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## BRIEF COMMUNICATION

A Fragment of the *Neurogenin1* Gene Confers Regulated Expression of a Reporter Gene In Vitro and In VivoRICHARD C. MURRAY,<sup>1</sup> STEPHEN J. TAPSCOTT,<sup>2,3</sup> JEAN W. PETERSEN,<sup>4</sup> ANNE L. CALOF,<sup>1\*</sup> AND MARY B. MCCORMICK<sup>4</sup><sup>1</sup>Department of Anatomy and Neurobiology and the Developmental Biology Center, University of California, Irvine, College of Medicine, Irvine, California<sup>2</sup>Fred Hutchinson Cancer Research Center, Seattle, Washington<sup>3</sup>Department of Neurology, University of Washington Medical Center, Seattle, Washington<sup>4</sup>Department of Medical Genetics, University of Wisconsin-Madison, Madison, Wisconsin

**ABSTRACT** The basic helix-loop-helix transcription factor *neurogenin1* is required for proper nervous system development in vertebrates. It is expressed in neuronal precursors during embryonic development and is thought to play a role in specifying neuronal fate. To investigate the regulation of *neurogenin1* expression, the transcriptional start site of the gene was identified and a 2.7-kb fragment ending in the first exon was shown to possess basal promoter activity. This 2.7-kb fragment was able to promote expression of reporter genes in P19 cells under conditions in which expression of endogenous *neurogenin1* was induced. Importantly, the 2.7-kb fragment was able to drive expression of a lacZ reporter gene in transgenic mice in most tissues in which *neurogenin1* is normally expressed, including those peripheral ganglia that fail to develop in *neurogenin1* “knockout” mice. These findings identify a regulatory region containing elements responsible for appropriate expression of a gene with a crucial role in generating the vertebrate nervous system. *Dev Dyn* 2000; 218:189–194. © 2000 Wiley-Liss, Inc.

**Key words:** *neurogenin1*; transgenic mice; P19 cells; neuronal precursor; neurogenesis; promoter; transcription factor; peripheral nervous system; central nervous system

## INTRODUCTION

In *Drosophila*, the proneural genes *achaete*, *scute*, and *atonal*, which encode basic helix-loop-helix (bHLH) transcription factors, control the determination of neural precursors. Recently, vertebrate homologues of *Drosophila* proneural genes have been cloned (Lee, 1997). One of these, *neurogenin1* [also known as *neuroD3* (McCormick et al., 1996) and *Math4C* (Cau et al., 1997)], is expressed specifically in neuronal precursor cells. Ectopic expression of *Xenopus neurogenin1* (X-

*ngnr-1*) in frog embryos induces neurogenesis in ectodermal cells that would not normally undergo neural differentiation (Ma et al., 1996). In mice lacking a functional *neurogenin1* gene, a subset of cranial sensory ganglia (Ma et al., 1998) and a subset of neurons in dorsal root ganglia (Ma et al., 1999) fail to form. Taken together, these data argue that *neurogenin1* plays a critical role in determination of specific subsets of neurons. Expression of *neurogenin1* appears to be tightly regulated during development (McCormick et al., 1996; Ma et al., 1998), suggesting that transcriptional control of *neurogenin1* is important in its regulation of neural patterning. To understand this regulation, we have identified an upstream region of the *neurogenin1* gene that appropriately regulates reporter gene expression in vitro. Experiments using transgenic mice imply that this region contains most of the cis-acting elements responsible for proper expression of endogenous *neurogenin1*.

## RESULTS AND DISCUSSION

Characterization of the *Neurogenin1* Transcription Start Site

Previous work has shown that the coding region of *neurogenin1* is contained in a single exon (McCormick et al., 1996; Cau et al., 1997; Ma et al., 1998). To determine the transcriptional start site, a primer near the beginning of the *neurogenin1* coding region was used to perform 5'RACE on total RNA isolated from P19 cells treated for 4 days with retinoic acid (RA), a condition known to induce expression of endogenous

