

Regulation of neurogenesis by interactions between HEN1 and neuronal LMO proteins

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SUMMARY

Basic-helix-loop-helix transcription factors regulate neurogenesis and neuronal differentiation by as yet unknown mechanisms. We show that an embryonic neuronal-specific basic-helix-loop-helix protein, HEN1 (also known as NSCL1 or NHLH), interacts with 'LIM only' proteins. Examination of the expression patterns of XHEN1 and XLMO-3, the *Xenopus* homologues of these human genes, reveals extensive overlap during early neurogenesis: at the onset of gastrulation on the dorsal side of the blastopore lip and, subsequently, in the prospective

neural plate. Binding of XLMO-3 increases the transcriptional activity of XHEN1 in vivo. Co-expression of these two genes in *Xenopus* embryos induces a cascade of expression of neuronal-specific basic-helix-loop-helix proteins that leads to neuronal differentiation. We propose that XHEN1, in concert with XLMO-3, is a critical regulator of neurogenesis.

Key words: *Xenopus*, HEN1, LMO, Neurogenesis

INTRODUCTION

A family of transcription factors distinguished by the inclusion of a basic-helix-loop-helix (bHLH) motif is implicated in regulating cellular determination (Jan and Jan, 1993; Chitnis and Kintner, 1995; Anderson, 1997). HEN1 is a neuronal-specific bHLH protein (Begley et al., 1992; Brown and Baer, 1994). It shares 71% nucleotide identity with SCL (Stem Cell Leukemia), a bHLH transcription factor required for formation of hematopoietic cell lineages (Porcher et al., 1996; Robb et al., 1996). The expression of HEN1 is restricted to the nervous system during the genesis of the neural tube and is localized in the subependymal layer of the neuroepithelium from which most neurons arise (Begley et al., 1992). Structural similarities between SCL and HEN1, and the spatial and temporal expression patterns of HEN1 are consistent with HEN1 playing an important role in neurogenesis.

In *Drosophila*, neurogenesis is regulated by the proneural and neurogenic gene products (Skeath and Carroll, 1994). Neurogenic gene products, such as Notch and its putative ligand, Delta, determine which precursor cells will pursue a neuronal fate by regulating the activities of the proneural genes (Lehmann et al., 1981; Lewis, 1996). The proneural genes, *achaete*, *scute* and *lethal of scute* from the *achaete-scute* complex (AS-C), encode bHLH proteins, which are expressed in overlapping subsets of the neurogenic region (Villares and

Cabrera, 1987; Campos-Ortega and Jan, 1991) and subsequently induce the expression of another AS-C gene, *asense*. *Asense* initiates early neuronal differentiation (Gonzalez et al., 1989; Brand et al., 1993; Jarman et al., 1993). The exact molecular mechanism by which proneural genes regulate gene expression is unclear. Most bHLH proteins regulate gene expression by forming homodimers or heterodimers that bind to E-box motifs (Murre et al., 1989; Brennan and Olson, 1990). During neurogenesis, the AS-C gene products may form functional dimers with the ubiquitously expressed *daughterless* gene product (Caudy et al., 1988). The mammalian homologues of *daughterless*, the E2A gene products, are not essential for neurogenesis (Zhuang et al., 1994), and neither HEN1/HEN1 homodimers nor HEN1/E2A heterodimers transactivate reporter constructs with multiple copies of an E-box despite binding to this specific E-box sequence (CAGCTG, Brown and Baer, 1994). Therefore, transcriptional activation by HEN1 requires interaction with additional, as yet unidentified factors.

To study the function of HEN1 during neuronal development, we first identified LMO-1 as a HEN1-binding protein using a modified yeast two-hybrid screen (Bao and Zervos, 1996). LMO-1 belongs to the 'LIM only' protein family that includes LMO-1, LMO-2, and LMO-3 (Sanchez-Garcia and Rabbitts, 1993). We next isolated *Xenopus* cDNA homologues of HEN1 and LMO-1 (XHEN1 and XLMO-3).

Expression of XHEN1 and XLMO-3 mRNAs was first detected at the onset of gastrulation. Following gastrulation, XHEN1 and XLMO-3 mRNAs were co-localized to the presumptive neural plate. Co-expression of XHEN1 and XLMO-3 in mammalian cells activated the expression of a reporter containing four copies of E-box elements. In *Xenopus*, co-injection of XHEN1 and XLMO-3 mRNAs induced expression of endogenous X-NGNR-1 (Xenopus Neurogenin-1), XNeuroD, N-CAM and N-tubulin, while downregulation of XHEN1 resulted in reduced neurogenesis. Thus XHEN1 association with XLMO-3 initiates a cascade of transcription factor expression that might constitute a key step in the regulation of neurogenesis.

MATERIALS AND METHODS

Yeast two-hybrid screen

An 'interaction trap two-hybrid system' was used as described previously (Bao and Zervos, 1996). The 'bait' was constructed by fusing the entire human HEN1 open reading frame to the LexA DNA-binding domain in the pEG202 vector by PCR amplification with the 5' primer (5' CCG GAA TTC ATG ATG CTC AAC TCA GAC ACC AT; *EcoRI* site underlined) and the 3' primer (5' CCG CTC GAG TCA GAC GTC CAG CAC GTG GTT CA; *XhoI* site underline). The selection of interactors was performed by plating 10^6 transformants of a human fetal brain cDNA library onto plates lacking leucine. The plates were incubated at 30°C for 4 days. The first 100 colonies that appeared on the plates were collected and further tested on X-gal-containing plates. The plasmids from 15 of 17 colonies that strongly activated *lacZ* expression were recovered and cDNA inserts were further analyzed by DNA sequencing.

To test the specific interaction between HEN1 and LMO-1, fragments of the human LMO-1 corresponding to amino acids 24–80 (LIM 1 domain) and 81–121 (LIM 2 domain) were cloned into pJG4-5, yielding plasmids L1 and L2. The fragment of the human HEN1 without HLH domain (amino acids 1–85) was subcloned into pEG202. The control 'baits' (DA, N-myc, C-fos, CYC) were as described previously (Bao and Zervos, 1996).

The full-length XLMO-3 was fused to the LexA DNA-binding domain in the pEG202 vector by PCR amplification with the 5' primer (5' CCG GAA TTC ATG CTT TCT GTT CAG CCA GAC; *EcoRI* site underlined) and the 3' primer (5' CCG CTC GAG TCA GCG CAC CTG GGC AGA ATA; *XhoI* site underline), and the full-length of XHEN1, X-NGNR-1, XNeuroD and XASH-3 were fused to the B42 DNA-activation domain of pJG4-5 (Zimmerman et al., 1993; Lee et al., 1995; Ma et al., 1996). Single yeast colonies were grown overnight and the activity of β -galactosidase was measured according to established method (Guarente, 1983).

Cloning of XHEN1 and XLMO-3

Human HEN1 and LMO-1 cDNAs were used to probe a stage-17 *Xenopus* embryo cDNA library (Kintner and Melton, 1987). Inserts from the positive clones were subcloned into pGEM3Z and sequenced. Sequence searches were performed using the BLAST program on the NCBI network server.

