REGULATION AND LINEAGE ANALYSIS OF NEUROG1 IN THE DEVELOPING SPINAL CORD

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DEDICATION

To my mom and Dad, Heman J. Quiñones and Ana L. Figueroa, for their love, support and encouragement and to my loving wife and daughters, Kenia G. Gandia, Ana and Diana, who have stood by my side every step of the way and who bring joy and meaning to my life.

REGULATION AND LINEAGE ANALYSIS OF NEUROG1 IN THE DEVELOPING SPINAL CORD

by

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ABSTRACT

The bHLH transcription factor Neurog1 is involved in neuronal differentiation and cell-type specification in distinct regions of the developing nervous system. I developed mouse models that efficiently drive expression of GFP or Cre recombinase in all Neurog1 (Ngn1, NeuroD3) domains. Deleting highly conserved sequences from a BAC containing 113kb 5' and 71kb 3' genomic sequence surrounding the *Neurog1* coding region allowed the identification of enhancer elements required to drive Neurog1 expression. I show that a 3.8 kb fragment located 4.2 kb 5' of *Neurog1* is required for efficient reporter expression in all *Neurog1* domains. This sequence contains previously identified enhancer elements for midbrain, hindbrain and dorsal neural tube, and has two sequences conserved from human to fish. A 16kb fragment containing 8.9 kb 5' and 5.2 kb 3' of the *Neurog1* coding sequence was not sufficient to drive expression in all domains. Reporter expression was observed in the dorsal neural tube, the midbrain, hindbrain and trigeminal ganglia, but was missing in the olfactory epithelium, dorsal root ganglia, dorsal telencephalon, and ventral neural tube. A 2.3 kb enhancer element located 8 kb 5' of the *Neurog1* coding region was identified that is necessary to direct expression in the ventral neural tube. In addition, these mouse models allowed both short-term and long-term lineage analyses. I show that derivatives of Neurog1-expressing progenitor cells in the neural tube largely comprise the interneuron populations dI2, dI6, V0, V1, and V2, and to a lesser extent motorneurons. This is seen in the co-expression of GFP driven by *Neurog1* regulatory sequences with the neuronal identity markers Brn3a, Islet1/2, Lhx1/5, Lhx3, Pax2, and Chx10. Genetic fate mapping in vivo using Cre recombinase reveals that although Neurog1-expressing cells primarily give rise to neurons, minor populations of oligodendrocytes and astrocytes are also identified in the lineage by adult stages in the spinal cord.

Adding temporal control to the fate mapping strategy demonstrates that the neurons are generated from Neurog1-expressing cells prior to E13, and glial cells after E13, placing Neurog1 in lineage restricted precursor cells during embryogenesis.

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PRIOR PUBLICATIONS

1. Quinones HI, Savage T, Johnson JE. Regulation of Neurog1 expression in the ventral neural tube is mediated by a distinct enhancer element. Manuscript in Preparation
2. Quinones HI, Battiste JB, Carlin E, Goodrich L, Johnson JE. Neurog1-expressing Cells Give Rise Preferentially to Interneurons in the Ventral Spinal Cord. Manuscript in Preparation
3. Sinha SK, Zachariah S, Quinones HI, Shindo M, Chaudhary PM. Role of TRAF3 and -6 in the activation of the NF-kappa B and JNK pathways by X-linked ectodermal dysplasia receptor. J Biol Chem. 2002 Nov 22; 277(47):44953-61.
4. Quinones HI, List AF, Gerner EW. Selective exclusion by the polyamine transporter as a mechanism for differential radioprotection of amifostine derivatives. Clin Cancer Res. 2002 May;8(5):1295-300.

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LIST OF DEFINITIONS

BAC- Bacterial Artificial Chromosome

bHLH- basic helix-loop-helix

BMP- Bone Morphogenetic Protein

Chlor- chloramphenicol

CNS- Central Nervous System

dI- dorsal interneuron

dIL- dorsal interneuron late

dP- dorsal progenitor

DRG- Dorsal Root Ganglion

HD- Homeodomain

HES- Hairy Enhancer of split

Kan- Kanamycin

M- Mantle

MN- Motor Neuron

Neurog1- Neurogenin 1

pMN- Motor Neuron Progenitor

PNS- Peripheral Nervous System

Shh- Sonic hedgehog

Strep- Streptomycin

TF- transcription factor

CHAPTER ONE:

Introduction

The survival of a species depends on how well these organisms are able to interact with their environment in such a way that they extend their life long enough for reproduction to occur. Evolution plays a pivotal role in species survival by selecting for phenotypes that ensure longevity and reproduction of the organism. Interaction with the environment and movement coordination were very difficult for the first multicellular organisms. However, evolution found a solution to these and other problems by way of a nervous system. The nervous system allowed for a more advanced environmental interaction which provided greater chances of survival and reproduction. As evolution progressed higher eukaryotes developed more complex systems comprised of organs, and the nervous system continued to adapt to control more and more functions. In more complex organisms, such as mammals, the nervous system is involved in ensuring the proper function of internal organs, in relaying sensory information from the periphery to the central nervous system, and then back to the motor neurons to activate the required movement, and many other functions. Thus, the nervous system is one of the most valuable evolutionary tools that ensure the survival and more extensive interaction between various species and their environment.

Correct sensory and motor processes require the proper communication between the peripheral and central nervous system, and this depends on the precise connections of neurons. The central nervous system is composed of the brain and spinal cord. The spinal cord is divided into two main regions, the grey matter and the white matter. The grey matter is composed primarily of thousands of neuronal cell types, which are classified by location, morphology, connections, and neurotransmitter phenotype (Kandel, 1991). Within the grey matter of the spinal cord are motor neurons and interneurons which facilitate movement and information relay. The sensory neuronal cell bodies are in the dorsal root ganglia (DRG), while the interneurons in the gray matter serve to integrate and process the sensory information. The grey matter is divided into two regions known

as the dorsal and the ventral horns, and these are further subdivided into various laminae, each of which contains a unique set of neurons (Altman and Bayer, 1984; Squire, 2003). The dorsal horn is organized into five laminae, which are composed of unique sets of neurons (Altman and Bayer, 1984). Lamina I or the marginal zone is primarily comprised of pyramidal neurons, flattened aspiny neurons, multipolar neurons, and fusiform spiny neurons. The substantia gelatinosa, or laminae II-III, contain small short-axoned golgi cells, larger stalked and islet cells. Lamina IV-V contain larger pyramidal neurons and medium sized neurons (Altmann and Brivanlou, 2001). This complex organization of distinct cell types with their proper connections between the spinal interneurons and sensory neurons from the periphery is dependent on the accurate specification of these cell types during neural development.

Neural specification has been studied extensively; however there is still much to be known as to how neural cell types are specified and how the molecules involved in this specification are regulated. During neurogenesis a number of extrinsic and intrinsic signals induce the formation of different neural cell types from the neural epithelium. Extrinsic factors have been shown to direct the rostro-caudal and dorso-ventral patterning of the neural tube, while neural specification is regulated by intrinsic molecules such as transcription factors. Loss-of function and gain-of function experiments have shed some light into how distinct transcription factors establish the identity of specific neuronal populations (Briscoe and Ericson, 2001; Caspary and Anderson, 2003; Helms and Johnson, 2003; Jessell, 2000). Lineage tracing experiments have also been pivotal in establishing which adult neural cell types are specified from distinct populations expressing these intrinsic factors (Machold and Fishell, 2005; Masahira et al., 2006). Understanding how transcription factors are regulated during neural cell type specification and what adult neural cells are derived from

neural progenitors expressing these factors during neurogenesis is an important question in developmental neuroscience.

Neurulation

During embryonic development, prior to neurogenesis, three main layers form during gastrulation. These layers are the ectoderm, the mesoderm, and the endoderm. The ectoderm will give rise to the epidermis, the peripheral nervous system (PNS) and central nervous system (CNS). The nervous system is derived from a group of ectodermal cells that receive signals from an underlying mesoderm derived structure, the notochord (Fig 1.1). These inductive signals from the notochord and flanking ectoderm cause the neural fold to elevate from the dorsal side of the embryo and form the neural fold. The dorsal edges continue to approach each other medially and initiate fusion, thereby forming the neural tube (Fig 1.1). Migratory cells from the dorsal fusion point, known as the neural crest cells, give rise to most of the craniofacial structures and PNS.

As neural development continues, extrinsic signals begin to induce a rostro-caudal and dorso-ventral pattern that divides the neural tube into distinct structures. The rostral portion of the neural tube gives rise to the forebrain, midbrain, and hindbrain while the caudal region forms the mature spinal cord. As the spinal cord develops it is characterized by two distinct populations; actively proliferating progenitor cells that are located close to the midline in the ventricular zone (VZ); and differentiated cells that have migrated laterally into the mantle. Within the spinal cord transcription factors begin to guide neural specification. These intrinsic signals are expressed in discrete regions of the spinal cord and the combination of transcription factors expressed within a given cell will determine the fate of that cell (Jessell, 2000; Kageyama et al., 1997; Tanabe and Jessell, 1996). Thus, the extrinsic and intrinsic signals guiding neural differentiation are essential in determining the fate of each individual neural cell type.

The neural tube produces distinct populations based on where these cells are born in relation to the dorso-ventral axis. Cells derived from progenitors in the dorsal domain of the neural tube will populate the dorsal horn and will become predominantly interneurons (Caspary and Anderson, 2003; Helms and Johnson, 2003). Neural cells in the dorsal horn are involved in relaying somatosensory information to the brain. However, cells that originate from the ventral region of the neural tube are differentiate into ventral interneurons and motor neurons (Briscoe and Ericson, 2001; Jessell, 2000). These cells will eventually mature into neurons in the ventral horn where they will be involved in motor outputs. The proper formation of these distinct populations is pivotal for the formation of a fully functional nervous system.

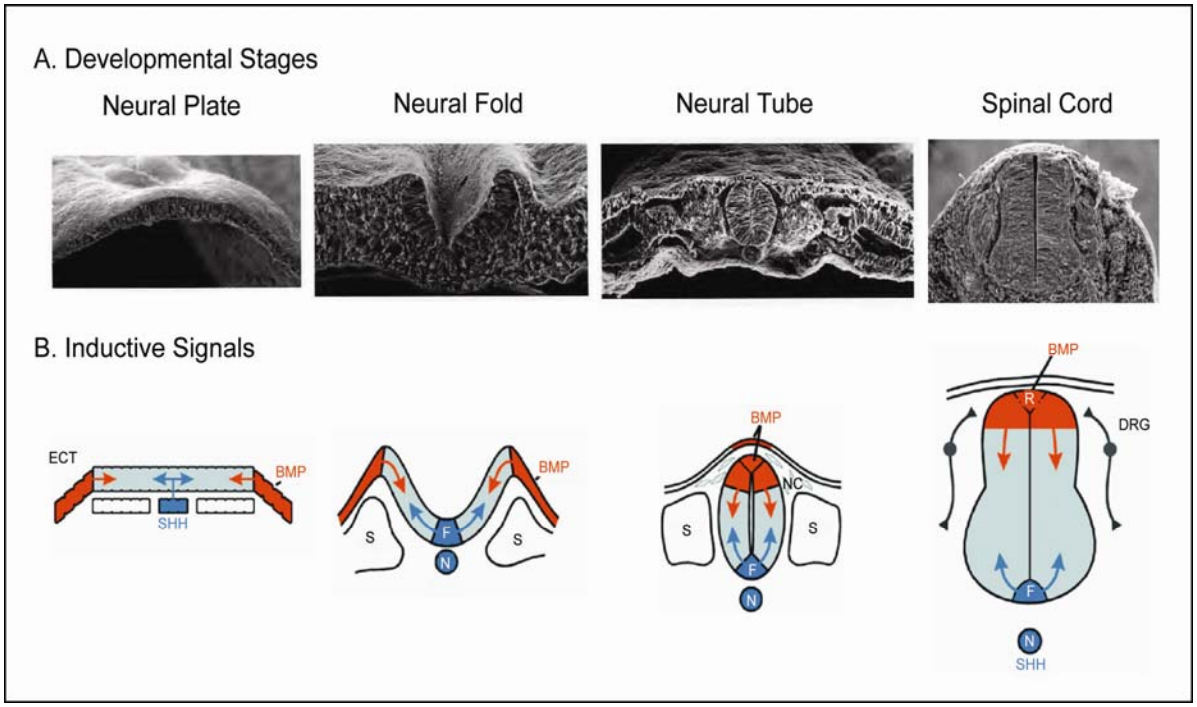


Figure 1.1 Neural Development

(A) Representative scanning EM images of different stages of neural tube formation. The neural plate is located above the notochord (N) and is flanked by the epidermal ectoderm (ECT). The neural plate begins to fold forming the neural groove. The folding edges continue to move upward and fuse creating the neural tube. Within the neural tube, cells located at the most dorsal end comprise the roof plate (R), while at the most extreme ventral end, floor plate (F) cells are found. (B) Two major classes of proteins provide the inductive signals that control differentiation along the dorsal-ventral axis of the developing spinal cord. Bone morphogenetic proteins (BMP) and Sonic hedgehog (Shh) are expressed in the roof plate and floor plate respectively, upon neural tube closure. The location of expression of these factors is represented at different developmental stages. Adapted from Kandel, 2001 and http://www.med.unc.edu/embryo_image.

BHLH Transcription Factor Family

Basic helix-loop-helix (bHLH) proteins have been implicated in haematopoiesis, neurogenesis, cardiac muscle development, mesodermal cell determination, dermal cell differentiation and skeletal development (Burgess et al., 1995; Cserjesi et al., 1995; Guillemot et al., 1993; Lee, 1997; Li et al., 1995; Porcher et al., 1996; Srivastava et al., 1995). During neural development bHLH transcription factors have a restricted expression pattern that reflects the dorso-ventral patterning of the neural tube (Altmann and Brivanlou, 2001; Helms and Johnson, 2003; Lupo et al., 2006; Shirasaki and Pfaff, 2002). BHLH factors have been shown to be transcriptional regulators of various developmental processes including cellular differentiation and lineage commitment. Their function is dependent upon protein dimerization and recognition of particular binding sequences on the DNA (Murre et al., 1989). bHLH transcription factors have been shown to activate transcription by forming homo- or heterodimers that bind E-boxes, with the consensus sequence CANNTG, in the promoter region of their target genes (Tsuchida et al., 1994). As their name implies, bHLH transcription factors contain a basic region and two alpha helices separated by a loop. The basic region of bHLH proteins mediates DNA binding, while the two alpha helices are required for protein-protein interactions (Ferre-D'Amare et al., 1993; Voronova and Baltimore, 1990).

BHLH transcription factors are separated into classes based on their DNA binding specificities, their dimerization capabilities, and their tissue distribution (Murre et al., 1994). There are seven Classes of bHLH transcription factors but I will only discuss two in this thesis as they are more applicable to this work. Class I bHLH proteins are composed of E-Proteins that are expressed ubiquitously and include factors E12, E47, HEB, and E2-2. These factors can form homodimers or heterodimers with Class II bHLH proteins which are expressed in a tissue specific manner. Class II

members include Mash1 (Ascl1), Math1 (Atoh1), Neurog1 (Ngn1), and Neurog2 (Ngn2), among others (Massari and Murre, 2000).

Neurogenesis and the role of bHLH factors

Class II bHLH proteins are an essential part of neural development. They are involved in determination and differentiation (Lee, 1997). Factors involved in differentiation are mainly expressed in post-mitotic cells later in development and are often expressed in adulthood (Bartholoma and Nave, 1994; Lipkowitz et al., 1992; Schwab et al., 1998). This subclass of bHLH proteins has been shown to promote the differentiated states by inducing cell-cycle arrest and neural specific markers (Farah et al., 2000; Lee, 1997). On the other hand, bHLH factors involved in determination are expressed earlier in development. Their expression is precisely timed and in a specific spatial pattern (Fig 1.2) (Altmann and Brivanlou, 2001; Gowan et al., 2001; Helms et al., 2005; Lupo et al., 2006; Shirasaki and Pfaff, 2002). These bHLH proteins are expressed within the ventricular zone of the developing neural tube, once progenitor cells migrate laterally and exit the cell cycle these bHLH proteins are no longer present.

BHLH transcription factors were originally discovered to be involved in cell fate determination during neurogenesis in *Drosophila*. During *Drosophila* development, bHLH proteins are expressed in the proneural cluster and promote these cells to become neural precursors (Jan and Jan, 1993). In *Drosophila*, the Achaete-Scute complex (AS-C) is involved in external organ formation (Campos-Ortega and Knust, 1990; Jan and Jan, 1993). Furthermore, AS-C and Atonal (ato) are required for the proper formation of components of *Drosophila* PNS and CNS (Campuzano and Modolell, 1992; Jarman et al., 1993). Loss-of-function experiments have demonstrated that in AS-C mutants, neuroectodermal cells fail to form proper neuroblasts and instead become epidermal cells, while other studies show that over-expression of AS-C promotes

hyperplasia (Campos-Ortega and Knust, 1990; Jimenez and Campos-Ortega, 1990). These results suggest that AS-C is required for neuroectodermal cells to properly form neuroblasts. Atonal is also required for the proper tissue specific development. Atonal mutants fail to form chordotonal organs and eyes while over-expression leads to an increased number of chordotonal organs (Jarman et al., 1994; Jarman et al., 1995).

In mammals, homologues of *Drosophila* bHLH genes have been characterized and shown to regulate neural development. *Mash1*, a homologue of Achaete and Scute, forms a heterodimer complex with E2A, a homologue of Da, and positively regulates neuronal differentiation (Johnson et al., 1990). During neural development, Mash1 is expressed in the neural tube, specific regions of the brain, and in progenitors of autonomic neurons and the olfactory epithelium (Guillemot and Joyner, 1993; Johnson et al., 1990). Mash1 mutants fail to form progenitors to primary olfactory neurons, autonomic neurons and some enteric neurons (Guillemot et al., 1993; Kageyama et al., 1997). In addition, the family of mammalian and *Xenopus* homologues of atonal (*Math1 / Math5)* have been identified and shown to induce neuronal differentiation (Andermann et al., 2002; Ben-Arie et al., 1997; Bermingham et al., 1999; Cau et al., 2002; Fritzsch, 2003; Kim et al., 2002; Lee, 1997; Ma et al., 1997; Sommer et al., 1996; Sun et al., 2001; Vetter, 2001; Zheng and Gao, 2000). Math1 expression is observed in the developing dorsal neural tube, brain stem, external granule cells of the cerebellum, rhombic lip of the brain's fourth ventricle, precursors to inner ear hair cells, and Merkel cells of the skin (Ben-Arie et al., 1997; Ben-Arie et al., 2000; Bermingham et al., 1999; Helms et al., 2000). Neurog1 and Neurog2 are homologs of TAP (target of poxn, also called biparous) and were first identified in vertebrates (Fode et al., 1998; Gautier et al., 1997; Ma et al., 1996). Finally, the Neurogenins (*Neurog*) were shown to be involved in rodent neural development

where over-expression of X-Ngnr1, a Neurog1 homolog, led to ectopic neurogenesis at the expense of ectodermal cells and activated X-NeuroD, another neural bHLH factor (Ma et al., 1996).

Neurog1 and Neurog2 have overlapping expression patterns and identical bHLH motifs (Sommer et al., 1996) that result in apparent redundant function in multiple neural domains (Gradwohl et al., 1996; Ma et al., 1996; Sommer et al., 1996). Neurog1 and Neurog2 are generally expressed during embryogenesis in proliferating neural progenitor cells initiating differentiation. Their expression is observed in specific dorsal and ventral regions of the neural tube, in the dorsal telencephalon, the olfactory epithelium, the sensory cranial ganglia, the dorsal root ganglia, and in specific regions of the midbrain and hindbrain (Cau et al., 2002; Lee, 1997; Ma et al., 1996; Ma et al., 1997; Sommer et al., 1996). However, Neurog1 and Neurog2 also have distinct functions that are highlighted by the loss of specific neuronal lineages in mice mutant for either *Neurog1* or *Neurog2* (Cau et al., 2002; Fode et al., 1998; Gowan et al., 2001). In particular, *Neurog1* mutants have defects in proximal cranial ganglia, olfactory neurons, and inner ear (Cau et al., 2002; Ma et al., 2000; Ma et al., 1998), while loss-of-function studies show that Neurog2 is required to form distal cranial sensory ganglia that are derived from epibranchial placodes and no expression of differentiation factors (Fode et al., 1998). Defects in other neural regions such as dorsal root ganglia, spinal neural tube, and dorsal telencephalon are more easily detected in *Neurog1/Neurog2* double mutants (Fode et al., 2000; Gowan et al., 2001; Ma et al., 1999; Scardigli et al., 2001). The partially overlapping expression between these closely related bHLH factors, and the complex mutant phenotypes, make it difficult to delineate the full complement of neural lineages derived from Neurog1-expressing cells.

Cell type specification in the spinal cord: role of bHLH and HD transcription factors

Determining the cell fate of a particular neural progenitor is pivotal in establishing a properly functioning nervous system. Gain- and loss-of function experiments have shown that bHLH transcription factors have determined roles in neurogenesis and cell type-specification, particularly Math1, Mash1, and Neurog1. During neural tube development cells within the ventricular zone express specific transcription factors which are turned off as the progenitor cells mature, exit the cell cycle and migrate laterally into the mantles. Once in the mantle these cells express specific markers which are used to establish their neural identities. The roles for bHLH transcription factors in specification of neurons have been extensively studied (Gowan et al., 2001; Nakada et al., 2004a; Parras et al., 2002; Perez et al., 1999). For example, previous studies in chick neural tube have demonstrated that Math1, Neurog1 and Mash1 can induce both neuronal differentiation and specification (Gowan et al., 2001; Nakada et al., 2004a). Overexpression of Math1 increased dI1 interneurons, Neurog1 increased dI2 interneurons, and Mash1 increased dI3 interneurons in the developing spinal cord (Gowan et al., 2001; Nakada et al., 2004a). Generation of a chimeric protein between Mash1 and Math1 revealed specification activity was restricted to the Helix2 of Math1, and both Helix1 and Helix2 of Mash1 (Nakada et al., 2004a). Additionally, over-expression studies have shown that Neurog1 is sufficient to induce neuronal differentiation in embryonic carcinoma P19 cells, cortical progenitors, Xenopus and zebrafish (Blader et al., 1997; Gowan et al., 2001; Lee, 1997; Ma et al., 1996; Nakada et al., 2004b; Sun et al., 2001). Gain- and loss-of function studies have shown Neurog1 can induce dI2 specification during neural development and is required for the formation of this population (Gowan et al., 2001). These data show the importance of these bHLH transcription factors during neurogenesis.

In the developing spinal cord bHLH transcription factors are expressed in discrete regions within the VZ. The expression boundaries of Mash1, Math1, and Neurog1 in the neural tube are sharply defined (Fig 1.2) (Gowan et al., 2001), as are the boundaries between Mash1 and Neurog1 in the developing forebrain (Ma et al., 1997). However, when one of these bHLH factors is not present, the dorso-ventral boundaries of the remaining factors are no longer respected. For example, loss-of function studies show that in the absence of Math1, the Neurog1 and Neurog2 expression boundaries are expanded dorsally. Furthermore, in *Neurog1/Neurog2* double knock-outs the boundary of Math1 expression broadens and is observed in the Neurog1/Neurog2 domains (Gowan et al., 2001). In addition, misexpression of Neurog1 in the chick neural tube suppressed Cath1 and Cash1 (chick homologs), while misexpression of Math1 reduced expression of Cash1 and cNeurog1 (Gowan et al., 2001). These data suggest that bHLH transcription factors in the developing neural tube inhibit each other's expression to ensure proper neural development by maintaining expression boundaries.

The expression of distinct transcription factors within the ventricular zone has been used to define specific progenitor populations within this region. During neurogenesis, between E10.5 to E11.5 of mouse development, in the dorsal neural tube there are six progenitor populations known as dP1, dP2, dP3, dP4, dP5 and dP6 (Fig 1.2). In the ventral region of the neural tube, the ventricular zone has been defined by five distinct progenitor populations, vP0, vP1, vP2, vP3 and the progenitor motor neuron domain (PMN). These populations express distinct combinations of transcription factors that are used to distinguish each population. The relationship between the progenitor population and interneurons found in the mantle is a direct one. For example, dP2 progenitor cells expressing markers Neurog1 and Pax3/7 will differentiate into dI2 interneurons expressing Lhx 1/5, Brn3a and Foxd3 (Fig 1.2).

