Development 128, 1335-1346 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV1608

Identification of NKL, a novel Gli-Kruppel zinc-finger protein that promotes neuronal differentiation

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Accepted 19 January; published on WWW 22 March 2001

SUMMARY

The proneural basic helix-loop-helix proteins play a crucial role in promoting the differentiation of postmitotic neurons from neural precursors. However, recent evidence from flies and frogs indicates that additional factors act together with the proneural bHLH proteins to promote neurogenesis. We have identified a novel zinc finger protein, neuronal Kruppel-like protein (NKL), that positively regulates neurogenesis in vertebrates. NKL is expressed in *Xenopus* primary neurons and in differentiating neuronal precursors in the intermediate zone of the mouse and chick neural tube. In frog embryos, *NKL* is induced by overexpression of *Neurogenin* (*Ngn*), arguing that *NKL* is downstream of the proneural determination genes. Our results show that NKL and a NKL/VP16 fusion protein promote differentiation of neuronal precursors in the embryonic chick spinal cord. Following in ovo misexpression of *NKL*, neuroepithelial cells exit the cell cycle and differentiate into neurons. Similarly, NKL/VP16 induces extra primary neurons in frogs and upregulates expression of the neural differentiation factors, Xath3 and MyT1, as well as the neuronal markers, *N-tubulin* and *elrC*. Our findings establish NKL as a novel positive regulator of neuronal differentiation and provide further evidence that non-bHLH transcription factors function in the neuronal differentiation pathway activated by the vertebrate neuronal determination genes.

Key words: NKL, Zinc-finger transcription factor, Neurogenesis, *Xenopus*, Chick

INTRODUCTION

Neurogenesis is a highly regulated process involving the sequential activation of proneural basic helix-loop-helix (bHLH) proteins that promote the differentiation of neurons from multipotential precursors (reviewed in Jan and Jan, 1993; Ludolph and Konieczny, 1995). In *Drosophila*, proteins encoded by the *achaete-scute* gene complex and *atonal* (Campos-Ortega, 1993; Modolell, 1997) initiate neurogenesis in selected precursors, a process that is negatively regulated by Notch signaling (Artavanis-Tsakonas et al., 1995). Similarly in vertebrates, the Atonal homologs Neurogenin 1, Neurogenin 2 and NeuroD drive the early 'determination' and late 'differentiation' phases of neurogenesis, respectively (Ma et al., 1996; Lee, 1997). This two step model of neurogenesis in which the neurogenins function as determination factors, while downstream bHLH proteins promote differentiation in determined neuronal precursors, reflects their temporal expression during development. *Ngn1* and *Ngn2* are expressed in the ventricular zone in dividing progenitors, whereas *NeuroD* and *Xath3/NeuroM* are expressed in neuroblasts located in the intermediate and mantle zones (Bartholomae and Nave, 1994; Ma et al., 1996; Sommer et al., 1996; Roztocil et al., 1997; Perez et al., 1999; Perron et al., 1998). The finding that the downstream bHLH genes *NeuroD* or *NSCL* are not activated in subsets of differentiating neurons in mice that lack *Ngn1* or *Ngn2* (Fode et al., 1998; Ma et al., 1998), thereby causing the loss of specific cranial ganglia, is also consistent with this two-step model.

In flies, amphibians and higher vertebrates misexpression of the proneural genes is sufficient to induce neurons. In *Drosophila*, overexpression of *atonal* induces the formation of ectopic chordotonal organs (Jarman et al., 1993), while expression of Ngn in frogs and zebrafish activates a full program of neurogenesis by inducing neuronal markers and components of the lateral inhibition machinery (Ma et al., 1996; Takke et al., 1999). Although the proneural bHLH proteins are able to promote ectopic neurogenesis when constitutively expressed, under normal circumstances this activity appears to be modulated by non-bHLH factors, thereby allowing the sequential generation of discrete neuronal populations in a organized manner. Some non-bHLH proteins act as co-factors. For example, the *Drosophila* proneural gene *scute*, which is autoregulated, requires Rel protein recognition sequences in addition to E-box sequences for maximal activation (Culi and Modolell, 1998). Similarly, the *Drosophila* C2H2 zinc-finger protein Senseless (Sens), which is activated in sensory organ precursors by the proneural genes, participates in a synergistic positive feedback loop with the proneural bHLH factors to induce PNS sense organ

differentiation (Nolo et al., 2000). To date, only a few vertebrate non-bHLH transcription factors have been identified that are downstream of Ngn and capable of modulating neurogenesis. Those include, the HLH protein Coe2 (Dubois et al., 1998) and the zinc-finger protein MyT1 (Bellefroid et al., 1996).

The Gli/Zic zinc-finger family of transcription factors play key roles in neural development, and many are expressed in dividing neural precursors, in temporal and spatial domains that overlap with the proneural bHLH genes (Aruga et al., 1994; Hui et al, 1994). Gli2 and Gli3 regulate neuronal specification, with Gli2 being necessary for the correct specification of floor plate cells (Matise et al., 1998), while Gli3 functions to repress ventral cell types (Litingtung and Chiang, 2000). The Zics, however, may function in the regulatory pathways controlling neurogenesis. Zic1 and Zic3 are able to promote neurogenesis when misexpressed in frogs (Nakata et al., 1997; Mizuseki et al., 1998), whereas Zic2 appears to inhibit neurogenesis by blocking both the activity and expression of *XNgn1* (Brewster et al., 1998). When misexpressed in frog, the Zic proteins also promote neural crest formation (Nakata et al., 1997; Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998; Nakata et al., 2000), suggesting they may also function as cell type determinants in the embryonic nervous system. Loss-of-function mutations in Zic2 or Zic3 have not been informative with respect to their role in neurogenesis and patterning, most probably because of genetic redundancy in the CNS (Nagai et al., 1997). Rather, these studies reveal that the Zic proteins are required for proper neurulation (Klootwijk et al., 2000; Nagai et al., 2000).

In this study we identify a novel C2H2 zinc finger protein, neuronal Kruppel-like protein (NKL), that shares homology with the Gli/Zic proteins and promotes neuronal differentiation. *NKL* is broadly expressed in the neural tube and peripheral nervous system as neural precursors are differentiating. The onset of NKL expression in the neural plate of *Xenopus* embryos coincides with primary neurogenesis, suggesting NKL functions in the pathway controlling the transition of neuronal precursors from a determined to a differentiated state. Overexpression of either NKL or a NKL/VP16 fusion protein in the embryonic chick spinal cord promotes the differentiation of neuronal precursors. In *Xenopus* embryos, overexpression of NKL/VP16 leads to an increase in primary neurons, which is accompanied by upregulation of *Xath3* and *MyT1*, while misexpression an antimorphic form of NKL, NKL/EnR, downregulates neuronal markers. These results argue that NKL functions as a positive regulator of neurogenesis and suggest that it may do so by facilitating the transition from determined neuronal precursor to postmitotic neuron.

MATERIALS AND METHODS

Animals

Xenopus laevis embryos were derived from adult frogs by hormone-induced egg-laying and fertilization in vitro using standard techniques. Staging was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). White Leghorn chick eggs were obtained from a local supplier and incubated at 38°C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Mouse embryos were generated from breedings in our animal facility with plug dates being designated as E0.5.

Isolation of mouse, *Xenopus* and chick NKL cDNAs

A 429 bp partial cDNA clone that partially encodes the zinc-finger domain of NKL was first identified in a two-hybrid screen searching for proteins that interact with Pax3; however, subsequent analyses indicated that this interaction was non-physiological. This cDNA fragment was then used to screen three different embryonic mouse cDNA libraries. A full-length mouse NKL cDNA sequence was constructed by splicing three overlapping clones together in frame. DNA sequencing was either performed with ^{35}S-dATP and a Sequenase kit or by the Salk Institute Sequencing Facility.

A cDNA encoding the mouse zinc fingers was used to screen a λgt10 *Xenopus laevis* Stage 17 cDNA library (Kintner and Melton, 1987) at moderate stringency. A single full-length cDNA clone, *XNKL(a)*, and multiple cDNAs encoding a highly homologous cDNA, *XNKL(b)*, were isolated and sequenced The full-length *XNKL(a)* clone is designated *Xenopus NKL* here. A mixture of the mouse and *Xenopus NKL* and *NKL* probes was used to screen several embryonic chick cDNA libraries at moderate stringency. These screenings yielded a single partial cDNA clone (0.6 kb) containing all five zinc fingers from a λgt10 chick E12 retina library (a gift from Drs Marc Ballivet and Linda Erkman). This cDNA was designated chick *NKL*.

