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Molecular characterization of *NSCL*, a gene encoding a helix–loop–helix protein expressed in the developing nervous system

(hemopoiesis/neurogenesis/transcription factor/genetic linkage)

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ABSTRACT **We report here the molecular cloning and chromosomal localization of an additional member of the helix–loop–helix (HLH) family of transcription factors, NSCL. The *NSCL* gene was identified based on its hybridization to the previously described hemopoietic HLH gene, *SCL*. Murine NSCL cDNA clones were obtained from a day 11.5 mouse embryo cDNA library. The coding region is 399 base pairs and encodes a predicted protein of 14.8 kDa. The nucleotide sequence shows 71% identity and the amino acid sequence shows 61% identity to murine SCL in the HLH domain. The NSCL protein-coding region terminates six amino acids beyond the second amphipathic helix of the HLH domain. Expression of NSCL was detected in RNA from mouse embryos between 9.5 and 14.5 days postcoitus, with maximum levels of expression at 10.5–12 days. Examination of 12- and 13-day mouse embryos by *in situ* hybridization revealed expression of NSCL in the developing nervous system. The *NSCL* gene was mapped to murine chromosome 1. The very restricted pattern of *NSCL* expression suggests an important role for this HLH protein in neurological development.**

The helix–loop–helix (HLH) proteins are a family of putative transcription factors, some of which have been shown to play an important role in growth and development of a wide variety of tissues and species. The family includes proteins critical to pigment determination in maize, neurological development in *Drosophila*, the centromere binding protein of yeast, enhancer binding proteins of lymphocytes, and genes critical to muscle differentiation (1–3). These proteins are able to form homodimers or heterodimers by virtue of their HLH motif (1, 4). Many members have a domain of basic amino acids, immediately upstream of the HLH domain, that determines DNA binding specificity (5).

Four members of this family have been clearly implicated in tumorigenesis via their involvement in chromosomal translocations in lymphoid tumors: *MYC*, *LYL1*, *E2A*, and *SCL* (8). We identified *SCL* because of its involvement in a 1;14 translocation in a stem cell leukemia (9, 10). Subsequently the involvement of *SCL* (also known as *TAL*) in translocation events has been confirmed by others (11, 12), and the frequency of *SCL* disruption in T-cell acute lymphoblastic leukemia is estimated at 25% (13).

In this paper we report the molecular cloning of an additional HLH gene, *NSCL*‖, which was identified because of its homology to *SCL*. This molecule is closely related to SCL in its HLH domain and is expressed in the developing nervous system (thus *N*eurological *SCL*).

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MATERIALS AND METHODS

Southern and Northern Blots. DNA and RNA blot hybridization analyses were performed by standard techniques. Genomic DNA (10 μg) was digested with restriction endonucleases, electrophoresed in 0.8% agarose gels, and transferred to nitrocellulose. Filters were prehybridized and hybridized in a hybridization mix containing either 6× or 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) (14). Filters were washed in 6×, 2×, or 0.2× SSC containing 0.1% $NaDodSO_4$ as indicated. Northern blots were performed with 2 μg of poly(A)$^+$ mRNA or 10 μg of total mRNA size fractionated on 1% formaldehyde agarose gels as described (15).

Library Screening. A human genomic SCL probe (1.0XX) (16), encompassing the HLH region, was initially used to screen a murine genomic library at low stringency (washing at 45°C in 0.1× SSC/0.1% $NaDodSO_4$). An additional murine genomic library (Clontech no. ML 1030j) was subsequently screened using murine NSCL probes (washing at 65°C in 0.2× SSC/0.1% $NaDodSO_4$). A murine cDNA library constructed from 11.5-day mouse embryo RNA (Clontech no. ML 1027a) was screened with murine genomic NSCL probes. Plasmid subclones were generated by ligating fragments into pBluescript vector (Stratagene) and were sequenced in both directions by using the dideoxy chain-termination method with Sequenase polymerase (United States Biochemical) and oligonucleotide primers.