Homeodomain (HD) factors in the developing neural tube have a similar non-overlapping expression pattern where Class I and Class II HD factors seem to rule specification (Briscoe et al., 2000). Neural progenitors express bHLH and HD transcription factors in a temporal and spatial pattern. These attributes also determine the HD factors these cells express as they become interneurons and mature into their respective adult cell types. These populations can be further subdivided by their dependence upon roof plate signals. Class A neurons (dI1-dI3) require roof plate signaling to form properly, while Class B (dI4-dI6) and $dIL^{A/B}$ neurons form independently of roof plate signals (Lee, 1997; Muller et al., 2002). In the ventral neural tube, interneurons (V0-V2 and V3) and motor neurons (MN) are born between E10.5-E13 (Fig 1.2).

Gene knockout studies have established a requirement for bHLH and HD factors in the proper formation of these specific neuronal populations. *Math1* is required for dI1 neurons (Bermingham et al., 2001; Gowan et al., 2001). In the absence of Math1 neurons no longer express the dI1 marker Lhx2/9; instead cells now express the dI2 marker Lhx1/5 (Gowan et al., 2001). Mash1 null embryos lack dI3 and dI5 as evident by the decrease in Islet1 and Lmx1b expression, respectively (Bermingham et al., 2001; Gowan et al., 2001). *Neurog1* mutants display a decrease in the dI2 markers Brn3a/Lhx1/5 but they are not completely missing, and a slight decrease in cells expressing MN markers (Gowan et al., 2001; Lee and Pfaff, 2003). *Neurog2* knock-out mice had no change in dorsal interneuron populations but showed a significant decrease in motor neuron populations at E12.5 (Gowan et al., 2001; Lee and Pfaff, 2003). However, mice lacking both *Neurog1* and *Neurog2* do not have dI2 interneurons and showed an even further decrease in cells expressing MN markers, suggesting there is some functional redundancy between Neurog1 and Neurog2 (Gowan et al., 2001; Lee and Pfaff, 2003).

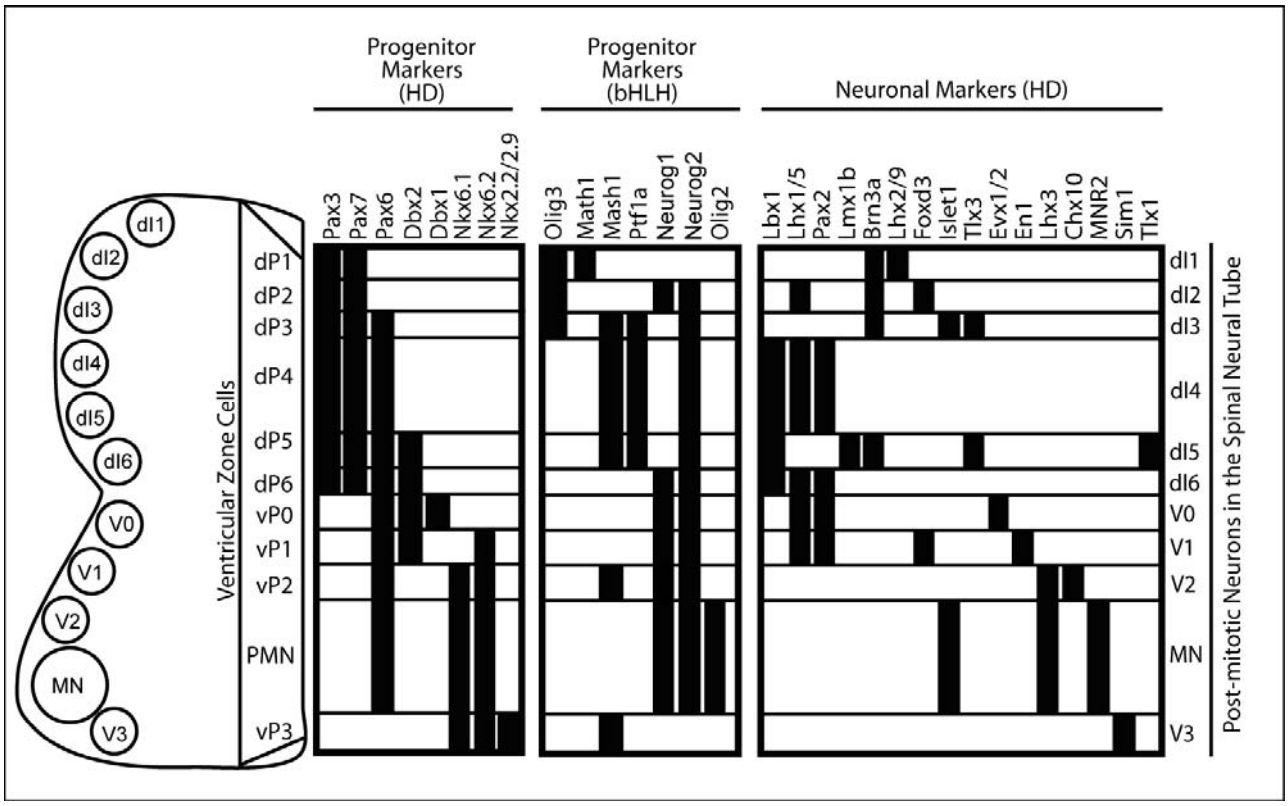


Figure 1.2 <u>Summary of Populations</u>.

Shown is a schematic representation of the homeodomain (HD) and bHLH transcription factors expressed in the early developing neural tube (E10.5-E11.5). Six early born (dI1-dI6) and five ventral populations (V0-V3 and MN) are derived from their respective ventricular zone progenitor domains shown to the left of the image. The HD and bHLH factor expression domains are represented by solid bars (middle). Similarly, the HD factors expressed in the post-mitotic neurons are displayed to the right. It is a combinatorial transcription factor code that progressively determines the identity of a spinal cord neuron. For, instance the unique transcription factor signature of a dP1 progenitor is: Pax3, Pax7, Olig3, Math1, Brn3a, and Lhx2/9. (Modified from Stacey Glasgow Dissertation, 2006.)

Regulation of the neural bHLH class of transcription factor

Regulation of bHLH transcription factors during neural development is complex and largely undefined. The molecular mechanisms involved in establishing discrete populations in the nervous system are beginning to be established; however, how these mechanisms are regulated is unknown. Recent studies have shed light into the regulation of neural bHLH proteins during development by isolating distinct cis-elements and molecular players involved in this complex process. For example, *Math1* regulation was found to be directed by a single enhancer element containing two discrete sequences located 3kb 3' of the *Math1* coding sequence (Helms et al., 2000). These sequences are conserved between mouse and human and were sufficient for driving reporter expression in all *Math1* domains (Helms et al., 2000). However, although these sequences were not identical they induced reporter expression in the same domains, suggesting these sequences have redundant activities (Helms et al., 2000). No separable enhancer elements that drive reporter expression in a tissue-specific manner have been identified for *Math1* (Ebert et al., 2003; Helms et al., 2000). The identification of the *Math1* element was essential for identifying a key player involved in *Math1* regulation. Zic1, a zinc-finger transcription factor, was found to repress *Cath1* (Chicken homolog of *Math1*) and the activity of an *Math1* enhancer-driven *lacZ* reporter when expressed in chick neural tubes (Ebert et al., 2003). This Zic1 repression was mediated by a conserved site found within the *Math1* enhancer element (Ebert et al., 2003). Autoregulation has also been shown to play a role in bHLH transcription factor regulation. In the case of *Math1*, initial expression is dependent upon BMP signaling from the roof plate, but sustained expression requires proper *Math1* expression (Lee et al., 2000). The previously identified enhancer element was shown to contain an E-box consensus binding site that was essential for *Math1* autoregulation (Ebert et al.,

2003; Helms et al., 2000). On the other hand, another neural bHLH, *Mash1* has been shown to negatively autoregulate (Horton et al., 1999; Meredith and Johnson, 2000).

Other neural bHLH transcription factors studied are regulated by separable tissue-specific enhancer elements (Blader et al., 2003; Gowan et al., 2001; Nakada et al., 2004b; Scardigli et al., 2001; Simmons et al., 2001). *Neurog2* is one such factor for which separable elements spanning over 20 kb of sequence have been identified that regulate Neurog2 expression to a number of its expression domains (Scardigli et al., 2001; Simmons et al., 2001). Elements 5' and 3' of the *Neurog2* coding region have reporter activity reflecting the spatial and temporal expression profile of Neurog2 (Simmons et al., 2001). A 4.4 kb fragment 5' of the *Neurog2* coding region that was sufficient to drive reporter expression in the ventral neural tube, and a 1 kb fragment located 3' of *Neurog2* that directed expression to both dorsal and ventral domains (Simmons et al., 2001). The identification of these sequences was also essential in identifying upstream regulators of *Neurog2*. Pax6, a homeodomain transcription factor involved in specifying the identity of ventral spinal cord progenitors, was found to bind to the ventral neural tube enhancer element (Scardigli et al., 2001). Finally, in the case of *Neurog2*, autoregulation does not seem to play a role in proper expression of this bHLH.

Previous studies using animal models have identified various cis-regulatory elements involved in regulating Neurog1 expression during neural development. A 7.5 kb fragment located 4.5 kb 5' of the *Neurog1* start site was shown to drive expression in most of the Neurog1 expression domains, including the midbrain, hindbrain, dorsal neural tube, ventral neural tube, trigeminal ganglia and olfactory epithelium (Gowan et al., 2001). However, reporter expression was missing in both the dorsal root ganglia and dorsal telencephalon (Gowan et al., 2001). Later studies identified separable cis-enhancer elements, located 5' and 3' of the *Neurog1* coding region,

that were sufficient to drive reporter expression to the dorsal neural tube, midbrain, and hindbrain (Blader et al., 2003; Nakada et al., 2004b). Expression in the trigeminal ganglia and ventral neural tube was observed repeatedly in some of the separable elements, pointing to redundancy in driving expression in these domains (Nakada et al., 2004b). Three sequences within the 7.5 kb region are highly conserved with sequences flanking the zebrafish *Neurog1* coding region; two of these overlap with the midbrain and dorsal neural tube enhancer elements (Blader et al., 2003; Gowan et al., 2001; Nakada et al., 2004b). In Zebrafish these sequences have been shown to be required to direct expression in the lateral stripe and the anterior plate (Blader et al., 2003). Thus, a reporter model that would fully recapitulate the Neurog1 expression profile has not been developed, and separable enhancer elements driving reporter expression in all domains of Neurog1 expression have not been identified.

Thesis Rationale and Goals

A fully functional nervous system requires the formation of the correct number of cells in specific regions and with appropriate connections, which occurs during neurogenesis. This is pivotal for the nervous system to be capable of receiving inputs from the periphery, correctly relaying the information to the higher brain structures, and sending correct motor responses back to the periphery. The formation of this complex neural network is dependent upon the signaling cascades involved in neural development. BHLH transcription factors are an important part of this process and their regulation during neural development is largely undefined. Identifying regulatory sequences involved in these processes will be key in identifying the upstream targets involved in regulating expression of these factors during development. Furthermore, the link between the distinct progenitor populations expressing neural bHLH proteins within the proliferative zone and the mature neural cell types is not clear. Tracing the adult neural cells derived from progenitors

expressing specific neural bHLH transcription factors will aid in deciphering how these mature cell types are specified.

Neurog1 is a bHLH transcription factor involved in neural development. Previous studies have identified distinct regulatory elements sufficient to drive reporter expression in a subset of Neurog1 expression domains. However, sequences driving expression to the dorsal telencephalon and dorsal root ganglia have not been identified. In addition, these sequences have not been properly studied to determine their requirement in driving Neurog1 expression to specific tissues. I hypothesize that cis-regulatory elements involved in Neurog1 expression are found within the highly conserved regions surrounding the *Neurog1* sequence. Here I assess conserved regions for their function in Neurog1 expression, and identify genomic boundaries for novel *Neurog1* regulatory sequences using BAC transgenic mice and interspecies sequence conservation. Identified elements are also tested for their ability to direct tissue specific expression. The importance of these sequences in Neurog1 expression at different stages of neural development will be studied.

The identity of developing interneurons and mature neural cell types derived from Neurog1-expressing progenitors is not completely defined. Previous studies have shown that roof-plate-dependent Neurog1 progenitor populations will become dorsal interneurons 2 (dI2) and differentiate into commissural interneurons (Gowan et al., 2001); however the fates of Neurog1-expressing cells in the ventral neural tube have not been established. Based on the expression pattern of Neurog1 it has been hypothesized that these give rise to most ventral interneurons but not motor neurons (Scardigli et al., 2001). Furthermore, Neurog1 has been shown to be an activator of neurogenesis and inhibitor of gliogenesis (Kim et al., 2002; Ma et al., 1999; Ma et al., 1997; Mueller and Wullimann, 2003; Sun et al., 2001). In this thesis I will determine if Neurog1-

expressing progenitors in the ventral neural tube will become discrete ventral interneurons and motor neurons populations using BAC transgenic mice. In addition I will perform long-term lineage tracing to establish the identity of mature neural cells derived from Neurog1-expressing progenitors.

CHAPTER TWO:

MATERIALS AND METHODS

BAC Handling and Preparation for Recombineering Protocols

Overview

In order to properly perform lineage tracing experiments I needed a good model in which reporter expression would mimic Neurog1 expression. Previous studies had isolated enhancer elements which drove expression to a number of Neurog1 expression domains, but did not fully recapitulate endogenous expression in all of its domains (Gowan et al., 2001; Nakada et al., 2004b). Hence, I needed to develop a model which would fully recapitulate Neurog1 expression in all of its domains. To accomplish this I decided to use bacterial artificial chromosomes, or BACs. These large pieces of DNA contain an average of 190 kilobases (kb) of DNA, whichis maintained by a generic backbone of DNA that allows it to replicate in E. coli as an artificial chromosome. The size of these BACs provides for the characterization of much larger regions of the genome. They are more stable than other large construct plasmids such as Yeast Artificial Chromosomes (YACs), which are much more susceptible to random recombination events and are more fragile than BACs. The ease of manipulation of the BAC compared to the YAC also makes the BAC an appealing choice. Furthermore, BAC reporters have been shown to recapitulate gene expression more faithfully that smaller plasmids (Aller et al., 2003; Hayashi and McMahon, 2002; Kim et al., 1998; Srinivas et al., 2001). Their expression is less dependent on position, probably due to large regions of insulating DNA, and their expression appears to be copy number dependent in a linear fashion. Therefore, BACs appear to be a good choice for the use of large genomic regions to report the expression of specific genes.

The Celera Genome Sequence Website was used to identify clones that contained the Neurog1 protein coding region. RP23-457E22 was chosen for further study. It contains a genomic

insert of 198 kb with 120 kb 5' and 78 kb 3' of the *Neurog1* translation start codon (Figure 3.1). The *Neurog1* coding region was precisely replaced by GFP (Clontech) with a nuclear localization sequence (NLS) added, or CRE using homologous recombination in bacteria (Yang et al., 1997a). The resultant modified BACs named $N1^{457}$-*GFP* and $N1^{457}$-*Cre*, respectively, were verified by Southern blot. The basic procedures used for these manipulations are detailed in the following sections.

Preparation of BAC DNA by MiniPrep

This is a rapid alkaline lysis MiniPrep method for isolating DNA from large BAC clones. It is a modification of a standard Qiagen-Tip method that uses no organic extractions or columns. The method works very well for doing analytical restriction digests of BAC clones and can be scaled up if necessary. First, using a sterile toothpick, inoculate a single isolated bacterial colony into 2 ml LB (or TB) media supplemented with 12.5 μg/ml chloramphenicol. Use a 14 ml snap cap polypropylene tube. Incubate overnight (up to 16 hr) shaking at 225-300 rpm at 37º C. Remove toothpicks using forceps. Centrifuge (in Sorvall SM24 or similar rotor) at 3,000 rpm for 10 min at 4ºC. Add 1 ml of culture to 1.5 ml Eppendorf tubes, spin at max rpm for 1 min, add second 1 ml, and spin at max again, all at 4 º C. Discard supernatant and resuspend (vortex) each pellet in 0.3 ml P1 (filter sterilized, 4º C: 15 Mm Tris, pH 8.0, 10 mM EDTA, 100 μg/ml RNase A). Add 0.3 ml of P2 (filter sterilized, Room Temp, 0.2 N NaOH, 1% SDS) solution and gently shake tube to mix contents. Allow to sit at room temperature for no more than 5 minutes. The suspension should change from very turbid to almost translucent. Slowly add 0.3 ml P3 (autoclaved, 4º C storage, but really needs to be fresh: 3 M KOAc, pH 5.5) solution to each tube and gently shake during addition. A thick white precipitate of protein and *E. coli* DNA will form. After adding P3 to every

tube, place tubes on ice for at least 5 min, 20 min is better. Centrifuge tubes at 10,000 g for 10 min at 4ºC. Cut tip of a P1000 (i.e. it has a wide bore) and use it to transfer to 1.5 ml Eppendorf tube with 0.8 mL ice-cold isopropanol. Avoid any white precipitate material. Mix by inverting 4-6 times. Place tubes on ice for 5 min. Samples can be left overnight at -20º C. Longer is better for precipitation, but no extra DNA is precipitated after over night incubation. Spin in 4º C Microfuge for 15 min at 14,000 rpm, remove supernatant and add 0.5 ml of 100% EtOH to each tube. Invert tubes several times to dissolve excess isopropanol, Spin in 4º C Microfuge to 5 min. Remove supernatant and add 0.5 ml of 70% EtOH to each tube. Invert tubes several times to dissolve excess salt, spin in 4º C Microfuge for 5 min. Carefully remove as much of supernatant as possible as some pellets may become dislodged. Use pipette, not vacuum. Air dry pellets at room temperature and when DNA pellet changes from white to translucent and ethanol smell is absent, resuspend in 40 µl of ddH_2O. Do not use normal narrow bore tips to mechanically resuspend DNA. Rather allow sample to sit in tube with occasional tapping. Resuspension may take more than 1 hour at room temperature. O/N resuspension is better.

Store resuspended BACs at 4ºC (BAC DNA may not be stable for more than 4-7 days at this temperature). So, fresh prep's are necessary. Use 5µl for digestion with NcoI, NotI or other restriction enzyme. There are NotI sites flanking the Sp6 and T7 promoter regions of the CYPAC2 vector; and therefore, this is a useful enzyme for analysis of insert size and for partial digest restriction mapping. Use 7-10µl for digest with a more frequent cutter such as BamHI or EcoRI. Constructs *N1*^{457}*-GFP* and *N1*^{457}*-Cre* contain a novel NcoI site at the ATG of *Neurog1*, such that Nco1 provides a nice change in restriction pattern between wild-type and modified BAC. Such restriction sites are helpful in general to identify differences in BAC preparations.

Purification of BAC DNA by Modified MidiPrep

This purification method can be used to generate a larger amount of BAC DNA at a higher purity than the MiniPrep above. The DNA can be used for characterization (digestion, PCR, etc.) or for pronuclear injection to establish new transgenic lines. The first day, add 2µl of BAC culture or pick single colony and inoculate into 5ml of LB+Chlor (12.5µg.ml) O/N at 37°C. After making glycerol stocks, add 1ml of O/N culture to 100ml of LB+Chlor (12.5µg/ml) in a 500ml Erlenmeyer flask and incubate at 37°C O/N in shaker.

The Next day pour the 100ml culture into large centrifuge tubes and spin at 6000g for 10min at 4°C. Pour off supernatant and add 20ml of chilled P1 buffer, let sit on ice for 5min and then vortex until there are no more clumps. Add 20 ml of P2 buffer and invert 6 times. After 90 seconds incubation at room temperature add 20ml of chilled P3 and invert six times, incubate on ice for 20 minutes. Then centrifuge at 11,000 rpm for 30min at 4°C. Filter supernatant with sterile gauze and transfer to pre-equilibrated columns (to equilibrate use 7ml of QBT), wash columns with 40ml of QC buffer. Elute DNA with 7ml pre-warmed QF buffer (65°C) and collect in fresh conical tube. Precipitate the DNA by adding isopropanol at room temperature (0.7 times the volume of the eluted sample) and invert tube six times. Aliquot into 2ml Eppendorf tubes until all sample is equally distributed, and incubate O/N at -20°C.

On the following day, centrifuge at 14,000 rpm for 30minutes at 4°C, pour off the isopropanol and wash with 100% ethanol. Spin at 14,000 rpm and wash with 70% ethanol. Spin at 14,000 rpm for 10min at 4°C and then discard supernatant. Allow air-drying, but do not over-dry as this will make it difficult to resuspend, and add 20-30ml of TE (digestion, sequencing), injection buffer (for pronuclear injection) or H2O (for electroporation using the Modified Copeland Protocol: See below). Concentration will range from 100ng/µl to 500ng/µl.

PCR analysis

PCR analysis of BAC DNA is mostly dependent on how clean and fresh your prep is. Use freshly purified BAC DNA, by either modified MiniPrep or MidiPrep as described above. Use approximately 50-100ng of your prep for the PCR reaction. Mix all ingredients on ice and hot start the PCR machine. The reaction mix consists of: DNA template (1.0µl); 10x Qiagen buffer (4.0µl); dNTP (2.0µl); Primer 1 (3.0µl); Primer 2 (3.0µl); Taq Polymerase (1.0µl of Expand Long-Template Taq from Qiagen); and H_20 (26.0µl). The current conditions were mostly used, changing expansion time based on the size of the PCR fragment: Hot Start, 94˚C; 94˚C, 5 min; Cycle 1, repeat 3 times: 94˚C, 1.0 min, 54˚C, 30s, 72˚C, 1.0 min; Cycle 2, repeat 35 times: 94˚C, 1.0 min, 58˚C, 45s, 72˚C, 1.0 min; Final: 72˚C, 10 min, 4˚C, hold.

BAC Reporter Generation: Adaptation and Optimization of BAC Recombination

Overview

BAC strategies were initiated by another graduate student in the lab, James Battiste. In order to construct the *N1*^{457}*-GFP* and *N1*^{457}*-Cre* BAC reporters required for lineage tracing, I adapted a protocol developed by the Heintz laboratory to precisely replace the *Neurog1* single coding exon with a reporter construct, either GFP or Cre (Yang et al., 1997b). I decided to use the RP23 457E22 as it contained the largest sequence 5' and 3' of *Neurog1*. To modify this BAC I used two constructs: *N1-GFP* which contains nGFP flanked by two homology regions precisely 5' and 3' of the *Neurog1* coding region; and *N1-Cre*, which contains Cre-recombinase flanked by the same homology regions (Figure 3). First generate a build vector (BV) containing the reporter of interest flanked by 1 kb of sequence immediately 5' of the *Neurog1* coding region and 1 kb of

sequence immediately 3' of *Neurog1*. The complete sequence of flanking DNA and reporter was cloned into the shuttle vector (SV) kindly provided by the Heintz lab using SalI enzyme. The shuttle vector was then transformed into bacteria containing the BAC clones and the BACs were recombined.

There were several modifications to the original Heintz protocol. At each step, two additional selections were added to increase the number of positive clones and decrease the false positives. The first selection was performed on solid media (included in the original protocol) and then colonies were picked and grown in liquid media for colony selection. These liquid cultures were diluted and plated on solid media in serial dilutions to achieve individual colonies. These additions increase the efficiency of the recombination method. In addition, products at each step were assayed by PCR and Southern blot to verify each clone had the appropriate orientation. Upon acquisition of the final product, the area of interest was sequenced to verify its proper integration.