The accession numbers for mouse *NKL*, *Xenopus NKL(a)* and *NKL(b)*, and chick *NKL* are AF249340, AF249341, AF249342 and AF249339, respectively.

Constructs

For expression studies, full-length and truncated forms of *Xenopus NKL* were constructed in pCS2 and pCS2-MT vectors (Turner and Weintraub, 1994). The NKL/VP16 and NKL/EnR fusion proteins were generated by ligating sequences encompassing the *Xenopus* NKL zinc-finger domain (residues 145-327) to pVP16 and pEnR, both a gift of Dr Sylvia Evans. Myc-tagged versions of VP16 and EnR constructs were generated in pCS2-MT. Tagged deletion constructs of *Xenopus* NKL contained the following amino acid residues: zinc finger, residues 145-327; N-terminal deletion, residues 145-492; and C-terminal deletion, residues 1-363.

EMSA

A double-stranded probe identical to the B1 probe (Kinzler and Vogelstein, 1990) was radiolabeled with [^{32}P]-dCTP by filling in with Klenow fragment. Double-stranded oligonucleotide Sp1 binding sites dsI and dsII (Jones et al., 1985) were used for competition experiments.

Myc-tagged zinc finger domains of both *Xenopus* and mouse NKL cloned into CS2 vectors were synthesized by coupled in vivo translation/transcription using Sp6 polymerase and a TNT kit (Promega). Unlabeled proteins were used in gel shift assays. An estimated 40-50 ng of synthesized protein was used in each binding assay. Proteins were pre-incubated with 10^5 cpm of probe in binding buffer containing 25 mM Hepes, pH 7.6, 0.5 mM EDTA, 0.5mM DTT, 50 mM NaCl, 10 μM $ZnCl_2$, 10% glycerol, plus 3 μg poly dI/dC (Boehringer-Mannheim) as nonspecific competitor. The probe was then added and incubated for 30 minutes at room temperature. For supershifts, 1 μl of the supernatant from the Myc1-9E10.2 hybridoma (ATCC) was added to the preincubation. Samples were loaded on a 5% acrylamide gel and run in 1×TBE at 300 V.

Misexpression in *Xenopus* and chick embryos

Capped transcripts of *XNgn1*, *NKL/VP16* and *NKL/EnR* were synthesized in vitro using SP6 polymerase (Kintner and Melton, 1987); 10 nl containing varying concentrations of each RNA was injected together with 400 pg of *lacZ* tracer RNA into one blastomere at the two- or four-cell stage (Coffman et al., 1990). Injection of *lacZ* RNA alone functioned as a negative control. Embryos were developed

to neurula and tailbud stages, at which time they were processed for in situ hybridization (Chitnis et al., 1995).

Windowed E2 chick embryos were electroporated with CS2-MT expression vectors at 5 μg/μl (Muramatsu et al., 1997) using a BTX electroporator (five 50 mseconds pulses at 25 V). Electroporations were targeted between somites 18-22. Eggs were sealed and allowed to develop to specified stages, at which time they were processed for immunohistochemistry.

Immunohistochemistry and in situ hybridization

Frog embryos were fixed in 4% paraformaldehyde and whole-mount in situ hybridizations were performed using digoxigenin-labeled RNA probes and alkaline phosphatase-conjugated anti-digoxigenin antibody (Chitnis et al., 1995). For sectioning, stained frog embryos were embedded in 3.5% agar/8% sucrose in PBS, and vibratomed at 70-100 μm. In situ hybridization of sectioned mouse and chick embryos was undertaken with [^{33}P]UTP-labeled (Amersham) RNA probes as described (Wilkinson, 1992).

Chick embryos were fixed for 3 hours in 4% paraformaldehyde, washed in PBS with 0.1% Triton (PBST), cryoprotected in 25% sucrose in PBS, and embedded in OCT (Sakura Finetek) for sectioning at 15 μm on a cryostat. For antibody stainings, slides were air-dried, washed three times with PBST, incubated with PBST with 10% fetal calf serum (FCS) for 1 hour, and incubated with the primary antibody in PBST plus 10% FCS for 1 hour at room temperature or overnight at 4º. Slides were then washed 3 times with PBST and incubated with Cy2-, Cy3- and HRP-conjugated secondary antibodies as required (Jackson ImmunoResearch) in PBST with 10% goat serum. For double-label experiments, this procedure was repeated sequentially to react the second primary antibody. Slides were dehydrated and coverslipped with DPX (BDH Laboratory Supplies). Samples were imaged by double-exposure photography on a Nikon epifluorescence microscope. Antibodies used in this study were Pax2, rabbit polyclonal (Dressler and Douglass, 1992); Isl1, mouse monoclonal (Developmental Studies Hybridoma Bank (DSHB)); Lim 1/2, mouse monoclonal 4F2 (DSHB); TuJ1, mouse monoclonal (Moody et al., 1987); BrdU, rat monoclonal (Harlan); and anti-Myc, mouse monoclonal 9E10 (ATCC) and rabbit polyclonal (a gift of Dr Sam Pfaff). For immunofluorescence, appropriate Cy3- and Cy2-conjugated secondary antibodies were used as recommended (Jackson ImmunoResearch).

For BrdU labeling, electroporated embryos were injected in the lumen of the neural tube with bromodeoxyuridine (50 mg/ml in 0.9% saline) and sacrificed 1 hour later. Immunocytochemistry was performed as described (Braisted and Raymond, 1992).

RESULTS

Identification of NKL, a zinc-finger protein related to Zic and Gli proteins

We identified a mouse cDNA encoding a novel five-fingered zinc-finger transcription factor homologous to Gli and Zic proteins (see Materials and Methods) and designated it NKL. Further screenings of embryonic cDNA libraries at high and moderate stringencies yielded several truncated but overlapping clones of a single mouse gene, a full-length *Xenopus* clone, and a single partial chick clone, the latter encoding primarily the zinc-finger domain (Fig. 1A). The zinc-finger domains of all three proteins are highly homologous, with chick NKL exhibiting 94% and 91% identity with the mouse and *Xenopus* proteins in this region. While the NKL proteins have conserved amino acid stretches outside the zinc-finger domain, the overall amino acid identity between full-length *Xenopus* and mouse NKL is only 67%, which is similar to the overall homology of other orthologous DNA-binding proteins in mouse and *Xenopus*.

A second highly homologous *Xenopus* gene that we attribute to that organism's pseudotetraploidy was also identified in these screens (see Materials and Methods); however, in no other species were homologues of NKL found. For instance, all the cDNAs we isolated, as well as the numerous entries of mouse NKL in the EST databases, encode a single gene, while database searches have identified only a single human EST (RT140), which encodes a NKL homolog and is 96% identical to mNKL in the zinc-finger domain. This gene (AC004789) maps to human chromosome 16p13.3. We have also identified a *Drosophila* EST clone AI456492 (62% identity between the zinc finger domains of mouse and *Drosophila*) that is distantly related to NKL.

The vertebrate NKL proteins exhibit significant homology to members of the Gli/Zic family of transcription factors and their invertebrate homologs, all of which have five tandem C2H2 zinc fingers. However, the primary sequence and spacing of the cysteines and histidines in the zinc-finger domain of NKL indicate that NKL represents a new structural member of this family distinct from the Gli and Zic proteins (Fig. 1B). While the mouse NKL zinc-finger domain is 52% and 49% identical to mouse Gli1 and *Xenopus* Zic1, respectively, NKL shows no significant homology to the Gli or Zic proteins outside the zinc-finger domain.

To determine whether NKL exhibits a similar DNA-binding specificity to other Gli/Zic proteins, we performed electrophoretic mobility shift (EMSA) assays using a Gli1 binding site (B1: Kinzler and Vogelstein, 1990) and the Myc-tagged zinc-finger domain of mouse NKL. Both the Gli and Zic proteins bind this CA-rich motif in vitro (Aruga et al., 1994). We found that the NKL zinc finger strongly shifts the B1 probe (Fig. 1C, lane 3), and that the NKL-specific band can be supershifted by an anti-Myc antibody (lane 4). Binding of NKL to B1 site is specific and is competed efficiently by cold B1 oligonucleotides (lanes 5 and 6), but not by an oligonucleotide that is bound by the zinc-finger protein Sp1 (Jones et al., 1985) (lanes 7, 8). These findings suggest that NKL possesses an in vitro binding specificity similar to other Gli/Zic proteins, further supporting its identification as a new member of the Gli/Zic class of transcription factors.

