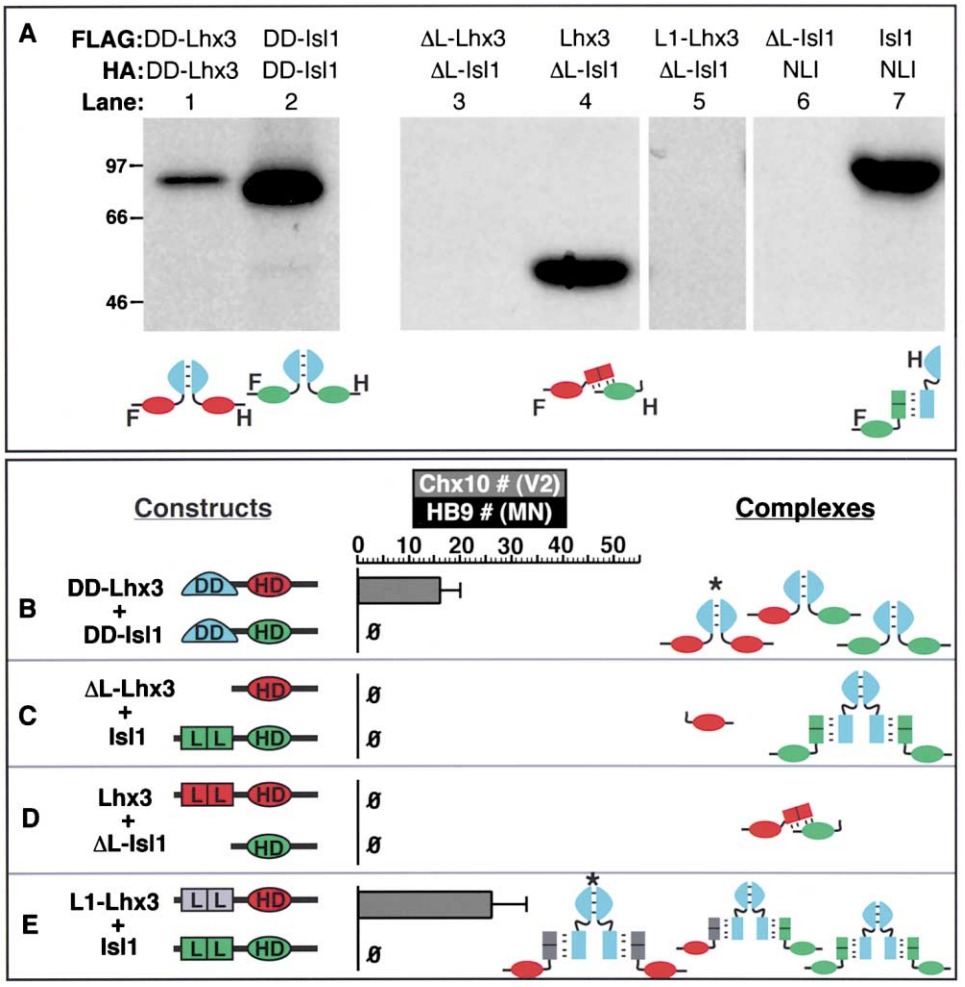

Figure 4. Lhx3 and Isl1 Dimers and Tetramers Are Insufficient for MN Specification

(A) Protein-protein interaction assay, as described in Figure 3. DD-Lhx3 and DD-Isl1 self-associate. The LIM domains specific to Lhx3 interact directly with the C terminus of Isl1.

(B–E) Table showing electroporated constructs, quantification of experimental outcome (MN or V2 IN), and primary complexes predicted to form. Mutant constructs are described in Figure 3 and as follows: ΔL-Isl1, Isl1 without LIM domains; DD-Isl1, fusion of the DD of NLI with the C-terminal end of Isl1. Ectopic MN and V2 IN generation was monitored by quantifying ectopic HB9 and Chx10 expression (average number of cells/section ± standard deviation), respectively, from ten sections in at least three electroporated HH stage 24 chick embryos in each case. Illustrated complexes are the most probable formed; many potential structures have been excluded for clarity. Asterisks denote complexes that are likely to be responsible for the observed positive IN activity. In several cases, endogenous NLI (blue) mediates complex formation (C and E).

interactions with NLI. In contrast, generic LIM domains cannot be used to replace those in Lhx3 for MN differentiation, correlating with the unique ability of Lhx3 to directly bind Isl1.

