

12-9-2004

# Lhx9 and lhx9alpha: differential biochemical properties and effects on neuronal differentiation

Bertrand Molle  
*Institut e Neurobiologie*

Stephane Pere  
*Institut e Neurobiologie*

Vieri Failli  
*Institut e Neurobiologie*

*See next page for additional authors*

Follow this and additional works at: [https://escholarship.umassmed.edu/pgfe\\_pp](https://escholarship.umassmed.edu/pgfe_pp)

 Part of the [Genetics and Genomics Commons](#)

## Repository Citation

Molle, Bertrand; Pere, Stephane; Failli, Vieri; Bach, Ingolf; and Retaux, Sylvie, "Lhx9 and lhx9alpha: differential biochemical properties and effects on neuronal differentiation" (2004). *Program in Gene Function and Expression Publications and Presentations*. 137. [https://escholarship.umassmed.edu/pgfe\\_pp/137](https://escholarship.umassmed.edu/pgfe_pp/137)

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in Program in Gene Function and Expression Publications and Presentations by an authorized administrator of eScholarship@UMMS. For more information, please contact [Lisa.Palmer@umassmed.edu](mailto:Lisa.Palmer@umassmed.edu).

---

# Lhx9 and lhx9alpha: differential biochemical properties and effects on neuronal differentiation

## **Authors**

Bertrand Molle, Stephane Pere, Vieri Failli, Ingolf Bach, and Sylvie Retaux

## **Rights and Permissions**

Copyright © 2004 Mary Ann Liebert, Inc. [Link to article on publisher's site.](#)

## *Lhx9* and *Lhx9 $\alpha$* : Differential Biochemical Properties and Effects on Neuronal Differentiation

BERTRAND MOLLÉ,<sup>1</sup> STÉPHANE PÈRE,<sup>1</sup> VIERI FAILLI,<sup>1</sup> INGOLF BACH,<sup>2</sup> and SYLVIE RÉTAUX<sup>1</sup>

### ABSTRACT

The *Lhx9* LIM-homeodomain transcription factor and its truncated isoform *Lhx9 $\alpha$*  are generated by alternative splicing of the *Lhx9* gene. Here we investigated the differential functional properties of these two isoforms. *Lhx9 $\alpha$* , which lacks parts of the homeodomain, was unable to bind DNA in EMSA experiments, but was able to associate with CLIM cofactors in GST pull-down assays. In transfection experiments in PC12 cells, *Lhx9 $\alpha$*  fusion constructs systematically showed a nuclear localization, as opposed to *Lhx9* fusion constructs, which also localized to the cytoplasm. Moreover, *Lhx9* increased NGF-induced neuronal differentiation of PC12 cells. *Lhx9 $\alpha$* , on the other hand, did not significantly increase neuronal differentiation but had an effect on the morphology of PC12 cells. Finally, as tested by RT-PCR experiments on transfected PC12 cells, *Lhx9* was not able to induce the transcription of *Lhx9 $\alpha$* . Our results show significantly different functional properties for *Lhx9* and *Lhx9 $\alpha$* , and suggest that *Lhx9 $\alpha$*  can compete away limiting amounts of nuclear CLIM cofactors. Thus, *Lhx9* and *Lhx9 $\alpha$*  isoforms could be implicated in regulating various aspects of neuronal differentiation.

### INTRODUCTION

LIM DOMAIN-CONTAINING PROTEINS represent a large family including LIM-homeodomain (LIM-HD) developmental transcription factors, LIM-only (LMO) nuclear or cytoplasmic factors, and LIM-kinases (reviewed in Bach, 2000; Rétaux and Bachy, 2002). The functions of these various LIM proteins are diverse, from patterning and cell specification during development to cytoskeletal regulation and control of cell division. Among them, the LIM-HD transcription factors and the LMO nuclear factors exert their function via an interaction of their LIM domains with cofactors named NLI/Ldb/CLIM (Agulnick *et al.*, 1996; Jurata *et al.*, 1996; Bach *et al.*, 1997) or with other factors (e.g., Bach *et al.*, 1999). Therefore, the LIM domains are generally regarded as protein-protein interaction motifs, which constitute scaffolds for the formation of higher order regulatory complexes.

There are 13 LIM-HD family members in mammals, which are distributed into six subgroups, and which all have orthologs in nonmammalian vertebrates and invertebrates (Failli *et al.*, 2000; Bachy *et al.*, 2002). They are expressed in highly spe-

cific patterns throughout embryonic development, particularly in the nervous system where they are involved in regional and neuronal cell-type specification. Among other examples, *Isl1* controls motorneuron specification in the spinal cord (Pfaff *et al.*, 1996), *Lmx1b* controls the serotonergic phenotype (Cheng *et al.*, 2003; Ding *et al.*, 2003), and *Lhx7* induces forebrain cholinergic neuron differentiation (Zhao *et al.*, 2003). To exert their transcriptional control on such various aspects of neuronal differentiation, LIM-HD factors interact with Ldb1/Ldb2 (or CLIM1/CLIM2), which are also expressed throughout the developing brain (Bach *et al.*, 1997). Due to their capacity of self-dimerization (Jurata and Gill, 1997), these cofactors can bridge two LIM-HD proteins together, and allow the formation of transcriptionally active heterotetrameric (or even hexameric) complexes composed of 2LIM-HD:2CLIM proteins. Studies in *Drosophila* have shown that any disruption of the LIM-HD:CLIM complex by factors such as LMO proteins which are able to compete with LIM-HD for CLIM interaction, also disrupts the normal function of the LIM-HD factor in a developmental event (Milan *et al.*, 1998; Milan and Cohen, 2000). Because LMO, LIM-HD and cofactors are often coexpressed in

<sup>1</sup>UPR 2197 "Développement, Evolution, Plasticité du Système Nerveux," Institut de Neurobiologie Alfred FESSARD, C.N.R.S, GIF-sur-YVETTE cedex, France.

<sup>2</sup>Center for Molecular Neurobiology Hamburg (ZMNH), University of Hamburg, Hamburg, Germany.

the same cells, a tightly regulated equilibrium between the expression levels and activity levels of the different partners is present for the developmental process to take place correctly (Weihe *et al.*, 2001; Becker *et al.*, 2002; Ostendorff *et al.*, 2002; Hiratani *et al.*, 2003).