Embryonic expression of mouse, *Xenopus* and chick NKL

To analyze *NKL* expression during neural development, mouse, chick and frog embryos were examined by in situ hybridization. *NKL* expression in the mouse begins at E9.5 in cranial ganglia, the dorsal root ganglia (DRG), and neural tube (data not shown). At E10.5 *NKL* is broadly expressed in the intermediate zone of the hindbrain (Fig. 2A) and spinal cord (Fig. 2B), and in the DRG. By E12.5, neurogenesis in the spinal cord has begun to wane and as a result, NKL expression becomes restricted to a narrow band of cells in the ventricular zone (Fig. 2C). A similar pattern of NKL expression is seen in the chick spinal cord at stage 25, where *NKL* is expressed in the intermediate zone (Fig. 2D), which is where newly postmitotic neurons are located. The temporal and spatial expression patterns of *NKL* in the mouse and the chick are reminiscent of the bHLH differentiation genes *NeuroD* and *NeuroM* (Lee et al., 1995; Roztocil et al., 1997), suggesting

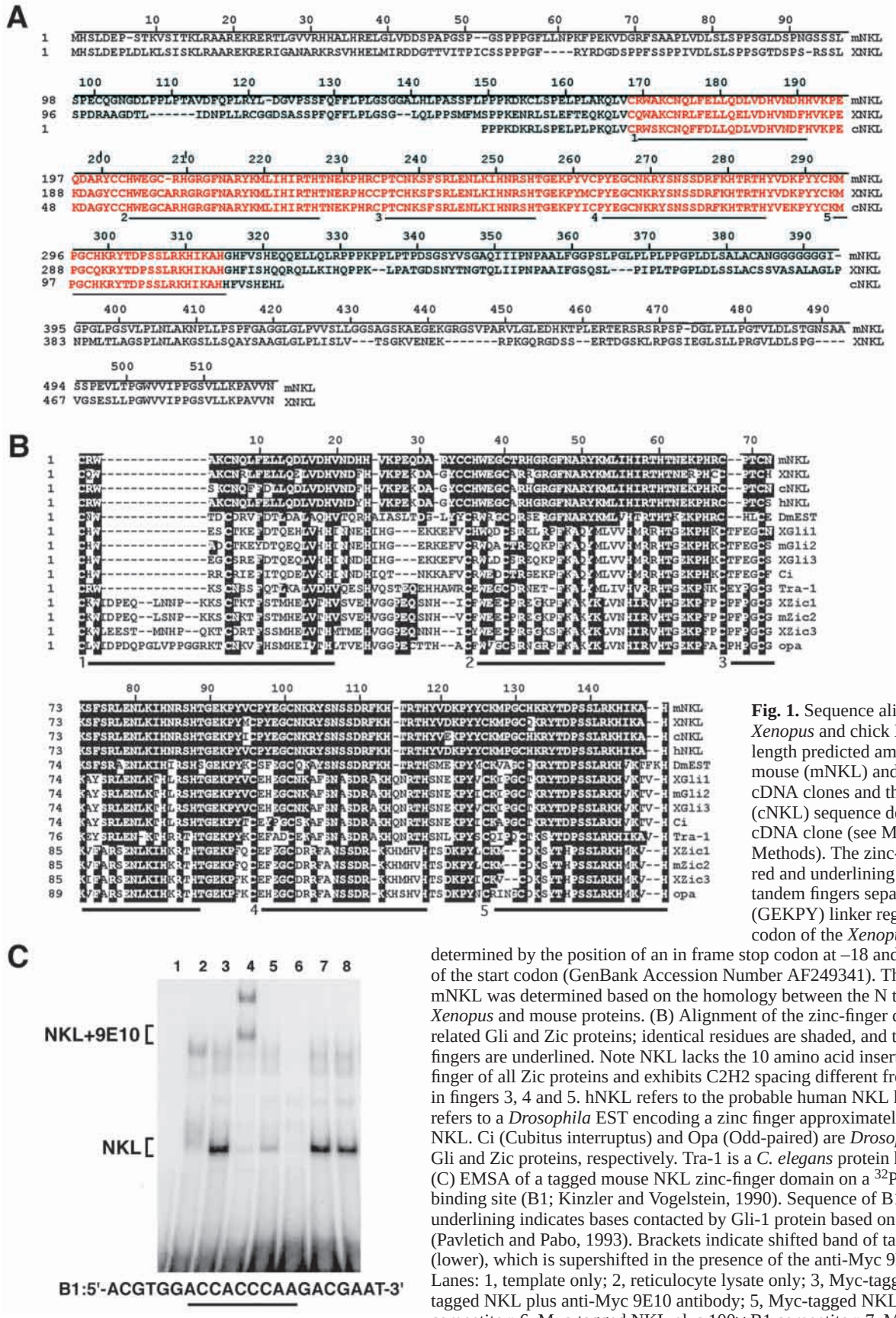

Fig. 1. Sequence alignment of mouse, *Xenopus* and chick NKL. (A) The full-length predicted amino acid sequence of mouse (mNKL) and *Xenopus* (XNKL) cDNA clones and the deduced chick (cNKL) sequence derived from a partial cDNA clone (see Materials and Methods). The zinc-finger domain is in red and underlining marks the five tandem fingers separated by consensus (GEKPY) linker regions. The initiation codon of the *Xenopus* clone was determined by the position of an in frame stop codon at –18 and the optimal context of the start codon (GenBank Accession Number AF249341). The initiation codon of mNKL was determined based on the homology between the N termini of the *Xenopus* and mouse proteins. (B) Alignment of the zinc-finger domain of NKL and related Gli and Zic proteins; identical residues are shaded, and the five tandem zinc fingers are underlined. Note NKL lacks the 10 amino acid insert found in the first finger of all Zic proteins and exhibits C2H2 spacing different from either Glis or Zics in fingers 3, 4 and 5. hNKL refers to the probable human NKL homolog; DmEST refers to a *Drosophila* EST encoding a zinc finger approximately 62% identical to NKL. Ci (Cubitus interruptus) and Opa (Odd-paired) are *Drosophila* homologs of Gli and Zic proteins, respectively. Tra-1 is a *C. elegans* protein homologous to Gli. (C) EMSA of a tagged mouse NKL zinc-finger domain on a ^{32}P-labeled Gli-1 binding site (B1; Kinzler and Vogelstein, 1990). Sequence of B1 template is shown; underlining indicates bases contacted by Gli-1 protein based on the crystal structure (Pavletich and Pabo, 1993). Brackets indicate shifted band of tagged NKL alone (lower), which is supershifted in the presence of the anti-Myc 9E10 antibody (upper). Lanes: 1, template only; 2, reticulocyte lysate only; 3, Myc-tagged NKL; 4, Myc-tagged NKL plus anti-Myc 9E10 antibody; 5, Myc-tagged NKL plus 10× B1 competitor; 6, Myc-tagged NKL plus 100× B1 competitor; 7, Myc-tagged NKL plus 10× GC-rich competitor; 8, Myc-tagged NKL plus 100× GC-rich competitor.