2NLI:2Isl1:2Lhx3 Hexamers Direct MN Generation

With the failure of tetramers to promote MN differentiation and the requirement for NLI and the LIM domains of Isl1 and Lhx3 in this process, we considered a possible role for higher-order LIM complexes. Both Lhx3 and Isl1 can participate in two interactions, with each other and with NLI, which theoretically could mediate the formation of hexameric complexes composed of two NLI:Isl1:Lhx3 subunits (Figure 5A). To test whether this hypothesized complex may be involved in MN generation, we first established that a hexamer analog could assemble in vitro by demonstrating that the DD-Isl1 chimera (dimer equivalent to a 2NLI:2Isl1 tetramer) could bridge two Lhx3 molecules (Figures 5F and 5G). As expected, this complex was specifically dependent on the LIM domains of Lhx3, as L1-Lhx3 was unable to interact with DD-Isl1 (Figures 5F and 5H). In parallel, spinal cord electroporations were performed with Lhx3 and DD-Isl1 or with L1-Lhx3 and DD-Isl1 to test the biological activity of these factors. The Lhx3 and DD-Isl1 combination was extremely effective at triggering MN generation, whereas the L1-Lhx3 and DD-Isl1 combination was not (Figures 5B, 5C, 5G, and 5H). Thus, MN specification correlated with the ability to form 2NLI:2Isl1:2Lhx3 hexamers or analogs recapitulating this higher-order complex. Furthermore, the intact homeodomains of both Lhx3 and Isl1 were necessary for MN induction, as indi-

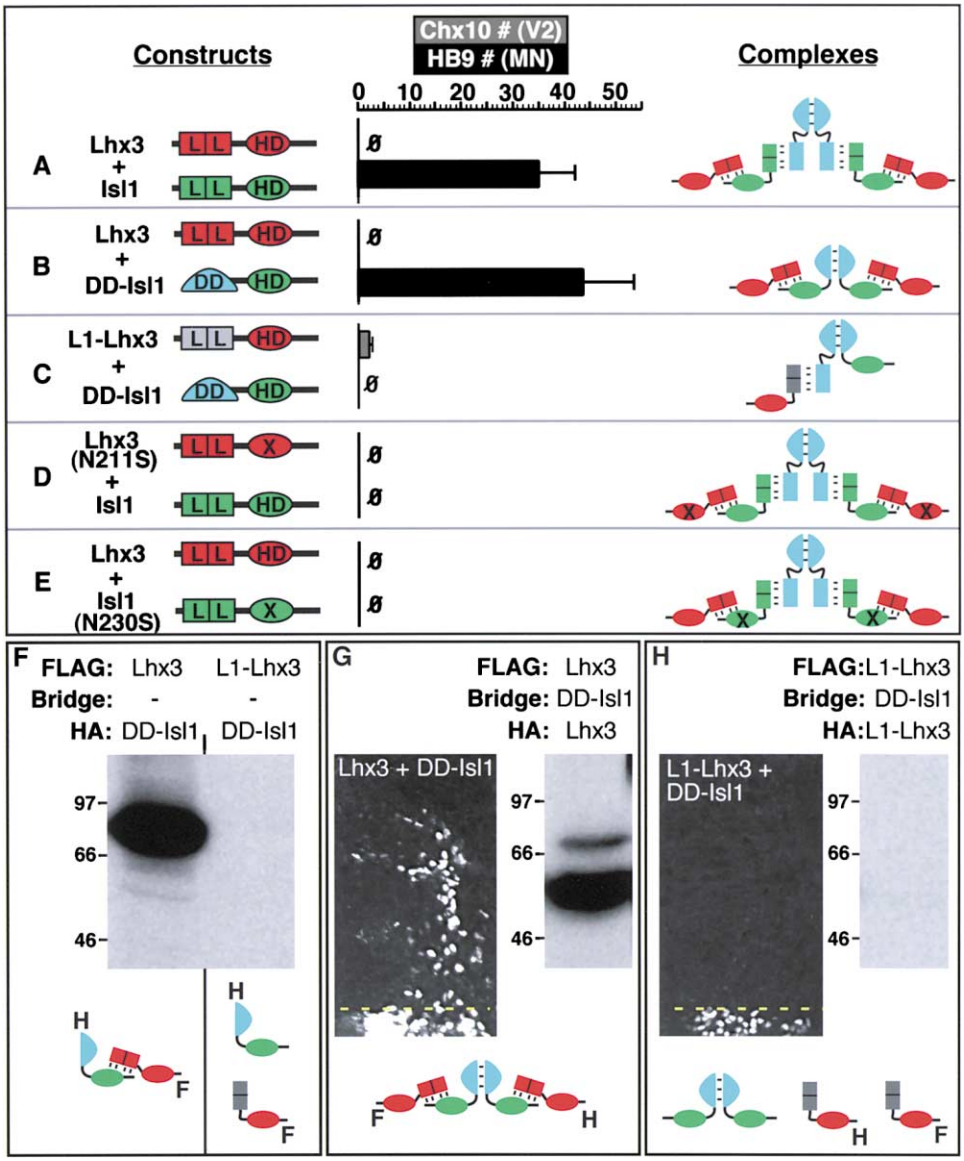

Figure 5. Lhx3 and Isl1 Hexameric Complexes Are Necessary for MN Specification

(A–E) Quantitative analysis of MN and IN differentiation as in Figure 4. Mutant constructs are described in Figures 3 and 4 and as follows: Isl1(N230S), a missense mutation that disrupts DNA binding by Isl1. Endogenous NLI (blue) mediates complex formation in several cases (A, C–E).

(F) Protein-protein interaction assay, as described in Figure 3. Lhx3 interacts with DD-Isl1, whereas L1-Lhx3 cannot.

