

# Tissue-specific Expression of the L1 Cell Adhesion Molecule Is Modulated by the Neural Restrictive Silencer Element

Pekka Kallunki, Gerald M. Edelman, and Frederick S. Jones

Department of Neurobiology, The Scripps Research Institute and The Skaggs Institute for Chemical Biology, La Jolla, California 92037

**Abstract.** The cell adhesion molecule L1 mediates neurite outgrowth and fasciculation during embryogenesis and mutations in its gene have been linked to a number of human congenital syndromes. To identify DNA sequences that restrict expression of L1 to the nervous system, we isolated a previously unidentified segment of the mouse L1 gene containing the promoter, the first exon, and the first intron and examined its activity *in vitro* and *in vivo*. We found that a neural restrictive silencer element (NRSE) within the second intron prevented expression of L1 gene constructs in nonneural cells. For optimal silencing of L1 gene expression by the NRSE-binding factor RE-1–silencing transcription fac-

tor (REST)/NRSF, both the NRSE and sequences in the first intron were required. In transgenic mice, an *L1lacZ* gene construct with the NRSE generated a neurally restricted expression pattern consistent with the known pattern of L1 expression in postmitotic neurons and peripheral glia. In contrast, a similar construct lacking the NRSE produced precocious expression in the peripheral nervous system and ectopic expression in mesenchymal derivatives of the neural crest and in mesodermal and ectodermal cells. These experiments show that the NRSE and REST/NRSF are important components in restricting L1 expression to the embryonic nervous system.

CELL adhesion molecules (CAMs)<sup>1</sup> play fundamental roles in the development of the nervous system. During embryogenesis, CAMs participate in neurite extension, fasciculation, axon guidance, and synapse formation (9, 10), and in the adult, they have roles in synaptic plasticity (34, 39). The neural cell adhesion molecule, N-CAM, is expressed in the neural tube before differentiation of neuroepithelial precursors, appears on the surface of neurons and glia, and is found on a number of nonneural tissues (9). In contrast, some CAMs appear at the time of neural differentiation and are expressed solely or predominantly in the nervous system (2, 12, 14, 21, 26, 36, 57).

These observations raise an important question: What restricts the expression of certain CAMs to the nervous system? To answer this question, we have attempted to

identify the DNA elements and protein factors that control expression of two particular CAMs that are restricted to the nervous system during development: the avian neuron–glia cell adhesion molecule (Ng-CAM) and mammalian L1 (2, 36). Ng-CAM and L1 are integral membrane proteins of the N-CAM subfamily characterized by six immunoglobulin-like domains and five fibronectin type-III repeats. Ng-CAM was first isolated from embryonic chicken brain (13), and L1 was identified by monoclonal antibodies prepared against mouse cerebellar membranes (44, 49). Both proteins have similar patterns of expression and appear on the surface of postmitotic neurons and peripheral glial cells (7, 24, 32, 38).

In previous work, we identified some of the regulatory regions of the chicken Ng-CAM gene (19), including the promoter and a region of the first intron that was found to contain five contiguous neural restrictive silencer elements (NRSEs). When linked to a reporter gene, the Ng-CAM promoter drove expression in both N2A neuroblastoma and NIH3T3 fibroblast cell lines. However, when the five NRSEs were included in these constructs, reporter gene expression was found in neuroblastoma cells, but not in fibroblasts (19).

The NRSE has been found in a number of genes for proteins that are restricted to the nervous system, including the type II sodium channel (33), synapsin I (46), and BDNF (51). A zinc finger protein known as the neural re-

Address all correspondence to Frederick S. Jones, Department of Neurobiology, The Scripps Research Institute, 10550 North Torrey Pines Rd., La Jolla, CA 92037. Tel.: (619) 784-2600. Fax: (619) 784-2646. e-mail: fjoness@scripps.edu

1. *Abbreviations used in this paper:* BMP, bone morphogenetic protein; CAM, cell adhesion molecule; CBP, CCAAT-binding protein; CNS and PNS, central and peripheral nervous system; E, embryonic day; HA, hemagglutinin; N-CAM, neural cell adhesion molecule; NF-1, nuclear factor-1; Ng-CAM, neuron–glia cell adhesion molecule; NRSE, neural restrictive silencer element; NRSF, neural restrictive silencer factor; RACE, rapid amplification of cDNA ends; REST, RE-1–silencing transcription factor; RLU, raw light units.

strictive silencer factor (NRSF) or the RE-1–silencing transcription factor (REST) binds to the NRSE and silences expression of genes containing NRSEs in nonneural cells (4, 47). REST/NRSF is expressed ubiquitously in nonneural cells and in neuronal precursors but not in postmitotic neurons (4, 47). Thus, downregulation of REST/NRSF is likely to be a key event in neural development in that it releases silencing and allows expression of several genes that are important for the establishment of the phenotype of neural cells.

Recently, a putative NRSE has been identified within an intron of the human and mouse L1 gene sequences (19, 48). Given the observations that the Ng-CAM and L1 genes both contain NRSEs, and that the expression patterns of these genes in their respective organisms are similar, we examined the role of the NRSE in regulating L1 gene expression both *in vitro* and *in vivo*. In the present study, we identify an additional segment of the mouse L1 gene containing the promoter and show that the NRSE within the L1 gene is critical for a neurally restricted pattern of L1 gene expression in the embryonic nervous system.

## Materials and Methods

### Isolation of L1 Genomic and cDNA Clones Corresponding to the 5' End of the mRNA

Genomic clones for mouse L1 were isolated from a library prepared from genomic DNA isolated from mouse embryonic stem cells in bacteriophage P1 (Genome Systems, St. Louis, MO) by PCR using primers derived from the mouse L1 gene sequence (22). Rapid amplification of cDNA ends (RACE) (11) on the L1 mRNA was performed using the Marathon cDNA amplification kit (CLONTECH Laboratories, Inc., Palo Alto, CA). RACE products and L1 genomic DNA fragments were subcloned into the pBluescriptII vector (Stratagene, La Jolla, CA). The sequence of both strands of L1 clones was determined (50). Sequencing data were compiled using the GCG package (University of Wisconsin, Madison, WI) and comparisons were made using FASTA (41). RNA start sites were located using an RNase protection assay (Ambion, Inc., Austin, TX).

### Preparation of Luciferase and lacZ Reporter Constructs

L1-1 through L1-6 were constructed by insertion of L1 genomic fragments into the XmaI and XhoI sites of pGL2basic (Promega Corp., Madison, WI). To prepare L1-5N and L1-5Nr plasmids, double-stranded oligonucleotides containing two copies of the NRSE from the L1 gene were inserted into the XmaI site upstream of the L1-5 promoter fragment. L1-7 through L1-12, L1lacZ, and L1lacZΔN were prepared using the vector CMVβ (CLONTECH Laboratories, Inc.), after replacing the human cytomegalovirus promoter with a polylinker containing the XmaI and XhoI sites, which allowed the insertion of the L1 genomic fragments. lacZ constructs were converted into luciferase reporters by removing the lacZ gene by digestion with NotI and replacing it with a modified luciferase gene cassette from pGEMluc (Promega Corp.) containing an SV-40 polyadenylation signal. A promoterless version of this vector, called lucpA, was also prepared to provide a negative control for the luciferase constructs L1-7 through L1-12.

Large L1 gene fragments were assembled as follows: A fragment containing the 5' untranslated sequences of exon 2 was inserted into the XhoI site downstream of exon 1. Fragments containing intron 1 were added at the BspI site at the 3' terminus of exon 1. A partial XbaI digest generated a construct that included the L1 promoter, exon 1, intron 1, and exon 2. The region containing the translated portion of exon 2, intron 2, exon 3, intron 3, and exon 4 was used in the generation of L1-8, L1-9, L1-11, and L1-12. The resulting fragment had Sse8387I restriction sites at either end and contained a SnaBI site at the 3' end of exon 4. A similar fragment lacking a segment of 20 bp that deleted the NRSE within intron 2 was generated by PCR. These fragments were inserted into L1 genomic constructs at the Sse8387I site downstream of the SV-40 polyA signal. The nuclear localization signal from the SV-40 large T antigen was inserted upstream

of the lacZ gene by replacing a XhoI–ClaI fragment with a Sall–ClaI fragment from the pnlacF vector (from J. Peschon, University of Washington, Seattle, WA).

### Preparation of REST/NRSF Expression Vectors and Cellular Transfection Experiments with L1 Constructs

The cDNA for REST/NRSF (4, 47) was generated by PCR from total human RNA isolated from HeLa and Jurkat cells using High Fidelity Taq polymerase (Boehringer-Mannheim Corp., Indianapolis, IN) and was inserted into the pCRII vector (Invitrogen, San Diego, CA). Expression of REST/NRSF was confirmed using an *in vitro* transcription/translation system (Promega Corp.). The REST/NRSF cDNA or a 5' segment encoding the NH<sub>2</sub>-terminal 423 amino acids containing the zinc finger DNA-binding domain (D-REST) was cloned in frame with the hemagglutinin (HA) tag in the mammalian expression vector SRα3. Expression of REST/NRSF and D-REST proteins was confirmed after transfection of COS-1 cells followed by immunoblot analysis of cell extracts with a monoclonal antibody to the HA tag. D-REST was ~50 kD, which corresponds to the predicted size of this protein fragment plus the HA tag. Expression of REST/NRSF produced two bands, one migrating at 200 kD and another at 120 kD, a size that corresponds to the reported size of REST/NRSF. The 200-kD band might represent a posttranslationally modified REST/NRSF protein, as suggested in previous studies (4).

NIH3T3, COS-1, and N2A cells were cultured in DMEM supplemented with either newborn or fetal calf serum and were transfected in equimolar amounts of DNA using lipofectamine (Life Technologies, Gaithersburg, MD). The lacZ reporter CMVβgal (CLONTECH Laboratories, Inc.) was cotransfected to normalize for transfection efficiency. For cotransfections, between 50- and 100-fold molar excess of either the REST/NRSF or D-REST expression vector was added to the transfection mixture. Cells were harvested after 48 h, and extracts were prepared, normalized for β-galactosidase activity, and assayed for luciferase activity, as described (17).