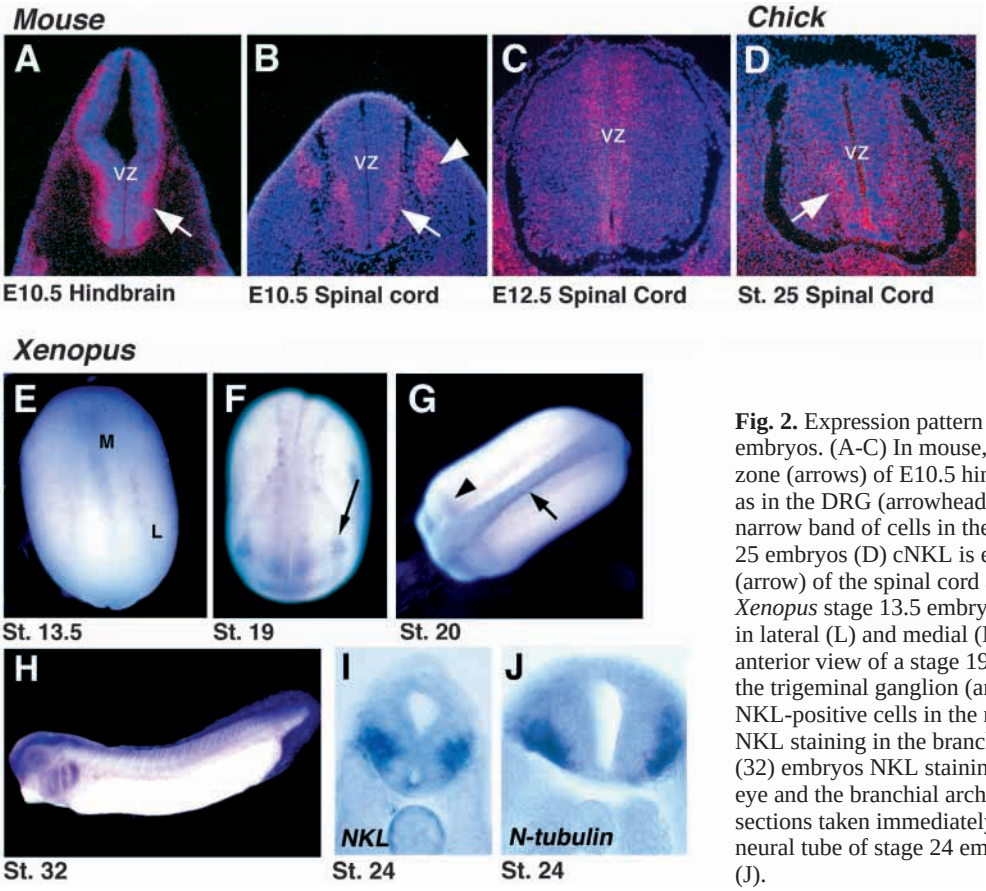

Fig. 2. Expression pattern of *NKL* in mouse, chick and *Xenopus* embryos. (A-C) In mouse, mNKL is expressed in the intermediate zone (arrows) of E10.5 hindbrain (A) and spinal cord (B), as well as in the DRG (arrowhead in B). At E12.5 mNKL is expressed in a narrow band of cells in the ventricular zone (vz) (C). In chick stage 25 embryos (D) cNKL is expressed in the intermediate zone (arrow) of the spinal cord as it is in the E10.5 mouse. (E) In *Xenopus* stage 13.5 embryos staining is apparent in the neural plate in lateral (L) and medial (M) stripes of primary neurons. (F) An anterior view of a stage 19 embryo indicates NKL hybridization in the trigeminal ganglion (arrow). (G) A dorsal view of columns of NKL-positive cells in the neuraxis (arrow). Note also the onset of NKL staining in the branchial arches (arrowhead). In tailbud stage (32) embryos NKL staining is seen in the neuraxis, the head, the eye and the branchial arches (H). (I,J) Transverse vibratome sections taken immediately posterior to the hindbrain through the neural tube of stage 24 embryos stained for *NKL* (I) and *N-tubulin* (J).

that *NKL* is temporally downstream of *Ngn1* and *Ngn2* (Perez et al., 1999).

In *Xenopus* neural plate stage embryos (Fig. 2E,F), *NKL* is seen in two lateral and two midline stripes, which correspond to precursors of primary sensory (Rohon-Beard) and motoneurons, respectively. As with mouse and chick, *Xenopus NKL* is expressed in a pattern similar to the bHLH differentiation genes *NeuroD* and *Xath3* (Lee et al., 1995; Takebayashi et al., 1997) and is temporally downstream of *XNgn1*, which is first expressed at stage 10.5. By the end of neurulation (Fig. 2G), *NKL* expression is apparent along the entire A-P axis in a pattern similar to *N-tubulin*, an early marker of neuronal differentiation (Papalopulu and Kintner, 1996). At tailbud stages, *NKL* expression persists in the neural tube and is upregulated in anterior structures such as the eye and brain and in the branchial arches (Fig. 2H). Transverse sections through the neural tube of tailbud stage embryos show *NKL*-positive cells in a pattern similar to that of *N-tubulin* (Fig. 2I,J), supporting the idea that *NKL* is expressed in newly postmitotic neurons.

Differentiating neurons upregulate *NKL* expression

The *NKL* expression pattern in mouse, chick and frog suggested to us that NKL might play a role in neurogenesis. We therefore asked whether *NKL* expression is upregulated when cells are induced to form neurons. In both P19 cells (data not shown) and *Xenopus* embryos, NKL expression was activated upon induction of neural differentiation (Fig. 3). Injection of *XNgn1* mRNA into *Xenopus* embryos induces

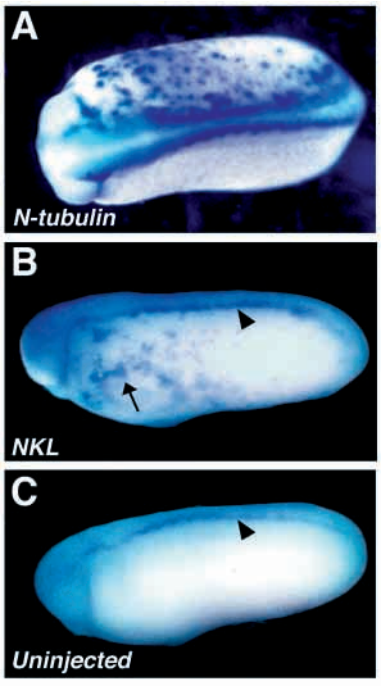

Fig. 3. *NKL* expression is induced by overexpression of *XNGN-1*. *Xenopus* embryos were injected in one blastomere of two-cell embryos with mRNAs encoding *XNGN-1* and a β-galactosidase tracer, and allowed to develop to the neural tube stage (stage 23). In all panels, anterior is to the left. (A) A dorsal view of a *XNGN-1*-injected embryo showing ectopic patches of *N-tubulin* expression on the injected (upper) side (65% (19/29) of embryos injected). (B) The injected side of a *XNGN-1*-injected embryo probed with antisense RNA for *NKL*. Note ectopic NKL staining in the ectoderm outside the neural tube (arrow), as well as a mild increase in the level of endogenous NKL staining in the neural tube (arrowhead). (C) The uninjected side, where hybridization signals indicate endogenous NKL expression (arrowhead). Ectopic NKL was seen in 60% (18/30) of embryos injected.

ectopic expression of neuronal markers such as *N-tubulin* (Chitnis et al., 1995) and several neuron-specific transcription factors such as MyT1 and NeuroD (Bellefroid al., 1996; Ma et al., 1996). Following injection of *XNgn1* transcripts into two-cell-stage embryos, we observed ectopic expression of *N-tubulin*, both within and outside the neural plate (Fig. 3A), as previously reported (Ma et al., 1996). Likewise, injection of *XNgn1* also promoted robust ectopic *NKL* expression outside the neural plate (Fig. 3B,C), thereby raising the possibility that *NKL* functions downstream of *XNgn1* to regulate neurogenesis.

Ectopic *NKL* promotes premature differentiation of chick neuronal precursors

The *NKL* expression pattern, together with its upregulation in RA-treated P19 cells (data not shown) and induction by *XNgn1* in frog embryos, led us to test whether NKL regulates neurogenesis in vertebrates. Plasmids encoding tagged forms of *Xenopus* NKL (MT-NKL) were overexpressed in neuronal progenitor cells in the spinal cord of stage 12 (embryonic day 2 (E2)) chick embryos by in ovo electroporation. The advantage of this approach is that neural precursors transfected at the onset of neurogenesis in the spinal cord can subsequently be assayed for their location, mitotic activity and expression of neuronal markers. When we electroporated embryos at E2 and analyzed them on E4 (stage 24), we found most MT-NKL-positive cells located lateral to the ventricular zone suggesting they were postmitotic (data not shown). To confirm this, we pulsed MT-NKL-electroporated embryos with BrdU for 1 hour and counted $Myc^+/BrdU^+$ cells (Fig. 4A-E). When embryos were electroporated at stage 12 and analyzed at stage 20 (E3.5), the majority of MT-NKL^+ cells (>95%) were $BrdU^-$ and these cells were located lateral to the ventricular zone (Fig. 4A-C). In contrast, cells transfected with a control vector expressing the Myc tag alone (CS2-MT) were distributed between the ventricular zone and mantle zone (Fig. 4D). Of these, approximately half (55%) were $BrdU^+$ (Fig. 4E). Thus, although fewer than 5% of the cells electroporated with MT-NKL were $BrdU^+$, 55% of control cells were $BrdU^+$ (summarized in Fig. 6F). Control embryos also contained $Myc^+/BrdU^-$ cells near the lumen of the neural tube (Fig. 4D,E). It is likely that many of these cells were in G1, G2, or M-phase during the short BrdU pulse and consequently were not labeled with BrdU, nor were they counted in this quantitative analysis even though they are still dividing. In contrast, Myc^+ cells were rarely seen adjacent to the lumen in MT-NKL-injected embryos 36 hours after electroporation, further arguing that the majority of MT-NKL^+ cells exit the cell cycle. When embryos were examined 24 hours after electroporation a marked lateral shift in the position of MT-NKL was already apparent (data not shown) and fewer than 20% of MT-NKL^+ cells co-labeled with $BrdU^+$. Together, these findings suggest that the induction of NKL in dividing neural progenitors promotes their rapid withdrawal from the cell cycle in a cell-autonomous manner.