(G) Electroporation of Lhx3 and DD-Isl1 triggers ectopic HB9^{+} MN differentiation. Protein interaction assays reveal that DD-Isl1 acts as a bridge for epitope-tagged Lhx3 molecules.

(H) L1-Lhx3 and DD-Isl1 fail to promote ectopic MN formation. This correlates with the inability of DD-Isl1 to mediate the bridging of L1-Lhx3 molecules.

cated by expressing mutant forms unable to bind DNA (Figures 5D and 5E). These data reveal a role for both Isl1 and Lhx3 homeodomains in MN specification.

These experiments demonstrated a requirement for the 2NLI:2Isl1:2Lhx3 hexamer in MN differentiation, but could not exclude an accessory role for the 2NLI:2Lhx3 tetramer in this process. We assessed the sufficiency of the 2NLI:2Isl1:2Lhx3 hexamer by using the nuclear LIM-only factor, LMO4 (Kenny et al., 1998), to compete for binding between NLI and Lhx3, thereby limiting the generation of 2NLI:2Lhx3 tetramers (Milan and Cohen, 1999; van Meyel et al., 1999, 2000). LMO4 was found to block the induction of V2 INs by Lhx3 (Figures 6A, 6B, and 6F), but under the same conditions it failed to inhibit MN specification by Lhx3 and DD-Isl1 (Figures 6C, 6D, and 6G). Use of the DD-Isl1 construct restricts the effects of LMO4 to NLI:Lhx3 interactions but does not inhibit the self-dimerization of DD-Isl1. Thus, MN differentiation occurs even when 2NLI:2Lhx3 complexes are inhibited from forming by LMO4.

The preceding observations predict that among the array of potential LIM complexes in MNs, the

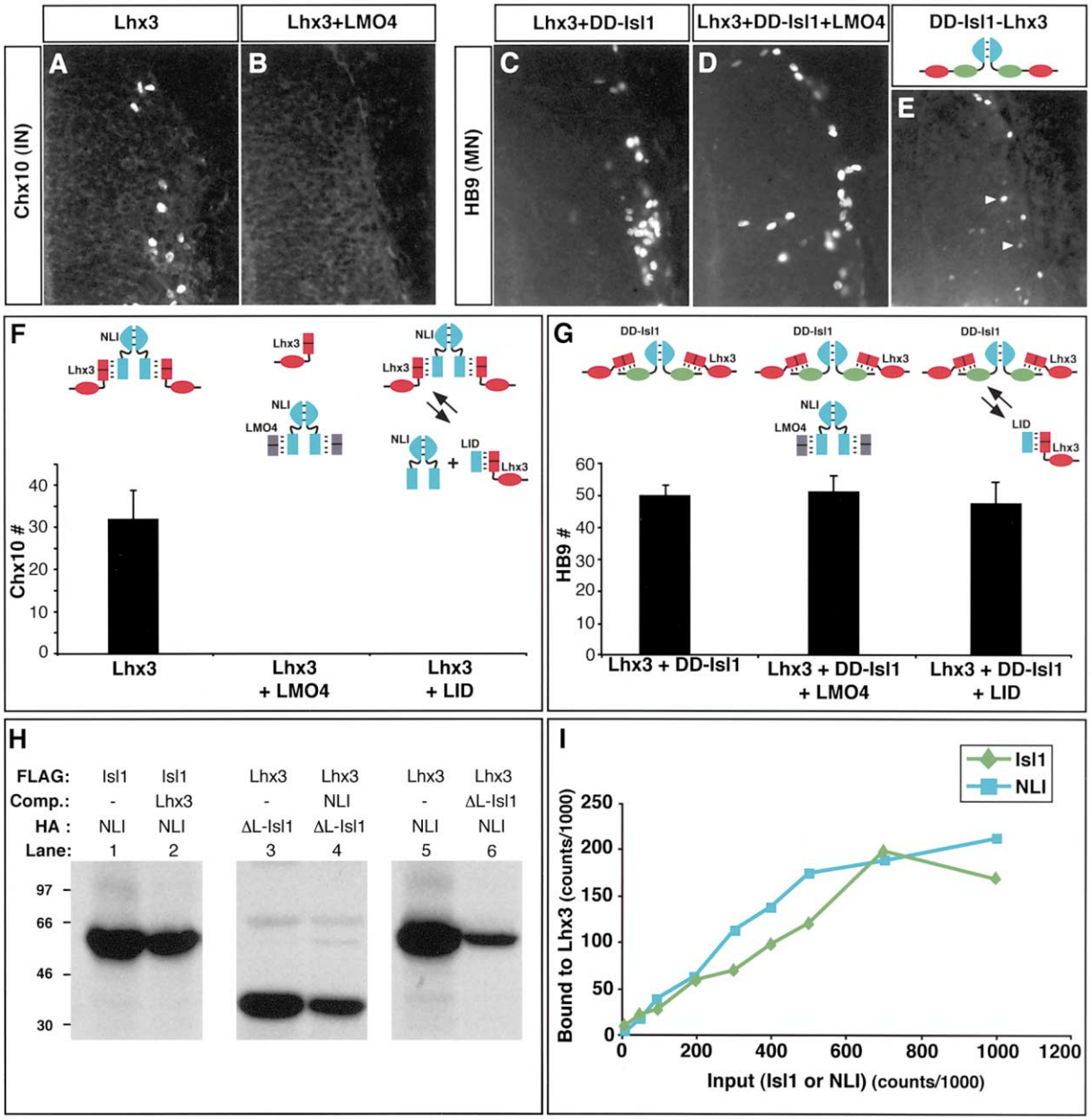

Figure 6. Hexamer Assembly Is Competitive with Tetramer Formation and Is Sufficient for MN Specification