The mouse *Lhx9* gene is able to generate two alternative transcripts encoding two isoforms, Lhx9 and Lhx9 $\alpha$  (Failli *et al.*, 2000). Whereas Lhx9 presents the classical features of a LIM–HD factor, Lhx9 $\alpha$  is truncated at the level of the third helix of its homeodomain. Moreover, the developmental expression patterns of the two transcripts show significant differences in levels and distribution. Therefore, we previously suggested that Lhx9 $\alpha$  could function as an endogenous dominant-negative form of Lhx9 during development, and could regulate and/or refine in space and time the transcriptional effects of Lhx9 (Failli *et al.*, 2000), in a manner similar to the competition between LIM–HD and LMO proposed in *Drosophila* (Milan *et al.*, 1998).

Here, we further characterize the biochemical properties of Lhx9 $\alpha$ , and show that this truncated isoform behaves like a nuclear LMO protein. In addition, we assess the respective effects of Lhx9 and Lhx9 $\alpha$  on neuronal differentiation in cell culture experiments, and show that they have distinct effects on the differentiation of PC12 cells.

## MATERIALS AND METHODS

### *In vitro* protein–protein interaction assays

PCR products of full-length Lhx9 and Lhx9 $\alpha$  were ligated in frame into the EcoRI/NcoI sites of the pGEX-KG bacterial expression vector to yield glutathione S-transferase (GST) fusion proteins. The *in vitro* protein–protein interaction assays with <sup>35</sup>S-methionine labeled, *in vitro* transcribed-translated CLIM1 and CLIM2 proteins were performed as described previously (Bach *et al.*, 1997).

### Electrophoretic mobility shift experiments (EMSA)

EMSA experiments and EMSA supershift assays were performed as described previously (Bach *et al.*, 1997) using <sup>32</sup>P-labeled oligonucleotides that encompass the Lhx3 binding site on the  $\alpha$ GSU promoter (Roberson *et al.*, 1994; Bach *et al.*, 1995) and bacterially expressed GST-fusion proteins.

### Culture of PC12 cells

PC12 cells were maintained in RPMI–glutamax culture medium containing 10% heat-inactivated horse serum and 5% heat-inactivated foetal veal serum. For NGF treatment (2.5S, Promega, Madison, WI, final concentration 50 ng/ml), the serum content was reduced to 1% total.

For transfection experiments, 2.10<sup>6</sup> cells were suspended in 400  $\mu$ l Opti-MEM and electroporated in the presence of 10  $\mu$ g DNA on a Biorad electroporator. Cells were then plated onto 24-well plates at a density of 1–5.10<sup>5</sup> cells/well on glass coverslips coated with Matrigel or with poly-L lysine/poly-ornithine/laminine. After 3 days of NGF treatment cells were fixed with 4% paraformaldehyde and processed for immunofluorescence and imaging.

For RT-PCR experiments, total RNA was extracted from

~10.10<sup>6</sup> cells using Trizol (Invitrogen, Carlsbad, CA). Random-primed, reverse-transcribed cDNA (AMV reverse transcriptase, Roche, Indianapolis, IN) was used as template for PCR using specific oligonucleotides primers (18–20 mers, sequence available on request) designed on the sequences of mouse (or rat when available) LIM-HD and CLIM cDNAs.

### *Lhx9* and *Lhx9 $\alpha$* expression constructs

The full-length coding sequences of Lhx9 and Lhx9 $\alpha$  were amplified by PCR and subcloned in frame as GFP or myc-tag fusions into the pEGFP-N3 (Clontech, Palo Alto, CA) or the pCS2-MT (a gift of David Turner) expression vectors.

### Immunofluorescence staining on PC12 cells

Fixed cells were rinsed with PBS and primary antibody incubation was performed during 2 h at room temperature in PBS containing 0.1% Triton and 0.5% BSA (PBT). A monoclonal mouse anti-GFP (Roche) was used at 1/500 and a monoclonal anti-myc (9E10) FITC-conjugate (Sigma, St. Louis, MO) was used at 1/200. Secondary antibodies were added in PBT (goat anti-mouse-Alexa488 or -Alexa594, Molecular Probes, 1/200) for 1 h and washed again. If needed, cells were counterstained with Alexa-594 phalloidin and DAPI (Molecular Probes). Coverslips were mounted with PPD-glycerol antifading medium and observed on a Nikon E800 fluorescence microscope equipped with a DXM1200 camera. Images were occasionally corrected for brightness/contrast and mounted using Adobe Photoshop (Adobe Systems, San Jose, CA).