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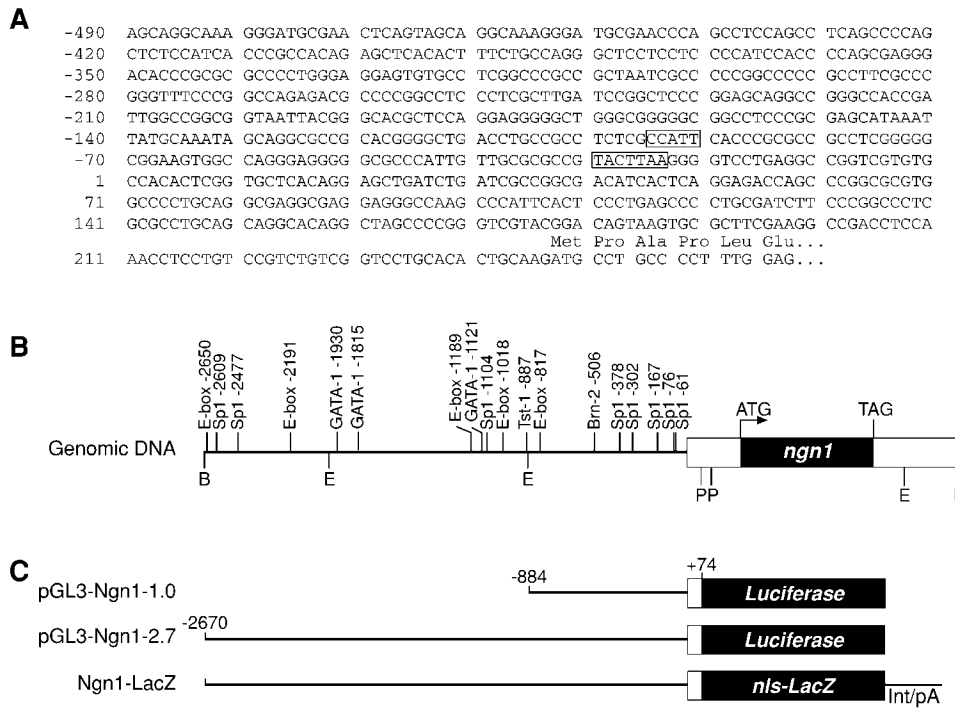


Fig. 1. **A:** Nucleic acid sequence surrounding the transcriptional start site (+1) of *neurogenin1*. The translational start site is indicated at (+248) and putative TATA and CCAAT sequences are boxed. **B:** Map of the *neurogenin1* gene. Consensus binding sites for transcription factors and restriction sites discussed in the text are indicated (B, BamHI; E, EcoRI; P, PstI). **C:** Luciferase reporter constructs (pGL3-Ngn1-1.0 and pGL3-Ngn1-2.7) and nuclear-targeted lacZ reporter construct (Ngn1-LacZ), which also contains intron and polyadenylation sequences [Int/pA] from the mouse *protamine 1* gene.

*neurogenin1* (McCormick et al., 1996). The transcriptional start site (numbered as +1 in Fig. 1A) was found to be 247 bp upstream of the translational start site. The 5' untranslated region identified here is 100% identical and 159 bp longer than the 5' UTR of the mouse *MATH4C* cDNA clone reported previously (Cau et al., 1997).

Sequence comparison indicates that the 247-bp 5' UTR and the entire coding region are both contained within exon 1 of the *neurogenin1* gene (Fig. 1A and B). This structure is similar to that reported for the *achaete-scute* homologue, *Mash1* (Verma-Kurvari et al., 1996). *Mash1* and *neurogenin1* are expressed in complementary domains in developing neuroepithelium and are both thought to act in neuronal precursors to specify neuronal fate (Ma et al., 1997). Two other neural bHLH genes, *neuroD* and *neuroD2* (Xu and Murphy, 1998; Kitamura et al., 1999), which appear to function later in development to direct neural differentiation, have a different gene structure that includes a non-coding first exon upstream of a second, coding, exon. These observations suggest the interesting possibility that similarity in function is echoed in similar structures of different subsets of bHLH genes.

For further analysis, 2.9 kb of genomic DNA including the transcriptional start site was sequenced. A putative TATA box at position -27 and a putative CCAAT box at position -93 were identified and are boxed in Figure 1A. These two sequences, as well as 26 bp surrounding the transcriptional start site, are 100% conserved in human *neurogenin1* (Genbank accession #AC005738). In addition, the 5'UTR iden-

tified here is 72% identical to sequences upstream of human *neurogenin1*, and 88% identical to the 5' UTR of rat *neurogenin1* cDNA (Ma et al., 1996), illustrating the high degree of conservation of this gene among mammals.