(A–G) Analysis of V2 IN and MN differentiation in HH stage 24 electroporated chick embryos.

(A) Lhx3 induces Chx10^{+} IN differentiation.

(B) LMO4 inhibits the induction of V2 INs by Lhx3.

(C) Lhx3 and DD-Isl1 induce HB9^{+} MNs.

(D) LIM:NLI tetramers are not necessary for MN development because LMO4 fails to inhibit the activity of DD-Isl1 and Lhx3.

(E) Electroporation of a triple fusion of the DD of NLI with the C-terminal ends of Isl1 and Lhx3 (DD-Isl1-Lhx3). Ectopic HB9^{+} MNs (arrowheads) are triggered to differentiate.

(F) Quantitative analysis of V2 IN development in the presence of dominant-negative LIM-only LMO4 or LIM-interaction domain (LID) of NLI.

(G) Quantitative analysis of MN development in the presence of dominant-negative LIM-only LMO4 or LIM-interaction domain (LID) of NLI.

(H) Relative protein-protein interactions in the presence or absence of a 2-fold excess of untagged competitor. (Lanes 1 and 2) Lhx3 does not block Isl1 from interacting with NLI. (Lanes 3 and 4) NLI is a competitor for Lhx3 binding to C-terminal Isl1. (Lanes 5 and 6) Likewise, the C-terminal end of Isl1 is a strong competitor for the interaction between Lhx3 and NLI.

(I) Binding curve analysis reveals the affinity between Lhx3:NLI and Lhx3:Isl1 are similar. Increasing amounts of ^{35}S-labeled LID from NLI or ΔL-Isl1 were titrated into a binding reaction with excess Lhx3. Bound proteins were identified by immunoprecipitation of Lhx3 followed by SDS-PAGE, and bound LID (from NLI) and ΔL-Isl1 were quantified by phosphoimage analysis. Representative of four experiments.

2NLI:2Isl1:2Lhx3 hexamer is the central regulator of MN identity. As a test of this hypothesis, we generated a triple fusion molecule linking the dimerization domain of NLI, the carboxyl terminus of Isl1, and the carboxyl terminus of Lhx3 (DD-Isl1-Lhx3). This structural analog of the putative hexameric complex was sufficient to initiate MN programs (Figure 6E), albeit with about one-fourth the efficiency of native Lhx3 and Isl1. The reduced level of MN induction probably relates to the inefficiency of mimicking the native structure of the 2NLI:2Isl1:2Lhx3 hexamer with the chimeric molecule. Taken together, our data demonstrate that distinct types of LIM complexes are involved in the specification of V2 INs and MNs.

Isl1 Converts Lhx3 to a MN-Promoting Factor

Though we found that 2NLI:2Lhx3 specifies V2 INs and 2NLI:2Isl1:2Lhx3 generates MNs, it remained unclear how particular LIM complexes were selected to function in MNs. Specifically, it seemed likely that MNs would assemble complexes for both IN and MN development since they share Lhx3 and NLI. We focused on the role of Isl1 because coexpression experiments had shown it could efficiently switch Lhx3 from an IN to a MN factor (Figure 1). It might be argued that the basis for preventing Lhx3 from activating V2 IN genes in MNs is that MN fate is simply dominant over IN identity. However, the regulation of Lhx3 seems to be separable from the general process of MN differentiation. This was demonstrated by showing that ΔL-Isl1 retains the ability of full-length Isl1 to inhibit V2 IN differentiation by Lhx3, yet ΔL-Isl1 lacks the ability to promote MN differentiation (Figure 4D). Therefore, the switching off of Lhx3's IN function by Isl1 can be dissociated from MN differentiation per se, suggesting that a more direct mechanism may underlie the cell-specific regulation of Lhx3.

We undertook additional biochemical experiments to determine which of the many possible complexes are most likely to actually form in MNs. In order for hexamers (2NLI:2Isl1:2Lhx3) to assemble, it is predicted that Isl1 simultaneously binds NLI on its N terminus and Lhx3 on its C terminus. Though competition for binding sites is detected, an excess of Lhx3 fails to block Isl1:NLI interactions (Figure 6H, lanes 1 and 2). Similarly, an excess of NLI competes with, but does not abolish, interactions between Lhx3 and Isl1 (Figure 6H, lanes 3 and 4). Since these interactions are not mutually exclusive, these data suggest that a ternary complex is capable of forming, with Isl1 binding simultaneously to NLI and Lhx3.