***In Situ* Hybridization.** Paraffin sections of 12- and 13-day C57BL/6J mouse embryos were examined as described (17). Probes were [^{35}S]UTP-labeled antisense RNA transcripts synthesized from two different NSCL cDNA clones in pBluescript by using either T7 or T3 RNA polymerase (BRL) according to the manufacturer's instructions. One cDNA clone [1.5 kilobases (kb)] extended to the *Eco*RI site in the 3′ untranslated region (UTR) and included the entire *NSCL* coding region and 5′ UTR. A second cDNA clone encompassed 600 base pairs (bp) of 3′ UTR sequence beginning at the *Eco*RI site. Both probes gave identical results. Hybridization of irrelevant sense probes showed no specific signal. Hybridized slides were exposed to Kodak NTB-2 emulsion for 10–30 days.

RESULTS

Detection of the *SCL*-Related Locus *NSCL*. While screening for murine *SCL* with a human SCL HLH probe under conditions of reduced hybridization stringency, one particular phage clone was identified that appeared to originate

Abbreviations: HLH, helix–loop–helix; UTR, untranslated region; CNS, central nervous system.

‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M82874).

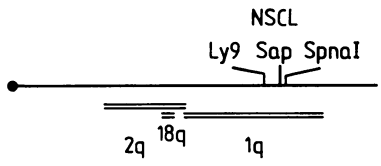

FIG. 1. Genetic linkage map of mouse chromosome 1. The location of genes is taken from refs. 19 and 20. Regions of synteny with human chromosomes are indicated below (double lines). The most likely order is *Ly-9–NSCL/Sap–Spna-1* locus on distal chromosome 1.

from a different gene. The region from this clone that hybridized to the *SCL* HLH domain also demonstrated a unique pattern when used to analyze Southern blots of murine and human genomic DNA (data not shown). Sequence analysis showed a related but distinct HLH region when compared to *SCL* (V.B. and I.R.K., unpublished observations). Moreover, chromosomal localization confirmed that this gene (*NSCL*) was localized to chromosome 1, in contrast to *SCL*, which is localized to chromosome 4 (18). This localization of *NSCL* was determined by genetic linkage using recombinant inbred mouse strains (data not shown). Comparison of the strain distribution pattern for alleles of the *NSCL* gene in the AKXL, BXD, SWXL, and BXH panels with that of other murine genes mapped using these recombinant inbred strains suggested that *NSCL* is located on the distal portion of chromosome 1 (Fig. 1), closely linked to the *Sap* locus (0 of 51 recombinants) (19, 20).

Cloning of a Murine NSCL cDNA. Expression of *NSCL* was detected in embryonic tissue (see below), and cDNA clones were therefore obtained from a day 11.5 embryo cDNA library screened by using a genomic NSCL HLH probe. A total of 29 clones containing inserts of between 0.6 and 1.5 kb were obtained from screening 1.5×10^6 recombinant plaques from the amplified library. Five overlapping classes of NSCL cDNA clones were identified and sequenced in both directions. No clones crossed the internal *Eco*RI restriction endonuclease site in the 3′ UTR, and analysis of genomic clones was used to confirm the sequence in this region.

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NSCL   TAKYRTAHATRERIRVEAENLAFAELRKLLPTLPPDKKLSKIEILRLAICYISYLNHVLDV

SCL    KVVRRIFTNSRERWRQQNVNGAFAELRKLIPTHPPDKKLSKNEILRLAMKYINFLAKLLND
LYL-1  KVARRVFTNSRERWRQQHVNGAFAELRKLLPTHPPDRKLSKNEVLRLAMKYIGFLVRLLRD
TWIST  LQTQRVMANVRERQRTQSLNEAFAALRKIIPTLPSDK.LSKIQTLKLAARYIDFLYQVLQS
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FIG. 3. Comparison of the amino acid sequence of murine NSCL, SCL, LYL1 (LYL-1) and twist proteins in the region of the HLH domain and upstream DNA-binding (basic) domain. Underlined residues indicate sites of consensus amino acids defining the HLH and basic domains.