As a positive control we also electroporated an expression plasmid that expresses a Myc-tagged version of XNgn1 (MT-XNgn1). As MT-XNgn1 misexpression promotes neurogenesis in *Xenopus*, we predicted that it would also promote differentiation of chick neuroepithelial cells. As expected, MT-$XNgn1^+$ cells were located in the lateral neural tube and were largely $BrdU^-$ (Fig. 4F). Thus, using this assay, misexpression of either wild-type NKL or XNgn1 produces indistinguishable phenotypes, suggesting that NKL (either by itself or in combination with other proneural genes) is sufficient to promote neurogenesis when overexpressed in the neural precursors (see Discussion).

NKL-overexpressing cells are neurons

Our observation that the MT-NKL-expressing cells migrating out of the ventricular zone are $BrdU^-$ suggests that they become postmitotic neurons. To test this further, we assayed MT-NKL^+ cells for the expression of neuronal markers. Chick embryos electroporated with either the control vector or with MT-NKL were stained with antibodies to the Myc epitope and to neuron-specific tubulin (TUJ1), a marker of newly postmitotic neuroepithelial cells and differentiating neurons (Memberg and Hall, 1994) (Fig. 5A,B). In embryos electroporated with the control vector, CS2-MT, Myc^+ cells were present throughout the ventricular zone and few, if any, of these cells were double-labeled (Fig. 5A). In contrast, embryos injected with MT-NKL showed Myc^+ staining in the mantle zone (Fig. 5B), and almost all of these cells were $TUJ1^+$. Quantification of this data is presented in Table 1.

Further examination of electroporated embryos with additional neuronal markers confirmed that the NKL-

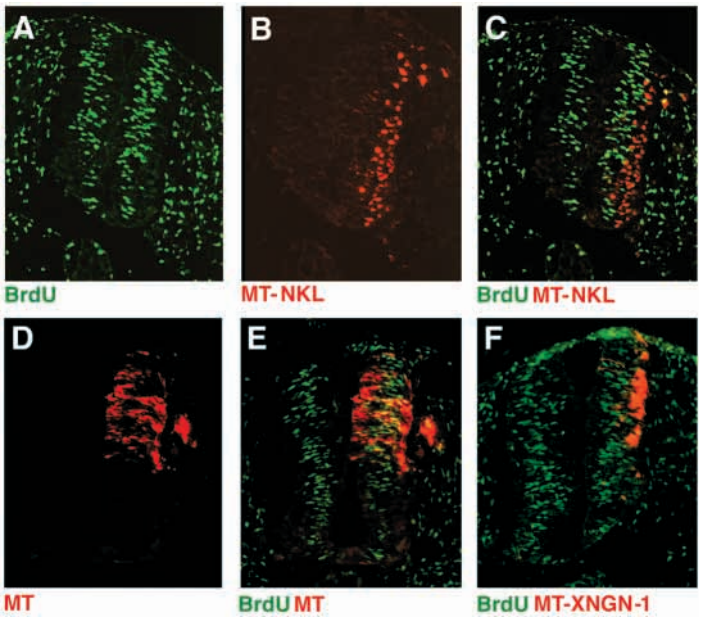

Fig. 4. Overexpression of NKL promotes premature differentiation of neuronal precursors in the chick neural tube. (A-F) Double-label immunofluorescence of Stage 18 (E3.5) chick embryos electroporated with MT-NKL (A-C), a construct expressing the nuclear localized Myc tag (MT) only (D,E), and a tagged form of XNGN-1 (MT-XNGN-1) (F). Electroporated embryos were stained with anti-Myc and BrdU: $BrdU^+$ cells are green, Myc^+ cells are red, and cells co-expressing both are yellow. All embryos were given a 1 hour pulse of BrdU in the neural tube before sacrificing. 10 μm cryostat sections through the forelimb level of the spinal cord were reacted with anti-Myc and anti-BrdU antibodies and the appropriate secondaries (see Materials and Methods). Note that MT-XNGN-1 and MT to a lesser extent are not restricted to the nucleus.

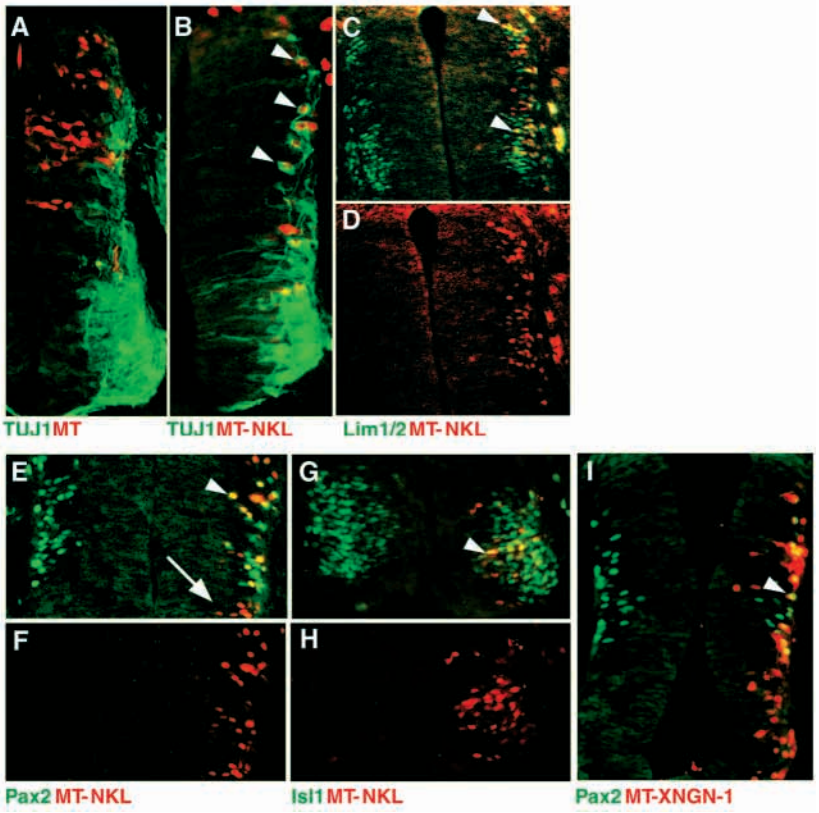

Fig. 5. NKL-overexpressing cells in the chick spinal cord are neurons. (A,B) Stage 18 embryos electroporated at stage 12 with the Myc tag alone (A) or MT-NKL (B) and stained with antibodies to both Myc (red) and TuJ1 (green). Only the electroporated sides are shown. (B) Note cluster of double-labeled MT-NKL/TuJ1-postive cells in the intermediate zone (arrowheads). (C,D) Embryos electroporated with MT-NKL are stained with anti-Myc (red nuclei) and Lim1/2 antibodies (green nuclei). Merged image is shown in (C) and double-labeled cells are indicated by arrowheads. Myc-only cells are shown in (D). Electroporated side is to the right, as in (E-I). MT-NKL constructs electroporated into the intermediate and ventral regions of the spinal cord are also Pax2 (E) and Isl1 (G) positive, respectively (arrowheads in E and G). Note that MT-NKL-positive cells ventral to the Pax2 domain in are Pax2 negative (arrow in E). In (E-H) Pax2- or Isl1-positive nuclei are seen as green; Myc-positive cells are red; and nuclei that co-express both are yellow. (I) Stage 18 embryos electroporated at stage 12 with MT-Ngn1 are reacted with antibodies to Pax2 (green) and Myc (red). As in (E), Myc-positive cells ventral to the Pax2 domain (compare to the untransfected side) are labeled with Myc only.

expressing cells were neurons and were properly patterned. Two interneuron markers, Lim1/2 (Ericson et al., 1996) and Pax2 (Burrill et al., 1997), as well as the motoneuron marker, Isl1 (Ericson et al., 1992) were used. In stage 19 embryos electroporated with MT-NKL, $Myc^+/Lim1/2^+$ cells were present throughout the Lim1/2 expression domain (Fig. 5C,D). Likewise MT-NKL, when electroporated into the medial or ventral regions of the spinal cord, co-labeled with $Pax2^+$ (Fig. 5E,F) or $Isl1^+$ (Fig. 5G,H) cells, respectively. Thus, in all three cases, when MT-NKL was electroporated into a given field of the neural tube, it was co-expressed with neuronal markers that are normally expressed within that domain. Furthermore, in no instance did NKL induce inappropriate expression of any marker examined. In positive control experiments, cells expressing XNgn1 also differentiated as neurons based on co-expression of Myc and Pax2 (Fig. 5I). From these data, we conclude that NKL promotes neurogenesis, and the resulting neurons then activate a differentiation program appropriate to their position in the spinal cord.