The activities of L1 luciferase constructs were determined in three separate sets of experiments. In the first set of experiments (see Fig. 2), the promoterless luciferase vector pGL2basic (Promega Corp.) served as the negative control, and the vector pGL2control (Promega Corp.), containing an SV-40 early promoter upstream of the luciferase gene, provided a positive control for promoter activity. The activities for pGL2control and constructs L1-1 through L1-6 were calculated by subtracting background activity in raw light units (RLU) produced by pGL2basic from the RLU values for each construct. For each cell line, the RLU values for pGL2basic and pGL2control were set at 0 and 100% activity, respectively. The relative luciferase activities for constructs L1-1 through L1-6 were then expressed as a percentage of SV-40 early promoter activity. For the second set of experiments (see Fig. 3 A), the background activity from pGL2basic vector was subtracted from the values for L1-5, L1-5N, and L1-5Nr. Activity levels for pGL2basic and the unsilenced L1 promoter construct L1-5 were set at 0 and 100%, respectively, in each of the four cellular transfection conditions (NIH3T3, N2A, NIH3T3 + D-REST, and N2A + REST). The relative luciferase activities for L1-5N and L1-5Nr were then expressed as a percentage of L1-5 promoter activity. For the third set of experiments (see Fig. 3 B), the background activity produced by the promoterless vector lucpA was subtracted from the raw RLU values for L1-7 through L1-12. The activity levels for lucpA and L1-7 were set at 0 and 100%, respectively, in each of the four transfection conditions. The relative luciferase activities were then expressed as a percentage of L1-7 promoter activity. The values for all of the relative luciferase activities in each experimental set are the average of at least six independent experiments.

### Generation and Analyses of Transgenic Mice Containing lacZ Constructs

To prepare transgenic mice, L1lacZ and L1lacZΔN transgenes were introduced into the RC6 mouse genome by standard oocyte microinjection techniques (16). Transgenes were excised from plasmids by digestion with restriction enzymes XmaI and SnaBI. Genomic DNA isolated from tails of F<sub>0</sub> progeny was screened for the presence of the transgene by PCR using the TissueAmp kit (Qiagen, Inc., Chatsworth, CA). Two different primer sets were designed for the PCR to discriminate between mice carrying the wild-type NRSE and those in which the NRSE was deleted.

Animals positive for the transgenes were mated to establish individual lines. Males from transgenic lines were mated with C57BL6 females, and pregnant females were sacrificed at different days of gestation to obtain embryos at different stages of development. At least one litter from each

of the transgenic lines produced in these studies was analyzed for  $\beta$ -galactosidase expression. To stain whole mounts, embryos were fixed in 0.2% glutaraldehyde/1% formaldehyde in PBS, washed in PBS, and immersed in staining solution containing 0.02% deoxycholate, 30 mM  $K_3Fe(CN)_6$ , 30 mM  $K_4Fe(CN)_6$ , and 0.5 mg/ml Bluogal (Life Technologies) in PBS overnight at 37°C. To detect  $\beta$ -galactosidase activity in tissue sections, embryos were fixed, transferred through an ascending gradient of sucrose to 24% sucrose/PBS, frozen in Tissue Tek (Miles, Inc., Elkhart, IN), and sectioned (40  $\mu$ m) on a cryomicrotome. Sections were attached to poly-L-lysine-coated microscope slides and stained for  $\beta$ -galactosidase as described above, mounted with 50% glycerol, and photographed with bright field optics.

## Results

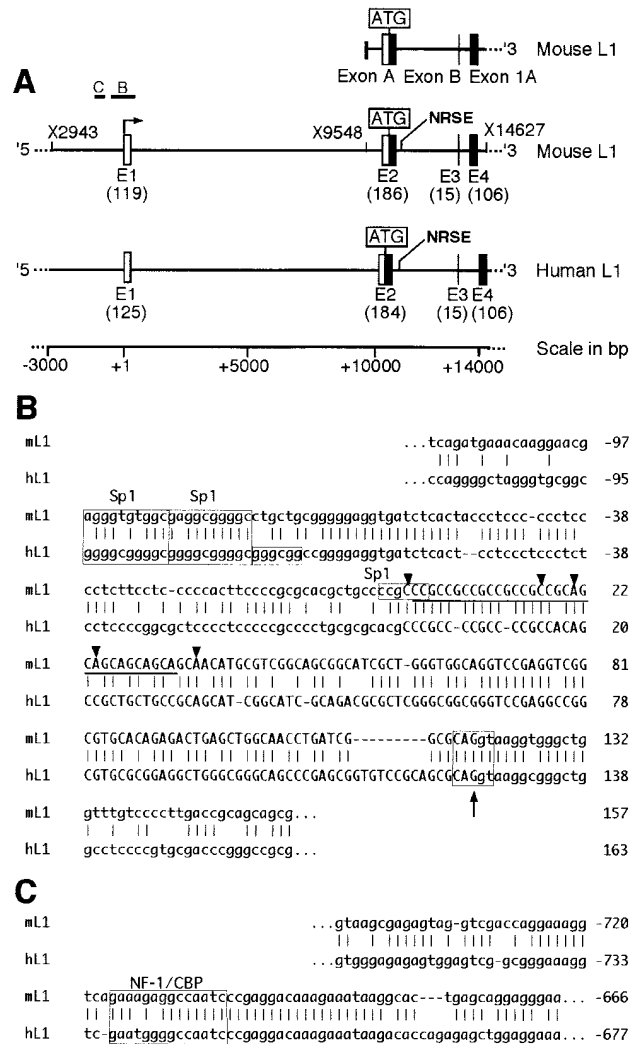
### Characterization of the 5' End of the Mouse L1 Gene

We previously characterized the structures of the genes for two avian neural CAMs that are closely related to mammalian L1: Ng-CAM (19) and Nr-CAM (unpublished data). Comparison of the 5' sequences of the Ng-CAM and Nr-CAM genes with those reported for the mouse L1 gene sequence (22) revealed a close correspondence in intron/exon structure (see Fig. 1 A). However, in both the Ng-CAM and Nr-CAM genes, the ATG codon is situated in the second exon, whereas in the L1 gene the ATG codon was located in a region proposed to be the 5'-most exon, called exon A (22). These contrasting comparisons suggested to us that there may be an additional exon and a promoter region upstream of exon A in the L1 gene. Primer extension analyses of mouse L1 mRNA transcripts in the original study (22) were in accord with this supposition.

To obtain cDNA sequences corresponding to the full-length 5' end of the L1 messenger RNA, we performed a RACE experiment (11) on RNA isolated from N2A cells. DNA sequencing of RACE clones revealed an additional segment of 5' untranslated sequences not detected in previous studies (22). No RACE products terminated at the 5' end of exon A, suggesting that, contrary to previous conclusions (22), the 5' end of exon A is not a major site for initiation of L1 gene transcription in N2A cells.

To characterize the 5' end of the L1 gene, we cloned and sequenced a 13-kb region of genomic DNA immediately upstream of the 5'-most sequences previously reported (22). These sequence data are available from GenBank/EMBL/DDBJ under accession number U91929. They include a 2,943-bp segment of 5' flanking sequence, the 119-bp first exon, and 9,429 bp of the first intron. The location of this gene segment relative to the previously characterized portion of the L1 gene (22) is shown in Fig. 1 A. The additional 5' untranslated sequences found in RACE experiments mapped the first exon  $\sim$ 10 kb upstream of exon A (Fig. 1 A). Given these new findings, we have redesignated exon A as exon 2.

Using RNase protection assays, multiple transcription initiation sites were mapped to the 5' end of the first exon. No transcripts were detected initiating at exon 2. Exon 1 was defined to be the 119 bp between the 5'-most RNA start site and the beginning of the 3' splice junction. When combined with the results from our RACE experiments, these data provide additional support for the conclusion that exon 2 (previously exon A) is not a site of transcription initiation.



**Figure 1.** (A) Schematic representations of the mouse and human L1 genes. (Top) Diagram of the 5'-most sequences established in previous studies (22). The nomenclature for the exons are those given by Kohl et al. in their study (22). The extended portion of the L1 gene that we describe in the present study is shown below. The lengths of the exon sequences in base pairs are indicated in parentheses. Coding and 5' untranslated sequences are indicated by black boxes and open boxes, respectively. The arrow indicates the 5'-most site of transcription initiation. The positions of XbaI (X) restriction sites are also indicated. The line segments shown above the promoter for the mouse L1 gene labeled B and C indicate regions that have a high degree of similarity with the human L1 gene. (B and C) Comparison of the sequences at the 5' end of the mouse (*mL1*) and human (*hL1*) L1 genes. Putative binding sites for NF-1/CBP and SP1 are indicated by boxes. First exon sequences in the mouse and human L1 genes are indicated by upper case letters. Arrowheads define transcription initiation sites for the mouse L1 gene. The trinucleotide repeats are underlined. The 3' splice junction is boxed and indicated by an arrow.

The sequences of 97 bp of the proximal promoter, the first exon, and a portion of the first intron are shown in Fig. 1 B. The RNA start sites initiated within a region containing several trinucleotide repeats of GCC and CAG (Fig. 1 B). Searches of the promoter and first exon for elements known to regulate gene expression revealed a bind-

ing site for the transcription factor SP1 (8) that overlaps the 5'-most transcription initiation site. In previous studies of the L1 gene (22), a binding site for homeodomain proteins was also identified 170 bp upstream of exon A (3, 22). Our analysis, however, now places this DNA element within the 3' end of the first intron. Most significant for the present experiments, the mouse L1 gene sequence contains a single NRSE immediately downstream of exon 2 (Fig. 1 A).

### Comparison of the Mouse and Human L1 Promoter Regions

A portion of the human L1 gene sequence is available from GenBank/EMBL/DDBJ under accession number U52112 and is part of a larger sequence from the X chromosome. We compared the sequence of the human X chromosome upstream of the existing human L1 gene with our sequence of the mouse L1 promoter and first exon and found that there was a high degree of similarity between the 5' end of the mouse and human L1 genes (Fig. 1 A). As is found in the mouse gene, the first exon of the human L1 gene is ~10 kb upstream of exon 2. As shown in Fig. 1, B and C, the sequences of the first exon, the 3' splice junctions, and the proximal promoters containing the SP1 sites are highly conserved between the mouse and human L1 genes. A region of the L1 promoter further upstream, containing a putative binding site for transcription factors of the NF-1 and CBP families, is also highly conserved between the two species (Fig. 1 C). The single NRSE in the second intron of the mouse L1 gene is also found in a comparable position in the human L1 gene. No other NRSEs were found in searches of the mouse and human L1 gene sequences.