Recombination Cassette Planning and Construction

I chose the pGEM plasmid as my BV to insert the reporters into the SV. The homology regions were flanked by one SalI site at the end farthest from the reporter (i.e. 5' homology region contained a SalI restriction site at its 5' end). These SalI sites facilitate insertion of the reporter, flanked by the 5' and 3' homologies, into the SV. Furthermore, I added an Nco1 restriction site at the ATG start codon of *Neurog1* to aid in Southern blot analyses. The nGFP or Cre coding sequences were inserted into the BAC precisely replacing the *Neurog1* sequence, effectively deleting the bHLH transcription factor sequence from the BAC. Therefore, homology sequences for the build vector consisted of 1kb immediately 5' and 1kb immediately 3' of the *Neurog1* coding region. The following primers were used: to amplify 5' homology region;

TCTAGACGGCAAACAGGAAAAT and CCATGGTGCAGTGTGCAGGACCGA; for 3' homology: TCCTGGGGTTCCGGGGCCGCTG and ATCAGGCCATCATGGCCTGTTTACAAATCAATTTCCTC. I cloned the homology regions into the BV by digesting the 5' fragment with XbaI and NcoI and the 3' sequence with BglII and SacI (See Appendix A). After cloning these homology regions into the pGEM containing either nGFP or Cre, I verified that they were inserted into the plasmid with the same orientation as those in the BAC by DNA sequencing.

Cloning of the Recombination Cassette into the Shuttle Vector

I isolated the recombination cassette (RC) from the BV and inserted it into the SV. The RC consists of the reporter flanked by the two homologous regions and can be cloned into the SV by digesting both the RC and SV with SalI (see previous section). This system is designed so that the SV has a temperature sensitive origin of replication that only replicates efficiently at 30˚ C and is resistant to tetracycline. It does not contain resistance genes for any other antibiotics. Furthermore, since the SV is a low copy plasmid. Yield ranged from 2-10µg SV DNA per 500 ml overnight culture. Digest and gel purify the RC from the BV. Digest with Sal1 and incubate at 37º C for 6 hours to overnight. Use the following amounts: 1-2µg of plasmid, 10µl H buffer, 5µl Sal I, and add ddH_20 to total volume of 100µl. Gel purify and ligate the RC into SV using the following conditions: 50 ng linearized SV, 100-200ng of isolated RC insert, 2µl 10X ligation buffer, 1µl of ligase, add H_2O to 20µl and incubate at 16º C overnight. Finally, transform the ligation product into DH5α by adding half of ligation reaction (10µl) to 100µl of ice cold chemically competent DH5α cells. Incubate 15 minutes on ice, then heat shock at 37º C for 2 minutes. Add 1 ml LB and shake at 30º C for 30 minutes. Plate at 1/10 and 9/10 dilutions onto LB-Tet (10µg/ml) plates and incubate

the plates at 30ºC for 16 to 36 hours. Pick colonies for MiniPrep. Isolate DNA and confirm integration of RC into SV. Orientation in SV does not matter because the BAC integration will be determined by proper homologous region orientation to reporter sequence.

Homologous Recombination into BACs

Transform 200 µl chemically competent BAC containing bacteria with the temperature sensitive SV-RC plasmid using 5-10µl of SV-RC MiniPrep DNA. Incubate 15 minutes on ice, then heat shock at 37º C for 2 minutes. Add 1 ml LB and shake at 30º C for 30 minutes. Plate cells at 1/10 and 9/10 dilutions onto LB-Tet (10µg/ml) / Chlor (12.5µg/ml) plates and grow overnight at 30º C. Pick 10 single colonies and grow in liquid LB- Tet (10µg/ml) / Chlor (12.5µg/ml) broth overnight at 30º C in shaker. This allows further selection for SV and BAC while allowing for additional time to recombine. Plate liquid bacterial suspension onto LB- Tet (10µg/ml) / Chlor (12.5µg/ml) plates and incubate overnight at 43ºC. Appropriate amount to plate varied and was optimized by serial dilutions to allow for identification of single colonies. Pick 20 of the larger regular size colonies and inoculate each colony into 2 ml LB- Tet (10µg/ml) / Chlor (12.5µg/ml) broth and plate same colony on an LB- Tet (10µg/ml) / Chlor (12.5µg/ml) plate. Incubate both at 43º C and save the master plate at 4º C for further use. MiniPrep DNA from 2 ml culture and analyze for co-integration of SV-RC by performing PCR for the presence of reporter (PCR reaction as described above). In addition, digest 5-10µl of BAC MiniPrep with NcoI using 2.5 mM spermidine to digestion mix for proper digestion of BAC DNA. Run digestion on 0.7% agarose gel at 30 volts overnight to separate fragments. Image the ladder and transfer to nitrocellulose membrane and hybridize with nGFP or Cre probes and with probes specific for the regions that

were recombined. Look for the appropriate size change indicating a co-integration event, and discard all other BAC clones. (Southern Blot Protocol, Maniatis).

The BACs that were confirmed to have recombined correctly were then allowed to resolve the SV out of the BAC. To achieve this pick single colonies from the appropriate Master plate, and inoculate in 5 ml of LB-Chlor (12.5μg/ml) at 43º C overnight shaking. The next day plate the liquid culture onto LB-Chlor (12.5μg/ml) plates and grow at 43º C overnight using serial dilutions to achieve a concentration that will produce single colonies. The purpose is to remove Tet selective pressure and allow the recombination event to resolve the SV out of the BAC. The high temperature selects against the temperature sensitive origin of replication on the isolated SV. Select for Tet sensitivity in the resolved BACs by picking 20 single colonies from the LB-Chlor (12.5μg/ml) plates. Streak them onto Fusaric Acid (FA) (10μg/ml) / Chlor (12.5μg/ml) plates and streak the appropriate controls: 1. Streak a Tet-resistant colony (from LB-Tet-Chlor co-integrate plates) – nothing should grow. 2. Streak a Tet-sensitive colony (from original BAC). Then incubate the FA+Chlor plates at 37ºC for 2-3 days and monitor the growth of any colonies. Long incubation time is necessary since the resolved colonies grow slowly on the toxic Fusaric Acid. The first cells that grow to a normal size on these FA plates should be those that properly resolved. Since the homology regions were the same size, 50% of the colonies contained the correctly integrated reporter. BAC clones can be screened by a variety of methods including colony hybridization, colony PCR, or full plasmid Southern analysis which can be found in Maniatis. To quickly screen for properly resolved colonies, perform a mini-culture dot blot. Pick 50-100 colonies and inoculated these into 1ml each of LB-Chlor (12.5μg/ml). Incubate overnight at 37º C shaking.

The next day place one 10 μl drop onto a nitrocellulose membrane in a grid pattern, allow them to dry, then lyse cells and probe for the presence of the reporter using the standard colony

hybridization protocol in Maniatis. Those BACs showing a strong positive for the reporter had recombined correctly. Purify the DNA using the modified MidiPrep (as described above). Confirmed correct reporter orientation by Southern blot analysis and sequencing reactions.

Generation of Deletions using Homologous Recombination: Adaptation and Optimization of BAC Recombineering Protocol

Overview

In order to test the aforementioned highly conserved regions for necessity in driving reporter expression to any of the Neurog1 domains, I modified the recombineering protocol previously published by the Copeland Laboratory (Copeland et al., 2001; Lee et al., 2001). I used the *N1*^{457}-*GFP* BAC, constructed using the modified Heintz Protocol (see above), to delete the regions of interest. This process requires four main steps: 1. Electroporating the *N1*^{457}*-GFP* BAC DNA into the appropriate bacteria; 2. Designing targeting vectors for deleting the regions of interest; 3. Generation of BAC recombinants; and 4. Purification of modified *N1*^{457}*-GFP* BAC DNA for pro-nuclear injection.

Electroporating the N1^{457}-GFP BAC DNA into EL250 bacterial cells

The Copeland lab constructed a number of DH10B derived E. coli strains that contain a defective λ prophage (Copeland et al., 2001; Lee et al., 2001). Since the *pBAC e3.6* BAC backbone contains a *loxP* site, I decided to use the *Flpe*-recombinase system, as opposed to the *Cre*-recombinase. The EL250 strain contains a *Flpe* gene under the tight control of *AraC* and *PBAD*; upon addition of arabinose in the medium, expression of *Flpe* is induced. This strain also contains a *PL* operon encoding *gam* and the *red* recombination genes, *exo* and *bet*, under tight control of the temperature sensitive λ repressor (*cI857*). This strain is therefore useful for removing the

FRTNeoFRT insert used for recombination. To transform the *N1*^{457}*-GFP* BAC into the EL250 bacteria, I picked a single colony from an LB-Strep (10 μg/ml) plate, no older than one week old, and inoculated in 5ml LB-Strep (10 μg/ml) at 32°C O/N (16-20 hrs, always grow these recombination strains at 32°C) to an OD_{600} = 0.5 – 0.7. The next day, I collected the cells by centrifuging at 5000 rpm at 0°C for 6 min using a 15 ml. Falcon® tube. I then resuspended the cell pellet with 888 μl of ice-cold water, transferred the cells to a 1.5ml Eppendorf tube (on ice) and centrifuged using a bench-top centrifuge at room temperature for exactly 20 seconds at 14,000 rpm. The tube was put on ice, aspirated the water without disturbing the pellet. Then resuspended the cell pellet with 888 μl of ice-cold water and centrifuged at 14,000 rpm. This process was repeated three times. Finally, the cell pellet was resuspended in 50 μl of ice-cold water and about 75 μl of the cell suspension were transferred to a disposable electroporation cuvette (0.1 cM gap). I then added 2 μl of *N1*^{457}*-GFP* BAC DNA (100ng) (purified within 48 hours using the Modified BAC MidiPrep, with no protein contamination) and electroporated it into the EL250 cells using the Bio-Rad GenePulser electroporator under the following conditions: 1.75kV, 25uF with the pulse controller set at 200ohms. The time constant should be around 4.0, anything bellow 3.7 or above 4.5 will hinder efficient electroporation. 1.0ml of SOC was added to each cuvette and incubated at 32°C for 1 hour. Cells were then plated onto LB-Chlor plates (12.5 μg/ml) for 24 hours. To verify BAC integrity, ten colonies were picked and the *N1*^{457}*-GFP* BAC DNA was purified using the Modified BAC MiniPrep. The BAC DNA was cut with *NotI* and *NcoI* enzymes and the restriction pattern was compared to the original *N1*^{457}*-GFP* BAC. To ensure no bacterial contamination was present, *EL250-N1*^{457}*-GFP* containing bacteria were inoculated in 5 ml of LB-Strep (10 μg/ml) / Chlor (12.5 μg/ml) overnight.

Designing targeting vectors for deleting the regions of interest

I designed four deletion targeting vectors to systematically delete the four highly conserved regions previously mentioned. This protocol should work with the deletion of *any* size fragment:

N1^{457}*-GFPΔ1*: I amplified a 321bp fragment that is found at the 5' end of the *N1*^{457}*-GFPΔ1* region to be deleted using the following primers: AAActcgagAGAGACTTTGTCCCACCCTGA, AAAaagcttTAGGCAACCCTAGAAACCTGA. These primers contain restriction sites for XhoI and HindIII, respectively. A 163bp fragment was amplified for a region found immediately after the 3' end of the *N1*^{457}*-GFPΔ1* sequence using the following primers: AAAgcggccgcTTTCTGCCCAAGAGCTGCTA, AAAccgcggTATCTGAGTGGTGGCCTCAGG, containing restriction sites for NotI and SacII, respectively. In a two step cloning protocol, both of these fragments were inserted into the previously described pL451 vector (Copeland et al., 2001; Lee et al., 2001) by digesting with the above mentioned restriction enzymes. The plasmid was then digested with the restriction enzymes XhoI and SacII to isolate a fragment containing the *FRT-Neo-FRT* sequence flanked by the 5' and 3' homologies.

N1^{457}*-GFPΔ2*: I amplified a 100bp fragment that is found at the 5' end of the *N1*^{457}*-GFPΔ2* region to be deleted using the following primers: AAActcgagCCCTTTGGGAATATTCAGG, GGAGCAAAGCTTCAAAGACA. These primers contain restriction sites for XhoI and HindIII, respectively. A 96bp fragment was amplified for a region found immediately after the 3' end of the *N1*^{457}*-GFPΔ2* sequence containing restriction sites for NotI and SacII using the following primers: AAAgcggccgcAAAAGGCTGCAAGGATGTGT, AAAccgcggGGAGAAAGATTAGATTAGGGC, containing restriction sites for NotI and SacII, respectively. In a two step cloning protocol, both of these fragments were inserted into the previously described pL451 vector (Copeland et al., 2001; Lee et al., 2001) by digesting with the

above mentioned restriction enzymes. The plasmid was then digested with the restriction enzymes XhoI and SacII to isolate a fragment containing the *FRT-Neo-FRT* sequence flanked by the 5' and 3' homologies.

$N1^{457}$-GFPΔ3: I amplified a 146bp fragment that is found at the 5' end of the *$N1^{457}$-GFPΔ3* region to be deleted using the following primers: AAActcgagTCCAGCTGTTCTTAAGGGTTC, AAAaagcttCAAGAGGGTTCCCTTTCTTCA. These primers contain restriction sites for XhoI and HindIII, respectively. A 153bp fragment was amplified for a region found immediately after the 3' end of the *$N1^{457}$-GFPΔ3* sequence containing restriction sites for NotI and SacII using the following primers: AAAgcggccgcATGTGGCCTCTGAAAAGGTCT, AAAccgcggAGCAGTGACAAATGCCCAAT, containing restriction sites for NotI and SacII, respectively. In a two step cloning protocol, both of these fragments were inserted into the previously described pL451 vector (Copeland et al., 2001; Lee et al., 2001) by digesting with the above mentioned restriction enzymes. The plasmid was then digested with the restriction enzymes XhoI and SacII to isolate a fragment containing the *FRT-Neo-FRT* sequence flanked by the 5' and 3' homologies.

$N1^{457}$-GFPΔ4: I amplified a 138bp fragment that is found at the 5' end of the *$N1^{457}$-GFPΔ4* region to be deleted using the following primers: AAActcgagAATCTCTCTTCCTGCCACCTT, AAAaagcttAGGCATTTAATTGGGGCTTG. These primers contain restriction sites for XhoI and HindIII, respectively. A 142bp fragment was amplified for a region found immediately after the 3' end of the *$N1^{457}$-GFPΔ4* sequence containing restriction sites for NotI and SacII using the following primers: AAAgcggccgcATATGGTCCATTTGAGCACCC, AAAccgcggTCCCCATCTTCTTTTCCAGT, containing restriction sites for NotI and SacII, respectively. In a two step cloning protocol, both of these fragments were inserted into the

previously described pL451 vector (Copeland et al., 2001; Lee et al., 2001) by digesting with the above mentioned restriction enzymes. The plasmid was then digested with the restriction enzymes XhoI and SacII to isolate a fragment containing the *FRT-Neo-FRT* sequence flanked by the 5' and 3' homologies.

Generation of BAC recombinants

A single colony of *EL250-N1^{457}-GFP* bacteria was picked from an LB-Strep (10 μg/ml) / Chlor (12.5 μg/ml) plate, no older than one week old, and inoculated in 5ml LB-Strep (10 μg/ml) / Chlor (12.5 μg/ml) at 32°C O/N (16-20 hrs, always grow these recombination strains at 32°C) to an OD_{600} = 0.5 – 0.7. The next day, 1.0ml of O/N culture was added into 20ml of LB in a 50 mL conical tube and inoculated at 32°C for 2-3 hrs or OD_{600}=0.5. 10ml of the cells were transferred to a new 50 mL conical and shaken in a 42°C water bath for 15 minutes. Immediately after the 42°C induction, the tube was put into wet ice and shaken on an orbital shaker to make sure the temperature dropped as fast as possible. The tube was left on wet ice for 20 min and then spun at 5000 rpm at 0°C for 6 min. The cell pellet was resuspended with 888μl of ice-cold water, transferred the cells to a 1.5ml Eppendorf tube (on ice) and centrifuged using a bench-top centrifuge at room temperature for exactly 20 seconds at 14,000 rpm. The tube was put on ice, aspirated the water without disturbing the pellet. Then resuspended the cell pellet with 888 μl of ice-cold water and centrifuged at 14,000 rpm. This process was repeated three times. Finally, the cell pellet was resuspended in 50 μl of ice-cold water and about 75 μl of the cell suspension were transferred to a disposable electroporation cuvette (0.1 cM gap). I then added 2 μl of the *5'-FRT-Neo-FRT-3'* (50ng), digested with XhoI and SacII and containing the respective 5' and 3' homologies for each of the four deletions, and electroporated it into the *EL250-N1^{457}-GFP* cells

using the Bio-Rad GenePulser electroporator as described above. 1.0 ml of SOC was added to each cuvette and incubated at 32°C for 1 hour. Cells were then plated onto LB-Chlor (12.5 μg/ml) / Kan (25 μg/ml) for 24-48 hrs.

To pop-out the floxed Neo *FRT-Neo-FRT* the *Flpe* recombinase needs to be transiently expressed. In this system, the *Flpe* recombinase is under the tight control of the *Ara* operon, hence 0.1 % arabinose will transiently induce its expression in the *EL250-N1^{457}-GFP* strain. Inoculate a single colony of the *EL250-N1^{457}-GFP* targeted cells in 5ml LB at 32°C O/N. The next day, to induce *Flpe* recombinase, 1.0ml of O/N culture was inoculated into 10ml of LB at 32°C for 2-3 hrs or OD_{600}=0.5. 100 μl of 10% L (+) arabinose (sigma A-3256) was added to the culture (0.1% final concentration) for one hour. 10^{-4} and 10^{-6} dilutions were plated into LB-Chlor (12.5 μg/ml) plates for 24 hours. Single colonies that grew in the LB-Chlor plates were picked and grown O/N in 5 mL of LB-Chlor at 32°C. These were streaked in LB-Chlor (12.5 μg/ml) and LB-Kan (25 μg/ml) plates and grown for 24 hours. Those colonies that grew in the LB-Chlor and not in the LB-Kan plates were then selected to verify BAC integrity as described above. PCR screening and sequencing with primers specific for each region were used to determine precise excision of the deletion target.

BAC Retrieval Protocol

In order to establish the sufficiency of smaller fragments of sequence, as compared to the 184 kb BAC, I decided to use a novel protocol that allows for the retrieval of large fragments of DNA from BACs (Liu et al., 2003; Zhang and Huang, 2003). Hence, I retrieved a 16kb fragment from the *N1^{457}-GFP* BAC flanked by sequences 5' and 3' of the *Neurog1* coding region (Fig 2.1). The following considerations must be taken into account: the *N1^{457}-GFP* BAC was in EL 250 cells; BAC was maintained with appropriate selection and not be kept longer than 1 week at 4 degrees;

all growth steps were performed at 32 degrees; successful retrieval was performed by PCR amplification of vector rather than linearizing by restriction digest (Fig 2.1); a 250 bp homology at both the 5' and 3' regions of sequence to be retrieved; and once retrieved, mini prepped DNA was transformed into electro-competent DH10B cells.

First, inoculate a 5mL culture of fresh, sterile LB (No Selection) with one colony of BAC cells around 2-3 PM day before retrieval, and grow at 32 degrees overnight to OD_{600} =1.2. Take 1 mL of overnight culture and inoculate 20 mL LB into sterile 250 mL flask at 32 degrees shaking at 180rpm for around 2 hours to OD_{600}=0.5-0.6. Transfer 10mL into a new flask for each electroporation and shake at 42 degrees 15 min. to induce rec proteins. After 15 min. at 42 degrees, put flasks in ice bath, swirl gently then leave in ice bath 15 min. Transfer 10 mL into 50 mL disposable corning tube and spin in JA17 at 4000rpm 0 degrees for 5 min., remove supernatant carefully and do not touch or suck up pellet. Resuspend in 888 µl ice cold sterile water and transfer to a pre-chilled 1.5 ml Microfuge tube, spin at 14 K rpm on benchtop at RT for 15 sec. Remove supernatant by vacuum and repeat washes two more times. Resuspend in 50 µl of ice cold water. Add 2-12 ng of linear vector to tube and transfer cells/vector (around 50-70 µl) to a 0.1 cm electroporation cuvette (*Bio-Rad*). Electroporate under the following conditions: 1.75kV, 25µF. 200 ohms, time constants should be at 4-4.5. Add 1 mL of sterile LB and grow at 32 degrees shaking for 1-2 hours then plate 200 µl on Carb. Spin remaining cells in swinging bucket centrifuge at 2500 rpm for 5 min. and remove supernatant. Resuspend cells in the remaining supernatant and plate, grow at 32 degrees overnight. The next day pick colonies and grow overnight in 2 mL LB/Carb at 32 degrees. Mini prep using STET prep and do PCR screening to make sure you have retrieved the correct fragment.

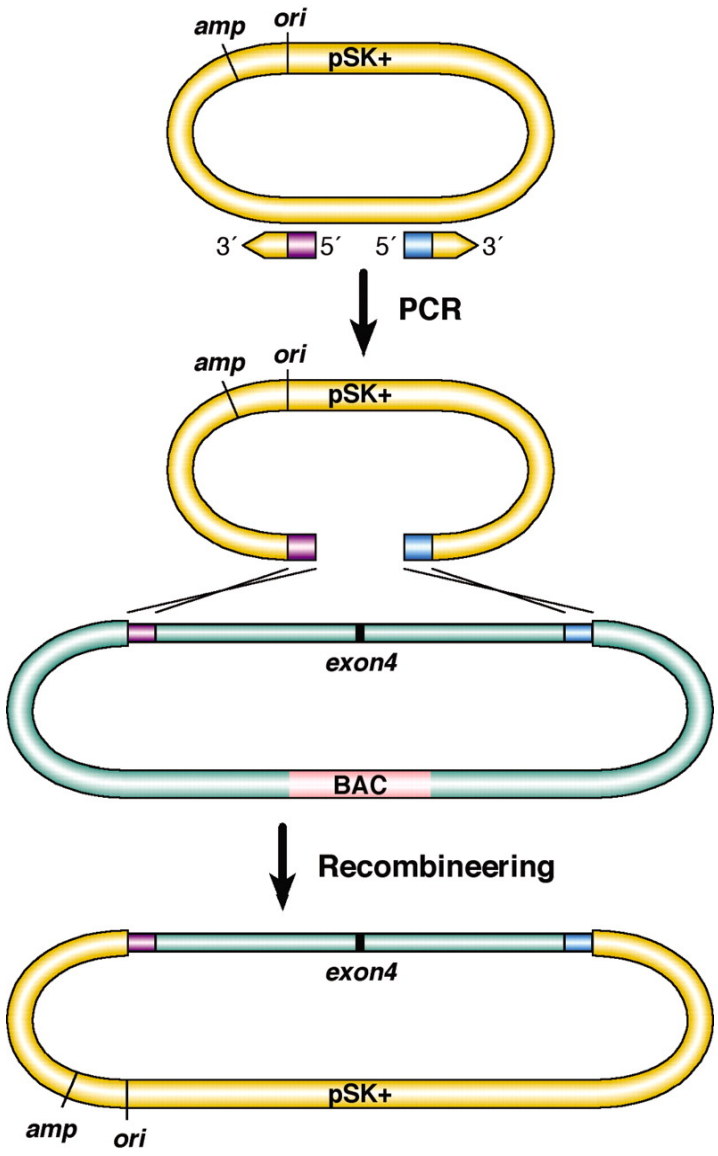


Figure 2.1 <u>Retrieval of large fragments of DNA from BACs.</u>

Subcloning a DNA fragment from a BAC into pBluescript (pSK^+) by gap repair with 100 bp homology arms via recombineering. Cloned regions of homology for 5' (purple) or 3' (blue) sequences flanking the fragment to be isolated. PCR primers with 22bp of homology to the inserted BAC regions are used to amplify pBluescript. The PCR-amplified, linearized pBluescript containing the two homology arms is then transformed into recombination-competent cells that carry the BAC (BAC backbone in pink color). Gap-repaired plasmids are selected by their ampicillin resistance. The black bar denotes the location of *Evi9* exon 4 in this diagram (Liu et al., 2003).