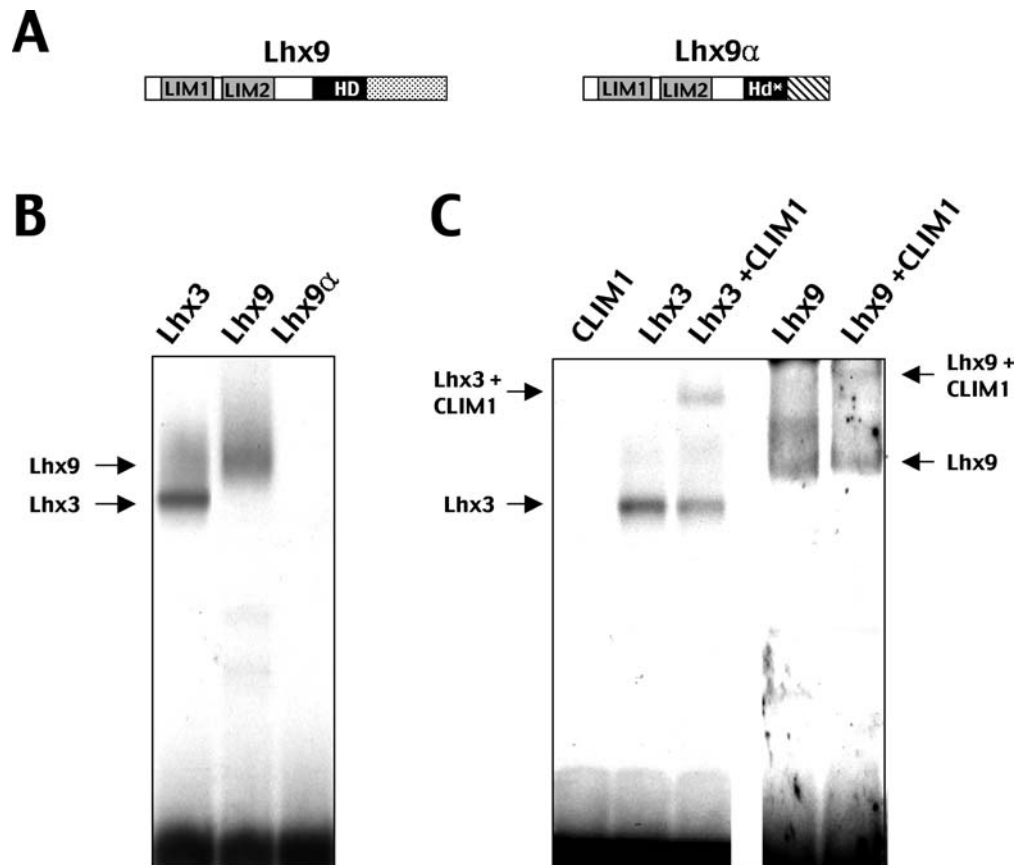
### Differentiation assay

For the neuronal differentiation assay of NGF-induced PC12 cells, a minimum of nine coverslips containing at least 150 transfected cells and obtained from three independent experiments were counted. Cells were considered to exhibit signs of neuronal differentiation if at least one of their neurite was equal in length or longer than the soma size. Statistical comparison was carried out using a Student's *t*-test.

## RESULTS

### *Lhx9 $\alpha$* is able to interact with cofactors but does not bind DNA

Lhx9 and Lhx9 $\alpha$  are identical in their N-terminal amino acid sequence including LIM domains, linker, and the two first helices of the homeodomain. The rest of their sequences is different, in that Lhx9 $\alpha$  is truncated at the level of the third helix of the homeodomain, and shows a distinct C-terminus sequence and 3'UTR sequence (Failli *et al.*, 2000, schematized in Fig. 1A). Because Lhx9 $\alpha$  lacks the third helix of the homeodomain which has previously been identified as crucial for homeodomain–DNA interactions (e.g., Kissinger *et al.*, 1990), we first tested and compared the DNA binding capacities of the two Lhx9 isoforms using EMSA experiments, with a probe containing the LIM–HD binding site of the  $\alpha$ GSU promoter. This sequence is recognized at least by Lhx2, the Lhx9 paralog, and by Lhx3 (Roberson *et al.*, 1994; Bach *et al.*, 1995). Indeed, similar to Lhx3, Lhx9 was not only able to interact with this sequence (Fig. 1B) but also to form slower migrating complexes



**FIG. 1.** DNA-binding capacities of *Lhx9* and *Lhx9α*. (A) A schematic representation of the structure of the two *Lhx9* isoforms. Both isoforms are identical in their LIM domains (LIM1 and LIM2, gray boxes) and the beginning of their homeodomains (HD, black box), as indicated by the gray shading between dotted lines. Note that the homeodomain of *Lhx9α* is truncated (asterisk) and that they differ in their C-terminal sequence (indicated by different fill patterns). (B) An EMSA experiment showing the interaction of *Lhx3* and *Lhx9* but not *Lhx9α* on the oligonucleotide probe. (C) Shows the formation of a  $^{32}\text{P}$ -labeled complex supershift in the presence of CLIM1 for both *Lhx3* and *Lhx9*.

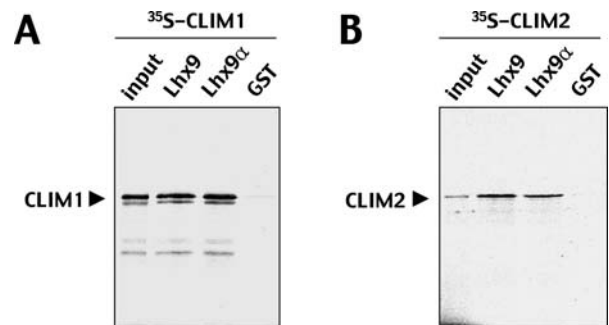
with the CLIM1 protein while bound to DNA in supershift experiments (Fig. 1C), indicating that *Lhx9* can form ternary complexes on DNA with CLIM cofactors. Conversely, *Lhx9α* was not able to form a complex with the oligonucleotide probe (Fig. 1B, right lane), showing that the absence of the third helix of the homeodomain abolishes its capacity to bind DNA.

Because *Lhx9* and *Lhx9α* both contain two identical LIM domains we tested their capacity to bind CLIM cofactors in GST pull-down experiments. The results demonstrate that both *Lhx9* and *Lhx9α* interact with CLIM1 and CLIM2 proteins with comparable affinity (Fig. 2A–B). Thus, similar to LMO proteins, *Lhx9α* associate with CLIM cofactors but does not bind to DNA.

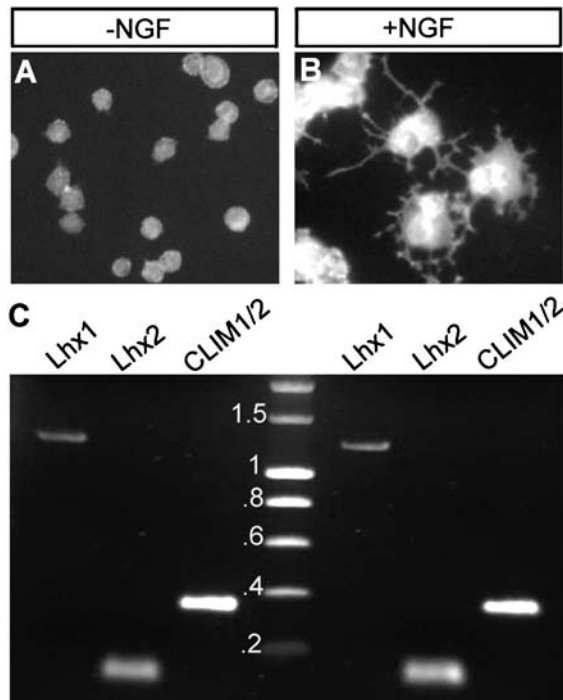
#### *Lhx9*, *Lhx9a*, and neuronal differentiation

We have previously reported that *Lhx9* and *Lhx9a* are mostly coexpressed in the same brain regions, *Lhx9a* being expressed at lower levels and significantly later in development. Given that LIM–HD factors are commonly involved in neuronal specification, we next sought to investigate whether the two *Lhx9* isoforms had differential effects on the neuronal differentiation process. To this end, we used PC12 cells (a rat pheochromocytoma

cell line), taking advantage of this cell line property to differentiate into neurons after NGF treatment (Fig. 3A–B). To validate the PC12 cell culture system as a tool to assess the effect of a LIM–HD factor, we first tested whether these cells express the



**FIG. 2.** CLIM-binding capacities of *Lhx9* and *Lhx9α*. (A, B) Present autoradiograms of GST pull-down experiments where  $^{35}\text{S}$ -labeled CLIM1 (A) or CLIM2 (B) strongly interact with bacterially expressed GST-fusion of *Lhx9* and *Lhx9α*. The input lane shows 10% of the total  $^{35}\text{S}$ -labeled protein input, and the control GST lane shows that GST itself does not interact with the CLIMs.