Additional transcription factor DNA-binding motifs were identified upstream of the transcriptional start site using Genomatix Matinspector (Quandt et al., 1995) and are indicated in Figure 1B. Interesting motifs include multiple Sp1 sites and five E-boxes, which are recognition sequences for bHLH transcription factors (Lee, 1997). The presence of E-boxes suggests that *neurogenin1* may be subject to autoregulation or cross-regulation by other bHLH transcription factors, as has been shown for *NEX-1* and *neuroD2* (Bartholoma and Nave, 1994; McCormick et al., 1996). Also noted were three GATA-1 binding sites. In *Drosophila*, the *pannier* gene, which encodes a zinc finger domain transcription factor homologous to GATA-1, has been shown to regulate expression of proneural genes and is thought to be involved in positioning proneural clusters in imaginal discs (Simpson et al., 1999). It will be interesting to see whether GATA-1 or a related gene has an analogous role in regulating sites of *neurogenin1* expression in vertebrates.

Consensus binding sites for the POU domain transcription factors Brn-2 and Tst-1 are also present. Like *neurogenin1*, expression of endogenous *Brn-2* is activated in P19 cells induced to undergo neural differentiation with RA (Hagino-Yamagishi et al., 1998), and *Brn-2* is expressed in neuronal progenitor cells in several regions in which *neurogenin1* is expressed (Al-

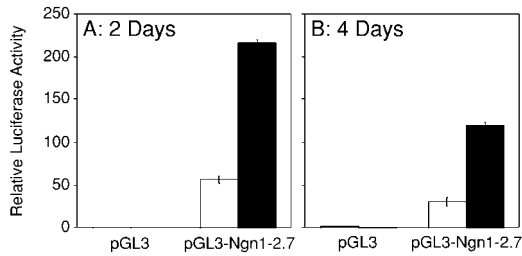


Fig. 2. P19 cells transfected with pGL3 or pGL3-Ngn1-2.7 were grown for either 2 (**A**) or 4 (**B**) days in the presence (black bars) or absence (white bars) of 0.1  $\mu$ M RA. The decrease in absolute level of luciferase activity between days 2 and 4 was likely due to loss of plasmid during the course of the transient transfection. Promoterless pGL3 showed only background levels of activity. Error bars represent standard errors of the mean from triplicate transfections.

varez-Bolado et al., 1995; Sommer et al., 1996). These observations suggest that *Brn-2* could be involved in regulating *neurogenin1* expression, at least in some regions of the developing nervous system.

### Regulation of Reporter Gene Expression In Vitro

To identify sequences likely to contain elements required for regulated expression, two fragments containing the *neurogenin1* transcriptional start site and extending 2.7 kb or 0.9 kb upstream were tested for their relative abilities to drive expression of a luciferase reporter gene in NSH neuroblastoma cells. These reporter constructs (pGL3-Ngn1-2.7 and pGL3-Ngn1-1.0, Fig. 1C) were tested in transient transfection assays as previously described (McCormick et al., 1996). The pGL3-Ngn1-2.7 construct showed approximately 5-fold greater activity than pGL3-Ngn1-1.0 (data not shown), and so was selected for use in subsequent experiments. To determine whether the 2.7 kb *neurogenin1* fragment directs reporter gene expression appropriately in vitro, the activity of pGL3-Ngn1-2.7 was measured in P19 cells undergoing RA-induced neural differentiation. Cells were transfected with pGL3-Ngn1-2.7 and grown aggregated in petri dishes for 2 or 4 days, in the presence or absence of 0.1  $\mu$ M RA. As shown in Figure 2, aggregation in the presence of RA for 2 or 4 days resulted a 3.8-fold increase in luciferase activity relative to aggregation alone. Since *neurogenin1* mRNA can be detected in P19 cells by Northern blot analysis after aggregation in the presence of RA at 2 and 4 days (McCormick et al., 1996), these data indicate that the 2.7-kb fragment directs appropriately-regulated expression of the luciferase reporter. The 2.7-kb fragment was then cloned into pnlacF (Mercer et al., 1991), a reporter construct useful for in vivo experiments (Ngn1-LacZ, Fig. 1C). When Ngn1-LacZ was transfected into P19 cells and tested in the RA-induction paradigm,  $\beta$ -galactosidase expression was induced in a manner similar to that observed using the luciferase reporter gene (data not shown). Thus, the Ngn1-