A composite nucleotide sequence for murine NSCL cDNA (2458 bp) and the predicted amino acid sequence are shown in Fig. 2. A long open reading frame begins at the CTG codon at 318 bp. This CTG codon is in a poor environment with respect to the consensus sequence required for translational initiation (21) and is unlikely to represent the translational start site. Within the long open reading frame, there are three methionine residues: at nucleotides 450, 453, and 471. The first and especially the third of these residues are good candidates for the translational initiation site (21). The presence of the stop codon (position 849) was also confirmed by sequence analysis of murine and human genomic NSCL clones. Although there is a potential open reading frame of 333 bp immediately beyond position 849, utilization of this region would require a frame shift. This is unlikely based on confirmatory sequence analysis of murine and human genomic NSCL clones (data not shown) and given the predicted amino acid sequence that defines the NSCL reading frame (see below).

The 3′ UTR is 1.6 kb with multiple stop codons. The polyadenylylation signal sequence ATTAAA is a well-recognized natural variant of the canonical motif (22). Twenty-two base pairs beyond this sequence the cDNA diverged from the genomic sequence and differed by the addition of a poly(A) tail, which delineated the 3′ extent of the gene. The 3′ UTR is relatively A+T-rich. This is particularly evident in the region commencing at 1813 bp, where the motif ATTTA occurs repeatedly. This sequence is associated with instability of mRNA and occurs in the 3′ UTR of a number of growth factors and oncogenes (23).