Because the LIM domains of Lhx3 can interact with either Isl1 or NLI, we next examined whether the relative affinities would allow for binding competition, thereby creating a sorting mechanism for the formation of particular complexes. To test this, we quantified the reduction in protein binding that occurs when competitor is added in slight excess. Using phosphoimage analysis to quantify the amount of protein pulled down, 2-fold NLI as a competitor reduced the Lhx3:ΔL-Isl1 interaction by 50%; similarly, 2-fold ΔL-Isl1 reduced the Lhx3:NLI interaction by 80% (Figure 6H, lanes 3–6). Comparable synthesis and stability of each protein was monitored by autoradiography of a sample of each translation reaction taken prior to immunoprecipitation (data not shown). To more accurately define the relative affinities between Lhx3 and either Isl1 or NLI, we next performed a binding curve analysis in which the bound fraction of protein was measured as a function of the input protein concentration (Figure 6I). This analysis revealed that Isl1 and NLI bind with similar affinities to the LIM domains of Lhx3, since the relationship between input concentration and bound fraction were similar for Isl1 and NLI.

Taken together, these data indicate that the interactions between NLI and Lhx3 and between Isl1 and Lhx3 are similar in affinity and stability and therefore are expected to be highly competitive with one another. In MNs, the binding of Isl1 to NLI is expected to compete with Lhx3's access to this cofactor (Figure 6H, lanes 1 and 2). This should diminish the amount of functional V2 tetramer in MNs based on the finding that competition for binding to NLI efficiently blocks V2 IN differentiation (i.e., competition between Lhx3 and LMO4; Figures 6A, 6B, and 6F). The displacement of Lhx3 from NLI is expected to facilitate the high-affinity interaction between Isl1 and Lhx3 for hexamer formation, thereby causing a shift from IN complexes to MN complexes in the presence of Isl1.

These in vitro biochemical findings lead to several predictions regarding the in vivo assembly of LIM complexes. Namely, Isl1 is expected to convert Lhx3 to a MN factor and simultaneously inhibit V2 IN formation due to the formation of MN hexamers at the expense of IN tetramers. To test whether the interactions between Lhx3, NLI, and Isl1 are competitive in vivo, we examined V2 IN and MN specification in the context of LIM domain binding proteins. The LID region of NLI was used as a competitor since it binds with high affinity to LIM domains. As expected, V2 IN differentiation was blocked by LID, presumably because it competes for the Lhx3:NLI interaction that is necessary to form functional tetramers (Figure 6F). Under similar expression conditions, however, LID was ineffective at inhibiting MN generation (Figure 6G). These findings provide in vivo evidence that the interactions for MN complexes (i.e., Isl1:Lhx3 binding) can compete with the interactions for IN complexes (i.e., NLI:Lhx3 binding). Thus, using cell differentiation as a readout to monitor the specific activities of the LIM complexes, it is clear that hexamer function is favored over tetramer activity.

LIM Complexes Involved in Neuronal Connectivity

In addition to their role in MN specification, Isl1 and Lhx3 have been implicated in the later regulation of motor column specification and axon pathfinding in both *Drosophila* and mouse (Sharma et al., 2000; Thor et al., 1999). In particular, Lhx3 expression is extinguished from all MNs except those that settle medially and innervate axial musculature (MMCm class). Consequently, the ectopic expression of Lhx3 in $Isl1^+$ MNs of the lateral motor column prevents their axons from entering the limb in mice, and instead many project to axial muscles. Similarly, expression of Lhx3 in the $Isl1^+$ preganglionic column repositions the cell bodies into the median motor column (Sharma et al., 2000).

Based on the relative binding interactions described above, it is expected that MMCm cells likewise form

hexamers (2NLI:2Isl1:2Lhx3) more readily than tetramers (2NLI:2Isl1, 2NLI:2Lhx3, 2NLI:Lhx3:Isl1). If this is the case, tetrameric complexes would not be expected to play a prominent role in the regulation of cell migration and axon navigation. To test this, we reconstituted the MMCm LIM code in non-MMCm cells using L1-Lhx3, which can only interact with NLI and therefore is limited to participating in tetrameric complexes, and we compared this to wild-type Lhx3, which favors the formation of hexameric complexes. Expression of GFP in MNs at brachial levels reveals axons entering the chick forelimb, whereas coexpression of GFP and Lhx3 recapitulates the mouse phenotype, with little or no GFP labeling beyond the plexus of the limb (Figures 7A and 7B). Similarly, MNs in the column of Terni (chick preganglionic column) fail to migrate to their normal dorsomedial position when expressing Lhx3 (Figures 7D and 7E). In contrast, the L1-Lhx3 fusion, which can form tetramers but not hexamers, was unable to significantly alter MN cell migration or axon guidance (Figures 7C and 7F). Thus, the hexameric complex appears to be a central regulator of the genetic pathways for both MN cell specification and the topographic organization of subsets of MNs that occurs later in development (Figure 7G).