**TABLE 1. Expression of Ngn1-LacZ in Transgenic Founder Embryos**

	Frequency of expression (% of founder embryos)
<b>PNS</b>	
Vestibulo-cochlear (VIII) ganglion <sup>a</sup>	63
Geniculate (VII) ganglion	50
Petrosal (IX) ganglion	38
Nodose (X) ganglion	38
Trigeminal ganglion <sup>a</sup>	25
Dorsal root ganglia <sup>a</sup>	25
Accessory ganglion <sup>a</sup>	25
Jugular ganglion <sup>a</sup>	25
Superior ganglion <sup>a</sup>	25
Otic vesicle	0
Olfactory epithelium	0
<b>CNS</b>	
Ventral neural tube	75
Mesencephalon <sup>b</sup>	50
Hindbrain <sup>b</sup>	50
Dorsal neural tube	25
Dorsal telencephalon <sup>b</sup>	25
Diencephalon <sup>b</sup>	25

<sup>a</sup>These structures, or subsets of neurons in them, are not formed in *neurogenin1*  $-/-$  mice (Ma et al., 1998; Ma et al., 1999).

<sup>b</sup>Staining was primarily in the ventricular zone.

LacZ construct is functional and the *lacZ* reporter gene behaves similarly to the luciferase reporter.

### Reporter Gene Expression In Vivo

To determine if the 2.7-kb fragment contains sequences required for correct expression of *neurogenin1* in vivo, transgenic mice were generated using the Ngn1-LacZ construct. Founder embryos were analyzed at day 11.5, a time when expression of endogenous *neurogenin1* has commenced in most locations (Sommer et al., 1996; Cau et al., 1997; Ma et al., 1998; Ma et al., 1999). A total of 51 transgenic embryos were obtained from 101 embryos generated in seven rounds of injections. Of the 51 transgenic embryos, eight embryos expressed *lacZ* as determined by X-gal staining.

The summary of expression patterns for all founder embryos is shown in Table 1. Examples of expression observed in the peripheral nervous system (PNS) are illustrated in Figure 3A–E. Regions of the PNS that normally express *neurogenin1* include otic vesicle, olfactory epithelium (OE), dorsal root ganglia (DRGs), and several cranial sensory ganglia (Ma et al., 1996; Sommer et al., 1996; Cau et al., 1997; Ma et al., 1997; Fode et al., 1998; Ma et al., 1998). In transgenic embryos, X-gal staining was seen in DRGs (arrowhead, Fig. 3A, B; arrows, Fig. 3C), which express *neurogenin1* from days 9–13 of embryonic development (Ma et al., 1999). This is significant, since a subset of DRG neurons is absent in animals that are homozygous null for *neurogenin1* (Ma et al., 1999). We also observed transgenic embryos in which trigeminal (V), vestibulo-cochlear (VIII), and accessory, jugular, and superior gan-