Co-immunoprecipitation of HEN-1 and LMO proteins

Full-length HEN1, tagged with an HA-epitope, (in pMT2, Bao and Zervos, 1996), was co-transfected into 293T cells with plasmids encoding each of the three mammalian LMO proteins tagged with a myc-epitope (gifts of Dr Rabbitts). 3 days after transfection, the cells were collected in 1 ml lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 100 mM NaF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin) on ice. The cells were then

homogenized and centrifuged at 12,000 *g* at 4°C for 10 minutes. Cell supernatants were precleared with Protein G-agarose. An aliquot of each was used to assess the expression levels of each protein by western blotting. To each 1 ml of cell supernatant, 10 μ l of HA- or Myc-epitope-specific antibody (Mannheim Boehringer) was added, and the mixture was incubated for 4 hours on ice. Protein G agarose was added to the antibody-sample mixture and incubated for 6 hours on ice. The protein G-agarose beads containing the antibody-antigen immunocomplexes were collected, washed three times and released from the beads by boiling in 50 μ l electrophoresis sample buffer. The immunoprecipitated complexes were separated by SDS-PAGE and epitope-tagged proteins were detected by immunoblotting (ECL, Amersham).

Luciferase assay

pNEX-167, which contains four E-box elements fused to the firefly luciferase gene (Bartholoms and Nave, 1994), was used as a plasmid reporter. The following plasmids were transfected into 293T cells by various combinations: 10 mg pNEX-167, 20 mg each of pCS-XHEN1, pCS-XHENA (XHEN1 with a truncation of the HLH domain), pCS-XLMO-3, pCMV-NEX and 2 mg of pCDNA3-GFP to determine the efficiency of transfection by counting GFP-positive cells. The total amount of transfected DNA was brought to identical levels by addition of the appropriate amounts of pC-DNA. 48 hours after transfection by lipofectine method, the luciferase activity was assayed by Luciferase Assay System (Promega).

Ribonuclease Protection Assay (RPA)

A modified non-radioactive RPA was used (Zhan et al., 1999). Total cellular RNA from embryos at the indicated stages were extracted using a Total RNA Extraction Kit (Qiagen). Dig-labeled RNA probes were prepared by using the RNA Labeling Kit (Boehringer Mannheim). Total RNA (50 μ g) from each stage was directly dotted onto a nylon membrane. The RNA was cross-linked to the nylon membrane with ultra-violet light and was washed twice in 2 \times SSC. Subsequent hybridization and visualization reactions were the same as described for the whole-mount in situ.

Whole mount in situ hybridization and immunocytochemistry

Whole-mount in situ hybridization was performed as described (Chitnis and Kintner, 1996). Embryos were fixed in MEMFA buffer (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO₄ and 3.7% formaldehyde) for 2 hours at room temperature. The MEMFA was replaced by methanol and the embryos were stored at -20°C. For immunostaining, the embryos were preincubated in 20% goat serum in PBS for 1 hour, and then incubated with XAN-3 antibody (1:100 dilution) at 4°C for 48 hours. For in situ hybridization, XLMO-3 and XHEN1 antisense RNA probes were prepared from their entire coding regions.

Overexpression assays

XLMO-3 and XHEN1 cDNAs were cloned into pCS2MT vector (Turner and Weintraub, 1994). The pCS constructs containing X-NGNR-1 or XNeuroD were used as originally described (Lee et al., 1995; Ma et al., 1996). Capped mRNA from cloned cDNA sequences was made using mMESSAGE mMACHINE (Ambion). For optimal effect, 10 nl of mRNA (0.5 μ g/ μ l) was injected (5 ng) into one or both cells of 2-cell embryos, however, the minimum dose needed to detect an effect was determined to be 500 pg. In some experiments, animal cap explants were used (Sudarwati and Nieuwkoop, 1971). Total RNA was extracted from 10 animal caps, and RT-PCR was used to monitor gene expression (Hemmati-Brivanlou and Melton, 1992).

Nucleotide sequence accession number

The XHEN1 (U94991) and XLMO-3 (U94992) nucleotide sequences are accessible from GenBank.

RESULTS

Identification of neuronal-specific partners for HEN1

We used a HEN1-LexA fusion protein to screen a human fetal brain cDNA library that was fused with a B42 activation domain (Bao and Zervos, 1996). The first 100 clones that grew on Leu-selection plates were picked. Among these clones, cDNAs from 15 clones that showed strong galactose-dependent blue color on X-gal plates were recovered. DNA sequence analysis of these 15 clones showed that five clones encoded a novel gene, four encoded E12, three encoded LMO-1, one encoded HEN1, and one encoded CBP. Because LMO-1 is expressed early during neuronal development and disruption of its hematopoietic counterpart, the LMO-2 gene (RBTN2, Ttg2), results in a phenotype strikingly similar to the SCL null phenotype (Warren et al., 1994), we focused subsequent analyses on the interaction between LMO-1 and HEN1 in early neuronal development.

The LIM domain of LIM or LMO proteins is believed to function as a specific protein-binding interface (Schmeichel and Beckerle, 1994). LMO proteins contain two LIM domains. As such, we next tested whether one or both of these domains were required for LMO-1 and HEN1 interaction. Although each LIM domain interacted with HEN1 (Fig. 1A), both LIM domains were required for the maximal interaction between LMO-1 and HEN1. LMO-1 binding required the HLH domain of HEN1. Deletion of the HLH region eliminated LMO-1 interactions and LMO-1 did not interact with the bHLH protein, daughterless, the bHLH-ZIP protein, N-Myc, the bZIP protein, c-Fos, or cyclin C (Fig. 1A).

To test if HEN1 and LMO-1 interact in mammalian cells, we transiently transfected human 293T cells with plasmids encoding HA-tagged HEN1 alone, or in combination with LMO-1, LMO-2 or LMO-3 (with a c-Myc epitope). 72 hours post-transfection, 293T cell lysates were incubated with the anti-myc antibody, and immunoprecipitates were analyzed by immunoblotting using the anti-HA antibody. LMO-1 as well as LMO-2 and LMO-3, co-precipitated with HEN1 (Fig. 1B) demonstrating that HEN1 can form stable complexes with each LMO protein. We determined that the levels of expression of each protein were similar by performing Western blots directly on the cell extracts prior to immunoprecipitation (data not shown).

Following the cloning of *Xenopus* homologues of HEN1 and LMO-1 (see below), we tested the ability of XLMO-3 to interact with XHEN1 as compared to other neuronal-specific *Xenopus* bHLH proteins in the yeast two-hybrid assay. Of the proteins tested, XLMO-3 and XHEN1 showed the strongest interaction (Fig. 1C). Quantification of β -galactosidase activities of five independent yeast transformants from each group showed that interaction between XLMO-3 and XHEN1 was

significantly stronger than the others, as determined from the quantification of four different experiments using a *t*-test ($P < 0.01$; Fig. 1D).