Discussion

The generation of cellular diversity frequently involves transcriptional regulators acting in a combinatorial manner. Although this represents a recurrent strategy in development, the mechanisms that allow individual factors to be used repeatedly yet specify different outcomes depending on their cellular context are not well defined. We have examined this issue with regard to the LIM transcription factor family. Our studies have defined a biochemical rationale for the early actions of two LIM homeodomain proteins, Isl1 and Lhx3, that have well-established functions in spinal neuron differentiation (Pfaff et al., 1996; Sharma et al., 1998; Tanabe et al., 1998). In the context of V2 INs, we find that Lhx3 interacts with the LIM-bridging molecule NLI to form tetrameric complexes for the specification of these neurons. The 2NLI:2Lhx3 complex in V2 cells represents the canonical architecture for LIM homeodomain factor complexes based on biochemical and genetic studies in *Drosophila* (Milan and Cohen, 1999; van Meyel et al., 1999, 2000). In MN differentiation, the potential to form LIM complexes is complicated by the expression of both Isl1 and Lhx3. Our studies indicate that it is not the composite activities of multiple hetero- and homomeric LIM tetramers, but rather the single action of a novel NLI-mediated hexameric LIM complex that drives MN specification.

LIM Factors as Regulators of Transcription

Although the assays used in these experiments centered on examining cell specification, it is implicit that the V2 complex and MN complex act differently by regulating distinct genes. This is predicted to occur through the ability of MN-hexamers to recognize different DNA elements from V2-tetramers (Figure 7G). The homeodomain of Lhx3 is required for the function of both the MN complex and V2 complex, and thus the specific architecture

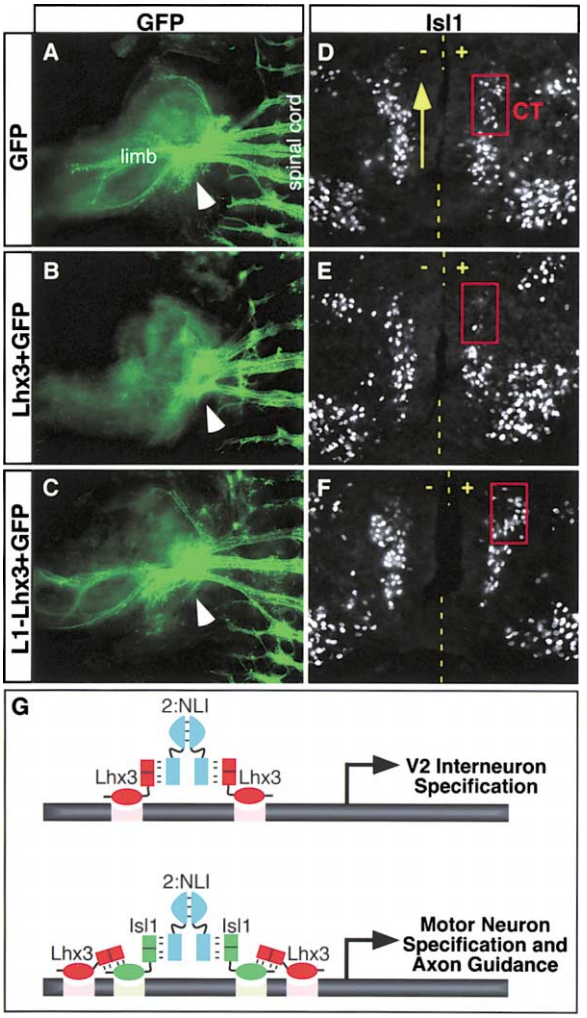

Figure 7. Lhx3:NLI Interactions Are Insufficient to Regulate Axon Guidance and Cell Migration

Embryos electroporated (+ side) at stage 12 with (A and D) GFP, (B and E) Lhx3 and GFP, and (C and F) L1-Lhx3 and GFP. Embryos were analyzed at HH stage 28 after axon outgrowth and motor column formation.

(A) GFP labels LMC motor axons extending from the spinal cord via ventral roots to the brachial plexus (white arrowhead) and into the distal limb.

(B) Lhx3 prevents LMC axons from projecting beyond the plexus to innervate the limb.

(C) L1-Lhx3 does not alter LMC axonal projections.

(D) Isl1 labels the sympathetic motor column of Terni (CT, boxed in red) in the medial spinal cord (33 ± 5 cells/half-section). The normal migration of CT MNs is indicated by a yellow arrow.

(E) Lhx3 prevents CT cell migration, resulting in the absence of dorsomedial Isl1$^+$ cells (7 ± 5 cells/half-section). These cells cluster with the somatic groups in the ventral spinal cord (not shown).

(F) L1-Lhx3 fails to mimic Lhx3 activity, leaving the CT unaltered (30 ± 4 cells/half-section). Images represent data from 5, 8, and 3 embryos, respectively.

(G) Summary depicting the putative LIM-signaling complexes bound to DNA and their developmental activities.

of the complexes must contribute to the way that Lhx3 is converted from activating V2 IN genes to MN genes in the presence of Isl1 and NLI.