Tamoxifen Induction of N1-CreER^{T2}

To determine if Neurog1 was expressed in progenitors to neurons and glia or in lineage restricted precursors I decided to use the tamoxifen-inducible system. The transgenic line *N1-CreER^{T2}* made by the Lisa Goodrich laboratory in Harvard. This line was made by substituting the *Neurog1* coding region for a tamoxifen inducible Cre-recombinase in the RP23 457 E22 BAC. They also modified this BAC further by digesting it with restriction enzymes that eliminated the 5' end starting at the Cxcl14 gene (see Fig 4.1 for gene location). In this mouse, recombinase activity is not on unless it is induced by the addition of tamoxifen to the system.

Tamoxifen Preparation

Tamoxifen must be in solution to administer it to mice. To make the suspension mix ethanol and oil, since it is a hydrophobic molecule, Tamoxifen is light sensitive hence it was always covered with aluminum foil. The standard stock solution had a concentration of 20 mg/ml. To prepare this solution dissolve 200mg of tamoxifen in 2mL 100% EtOH then vortex very well until in solution. To fully dissolve the tamoxifen dip the mixture into a 37°C water bath in 30 sec intervals and vortexing between intervals. Then add 18mL of sunflower oil and gently invert several times to homogenize the mixture. To get everything into solution and remove bubbles sonicate 3-5min using a beaker with ice. Freeze in 1 ml aliquots at –20°C until ready to use (TM is stable for several months at this temperature). To thaw, dip in 37°C water bath for 5 min or until gavaging the animal. This prevents precipitation and makes injection much better. Use an 18 gauge gavage needle to feed TM to the subjects.

Tamoxifen Gavage Feeding

In order to administer tamoxifen (TM) in a consistent fashion to every animal, I chose to perform gavage feeding of the TM solution. Gavage administration delivered a more consistent dose. I administered TM at a standard concentration of 2 mg/40 g body weight of the animal. After weighing the animal and determining the amount of TM to be given, I use a 1 ml syringe with an 18 gauge gavage needle. I then grab the mouse by scruff of the neck and hold the tail. Then I present the gavage needle to the mouth of the animal and allow it to open the mouth. Slowly allow the needle to continue down the esophageal track, without pushing or make any sudden movements. Once the needle is resting in the stomach, then slowly push the TM-oil mix until you add the required amount. I was careful not to hurt the animal by perforating the stomach or esophagus. Then I wash the needles with ethanol and water to clear any TM-oil residue and re-use these for later inductions. I recorded the amount of TM injected, time, weight and animal number in the appropriate log. I only reused unused portions of TM once by freezing it to -20º C immediately after use.

Transgenic Mouse Generation and Mouse Mutant Strains

Transgenic mice were generated by standard procedures (Hogan et al., 1986) using fertilized eggs from B6D2F1 (C57B1/6 x DBA) crosses. *N1*^{457}*-GFP* and *N1*^{457}*-Cre* BACs were prepared using the modified Qiagen Midi Prep procedure (see above). These BACs were then injected into the pronucleus of fertilized mouse eggs at 0.3-3ng/µl in 10mM Tris pH7.5, 0.1mM EDTA, 100mM NaCl. BAC DNA should be purified the day before injection and stored at 4º C until it is ready to be injected. The *N1*^{457}*-CreER*^{T2} animals were kindly provided by Dr. Goodrich, from Harvard Medical School. The pronuclear injections were performed by Adil Omar-Abdalla, or

the University of Texas, Southwestern Medical Center Transgenic Facility. They use standard procedures including spin column filtration to remove any particulates.

Transient transgenic mice were generated by standard procedures (Hogan et al., 1986) using fertilized eggs from B6D2F1 (C57B1/6 x DBA) crosses and collected at E11.5. For TgN1-17 the following primers were used: AAAAGATCTGTCATTCATGAAATTCTTCCACCCGC and AAAAGGATCCTGAGCCCTGGAGACAGAAGA and was cloned into BgnGFP using BglII and BamHI (See Appendix A: BgnGFP-TgN1-17). TgN1-18 was cloned using the following primers: AAAGGATCCTGACAAATGCCCAAT and CCCTCGAATTCTGTTCTTAAGGGTTC and was cloned into BgnGFP using BamHI and EcoRI (See Appendix A: BgnGFP-TgN1-18). Transgenes were then isolated using

Animal Husbandry

The *N1*^{457}*-GFP* and *N1*^{457}*-Cre* animals were developed as describe above and the *N1*^{457}*-CreER*^{T2} transgenic mice were kindly provided by Dr. Lisa Goodrich; these mice have a tamoxifen-inducible Cre-recombinase replacing the Neurog1 protein coding region in the RP23-457E22 BAC. The reporter strains *R26R-YFP* (Srinivas et al., 2001), *R26R-LacZ* (Soriano, 1999) (Gt(ROSA)26Sor^{tmlSor}) and *Z/EG* (Novak et al., 2000) were used to visualize cells and their progeny that were exposed to Cre recombinase activity. Genotyping was performed using PCR with primers 5'-TTACTTGTACAGCTCGTCCATGCC-3'; 5'-GTGAGCAAGGGCGAGGAGCTGTT-3'for GFP locus, and with 5'-GCATAACCAGTGAAACAGCATTGCTG-3' and 5'-GGACATGTTCAGGGATCGCCAGGCG-3' for *Cre*-recombinase. PCR conditions for GFP and Cre reactions were: 95°C-5min; (95°C-30sec,

60°C-30sec, 72°C-1min) X 4 cycles; (94°C-30sec, 57°C-1min, 72°C-1min) X 36 cycles; 72°C-10min; 4°C hold. R26R mice were genotyped with 5'-AAAGTCGCTCTGAGTTGTTAT-3'; 5'-GCGAAGAGTTTGTCCTCAACC-3'; 5'-GGAGCGGGAGAAATGGATATG-3' and 5'-TTACTTGTACAGCTCGTCCATGCC-3'; 5'-GTGAGCAAGGGCGAGGAGCTGTT-3'for *Z/EG*. PCR conditions for R26R and Z/EG reactions were: 98°C-2min; (95°C-30sec, 60°C-1min, 72°C-1min) X 4 cycles; (94°C-30sec, 57°C-1min, 72°C-1min) X 36 cycles; 72°C-10min; 4°C hold.

Tissue Preparation

Embryos were dissected at appropriate stages and dissected in ice-cold 0.1M Sodium Phosphate buffer pH7.4. These were fixed at 4°C in 4% formaldehyde in 0.1M Sodium Phosphate buffer pH7.4. E10.5-12.5 embryos were fixed for 2 hours. Embryos staged E15.5-E18.5 were more thoroughly dissected by removal of skin, visceral tissue, and most bone including the ribs so that spinal cords were contained solely within the spinal column. E15.5-E18.5 spinal cords were fixed overnight in 4% formaldehyde at 4°C, washed twice in 0.1M Sodium Phosphate buffer pH7.4 for 30 minutes and then again overnight. Tissues were then sunk in 30% sucrose overnight.

To obtain adult spinal cords I performed perfusions with 4% formaldehyde after anesthetizing mice with Avertin (0.024mls/gm). The perfusion apparatus was flushed with roughly 100ml of cold 0.1M Sodium Phosphate buffer pH7.4. Then I made an incision in the anesthetized mice across the lower abdomen and up the ribcage to expose the chest cavity. A 25 gauge needle connected to the perfusion apparatus was inserted into the lower left quadrant of the heart and an incision was made in the upper right quadrant of the heart. 0.1M Sodium Phosphate buffer pH7.4 was circulated through the mouse until the heart turned white. 4% paraformaldehyde was then circulated through the mouse for 10 minutes. Spinal cords were then dissected and retained within the spinal column and post-fixed overnight at 4°C in 4% formaldehyde in 0.1M Sodium Phosphate

buffer pH7.4, and washed three times in 30ml of 0.1M Sodium Phosphate buffer pH7.4 for 30 minutes. All tissues were sunk in 30% sucrose in 0.1M Sodium Phosphate buffer pH7.4 overnight, embedded in OCT compound (Tissue Tek) and cryosectioned.

β-gal staining of mouse embryos

To perform β-gal staining of mouse embryos I fixed these for 30 minutes in 4% formaldehyde in 0.1M Sodium Phosphate buffer pH7.4 at room temperature and washed three times in 0.1M Sodium Phosphate buffer pH7.4 for 30 minutes each. Embryos were then incubated at 32°C for 6 hours in X-gal staining solution (PBS/5mM Kferricyanide, 5mM Kferrocyanide, 2mM $MgCl_2$, 1mg/ml X-gal), washed three times in 0.1M Sodium Phosphate buffer pH7.4 for 20 minutes, and post-fixed in 4% paraformaldehyde.

For staining of β-gal on cryosections, mice were fixed, 2 hours in 4% paraformaldehyde at 4°C then processed and sectioned. Sections were collected on pre-warmed slides. A Fisher slide warmer was used to maintain the temperature of the slide between 37°C and 40°C throughout the sectioning process. If Histobond slides were used, sections were incubated on the slide warmer for an additional 10-15min post-collection to achieve proper adhesion of the sections to the slides. Slides containing sections were washed twice in 0.1M Sodium Phosphate buffer pH7.4 for 10 minutes and incubated overnight in X-gal staining solution as above. Slides were then washed in 1XPBS and coverslipped for imaging.

Counterstaining

Nuclear fast red staining was performed on cryosections and vibratome sections. Slides were air dried for 30 minutes at room temperature. The slides were dipped 5 times in water,

incubated for 5 minutes in filtered nuclear fast red stain, dipped 7 times in water, dipped 7 times in fresh water, and dehydrated stepwise in ethanol (30%, 50%, 75%, 95%, and 100%) for 2 minutes each. Slides were treated three times for 2 minutes in xylene and mounted with permount.

Immunohistochemistry

Single and double immunohistochemistry were performed under the same conditions. Slides were washed three times for 15 minutes in 1X PBS to remove OCT. Slides were then incubated in 1X PBS/1%goat serum/0.1%Triton-X-100 for 30 minutes. The blocking solution was removed and the appropriate primary antibody, diluted in 1XPBS/1%goat serum/0.1%Triton-X-100, was added to slides for incubation overnight at 4°C. Primary antibody was removed and saved for future use; slides were washed three times for 15 minutes in 1XPBS; and diluted secondary antibodies were added for incubation at room temperature for one hour in the dark. Slides were then washed three times in 1XPBS for 15 minutes and mounted with Vectashield for imaging. Double immunohistochemistry was performed by co-incubation with both primary antibodies. Embryos processed for immunohistochemistry were cryosectioned at 30μm.

Immunofluorescence was performed using the following primary antibodies: mouse anti-Lhx1/5 (4F2) (Developmental Studies Hybridoma Bank), rabbit anti-Neurog1 (Johnson Lab), mouse anti-Mash1 (Lo et al., 1991), rabbit anti-GFP (Molecular Probes), rabbit anti-Islet1/2 (Tsuchida et al., 1994), rabbit anti-Math1, rabbit anti-Olig2 (C. Stiles). For a more detailed summary see Table 2.1.

Immunofluorescence analysis was performed using a Bio-Rad MRC 1024 confocal microscope. Images were processed and analyzed using Adobe Photoshop.

Antibody	Reactivity	Dilution	Source	Antigen or Cell type Marked
Brn3a	Guinea pig	1:250	E. Turner	dI1-3. dI5 interneurons
Chx10	Rabbit Poly	1:4000	DSHB	V2
Cre	Rabbit Poly	1:500	Sigma-Aldrich	Cre Recombinase
Evx1/2	Guinea Pig	1:8000	DSHB	V0
En1	Mouse Mono	1:50	DSHB	V1
GFP	Rabbit poly	1:500	Molecular Probes	Green Fluorescence Protein
NeuN	Mouse Mono	1:1000	Chemicon	Neurons
APC	Mouse Mono	1:100	Calbiochem	Oligodendrocytes
GFAP	Mouse Mono	1:400	Sigma-Aldrich	Astrocytes and radial glia progenitors
S100β	Mouse Mono	1:2000	Sigma-Aldrich	Subtypes of astrocytes and oligodendrocytes
Olig2	Rabbit Poly	1:1000	C. Stiles	Oligodendrocytes and Oligodendrocyte precursor cells
Sox10	Guinea Pig	1:1000 – 1:2000	M. Wegner	Oligodendrocytes esp. at E17/E18
HB9	Mouse Mono	1:100	Chemicon	Motor Neurons
Islet 1/2	Rabbit Poly	1:50	DSHB	dI3 and Motor Neurons
Lhx1/5	Mouse Mono	1:50	DSHB	dI2, dI4, dI6, V0-V2, Motor Neurons
Lhx2/9	Rabbit Poly	1:8000	T. Jessell	dI1
Mash1	Mouse Mono	1:100	Anderson	dP3-dP5
Math1	Rabbit Poly	1:100	Johnson	dP1
Nkx2.2	Mouse Mono	1:50	DSHB	Oligodendrocytes
Ngn1	Rabbit Poly	1:5000	Johnson	dP2, dP6, V0-V3, Motor Neurons

Table 2.1. Summary of Antibodies.
Shown is the Antibodies used in this research, dilution, source and antigen/cell type recognized by the antibody.

CHAPTER THREE:

GENOMIC BOUNDARIES OF NEUROG1 REGULATORY SEQUENCE USING BAC TRANSGENIC ANIMALS AND INTERSPECIES SEQUENCE CONSERVATION

Summary

The bHLH transcription factor Neurog1 is involved in neuronal differentiation and cell-type specification in distinct regions of the developing nervous system. I developed mouse models that efficiently drive expression of GFP in all Neurog1 (Ngn1, NeuroD3) domains. I show that a 184 kb BAC containing 113kb 5' and 71kb 3' genomic sequence surrounding the *Neurog1* coding region drives reporter expression in all *Neurog1* domains. A 3.8 kb fragment located 4.2 kb 5' of *Neurog1* is required to direct reporter expression in all *Neurog1* domains. This fragment contains previously identified enhancer elements for midbrain, hindbrain and dorsal neural tube and two sequences conserved from human to fish. A 16kb fragment containing 8.9 kb 5' and 5.2 kb 3' sequence flanking the *Neurog1* coding region was not sufficient to drive expression in all domains. Reporter expression was observed in the dorsal neural tube, the midbrain, hindbrain and trigeminal ganglia, but was missing in the olfactory epithelium, dorsal root ganglia, dorsal telencephalon and ventral neural tube. Furthermore, a 2.3 kb enhancer element located 8 kb 5' of the *Neurog1* coding region was identified that is necessary to drive reporter expression in the ventral neural tube. Thus, separable enhancer elements that are required for efficient reporter expression in all *Neurog1* domains have been identified. These regions can aid in identifying upstream targets involved in *Neurog1* regulation.

Introduction

Proper neural function depends on development of the correct number of cells with the correct identity for accurate assembly of neuronal circuits. This process is regulated by a wide range of molecules that induce neural differentiation. Neural bHLH transcription factors are an essential part of this process. A subset of neural-bHLH transcription factors are transiently expressed in proliferating cells, but is no longer expressed as these cells differentiate into more mature neural cell types (Lee, 1997). This subclass of bHLH transcription factors include Neurogenin 1 (Neurog1), Neurogenin 2 (Neurog2), Math1, and Mash1. These factors have a distinct expression pattern during neurogenesis. Loss of function studies have shown them to be important in the formation of specific neuronal populations (Bertrand et al., 2002; Gowan et al., 2001). Hence, precise spatial and temporal expression of these bHLH transcription factors is essential for the proper formation of a functional nervous system. In this study I use transgenic mice to map specific elements involved in regulating one of these bHLH factors, Neurog1.

During neurogenesis Neurog1 is expressed in progenitor cells within the dorsal telencephalon, midbrain, hindbrain, trigeminal ganglia, dorsal root ganglia, olfactory epithelium, dorsal and ventral neural tube (Cau et al., 2002; Lee, 1997; Ma et al., 1996; Sommer et al., 1996). Gain- and loss-of function studies have been valuable in understanding *Neurog1* function during neural development. Overexpression studies have shown that Neurog1 is sufficient to induce neuronal differentiation in embryonic carcinoma P19 cells, cortical progenitors, chick neural tube, Xenopus and zebrafish (Blader et al., 1997; Gowan et al., 2001; Lee, 1997; Ma et al., 1996; Nakada et al., 2004b; Sun et al., 2001). Furthermore, Neurog1 has been shown to play a role in specifying neuronal subtype in neural crest derivatives and in chick dorsal neural tube where ectopic expression induced sensory neuron-appropriate markers in non-sensory crest derivatives

and excess dI2 dorsal interneurons, respectively (Gowan et al., 2001; Perez et al., 1999). Loss-of-function studies in mouse have shown that *Neurog1* is required for the formation of olfactory neurons and cranial sensory ganglia (Cau et al., 2002; Ma et al., 1998; Ma et al., 1999). Furthermore, Neurog1 and the related factor Neurog2 together have been shown to be required for the proper development of dorsal root ganglia, dorsal interneuron population dI2 in the spinal cord, and glutamatergic neurons in cerebral cortex (Gowan et al., 2001; Ma et al., 1999; Nieto et al., 2001; Schuurmans et al., 2004). Taken together these studies show that Neurog1 can induce general neuronal differentiation and specify neuronal subtype in the peripheral and central nervous system (Gowan et al., 2001; Perez et al., 1999). Hence, understanding how Neurog1 expression is regulated during neurogenesis is an important part of identifying the mechanisms involved in the accurate assembly of neuronal circuits.

Previous studies using animal models have identified various cis-regulatory elements involved in regulating Neurog1 expression during neural development. A 7.5 kb fragment located 4.5 kb 5' of the *Neurog1* start site was shown to drive expression in most of the Neurog1 expression domains, including the midbrain, hindbrain, dorsal neural tube, ventral neural tube, trigeminal ganglia and olfactory epithelium (Gowan et al., 2001). However, reporter expression was missing in both the dorsal root ganglia and dorsal telencephalon (Gowan et al., 2001). Three sequences within the 7.5 kb region are highly conserved with sequences flanking the zebrafish *Neurog1* coding region, two of these overlap with specific enhancer elements identified for the midbrain and dorsal neural tube (Fig 3.1 and Fig 3.6)(Blader et al., 2003; Nakada et al., 2004b). In Zebrafish these sequences have been shown to be required to direct expression in the lateral stripe and the anterior plate (Blader et al., 2003). A dorsal neural tube enhancer element was identified within a 0.7kb sequence that is sufficient to drive dorsal neural tube expression in mouse (Nakada

et al., 2004b). Additional regulatory sequences located 3' of the *Neurog1* coding region have been shown to direct expression to the midbrain and hindbrain (Fig 3.1)(Nakada et al., 2004b; Simmons et al., 2001). Thus, tissue specific expression of Neurog1 is regulated by separable enhancer elements with some redundancy for midbrain and hindbrain expression.

Transgenic models have played an important role in the study of neural bHLH transcriptional regulation. Similar to Neurog1, the evolutionarily related bHLH, Neurog2, has been shown to be regulated by separable enhancer elements. Distinct elements spanning over 20 kb of sequence have been identified that regulate *Neurog2* expression to a number of its expression domains (Scardigli et al., 2001; Simmons et al., 2001). Also, a 1.1 kb sequence located 7kb 5' of *Mash1* has been shown to drive reporter expression to a subset of *Mash1* expressing tissues (Verma-Kurvari et al., 1998). However, this fragment is not sufficient to drive expression in all *Mash1* expression domains (Verma-Kurvari et al., 1998), suggesting other regulatory elements exist at more distant positions. On the other hand, no separable enhancer sequences have been implicated in directing tissue-specific expression of *Math1*. Rather a single enhancer element located 3kb 3' of the *Math1* coding sequence was found to be sufficient to drive reporter expression in all of the *Math1* expression domains (Ebert et al., 2003; Helms et al., 2000).

Previous studies addressing Neurog1 transcriptional regulation have identified separable enhancer elements involved in Neurog1 expression. However, regulatory elements involved in regulating Neurog1 expression in the dorsal telencephalon and dorsal root ganglia have not been established. I hypothesized that large genomic regions from BAC clones would contain all of the cis-elements involved in Neurog1 regulation and hence drive reporter expression in all of the Neurog1 expression domains. Through BAC recombineering the *Neurog1* coding sequence was precisely replaced with EGFP in a BAC containing large genomic regions flanking the *Neurog1*

coding region. Reporter expression in transgenics containing the modified BAC fully recapitulated the Neurog1 expression profile, including the dorsal root ganglia and dorsal telencephalon. Using targeted deletions in the BAC sequence, I identified two regions that were necessary for directing reporter expression in the *Neurog1* pattern. Deletion of one sequence resulted in loss of expression across all Neurog1 domains at e11.5, while the second resulted in a specific loss in ventral neural tube expression, identifying a novel ventral neural tube enhancer. Thus this study supplies tools for studying Neurog1 regulation in a tissue specific manner, and provides the framework for identifying upstream regulatory molecules involved in Neurog1 expression.

BAC RP23 457E22 contains sufficient cis*-regulatory sequence for directing accurate Neurog1 expression*

Previous studies testing individual DNA fragments of *Neurog1* flanking sequence across ~15 kb identified multiple sequences sufficient to direct expression of reporter genes to a subset of the Neurog1 expression domain (Blader et al., 2003; Gowan et al., 2001; Murray et al., 2000; Nakada et al., 2004b). However, not all enhancer elements were defined particularly those sufficient to direct efficient expression in the dorsal telencephalon and dorsal root ganglia. Conservation between human and mouse sequences in 50 kb surrounding the *Neurog1* coding region (Fig. 3.1) reveals multiple blocks of conserved sequence outside the 15 kb previously tested. A subset of these sequence blocks show conservation with chicken (Fig. 3.1, orange ovals) and zebrafish (Fig. 3.1, yellow ovals). In this study, I test these additional conserved sequence blocks to determine if they are sufficient to direct expression to a Neurog1 expression domain. In addition, I test whether previous identified enhancer regions are required for Neurog1 expression.

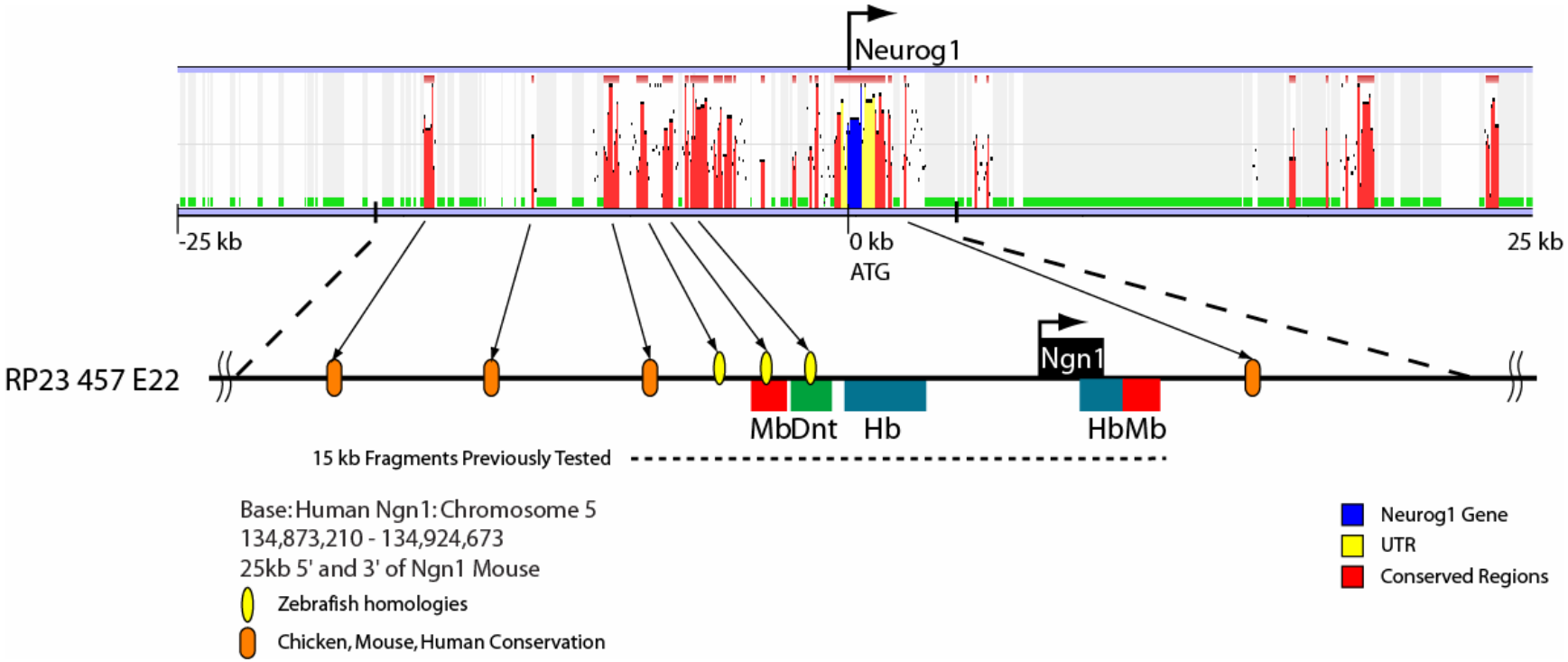


Figure 3.1 <u>Sequence conservation 25kb 5' and 3' of Neurog1.</u>

Figure 3.1 Sequence conservation 25kb 5' and 3' of Neurog1.