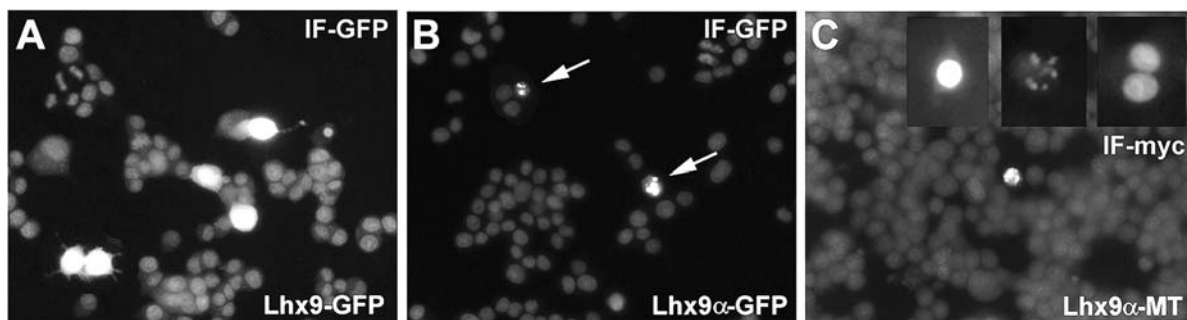
**FIG. 3.** PC12 cells express proteins of the LIM-HD network. (A, B) Show high magnification fluorescence photomicrographs of PC12 cells not treated (A) or treated (B) with NGF during 72 h. Cells were counterstained with Alexa-594 phalloidin to visualize the actin cytoskeleton and the neurites, together with a DAPI nuclear stain. (C) Shows the result of RT-PCR experiments performed on total RNA extracted from control or NGF-treated PC12 cells. The identity of PCR products are indicated and run at the expected size according to the pairs of primers used (1.2 kb for *Lhx1*, 160 bp for *Lhx2*, and 350 bp for *CLIM*).

components of the LIM-HD protein network, that is, CLIM cofactors and at least some LIM-HD factors. RT-PCR analysis on PC12 cells with or without NGF treatment showed that under both conditions these cells express mRNAs encoding CLIM cofactors (the oligonucleotide primers used for PCR amplify both CLIM1 and CLIM2) and the LIM-HD factors *Lhx1* and *Lhx2*

(Fig. 3C). Other LIM-HD members tested included *Lhx5*, *Lhx9*, *Lhx9 $\alpha$* , and *Lmx1a*, which were not detected in these RT-PCR experiments (data not shown and Fig. 6B).

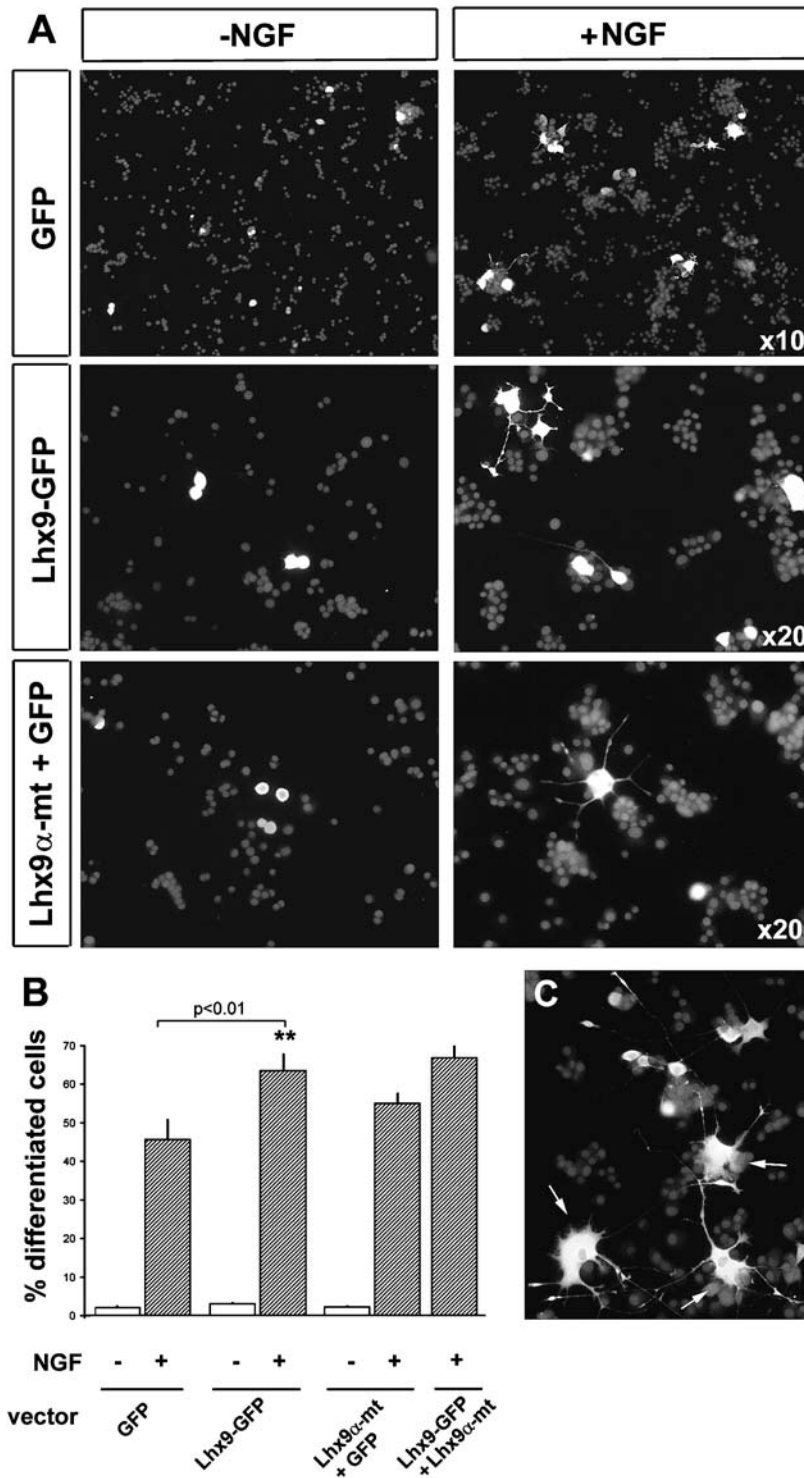
In a first series of experiments, and to further characterize the functional properties of the two isoforms, *Lhx9*-GFP and *Lhx9 $\alpha$* -GFP fusion constructs were electroporated into PC12 cells. We found a clearly distinct subcellular localization of the two fusion proteins after GFP immunofluorescence staining: *Lhx9*-GFP was found abundantly throughout the cell nucleus, cytoplasm, and neurites, whereas *Lhx9 $\alpha$* -GFP was exclusively restricted to a punctate-like nuclear staining, both in the presence or absence of NGF (Fig. 4A and B). To discard the possibility of a distinct localization of *Lhx9 $\alpha$*  due to the GFP tag, we transfected a *Lhx9 $\alpha$* -myc-tag construct. We found a similar, although slightly more diffuse, nuclear punctate-like staining after myc immunofluorescence labeling of the *Lhx9 $\alpha$* -myc fusion protein (Fig. 4C). The same results were observed when mouse primary cortical neurons were transfected with *Lhx9*-GFP or *Lhx9 $\alpha$* -myc-tag, respectively (data not shown). Thus, the strict nuclear localization of *Lhx9 $\alpha$*  appeared like a specific feature of the truncated isoform. This conclusion was further supported by the fact that another LIM-HD protein, *Lhx7*, was also found to be distributed throughout the nucleus and cytoplasm of PC12 cells when fused to a myc epitope-tag (S. Rétaux and I. Bachy, unpublished observations).