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GTGACTGTGTGGGTGTGTGTATGTATGTATGTGTGAGTGTGGGTGACTCCAGTTCTGGACTAAGTAAGAGCATCCCTTTTGCCTAGGCCTTACAAATCCTCATCATGT      108
GTACCCTTCTCATCTGTCCTGGATCCTGTTCAGCCACAAGCTGCCCCTAAGAGCTCTAGAAACTGCTCACTAACTTTGCAGACAGATGACCCTTGAGCAGCGAGAAGA      216
GACTGGGGCTCTCTACGCTGTCCCTCTCACTTGCCAGCTTGGATCCCTTGGCAGAGTCCTAGGCTCCAGGCTGGAATCTTGACCATGGAACTTTGAAGTAACTGCTTC      324
CCAGAGCTCAGTGGCAGACCCCGGCACTCCAGGCCCTTGCTGCTTTCCTCCTAACCCTCTTTGAAGCTCCTAAAGGGAGGGGTGGGGAGAGGATCCGCATTTCCCTAG      432
                  Met Met Leu Asn Ser Asp Thr Met Glu Leu Asp Leu Pro Pro Thr His Ser Glu Thr Glu Ser Gly
GTGGGGGGTTTTCCACC ATG ATG CTC AAC TCC GAT ACC ATG GAG CTG GAC CTG CCT CCC ACC CAC TCG GAG ACC GAG TCG GGC      515
 Phe Ser Asp Cys Gly Gly Gly Pro Gly Pro Asp Gly Ala Gly Ser Gly Asp Pro Gly Val Val Gln Val Arg Ser Ser Glu
 TTT AGC GAC TGT GGG GGC GGA CCG GGC CCC GAT GGT GCT GGA TCC GGG GAT CCA GGA GTG GTC CAG GTC CGG AGC TCA GAG      596
 Leu Gly Glu Ser Gly Arg Lys Asp Leu Gln His Leu Ser Arg Glu Glu Arg Arg Arg Arg Arg Ala Thr Ala Lys Tyr
 CTT GGA GAG TCC GGC CGC AAA GAC CTG CAG CAC TTG AGT CGT GAG GAG CGC AGG CGC CGG CGC GCC ACG GCC AAG TAC      677
 Arg Thr Ala His Ala Thr Arg Glu Arg Ile Arg Val Glu Ala Phe Asn Leu Ala Phe Ala Glu Leu Arg Lys Leu Leu Pro
 CGC ACG GCA CAC GCC ACG CGG GAG CGC ATC CGC GTG GAA GCC TTC AAC CTA GCC TTC GCC GAG CTG CGC AAG CTG CTG CCC      758
 Thr Leu Pro Pro Asp Lys Lys Leu Ser Lys Ile Glu Ile Leu Arg Leu Ala Ile Cys Tyr Ile Ser Tyr Leu Asn His Val
 ACT CTG CCC CCG GAC AAG AAG CTC TCT AAG ATT GAG ATC CTA CGC CTG GCC ATC TGC TAT ATC TCC TAC CTG AAC CAT GTG      839
 Leu Asp Val ***
 CTG GAC GTC TGA ACTCAGCCCGCATCCCACCCTGGATTTTCCCCATTTCCCTGGGCCTCTCCAGAGCCCCCTGTCACCATACATTACTTAGAATGGCCGGCCC      942
CCTCCCATCCCAGAGGACCAGGCTCACATCGCTAGTCCTGAAGGCGGTTTCTTTTCATTGGCCCAGGAATGTGAAGGATGTTCTTATGGGTTCCTTCAGAGAGTTGTT     1050
CCGGCCTAGCTTGGTCAGAGTTGCTTGCCAGAGTGGTCAGAATGGAGTCCATGCTGGGTTTACTAAGCCTAGCCTCTCTGCACAGCTGCTGCCTCATCTATCCTATGC     1158
CCAAGTTTTCAGAGACCCCAGACCCTAACTCTCTTTCTGGCAATGGTGGGACTCAAAATCGGAATGGGAACTGACTAGCGGCAGAGGTTGAAGGAGCGGGTCCCCAAC     1266
TCCCAGACTTTTCGGTCCATTGGGGGGGGGCAGTGGCGGGGGTGGGGGGGAGGAATGGGCTCTTGGGATGGCAGAGAGATGACGGAGGTGAATCCTGGGTAGCCAGGG     1374
ATTATAGGAAAATTCTGCCTTTCTTTTGTCTCTCTCAGTCCTGAGGCTTAGGATATACAGACCTCAGATCTAACTTGGTAGTGAGTGCCTTGCCTTCTTGGAGCTGTC     1482
CGGCCGCTCCCGGTCCCCTCCCACCCCTGCCACCCAGAATTCTCCCTTTCCCTGTGCCGGCTAGAAAAAGGAAAACCTTAGTCCTGGGATAAGGATGACACCCCCAAA     1590
CAGCCCAGGGCACCTTGCAGAAGGCTCAGGCCCTGGGTGGGGCCGCTCCACAGCAGCTCCTTCCATCCCAGGGGACCCTTGAAAACATGCAAACCCCATCAGCTCCTC     1698
CCTCAGATGCAAGGGCATGCCTGCTACTGCCCTCTACGAATGCTCTAGGGCCTTCTTATTACATTTTCCCCCTTCCTTTCTTAAACCGCCGTGGGAAGGAAGGTGACC     1806
CGCCCATGCTGCTGAGCCCCACCCACCTGCCCATTGTAGCCTTGCGACCCAGAGTCTATGGGCCTTAAATTCCCTGTAGAAGAACTCACTGCTTTTCTCTGCTCCATC     1914
CATCCTCACACCAAACTCCTAGCTCTACAAGGATATTTATTTATGTATTTATTTATTCACTTATTTATTTATGTATTTATTTATTTATAAATATTACTATTTATTACC     2022
GAGTTATGCACTTTGGGGTAGGGTGAGGGGGGCTCCTTGCAGCTTGCTTTAGCTGAGGTCCTCTTGCTCTCTCCCGGGTCACTTCTCTTCTTCTTTCTCTAGTGCAAG     2130
TATGTGTGGGGATCCCTTCACCCACCATACTGGTACCCCTTCTTCAGCTCCATCTGTCCCTATCCCCTTTCCTCTGGAATAGTGTCCCTTCTCAGCCTCCCAGCTTCT     2238
AGGGGGTCCTTTCTCAATCTCTCCCTGCTGCCCCCGCCCCGACAATCCCCCTCCAGTCCTGTGTACTCCATTCCTCTTACCAGCCCTGCTTTTCCCCAAACCACCAAG     2346
AGCAGACCCTGGGATCTGTGTCTGGTGTCCTGTGTGTCTTTGTCTGGTTGCATTCCTAATTTCCTACAAAAAGAAAAATTAAAGTGACCTCGATCTAGTACCAGAAAA     2454
AAAA                                                                                                              2458
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FIG. 2. Nucleotide and amino acid sequence for a murine NSCL cDNA. Overlapping clones from a mouse embryo cDNA library were sequenced with oligonucleotide primers, and the nucleotide sequence is shown. A long open reading frame begins at position 318. The first in-frame methionine is at position 450. The open reading frame terminates at position 849. The polyadenylylation signal is underlined.