Isolation of the *Xenopus* homologues of HEN1 and LMO-1

Human HEN1 and LMO-1 cDNA probes were used to screen a *Xenopus* cDNA library (Kintner and Melton, 1987). The XHEN1 cDNA was predicted to encode a protein of 128 amino acids with 75% sequence identity to human HEN1 and 65% identity to human HEN2 (Fig. 2A). Within the bHLH domain, XHEN1 is 99% identical to human HEN1. Sequence comparison of XHEN1 with other *Xenopus* bHLH proteins implicated in neurogenesis

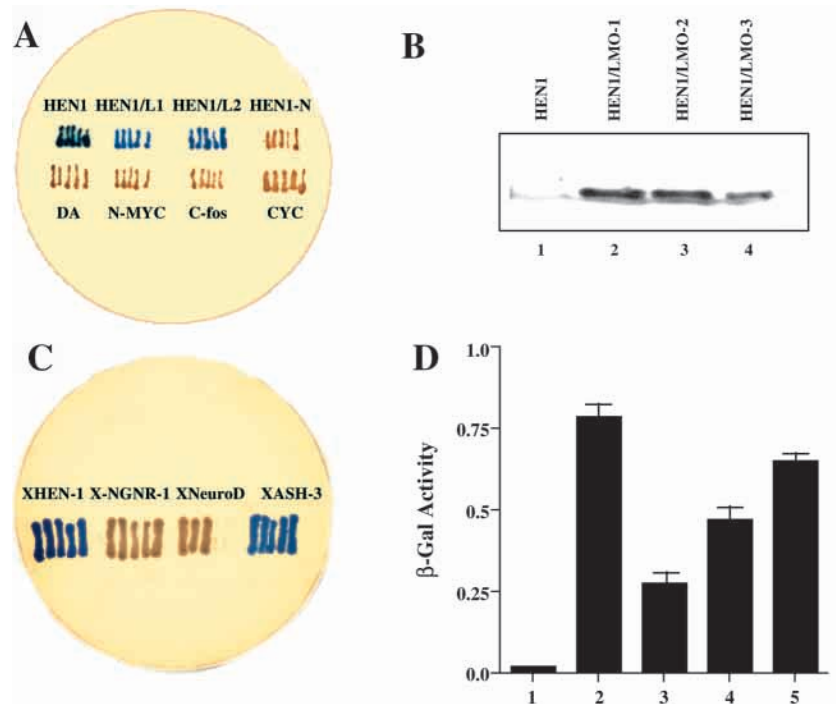


Fig. 1. Specific interaction between HEN1 and LMO-1. (A) Testing the specific interaction of HEN1 with LMO-1 in yeast. The full-length LMO-1, or only one of its LIM domains (L1 or L2) fused in-frame with the transcription activator B42 in pJG4-5 vector, was transformed into the EGY48 yeast strain containing one of the following baits fused to LexA DNA-binding domain: HEN1 (full-length HEN1), HEN1-N (N-terminal of HEN1 without bHLH domain), DA (daughterless), N-MYC, C-fos and CYC (cyclin C). Transformants were tested for specific interaction on glucose or galactose X-gal plates (galactose plate is shown here). The blue color indicates a positive protein-protein interaction. (B) Detection of complexes between HEN1 and LMO proteins by immunoprecipitation. Complexes were immunoprecipitated with anti myc-tag antibody from 293T mammalian cells co-transfected with HA-tagged HEN1 and one of the myc-tagged LMO proteins. The complexes were separated on the SDS-PAGE, and HEN1 protein was detected with anti-HA antibody. (C) Testing the binding of XLMO-3 to *Xenopus* neuronal-specific bHLH factors. The full-length XLMO-3 fused in-frame with the B42 in pJG4-5 was transformed into EGY48 yeast containing one of the following baits fused to LexA DNA-binding domain: XHEN1, X-NGNR-1, XNeuroD and XASH-3. Transformants were tested for specific interaction on galactose X-gal plates. The blue color indicates a positive protein-protein interaction. (D) Quantification of the strength of the interaction of XLMO-3 with *Xenopus* bHLH factors by measuring β -galactosidase activity. Yeast with XLMO-3 bait were transformed with Cyclin C (1), XHEN1 (2), X-NGNR-1 (3), XNeuroD (4) and XASH-3 (5). Five independent transformants from each group were picked and grown over 48 hours, and β -galactosidase activity was measured.

showed (Fig. 2B) that XHEN1 is phylogenetically distant from XASH-3 (Zimmerman et al., 1993; Turner and Weintraub, 1994), X-NGNR-1a, X-NGNR-1b (Ma et al., 1996), XNeuroD (Lee et al., 1995) or XATH-3 (Takebayashi et al., 1997).

Two *Xenopus* cDNAs that hybridized with LMO-1 probe encode the same protein of 156 amino acids with 96% sequence identity to human LMO-3, 84% to LMO-1 and 49% to LMO-2 (Fig. 2C). Within the two LIM domains, it is 99% identical to LMO-3, 97% to LMO-1 and 66% to LMO-2. As it is most similar to human LMO-3, we named this gene XLMO-3. It is not known if there is another LMO protein more closely related to LMO-1 than to LMO-3 in *Xenopus*.

Expression patterns of XHEN1 and XLMO-3 during neurogenesis

The expression patterns of XHEN1, XLMO-3 and X-NGNR-1 mRNA were compared throughout gastrulation and later in development. Although no expression of these genes was

detected prior to gastrulation, XHEN1 and XLMO-3 mRNAs were detected by RNase protection assay just prior to the onset of gastrulation (stage 10); while mRNA encoding X-NGNR-1 was not detected at this stage, in agreement with published data (Bellefroid et al., 1996; Ma et al., 1996). At the neurula stage (stage 16), all three genes were expressed at comparable levels (Fig. 3A). Comparison of the expression patterns of XHEN1, XLMO-3 and X-NGNR-1 by whole-mount in situ hybridization, confirmed the results of the RNase protection assay (Fig. 3B). The spatial expression patterns of XHEN1 and XLMO-3 were almost identical at early developmental stages (Fig. 3B-J). Expression of XHEN1 and XLMO-3 was detected at the onset of gastrulation (stage 10.5) exclusively on the dorsal side of the dorsal lip of the blastopore (Fig. 3B,C) similar to the previously reported expression of X-Delta (Ma et al., 1996). In the same batch of embryos, X-NGNR-1 was expressed more weakly and laterally in an area corresponding to the margins of the prospective neural plate. As gastrulation