In a next series of experiments, the effect of *Lhx9* and *Lhx9 $\alpha$*  on NGF-induced neuronal differentiation of PC12 cells was tested (Fig. 5). After 72 h of NGF treatment, PC12 cells transfected with a *Lhx9*-GFP fusion construct showed a significant 40% increase in neuronal differentiation compared to their GFP-transfected controls (Fig. 5A and B). Because *Lhx9 $\alpha$* -GFP fusion localized to the nucleus and therefore did not allow the visualisation of neuritic extensions, the effect of *Lhx9 $\alpha$*  on neuronal differentiation was tested upon cotransfection of a *Lhx9*-myc-tag and a GFP construct, and was found intermediate and not significant compared to GFP and *Lhx9*-GFP in terms of neuronal differentiation (Fig. 5). However, it came out upon microscopic analysis that *Lhx9*- and *Lhx9 $\alpha$* -transfected cells presented significantly different morphological features. *Lhx9*- (as well as GFP-) transfected cells had “classical” neuronal characteristics, showing one or several neuritic extensions and a round or ovoid cell body, whereas a significant proportion of *Lhx9 $\alpha$* -transfected cells showed a flattened aspect (Fig.



**FIG. 4.** Subcellular localization of *Lhx9* and *Lhx9 $\alpha$* . (A–C) Immunofluorescence photomicrographs of PC12 cells transfected with a *Lhx9*-GFP (A), *Lhx9 $\alpha$* -GFP (B), or *Lhx9 $\alpha$* -myc tag (C, and insets in C) fusion constructs, in the absence of NGF treatment, and counterstained with DAPI nuclear staining. Immunofluorescence is directed against GFP (A,B) or the myc epitope-tag (C) and reveals a clear nuclear localization for *Lhx9 $\alpha$* .





**FIG. 5.** Effect of *Lhx9* and *Lhx9α* on NGF-induced differentiation of PC12 cells. **(A)** Immunofluorescence photomicrographs of PC12 cells transfected with the indicated constructs and cultured in the presence or absence of NGF. GFP immunofluorescence (IF-GFP) was used to outline the morphology of transfected cells. Cell nuclei were counterstained with DAPI. The magnification power of photomicrographs are indicated. **(B)** Histogram showing the quantification of the effect. **(C)** High-power photomicrograph showing a representative field where several *Lhx9α*-transfected cells with peculiar “flattened” morphology are present (indicated by arrows).

5C). Quantification showed that 24% of *Lhx9 $\alpha$* -transfected cells versus 10% of *Lhx9*-transfected cells presented this flattened morphological feature ( $P < 0.01$ , Student *t*-test). Thus, *Lhx9* and *Lhx9 $\alpha$*  had differential effects on the process of NGF-induced neuronal differentiation of PC12 cells.

Finally, to test a possible dominant-interfering action of *Lhx9 $\alpha$*  on *Lhx9*, we cotransfected the two isoforms into PC12 cells and assessed NGF-induced neuronal differentiation. Upon cotransfection, we observed a 46% increase in neuronal differentiation which was not significantly different from the 40% increase observed with *Lhx9* alone (Fig. 5B). In addition, 24% of cotransfected cells presented the “flat” phenotype after cotransfection.

#### *Does Lhx9 regulate the transcription of Lhx9 $\alpha$ ?*

During *Drosophila* wing disk development, the LIM–HD selector gene *apterous*, which is the *Lhx9* ortholog, induces the LMO inhibitor (dLMO) to terminate its own effect, and this regulation is crucial for the proper development of the wing (Milan and Cohen, 2000). In a last series of experiments, we tested whether a similar mechanism could be at work in our system of neuronal differentiation, that is, whether *Lhx9* could positively regulate the transcription of *Lhx9 $\alpha$* . PC12 cells, which do not normally express either of *Lhx9* or *Lhx9 $\alpha$*  transcripts (Fig. 6B), were transfected with *Lhx9* or *Lhx9 $\alpha$*  expression vectors and cultured in the presence of NGF before RT-PCR analysis. PCR primers were chosen either in the cod-