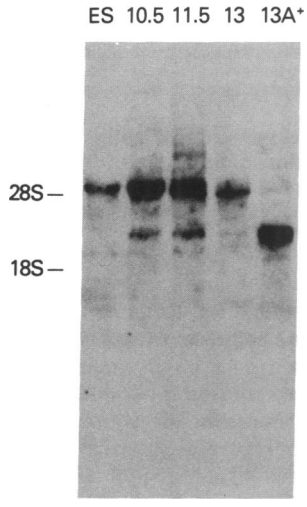

FIG. 4. The *NSCL* gene is expressed in mouse embryonic tissue. Total mRNA from embryonic stem cells (ES) and murine embryonic tissue at 10.5, 11.5, and 13 days postcoitus was probed with a 1.7-kb NSCL genomic *Bam* fragment containing the *NSCL* coding region and 1.3 kb of 3′ UTR. The probe crossreacts with the 28S ribosomal band and detects a message of ≈3 kb. Poly(A)$^+$ mRNA from day 13 mouse embryo (13A$^+$) is also shown.

The *NSCL* nucleotide sequence shows homology to the nucleotide sequence of several HLH genes. In the HLH domain, there is a region of 71% identity to *SCL* (between 688 and 838 bp) (18), 62% identity to *LYL1* (697–918 bp) (24), and 62% identity to the murine *twist* gene (608–844 bp), a regulator of mesoderm and neural crest development (25).

NSCL Encodes an Additional HLH Protein. The predicted NSCL protein consists of 133 amino acids with a molecular weight of 14.8 kDa. The protein has an HLH motif that is most closely related to SCL (61% identity in a 56-amino acid overlap). There is also homology with LYL1 (60% identity) and twist (57% identity) and lesser homology with other HLH proteins in this region (Fig. 3).

Immediately upstream of the HLH motif there is a highly basic domain (likely to confer DNA binding) that shows 5 of 13 amino acids identical to SCL and LYL1. The basic region is particularly prominent in the NSCL protein and extends for 25 amino acids 5′ of the HLH domain. In this region there are six consecutive arginine residues. The NSCL protein is also unusual for HLH proteins in that it terminates 6 amino acids beyond the second amphipathic helix.

***NSCL* Displays a Restricted Pattern of Expression.** A wide variety of adult murine hemopoietic cell lines and tissues