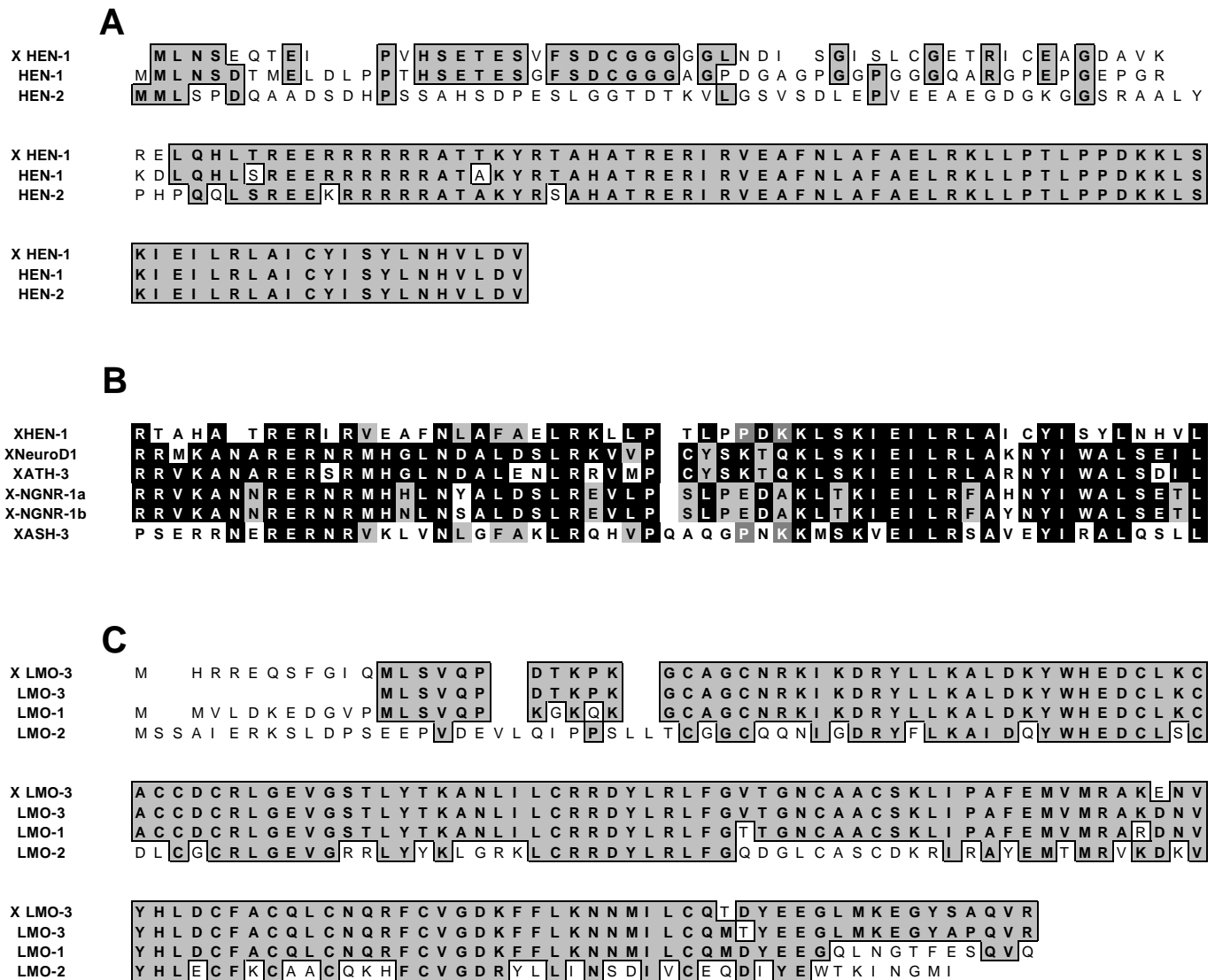


Fig. 2. Sequence of XHEN1 and XLMO-3. (A) Alignment of the entire predicted amino acid sequences of *Xenopus* XHEN1 and human HEN1 and HEN2. Grey boxes indicate conserved amino acids. (B) Alignment of the bHLH regions of six *Xenopus* neuronal-specific bHLH proteins. (C) Alignment of the entire predicted amino acid sequences of *Xenopus* XLMO-3 and human LMO-1, LMO-2, LMO-3. Grey boxes indicate identical amino acids.

proceeded from stage 10 to stage 12, expression of all three genes became more localized to the dorsal region, subsequently extending in an anterior direction as the neural plate formed. At stage 11-11.5, two stripes on each side of the dorsal midline were observed for both XHEN1 and XLMO-3, with the intensity of the staining decreasing along the anteroposterior axis. A similar staining pattern was observed for X-NGNR-1 at this stage, although the expression was more diffuse (Fig. 3E-G). By stages 12-12.5, the two medial stripes of expression became evident for X-NGNR-1, and strong expression of X-NGNR-1 was observed in the trigeminal placode (Ma et al., 1996; Bellefroid et al., 1996). No XHEN1 or XLMO-3 mRNA was detected in the region of the trigeminal placode (Fig. 3H-J).

At stage 14, expression of XHEN1 and XLMO-3 was restricted to the neural plate and extended anteriorly in a fan-shaped pattern. Posteriorly, the staining was restricted to the medial region of the neural plate that subsequently gives rise to motoneurons and interneurons (Fig. 4K,L, arrowheads). N-tubulin (a neuronal differentiation marker) was detected in the same area as well as more laterally within the neural plate in the region destined to give rise to sensory neurons (Fig. 4D,H, arrowheads). Transverse sections of stage 20 embryos at the trunk level show that both XHEN1 and XLMO-3 were localized in the ventral part of the neural tube (Fig. 4B). XLMO-3 also was expressed in some mesodermal tissue. Following neural tube closure (stage 25), XHEN1 and XLMO-3 mRNAs were detected in the spinal cord, the brain and the cranial neural crest in a similar pattern to X-NGNR-1 (Fig. 4E-H) and XNeuroD (Lee et al., 1995). At stage 35, XLMO-3 expression persisted throughout the nervous system although weakly in the posterior region, whereas XHEN1 expression became limited to the brain region (Fig. 4I,J).

Transcriptional activation of XHEN1 by XLMO-3

To assess the functional significance of the interaction between XHEN1 and XLMO-3, we tested the ability of XHEN1 and XLMO-3, separately or in combination, to activate transcription *in vivo*. We co-expressed XHEN1 and XLMO-3 in mammalian 293T cells along with a reporter plasmid (pNEX-167). pNEX-167 is made of 5' regulatory region (167 bp) of NEX-1 gene fused to the firefly luciferase gene followed by the SV40 polyadenylation signal (Bartholoms and Nave, 1994). The NEX-1 promoter regions contains four E-box elements (E1, CAGATG; E2, CACATG; E3, CAGATG; E4, CAGTTG). CAG is the well-defined half-site for HEN-1 binding (Brown and Baer, 1994). Fig. 5A shows that XHEN1 was able to transactivate the reporter plasmid only when co-expressed with XLMO-3, supporting the idea that these two genes work *in vivo* in a cooperative manner. The transactivation required the HLH domain of XHEN1 since co-expression of XLMO-3 with a XHEN1 construct lacking its HLH domain (Δ XHEN1) was unable to activate transcription of the reporter construct (Fig. 5A).

Ectopic induction of N-CAM by XHEN1 and XLMO-3 overexpression

To determine if XHEN1 and XLMO-3 might regulate neurogenesis, we micro-injected XHEN1 and XLMO-3 mRNAs into 2-cell embryos. Expression of the early neuronal marker, N-CAM, was examined in isolated presumptive

ectoderm ('animal caps'). N-CAM was detected in animal caps from embryos co-injected with XHEN1 and XLMO-3 and harvested when control embryos had reached stage 11. Lower levels (2.4 fold) of N-CAM mRNA were present in the animal caps injected with XHEN1 alone. XLMO-3 alone did not induce detectable N-CAM expression. The induction of N-CAM by XHEN1 and XLMO-3 was not a secondary consequence of induction of mesodermal tissues since muscle actin, a mesodermal marker, was not found in animal caps from embryos injected with these transcription factors (Fig. 5B).

The induction of neural markers by XHEN1/XLMO-3 expression was confirmed by whole-mount immunostaining of embryos with XAN-3, an antibody that recognizes N-CAM (Sakaguchi et al., 1989). XAN-3 staining was not detectable in uninjected, stage 10 embryos (data not shown). Little or no staining was observed in embryos injected with XHEN1 or XLMO-3 alone. Strong staining was seen in all embryos co-injected with XHEN1 and XLMO-3 (Fig. 5C), consistent with the measurements of N-CAM mRNA in animal caps. By stage 25, ectopic XAN-3 immunostaining was never observed outside the neural tube.

A cascade of transcription factor activation by XHEN1 and XLMO-3

During development, XHEN1 is first expressed at stage 10 (see Fig. 3A) while X-NGNR-1 and XNeuroD mRNAs are first detectable at stage 10.5 and 13.5, respectively (Lee et al., 1995; Ma et al., 1996). Because of the sequential expression of these genes, we examined whether overexpression of XHEN1 and XLMO-3 was sufficient to induce the expression of endogenous X-NGNR-1, and subsequently XNeuroD (Ma et al., 1996). We injected XHEN1 and/or XLMO-3 mRNAs into one blastomere of 2-cell-stage embryos and examined the effect on X-NGNR-1 or XNeuroD expression.