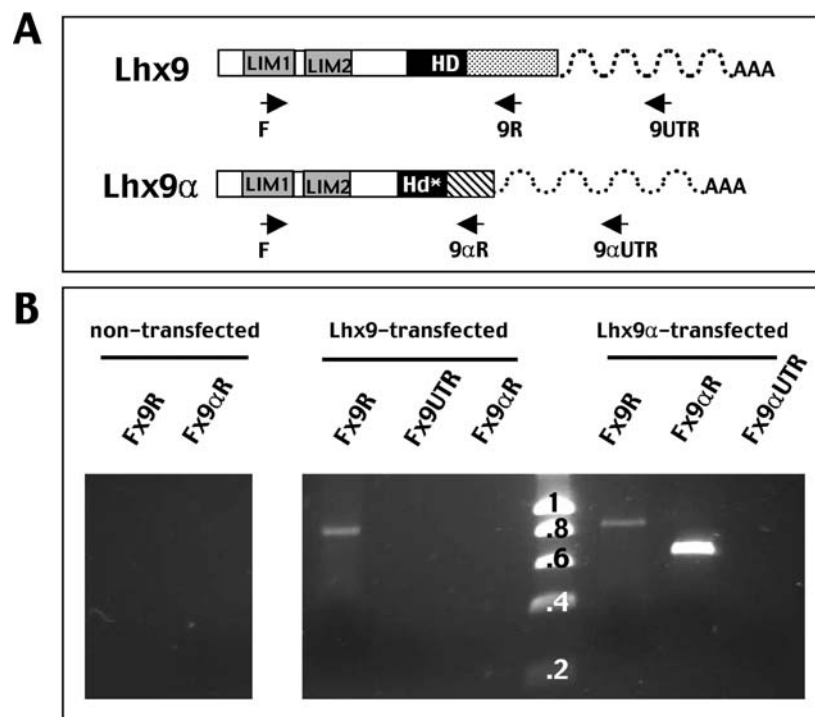
ing sequence or in the 3'UTR sequence of *Lhx9/9 $\alpha$*  mRNAs, to discriminate between expression due to the transfected expression vector or to an endogenous transcriptional regulation (Fig. 6A). The results show that in the PC12 cell culture system, *Lhx9* was not able to induce its own transcription or that of its isoform. However, and quite unexpectedly, in *Lhx9 $\alpha$* -transfected cells, a PCR product corresponding to the *Lhx9* transcripts was detected (Fig. 6B).

## DISCUSSION

In this paper we have begun to investigate the possible functional and physiological relevance of the existence of two alternatively spliced isoforms for the *Lhx9* transcription factor.

#### *Lhx9 $\alpha$ behaves like a nuclear LMO protein*

With its two LIM domains and its truncated homeodomain, we had previously suggested that *Lhx9 $\alpha$*  could be an endogenous dominant-negative or competitor isoform of the *Lhx9* transcription factor. We now demonstrate that *Lhx9 $\alpha$*  is able to bind the CLIM cofactors but not DNA, and although the subcellular localization information given by transfected epitope-tagged constructs is not fully dependable, the short isoform seems to localize preferentially to the nuclear compartment. Thus, *Lhx9 $\alpha$*  has the capacity and the location to compete away limiting amounts of CLIM cofactors, similarly to nuclear LMO proteins



**FIG. 6.** Autoregulatory effect of *Lhx9* and *Lhx9 $\alpha$*  on their own transcription. (A) Schematic representation of the two transcripts, showing the localization of PCR primers chosen to discriminate between expression due to the transfected vector or to endogenous expression (drawing not to scale). (B) RT-PCR analysis of transcripts present in NGF-treated PC12 cells nontransfected or transfected with the indicated construct. A PCR product is present at the expected size in control lanes and in the experimental situation where *Lhx9 $\alpha$* -transfected cells induce the expression of *Lhx9* transcripts.



as previously reported (Milan *et al.*, 1998). More specifically, the punctate nuclear staining indicates that *Lhx9 $\alpha$*  executes its regulation in specific nuclear subcompartments. Thus, the role of *Lhx9 $\alpha$*  for functions of PML bodies and/or for nucleolar functions (reviewed in Eskiw and Bazett-Jones, 2002; Olson *et al.*, 2002) now needs further investigation.

In homeodomain-containing transcription factors, the third helix of the homeodomain has been classically recognized with a DNA-binding activity. The result of our gel-shift experiments are fully compatible with this idea. However, and interestingly, the homeodomain of the antennapedia and engrailed homeoproteins have also been implicated in the possible nuclear export and secretion process of homeoproteins (Joliot *et al.*, 1991, 1997, 1998). As deduced from mutation and deletion analysis, the third helix of the homeodomain seems to be responsible and necessary for this export mechanism. Our experiments bring striking support to this hypothesis, with the observation that *Lhx9* distributes to the entire cell (nucleus and cytoplasm), whereas *Lhx9 $\alpha$* , which precisely misses the third helix of the homeodomain, is restricted to the nucleus. Although we have not observed cell-to-cell transfer of the *Lhx9* protein as reported for antennapedia or engrailed (e.g., Joliot *et al.*, 1998), we provide to our knowledge the first evidence for differential nuclear localization properties of two endogenously and naturally occurring isoforms of a homeodomain factor.

#### *Lhx9/Lhx9 $\alpha$* and neuronal differentiation

Nuclear LIM domain-containing factors (LIM–HD and nuclear LMO) are involved in cell specification and differentiation (Bach, 2000). On the other hand, some LMO factors are also powerful oncogenes (Rabbitts, 1998). It has therefore been hypothesized that in some human cancers caused by chromosomal LMO translocations the deregulation of LIM–HD activity by dominant-interfering LMO overexpression alters the proper maintenance of the differentiated state, and leads to a failure in control of cell proliferation. Indeed, in various systems, nuclear proteins consisting mainly of LIM domain proteins are implicated in the positive or negative control of cell differentiation: the mouse FHL2 factor promotes the differentiation of myoblasts (Martin *et al.*, 2002) and *Xenopus* LMO3 increases neurogenesis (Bao *et al.*, 2000), whereas LMO2 and LMO4 negatively regulate erythroid and mammary cell differentiation, respectively (Visvader *et al.*, 1997, 2001). The differential effects of *Lhx9* and *Lhx9 $\alpha$*  observed in this report regarding neuronal differentiation of PC12 cells are therefore consistent with a general role of the LIM–HD/LMO protein network in the control of cell differentiation, and also with their differential biochemical properties and subcellular localization: *Lhx9* promotes neuronal differentiation, which fits well with the recent proposal that LIM–HD factors, together with proneural bHLH genes, could participate in the synchronization of cell cycle exit and cell specification (Lee and Pfaff, 2003), whereas *Lhx9 $\alpha$*  rather has an effect on an aspect of cellular morphogenesis. In addition, in our experimental culture system, *Lhx9 $\alpha$*  does not show any dominant-interfering effect on *Lhx9*-induced increase in neuronal differentiation, and *Lhx9* does not interfere with *Lhx9 $\alpha$* -induced change in cell morphology, therefore suggesting an absence of competition between the two isoforms, and reinforcing the idea that they function in distinct