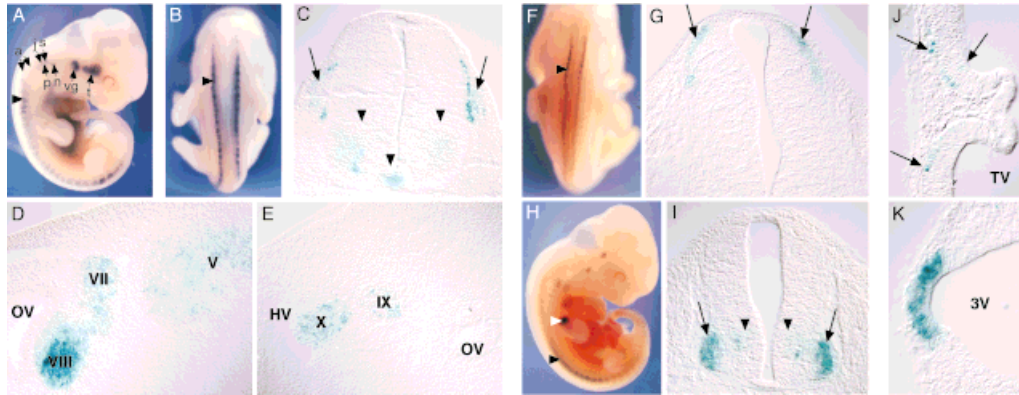


Fig. 3. **A:** Lateral view of founder embryo showing expression in trigeminal (t), vestibulo-cochlear and geniculate (vg), nodose (n), petrosal (p), superior (s), jugular (j), accessory (a), and dorsal root (arrowhead) ganglia. **B:** Dorsal view of embryo in (A) to show expression in dorsal root ganglia (arrowhead). **C:** Transverse section of founder embryo showing staining in the dorsal root ganglia (arrows) and ventral neural tube and floor plate (arrowheads). **D:** Horizontal section of founder embryo showing staining in trigeminal (V), geniculate (VII), and vestibulo-cochlear (VIII) ganglia. OV = otic vesicle, anterior = right, lateral = top of the figure. **E:** Horizontal section of founder embryo showing staining in petrosal (IX) and nodose (X) ganglia. OV = otic vesicle, HV = primary head vein, anterior = right, lateral = top of figure. **F:** Dorsal view of founder embryo showing dorsal neural tube expression (arrowhead). **G:** Trans-

verse section of embryo shown in (F) at level indicated by arrowhead. X-gal staining is present in commissural neurons in dorsal neural tube (arrows). **H:** Lateral view of founder embryo showing ventral neural tube expression (black arrowhead). White arrowhead indicates ectopic *lacZ* expression in proximal limb bud. **I:** Transverse section of embryo shown in (H) at level indicated by black arrowhead. X-gal staining is present in ventricular zone of neural tube (arrowheads) and in ventral horn motor neurons (arrows). **J:** Transverse section of founder embryo showing staining in ventricular zone of dorsal telencephalon (arrows), TV = telencephalic vesicle, dorsal = left of figure. **K:** Transverse section of founder embryo showing staining in ventricular zone of diencephalon, 3V = third ventricle, dorsal = left of figure.

glia expressed *lacZ* (Fig. 3A, D, E); these cranial ganglia are dependent on *neurogenin1* for proper development (Ma et al., 1998). The geniculate (VII), petrosal (IX), and nodose (X) ganglia, which have been reported to express *neurogenin1* at E9.5 (Fode et al., 1998; Ma et al., 1998), also expressed *lacZ* at the E11.5 timepoint studied in our experiments (Fig. 3A, D, E). In the central nervous system (CNS), expression of *neurogenin1* commences around E8.5 and mostly disappears by E16–17 (Sommer et al., 1996; Ma et al., 1998). At E11.5, *neurogenin1* is expressed in the ventricular zone of dorsal telencephalon, diencephalon, mesencephalon, and hindbrain (Ma et al., 1996; Sommer et al., 1996; Ma et al., 1997). *Neurogenin1* is also expressed in the ventral half of the neural tube, as well as a narrow domain below the roof plate (Ma et al., 1996; Sommer et al., 1996). In the CNS of transgenic embryos, *lacZ* expression was observed in the ventricular zone of the dorsal telencephalon, diencephalon, mesencephalon, and hindbrain (Fig. 3J, K and Table 1), as well as dorsal and ventral neural tube (Fig. 3G, I). Altogether, *lacZ* expression was observed in 15 of 17 sites in which *neurogenin1* is expressed at this developmental age. Notably, *lacZ* expression was never observed in regions such as the enteric and sympathetic ganglia of the PNS or the striatum of the CNS; these are regions that also fail to express endogenous *neurogenin1* (Ma et al., 1997).