Table 1. Analysis of MT-XNKL-expressing cells for TuJ1

	Treatment	
Counted*	MT-XNKL	Control (MT)
Total Myc-positive cells	235	232
Myc/TuJ1-positive cells	211	20
%Myc/TuJ1-positive cells	89	9

*MT-XNKL and control (MT) counts represent sections taken from four and three embryos, respectively. Only the dorsal two-thirds of the spinal cord was analyzed, owing to the density of TuJ1 staining in the ventral horn.

NKL /VP16 phenocopies the neuron-inducing activity of wild-type NKL

To further test whether NKL regulates neurogenesis, constructs containing either the Engrailed repressor (EnR) or VP16 activation domain fused to the NKL zinc-finger domain were generated. In view of the ability of NKL to promote neurogenesis, we were particularly interested in generating an antimorphic version of NKL and asking whether it could block neurogenesis in the chick assay system. Our molecular characterization of NKL had revealed the presence of proline-rich domains in the N and C termini of the mouse and *Xenopus* proteins, which in other transcription factors often function as transcriptional activation domains. Consistent with this finding, in embryos electroporated with MT-NKL/VP16, Myc^+/ $BrdU^-$ cells were observed lateral to the ventricular zone (Fig. 6A), phenocopying the activity of wild-type MT-NKL. In contrast, when a form of NKL that contained the zinc-finger domain alone (MT-NKL/zf) was electroporated (Fig. 6B) and analyzed for its ability to promote differentiation, 44% of the Myc^+ cells co-labeled with BrdU after a 1 hour pulse (see Fig. 6F, zf).

To further map the domains that are required for the neuron inducing activity of NKL, we deleted either the N or C terminus of NKL, leaving the zinc finger intact. Deletion of the N terminus produced a loss of function equivalent to that seen with the zinc finger alone or the Myc control (Fig. 6C), while a mutant lacking the C terminus promoted differentiation as potently as did MT-NKL (Fig. 6D). Together these findings indicate that the NKL protein possesses transactivator activity and that this activity maps to the N-terminal 18kD of the protein.

To examine whether a Myc-tagged form of NKL/EnR (MT-

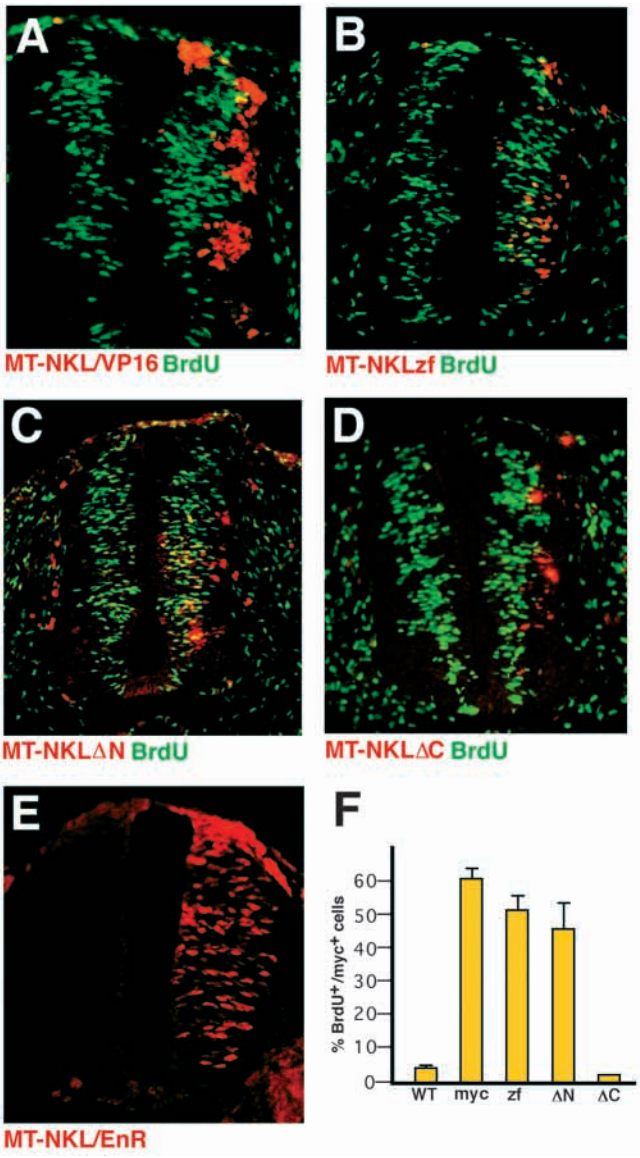

Fig. 6. Overexpression of NKL/VP16 phenocopies overexpression of wild-type MT-NKL in the chick neural tube. (A) Chick embryos were electroporated with MT-NKL/VP16, given a 1 hour BrdU pulse, and analyzed for Myc (red) and BrdU (green) expression as described in Fig. 4. Note that the majority of Myc-positive cells are $BrdU^-$. (B) Embryos electroporated with the Myc-tagged NKL zinc-finger domain (MT-NKLzf) and analyzed by the protocol described for (A). (C) Embryos electroporated with a tagged deletion mutant of NKL containing an intact zinc-finger domain but lacking the N-terminal 144 amino acids of the protein (MT-NKLΔN). (D) Embryos electroporated with a deletion mutant lacking the C terminal 128 amino acids but retaining the intact zinc-finger domain (MT-NKLΔC). (E) Chick embryos electroporated with MT-NKL/EnR at Stage 10, sacrificed at Stage 14, and reacted with antibodies to Myc only. Note what appear to be clones of Myc-positive cells, indicative of proliferating cells. (F) Quantification of cell counts from data presented here and in Fig. 4. The *y*-axis shows the total number of $Myc^+/BrdU^+$ (yellow) cells divided by the total number of Myc-positive (red) cells. Cells lining the lumen of the neural tube and migrating crest cells were excluded from the analysis. Injected constructs include: WT (full-length NKL), myc (Myc only), zf (tagged NKL zinc-finger only), ΔN (tagged NKL N-terminal deletion) and ΔC (tagged NKL C-terminal deletion). Three or more embryos were analyzed for each construct. Numbers of cells counted were 593 (WT), 539 (myc), 390 (zf), 245 (ΔN), and 196 (ΔC).

NKL/EnR) can function as an antimorph, stage 12 embryos were electroporated and analyzed at various times. Embryos examined at stage 20 showed very faint Myc staining (data not shown); however, embryos electroporated at stage 15 showed staining in large numbers of cells in the ventricular zone (Fig. 6E), suggesting that cells expressing NKL/EnR continue to divide. The reason for this loss of Myc expression in older embryos is unclear. It is possible that the MT-NKL/EnR protein is unstable or that expressing cells die prematurely. Nevertheless, our observation that NKL/EnR cells remain in the ventricular zone could reflect their inability to differentiate.