Smooth-graph and dot-plots constructed by zPicture (Ovcharenko et al., 2004) for a 50 kb region containing 25 kb 5' and 3' of the *Neurog1* coding region. The automatic sequence upload feature extracted the human sequences and the RefSeq annotation files from the UCSC database. Default parameters (>100 bp/>70% ID) were used to highlight intragenic (red) conserved elements. Untranslated Regions (UTRs) are colored in yellow, and exons are in blue. Inverted regions are shaded in gray. The RP23 457 E22 BAC is diagrammed below the zPicture to show the location of the *Neurog1* coding region and previously studied enhancer elements for midbrain, hindbrain and dorsal neural tube expression (red, blue, and green, respectively). Sequence conserved between mouse and human with zebrafish (yellow ovals), and chicken (orange ovals) is indicated. Dnt: dorsal neural tube; Mb: midbrain; Hb: hindbrain. zPicture generated from http://rvista.dcode.org/ (Loots and Ovcharenko, 2004; Ovcharenko et al., 2004).

To begin testing the function of additional cis-regulatory regions for *Neurog1,* I generated a transgenic mouse that drives GFP reporter in all Neurog1 domains. Using BAC recombineering techniques (Gong et al., 2002; Lee et al., 2001; Yang et al., 1997), the *Neurog1* coding sequence was precisely replaced with a nuclear localized EGFP in the BAC RP23 457E22 (Fig. 3.2A). This modified BAC containing 113kb 5' and 71kb 3' genomic sequence flanking the *Neurog1* coding region was injected into the pronuclei of single cell mouse embryos to generate a stable transgenic strain, or transgenic embryos were harvested at E11.5 and assayed for GFP expression. Characterization of the stable transgenic line, *N1^{457}-nGFP*, is described in detail in Chapter 4. This transgenic strain was contributed to the GENSAT project and the standard GENSAT characterization at E15.5, P7 and adult is available at http://www.ncbi.nlm.nih.gov/.

In *N1^{457}-nGFP* transgenic embryos at E11.5, GFP expression is detected in all *Neurog1* domains including dorsal and ventral neural tube, olfactory epithelium, trigeminal ganglia, midbrain, hindbrain, and notably the dorsal root ganglia and dorsal telencephalon (Fig 3.2D,E; Figure 3.3). I examined whether expression from the *N1^{457}-nGFP* transgene respected the endogenous boundaries of Neurog1 expression in the dorsal neural tube where Neurog1 is present precisely in progenitors to the dorsal interneuron population 2 (dI2), non-overlapping with the closely neighboring progenitors marked by the bHLH factors Math1 and Mash1. GFP overlaps with Neurog1 immunofluorescence (Fig. 3.4) but not Math1 and Mash1. Furthermore, GFP persists into differentiating interneurons, and overlap is seen with Brn3a but not Lhx2/9 consistent with expression precisely in dI2 but not dI1 neurons (Fig. 3.4). Thus, the BAC transgene, *N1^{457}-nGFP*, reliably reports Neurog1 expression domains when tested in transgenic transient embryos at E11.5.

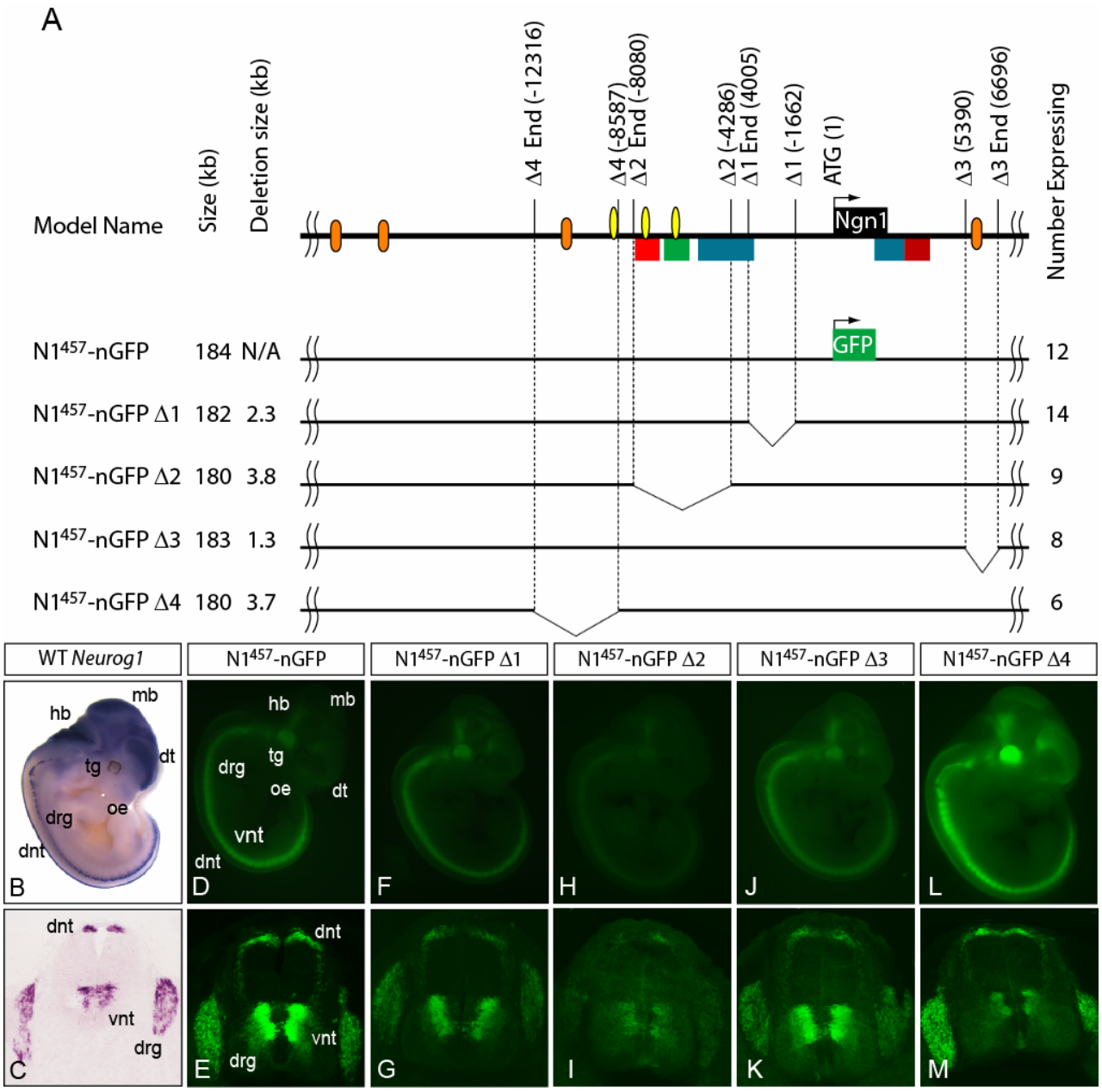


Figure 3.2 Specific regulatory elements are required to drive proper Neurog1 expression.

Figure 3.2 <u>Specific regulatory elements are required to drive proper Neurog1 expression.</u>

(A) Schematic illustrating the modified *EGFP* BACs *N1^{457}-nGFP- N1^{457}-nGFPΔ4* tested in transgenic mouse embryos. The black line at the top represents the *Neurog1* gene with the coding sequence represented by black box. Numbers in parentheses refer to the number of nucleotides 5′ (negative numbers) or 3′ (positive numbers) of the ATG of *Neurog1*, which is set to 1. Number Expressing is the number of independently derived transgenic embryos expressing the *EGFP* transgene. In-situ hybridization with probe against *Neurog1* mRNA in (B) whole-mount and (C) cross-section of neural tube at forelimb level at E11.5. (D-M) GFP expression in transgenic embryos is shown in whole mount and in cross section. Whole-mount images were taken using identical exposure times (D, F, H, J, and L). Cross sections (E, G, I, K, M) through the spinal cord of transgenic embryos were obtained using confocal microscopy with identical Power, Gain, Offset and Iris settings (with the exception of Δ2, as I used a larger Iris as expression levels were notably lower). At least four embryos for each transgene were analyzed by sectioning and confocal imaging. dnt: dorsal telencephalon; dt: dorsal telencephalon; drg: dorsal root ganglia; hb: hindbrain; mb: midbrain; oe: olfactory epithelium; tg: trigeminal ganglia; vnt: ventral neural tube.

Some sequences conserved across multiple mammalian species and chicken are not required for Neurog1 expression pattern at E11.5

The reliable expression of *N1[457]-nGFP* provides a tool to test whether the enhancer sequences previously described as sufficient for directing expression to specific subsets of the Neurog1 pattern are also required. To do this, four deletions were made in the *N1[457]-nGFP* BAC using BAC recombineering techniques (Copeland et al., 2001). Three deletions covered conserved sequences 5' of *Neurog1* and one was 3' of the gene (Fig. 3.2A). Each BAC deletion was used to generate transgenic embryos that were assayed at E11.5. To control for unplanned, undetected rearrangements in the BAC sequences, two independently derived BAC constructs for each deletion were used to generate multiple transgenic embryos. The GFP fluorescence from embryos shown in Figs. 3.2 and 3.3 using different transgenes were obtained using comparable exposure times and gain settings. Two of the deletions, *N1[457]-nGFPΔ1* and *N1[457]-nGFPΔ3,* although containing sequence conserved across multiple mammalian species and chicken, had no detectable alteration in the expression of the reporter gene at E11.5 (Fig 3.2A, J, K; Fig 3.3). Even the precision in the dI2 progenitors was maintained (Fig. 3.4). *N1[457]-nGFPΔ1* lacks a 2.3 kb fragment located 1.6 kb 5' of the *Neurog1* coding sequence and predicted to be 5' of the transcription start site (Fig 3.2F, G). A transgene containing this region worked only inefficiently at directing expression of a reporter to Neurog1 domains (Murray et al., 2000; Nakada et al., 2004b)(See TgN1-1). *N1[457]-nGFPΔ3* lacks a 1.3 kb sequence 5.4 kb 3' of the *Neurog1* start site (Fig 3.2J, K). This sequence was also not sufficient to direct expression of a reporter gene at E11.5 (Fig. 3.6A). These results indicate that the conserved sequences in these two deletions are not required for accurate expression of Neurog1 at E11.5. However, the inability to detect a loss of activity at E11.5 does not

preclude the possibility that the conserved sequences deleted in Δ*1* and Δ*3* are required for Neurog1 expression at another developmental stage.

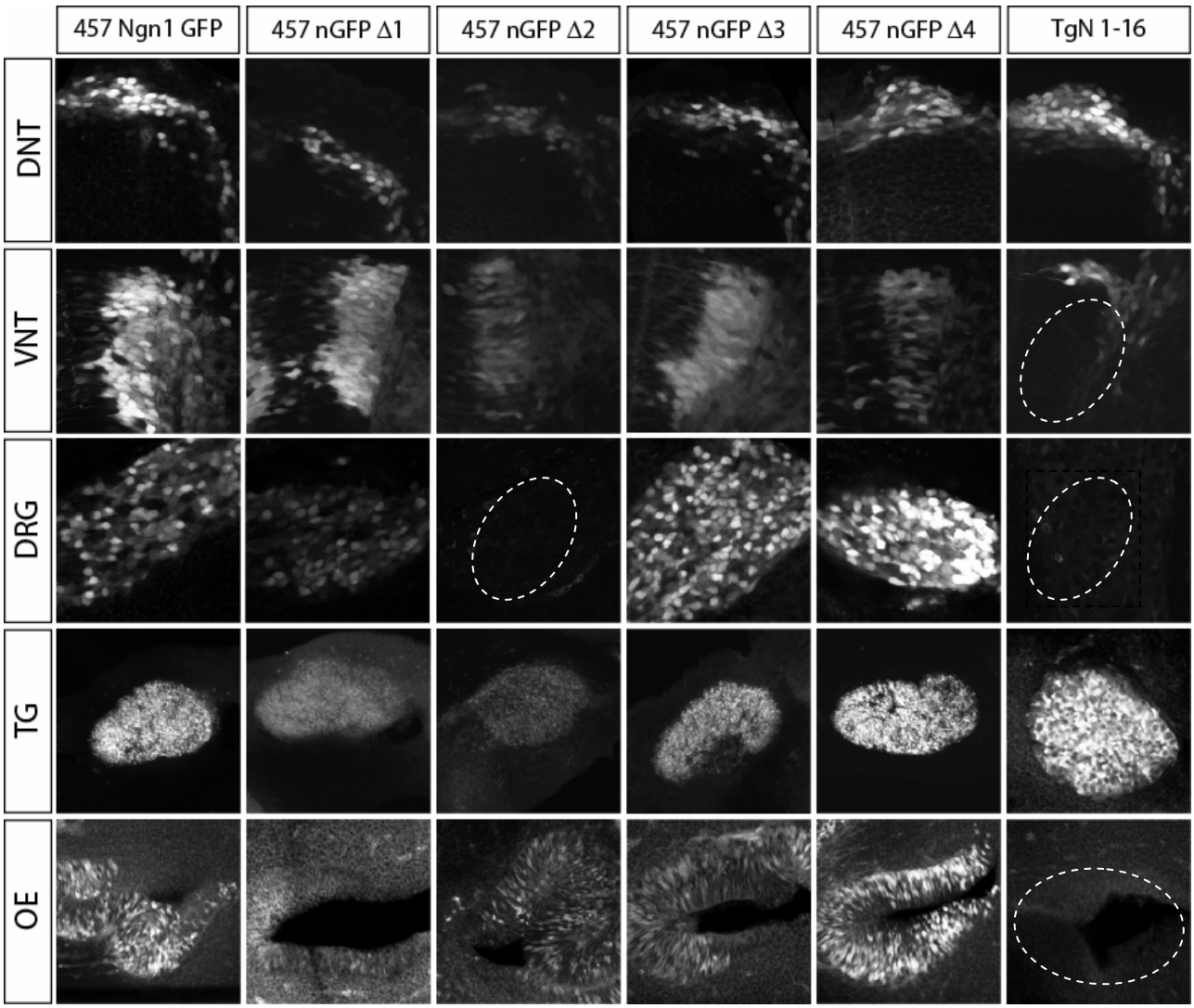


Figure 3.3 <u>Specific regulatory elements are required to drive tissue-specific Neurog1 expression.</u>

Figure 3.3 Specific regulatory elements are required to drive tissue-specific Neurog1 expression.

Tissue-specific *EGFP* reporter expression of indicated transgenic mouse models. Images were obtained using different confocal settings for Power, Iris, Gain and offset. DNT: dorsal telencephalon; DRG: dorsal root ganglia; OE: olfactory epithelium; TG: trigeminal ganglia; VNT: ventral neural tube. Dashed ovals showing lack of expression in the ventral neural tube, dorsal root ganglia and olfactory epithelium.

A 3.8 kb sequence 5' of Neurog1 is required for efficient expression in all Neurog1 domains at E11.5

The deletion in *N1[457]-nGFPΔ2* includes enhancer sequence shown previously to be sufficient to drive reporter expression to the dorsal neural tube, midbrain, and hindbrain regions, and contains two highly conserved zebrafish homologies (Blader et al., 1997; Gowan et al., 2001; Nakada et al., 2004b)(also see Fig. 3.6, TgN1-2). Indeed, a dorsal tube enhancer element was identified within a 0.7 kb fragment that is sufficient to direct expression specifically to the dorsal neural tube domain, and is required within the 7.5 kb TgN1-2 enhancer for the dorsal neural tube expression (Nakada et al., 2004b). Thus, it was predicted that at minimum, *N1[457]-nGFPΔ2* would lack expression in the dorsal neural tube. Surprisingly, *N1[457]-nGFPΔ2* dorsal neural tube expression was not specifically lost. Rather, there was a dramatic decrease in overall reporter expression levels across all domains when compared to *N1[457]-nGFP* (Fig. 3.6). Although higher gains were required to detect the GFP, expression in all Neurog1 domains was detected (Fig. 3.3) and the precise dI2 progenitor boundaries were maintained (Fig. 3.4). This dramatic decrease in overall expression demonstrates the presence of an important general enhancer component within the conserved sequence in *Δ2*. Furthermore, the fact that low expression remains in all Neurog1 domains suggests that an autoregulatory component may exist in controlling gene expression from this locus. Alternatively, regulation of Neurog1 may be controlled by redundant enhancer cassettes, such as the existence of an additional dorsal neural tube element elsewhere within the BAC sequence.

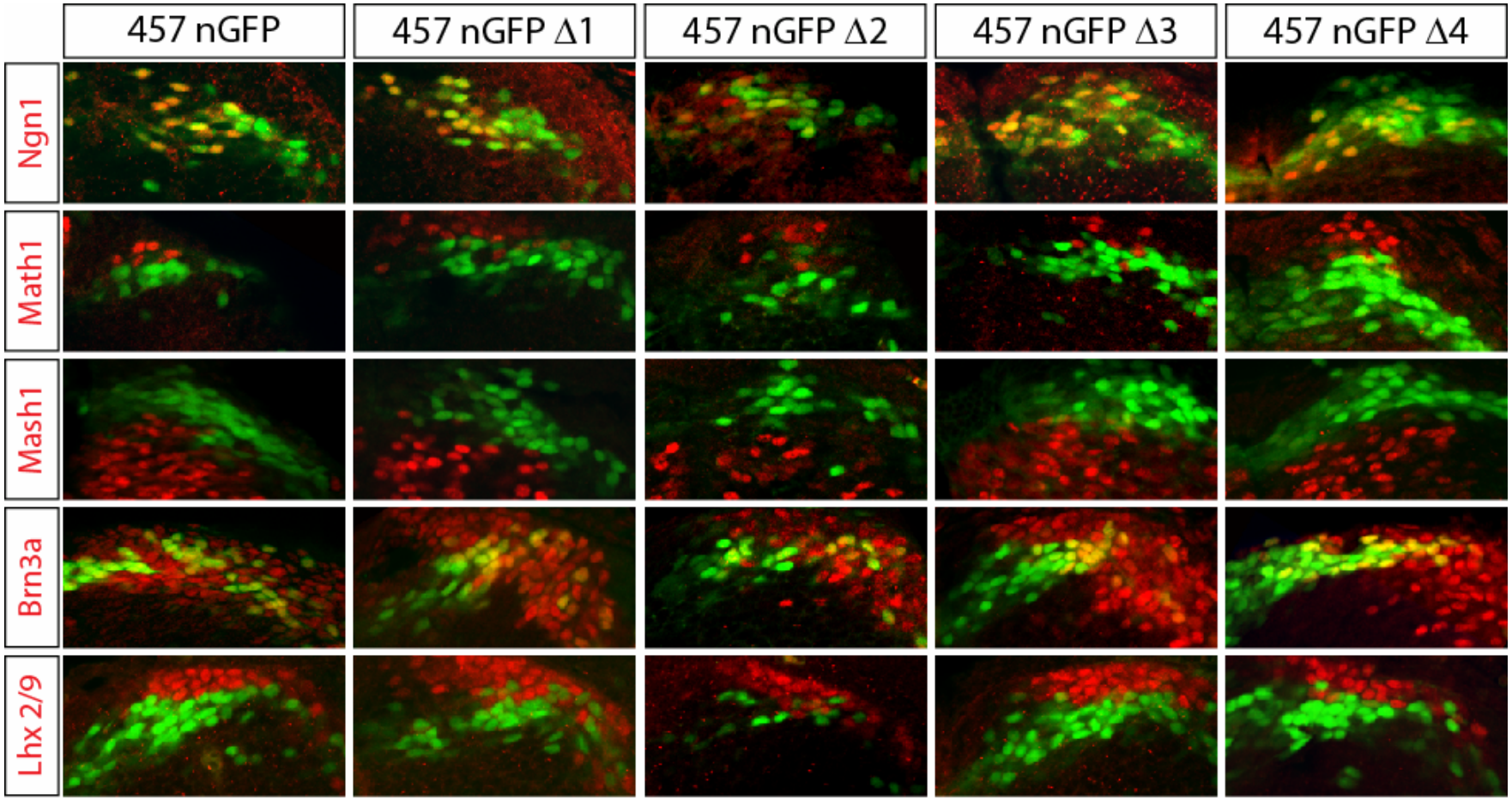


Figure 3.4 Reporter expression respects spatial boundaries of Neurog1.

Figure 3.4 <u>Reporter expression respects spatial boundaries of Neurog1.</u>

Immunofluorescence with multiple bHLH and HD transcription factor markers and GFP (green) in *N1^{457}-nGFP* - *N1^{457}-nGFPΔ4* dorsal neural tubes at E11.5. Antibodies are indicated and recognize Neurog1, Math1 (Math1), Mash1 (Mash1), Brn3a, Lhx2/9. GFP expression co-localizes with Neurog1 in the dP2 population as seen by the yellow cells. GFP, Math1 and Mash1 showed no co-localization in any of the animal models. GFP and Brn3a overlap in the dI2 domain as seen by the yellow cells. GFP did not overlap with Lhx2/9 in the dI1 domain.

A ventral neural tube specific enhancer is identified

The only deletion that resulted in a dramatic loss of expression in a specific subset of the Neurog1 pattern was *N1*^{457}*-nGFPΔ4* (Fig. 3.2 L, M; Fig 3.3; and Fig 3.5). Like the other deletions, *Δ4* contains sequence highly conserved across vertebrate species, and in this case, including zebrafish. (Fig. 3.2 A). With this deletion, there is a dramatic decrease in GFP levels specifically in the ventral neural tube. This is evident by comparing the levels in the dorsal root ganglia to the levels in the ventral neural tube in the wild type *N1*^{457}*-nGFP* versus the *N1*^{457}*-nGFPΔ4* transgenic embryos (Fig. 3.2D,E, vs. L,M), and highlighted by normalizing the imaging of the sections to the levels of GFP in the dorsal root ganglia (Fig. 3.5). From these data, it is clear that sequence included in the *Δ4* deletion is required for efficient expression in the ventral neural tube but not other domains (Fig. 3.2 for comparison). Again, the low level expression remaining in the ventral neural tube could reflect the existence of a redundant specific enhancer element, or the general autoregulatory component suggested by *N1*^{457}*-nGFPΔ2* (see previous section).