Although virtually all expression in transgenic animals was seen in regions where *neurogenin1* is normally expressed, there were two regions where expression should have been observed but was not. There was

no X-gal staining observed in otic vesicle or OE (Table 1, Fig. 3D). Since *neurogenin1* expression in otic vesicle occurs transiently in young embryos [E9–10.5 (Sommer et al., 1996; Ma et al., 1998)], our failure to observe Xgal staining in this structure was likely due to the age at which our embryos were analyzed (E11.5). This is supported by our frequent observation of X-gal staining in the vestibulo-cochlear ganglion (Table 1, Fig. 3D), a derivative of the otic vesicle. OE expresses *neurogenin1* for about one week during embryonic development [E10.5–17.5 (Cau et al., 1997)]. It is possible that the 2.7 kb fragment is capable of driving expression in OE, but at such a low frequency that it was not detected in our experiments. Alternatively, lack of expression may be due to absence in this fragment of cis-regulatory elements necessary for OE expression. Since *neurogenin1* upstream sequences distal to the 2.7-kb fragment used in our experiments appear to be capable of directing reporter gene expression in OE (Jane E. Johnson, personal communication), element(s) necessary for OE expression are likely located at some distance from the proximal elements that direct most tissue-specific expression of *neurogenin1*. Interestingly, this has also been observed for another proneural gene homologue, *Mash1* (Verma-Kurvari et al., 1998).

In several animals with X-gal staining in the neural tube, staining was not confined to neural precursors in the germinal epithelium, where endogenous *neurogenin1* mRNA is normally expressed (Ma et al., 1996; Sommer et al., 1996; Ma et al., 1997). Instead, additional X-gal staining was observed in populations of neurons located lateral to, and presumably derived

from, precursors in the proliferative zone. Two examples of such staining are shown in Figure 3. In one embryo, staining was observed in presumptive commissural neurons in the dorsal neural tube (Fig. 3F and G); in a second embryo, staining was observed in ventral horn motor neurons, as well as adjacent ventral germinal neuroepithelium (Fig. 3H and I). These observations suggest that such staining is due to persistence of the  $\beta$ -galactosidase enzyme in neurons that are lineally related to *neurogenin1*-expressing precursors in the adjacent germinal neuroepithelium. This in turn implies that *neurogenin1* may be a molecular marker for precursors of both commissural and motor neurons (cf., Lee et al., 1998). Thus, these transgenic animals may provide a useful tool for studies to determine the fate of *neurogenin1*-expressing neural precursors.

### Elements Regulating Expression of *Neurogenin1*

The 2.7-kb fragment of *neurogenin1* described here contains information required to direct reporter gene expression in virtually the complete tissue-specific pattern of the endogenous gene. However, we did observe variability in the pattern of expression in individual founders. Such variability is expected with pronuclear injection experiments, due to variation in transgene insertion site, copy number, and potential mosaicism of transgene integration, and has been observed for other neural-specific promoters tested in similar experiments (Gloster et al., 1994; Verma-Kurvari et al., 1996). It may be that regulatory element(s) present in such promoter fragments are sensitive to influences from DNA at the integration site surrounding the transgene. Another possibility is that the 2.7-kb fragment contains the information required for tissue specificity, but additional enhancer elements are required for fully penetrant expression. Previous work on bHLH transcription factor genes has shown that elements required for robust tissue-specific expression can be located many kilobases upstream of the transcriptional start site. For example, although a proximal fragment of the *myoD* gene contains tissue-specific regulatory sequences, expression in vivo requires additional enhancer elements located at approximately 5 kb and 23 kb upstream of the coding region (Goldhamer et al., 1992; Tapscott et al., 1992; Asakura et al., 1995).

These data represent an important first step in characterizing the cis-acting regulatory elements responsible for tissue-specific regulation of *neurogenin1* expression. The 2.7-kb *neurogenin1* gene fragment we have identified should provide an important tool for identifying upstream genes that are involved in regulating the process of neural determination. In addition, this fragment will allow the generation of transgenic mice in which gene function can be assessed in specific populations of neural precursors.

## EXPERIMENTAL PROCEDURES

### 5' RACE and Sequencing

The 5' end of the *neurogenin1* cDNA was identified using the SMART RACE cDNA amplification kit (Clontech, Inc., Palo Alto). 5' RACE was performed using 1  $\mu$ g of total RNA isolated from P19 cells aggregated for 4 days in RA (all-trans; 0.1  $\mu$ M) as described (McCormick et al., 1996). The gene-specific primer (5'-TC-GAGATCAGAGATGCAGGT-3') was designed to hybridize 19 bp downstream of *neurogenin1*'s start ATG. PCR products were gel purified and cloned into a TA vector (Invitrogen, La Jolla, CA) for sequencing; ten independent clones were sequenced.