pathways. In this sense, one could have hypothesized that *Lhx9 $\alpha$* , which is expressed *in vivo* at later embryonic stages than *Lhx9*, could be responsible for the refinement of the neuronal phenotype specified by the LIM–HD *Lhx9*. This is one of the reasons that prompted us to investigate whether *Lhx9* was itself responsible for turning on the transcription of its alternative transcript. In our experimental conditions however, we did not detect any direct positive transcriptional activation of *Lhx9 $\alpha$*  by *Lhx9*. However, although the molecular mechanisms remain unknown, our results suggest that *Lhx9 $\alpha$*  may play a role in *Lhx9* expression. Since mRNA encoding *Lhx9 $\alpha$*  is transcribed at later stages of mouse embryonic development when compared directly with *Lhx9* (Failli *et al.*, 2000), the effects of *Lhx9 $\alpha$*  on *Lhx9* mRNA expression appear to be most likely at the maintenance level.

## CONCLUSION

The LIM–HD protein network is a complex system comprising multiple partners and involved in the regulation of many developmental events. This complexity is reinforced by the possibility to generate isoforms by alternative splicing mechanisms. Such isoforms have been described for other LIM–HD members: *Lhx7a*, truncated in the homeodomain similarly to *Lhx9 $\alpha$* , has not been functionally studied (Grigoriou *et al.*, 1998); *Isl1 $\alpha$*  and *Isl1 $\beta$*  differ by their carboxy-terminal sequence, have differential transactivation activities and undergo differential phosphorylation (Ando *et al.*, 2003). There are also N-terminal alternatively spliced *Lhx3* isoforms, which differ in terms of gene activation properties (Sloop *et al.*, 1999). The example of *Lhx9/Lhx9 $\alpha$*  reported here provide insights into the functional/physiological significance of the existence of these multiple isoforms.

## ACKNOWLEDGMENTS

This work supported by a CNRS-ACI “Biologie du Développement et Physiologie Intégrative” to S.R., and by the Deutsche Forschungsgemeinschaft (DFG) to I.B. We wish to thank Arnaud Menuet for his scientific interest and support.

## REFERENCES

- AGULNICK, A.D., TAIRA, M., BREEN, J.J., TANAKA, T., DAWID, I.B., and WESTPHAL, H. (1996). Interactions of the LIM-domain-binding factor *Ldb1* with LIM homeodomain proteins. *Nature* **384**, 270–272.
- ANDO, K., SHIODA, S., HANDA, H., and KATAOKA, K. (2003). Isolation and characterization of an alternatively spliced variant of transcription factor *Isl1*. *J. Mol. Endocrinol.* **31**, 419–425.
- BACH, I. (2000). The LIM domain: Regulation by association. *Mech. Dev.* **91**, 5–17.
- BACH, I., RHODES, S.J., PEARSE, R.V., 2nd, HEINZEL, T., GLOSS, B., SCULLY, K.M., SAWCHENKO, P.E., and ROSENFELD, M.G. (1995). P-Lim, a LIM homeodomain factor, is expressed during pituitary organ and cell commitment and synergizes with Pit-1. *Proc. Natl. Acad. Sci. USA* **92**, 2720–2724.
- BACH, I., CARRIERE, C., OSTENDORFF, H.P., ANDERSEN, B.,