NKL/EnR and NKL/VP16 have opposing effects on primary neurogenesis in *Xenopus* embryos

To further assess whether NKL/EnR blocks neurogenesis, we examined the effect of overexpressing NKL/EnR on primary neurogenesis in *Xenopus* embryos. In embryos injected with *XNKL/EnR*, there was a clear reduction in *N-tubulin* expression in 61% (*n*=35/57) of the embryos tested (Fig. 7A). This reduction in *N-tubulin* was also mirrored by the later loss of *NF-M* expression in tailbud stage embryos (*n*=10/20) (Fig. 7C). Likewise, misexpression of *XNKL/EnR* resulted in loss of *Xath3* in 52% (*n*=23/40) of the embryos injected (Fig. 7B). Thus, it appears that NKL/EnR functions as an antimorph in frog embryos and prevents neural precursors from differentiating as neurons.

In marked contrast to the inhibitory activity of NKL/EnR, injection of low levels of *XNKL/VP16* RNA (40 pg) resulted in a significant increase in the density of *N-tubulin*-positive cells within the stripes of primary neurons (Fig. 7D) as well as a broadening of the *N-tubulin*-positive field (Fig. 7E). Such increases were observed in 63% (*n*=64/102) of the embryos examined. Similar increases in expression of the pan-neuronal marker *elrC* (Bang, 1999) were also seen in 72% (*n*=31/43) of the embryos injected with *XNKL/VP16* RNA (Fig. 7G). Injection of one-fifth the amount of *XNKL/VP16* RNA (8 pg) also produced increases in *elrC* expression in 55% of the injected embryos (17/31, data not shown). At tailbud stages, 60% (*n*=15/25) of the injected embryos showed increases in *NF-M* expression either within the neural tube or in patches of cells lying immediately adjacent to the neural tube (Fig. 7F). *XNKL/VP16* misexpression also upregulated expression of two neurogenin targets, *MyT1* (Fig. 7H) and *Xath3* (Fig. 7I) in 29/52 and 21/44 embryos, respectively. The expression pattern of NKL in *Xenopus* embryos and its induction by XNgn1 indicates NKL functions downstream of the early determination genes. Interestingly, we did not observe changes in XNgn1 expression in *Xenopus* embryos injected with either NKL/VP16 or NKL/EnR (data not shown), an observation consistent with NKL functioning downstream of neuronal determination genes. Taken together, these data show that overexpression of *XNKL/EnR* and *XNKL/VP16* in frog embryos has consistent and opposing effects on neuronal differentiation: *XNKL/VP16* promotes increases in *N-tubulin*, *elrC*, *Xath3*, *MyT1*, and *NF-M* expression, while *XNKL/EnR* decreases expression of *N-tubulin*, *Xath3*, and *NF-M*. Like

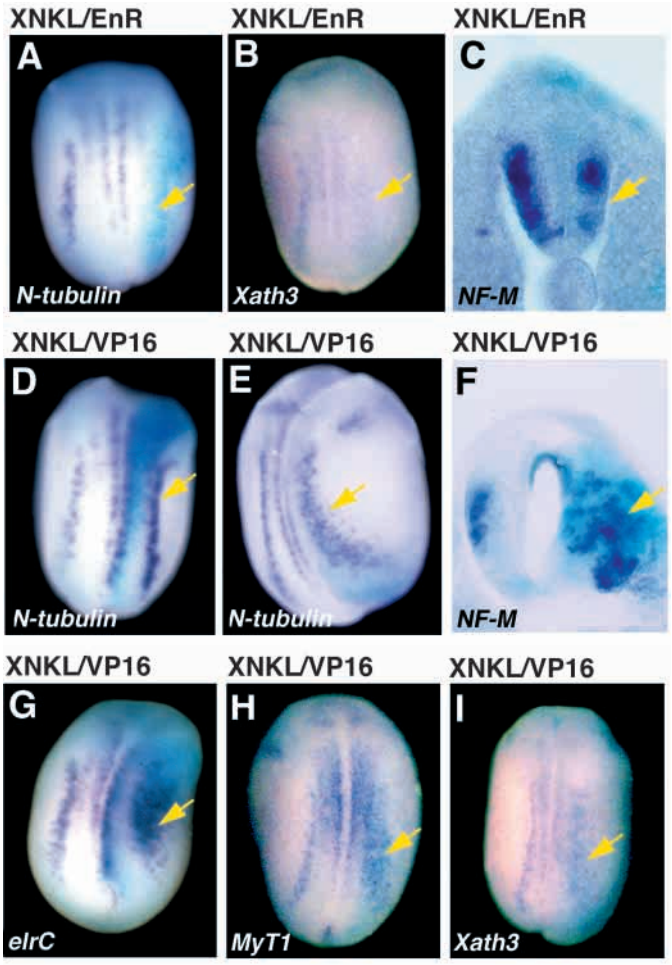

Fig. 7. Overexpression of NKL/EnR and NKL/VP16 fusion proteins promotes changes in neuronal markers in *Xenopus* embryos. Xenopus embryos were injected with mRNA encoding a β-galactosidase tracer and mRNAs encoding NKL/EnR (A-C), or NKL/VP16 (D-I) fusion proteins. Embryos were injected with 40 pg of test RNA. RNA was injected into one blastomere at the two-cell stage and probed with antisense RNAs for the indicated neuronal markers. (A,B,D,E,G-I) Expression of N-tubulin, Xath3, elrC and MyT1 expression in representative embryos at neural plate stages. (C,F) Show neurofilament expression in cross sections through stage 26 embryos. In all embryos the injected side is to the right. Changes in gene expression are indicated by arrows.

overexpression of wild-type and activated forms of NKL in chick, *XNKL/VP16* induces a neurogenic phenotype in frogs, while the antimorphic form of the protein, *XNKL/EnR*, inhibits expression of neuronal markers.

DISCUSSION

In this study we show that NKL, a zinc-finger protein expressed in newly postmitotic neurons, can function as a regulator of neurogenesis in vertebrates. In mouse and chick, *NKL* expression in the intermediate zone follows that of *Ngn1* and *Ngn2*, which are expressed in the ventricular zone in dividing precursors (Sommer et al., 1996). Likewise, in *Xenopus*, *NKL* transcripts first appear at stage 12, after *XNgn1*, which is expressed at stage 10.5, but prior to *Xath3* and *NeuroD*, which are expressed from stage 14 onwards (Lee et al., 1995). Thus, *NKL* is expressed downstream of the proneural determination genes, where its expression overlaps with *Xath3* and *NeuroD*. NKL is also co-expressed with *MyT1*, which is expressed in differentiating neural precursors from stage 11.5 onwards. In tailbud stage *Xenopus* embryos, NKL expression overlaps with markers of neuronal differentiation such as N-tubulin and neurofilament (Fig. 2). This finding together with the observation that NKL/VP16 induces *Xath3* and *MyT1* but not *XNgn1* positions NKL downstream of the neural determination genes and upstream of the early differentiation genes.

Our finding that overexpression of *XNgn1* in *Xenopus* embryos upregulates NKL in an apparently cell-autonomous manner is suggestive of NKL being a downstream target of Ngn activity. Interestingly, the induction of *NKL* by *XNgn1* is similar to that seen with other neuronal differentiation proteins, such as NeuroD, MyT1, Xath3 and the Notch ligand Delta (Bellefroid et al., 1996; Ma et al., 1996; Perron et al., 1999). We propose that NKL is part of the downstream transcription factor cascade activated in determined precursors, leading us to suggest that NKL may mediate some of the proneural effects of neurogenin.

Neuronal differentiation phenotypes in chick

In keeping with its expression in differentiating neuroblasts, when NKL is misexpressed in the ventricular zone of the chick neural tube, where it is normally not expressed, it causes progenitor cells to withdraw from the cell cycle and differentiate as neurons. This finding raises the possibility that one function of NKL is to facilitate the transition from determined neural precursor to postmitotic neuron. Support for this hypothesis comes from the cell cycle status of transfected cells in embryos electroporated with control and NKL expression vectors. In electroporated embryos pulsed with BrdU, more than 55% of the cells expressing the control CS2-MT expression vector were adjudged as still dividing, whereas 95% of the cells expressing MT-NKL had withdrawn from the cell cycle and migrated radially into the mantle zone. Furthermore, this analysis most likely underestimates the number of $Myc^+/BrdU^+$ cells that remain in the cell cycle in control CS2-MT electroporated embryos, as the length of the BrdU pulse (1 hour) used was only 25% the length of S phase. The premature withdrawal of NKL^+ cells from the cell cycle, coupled with their expression of neuron-specific tubulin and other neuronal markers, shows that NKL is capable of inducing dividing neural progenitors to differentiate as neurons.