The 2,744-bp BamHI-PstI fragment upstream of the *neurogenin1* coding region was subcloned into pBS SK(-) and cycle sequenced using AmpliTaq DNA polymerase, FS (PE Applied Biosystems), using 1  $\mu$ g of double stranded DNA template and 6.4 pmol of primer. Sequencing primers were T3, T7 and the following gene specific primers: forward primers -2139 (5'-CCACCAC-CTCCTGGCCACCT-3'), -1668 (5'-CAGGACATCCAG-GAG-3'), -1272 (5'-CTGCCCAAGAGCTG-3'), -879 (5'-CCACTGTGTGCTTGCT-3'), -580 (5'-TAACCTTGGC-GGAGC-3'), -192 (5'-GGGCACGCTCCAGGAG-3'); and reverse primers -2409 (5'-GTACTTGAAACGCAGG-3'), -1970 (5'-GAGAATTCACCTCCCGG-3'), -1785 (5'-GATGCTCTAGGCTC-3'), -1369 (5'-GACAGATCCT-TGGGCATC-3'), -1035 (5'-GGAGCAAACCTGGATCG-3'), -730 (5'-TCTTGCTCTCTCCATTTC-3'), -329 (5'-CCTCGCTGGGGTGGATGG-3'), -120 (5'-GCGGCG-CCTGCTATTTG-3'). The entire fragment was sequenced twice in both directions to ensure accuracy. The sequence has been submitted to Genbank (Accession: AF225969).

### Constructs

The pGL3-Ngn1-2.7 and pGL3-Ngn1-1.0 luciferase reporter constructs were generated by subcloning the 2,744-bp BamHI-PstI and 958-bp EcoRI-PstI fragments, which terminate in the first exon of *neurogenin1*, into pGL3 (Promega, Madison, WI). Ngn1-LacZ was generated by subcloning the 2744-bp BamHI-PstI fragment of *neurogenin1* into pnlacF (Mercer et al., 1991).

### Tissue Culture and Transfections

Human NSH neuroblastoma cells and P19 embryonal carcinoma cells were cultured as described (Jones-Villeneuve et al., 1983; Azar et al., 1990). Cells were co-transfected with indicated luciferase reporter constructs and pCMV-lacZ reporter using Fugene (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Luciferase assays were performed using standard protocols (Ausubel, 1988). Data are expressed as the ratio of luciferase activity to  $\beta$ -galactosidase activity in a given cell extract [relative luciferase activity (Walters et al., 1995)].

## Transgenic Mice

Transgenic embryos were generated by injection of the linearized ND3-LacZ construct into pronuclei of fertilized mouse ova (CB6 F2) using standard techniques (Hogan et al., 1994). Founder embryos were harvested at day 11.5 of gestation (E11.5) and fixed for 90 min in 1% formaldehyde, 0.2% glutaraldehyde, 5 mM EGTA, 2 mM  $\text{MgCl}_2$ , 0.02% Nonidet P-40, 0.01% deoxycholate in PBS (pH 7.4) at 4°C.  $\beta$ -galactosidase staining was performed overnight in 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside), 5 mM  $\text{K}_3\text{Fe}(\text{CN})_6$ , 5 mM  $\text{K}_4\text{Fe}(\text{CN})_6$ , 2 mM  $\text{MgCl}_2$ , 0.02% Nonidet P-40, 0.01% deoxycholate in PBS at 37°C. Transgenic founder animals were identified by PCR of yolk sac DNA using primers (5'-TGATGAAAGCTGGC-TACAG-3' and 5'-ACCACCGCACGATAGAGATT-3') specific for the *lacZ* transgene. For analysis of sections, embryos were equilibrated overnight in 30% sucrose in PBS at 4°C, sectioned on a cryostat at 30  $\mu\text{M}$ , and mounted on gelatin-coated glass slides. Slides were rapidly dehydrated through xylenes and coverslipped with Protexx (Lerner Laboratories). Sections were viewed with Nomarski optics on a Zeiss Axiophot microscope and images captured using a digital Spot camera (Diagnostic Instruments).

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