- and ROSENFELD, M.G. (1997). A family of LIM domain-associated cofactors confer transcriptional synergism between LIM and Otx homeodomain proteins. *Genes Dev.* **11**, 1370–1380.
- BACH, I., RODRIGUEZ-ESTEBAN, C., CARRIERE, C., BHUSHAN, A., KRONES, A., ROSE, D.W., GLASS, C.K., ANDERSEN, B., IZPISUA BELMONTE, J.C., and ROSENFELD, M.G. (1999). RLIM inhibits functional activity of LIM homeodomain transcription factors via recruitment of the histone deacetylase complex. *Nat. Genet.* **22**, 394–399.
- BACHY, I., FAILLI, V., and RETAUX, S. (2002). A LIM-homeodomain code for development and evolution of forebrain connectivity. *Neuroreport* **13**, A23–A27.
- BAO, J., TALMAGE, D.A., ROLE, L.W., and GAUTIER, J. (2000). Regulation of neurogenesis by interactions between HEN1 and neuronal LMO proteins. *Development* **127**, 425–435.
- BECKER, T., OSTENDORFF, H.P., BOSSENZ, M., SCHLUTER, A., BECKER, C.G., PEIRANO, R.I., and BACH, I. (2002). Multiple functions of LIM domain-binding CLIM/NLI/Ldb cofactors during zebrafish development. *Mech. Dev.* **117**, 75–85.
- CHENG, L., CHEN, C.L., LUO, P., TAN, M., QIU, M., JOHNSON, R., and MA, Q. (2003). Lmx1b, Pet-1, and Nkx2.2 coordinately specify serotonergic neurotransmitter phenotype. *J. Neurosci.* **23**, 9961–9967.
- DING, Y.Q., MARKLUND, U., YUAN, W., YIN, J., WEGMAN, L., ERICSON, J., DENNERIS, E., JOHNSON, R.L., and CHEN, Z.F. (2003). Lmx1b is essential for the development of serotonergic neurons. *Nat. Neurosci.* **6**, 933–938.
- ESKIW, C.H., and BAZETT-JONES, D.P. (2002) The promyelocytic leukemia nuclear body: Sites of activity? *Biochem. Cell Biol.* **80**, 301–310.
- FAILLI, V., ROGARD, M., MATTEI, M.G., VERNIER, P., and RETAUX, S. (2000). Lhx9 and Lhx9alpha LIM-homeodomain factors: Genomic structure, expression patterns, chromosomal localization, and phylogenetic analysis. *Genomics* **64**, 307–317.
- GRIGORIOU, M., TUCKER, A.S., SHARPE, P.T., and PACHNIS, V. (1998). Expression and regulation of Lhx6 and Lhx7, a novel subfamily of LIM homeodomain encoding genes, suggests a role in mammalian head development. *Development* **125**, 2063–2074.
- HIRATANI, I., YAMAMOTO, N., MOCHIZUKI, T., OHMORI, S.Y., and TAIRA, M. (2003). Selective degradation of excess Ldb1 by Rnf12/RLIM confers proper Ldb1 expression levels and Xlim-1/Ldb1 stoichiometry in *Xenopus* organizer functions. *Development* **130**, 4161–4175.
- JOLIOT, A., PERNELLE, C., DEAGOSTINI-BAZIN, H., and PROCHIANTZ, A. (1991). Antennapedia homeobox peptide regulates neural morphogenesis. *Proc. Natl. Acad. Sci. USA* **88**, 1864–1868.
- JOLIOT, A., TREMBLEAU, A., RAPOSO, G., CALVET, S., VOLOVITCH, M., and PROCHIANTZ, A. (1997). Association of Engrailed homeoproteins with vesicles presenting caveolae-like properties. *Development* **124**, 1865–1875.
- JOLIOT, A., MAIZEL, A., ROSENBERG, D., TREMBLEAU, A., DUPAS, S., VOLOVITCH, M., and PROCHIANTZ, A. (1998). Identification of a signal sequence necessary for the unconventional secretion of Engrailed homeoprotein. *Curr. Biol.* **8**, 856–863.
- JURATA, L.W., and GILL, G.N. (1997). Functional analysis of the nuclear LIM domain interactor NLI. *Mol. Cell Biol.* **17**, 5688–5698.
- JURATA, L.W., KENNY, D.A., and GILL, G.N. (1996). Nuclear LIM interactor, a rhombotin and LIM homeodomain interacting protein, is expressed early in neuronal development. *Proc. Natl. Acad. Sci. USA* **93**, 11693–11698.
- KISSINGER, C.R., LIU, B.S., MARTIN, B.E., KORNBERG, T.B., and PABO, C.O. (1990). Crystal structure of an engrailed homeodomain–DNA complex at 2.8 Å resolution: A framework for understanding homeodomain–DNA interactions. *Cell* **63**, 579–590.
- LEE, S.K., and PFAFF, S.L. (2003). Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors. *Neuron* **38**, 731–745.
- MARTIN, B., SCHNEIDER, R., JANETZKY, S., WAIBLER, Z., PANDUR, P., KUHLE, M., BEHRENS, J., VON DER MARK, K., STARZINSKI-POWITZ, A., and WIXLER, V. (2002). The LIM-only protein FHL2 interacts with beta-catenin and promotes differentiation of mouse myoblasts. *J. Cell Biol.* **159**, 113–122.
- MILAN, M., and COHEN, S.M. (2000). Temporal regulation of apterous activity during development of the *Drosophila* wing. *Development* **127**, 3069–3078.
- MILAN, M., DIAZ-BENJUMEA, F.J., and COHEN, S.M. (1998). Beadex encodes an LMO protein that regulates Apterous LIM-homeodomain activity in *Drosophila* wing development: A model for LMO oncogene function. *Genes Dev.* **12**, 2912–2920.
- OLSON, M.O., HINGORANI, K., and SZEBENI, A. (2002). Conventional and nonconventional roles of the nucleolus. *Int. Rev. Cytol.* **219**, 199–266.
- OSTENDORFF, H.P., PEIRANO, R.I., PETERS, M.A., SCHLUTER, A., BOSSENZ, M., SCHEFFNER, M., and BACH, I. (2002). Ubiquitination-dependent cofactor exchange on LIM homeodomain transcription factors. *Nature* **416**, 99–103.
- PFAFF, S.L., MENDELSON, M., STEWART, C.L., EDLUND, T., and JESSELL, T.M. (1996). Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. *Cell* **84**, 309–320.
- RABBITS, T.H. (1998). LMO T-cell translocation oncogenes typify genes activated by chromosomal translocations that alter transcription and developmental processes. *Genes Dev.* **12**, 2651–2657.
- RETAUX, S., and BACHY, I. (2002). A short history of LIM domains (1993–2002): from protein interaction to degradation. *Molec. Neurobiol. Rev.*, **26**, 269–281.
- ROBERSON, M.S., SCHODERBEK, W.E., TREMML, G., and MAURER, R.A. (1994). Activation of the glycoprotein hormone alpha-subunit promoter by a LIM-homeodomain transcription factor. *Mol. Cell Biol.* **14**, 2985–2993.
- SLOOP, K.W., MEIER, B.C., BRIDWELL, J.L., PARKER, G.E., SCHILLER, A.M., and RHODES, S.J. (1999). Differential activation of pituitary hormone genes by human Lhx3 isoforms with distinct DNA binding properties. *Mol. Endocrinol.* **13**, 2212–2225.
- VISVADER, J.E., MAO, X., FUJIWARA, Y., HAHM, K., and ORKIN, S.H. (1997). The LIM-domain binding protein Ldb1 and its partner LMO2 act as negative regulators of erythroid differentiation. *Proc. Natl. Acad. Sci. USA* **94**, 13707–13712.
- VISVADER, J.E., VENTER, D., HAHM, K., SANTAMARIA, M., SUM, E.Y., O'REILLY, L., WHITE, D., WILLIAMS, R., ARMES, J., and LINDEMAN, G.J. (2001). The LIM domain gene LMO4 inhibits differentiation of mammary epithelial cells in vitro and is overexpressed in breast cancer. *Proc. Natl. Acad. Sci. USA* **98**, 14452–14457.
- WEIHE, U., MILAN, M., and COHEN, S.M. (2001). Regulation of Apterous activity in *Drosophila* wing development. *Development* **128**, 4615–4622.
- ZHAO, Y., MARIN, O., HERMESZ, E., POWELL, A., FLAMES, N., PALKOVITS, M., RUBENSTEIN, J.L., and WESTPHAL, H. (2003). The LIM-homeobox gene Lhx8 is required for the development of many cholinergic neurons in the mouse forebrain. *Proc. Natl. Acad. Sci. USA* **100**, 9005–9010.

Address reprint requests to:

Sylvie Rétaux, Ph.D.

UPR 2197 “Développement, Evolution  
Plasticité du Système Nerveux”

Institut de Neurobiologie Alfred FESSARD, C.N.R.S.

Avenue de la Terrasse

91198 GIF-sur-YVETTE cedex, France

E-mail: Sylvie.Retaux@iaf.cnrs-gif.fr

Received for publication February 5, 2004; received in revised form May 19, 2004; accepted June 3, 2004.