### The NRSE and the First Intron Are Required for Silencing of the L1 Gene Expression by REST/NRSF in Cellular Transfection Experiments

To examine whether the DNA sequences upstream of exon 1 in the mouse L1 gene had promoter activity, we prepared six luciferase reporter constructs containing exon 1 together with varying lengths of 5' flanking sequence from the gene and examined their activity in either NIH3T3 fibroblasts or N2A neuroblastoma cells. All six L1 gene fragments (containing as few as 70 and as many as 2,943 bp upstream from the transcription initiation sites) showed promoter activity (Fig. 2). L1-5 and L1-1 were the most active promoter constructs in NIH3T3 and N2A cells, respectively. In general, the luciferase activities of L1-1 through L1-6 were comparable or greater than the activity of the SV-40 early promoter and on average were between 13- and 8-fold greater than those produced by the promoterless luciferase vector in NIH3T3 cells and N2A cells, respectively. These findings indicate that there is a promoter upstream of exon 1 that is constitutively active in both NIH3T3 and N2A cells.

**Tests of NRSE Function.** The fact that reporter constructs containing the L1 promoter and the first exon were equally active in neural (N2A) and nonneural (NIH3T3) cells indicated that cell type-specific expression required other regions of the gene. To assess whether the NRSE within the second intron of the L1 gene played a role in the silencing of L1 promoter in nonneural cells, we pre-

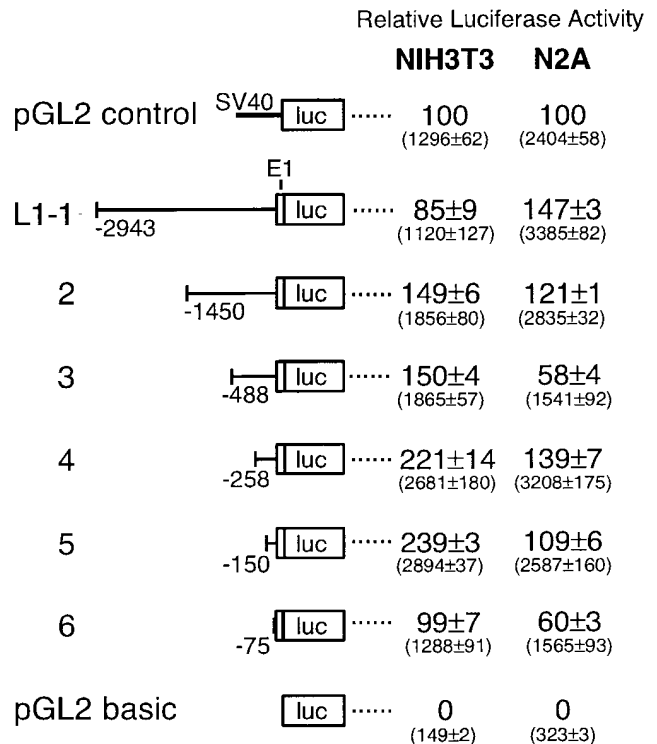
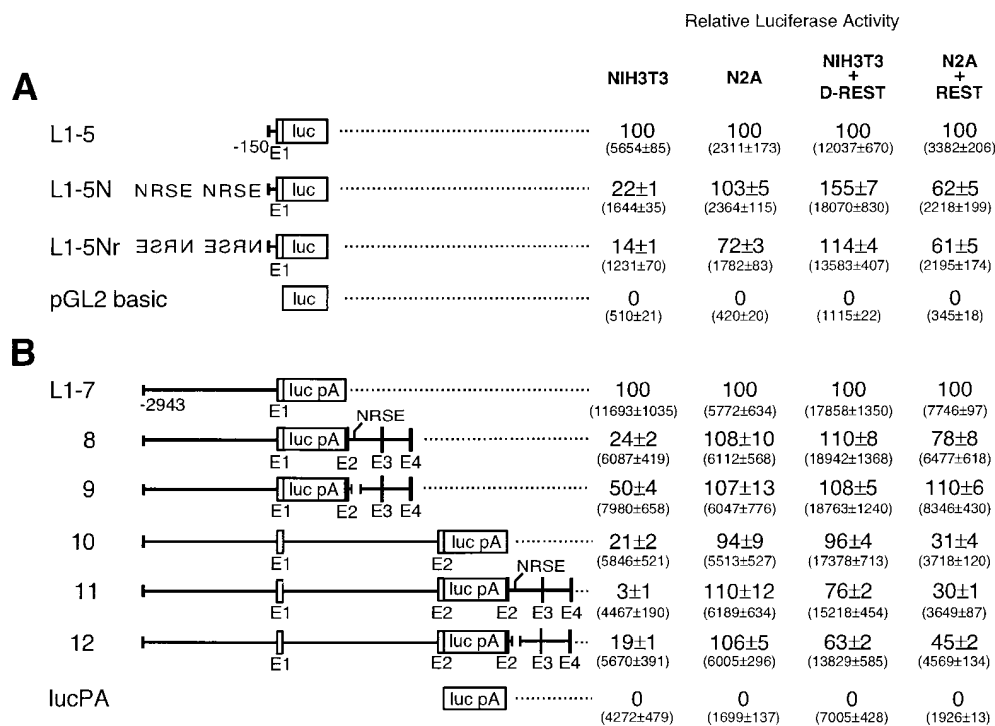


Figure 2. Activity of L1 promoter constructs in cellular transfection experiments of NIH3T3 and N2A cells. The relative luciferase activities of L1 constructs are expressed as a percentage of SV-40 promoter activity produced by the PGL2control vector. The values for pGL2control and pGL2basic were set at 100 and 0% activity, respectively. The corrected values for activities were derived by subtracting the background activity in RLU produced by the promoterless vector pGL2basic from the RLU values for each construct. The numbers in parentheses are the mean RLU values produced by each construct and the standard errors for both percentage activity and RLU are shown ( $n = 6$ ).

pared a number of L1 gene constructs containing or lacking the NRSE and examined their activity in cellular transfection experiments.

Two constructs, L1-5N and L1-5Nr, were prepared by inserting two copies of the NRSE from the L1 gene in either forward or reverse orientation upstream of the most active L1 promoter, L1-5. These constructs were transfected into NIH3T3 or N2A cells and examined for activity. The presence of NRSEs in either orientation led to a significant reduction in L1 promoter activity in NIH3T3 cells, but not in N2A cells (Fig. 3 A). These data indicate that the NRSEs are sufficient for silencing L1 promoter activity in a cell type-specific manner.

To assess the role of the NRSE in the context of the native L1 gene, six constructs containing up to 18 kb of the mouse L1 gene (L1-7 through L1-12; Fig. 3 B) were prepared and examined for their activity in NIH3T3 and N2A cells. To compare the relative level of silencing by downstream segments of the gene, the activity of L1-7 was set at 100%. In NIH3T3 cells, addition to the promoter of an L1 gene segment containing exons 2-4 including introns and the NRSE (L1-8) reduced the activity to 24% of control (L1-7). Deletion of the NRSE in L1-8 (L1-9) resulted in 50% of the control activity, indicating that the NRSE was



vector were subtracted from the values of L1-5, L1-5N, and L1-5Nr in each of the four experimental conditions. The activity levels produced by pGL2basic were set at 0%, and the levels for the unsilenced L1 promoter construct L1-5 were set at 100%. (B) The levels of background activity produced by the control vector lucpA were subtracted from the RLU values for each construct, L1-7 through L1-12. The RLU values for the promoterless vector lucpA were set at 0% activity, and values for the unsilenced L1 promoter construct L1-7 were set at 100% activity. Standard errors for both percentage activity and RLU values are shown,  $n = 6$  for A, and  $n = 10$  for B.

responsible for only a portion of the silencing found in this DNA fragment. Addition of the first intron and second exon of the L1 gene (L1-10) showed a reduction in activity (21% of L1-7) that was approximately equal to inserting the L1 genomic segment containing the NRSE (L1-8). This result suggests that the first intron contains a silencer that is as effective as the NRSE. Addition of the entire L1 genomic region between intron 1 through exon 4 (including the NRSE) (L1-11) resulted in the most significant reduction in activity (3% of L1-7). Deletion of the NRSE in L1-11 yielded L1-12. L1-12 had reduced activity (19% of L1-7) that was approximately equal to L1-10, further indicating that a silencing activity within the first intron was still effective even in the absence of the NRSE.

In N2A cells, the activities of L1-8 through L1-12 were indistinguishable from that of L1-7 (Fig. 3 B). Overall, the results from the transfection experiments indicate that the NRSE and an additional silencer present in the first intron, both of which function in NIH3T3 cells, are not active in N2A cells.