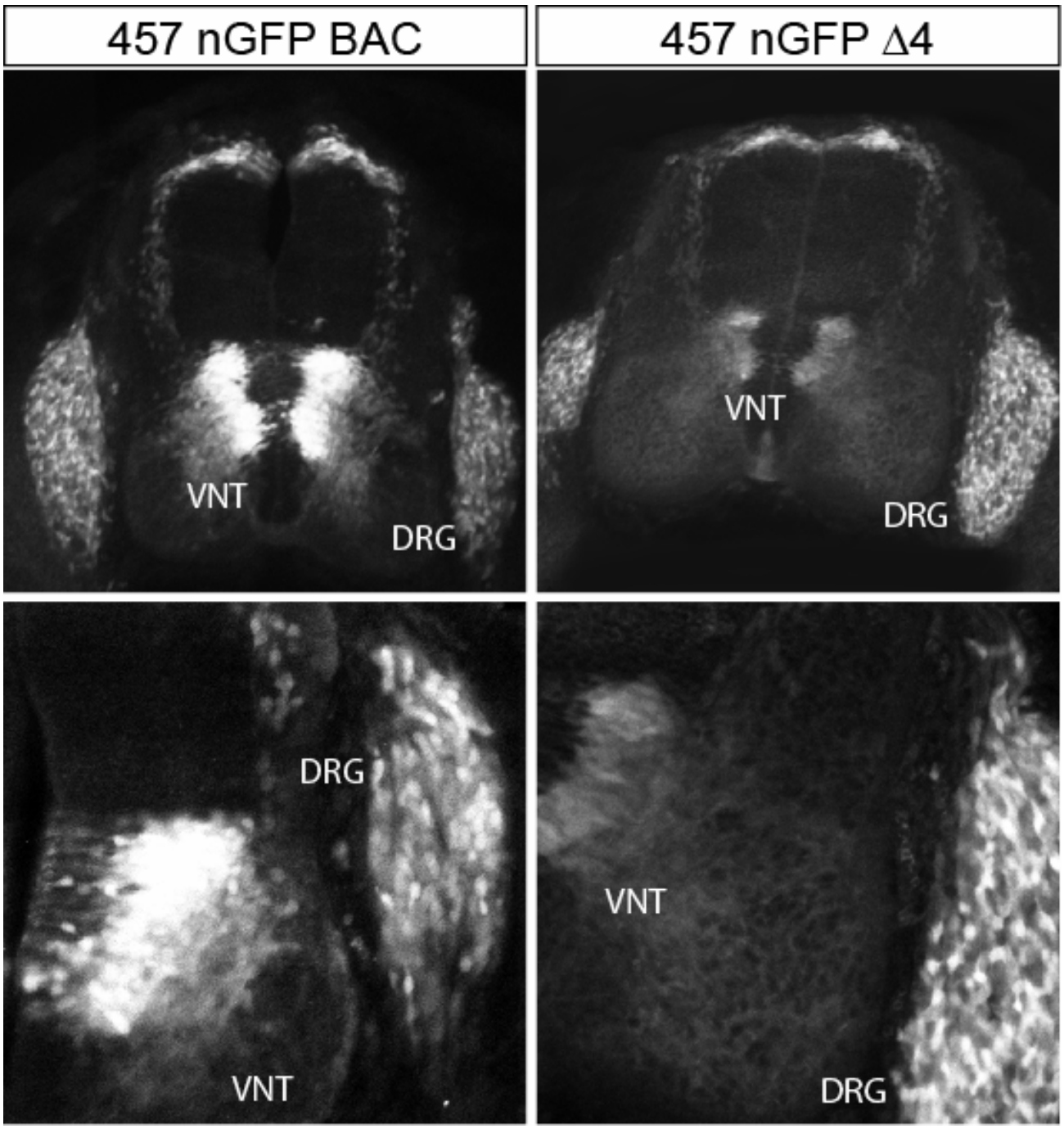


Figure 3.5 VNT enhancer is necessary for ventral neural tube expression.

Figure 3.5 <u>VNT enhancer is necessary for ventral neural tube expression.</u>

Cross sections of $N1^{457}$-nGFP and $N1^{457}$-nGFPΔ4 at forelimb level through the spinal cord of transgenic embryos. Images were obtained using confocal microscopy and normalizing for GFP expression on DRG. DRG: dorsal root ganglia; VNT: ventral neural tube.

Analyzing ventral neural tube expression from additional transgenic constructs identified the location of the ventral neural tube enhancer. First, a 16 kb sequence including 9 kb 5' and 7 kb 3' of *Neurog1* was retrieved from *N1^{457}-nGFP* using the Copeland retrieval method (Zhang and Huang, 2003), and this 16 kb transgene was tested for activity in transgenic mice. This was done to combine for the first time contiguous sequence from the 5' and 3' regions to determine if the combination is what was required for expression in all Neurog1 domains. GFP expression from this construct mimics Neurog1 expression in midbrain, hindbrain, dorsal neural tube, and trigeminal ganglia but expression in the ventral neural tube, dorsal root ganglia, dorsal telencephalon, and olfactory epithelium is lost (Fig. 3.6; TgN 1-16). This clearly places required enhancer elements for these last four domains outside the 16 kb tested. For the ventral neural tube, the difference between the 5' end of the 16 kb transgene that lacks ventral neural tube expression, and the previously reported TgN1-2 that drives ventral neural tube expression (Nakada et al., 2004b and Fig. 3.6 here), is a 1.2 kb region that contains a sequence conserved from human to chicken. A transcription factor consensus binding site search reveals multiple possible sites for Nkx2.2, a HD transcription factor shown to induce gliogenesis, which might be involved in maintaining the ventral boundary of expression through repression (Aguirre and Gallo, 2004; Fu et al., 2002; Gulacsi and Anderson, 2006; Ohori et al., 2006). Other regions containing cross species conserved sequences were also tested but showed no reporter activity at E11.5. Therefore, these sequences could be responsible for modulating levels or functioning at different stages of development not detected in these analysis (Fig 3.6: TgN 1-17; TgN1-18). This neural tube enhancer element can be used to study tissue-specific expression and identify specific binding sites for potential upstream regulatory molecules involved in regulating Neurog1 expression.

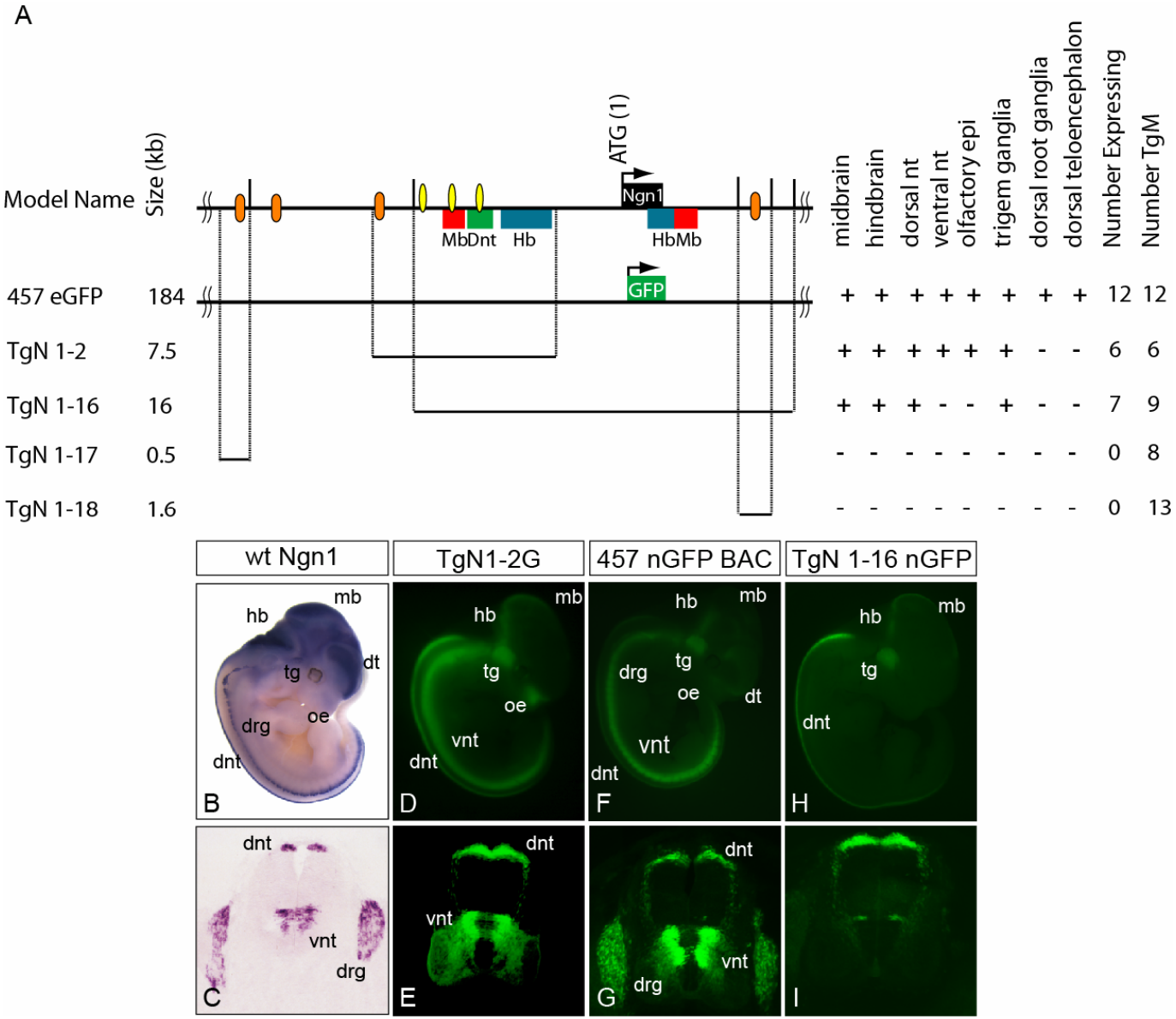


Figure 3.6 Ventral neural tube enhancer is necessary to drive tissue-specific expression.

Figure 3.6 Ventral neural tube enhancer is necessary to drive tissue-specific expression.

(A) Schematic illustrating the *GFP* transgenes $N1^{457}$*-nGFP*, TgN1-2 to TgN1-18 tested in transgenic mouse embryos. The black line at the top represents the *Neurog1* gene with the coding sequence represented by black box. Number TgM is the number of independently derived transgenic embryos examined, and Number Expressing is the number of embryos expressing the *GFP* transgene in the tissues indicated. In-situ hybridization with probe against *Neurog1* mRNA in (B) whole-mount and (C) cross-section of neural tube at forelimb level at E11.5. Whole-mount images were taken using the same exposure times for $N1^{457}$*-nGFP* and TgN 1-18 (F, H). Cross sections (G, I) through the spinal cord of transgenic embryos were obtained using confocal microscopy with the same Power, Gain, Offset and Iris. TgN1-2 was previously published (Nakada et al., 2004b). dnt: dorsal telencephalon; dt: dorsal telencephalon; drg: dorsal root ganglia; hb: hindbrain; mb: midbrain; oe: olfactory epithelium; tg: trigeminal ganglia; vnt: ventral neural tube.

CHAPTER FOUR:

NEUROG1-EXPRESSING CELLS GIVE RISE PREFERENTIALLY TO INTERNEURONS IN THE VENTRAL SPINAL CORD

Summary

I developed mouse models that efficiently drive expression of GFP or Cre recombinase in all Neurog1 domains. Using both short-term and long-term lineage analyses, derivatives of Neurog1-expressing progenitor cells in the neural tube largely comprise the interneuron populations dI2, dI6, V0, V1, and V2, and to a lesser extent motorneurons. This is seen in the co-expression of GFP driven by *Neurog1* regulatory sequences with the neuronal identity markers Brn3a, Islet1/2, Lhx1/5, Lhx3, Pax2, and Chx10. Genetic fate mapping in vivo using Cre recombinase reveals that although Neurog1-expressing cells primarily give rise to neurons, minor populations of oligodendrocytes and astrocytes are also identified in the lineage in the adult spinal cord. Adding temporal control to the fate mapping strategy demonstrates that the neurons are generated from Neurog1-expressing cells prior to E13, and glial cells after E13, placing Neurog1 in lineage restricted precursor cells during embryogenesis.

Introduction

Proper neural function depends on development of the correct number of cells with the correct identity for accurate assembly of neuronal circuits. The ventricular zone (VZ) of the embryonic neural tube gives rise sequentially to the neurons, oligodendrocytes, and astrocytes that comprise the adult nervous system. Two important families of regulators define distinct cells in the VZ, and these regulators act to confer diversity to the emerging nervous system; the bHLH and HD transcription factors (Allan and Thor, 2003; Lee and Pfaff, 2003). Combinations of HD factors define progenitor cells and instruct their fate into specific neuronal subtypes (Allan and Thor, 2003; Lee and Pfaff, 2003). In contrast, the bHLH proteins have traditionally been viewed as "generic" proneural factors, acting within the Notch pathway to single out neuronal progenitors and promoting their subsequent differentiation within the ventricular zone (Bertrand et al., 2002). However, increasing evidence points to a more specific role for bHLH genes in the establishment of unique cell fates (Gowan et al., 2001; Lee and Pfaff, 2003; Nakada et al., 2004b; Perez et al., 1999; Scardigli et al., 2001). The bHLH factor Neurog1 (Ngn1, NeuroD3) defines progenitors that give rise to dI2 dorsal interneurons but it is also expressed broadly in the ventral neural tube. Here I use in vivo genetic fate mapping techniques to define the neural lineages in the spinal cord arising from cells expressing the bHLH factor Neurog1.

bHLH factors involved in neural development can be subdivided into different subclasses including the Neurogenin family (Neurog1,2,3) which is most closely related to *Drosophila* Tap (Gautier et al., 1997; Ledent et al., 1998). Neurog1 and Neurog2 have overlapping expression patterns and identical bHLH motifs (Sommer et al., 1996) that result in apparent redundant function in multiple neural domains (Frowein et al., 2002; Helms et al., 2005; Sommer et al., 1996). However, Neurog1 and Neurog2 also have distinct functions that are highlighted by the loss of

specific neuronal lineages in mice mutant for either *Neurog1* or *Neurog2* (Scardigli et al., 2001; Sommer et al., 1996). In particular, *Neurog1* mutants have defects in proximal cranial ganglia, olfactory neurons, and inner ear (Cau et al., 2002; Ma et al., 2000; Ma et al., 1998). Defects in other neural regions such as dorsal root ganglia, spinal neural tube, and dorsal telencephalon are more easily detected in *Neurog1/Neurog2* double mutants (Fode et al., 2000; Gowan et al., 2001; Ma et al., 1999; Scardigli et al., 2001). The partially overlapping expression between these closely related bHLH factors, and the complex mutant phenotypes, make it difficult to delineate the full complement of neural lineages derived from Neurog1-expressing cells.

Over expression of multiple neural bHLH factors including Neurog1 is sufficient to induce neuronal differentiation in embryonic carcinoma P19 cells, cortical progenitors, and chick neural tube (Farah et al., 2000; Lee, 1997; Lee and Pfaff, 2003; Sun et al., 2001). In cortical progenitors, Neurog1 over-expression also inhibits gliogenesis, and unlike its function in neurogenesis, this inhibition is DNA binding independent (Sun et al., 2001). That Neurog1 plays an additional role in specifying neuronal subtype has been demonstrated in neural crest derivatives and in chick dorsal neural tube where ectopic expression induced sensory neuron-appropriate markers in non-sensory crest derivatives and excess dI2 dorsal interneurons, respectively (Gowan et al., 2001; Perez et al., 1999). Together, these data define Neurog1 as an important player in the formation of neurons over glia, and in some cases in contributing to neuronal type specification in multiple regions of the developing nervous system.

Neurog1 expression, like other bHLH transcription factors, is transient and restricted to specific subsets of neural progenitors within the ventricular zone (VZ). As these cells migrate laterally away from the VZ, exit the cell cycle, and differentiate into specific neural cell types, Neurog1 expression is lost. Hence, this conundrum poses a limitation in determining which mature

neural cells are derived from Neurog1-expressing progenitors. I set out to solve this issue using in vivo genetic fate mapping of the cell types in the spinal cord derived from Neurog1-expressing progenitors. Three transgenic mouse models were generated to address these issues. Each transgenic mouse strain contains a 184kb BAC that has the centrally located *Neurog1* coding sequence replaced by that for GFP, Cre, or $CreER^{T2}$. Here I show that Neurog1-expressing progenitors give rise to dorsal interneuron populations dI2 and dI6, and to ventral interneuron populations V0, V1, V2 as well as a subset of motor neurons in the spinal cord. Although the vast majority of Neurog1 derivatives are neurons born before E13, after this time the remaining Neurog1 cells generate glial cells suggesting inhibition of this cell-type by Neurog1 is overcome.

A 184 kb BAC contains regulatory information for directing Neurog1-like expression

The transient nature of expression of the bHLH transcription factor Neurog1 in VZ cells, which is extinguished after the cells leave the VZ and differentiate, makes it difficult to study which mature neural cell types are derived from these cells. To overcome this hurdle, I generated transgenic mouse models to perform short and long term lineage tracing of these cells. The bacterial artificial chromosome (BAC) RP23-457E22 has the *Neurog1* coding region flanked by 113kb 5' and 71kb 3' genomic sequence (Fig. 4.1A). Using BAC recombineering strategies (Yang et al., 1997a), the *Neurog1* coding sequence was replaced with that encoding nGFP or Cre-recombinase. A third BAC replacing *Neurog1* with a tamoxifen-inducible Cre (Cre^{T2}) was generated by Lisa Goodrich's laboratory at Harvard (Fig. 4.1A). These modified BACs were then used to generate transgenic animals $N1^{457}$*-nGFP*, $N1^{457}$*-Cre*, or $N1^{457}$*-*$CreER^{T2}$, from which I chose one line of each to complete the analyses.

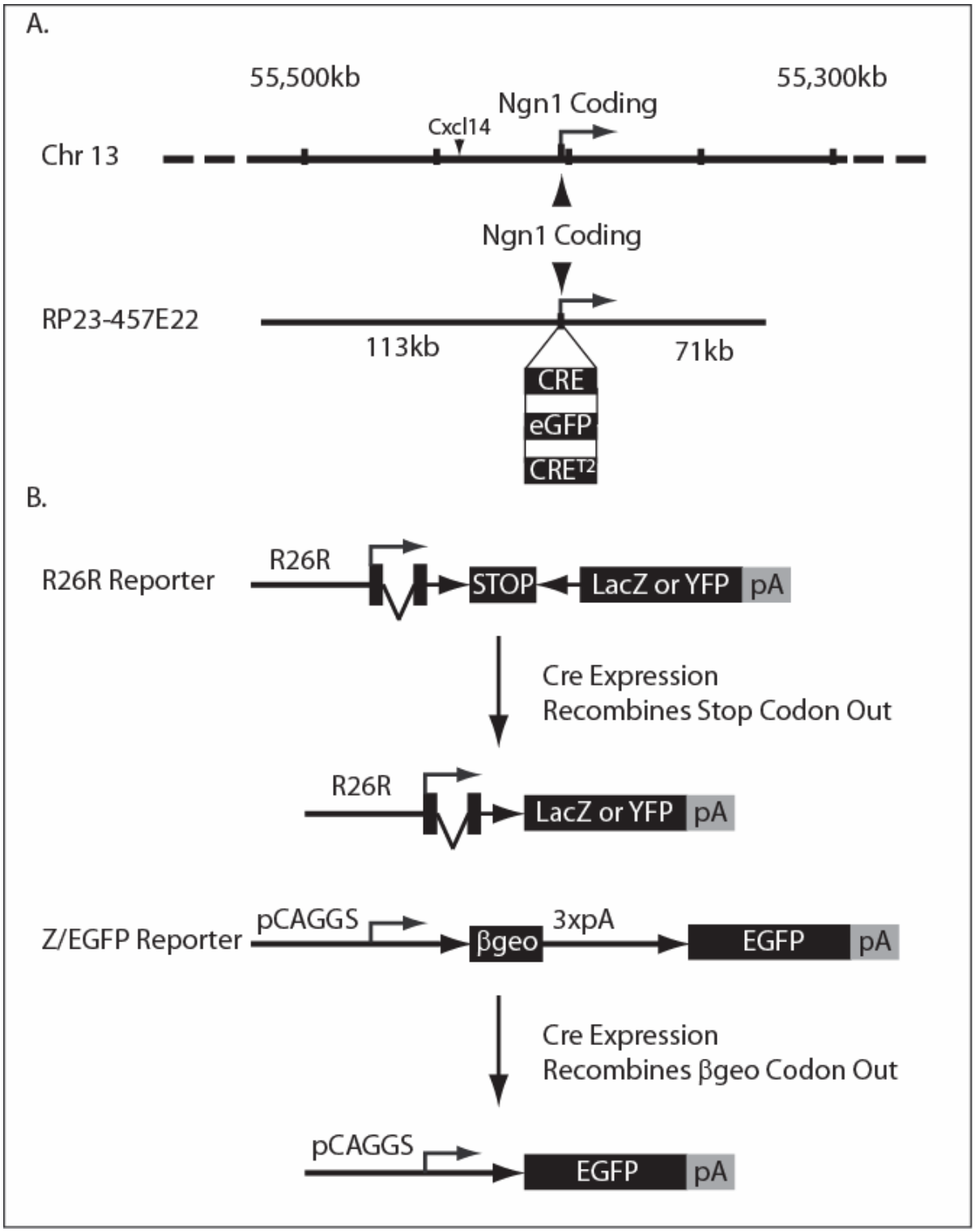


Figure 4.1 Diagram of BAC Constructs for Transgenic Mice and Reporters R26R and Z/EG.

Figure 4.1 Diagram of BAC Constructs for Transgenic Mice and Reporters R26R and Z/EG.

Neurog1 locus is found on chromosome 13 in the mouse genome. The RP23-457E22 BAC contains 113kb of sequence 5' of the *Neurog1* coding region and 71kb 3' (orientation relative to *Neurog1*). The kidney-expressed chemokine *Cxcl14* is ~40kb 5' of *Neurog1* and is contained within the RP23-457E22 BAC. The *Neurog1* coding sequence was precisely recombined out of the BAC and replaced by Cre, nGFP or $CreER^{T2}$. The R26R-reporter and Z/EG-reporter mice were previously described (Novak et al., 2000; Soriano, 1999; Srinivas et al., 2001). Protein from the R26R reporter lines is only made after the STOP sequence is recombined out by Cre recombinase activity. In the Z/EG line all cells express lacZ until Cre is active, at which time the lacZ is recombined out and the GFP reporter protein is expressed.

I characterized GFP expression in the *N1[457]-nGFP* mice to determine if the RP23-457E22 contains all regulatory elements responsible for driving Neurog1 expression in the developing mouse. High expression levels of GFP enriched in nuclei were detected in all Neurog1 domains examined (Fig. 4.2). Whole mount GFP in an E11.5 embryo is detected in the dorsal and ventral neural tubes, dorsal root ganglia, dorsal telencephalon, hindbrain, olfactory epithelium, and trigeminal ganglia, comparable to the domains of Neurog1 detected by whole mount mRNA in situ hybridization (Fig. 4.2A, C). In cross section, the GFP pattern in the dorsal and ventral neural tube VZ, and the dorsal root ganglia mimic the Neurog1 pattern (Fig. 4.2B, D). Notably, the BAC contains regulatory information for directing reporter gene expression to dorsal root ganglia and dorsal telencephalon, information not found in previously identified enhancers (Nakada et al., 2004b).

Cre expression and activity in the *N1[457]-Cre* line also reflects endogenous Neurog1. Immunofluorescence using antibody to Cre on a section through the neural tube of an E10.5 embryo shows Cre in the neural tube and dorsal root ganglia in the Neurog1 pattern (Fig. 4.2B, F). Evidence that the Neurog1 lineage is genetically marked established by crossing *N1[457]-Cre* to the Cre reporter mouse strains *R26R-YFP* and *R26R-LacZ* (Soriano, 1999; Srinivas et al., 2001). The pattern of YFP and X-gal in *N1[457]-Cre;R26R-YFP and N1[457]-Cre;R26R-lacZ* E11.5 embryos reflected a Neurog1-like pattern and was identical to that of GFP in the *N1[457]-GFP* (Fig. 4.2A, C, E,G). X-gal staining of a cross-section of a *N1[457]-Cre;R26R-LacZ* E10.5 confirmed Cre activity in Neurog1 domains and shows the persistence of the ß-gal in lineages derived from Neurog1 expression cells (Fig. 4.2B, H).