In contrast to the experiments where *XNKL/VP16* was misexpressed in frog embryos, no significant increase was observed in the number of cells expressing neuronal markers after electroporation of MT-NKL in chicks. While the exact reason for this is unclear, several factors may be responsible. First, the expression of electroporated DNAs in the chick neural tube is mosaic, making quantitative changes in pan-neuronal markers difficult to detect. Second, any increase in the number of postmitotic neurons may be counteracted by depletion of the pool of dividing precursors. Third, NKL^+ cells that emerge from the ventricular zone may prevent other progenitors from differentiating by increasing lateral inhibition. Phox2b for example, has recently been shown to promote premature differentiation of motoneurons (Dubreuil et

al., 2000), and in this study, increased expression of Delta1 was seen 24 hours after electroporation of Phox2b. However, this increase was transient and the overall numbers of electroporated TuJ1$^+$ cells observed 2 days after electroporation were apparently equivalent in electroporated neural tubes and controls. Finally, when we electroporated Ngn, a potent activator of neurogenesis, into the chick neural tube under conditions similar to NKL, no significant increase in neurons on the electroporated side of the neural tube was observed. It is therefore likely that the overall number of postmitotic neurons generated in the chick neural tube under these conditions reflects the combined outcome of multiple competing processes, including differentiation, lateral inhibition and depletion of a precursor pool, which in some circumstances may even lead to fewer neurons being generated. In fact, we have observed that neural tubes with extensive NKL hits often showed a small decrease in the number of cells that express neuronal markers such as Pax2 on the electroporated side, when compared with the unelectroporated side (data not shown). A similar depletion of neurons was also noted following electroporation of MT-XNgn1 (E. L., C. K. and M. G., unpublished).

How does NKL promote neuronal differentiation?

Our observation that cells expressing NKL exit the cell cycle raises the possibility that NKL promotes generalized cell cycle arrest, which appears to be obligatory for neurogenesis (Farah et al., 2000) and myogenesis (Ludolph and Konieczny, 1995). However, we favor the idea that that cell cycle arrest occurs as a consequence of the differentiation activity of NKL for the following reasons. Studies in *Xenopus* show that injection of cell cycle inhibitors into frog embryos at levels sufficient to block cell division has little effect on the expression level of neuronal markers in the neural plate (Hardcastle and Papalopulu, 2000). Furthermore, misexpression of the cell cycle inhibitor p28 in the chick neural tube at stages comparable with those analyzed here, while able to induce cell cycle arrest, does not induce neuronal differentiation (N. Koyano-Nakagawa and C. K., unpublished). In contrast, a substantial portion of NKL-expressing cells in electroporated embryos differentiate as neurons, based on the expression of the pan neural marker TuJ1 and other neuronal markers (Fig. 5). Taken together, these observations suggest that cell cycle arrest alone is not sufficient to promote the differentiation of dividing progenitor cells into mature neurons.

Alternatively NKL may function to relieve neural progenitors from lateral inhibition. In *Xenopus* and zebrafish, Ngn1 activates lateral inhibition to restrict the number of cells that activate downstream bHLHs, resulting in a 'salt-and-pepper' pattern of neuronal differentiation. When lateral inhibition is blocked, more precursors differentiate and more *N-tubulin*-positive cells are observed in stripes of primary neurons. *XNKL/VP16* misexpression in *Xenopus* increases the density of differentiated primary neurons in neurogenic domains. However, *XNKL/VP16* also induces premature neuronal differentiation between the neurogenic stripes, where neurons do not form even when lateral inhibition is disabled (Chitnis et al., 1995), suggesting that XNKL/VP16 does more than interfere with lateral inhibition.

Our studies show that electroporation of tagged forms of NKL and XNgn1 into the neuroepithelium of the chick neural tube generates similar phenotypes. Both MT-NKL$^+$ and MT-XNgn1$^+$ cells delaminate from the ventricular zone, move laterally into the intermediate and mantle zones, and become postmitotic neurons (Fig. 4). While NKL is clearly able to induce neurogenesis in both chick and frog embryos, its activity appears to be more limited than that of XNgn1. Neurogenin can induce ectopic neurogenesis in non-neuralized tissues and mesoderm-derived tissues (Lee et al., 1995; Ma et al., 1996; Perez et al., 1999), whereas the neurogenic activity of NKL is restricted to the neural plate/tube. This difference in NKL activity is also consistent with the inability of NKL/VP16 to induce *XNgn1* expression in frog embryos, and together with the late onset of NKL expression, makes it unlikely that NKL plays a determination role in neurogenesis.

The actions of NKL in frog and chick embryos are more consistent with NKL playing a cooperative role in neurogenesis, either by increasing the potency of the proneural bHLH proteins or by activating downstream differentiation genes such as *Xath3* and *MyT1*. At the time NKL is electroporated into the chick neural tube (stages 11-12), cells in the ventricular zone already express *Ngn1* and *Ngn2* at varying levels (Perez et al., 1999). Thus, in the chick, NKL may potentiate subthreshold levels of Ngn, thereby promoting neuronal differentiation. In frog embryos, overexpression of an antimorphic form of NKL, NKL/EnR, results in losses of the neurogenin target *Xath3* and markers of neuronal differentiation, such as N-tubulin and neurofilament, while overexpression of NKL/VP16 upregulates expression of neuronal markers and neurogenin targets (Fig. 7). These findings suggest that NKL may synergize with XNgn1 to induce its targets. Although NKL is genetically downstream of Ngn, our findings suggest that upregulation *Xath3* and or *MyT1* depends on factors other than NKL that are present in the neural plate/tube. Our results do not define the molecular mechanism that underlies the neurogenic activity of NKL. Nevertheless, other studies that show the zinc finger protein Sens cooperates with Atonal or Scute to promote bristle formation in flies (Nolo et al., 2000), and that MyT1 synergizes with the proneural protein Xash3 to induce expression of *N-tubulin* (Bellefroid et al., 1996) indicate transcriptional cooperativity may be required for neuronal differentiation, as is the case for myogenic differentiation (Cheng et al., 1993). Based on those precedents, it is therefore possible that NKL functions as co-factor for the proneural bHLH proteins.

The similar DNA-binding specificity of NKL and other members of the Gli/Zic family of transcription factors raises the interesting prospect that NKL promotes neurogenesis by competing with homologous but antagonistic DNA binding proteins, such as Zic2. In mouse, the *Zic* genes (*Zic1*, *Zic2* and *Zic3*) are expressed in undifferentiated cells in the ventricular zone, while in frog, *Zic2* transcripts are restricted to non-neurogenic stripes of the neural plate (Brewster et al., 1998). *NKL* is expressed in the inverse pattern in both mouse and frog, i.e. immediately outside the ventricular zone in mouse and in neurogenic stripes in the frog. Although it has been reported that the Zic proteins induce expression of early neuronal markers (Nakata et al., 1997; Mizuseki et al., 1998), it has also been shown that expression of wild-type *Zic2* inhibits both *XNgn-1* activity and its expression in frogs, thereby inhibiting neurogenesis (Brewster et al., 1998). In inducing neuronal differentiation, NKL activity is opposite to the proposed anti-

neurogenic role of Zic2, raising the possibility that both proteins bind to a common site in the enhancers of neuronal differentiation or determination genes, where they might function antagonistically to control the extent or timing of neuronal differentiation. However, the phenotype that we observe with NKL/VP16 and that described for Zic2/VP16 differs in two important aspects: NKL/VP16 does not induce expression of *XNGN-1*, nor does it induce neurons outside of the neural plate. Thus, it is unlikely that NKL merely inteferes with Zic2 activity in the embryo. Further insights into the mechanism by which NKL induces cell cycle exit and neurogenesis will require a more detailed characterization of the enhancers that regulate neuronal determination and differentiation in vertebrates.

We thank members of the Goulding and Kintner labs for valuable input and acknowledge major contributions made to this project by Dr John Burrill. We thank Kim Thamatrakoln for technical assistance and Ed Han for sharing preliminary observations of neurogenin activity in chicks. We thank Drs Anne Bang, Gisele Deblandre, Sylvia Evans, Naoko Koyano-Nakagawa, Sam Pfaff, Laura Rivard, and Kamal Sharma for critical reading of the manuscript. E. L. thanks Dr Chuck Stevens for suggestions and encouragement. This work was supported by grants to M. G. from NIH, the Pew Charitable Trust and the Fritz Burns Foundation, and to C. K. from the NIH. E. L. was supported in part by pre-doctoral fellowships from the American Heart Association and the Chapman Foundation.

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