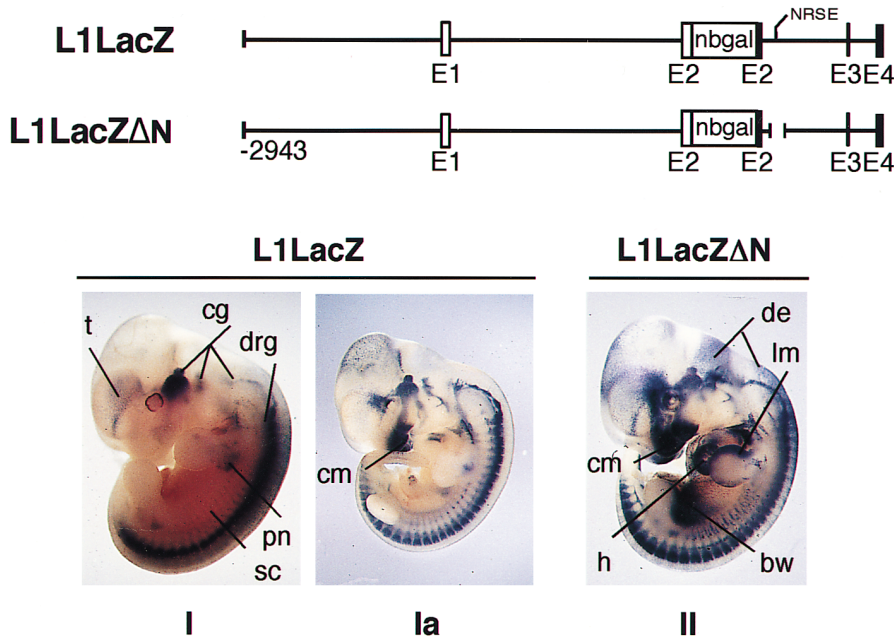
**Effects of Dominant Negative and Native Forms of REST.** To demonstrate that the NRSE in the L1 gene was a target for silencing via REST/NRSF, we performed two types of cellular cotransfection experiments. In the first experiment, a plasmid directing the expression of a truncated REST/NRSF protein (called D-REST) containing the DNA-binding zinc fingers but not the silencer domain (4,

Figure 3. Relative luciferase activities of L1 gene constructs in transfection experiments of NIH3T3 and N2A cells and in cotransfection experiments with constructs expressing either a dominant negative form of REST/NRSF (designated D-REST) or the full-length REST/NRSF. The relative activities represent a percentage within the range that is established between the background activity of a promoterless control vector (which is set at 0%) and an unsilenced L1 promoter construct (which is set at 100%). The numbers in parentheses are the mean values for RLU produced by each construct in each of the four experimental conditions (NIH3T3, N2A, NIH3T3 + D-REST, and N2A + REST). To derive the relative activities shown in A, the RLUs for the promoterless pGL2basic

47) was cotransfected into NIH3T3 cells together with various L1 gene constructs to examine whether a dominant negative form of the REST/NRSF protein could prevent silencing of L1 promoter activity. In the second experiment, N2A cells were cotransfected with L1 constructs and a plasmid expressing the fully active REST/NRSF protein to determine whether ectopic expression of REST/NRSF in cells that normally contain low levels of REST/NRSF activity could reduce the activity from the L1 constructs.

D-REST released silencing of all L1 gene constructs that showed silencer activity in NIH3T3 cells (L1-5N, L1-5Nr, L1-8, L1-9, L1-10, L1-11, and L1-12) (Fig. 3, A and B). Expression of the full-length REST protein in N2A cells led to partial silencing of the activities of L1-5N, L1-5Nr, L1-8, L1-10, L1-11, and L1-12 but did not affect the activities of L1-7 and L1-9 (Fig. 3, A and B). Thus, REST reduced the activity of all L1 gene constructs containing the NRSE, the first intron alone, or the two elements in combination.

We conclude that (a) the NRSE in the L1 gene responds to REST/NRSF; (b) the first intron contains a silencer that can function without the NRSE, but nonetheless acts in response to REST/NRSF; and (c) optimal silencing of the L1 gene by REST/NRSF is achieved when both the first intron and the NRSE are combined. These in vitro findings prompted an analysis in vivo of the modulation of L1 gene expression by the NRSE.



**Figure 4.** (Top) Diagram of the L1lacZ and L1lacZΔN constructs that were used to generate transgenic mice. A detailed description of the construction of these transgenes is provided in Materials and Methods. (Bottom) β-galactosidase expression patterns of individual lines carrying either the L1lacZ or L1lacZΔN transgenes in whole mounts of E10.5 embryos. All transgenic lines (lines I, Ia, and II, as examples) showed identical expression patterns in structures within the central and peripheral nervous system. Most L1lacZ lines showed a completely neurally restricted pattern of β-galactosidase expression (line I). Some L1lacZ lines also showed some non-neural expression at a reduced level in a subset of tissues in which the L1lacZΔN transgene was strongly expressed (line Ia). All L1lacZΔN lines (II, as an example) had extensive extraneural staining patterns of β-galactosidase that were identical. Abbrevia-

tions for anatomical structures: *bw*, body wall; *cg*, cranial ganglia; *cm*, cephalic mesenchyme; *de*, dorsal ectoderm; *drg*, dorsal root ganglia; *h*, heart; *pn*, peripheral nerve; *sc*, sympathetic chain; *t*, telencephalon.

### Production of Transgenic Mice Containing L1 Gene Constructs

To determine whether the 5' end of the L1 gene was sufficient to direct expression of the gene to the nervous system *in vivo* and to examine the effect of NRSE removal on L1 expression, transgenic mice were generated containing two different L1-*lacZ* reporter constructs (Fig. 4). The two transgenes, designated L1lacZ and L1lacZΔN, were identical to L1-11 and L1-12 (Fig. 3 B), except that the luciferase gene was replaced by a *lacZ* gene cassette containing a nuclear localization signal. The only difference between these L1 transgenes was that L1lacZ contained the NRSE, whereas L1lacZΔN did not.

33 transgenic lines were established for the L1lacZ transgene. 15 showed no expression of β-galactosidase, suggesting that in these cases, the transgene most likely integrated into genomic regions that completely silenced its expression. Of the 18 expressing lines, 12 showed neurally restricted β-galactosidase expression patterns that were identical (Fig. 4, pattern I). The remaining six lines showed the same neural pattern but in addition displayed some staining outside of the nervous system. For example, the line shown in Fig. 4 called Ia has the same neural pattern as I but has additional staining in the cephalic mesenchyme. The other lines were similar to Ia but differed slightly in the locations in which the nonneural staining was seen. In all cases, the nonneural staining appeared in locations that were a subset of those carrying the L1lacZΔN transgene (see below). Such patterns might arise from a partial release of silencing of L1 gene expression upon integration of the transgene in a particularly active region of the genome.

For the L1lacZΔN transgene that lacked the NRSE, seven transgenic lines were obtained, and four of these

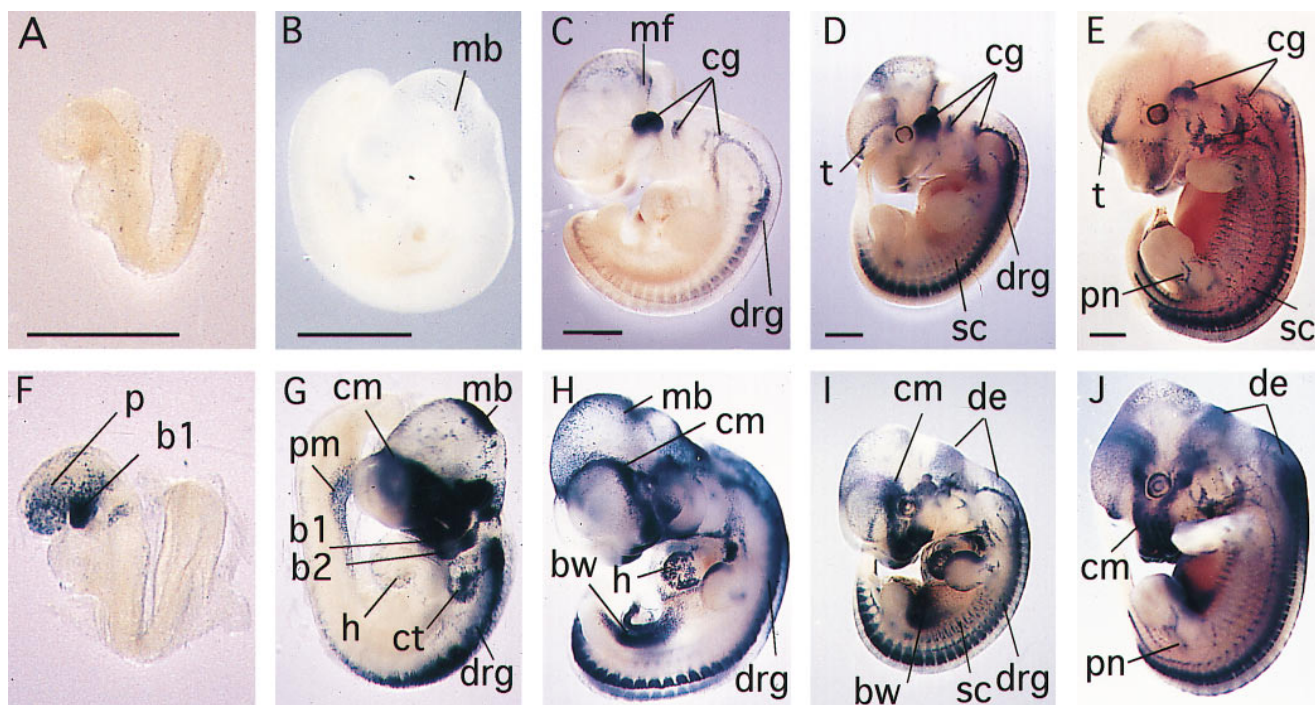
showed β-galactosidase expression. All four lines had an identical expression pattern that included the neural pattern characteristic of the L1lacZ lines, but in addition had intense and expansive β-galactosidase staining outside of the nervous system (Fig. 4, pattern II).

To examine in detail the differences between neurally restricted and unsilenced patterns of L1 gene expression, one L1lacZ line showing the neurally restricted pattern I and one L1lacZΔN line showing the extensive nonneural staining pattern II were selected. The expression patterns of both transgenes in whole mounts were first compared at each day of embryonic development between E8.5 and E12.5 (Fig. 5).

### Expression of the L1lacZ Transgene Is Coincident with Neural Differentiation and Is Restricted to the Nervous System

Expression of the L1lacZ transgene was not detected before neural differentiation at E8.5 (Fig. 5 A) and was first observed at E9.5 in the central nervous system (CNS) within the midbrain and in the peripheral nervous system (PNS) within the trigeminal ganglion (Fig. 5 B; *mb*). At E10.5, the punctate β-galactosidase expression in the midbrain became more intense, showing a distinct posterior boundary at the mesometencephalic fold (Fig. 5 C; *mf*). At E11.5, expression of the L1lacZ transgene extended to more rostral locations of the CNS and was observed in the telencephalon (Fig. 5 D; *t*). In the PNS starting at E10.5, expression of β-galactosidase was observed in cranial and dorsal root ganglia and along the nerves emanating from these ganglia (Fig. 5 C; *cg* and *drg*). Between E11.5 and 12.5, the cranial and dorsal root ganglia and the sympathetic chain showed strong L1lacZ transgene expression (Fig. 5, D and E; *cg*, *drg*, and *sc*).