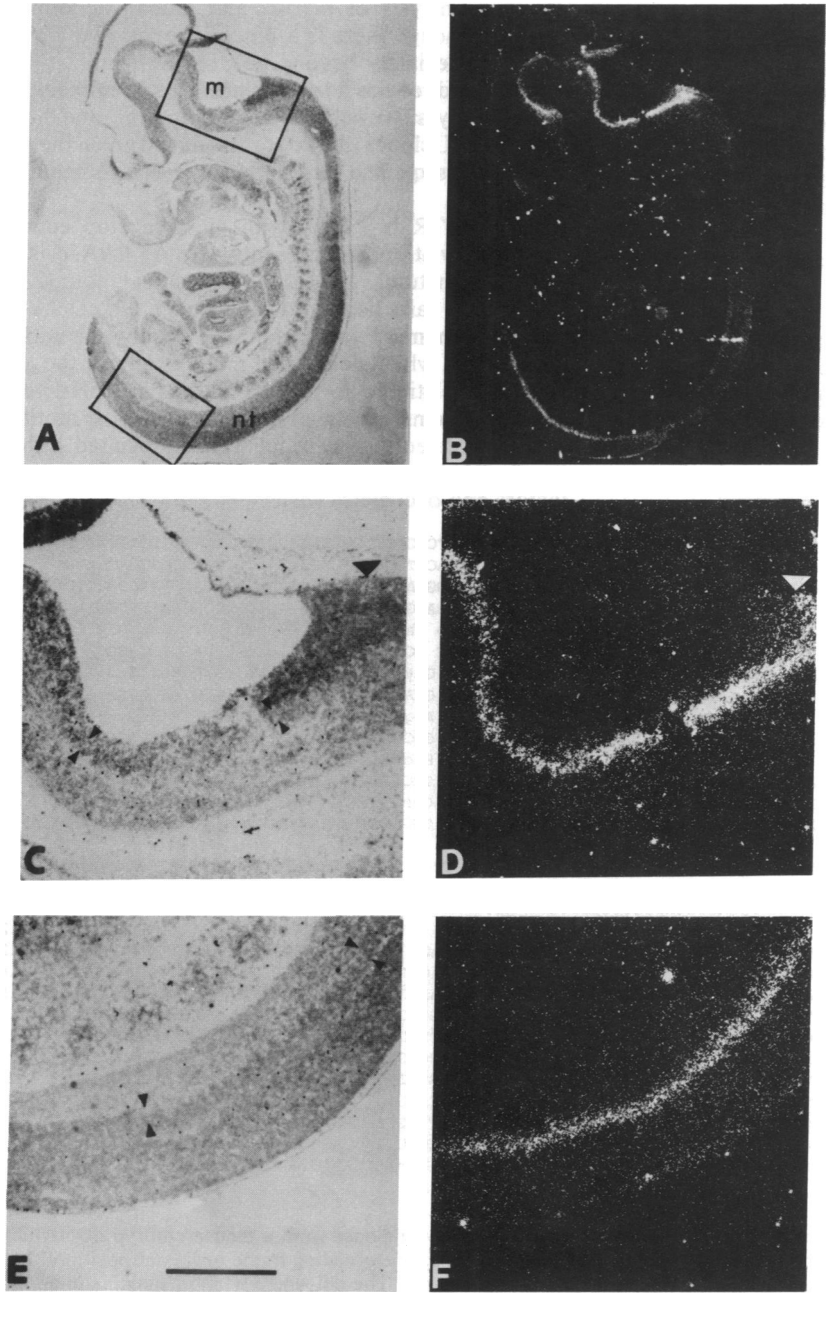

FIG. 5. Expression of NSCL in the developing CNS: representative sagittal section of a day 13 mouse embryo hybridized *in situ* to an ^{35}S-labeled antisense NSCL probe. (*A* and *B*) Boxed areas indicate the regions of the myelencephalon (m) and neural tube (nt), which are shown at higher magnification in *C* and *D* (myelencephalon) and *E* and *F* (neural tube). Expression is seen throughout the CNS and is confined to a narrow strip of cells in the subependymal layer of the neuroepithelium (indicated by the small arrowheads). The large arrowhead denotes the boundary of expression in the posterior myelencephalon. (*A*, *C*, and *E*) Brightfield optics. (*B*, *D*, and *F*) Darkfield optics. (*A* and *B*, bar = 1.5 mm; *C–F*, bar = 800 μm.)

were examined by using Northern analysis of poly(A)$^+$ mRNA. *NSCL* expression was not detected in hemopoietic cells (T-lymphocyte, B-lymphocyte, myeloid, and erythroid cell lines) or in a variety of nonhemopoietic tissues and cell lines (data not shown).

However expression of *NSCL* was detected by using mRNA from mouse embryonic tissue. *NSCL* expression was studied using RNA from 8.5- to 14.5-day mouse embryos, and the transcript was first detected at 9.5 days postcoitus. Maximum levels of expression were seen in embryonic tissue at day 10.5–12.5 postcoitus. *NSCL* expression was reduced and only just detectable in mRNA from total embryonic tissue at day 13–14.5 postcoitus. However analysis of poly(A)$^+$ mRNA from day 13 embryo revealed significant *NSCL* expression at this time. Of interest, both total mRNA and poly(A)$^+$ mRNA from embryonic stem cells showed no detectable *NSCL* expression (Fig. 4).