Injection of XHEN1 and XLMO-3 mRNAs consistently resulted in precocious expression of X-NGNR-1 in the injected side of stage 9 embryos (Fig. 6C). Weaker but clear expression of X-NGNR-1 was detected in embryos injected only with XHEN1 mRNA (Fig. 6B). No expression was detected in embryos injected only with XLMO-3 mRNA (Fig. 6A). A similar pattern of XNeuroD expression was detected at the same stage, although at lower levels (Fig. 6E,F). To test whether overexpression of X-NGNR-1 or XNeuroD could reciprocally activate the endogenous expression of XHEN1 or XLMO-3, we injected one blastomere of two-cell embryos with either X-NGNR-1 or XNeuroD mRNA. No ectopic expression of XHEN1 or XLMO-3 was found in stage 9 embryos injected with either X-NGNR-1 or XNeuroD mRNA (Fig. 6H-K). As demonstrated by Ma et al. (1996), XNeuroD expression was induced in the half of the embryos injected with X-NGNR mRNA (Fig. 6G). Therefore, XHEN1 and XLMO-3 activate a unidirectional cascade of neuronal transcription factor expression.

Neuronal differentiation induced by XHEN1 and XLMO-3

To determine if induction of X-NGNR-1 and XNeuroD could lead to terminal neuronal differentiation, we examined the expression of N-tubulin in embryos injected with either XHEN1 and/or XLMO-3 mRNAs by using whole-mount *in situ* hybridization (Oschwald et al., 1991). By stage 14,

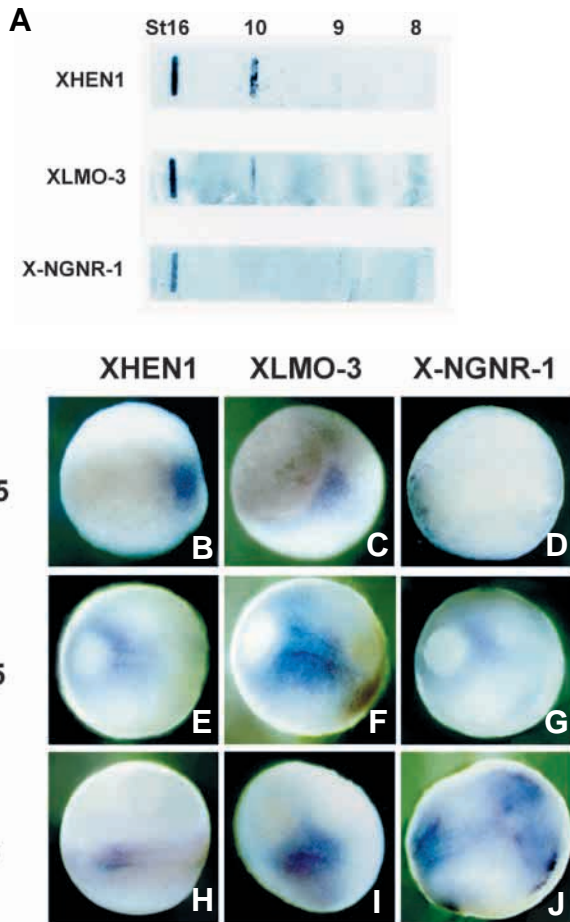
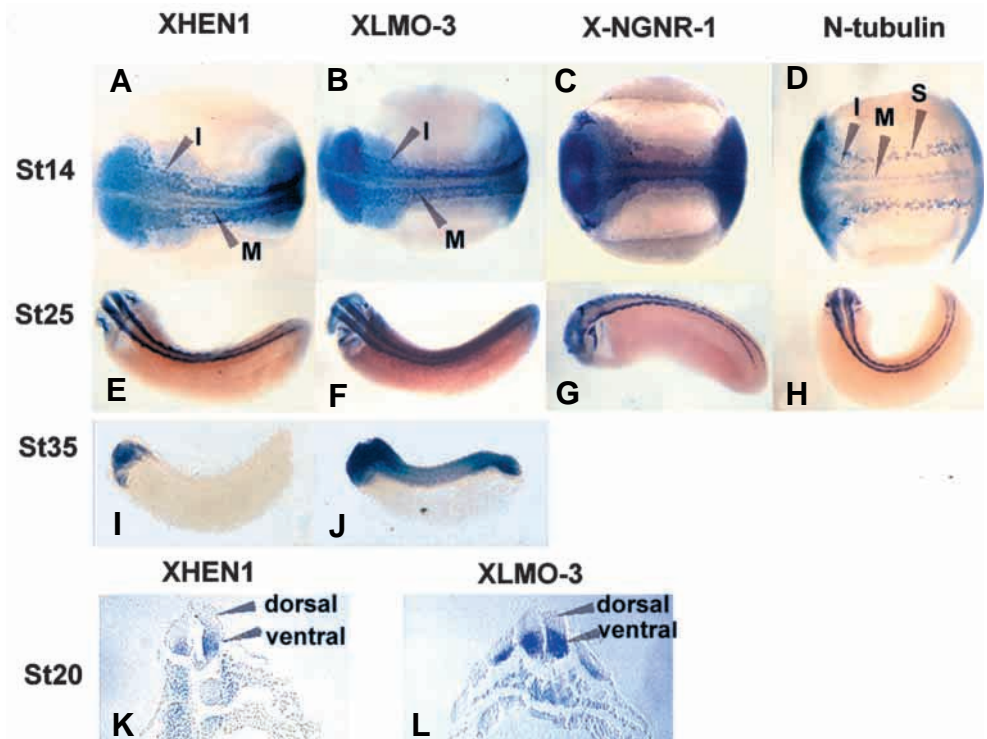


Fig. 3. Spatial and temporal expression of XHEN-1 and XLMO-3 during early neurogenesis. (A) Detection of XHEN1, XLMO-3 and X-NGNR-1 during early development by RPA. The probes as indicated on the left side were made by the Dig-RNA labeling kit. XLMO-3 and XHEN1 are expressed earlier than X-NGNR-1. (B-J) Stage 10, 11.5 and 12.5 *Xenopus* embryos were hybridized with probes for XHEN1, XLMO-3 and X-NGNR-1. Uncleared embryos are shown in a dorsal view, with anterior to the left. XLMO-3 and XHEN1 are expressed in the same location.

Fig. 4. Comparison of the expression patterns of XHEN1, XLMO-3, X-NGNR-1 and N-tubulin. (A-J) Stage 14, 25 and 35 *Xenopus* embryos were hybridized with probes for XLMO-3, XHEN1, X-NGNR-1 and N-tubulin. Embryos are shown in a dorsal (stage 14), dorsal-lateral (stage 25) or lateral (stage 35) view, with anterior to the left. At stage 14, XLMO-3 and XHEN1 are expressed only in two domains which will become interneurons (I) and motoneurons (M) of spinal cord, while X-NGNR-1 and N-tubulin are expressed also in the domains which will form sensory neurons (S) besides of these two medial domains. At stage 25, the expression patterns for all four probes are very similar. At stage 35, XHEN1 is only expressed in the brain region. (K,L) Transverse sections of *Xenopus* embryos hybridized with probes for XHEN1 and XLMO-3 at stage 20. In the neural tube, both of XHEN1 and XLMO-3 are located in the ventral part of spinal cord which is formed by interneurons and motoneurons.



overexpression of XHEN1, but not XLMO-3, induced N-tubulin expression (Fig. 7A). Stronger ectopic expression of N-tubulin was found in the dorsal part of the embryo near the neural tube following co-injection with XHEN1 and XLMO-3 mRNAs. In comparison, both ventral and dorsal cells on the side of the embryos injected with X-NGNR-1 expressed N-tubulin (Fig. 7A). At stage 25, a similar pattern of ectopic expression of N-tubulin was found. However, the extent of ectopic expression was decreased for all groups. Most interestingly, the ectopic expression of N-tubulin induced by XHEN1 and XLMO-3 was restricted to the dorsal half of the embryo, while ectopic expression of N-tubulin induced by X-NGNR-1 was relatively high, widespread, extending to the ventral part of embryos (Fig. 7A).