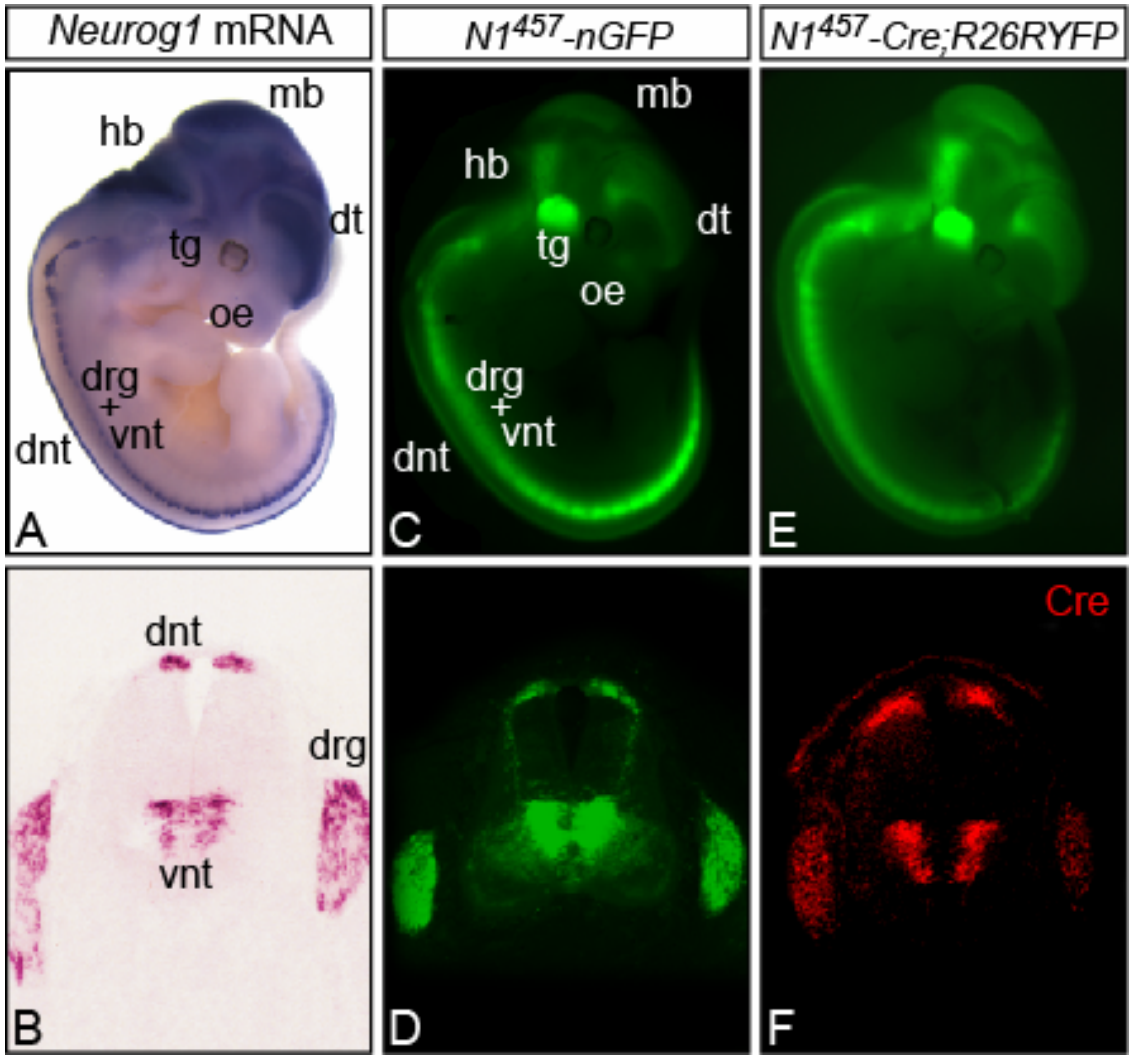


Figure 4.2 Neurog1-BAC transgenic mouse lines accurately express reporter genes in the Neurog1 pattern.

Figure 4.2 Neurog1-BAC transgenic mouse lines accurately express reporter genes in the Neurog1 pattern.

mRNA in-situ hybridization of *Neurog1* at E11.5 in the whole embryo (A) and in transverse section at limb level (B). (C-D) GFP expression in *N1*^{457}*-nGFP* transgenic embryos at E11.5. GFP mimics Neurog1 in the ventricular zone of the dorsal and ventral neural tube (dnt and vnt), and in dorsal root ganglia (drg). (E) YFP or (F) immunofluorescence for Cre at E11.5 in an *N1*^{457}*-Cre*;*R26RYFP* embryo. dt: dorsal telencephalon; hb: hindbrain; mb: midbrain; oe: olfactory epithelium; tg: trigeminal ganglia.

One hallmark of Neurog1 expression in neural tube is its spatial restriction and non-overlap with two other neural bHLH factors, Math1 and Mash1 (Gowan et al., 2001; Sommer et al., 1996). To determine if the BAC sequences direct expression of GFP with the same precision as endogenous Neurog1 I performed double immunocytochemistry with antibodies to Neurog1, Math1, and Mash1 (Fig. 4.3). GFP overlaps with Neurog1 in the dorsal and ventral neural tube but with Neurog1 positive cells located closest to the midline having lowest GFP levels (Fig. 4.3B, C) suggesting a delay in GFP detection relative to Neurog1. As VZ cells differentiate and migrate laterally into the mantle, the cells lose Neurog1 but retain GFP (Fig. 4.3B, C: green cells), a phenomena commonly seen, and likely reflect differences in stability of the proteins. Although the dynamics of GFP expression over time varies from endogenous Neurog1 as detected in the specifics of the medial/lateral patterning, in the dorsal/ventral axis, the GFP appears restricted to the Neurog1 domain with no overlap with Mash1 (Fig. 4.3D-F) or Math1 (Fig. 4.3G). Together, these results illustrate that the BAC RP23-457E22 contains the cis-regulatory information for directing transcription of Neurog1, and thus, GFP and Cre recombinase in the transgenic mouse lines generated reflect expression of endogenous Neurog1.

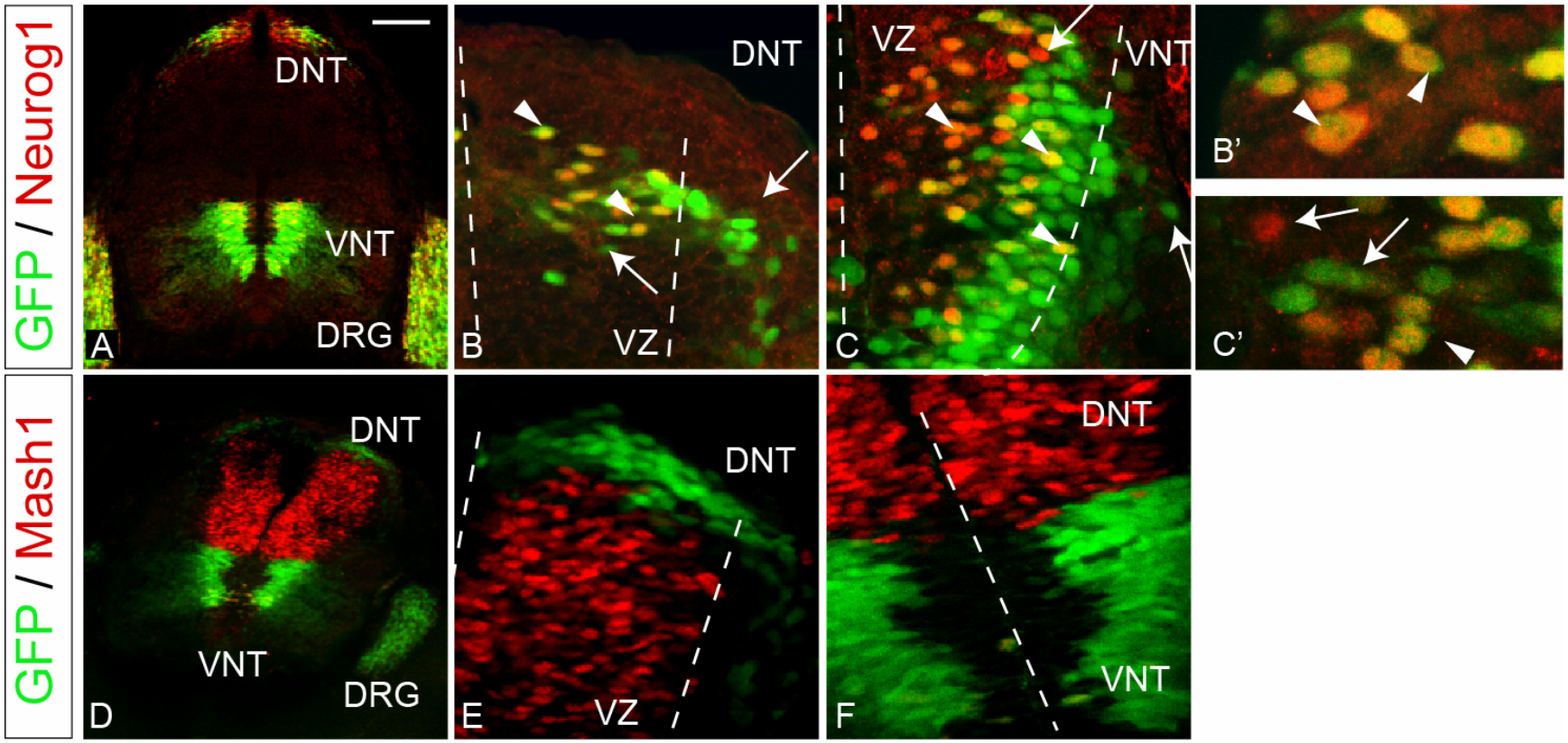


Figure 4.3 N1^{457}-nGFP reporter expression respects Neurog1 boundaries of expression in the neural tube.

Figure 4.3 N1^{457}-nGFP reporter expression respects Neurog1 boundaries of expression in the neural tube.

Confocal imaging of an *N1^{457}-nGFP* E11.5 neural tube showing GFP (green) and Neurog1, Mash11, or Math1 immunofluorescence (red). (A-C) Close overlap is seen between Neurog1 and GFP (arrowheads) although a slight delay in GFP accumulation and then persistence as the cells differentiate and move laterally (arrows) is seen. (B'-C') high magnification of boxed regions in (B-C). (D-G) like endogenous Neurog1, GFP respects precise borders with Mash1 and Math1. No overlap in GFP and Mash1 is detected at the dorsal (D, E) or ventral (D, F) boundaries where Neurog1 and Mash1 meet. No overlap in GFP and Math1 is detected at the boundary where Neurog1 and Math1 meet (G). Midline is to the left in (B-C, E,G) and middle in (F). (B,C,E) Dashed lines denote VZ. drg: dorsal root ganglia; dnt: dorsal neural tube; vnt: ventral neural tube ; VZ: ventricular zone. Scale bars 150 μm in (A,D), 40 μm in (B,C,E-G), 15 μm in (B',C').

Neurog1-expressing progenitors become dI2, dI6, V0, V1 and V2 interneuron populations

The transient expression of the *Neurog1* gene and relatively fast degradation of its protein product makes it difficult to determine which neural cell types arise from Neurog1-expressing cells. Based on its VZ expression pattern in the neural tube, it has been inferred that progenitors expressing this protein will give rise to ventral interneurons and motor neurons. To determine precisely which ventral neurons are in the Neurog1 lineage, I exploited the GFP stability in the *N1^{457}-nGFP* mice to perform short-term lineage tracing by co-labeling with markers specific for various neuronal populations. As previously published (Gowan et al., 2001), the small domain of Neurog1 in the dorsal neural tube gives rise to dorsal interneuron population 2 (dI2) shown here by the co-localization of GFP and antibodies recognizing Brn3a and Lhx1/5 (Fig. 4.4A,B: white cells; 4H: turquoise). GFP did not overlap with *all* cells expressing Brn3a and Lhx1/5 consistent with a partial loss of this population in the Neurog1 null (Gowan et al., 2001). Neurog1 and GFP have a broader expression domain ventrally. Here GFP and Lhx1/5 are co-expressed in interneurons dI6 (dorsal interneuron population 6), V0, V1, and V2 (ventral interneuron populations) (Fig. 4.4D, E). GFP did not overlap with Brn3a positive cells in the region defining dI5 interneurons consistent previous reports that these neurons are derived from Mash1 progenitors (Helms et al., 2005) (Fig. 4.4C). GFP did not co-localize with Pax2;Lhx1/5 in the dI4 domain (Fig. 4.4H,I) but did in the dI6-V1 domains (Fig. 4.4J-L). Chx10 at this stage marks V2 interneurons, thus the overlap of Chx10 and GFP defines V2 as a Neurog1 derivative (Fig. 4.4F, G). GFP co-localizes with Evx1/2 (V0), En1 (V1), and Lhx3 (V2 and a subset of motor neurons) (data not shown). These results demonstrate that Neurog1-expressing progenitors differentiate into dI2, dI6, V0, V1 and V2 interneuron populations but not dI1, dI3, dI4 or dI5.

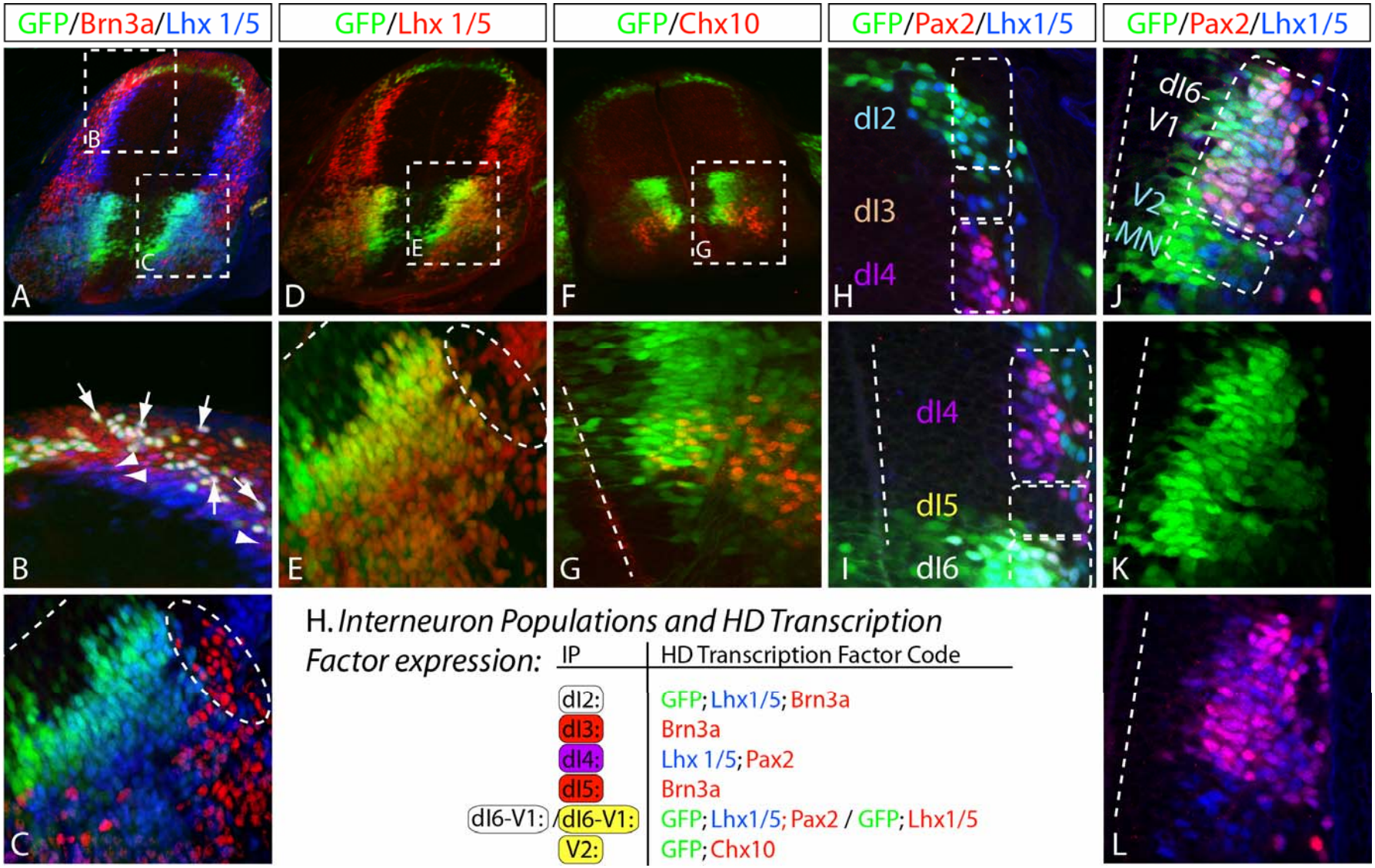


Figure 4.4 Neurog1-expressing cells give rise to interneuron populations dI2, dI6, V0, V1, and V2.

Figure 4.4 Neurog1-expressing cells give rise to interneuron populations dI2, dI6, V0, V1, and V2.

Immunofluorescence with multiple HD transcription factor markers and GFP (green) in *NI^{457}-nGFP* neural tubes at E11.5. Antibodies are indicated and recognize Brn3a (A-C, red), Lhx1/5 (A-C, H-L, blue: D-E, red), Chx10 (F-G, red), Pax2 (H-L, red). GFP, Brn3a and Lhx1/5 overlap in the dI2 domain as seen by the white cells (A-B, arrows) and turquoise cells (H). There are a few dI2 cells that are not GFP (+) and are seen as pink (Brn3a;Lhx1/5, arrowheads B). Ventrally, GFP co-localizes with Lhx1/5 from dI6-V2 (turquoise in (A, C) yellow in (D, E)) but not with Brn3a, which marks the dI5 population (A, oval in C). (F, G) GFP overlaps with Chx10 which marks V2. (H-L) use Pax2 and Lhx1/5 to show GFP does not overlap in dI4 (pink in H-I) and confirms overlap in dI6-V2 (white in J, turquoise in K). (M) Table showing the pattern of expression of HD transcription factors in the different interneuron populations (IP). dI2-dI6, dorsal interneuron populations; V0-V2, ventral interneuron populations; MN, motorneurons. Scale bars 150 μm in (A, D), 40 μm in (B, C, E-L).

Neurog1 gives rise to a small subset of motor neurons

Analysis of the *N1^{457}-nGFP* revealed that a majority of ventral interneurons are derived from Neurog1 progenitors, with much less GFP detected in the motor neuron domain (MN) (Fig. 4.4). Since GFP from *N1457-nGFP* is transient, it is possible that the earlier formed MN populations lost their GFP by E11.5. Examination of the embryos at E10.5 still did not reveal extensive GFP in the MN population (data not shown). However, to assess the possibility that the Neurog1 cells preferentially give rise to ventral interneurons over MN, I used in vivo genetic fate mapping with Cre recombinase (Kim et al., 1998; Novak et al., 2000). I crossed the *N1^{457}-Cre* mice with the *Z/EG* reporter line (Novak et al., 2000) that expresses GFP in cells where Cre recombinase is active and LacZ in cells in the absence of recombination. In the E12.5 neural tube, I observed that GFP expression was predominant in ventral interneuron domain, consistent with the results in the previous section, but were notably sparse in motor neuron domains (Fig. 4.5A, C). Indeed, the LacZ expression pattern in this region was complementary to that of GFP, where LacZ was found predominantly in the motor neuron domain and to a lesser extent in the interneuron domain (Fig. 4.5B, D compared with A, C). These results show that Neurog1+ cells largely contribute to the ventral interneuron populations, and to a much lesser extent to MN. This finding was verified by co-labeling GFP from the ZEG locus with MN markers Hb9, Isl1/2, and Lhx3. Again, Neurog1 lineage cells have broad overlap with the interneuron marker Lhx1/5 (Fig. 4.5E, G; yellow), while only a small subset overlapped with the MN marker Isl1/2 (Fig. 4.5E, G; turquoise). Consistent with this, only a small subset of Hb9+ MN expressed GFP (Fig. 4.5I). To determine if the Neurog1 lineage motor neurons belonged to a specific MN pool, I compared the overlap of GFP with Lhx3 and Isl1/2 combined. A small subset of Lhx3+ cells in the MMC_m (Fig. 4.5F, J) and a small subset of Isl1/2 in the LMC_l (Fig. 4.5F, H) co-localize with GFP. However, GFP did not co-localize with

Isl1/2 in the LMC_m. Thus, a subset of MN fall within the Neurog1 lineage, and even within this subset, there is a bias towards specific neuronal subtypes.

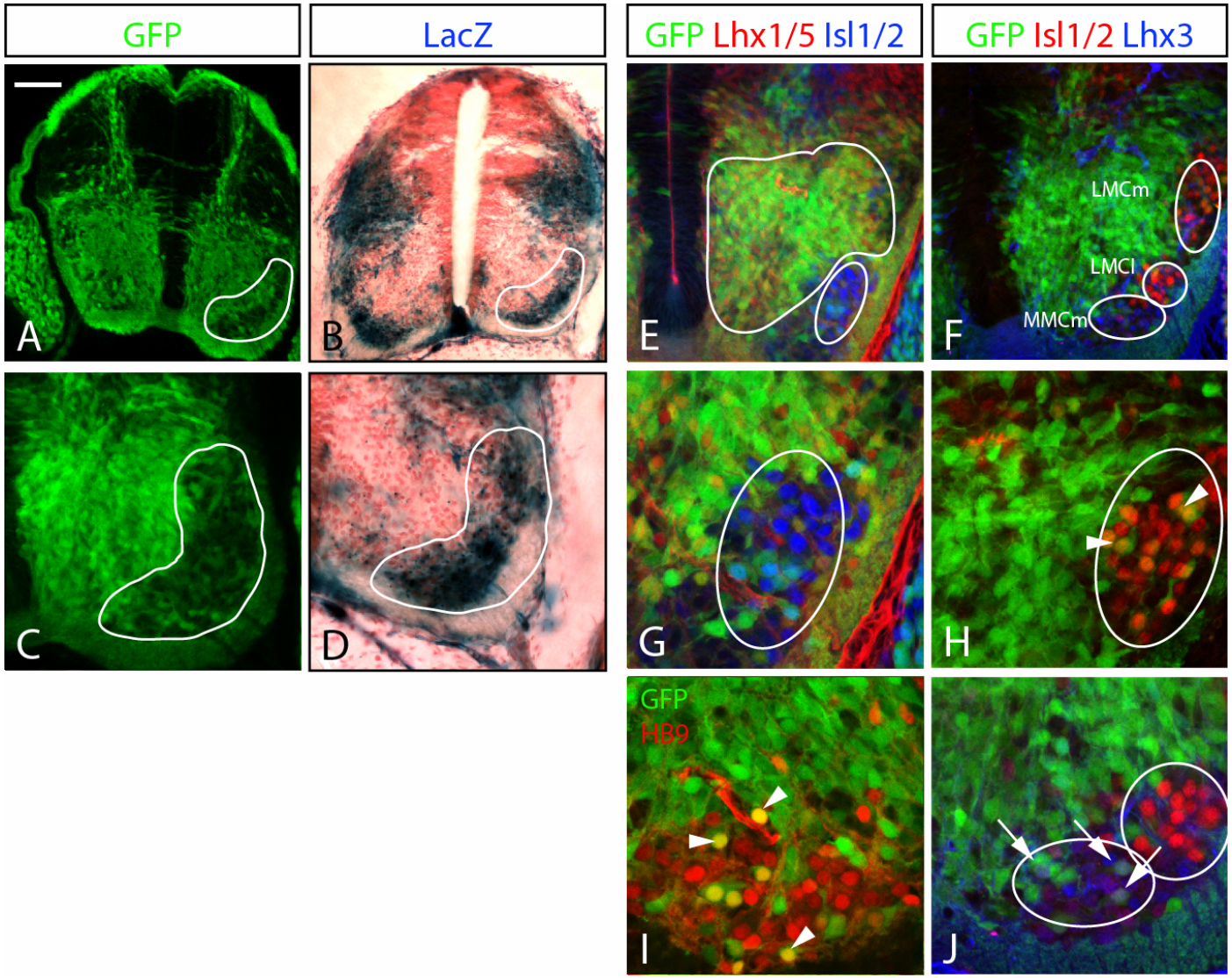


Figure 4.5 Neurog1-expressing cells give rise to a subset of motorneurons.

Figure 4.5 *Neuroq1*-expressing cells give rise to a subset of motorneurons.