NSCL Is Expressed in Developing Neurological Tissue. *In situ* hybridization analysis was performed to determine the spatial expression pattern of *NSCL* during development. Sections of mouse embryos (12 and 13 days) were hybridized to two different ^{35}S-labeled antisense probes from NSCL cDNA clones with identical results. At both stages, *NSCL* was strongly expressed in the developing central nervous system (CNS) in the brain and neural tube (day 12 not shown). Transcripts were exclusively localized to a narrow strip of cells in the subependymal layer of the neuroepithelium (Fig. 5). NSCL transcripts were not detected in the mitotically active germinal layer adjacent to the ventricle or in the outer layers of the brain or neural tube. Transcripts of NSCL were also localized to the developing peripheral nervous system. Strong expression was found in the dorsal root ganglia, the trigeminal ganglia (Fig. 6), and other cranial ganglia (not shown). Specific hybridization was also detected in the sensory nasal epithelium (Fig. 6) and the sensory layer of the developing optic cup (not shown).

DISCUSSION

In this paper we describe the molecular characterization of an additional HLH locus, *NSCL*. Members of this newly recognized family of genes have been shown to play a critical role in development and differentiation events in a wide variety of tissues and species (1–3). Of the known HLH

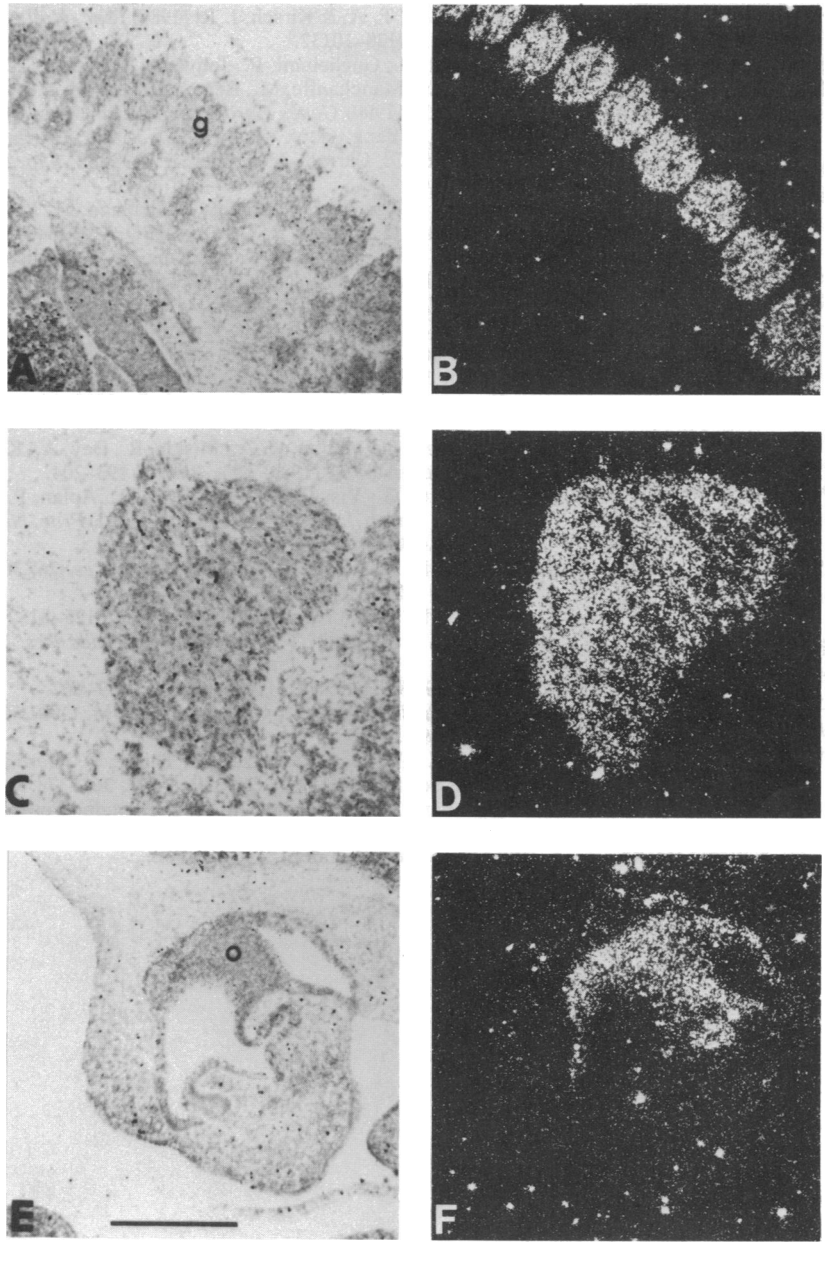

FIG. 6. Localization of NSCL transcripts in the peripheral nervous system and sensory epithelium of day 13 embryos. Transcripts of NSCL were detected in the dorsal root ganglia (*g*) (*A* and *B*), the trigeminal ganglion (*C* and *D*), and the olfactory sensory epithelium (o) (*E* and *F*). Sagittal sections are shown. (*A*, *C*, and *E*) Brightfield optics. (*B*, *D*, and *F*) Darkfield optics. (*A*, *B*, *E*, and *F*, bar = 800 μm; *C* and *D*, bar = 530 μm.)

genes, *NSCL* is closely related to *SCL* (61% identity in the HLH domain at the amino acid level) and was identified based on nucleotide homology. It is also similar to LYL1 in the HLH domain (60% identity) and the mouse *twist* gene (57% identity). The predicted NSCL protein contains both a typical HLH motif and an upstream basic domain. The NSCL basic region is particularly prominent, extending 25 amino acids upstream of the HLH domain and including a stretch of six consecutive arginine residues. The presence of this basic region suggests a role for NSCL as a positive regulator of the HLH class. The *NSCL* gene was mapped to a region of murine chromosome 1 known to be syntenic with human chromosome 1q, suggesting that the human *NSCL* gene may be located in the region 1q12-23.