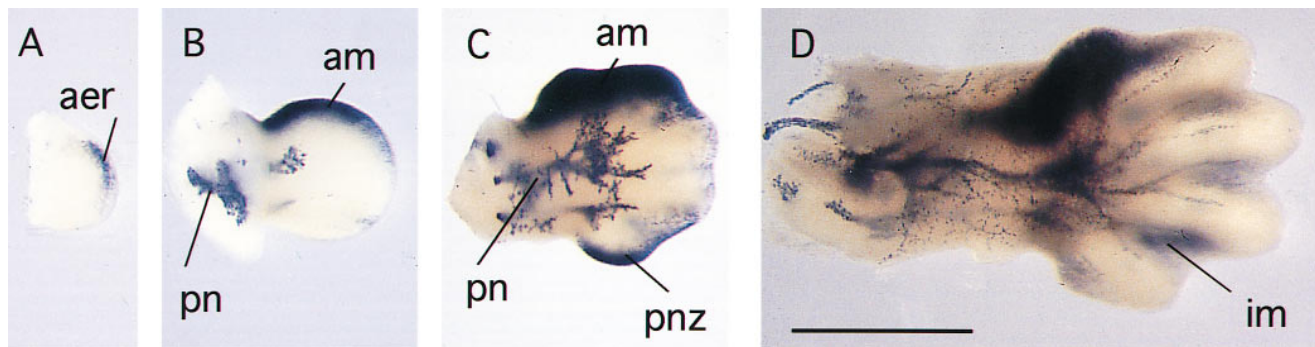
**Figure 5.** Analyses of the  $\beta$ -galactosidase staining patterns in whole mount from mouse embryos carrying either L1lacZ (A–E) or L1lacZ $\Delta$ N (F–J) transgenes. The stages of embryonic development are as follows: E8.0–8.5 (A and F), E9.5 (B and G), E10.5 (C and H), E11.5 (D and I), and E12.5 (E and J). Abbreviations for anatomical structures: b1, branchial arch 1; b2, branchial arch 2; bw, body wall; cg, cranial ganglia; cm, cephalic mesenchyme; ct, circumpharyngeal tract; de, dorsal ectoderm; drg, dorsal root ganglia; h, heart; mb, midbrain; p, prosencephalon; pm, posterior mesoderm; pn, peripheral nerve; sc, sympathetic chain; t, telencephalon; tg, trigeminal ganglion. Bars, 1 mm (apply to each stage of development).

### **Expression of the L1lacZ $\Delta$ N Transgene Occurs before Neural Differentiation and Appears at Several Nonneural Sites**

Expression of L1lacZ $\Delta$ N transgene was observed first at E8.5, a full day earlier than in embryos carrying the L1lacZ transgene (compare Fig. 5, F and G, with A and B). This initial expression of the L1lacZ $\Delta$ N transgene was found within the first branchial arch and in the prosencephalon (Fig. 5 F; b1 and p). These areas contain migratory neural crest cells from the regions of the developing

hindbrain and mesencephalon that differentiate into both PNS and craniofacial mesenchymal tissues.

The most dramatic differences in the intensity of  $\beta$ -galactosidase expression patterns between the L1lacZ and L1lacZ $\Delta$ N transgenes were apparent at E9.5. While L1lacZ embryos showed faint expression of  $\beta$ -galactosidase in the midbrain and trigeminal ganglion (Fig. 5 B), L1lacZ $\Delta$ N embryos showed intense  $\beta$ -galactosidase expression in the head and trunk (Fig. 5 G). In the head, the L1lacZ $\Delta$ N transgene was expressed in the prosencephalic region sur-



**Figure 6.** Analyses of the  $\beta$ -galactosidase staining patterns produced by the L1lacZ $\Delta$ N transgene during the development of the forelimb. The dorsal side of the limb is shown at each stage of development: E10.5 (A), E11.5 (B), E12.5 (C), and E13.5 (D). Abbreviations for structures: aer, apical ectodermal ridge; am, anterior mesoderm; im, interdigital mesenchyme; pn, peripheral nerve; pnz, posterior necrotic zone. Bar, 1 mm.



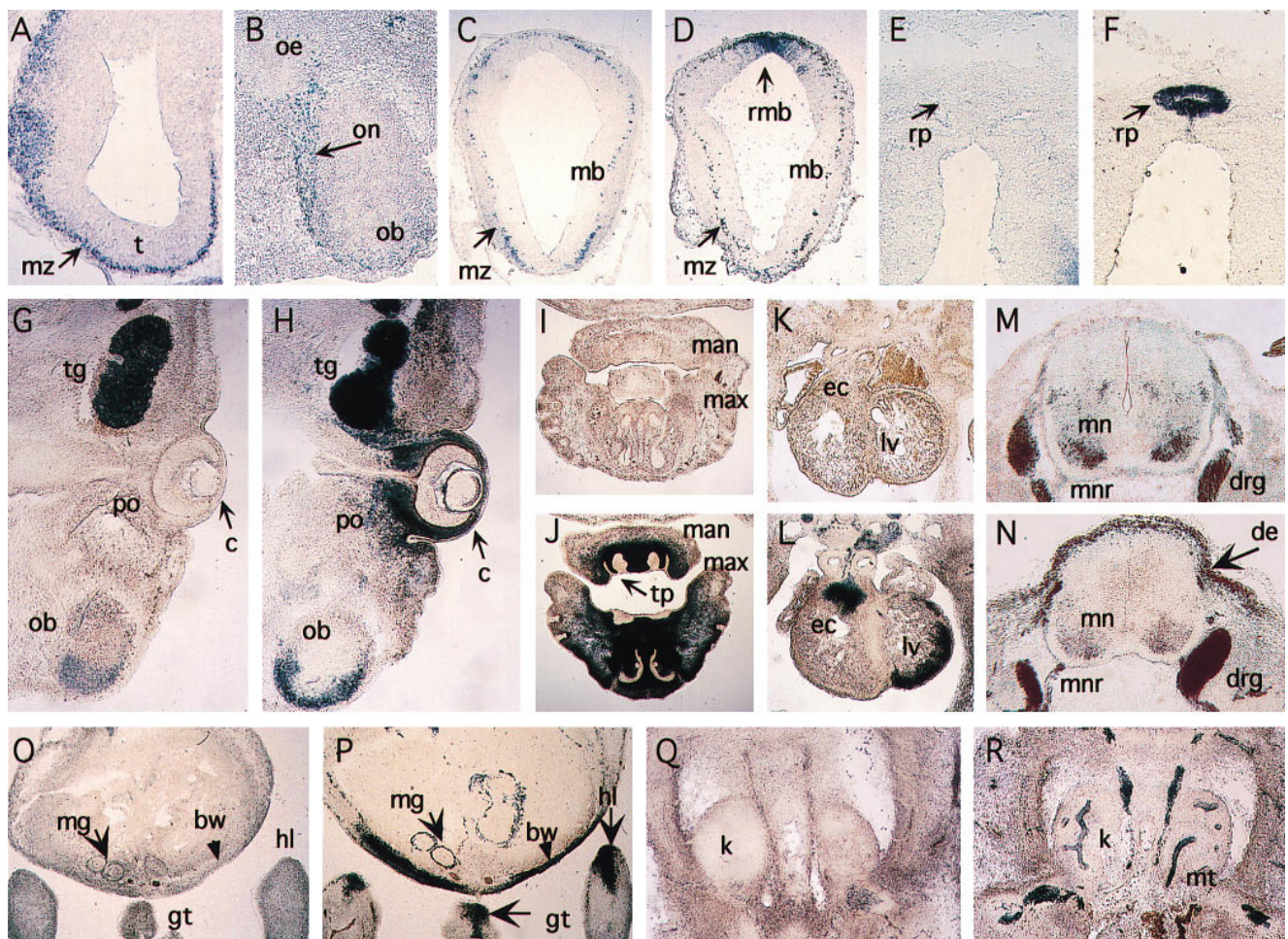
rounding the eye and in the frontonasal mass. Expression was widespread in the cephalic mesenchyme, branchial arches 1 and 2, and in the circumpharyngeal tract (Fig. 5 G; *cm*, *b1*, *b2*, and *ct*). In the trunk, expression of L1lacZΔN was also evident in the posterior mesoderm (Fig. 5 G; *pm*). In the PNS, expression of the L1lacZΔN was observed earlier than that of the L1lacZ transgene appearing at E9.5–10.5, when neural crest cells were condensing into the primordia of the cranial and dorsal root ganglia (Fig. 5, G and H). In the CNS, expression of the L1lacZΔN transgene appeared in the midbrain, telencephalon, and spinal cord in a pattern that was very similar to that of the L1lacZ transgene (compare Fig. 5, H–J, to C–E).

Between E10.5 and E12.5 the L1lacZΔN transgene continued to be expressed during the differentiation of neural crest cells into both neural and mesenchymal tissues. Expression of the transgene persisted in the periorcular re-

gion, the snout, and in dorsal cranial regions, particularly within an area overlapping the telencephalon and midbrain, and over the hindbrain (Fig. 5, H–J). In the PNS between E11.5 and E12.5, the L1lacZΔN transgene was expressed in the fully differentiated cranial and dorsal root ganglia, the sympathetic chain, and in peripheral nerves (Fig. 5, I and J; *drg*, *sc*, and *pn*). Between E10.5 and E12.5, expression of L1lacZΔN was prominent in the dorsal ectoderm, particularly over the hindbrain and cervical spinal cord, and extended over the entire ventral body wall and into the limbs (Fig. 5, H–J; *de* and *bw*).