What role does NLI play in the LIM complexes? Our

studies indicate that the dimerization of NLI is necessary for the proper function of the complexes involved in both IN and MN specification. It remains unclear whether NLI dimerization is necessary for the LIM factors to bind DNA, to activate transcription, or to interact with other factors. In addition to the LIM genes, NLI has been reported to interact with a variety of other transcription factors (Bach et al., 1997; Ramain et al., 2000; Torigoi et al., 2000). In *Drosophila*, lower-affinity non-LIM interactions have been found to occur in a region of Chip located between residues 439 and 456 (Torigoi et al., 2000). The homologous region was included with the amino-terminal region of NLI containing the dimerization domain when creating the chimeric NLI-LIM proteins for our analyses. Therefore, it is possible that in addition to dimerization, NLI also serves as a docking site for additional cofactors involved in neuronal specification. Nevertheless, the NLI:LIM complexes described here were able to specify particular neurons at all dorsal-ventral locations, so any important cofactors for the LIM complexes must be present throughout the neural tube and cannot account for their specific functions.

The Specificity Problem

Given the dimerization properties of the LIM factors and NLI, any cell expressing two or more of these proteins is confronted with the possibility of assembling a multitude of complexes (Jurata et al., 1998). A priori, different cells expressing some of the same LIM factors are expected to assemble overlapping arrays of related transcriptional complexes, thereby creating the potential for activation of inappropriate genes. Not surprisingly, in the developing spinal cord, where numerous transcriptional codes operate to control the acquisition of cell identities, many studies of genetic mutants have detected examples of hybrid cell fates (Arber et al., 1999; Ericson et al., 1996; Sander et al., 2000; Thaler et al., 1999; Vallstedt et al., 2001). Clearly the normal mechanisms that control gene expression in the developing spinal cord are designed to restrict inappropriate genes from becoming expressed in order to establish proper cell identity.

Transcriptional synergy between overlapping combinations of transcription factors appears to be one general mechanism for achieving cell type-specific gene regulation (Dasen and Rosenfeld, 1999). Here, we have described a mechanism for generating specific transcriptional responses from overlapping LIM-homeodomain transcription factor codes—through the competitive formation of neuronal subtype-specific transcription complexes. Thus, the activity of Lhx3 is regulated by forming different types of complexes in each cellular environment that select different DNA targets to activate. This contrasts with the synergistic mechanism for context-dependent gene activation, where transcription factors are thought to interact with many targets but only activate the subset of genes with the appropriate ensemble of factors present.

The specificity mechanism described above raises the question of how cell type-specific LIM complexes might be generated. Several features of Lhx3 and Isl1 appear to be responsible. First, Lhx3 can interact with both NLI and Isl1. Second, Isl1 directly gates the activity of Lhx3, suppressing its IN activity and activating its MN function. Lastly, a bias in the formation of LIM complexes in MNs is predicted due to the displacement of Lhx3 from NLI by Isl1 and the presence of a high-affinity binding site for Lhx3 on the C-terminal end of Isl1. Therefore, the formation of hexamers depletes Lhx3 from IN-tetramers. Likewise, the direct binding of Lhx3 to Isl1 reduces the number of 2NLI:2Isl1 tetramers in MNs. Whether 2NLI:2Isl1 complexes have the potential to regulate inappropriate genes in somatic MNs remains unknown. However, hindbrain visceral MNs, dorsal root ganglion sensory neurons, and forebrain neurons express Isl1 in the absence of Lhx3 (Thor et al., 1991), suggesting 2NLI:2Isl1 tetramers may be involved in gene regulation within these neurons and therefore may represent an undesirable LIM complex in somatic MNs. The use of multiple interactions involving competition for the same binding site, in this case the LIM domains of Lhx3, represents an additional strategy for using factors in multiple cellular contexts.

Another mechanism that is known to contribute to the clean switch in Lhx3's activity is the involvement of feedback regulatory interactions in MNs by HB9, whose expression is triggered by the MN-hexamer. HB9 probably contributes to the silencing of V2 IN genes in MNs using at least two mechanisms. Studies of mouse mutants indicate that HB9 is necessary for maintenance of high levels of Isl1 in MNs (Arber et al., 1999; Thaler et al., 1999) needed to compete with Lhx3 for binding in V2 IN complexes. In addition, HB9 appears to function as a transcriptional repressor that can silence V2 IN genes (Muhr et al., 2001; Tanabe et al., 1998). Therefore, the initial bias in hexamer formation appears to be sufficient to initiate a cascade of gene expression involving HB9 that serves to further refine the appropriate pattern of gene expression.