In situ hybridization demonstrated expression of the *NSCL* gene in the subependymal layer of the neuroepithelium throughout the CNS, in the dorsal root ganglia, in the trigeminal and other cranial ganglia, in the sensory nasal epithelium, and in the sensory layer of the developing optic cup. Although NSCL is localized to developing neural structures in the CNS and peripheral nervous system, it cannot be definitely ascertained by *in situ* hybridization if expression is restricted to developing neurons or to support cells. However, these neural tissues have distinctly different origins (CNS, neural crest, or placode), and the most striking common feature of these tissues is the presence of differentiating neurons (26). In addition, the ganglia at this stage are almost exclusively neuronal cell bodies. Thus it is likely that the *NSCL* gene is expressed in developing neurons. Development of the CNS takes place through cell proliferation in the ventricular (ependymal) layer followed by migration to the outer layers. This migration is accompanied by cellular differentiation (6). The restriction of *NSCL* expression to the subependymal layer is intriguing since this indicates a role for this gene in the differentiation of the CNS.

The transient expression of *NSCL* in embryonic tissue and predominantly in the developing nervous system clearly suggests a role for the NSCL protein in nervous system development. Other members of the HLH family are known to be critical in nervous system differentiation (7), but the low degree of homology between them and NSCL (30–40% identity in the HLH domain with members of the achaete-scute subfamily) suggests that NSCL is not a member of this subfamily of HLH proteins. In contrast, it is intriguing to note the high degree of homology between *NSCL* and two genes expressed predominantly in hemopoietic tissue, *SCL* and *LYL1*.

In summary we describe here an additional HLH gene, NSCL, that is closely related to *SCL* in the HLH domain. The restricted pattern of *NSCL* expression primarily in the developing brain suggests a role for *NSCL* as a regulator of neurological differentiation and development.

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