Further evidence for the role of XHEN1 in activating a signaling cascade leading to neuronal differentiation comes from loss-of-function experiments. We made a XHEN1 mutant by fusing a WRPW peptide at its C-terminal end. The C-terminal WRPW motif is present in all hairy-related bHLH proteins, and this class of bHLH proteins is thought to repress transcription through direct binding of Groucho with this WRPW motif (Paroush et al., 1994). We anticipated that this construct might work in a dominant negative manner and

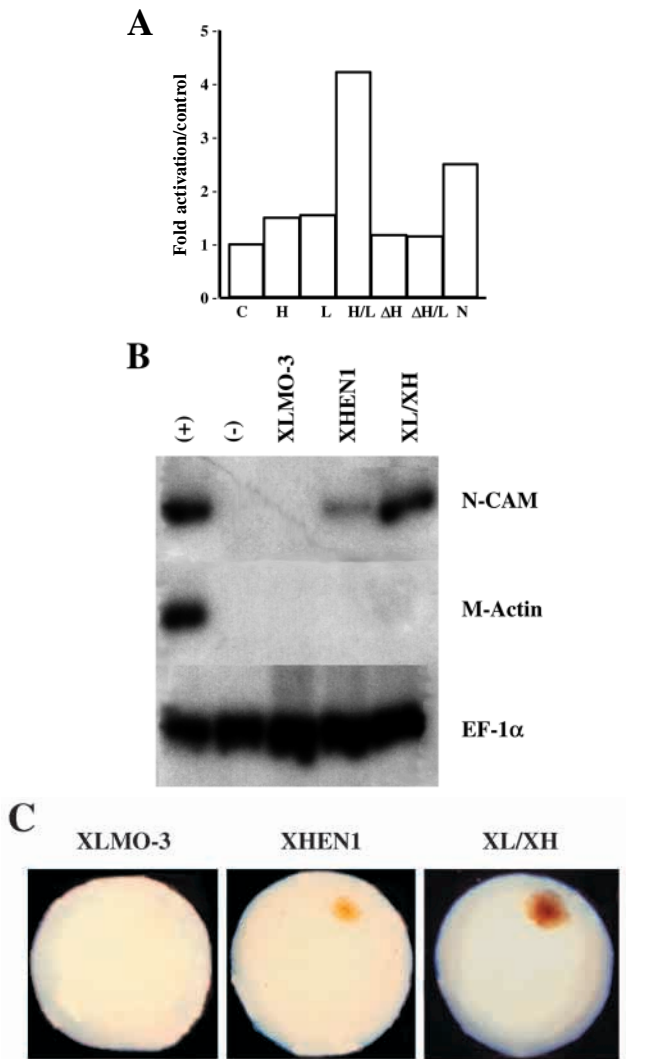


Fig. 5. Cooperative role of XHEN1 and XLMO-3 in vivo. (A) Transcriptional activity of XHEN1. The following plasmids were co-transfected with 10 μg pNEX-167, a reporter plasmid made of 5' regulatory region (167 bp) of NEX-1 gene fused to the firefly luciferase gene. C, control, reporter alone; H, 20 μg XHEN1; L, 20 μg XLMO-3; H/L, 20 μg each XHEN1 and XLMO-3; ΔH, 20 μg truncated XHEN1; ΔH/L, 20 μg each truncated XHEN1 and XLMO-3; N, 20 μg NEX. (B) Induction of N-CAM in animal caps by over-expression of XHEN1 and XLMO-3. EF-1α is a ubiquitously expressed mRNA and serves as a control for RT-PCR and loading. The bands from lanes of XHEN1 and XHEN1/XLMO-3 were quantified by NIH image 1.6. The optical density for the N-CAM and EF-1α band is 26.74 and 95.12 pixels/mm² in the XHEN1 lane, 62.73 and 91.61 pixels/mm² in XHEN1/XLMO-3 lane. After calibrating with the optical density of EF-1α band (the loading control), about 2.4 times more N-CAM was found to be induced by co-injection of XHEN1 and XLMO-3 than injection of XHEN1 alone. The mesodermal marker, muscle actin (M-Actin), is only induced in the positive control which are uninjected animal caps treated with activin (+). Untreated animal caps from uninjected eggs were used as the negative control (-). (C) Whole mount immunostaining with XAN-3 antibody of embryos injected with XHEN1 or/and XLMO-3. The main antigen for XAN-3 is N-CAM. The embryos were collected at stage 10 following the injection. There was no staining in the embryos injected only with XLMO-3 (left). Weak staining was found at stage 10 in the embryos injected only with XHEN1 (center), whereas strong staining was observed in the embryos co-injected with both XHEN1 and XLMO-3 (right).

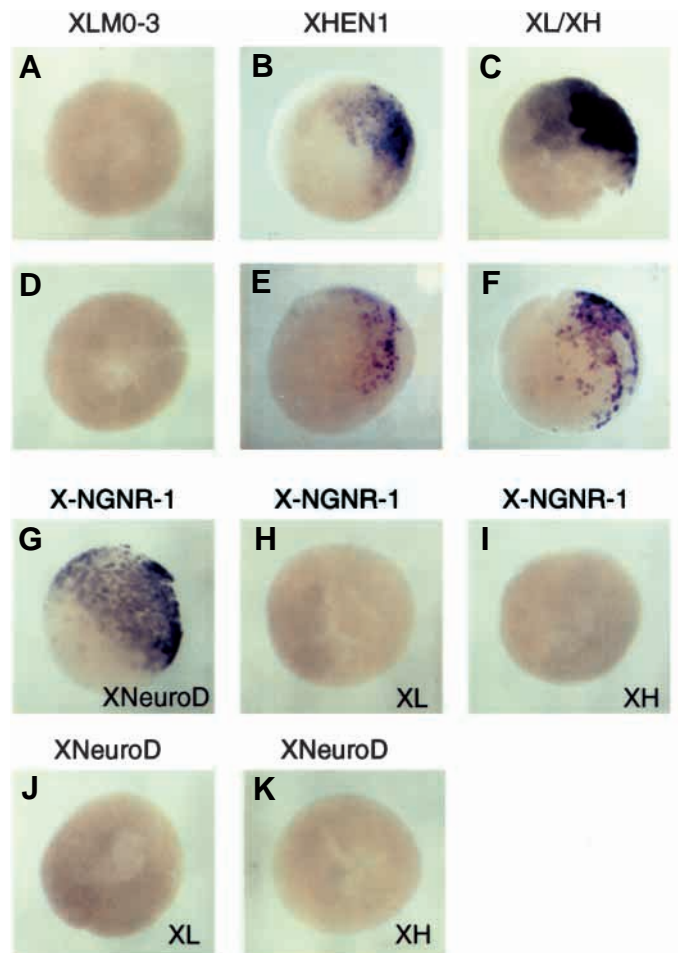


Fig. 6. Induction of X-NGNR-1 and XNeuroD Expression by XHEN1 and/or XLMO-3. (A-F) Embryos injected on one side with XLMO-3 (A,D), XHEN1 (B,E), or both (C,F) were hybridized at stage 9 with X-NGNR-1 (A-C) or XNeuroD (D-F) probe. Injection of XHEN1 mRNA induced endogenous X-NGNR-1 expression as well as endogenous XNeuroD expression, while co-injection of XHEN1 and XLMO-3 mRNAs induced more endogenous X-NGNR-1 and XNeuroD. (G-K) Embryos injected on one side with X-NGNR-1 (G-I) or X-NeuroD (J,K) mRNAs were processed by in situ at stage 9 with probes for XNeuroD (G), XLMO-3 (XL; H,J) and XHEN1 (XI; I,K). X-NGNR-1 induced endogenous XNeuroD expression, but not endogenous XHEN1 and XLMO-3 expression.

inhibit neuronal differentiation when overexpressed in *Xenopus* embryo. As shown in Fig. 7B, expression of XHEN1-WRPW in one side of *Xenopus* embryo eliminated N-tubulin expression at stage 13/14. Since a high proportion of the embryos died before reaching stage 14, it is possible that overexpression of XHEN1-WRPW repressed other genes in addition to XHEN1. The inhibition of N-tubulin expression strengthens the idea that XHEN1 is a physiological regulator of neurogenesis.