#### Expression of the L1lacZΔN Transgene during Limb Development

As an example of ectopic extraneural expression, the pattern of appearance of the L1lacZΔN transgene was fol-



**Figure 7.** Comparison of the patterns of  $\beta$ -galactosidase expression in sections of embryos carrying either the L1lacZ transgene (A–C, E, G, I, K, M, O, and Q) or the L1lacZΔN transgene (D, F, H, J, L, N, P, and R) transgenes. All transverse sections were taken from E12.5 embryos, except for those taken at the level of the mandibular and maxillary processes (I and J), which were taken from E13.5 embryos. Sections were taken at the level of the telencephalon (A), the olfactory nerve (B), the midbrain (C and D), diencephalon and Rathke's pouch (E and F), eye and trigeminal ganglion (G and H), heart (K and L), thoracic spinal cord (M and N), hindlimbs (O and P), and the kidneys (Q and R). Abbreviations for anatomical structures: *bw*, body wall; *c*, cornea; *de*, dorsal ectoderm; *drg*, dorsal root ganglia; *ec*, endocardial cushion; *gt*, genital tubercle; *hl*, hindlimb; *k*, kidney; *lv*, left ventricle of the heart; *man*, mandibular process; *max*, maxillary process; *mg*, midgut; *mn*, motoneurons; *mnr*, motoneuron roots; *mt*, metanephric tubule; *mz*, marginal zone; *ob*, olfactory bulb; *oe*, olfactory epithelia; *on*, olfactory nerve; *po*, periocular skeleton; *rmb*, roof of midbrain; *rp*, Rathke's pouch; *tg*, trigeminal ganglion; *tp*, tooth primordium.



Table I. Cellular Expression of L1lacZ and L1lacZΔN Transgenes

		L1lacZ	L1lacZΔN
Neural Crest			
Neural (PNS)	Cranial sensory ganglia + nerves	+	+
	Spinal DRG	+	+
	Sympathetic ganglia	+	+
	Adrenal medulla	+	+
Mesenchymal	Cephalic mesenchyme	-	+
	Heart : endocardial cushion	-	+
	Mandibular + maxillary processes	-	+
CNS	Postmitotic neurons in brain, spinal cord	+	+
	Rathke's pouch	-	+
Mesoderm	Limb mesenchyme	-	+
	Nephric mesenchyme	-	+
	Ventral body wall	-	+
	Genital tubercle	-	+
Ectoderm	AER	-	+
	Dorsal ectoderm	-	+

lowed in more detail in limbs that were isolated from embryos between E10.5 and E13.5 (Fig. 6, A–D). In the anterior limb bud at E10.5, expression was seen in the apical ectodermal ridge (Fig. 6 A; *aer*). At E11.5, expression became more prominent in the anterior portion of the limb and also along the nerves (Fig. 6 B; *am*, *pn*). At E12.5, expression of β-galactosidase was observed in both anterior and posterior portions of the limb (Fig. 6 C; *am*, *pnz*). At this time, extensive staining was observed in the nuclei of cells along peripheral nerves that penetrate and ramify within the limb. Since the lacZ reporter gene contained the nuclear localization signal, much of the staining along the nerves is likely to be in the nuclei of the precursors of peripheral glial cells ensheathing the nerves. By E13.5, expression of the L1lacZΔN transgene was prominent in the interdigital mesenchyme and persisted in cells ensheathing the nerves (Fig. 6 D; *im*). Such apical ectodermal ridge and interdigital β-galactosidase staining was not observed in embryos carrying the L1lacZ transgene (data not shown).

#### Deletion of the NRSE Releases Silencing of L1 Gene Expression in Nonneural Derivatives of Neural Crest and in Mesodermal and Ectodermal Cells

To provide further histological analysis of the cell populations within differentiating tissues in which the L1lacZ and L1lacZΔN transgenes showed differences in their expression, transverse sections were taken from embryos at various positions along the anteroposterior axis and examined for cellular expression of β-galactosidase. All tissue sites showing differences in the cellular expression patterns between these two transgenes are illustrated in Fig. 7. The common and ectopic sites of expression for the two transgenes are also summarized in Table I.

Embryos carrying the L1lacZ transgene showed a neurally restricted pattern of β-galactosidase expression that is consistent with the known pattern of L1 expression in

postmitotic neurons and peripheral glia. The L1lacZ transgene was expressed in the marginal zones of the telencephalon and midbrain (Fig. 7, A and C; *mz*), in the olfactory nerve (Fig. 7 B; *on*), and in the intermediate zone of the olfactory bulb (Fig. 7, B and G; *ob*). In the spinal cord, L1lacZ was expressed in the mantle layer in motoneurons and in cells surrounding the motor and sensory roots (Fig. 7 M; *mn* and *mnr*). The pattern of L1lacZΔN transgene expression was identical to the L1lacZ pattern in all of these neural cells (for examples, see Fig. 7, D, H, and N; *mz*, *ob*, *mn*, and *mnr*). In addition to these common regions of expression, the L1lacZΔN transgene showed intense expression in the neuroepithelia within the roof of the midbrain and in Rathke's pouch (Fig. 7, compare C and D, E and F; *rm* and *rp*).

In the PNS, both transgenes were expressed in the trigeminal (Fig. 7, G and H; *tg*), facio-acoustic, and glossopharyngeal ganglia. Expression of both transgenes was observed in the sympathetic chain and the vagus nerve, although the expression by the L1lacZΔN was significantly more intense than that of the L1lacZ transgene (data not shown). Both transgenes were also expressed in dorsal root ganglia and in the nuclei of cells ensheathing fiber bundles that project toward the periphery (Fig. 7, M and N; *drg*).

In contrast to L1lacZ embryos, mice carrying the L1lacZΔN transgene showed extensive β-galactosidase staining in nonneural tissues. The L1lacZΔN transgene showed intense expression in craniofacial mesenchymal tissues, particularly in the periocular skeleton and the cornea, and in the mandibular and maxillary processes. L1lacZ embryos showed no β-galactosidase expression in these areas (Fig. 7, compare G and H, and I and J; *po*, *c*, *man*, and *max*). The heart revealed little if any expression of the L1lacZ transgene (Fig. 7 K) but showed intense expression of the L1lacZΔN transgene in the endocardial cushion tissue and in the wall of the left ventricle (Fig. 7 L; *ec* and *lv*). In addition to these sites, the L1lacZΔN transgene was expressed in the dorsal ectoderm overlying the spinal cord (Fig. 7 N; *de*).

In the abdominal region, both transgenes were expressed in the presumptive enteric ganglia surrounding the esophagus, stomach, midgut, and duodenum. However, the L1lacZΔN transgene showed more intense expression in the cells surrounding these tissues (Fig. 7, compare O and P; *mg*). The L1lacZΔN transgene was also expressed in the mesoderm of the abdominal body wall, the genital tubercle, and the hindlimb (Fig. 7 P; *bw*, *gt*, and *hl*). Expression of both transgenes was observed in the abdominal paraganglia and later (at E13.5) in the adrenal medulla (data not shown). At E12.5, the L1lacZΔN transgene was expressed in the kidney within the metanephric tubules and the ureter (Fig. 7 R; *k* and *mt*). Such expression was not observed in embryos carrying the L1lacZ transgene (Fig. 7 Q).

#### Discussion

The present studies provide evidence that the tissue-specific expression of the L1 adhesion molecule is modulated by an NRSE that silences the promoter of the L1 gene. In initial studies, we identified and sequenced a previously

uncharacterized segment of 13 kb at the 5' end of the mouse L1 gene. Our results show that the first exon of the L1 gene is actually located 10 kb further upstream of exon A, the previously identified first exon (22). Two types of analysis indicate that this is in fact the first exon of the L1 gene. First, all of the sequences obtained from cDNA clones generated by RACE were included in this exon. Second, RNase protection analyses showed multiple start sites in the L1 genomic sequence that were immediately upstream of the sequence found in our cDNA clones produced by RACE.

By searching the human L1 gene sequence, we identified an exon comparable to the one we defined for mouse; it too is about 10 kb upstream of the 5'-most exon that was assigned previously. The mouse and human L1 genes are very similar in the region of the proximal promoter and first exon. Both genes contain binding sites for the transcription factor SP1 and share a segment of ~60 bp that include binding sites for nuclear factor 1 (NF-1) and the CCAAT-binding protein (CBP). Further analyses of these DNA elements and use of transcription initiation sites will be required to assess their role in the regulation of L1 gene expression.

The human L1 gene is located on the X chromosome near the region known to cause the fragile X syndrome, and mutations in the human L1 gene have recently been linked to three syndromes: X-linked spastic paraplegia (SPG1); mental retardation, aphasia, shuffling gait, and adducted thumbs (MASA); and X-linked hydrocephalus (HSAS) (18, 45, 53). So far, the L1 mutations that give rise to these disorders have been mapped to the coding exons of the L1 gene. It may be of interest to determine whether mutations in the promoter might also contribute to some of these syndromes. For example, transcriptional start sites for the L1 gene include two sets of trinucleotide repeats, and expansion of such repeats has been associated with a number of congenital disorders of the nervous system (25).

### ***The NRSE and REST/NRSF Silence Expression of the Mouse L1 Gene In Vitro***

In cellular transfection experiments, the L1 promoter by itself was expressed in both neural (N2A) and nonneural (NIH3T3) cells. However, when combined with downstream segments of the gene that included the first intron and the NRSE, the promoter was active only in neural (N2A) cells. Two copies of the NRSE were sufficient to silence the activity of L1 gene constructs in NIH3T3 cells. This silencing was eliminated upon expression of a truncated version of REST/NRSF (called D-REST), which is known to release silencing by the NRSE (4). Similarly, expression of the full-length REST/NRSF protein in N2A cells resulted in a reduction in the activity of NRSE-containing constructs. These data are in accord with the conclusion that the NRSE mediates silencing of L1 gene expression in nonneuronal cells that normally express REST/NRSF.

In addition to the NRSE, sequences in the first intron partially silenced L1 gene expression in NIH3T3 cells, and optimal silencing was observed when both the NRSE and the first intron were included in L1 gene constructs. The

DNA sequence of the entire first intron does not contain any additional NRSEs, but expression of REST/NRSF silenced the activity of a construct containing this intron alone. These results suggest that there may be an unidentified sequence in the first intron, not related to the NRSE, that binds to REST/NRSF. Alternatively, a silencer protein other than REST/NRSF may bind to sequences in the first intron and silence L1 gene expression by interacting with REST/NRSF.