Differentiation Pathways and LIM codes

Twelve LIM homeodomain genes and four LIM-only genes have been identified in higher vertebrates to date, and many have been implicated in the development of both neuronal and nonneuronal cell types (Hobert and Westphal, 2000). Within the spinal cord and hindbrain, a number of striking examples of combinatorial LIM codes have been found to label discrete cell populations (Appel et al., 1995; Tsuchida et al., 1994; Varela-Echavarria et al., 1996). Recent studies have also indicated that LIM codes are involved in the specification of particular cell types in the forebrain region of the CNS (Marin et al., 2000; Nakagawa and O'Leary, 2001). Like the example of Isl1 and Lhx3 in this report, cells using multiple LIM factors are confronted with the possibility of assembling a number of LIM complexes with NLI. In MN differentiation we find that this apparent paradox is resolved through the use of multiple protein-protein interactions mediated by the specific LIM domains of Lhx3. The number of cell types and combinatorial codes in which the LIM factors have been implicated suggests these factors may participate in many more cell-specific protein-protein interactions than have currently been identified. In the case of Lhx3, which is also involved in pituitary development (Sheng et al., 1996), an additional interaction has been found with SLB in these cells (Howard and Maurer, 2000). The LIM factors may be particu-

larly well suited to act in combinatorial codes, because LIM domains represent a robust module for mediating numerous protein-protein interactions (Dawid et al., 1998).

Our studies also provide insight into the transcriptional cascades involved in MN and IN development. The LIM homeodomain complexes involved in V2 IN and MN specification are capable of overriding extrinsic signals and progenitor genetic programs in the dorsal neural tube. The relatively late expression and sufficiency of Lhx3/Isl1 codes in directing cell fate decisions provides evidence that combinatorially expressed LIM homeodomain factors execute the progenitor cell repressor programs (Briscoe et al., 2000; Marquardt and Pfaff, 2001). This model, however, suggests that MN identity remains undefined beyond the final cell division as Isl1 is expressed exclusively by postmitotic cells (Ericson et al., 1992; Pfaff et al., 1996). Similarly, V0 IN specification requires the postmitotic protein, Evx1 (Moran-Rivard et al., 2001). The identification of relatively late-acting factors with the capacity to override the initiated preprograms in embryonic progenitor cells may provide the means to generate specific classes of MNs and INs from neural stem cells.

Experimental Procedures

DNA Constructs

Rat Isl1, HB9; mouse Lhx3, NLI and LMO4; and GFP cDNAs were cloned into pCS2 (Turner and Weintraub, 1994). ΔL-Isl1 (aa 110–349) and ΔL-Lhx3 (aa 151–402) were used to generate the DD (NLI aa 1–300) fusions, DD-Isl1, DD-Lhx3, and DD-Isl1-Lhx3. The LIM domains of Lhx1 (aa 1–117) were added to the ΔL-Lhx3 to create the chimeric L1-Lhx3 molecule, and site-directed mutagenesis was used to generate DNA binding mutants Lhx3 (N211S) and Isl1 (N230S). DD (NLI aa 1–300) and LID (NLI aa 300–376) were also cloned into pCS2.

Immunostaining

Immunohistochemistry was performed as described previously (Jurata et al., 1996; Thaler et al., 1999). The following antibodies were used: mouse anti-Lhx3 (4E12, DSHB), rabbit anti-Lhx3 (Sharma et al., 1998), mouse anti-Isl1/2 (Tsuchida et al., 1994) (4D5, DSHB), rabbit anti-Isl1/2 (Ericson et al., 1992), rabbit anti-NLI (Jurata et al., 1996), rabbit anti-Chx10 (Thaler et al., 1999), guinea pig anti-Chx10 (Thaler et al., 1999), mouse anti-Isl2 (Tsuchida et al., 1994) (4H9, DSHB), mouse anti-MNR2/HB9 (Tanabe et al., 1998) (5C10, DSHB), rabbit anti-Nkx6.1 (Briscoe et al., 2000), guinea pig anti-Olig2, and mouse anti-SC1.

In Ovo Electroporation

Expression constructs encoding truncated, hybrid, and tagged versions of Lhx3, Isl1, HB9, NLI, and GFP were injected into the lumens of Hamburger and Hamilton (HH) stage 12–14 chick embryonic spinal cords (Hamburger and Hamilton, 1951). Electroporation was performed using a square wave electroporator (BTX) as previously described (Nakamura and Funahashi, 2001). Coelectroporation results in >80% of cells expressing both constructs. Incubated chicks were harvested and analyzed at stage 22–30. GFP-transfected chicks were dissected as half-mounts, cleared in glycerol, and photographed using a Zeiss Stemi SV fluorescent dissecting microscope.

Protein Interactions

Isl1, Lhx3, NLI, and their derivates were subcloned into hemagglutinin-tag and FLAG-tag versions of pcDNA (Invitrogen). TNT reticulocyte lysate (Promega) was used to produce [^{35}S]methionine-labeled proteins. Anti-FLAG M2 antibody (Eastman Kodak) and anti-HA antibody 12CA5 and HA.11 (Berkeley Antibody Co.) were used for immunoprecipitations. Following the production of in vitro transcribed and translated proteins, LIM complexes were first immunoprecipitated with anti-FLAG antibodies and protein A-sepharose, washed, dissociated, and then reprecipitated with anti-HA antibodies prior to electrophoresis on 12.5% SDS-polyacrylamide gels and fluorography (Jurata et al., 1998).

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