To further characterize the time course of neuralization and neural differentiation induced by XHEN1 and XLMO-3, we injected both blastomeres of 2-cell-stage embryos with XNeuroD, X-NGNR-1 or XHEN1/XLMO-3 mRNAs. Batches of ten animal caps were isolated for each group and collected at 4, 12, 24 and 48 hours, then assayed for expression of

various mRNAs by RT-PCR. N-CAM, an early neural marker, was found in all three injected groups after animal caps were maintained *in vitro* for 12 hours, although its expression level decreased after longer incubation time in the X-NGNR-1- or XHEN1/XLMO-3-injected groups. In contrast, N-tubulin was induced in animal caps injected with X-NGNR-1 or XHEN1/XLMO-3, but not in the group injected with XNeuroD, which was only able to induce N-tubulin expression at later incubation times (24 and 48 hours). Moreover, N-tubulin expression had greatly decreased or disappeared at 48 hours in animal caps injected with X-NGNR-1 or XHEN1/XLMO-3 (Fig. 7C).

DISCUSSION

The main findings of this study are that two neural-specific gene products, XHEN1 and XLMO-3, physically and functionally interact. During development, expression of both of these genes overlaps, initially near the dorsal lip of the blastopore, and subsequently in the neural plate. When overexpressed, XHEN1/XLMO-3 induced a transcription cascade involving X-NGNR-1 and XNeuroD that resulted in N-CAM and N-tubulin expression. Thus a XHEN1:XLMO-3 complex appears to act early in *Xenopus* embryogenesis to promote ectodermal cells to acquire a neural fate.

Specific interaction between HEN1 and LMO-1

Several lines of evidence suggest that XHEN1/XLMO-3 interaction is a functional one. *In vivo* transcription by XHEN1 is dependent on the presence of XLMO-3. Furthermore, co-injection of XLMO-3 in *Xenopus* embryos significantly potentiates the neurogenic activity of XHEN1 (2.4-fold), as seen by the induction of NCAM mRNA in animal caps. This potentiation could be due to a direct physical interaction between XHEN1 and XLMO-3 leading to more active protein complexes. Potentiation of ectopic neurogenesis induced by XASH-3 or Mash-1 with non-bHLH proteins has also been shown (Zimmerman et al., 1993; Ferreira et al., 1994; Turner and Weintraub, 1994; Mao and Nadal-Ginard et al., 1996). It is not known how binding of non-bHLH proteins potentiates the neurogenic activity of bHLH proteins. 'LIM-only' proteins such as XLMO-3 lack DNA-binding homeodomains (Boehm et al., 1991). The LIM domain, a cysteine-rich sequence that coordinates zinc atoms, provides an interface for protein-protein interaction, but does not directly bind DNA (Schmeichel and Beckerle, 1994). It is interesting to note that LMO-2, SCL and GATA1 (and possibly E47 and Ldb1) form a complex in mammalian cells (Osada et al., 1995; Wadman et al., 1997), and loss-of-function studies found that these proteins can serve similar roles in hematopoietic development (Pevny et al., 1991; Warren et al., 1994; Porcher et al., 1996; Robb et al., 1996). Thus, LMO-2 might regulate transcription by interacting with other transcription factors that, in turn, bind to DNA regulatory elements (Wadman et al., 1997). It is possible that LMO-1 regulates the activity of HEN1 by a similar mechanism. Each of the three LMO proteins (LMO-1, LMO-2, and LMO-3) can interact with HEN1. The precise spatial and temporal expression patterns of the three LMO genes are not yet known although, since all three are expressed in the

nervous system (Greenberg et al., 1990, McGuire et al., 1991), it is possible that HEN1 forms complexes with other LMO proteins during neurogenesis in addition to LMO-1.

We found that XLMO-3 binds to XASH-3 in the yeast two-hybrid assay, although more weakly than it does to XHEN1 (Fig. 1C). This raises the possibility that XASH-3 and XLMO-3 might interact *in vivo* during the development of the CNS. We found that XLMO-3 and XASH-3 were expressed in different parts of the neural tube at the time of neural tube closure: the expression of XASH-3 is limited in the sulcus limitans (Zimmerman et al., 1993), while XLMO-3 is expressed in the ventral part of the neural tube (Fig. 4B), ruling out the possibility that these two proteins interact at that stage. However, we cannot rule out the possibility that XLMO-3 and XASH-3 could interact at different stages of development or in other regions of the nervous system.

Cascades of transcription factor activation during neurogenesis in *Xenopus*

Neural induction requires the successive activity of secreted factors (i.e. noggin or follistatin), proneural gene products (bHLH factors) and neurogenic gene products (such as Notch). The exact contribution of each group to the ultimate formation of the nervous system is not completely clear (Harland, 1994, Chitnis and Kintner, 1995). Our work identifies one of the molecular pathways in which XHEN1/XLMO-3 initiate the cascade of expression of bHLH genes during early neurogenesis.

Several investigators have shown that different bHLH factors determine specific neuronal lineages (Guillemot, 1995; Ma et al., 1996, 1998; Fode et al., 1998). For example, a cascade involving Mash1, Math4, neurogenin1 and NeuroD has been implicated in the development of olfactory progenitors and another cascade involving Xash3, NeuroD and Xath5 has been implicated in the development of retinal progenitors (Cau et al., 1997; Kanekar et al., 1997). The present study indicates that XHEN1/XLMO-3 start a regulatory cascade that leads to the sequential expression of X-NGNR-1 and XNeuroD. Both XHEN1 and XLMO-3 were expressed on the dorsal side of the dorsal lip of the blastopore earlier than X-NGNR-1. In addition, XHEN1 and XLMO-3 can induce the expression of X-NGNR-1, and, in turn, XNeuroD. Induction of X-NGNR-1 expression was observed as early as stage 9, just following the activation of zygotic transcription (stage 8). Thus, the expression of X-NGNR-1 is a close downstream target, if not a direct target, regulated by XHEN1 and XLMO-3. However, a different temporal sequence of NGNR, NeuroD and HEN1 expression has been reported in rat, which was confirmed by the phenotype of a null mutation of NGN-1 in mice (Ma et al., 1998). Temporal differences in the cascade of bHLH factors leading to myogenesis have been reported between *Xenopus* and mouse (Sassoon, 1993; Rawls and Olson, 1997).