### ***The Transgene L1lacZ Has a Neurally Restricted Pattern of Expression and Deletion of the NRSE Leads to Premature and Ectopic Expression in Nonneural Cells***

L1 is found predominantly on postmitotic neurons in the CNS and PNS and is also expressed by premyelinating Schwann cells in the peripheral nervous system (35, 38, 44, 49). A segment of the L1 gene that included the promoter, the first four exons, and the first three introns (L1lacZ) was sufficient in 12 independent lines of transgenic mice to give an identical neurally restricted pattern consistent with the normal pattern of L1 expression. In addition to the neurally restricted pattern obtained with L1lacZ, six lines showed some nonneural expression of  $\beta$ -galactosidase in a subset of the tissues (primarily the cephalic mesenchyme) in which the construct lacking the NRSE, L1lacZ $\Delta$ N, was also expressed. These results suggest that silencing of L1lacZ expression may be partially released when the transgene is integrated in some genomic locations.

Absence of the NRSE in the L1lacZ $\Delta$ N construct led to extensive extraneural expression of  $\beta$ -galactosidase in the embryo. All four of the L1lacZ $\Delta$ N lines had identical patterns of expression in nonneural cells; none of these lines showed the neurally restricted pattern that was seen with the L1lacZ transgene. These cells included nonneural derivatives of the neural crest and some cells of mesodermal and ectodermal origin (see Table I). However, in the CNS, expression of the L1lacZ $\Delta$ N transgene was indistinguishable from expression of the unmutated L1lacZ transgene, and both transgenes were expressed in neural derivatives of the neural crest, including neurons and glia of the dorsal root, sympathetic, parasympathetic, and enteric ganglia and chromaffin cells of the adrenal medulla.

The L1lacZ $\Delta$ N transgene was expressed earlier than the L1lacZ transgene in the neural crest cells of the head and trunk, before their differentiation into the peripheral nervous system and mesenchymal tissues. This precocious expression was accompanied by the ectopic expression of the L1 gene in mesenchymal derivatives of the neural crest, such as the connective tissue cells of the head and face and the cells that make up part of the aorticopulmonary septum of the heart. In addition, our preliminary studies of newborn mice indicate that the L1lacZ $\Delta$ N, but not the L1lacZ transgene, is expressed in the precursors of melanocytes (unpublished data). These results indicate that the NRSE and REST/NRSF are likely to play a key role in the further differentiation of the neural lineage from the ectomesenchymal lineage of neural crest.

The precocious expression of the L1lacZ $\Delta$ N transgene in neural crest cells also resulted in a more intense level of  $\beta$ -galactosidase expression than the L1lacZ transgene

along several tracts of peripheral nerves, such as those innervating the limb and gut. Although more thorough analyses using specific molecular markers are required, the cells showing the elevated  $\beta$ -galactosidase expression appear to be Schwann cell precursors. Recent studies have shown that L1 is expressed early during the differentiation of immature, premyelinating Schwann cells but is downregulated in mature Schwann cells (15). The more intense expression of L1 that we observed in the PNS of the limb upon deletion of the NRSE may represent a release from silencing of the L1 gene in myelinating Schwann cells. The NRSE in the L1 gene may therefore play a role in the downregulation of L1 expression that occurs in mature Schwann cells.

### **Multiple Silencers May Be Required to Prevent Expression of L1 in the Full Spectrum of Nonneural Tissues**

REST/NRSF mRNA and protein are present in a majority of nonneuronal cell types (4, 23, 37, 47) and in undifferentiated neural precursors (4, 47), suggesting that the presence of REST/NRSF prevents precocious or full expression of the neuronal phenotype during early neurogenesis. Deletion of the NRSE sequence from neuronal genes might therefore be expected to lead to expression of neuronal genes in both neuronal precursors and nonneuronal cells. Previous studies of the NRSE in the SCG10 gene (54) and the rat gene for the Na,K-ATPase  $\alpha$ 3 subunit (40) showed that deletion of the element led to expression of these genes in a number of nonneural tissues, such as heart, liver, kidney, lung, and ovaries. However, analyses of the expression of these genes at the cellular level were not performed.

As indicated above, our results demonstrate that deletion of the NRSE in the L1 gene leads to precocious expression of L1 in those neural crest cells that ultimately form the peripheral nervous system and to ectopic expression in all nonneural derivatives of the neural crest as well as in cells of mesodermal and ectodermal origin. The data suggest that the REST/NRSF protein may play a role in the differentiation of neural crest cells, restricting the expression of certain genes to neural and glial derivatives. We did not see ectopic expression in a number of other tissues, such as liver, spleen, or ovaries, or in neural precursors of the central nervous system, all of which express REST/NRSF. Therefore, elimination of the NRSE does not lead to L1 expression in the full spectrum of tissues in which REST/NRSF is expressed. Recent evidence suggests that multiple restriction points are likely to exist during the differentiation of the CNS (5, 28, 52). It is therefore likely that REST/NRSF is only one of the many factors that restrict the expression of certain genes to neurons. These observations and our *in vitro* studies suggest that there are additional silencers in the L1 gene that act either independently or in concert with the NRSE and REST/NRSF. At least one such element is localized to the first intron. It will be of interest to identify this silencer and the regulatory proteins to which it binds. Such studies may reveal specific combinations of factors required for silencing of L1 gene expression in different tissues.

A question arises about the factors that activate the L1 promoter in the nonneural tissues of the mice containing

the transgene in which the NRSE is deleted. There is a striking correlation between the tissue patterns and cells that express the L1lacZ $\Delta$ N transgene and the expression patterns of the Msx homeodomain proteins (29–31). A tenable hypothesis is that in the absence of the NRSE, expression of the L1 gene may be induced directly by Msx homeodomain proteins or indirectly by factors that induce the expression of Msx genes, such as members of the bone morphogenetic protein (BMP) family (1, 6, 27, 56). In support of this possibility, BMP-2, -4, and -7 have been shown to induce expression of L1 in neuroblastoma–glioma NG-108-15 cell line (42, 43). In addition, deletion of the NRSE may allow the activation of L1 gene expression by BMPs outside of the nervous system. For instance, BMP-4 and its receptors are expressed in a pattern that closely resembles the expression of L1lacZ $\Delta$ N transgene in apical ectodermal ridge, the anterior mesenchyme, and interdigital zone of the limb (20, 55, 56).

The present studies provide a transgenic mouse model for understanding the mechanisms underlying activation of the L1 gene by such factors and for further analysis of how this activation might be silenced by the NRSE and REST/NRSF. They also provide a basis for future studies of the components that positively regulate the neuronal expression of the L1 gene as well as those that may misregulate its expression, resulting in abnormal neural development and function.

We are grateful to Madhu Katragadda for excellent technical assistance. We thank Drs. Bruce Cunningham, Kathryn Crossin, Joseph Gally, Leslie Krushel, and George Miklos for critical reading of the manuscript.

This work was supported by U.S. Public Health Service Grants HD33576 (to G.M. Edelman) and NS34493 (to F.S. Jones) and a grant from the G. Harold and Leila Mathers Charitable Trust. G.M. Edelman is a consultant to Becton Dickinson and Company.

Received for publication 3 April 1997 and in revised form 19 June 1997.

### **References**

1. Arkell, P., and R.S.P. Beddington. 1997. BMP-7 influences patterns and growth of the developing hindbrain of mouse embryos. *Development (Camb.)* 124:1–12.
2. Burgoon, M.P., M. Grumet, V. Mauro, G.M. Edelman, and B.A. Cunningham. 1991. Structure of the chicken neuron–glia cell adhesion molecule, Ng-CAM: origin of polypeptides and relation to Ig superfamily. *J. Cell Biol.* 112:1017–1029.
3. Chalepakis, G., J. Wijnholds, P. Giese, M. Schachner, and P. Gruss. 1994. Characterization of Pax-6 and Hoxa-1 binding to the promoter region of the neural cell adhesion molecule L1. *DNA Cell Biol.* 13:891–900.
4. Chong, J.A., J. Tapia-Ramírez, S. Kim, J.J. Toledo-Aral, Y. Zheng, M.C. Boutros, Y.M. Altshuler, M.A. Frohman, S.D. Kraner, and G. Mandel. 1995. REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. *Cell* 80:949–957.
5. Davis, J.Q., and V. Bennett. 1994. Ankyrin binding activity shared by the neurofascin/L1/NrCAM family of nervous system cell adhesion molecules. *J. Biol. Chem.* 269:27163–27166.
6. Dewulf, N., K. Verschuere, O. Lonnoy, A. Moren, S. Grimsby, K.V. Spiegel, K. Miyazono, D. Huylebroeck, and P.T. Dijke. 1995. Distinct spatial and temporal expression patterns of two type II receptors for bone morphogenetic proteins during mouse embryogenesis. *Endocrinology* 136:2652–2663.
7. Dodd, J., and T.M. Jessell. 1988. Axon guidance and the patterning of neuronal projections in vertebrates. *Science (Wash. DC)* 242:692–699.
8. Dynan, W.S., and R. Tjian. 1985. Control of eukaryotic mRNA synthesis by sequence-specific DNA binding proteins. *Nature (Lond.)* 316:774–778.
9. Edelman, G.M., and K.L. Crossin. 1991. Cell adhesion molecules: implications for a molecular histology. *Annu. Rev. Biochem.* 60:155–190.
10. Fields, D., and K. Itoh. 1996. Neural cell adhesion molecules in activity-dependent development and synaptic plasticity. *Trends Neurosci.* 19:473–480.
11. Frohman, M.A., M.K. Dush, and G.R. Martin. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single



- gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA*. 85: 8998–9002.
12. Furley, A.J., S.B. Morton, D. Manalo, D. Karagogeos, J. Dodd, and T.M. Jessel. 1990. The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite outgrowth-promoting activity. *Cell*. 61: 157–170.
  13. Grumet, M., and G.M. Edelman. 1984. Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by a specific cell adhesion molecule. *J. Cell Biol.* 98:1746–1756.
  14. Grumet, M., V. Mauro, M.P. Burgoon, G.M. Edelman, and B.A. Cunningham. 1991. Structure of a new nervous system glycoprotein, Nr-CAM, and its relationship to subgroups of neural cell adhesion molecules. *J. Cell Biol.* 113:1399–1412.
  15. Guenard, V., L.A. Gwynn, and P.M. Wood. 1995. Transforming growth factor- $\beta$  blocks myelination but not ensheathment of axons by Schwann cells in vitro. *J. Neurosci.* 15:419–428.
  16. Hogan, B., F. Costantini, and E. Lacy. 1986. *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1–323.
  17. Jones, F.S., C. Kiousi, D.W. Copertino, P. Kallunki, B.H. Holst, and G.M. Edelman. 1997. *Barx2*, a new homeobox gene of the *Bar* class is expressed in neural and craniofacial structures during development. *Proc. Natl. Acad. Sci. USA*. 94:2632–2637.
  18. Jouet, M., A. Rosenthal, G. Armstrong, J. MacFarlane, R. Stevenson, J. Paterson, A. Metzner, V. Ionasescu, K. Temple, and S. Kenrick. 1994. X-linked spastic paraplegia (SPG1), MASA syndrome and X-linked hydrocephalus result from mutations in the *L1* gene. *Nat. Genet.* 7:402–407.
  19. Kallunki, P., S. Jenkinson, G.M. Edelman, and F.S. Jones. 1995. Silencer elements modulate the expression of the gene for the neuron-glia cell adhesion molecule, Ng-CAM. *J. Biol. Chem.* 270:21291–21298.
  20. Kawakami, Y., T. Ishikawa, M. Shimabara, N. Tanda, M. Enomoto-Iwamoto, M. Iwamoto, T. Kuwana, A. Ueki, S. Noji, and T. Nohno. 1996. BMP signaling during bone pattern determination in the developing limb. *Development (Camb.)*. 122:3557–3566.
  21. Kayyem, J.F., J.M. Roman, E.J. delaRosa, U. Schwartz, and W.J. Dreyer. 1992. Bravo/Nr-CAM is closely related to the cell adhesion molecules L1 and Ng-CAM and has a similar heterodimer structure. *J. Cell Biol.* 118: 1259–1270.
  22. Kohl, A., K.P. Giese, M.H. Mohajeri, D. Montag, M. Moos, and M. Schachner. 1992. Analysis of promoter activity and 5' genomic structure of the neural cell adhesion molecule L1. *J. Neurosci. Res.* 32:167–177.
  23. Kraner, S.D., J.A. Chong, H.-J. Tsay, and G. Mandel. 1992. Silencing the type II sodium channel gene: a model for neural-specific gene regulation. *Neuron*. 9:37–44.
  24. Krushel, L.A., A.L. Prieto, B.A. Cunningham, and G.M. Edelman. 1993. Expression patterns of the cell adhesion molecule Nr-CAM during histogenesis of the chick nervous system. *Neuroscience*. 53:797–812.
  25. La Spada, A.R., H.L. Paulson, and K.H. Fischbeck. 1994. Trinucleotide repeat expansion in neurological disease. *Ann. Neurol.* 36:814–822.
  26. Lemmon, V., and S.C. McLoon. 1986. The appearance of an L1-like molecule in the chick primary visual pathway. *J. Neurosci.* 6:2987–2994.
  27. Luo, G., C. Hofmann, A.L. Bronckers, M. Sohocki, A. Bradley, and G. Karsenty. 1995. BMP-7 is an inducer of nephrogenesis, and is also required for eye development and skeletal patterning. *Genes Dev.* 9:2808–2820.
  28. Luskin, M.B. 1993. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron*. 11:173–189.
  29. Lyons, G.E., D. Houzelstein, D. Sassoon, B. Robert, and M.E. Buckingham. 1992. Multiple sites of Hox-7 expression during mouse embryogenesis: comparison with retinoic acid receptor mRNA localization. *Mol. Reprod. Dev.* 32:303–314.
  30. MacKenzie, A., M.W.J. Ferguson, and P.T. Sharpe. 1992. Expression patterns of the homeobox gene, *Hox-8*, in the mouse embryo suggest a role in specifying tooth initiation and shape. *Development (Camb.)*. 115:403–420.
  31. Mackenzie, A., G.L. Leeming, A.K. Jowett, M.W.J. Ferguson, and P.T. Sharpe. 1991. The homeobox gene *Hox 7.1* has specific regional and temporal expression patterns during early murine craniofacial embryogenesis, especially tooth development *in vivo* and *in vitro*. *Development (Camb.)*. 111:269–285.
  32. Martini, R., and M. Schachner. 1986. Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM and MAG) and their shared carbohydrate epitope and myelin basic protein in developing sciatic nerve. *J. Cell Biol.* 103:2439–2448.
  33. Maue, R.A., S.D. Kraner, R.H. Goodman, and G. Mandel. 1990. Neuron-specific expression of the rat brain type II sodium channel is directed by upstream regulatory elements. *Neuron*. 4:223–231.
  34. Mayford, M., A. Barzilai, F. Keller, S. Schacher, and E.R. Kandel. 1992. Modulation of an NCAM-related adhesion molecule with long-term synaptic plasticity in *Aplysia*. *Science (Wash. DC)*. 256:638–644.
  35. Mirsky, R., K.R. Jessen, M. Schachner, and C. Goridis. 1986. Distribution of the adhesion molecules N-CAM and L1 on peripheral neurons and glia in adult rats. *J. Neurocytol.* 15:799–815.
  36. Moos, M., R. Tacke, H. Scherer, D. Teplow, K. Fruh, and M. Schachner. 1988. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature (Lond.)*. 334:701–703.
  37. Mori, N., C. Schoenherr, D.J. Vandenberg, and D.J. Anderson. 1992. A common silencer element in the SCG10 and type II Na<sup>+</sup> channel genes binds a factor present in nonneuronal cells but not in neuronal cells. *Neuron*. 9:45–54.
  38. Moscoso, L.M., and J.R. Sanes. 1995. Expression of four immunoglobulin superfamily adhesion molecules (L1, Nr-CAM/Bravo, neurofascin/ABGP, and N-CAM) in the developing mouse spinal cord. *J. Comp. Neurol.* 352:321–334.
  39. Muller, D., C. Wang, G. Skibo, N. Toni, H. Cremer, V. Calaora, G. Rougon, and J.Z. Kiss. 1996. PSA-NCAM is required for activity-induced synaptic plasticity. *Neuron*. 17:413–422.
  40. Pathak, B.G., J.C. Neumann, M.L. Croyle, and J.B. Lingrel. 1994. The presence of both negative and positive elements in the 5'-flanking sequence of the rat Na, K-ATPase  $\alpha 3$  subunit gene are required for brain expression in transgenic mice. *Nucleic Acids Res.* 22:4748–4755.
  41. Pearson, W.R., and D.J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA*. 85:2444–2448.
  42. Perides, G., R.M. Safran, D.C. Rueger, and M.E. Charness. 1992. Induction of the neural cell adhesion molecule and neuronal aggregation by osteogenic protein 1. *Proc. Natl. Acad. Sci. USA*. 89:10326–10330.
  43. Perides, G., R.M. Safran, L.A. Downing, and M.E. Charness. 1994. Regulation of neural cell adhesion molecule and L1 by the transforming growth factor- $\beta$  superfamily. Selective effects of the bone morphogenetic proteins. *J. Biol. Chem.* 269:765–770.
  44. Rathjen, F.G., and M. Schachner. 1984. Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1–10.
  45. Rosenthal, A., M. Jouet, and S. Kenrick. 1992. Aberrant splicing of neural cell adhesion molecule L1 mRNA in a family with X-linked hydrocephalus. *Nat. Genet.* 2:107–112.
  46. Sauerwald, A., C. Hoesche, R. Oschwald, and M.W. Kilimann. 1990. The 5'-flanking region of the synapsin-I gene—a G+C-rich, TATA-less and CAAT-less, phylogenetically conserved sequence with cell type-specific promoter function. *J. Biol. Chem.* 265:14932–14937.
  47. Schoenherr, C.J., and D.J. Anderson. 1995. The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. *Science (Wash. DC)*. 267:1360–1363.
  48. Schoenherr, C.J., A.J. Paquette, and D.J. Anderson. 1996. Identification of potential target genes for the neuron-restrictive silencer factor. *Proc. Natl. Acad. Sci. USA*. 93:9881–9886.
  49. Stallcup, W.B., L.L. Beasley, and J.M. Levine. 1985. Antibody against nerve growth factor-inducible large external (NILE) glycoprotein labels nerve fiber tracts in the developing rat nervous system. *J. Neurosci.* 5: 1090–1101.
  50. Tabor, S., and C.C. Richardson. 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. USA*. 84: 4767–4771.
  51. Timmusk, T., K. Palm, M. Metsis, T. Reintam, V. Paalme, M. Saarma, and H. Persson. 1993. Multiple promoters direct tissue-specific expression of the rat BDNF gene. *Neuron*. 10:475–489.
  52. Williams, B.P., and J. Price. 1995. Evidence for multiple precursor cell types in the embryonic rat cerebral cortex. *Neuron*. 14:1181–1188.
  53. Wong, E.V., S. Kenrick, P. Willems, and V. Lemmon. 1995. Mutations in the cell adhesion molecule L1 cause mental retardation. *Trends Neurosci.* 18:168–172.
  54. Wuenschell, C.W., N. Mori, and D.J. Anderson. 1990. Analysis of SCG10 gene expression in transgenic mice reveals that neural specificity is achieved through selective depression. *Neuron*. 4:595–602.
  55. Yokouchi, Y., J.-i. Sakiyama, T. Kameda, H. Iba, A.U. Suzuki, N. Ueno, and A. Kuroiwa. 1996. BMP-2/-4 mediate programmed cell death in chicken limb buds. *Development (Camb.)*. 122:3725–3734.
  56. Zou, H., and L. Niswander. 1996. Requirement for BMP signaling in interdigital apoptosis and scale formation. *Science (Wash. DC)*. 272:738–741.
  57. Zuellig, R.A., C. Rader, A. Schroeder, M.B. Kalousek, F. Von Bohlen und Halbach, T. Osterwalder, C. Inan, E.T. Stoekli, H.U. Afolter, and A. Fritz. 1992. The axonally secreted cell adhesion molecule, axonin-1: primary structure, immunoglobulin-like and fibronectin-type-III-like domains and glycosyl-phosphatidylinositol anchorage. *Eur. J. Biochem.* 204: 453–463.