The amount of N-tubulin induced by XHEN1/XLMO-3 overexpression was significantly lower than the level induced by overexpression of X-NGNR-1 or XNeuroD. This could be due to the inability of XHEN1/XLMO-3 to induce endogenous X-NGNR-1 and XNeuroD expression to levels comparable to that achieved by the mRNA injection. Alternatively, XHEN1/XLMO-3 might activate a parallel inhibitory pathway similar to what has been described with

XASH-3 (Kanekar et al., 1997). In preliminary experiments, co-injection XHEN1 with X-NGNR-1 markedly decreased the ectopic neurogenesis induced by X-NGNR-1 (data not shown). These data support a role for XHEN1 in both inducing an early neuralizing response and a delayed inhibitory response. It is also possible that XHEN1 and X-NGNR-1 have common, yet unidentified, rate-limiting partners that would be titrated out in these co-injection experiments.

Multiple regulators of the activity of bHLH factors during neuronal development

The activity of neuronal bHLH factors is differentially regulated by undefined factors. For example, X-NGNR-1 and XNeuroD induce N-tubulin expression in both dorsal and ventral regions, while N-tubulin induction by XHEN1/XLMO-3 or XASH-3 is restricted to dorsal regions (Fig. 7A). XASH-3 is sensitive to lateral inhibition by X-Delta-1/X-Notch-1 signals (Chitnis and Kintner, 1996). HEN-1 might be subjected to similar lateral inhibition, since HEN-1 expression is ectopically induced in *Notch-1*^{-/-} mice (Pompa et al., 1997). The relative insensitivity of XNeuroD to lateral inhibition might account, at least in part, for the differences in the ectopic expression pattern of N-tubulin induced by XNeuroD compared to the pattern induced by XHEN1/XLMO-3. In addition, neuronal-specific bHLH proteins might be differentially sensitive to signals, such as BMPs, thereby yielding distinct patterns of neuronal marker expression (Hemmati-Brivanlou and Melton, 1992; Calof, 1995).

The major conclusion of this study is that the bHLH transcription factor HEN1 in collaboration with LMO proteins, forms a complex that initiates a cascade of transcriptional activation leading to neurogenesis and early neuronal differentiation. A role of their *Xenopus* homologues, XHEN1 and XLMO-3, in early neurogenesis is supported by the early overlapping expression pattern of both genes. The functional significance of the XHEN1 and XLMO-3 interaction is supported by the facts that: (1) XHEN1 transcriptional activity is dependent upon XLMO-3, (2) co-expression of XHEN1 and XLMO-3 leads to the sequential induction of X-NGNR-1 and XNeuroD, as well as the ectopic expression of the neuronal markers, N-CAM and N-tubulin, and (3) XHEN1 loss of function results in reduced neurogenesis as seen by the

expression of N-tubulin. The temporal and spatial limitations of the expression patterns of ectopic neurogenesis induced by XHEN1/XLMO-3 suggests that the neurogenic activity of this complex is subject to further regulation by other neurogenic factors.

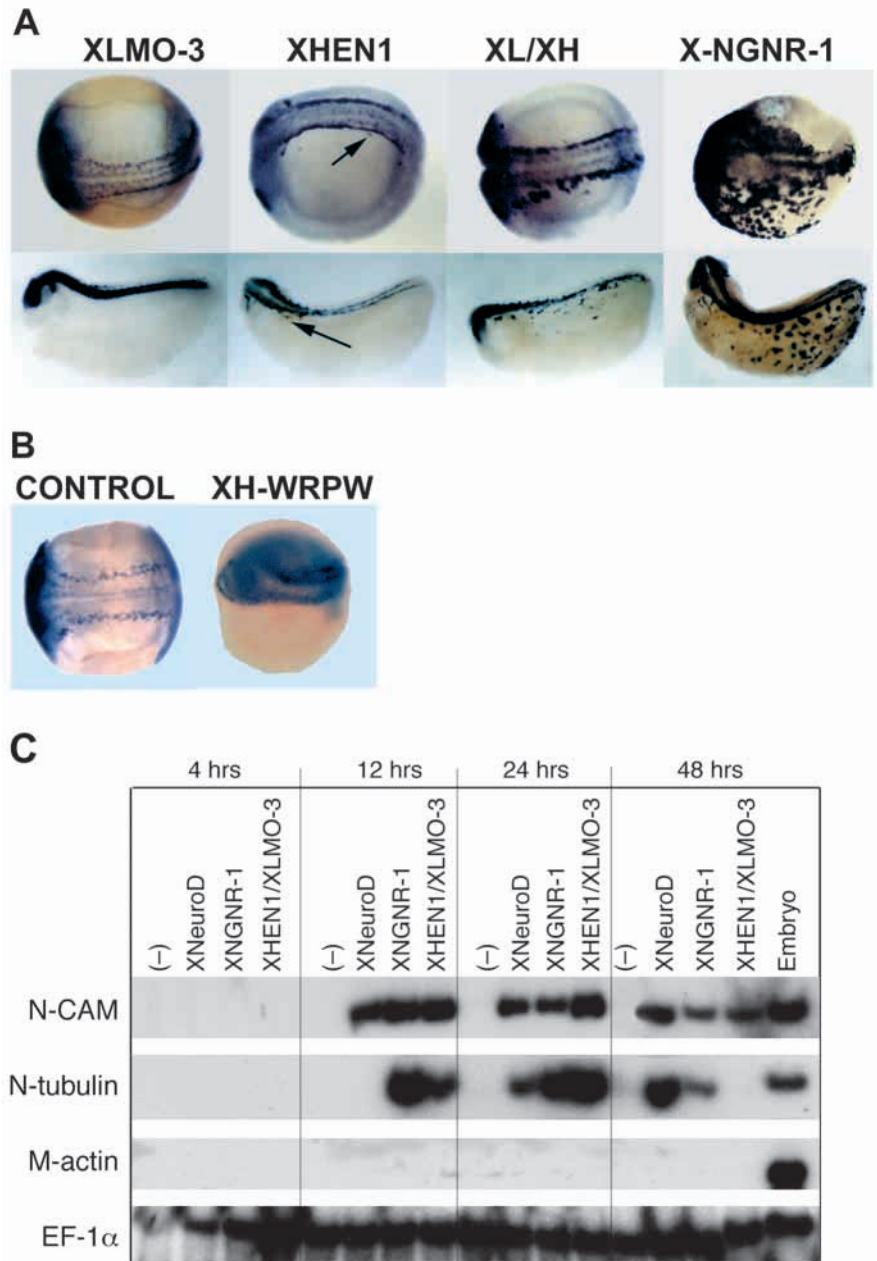


Fig. 7. Ectopic neurogenesis induced by XHEN1 and/or XLMO-3 mRNAs. (A) Ectopic neurogenesis was visualized at stage 14 and stage 25 embryos by whole mount in situ hybridization with an N-tubulin probe. Embryos were injected on one side (left side at the bottom) with XLMO-3, XHEN1, XHEN1/XLMO-3 or X-NGNR-1. All embryos are shown with anterior to the left. Only the injected half of stage 25 embryos is shown. (B) Downregulation of neurogenesis by overexpression of XHEN1-WRPW. Ectopic neurogenesis was visualized at stage 14 by N-tubulin staining. One cell of the 2-cell stage embryos were injected with 500 pg of mRNA encoding for XHEN1-WRPW. The injected half (bottom half) showed little N-tubulin expression (right panel) when compared to the uninjected control (left panel). (C) Temporal induction of N-CAM and N-tubulin in animal caps by over-expression of XHEN1 and XLMO-3. EF-1α is a ubiquitously expressed mRNA and serves as a control for RT-PCR and loading.

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