E12.5 neural tube from *N1*^{457}*-Cre*;*Z/EG* embryos. (A, C) GFP expression is observed predominantly in the interneuron domain and to a lesser extent in the motor neuron domain (marked area in A and C). (B, D) X-gal staining in the dorsal interneuron domain and MN region illustrates populations where there was no Cre activity. Antibodies recognizing Lhx1/5 (red: E, G), Isl1/2 (blue: E, G); red: F, H, I), and Lhx3 (blue: F, H, I). (E, G) Essentially all Lhx1/5 co-localizes with GFP (area marked), however, only a subset of Isl1/2+ cells are GFP+ (oval in E, G). (F, J) Lhx3+ cells in the MMC_m domain are GFP+ (oval and arrows) while only a subset of Isl1/2+ cells in the LMC_l and LMC_m co-localize with GFP (circle, oval and arrowheads: F, H, J). (I) GFP overlaps with the broad MN marker Hb9 (arrowheads, I). MN: motor neurons; MMC_m: medial medial motor column; LMC_l: lateral lateral motor column; LMC_m: medial lateral motor column. Scale bars 150 μm in (A, B), 75 μm in (C-F) 40 μm in (G-J).

Neurog1-expressing cells give rise to some glia in the spinal cord

Previous studies have demonstrated that over-expressing Neurog1 induces neuronal differentiation in embryonic carcinoma P19 cells, cortical progenitors, and chick neural tube (Farah et al., 2000; Nakada et al., 2004b; Sun et al., 2001). Furthermore, Neurog1 not only promotes neurogenesis but it inhibits gliogenesis (Sun et al., 2001). These studies would predict that the Neurog1 lineage is restricted to neurons. To evaluate the full complement of Neurog1 derived populations throughout development, I examined adult spinal cords from *N1^{457}-Cre*;*Z/EG* and *N1^{457}-Cre*;*R26RLacZ* mice. In both cases, the Cre reporter (GFP or LacZ, respectively) was detected extensively in the lateral and ventral horn gray matter with some scattered cells in white matter (Fig. 4.6A, B). The extensive staining in the lateral and ventral horns is consistent with the embryonic data showing Neurog1 progenitors give rise to most ventral interneurons and some motor neurons. The scattered GFP and LacZ expressing cells detected in the white matter are distributed mainly in the lateral and ventral funiculus. Cell-type identity of these cells was determined using double label immunofluorescence with GFP (+) in *N1^{457}-Cre*;*Z/EG* spinal cords. As expected, a majority of the GFP cells co-labeled with the broad neuronal marker *NeuN* (Fig. 4.6C). However, the scattered cells in the white matter are not *NeuN*+ but co-localize with the oligodendrocyte markers *Olig2*, *APC* and *Nkx2.2* (Fig. 4.6D, E, and data not shown). In addition, there is also overlap with the astrocyte marker *GFAP* detected (Fig. 4.6F). These results demonstrate that in the spinal cord Neurog1 positive progenitors largely mature into neurons, with low level contribution to oligodendrocytes, and astrocytes by postnatal day 30.

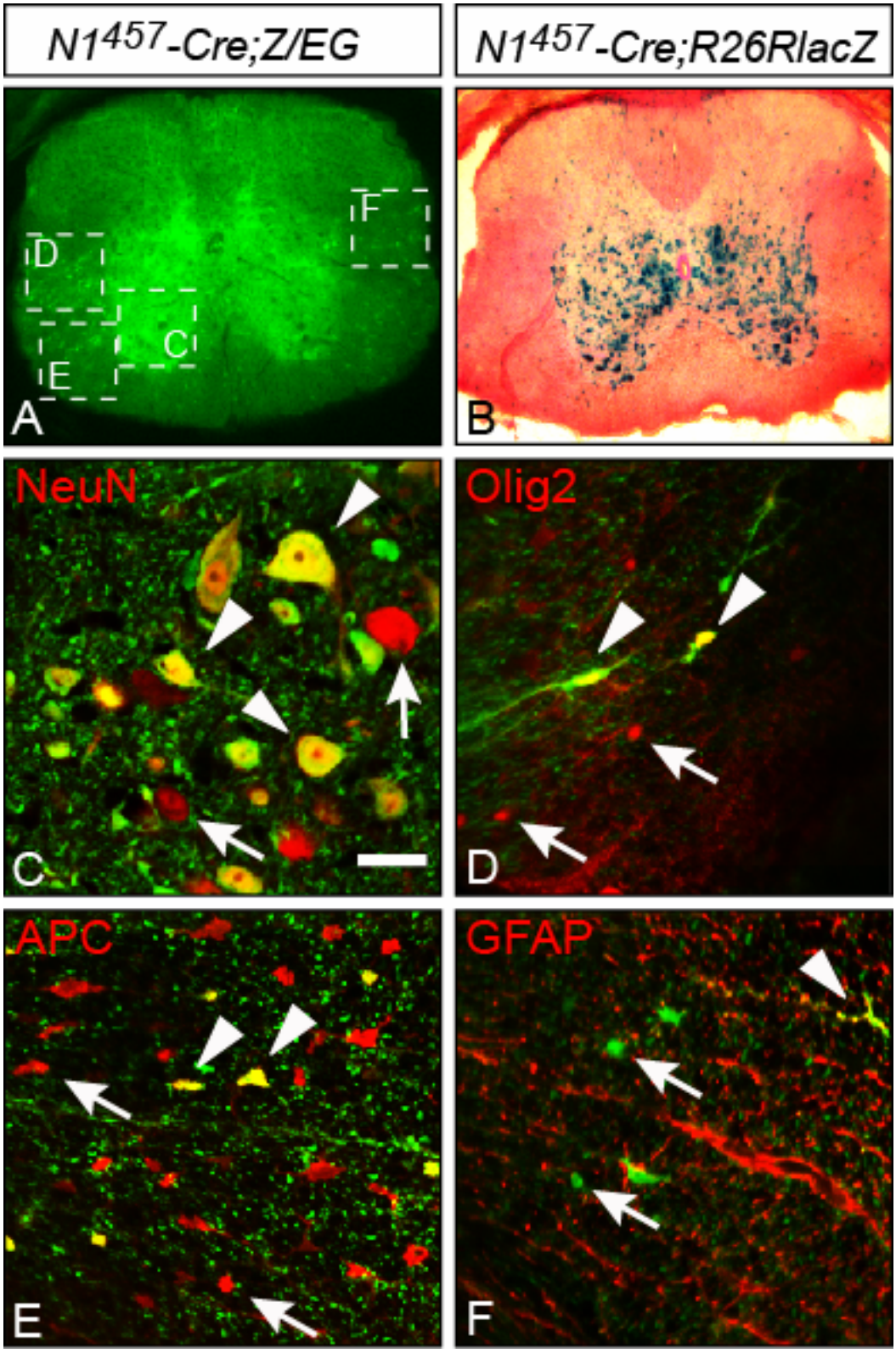


Figure 4.6 **Neurog1-expressing cells give rise to neurons and glia in the spinal cord.**

Figure 4.6 <u>Neurog1-expressing cells give rise to neurons and glia in the spinal cord.</u>

Spinal cords of *N1*^{457}*-Cre*; *Z/EG* and the *N1*^{457}*-Cre*;*R26R-LacZ* were examined at P30. (A) GFP expression in *N1*^{457}*-Cre;Z/EG* spinal cords and (B) X-gal staining in *N1*^{457}*-Cre*;*R26R-LacZ* are detected predominantly in the deep dorsal, lateral, and ventral horns. Reporter gene expression was also observed in the white matter, mainly concentrated in the Lateral funiculus and Ventral Funiculus (I question this as I don't really see it). Antibodies recognizing the neuronal marker *NeuN* (A), the oligodendrocyte markers *Olig2* (B) and *APC* (C), and the astrocyte marker GFAP all co-localized with GFP expression in *N1*^{457}*-Cre x Z/EG* spinal cords. Arrowheads point to co-localization, arrows point to non-overlapping cells expressing GFP or indicated marker. High magnification images in C-F are taken from approximate regions boxed in A. Scale bars 40 μm in (C-F).

Neurog1-expressing cells prior to E12 are lineage restricted neuronal precursors

Previous studies have shown that cells expressing the neural bHLH factor Mash1+ are restricted to the neuronal lineage or the oligodendrocyte lineage depending on the stage of development of the spinal cord (Battiste JB, 2007). To test if the low level contribution of Neurog1 cells to the glial lineages was due to expression in an early common progenitor, or in lineage restricted progenitors as was seen with Mash1, I utilized *N1*^{457}*-CreER*^{T2} mice that provides temporal control of Cre activity through treatment with Tamoxifen (TM) (Feil et al., 1996; Hayashi and McMahon, 2002). *Neurog1-Cre*^{T2} mice were crossed with *R26RYFP*, the pregnant females treated with TM at E10.5, E12.5, or E15.5, and embryos collected at E18 for analysis. The TM activates Cre beginning by 6 hours with a peak between 12-24 hours after the treatment (Hayashi and McMahon, 2002). E18 spinal cord cells expressing Neurog1 at E10.5 co-localized with NeuN, but not Olig2, APC or GFAP (Fig. 4.7A-C). Neurog1-expressing cells from TM treatment at E12.5 and E15.5 showed the YFP co-localizing with GFAP but not NeuN or Olig2 (Fig. 4.7B-I). Collectively, these results show that Neurog1 is not in an early common progenitor, but rather, Neurog1+ cells present in the spinal cord prior to E12 are restricted to the neuronal lineage but after E13 they are restricted to the glial fates.

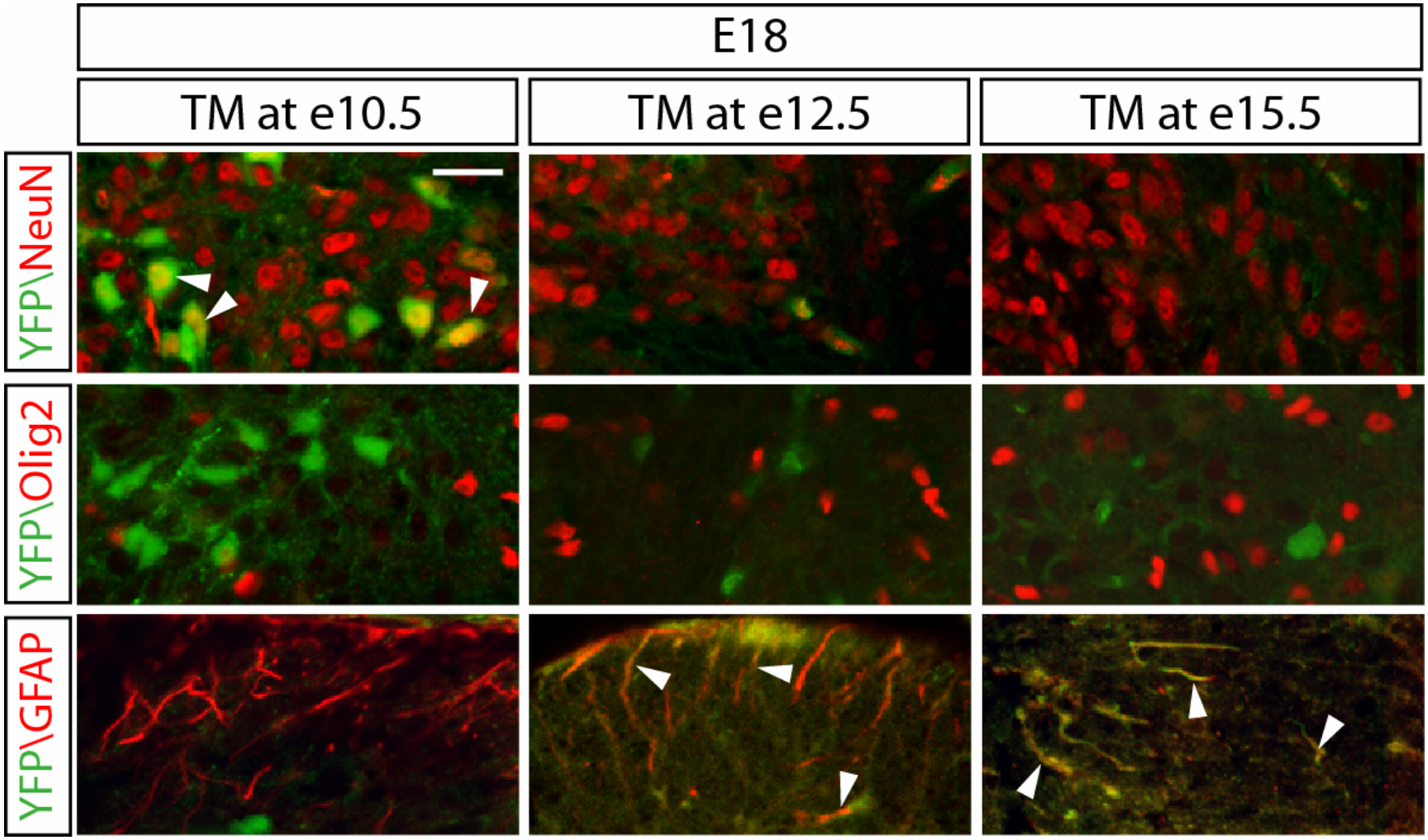


Figure 4.7 Neurog1-expressing cells are restricted to neuronal or glial lineages at different embryonic stages.

Figure 4.7. <u>Neurog1-expressing cells are restricted to neuronal or glial lineages at different embryonic stages.</u>

N1-Cre^{T2};R26R-YFP spinal cords were examined at E18 after TM induction at E10.5 (A-C), E12.5 (D-F) or E15.5 (G-I). YFP (green) and antibodies recognizing markers of neurons (NeuN; A, D, G), oligodendrocytes (Olig2; B, E, H), and astrocytes (GFAP; C, F, I). TM treatment at E10.5 results in marking Neurog1-expressing cells that give rise solely to neurons (A, arrowheads). In contrast, TM at E12.5 and E15.5 result in marking Neurog1-expressing cells that give rise solely to GFAP expressing cells (F,I, arrowheads). Scale bars 40 μm.

CHAPTER FIVE:

DISCUSSION

Distinct sequences are required for Neurog1 tissue specific expression

The RP23 457 BAC contains all of the regulatory elements required to control reporter expression in all of the Neurog1 expression domains. This shows that Neurog1 regulation is dependent on cis-elements contained within a region encompassing 113kb 5' and 71kb 3' of the *Neurog1* coding region. A 3.8 kb deletion in this BAC 4.2 kb 5' of the *Neurog1* coding region had decreased reporter expression levels in all expression domains (*N1^{457}-nGFP Δ2*), indicating this sequence is vital for proper Neurog1 expression in all domains at E11.5. However, a fragment containing the 3.8kb was not sufficient to direct expression in all Neurog1 expression domains.

While the importance of *N1^{457}-nGFP Δ2* in overall regulation of Neurog1 expression is clear, this result was surprising as this deletion was predicted to be important in tissue specific expression. This deletion eliminated three enhancer elements, one responsible for directing expression to the midbrain, one for the hindbrain, and one for the dorsal neural tube (Nakada et al., 2004b). The first two elements, however, were shown to be redundant, as two sequences 3' of the *Neurog1* coding region were shown to drive expression to midbrain and hindbrain as well. Hence, the loss of expression in these tissues was not expected. The dorsal neural tube element absent in *N1^{457}-nGFP Δ2* is sufficient for directing Neurog1 expression in the dorsal neural tube, and is necessary within a 7.5 kb fragment they tested (Nakada et al., 2004b). Thus, a complete loss of expression in this domain was expected rather than the decrease expression observed. Surprisingly, expression in the dorsal root ganglia and dorsal telencephalon was also affected, even though the sequence within this region is not sufficient to direct expression to these tissues (Nakada et al., 2004b).

The *N1^{457}-nGFP Δ4* deletion helped identify a ventral neural tube enhancer, but did not eliminate all ventral neural tube expression. This low level expression remaining in Neurog1

domains in *N1[457]-nGFP Δ2* and *N1[457]-nGFP Δ4* suggest that other elements involved in regulating Neurog1 expression at E11.5 are contained in other regions within the RP23 457 BAC.

Why is there low level expression remaining in *N1[457]-nGFP Δ2* and *N1[457]-nGFP Δ4* if important elements are deleted? There are two possibilities that would explain the remaining expression of these transgenes. First, there could be redundant elements that drive expression to both the dorsal and ventral neural tube, hence explaining why there is not a complete loss of expression in these domains when the respective enhancer elements are deleted. Enhancer elements for trigeminal ganglia, midbrain, and hindbrain are redundantly encoded (Nakada et al., 2004b), so what about dorsal and ventral neural tube enhancer elements? One possibility is that there are redundant elements for each expression domain that has low activity. A more parsimonious explanation is that there is an autoregulatory sequence through which Neurog1 inefficiently activates its own expression. Basic helix-loop-helix transcription factors are notorious for autoregulating their activities. *Mash1* negatively autoregulates while *Math1* has been shown to depend on autoregulation to maintain its expression (Ebert et al., 2003; Helms et al., 2000; Meredith and Johnson, 2000). Perhaps Neurog1 autoregulates its own expression through element(s) contained in the BAC but outside the deletions tested. Thus, the low level expression could be due to inefficient Neurog1 self activation. Since low level expression is not maintained in the ventral neural tube, dorsal root ganglia, olfactory epithelium and dorsal telencephalon in the 16kb transgene, this element(s) would have to be outside this region as well. This idea could be tested by generating stable mouse lines with *N1[457]-nGFPΔ2* and *N1[457]-nGFPΔ4* and crossing them with *Neurog1* knock out mice. If GFP is lost in *N1[457]-nGFPΔ2* and lost in the ventral neural tube in *N1[457]-nGFPΔ4* then the remaining expression seen in *N1[457]-nGFPΔ2* and *N1[457]-nGFPΔ4* is likely due to an autoregulatory sequence encoded elsewhere in the BAC. However, if low-levels of

expression are still maintained that would suggest the remaining expression is not due to autoregulation and that other redundant elements are contained within the BAC.

Accurate spatial and temporal gene expression is critical for the proper development of the nervous system. Hence, having distinct elements involved in activation or downregulation of expression is necessary for each tissue. On the other hand, autoregulation may also play an important role in the basic expression profile of Neurog1 and may be temporally and spatially regulated as well. Identifying sequences involved in regulating Neurog1 tissue-specific expression in the dorsal and ventral neural tube and discovering which transcription factors are involved in interacting with these regulatory elements is pivotal in understanding the molecular mechanisms involved in neurogenesis.

Separable enhancer elements are sufficient to drive tissue-specific expression

Separable enhancer elements have been previously reported to drive Neurog1-like reporter expression to distinct domains (Nakada et al., 2004b). In this previous report, multiple sequences were tested individually rather than a contiguous unit. I test these elements as a contiguous unit in the 16kb TgN 1-16 (Fig. 3.6) to test whether expression domains lacking in previous reports required a combination of elements found 3' and 5' of the *Neurog1* coding region. The *N1*^{457}*-nGFP* contains all of the regulatory elements involved in Neurog1 expression. However, a 16kb fragment containing most of the previously isolated enhancer elements was not sufficient to drive reporter expression in all domains, lacking expression in the dorsal telencephalon, dorsal root ganglia and ventral neural tube. This suggests that regulatory sequences driving expression to these regions are found outside of this fragment but contained within the BAC. Furthermore, it shows that expression to these domains is not mediated by the additive effects of sequences contained by the 16 kb

fragment. A 2.3 kb ventral neural tube enhancer element, that was missing in the 16 kb transgene and deleted in the *N1*^{457}*-nGFPΔ4*, was sufficient to drive reporter expression in this domain. Separable enhancer elements driving Neurog1 expression to discrete tissues is a feature that has been previously observed in Neurog1 and other neural bHLH factors. This configuration is similar to that of the evolutionarily related N*eurog2*, where tissue-specific cis-elements have been isolated (Scardigli et al., 2001; Simmons et al., 2001). However, not all bHLH transcription factors possess separable tissue-specific cis-elements, *Math1* expression has been shown to be directed by a single enhancer in all of the *Math1* domains and no specific tissue elements have been identified (Helms et al., 2000). For *Mash1*a single enhancer element has also been shown to drive reporter expression to a subset of *Mash1* expressing tissues (Verma-Kurvari et al., 1998). However, this fragment is not sufficient to drive expression in all *Mash1* expression domains (Verma-Kurvari et al., 1998), suggesting other regulatory elements exist at more distant positions.

Discrete elements driving the expression of Neurog1 in the dorsal and ventral neural tube are sufficient to drive proper tissue specific expression. Expression mediated by these enhancer elements is likely to be directly or indirectly regulated by signaling molecules released from the roof plate and floor plate, respectively. The dorsal neural tube enhancer element is likely to respond to BMP-dependent signaling molecules while the ventral neural tube element is prone to be regulated by Shh-dependent factors. However, if BMP and Shh signaling are regulating these individual enhancers they may be expected to contain consensus binding sites for transcription factor effectors of these pathways. The dorsal neural tube and ventral neural tube enhancers do not contain any recognizable Smad or Gli binding sites suggesting that if they are regulated by BMP and Shh signaling it may be indirect. The identification of transcription factor binding in these distinct elements will be pivotal in understanding how neurogenesis is regulated.

Neurog1 is predominantly in interneurons and a subset of motor neurons

Previous Neurog1 loss of function studies have shown a loss of the dI2 populations while no significant changes were observed in ventral neural tube interneuron populations (Gowan et al., 2001; Scardigli et al., 2001). However in *Neurog1/Neurog2* double knock-outs ventral interneuron and motor neuron populations were severely affected (Scardigli et al., 2001). Furthermore, Neurog1 expression is limited to the ventricular zone in the developing neural tube, and due to its transient expression, it has been very difficult to identify the Neurog1-derived ventral interneuron populations. To determine which ventral neural populations are derived from Neurog1-expressing cells, I used BAC recombineering to replace the *Neurog1* coding region with an *nGFP* reporter and then developed transgenic mouse lines using this BAC. This data shows that Neurog1 expression overlaps with that of the reporter, but rather than reporter expression being confined to the ventricular zone, it persists into the mantle, allowing short-term lineage tracing. In addition, reporter expression respects the dorso-ventral boundaries of bHLH expression as GFP does not overlap with other bHLH transcription factors within the ventricular zone. Based on the neural tube expression pattern of Neurog1, previous studies have suggested that cells expressing this bHLH transcription factor may give rise to dI6 and most ventral interneurons (Gowan et al., 2001). Consistent with this idea, using the HD transcription factor code, I show that Neurog1-expressing progenitors differentiate into dI6, V0, V1, V2, and at least a subset of motor neurons. Other studies have placed *Neurog2* as a key player in motor neuron development, while Neurog1 is believed to not be as important in those lineages (Scardigli et al., 2001). These studies showed a significant decrease in the formation of *Isl1/2* (+) motor neurons in *Neurog2* knock outs, while very little change in this population in Neurog1 deficient animals (Scardigli et al., 2001). Consistent with this

most motor neurons are not derived from a Neurog1 progenitor, rather only a small subset of these motor neurons, predominantly found within the MMC_m and LMC_m, come from a Neurog1-expressing progenitor. This mouse model in which reporter expression fully recapitulates Neurog1 expression throughout development is a valuable tool in the study of Neurog1-related neurogenesis.

Neurog1 in lineage-restricted precursors to neurons and glia

Using a model in which *Cre*-recombinase was replaced for the *Neurog1* coding region, through BAC recombineering, I traced the lineage into the mature spinal cord. I show that Neurog1-expressing progenitors can differentiate into mostly neurons, and a small subset of oligodendrocytes and astrocytes. This differs from the fate of other progenitors expressing another bHLH transcription factor, *Mash1*. In this case, cells derived from Mash1 (Mash1) (+) progenitors contributed heavily to neurons and oligodendrocytes, but not astrocytes. (Battiste et. al., 2006).

P30 spinal cords of the $N1^{457}$-*Cre* x *Z/EG* showed that *GFP* (+) neurons were mainly located in layers V through IX, with very little *GFP* expression in the dorsal horn. This is consistent with the fact that dI2 interneurons migrate ventrally to the deep dorsal horn while the dI6 population migrates to the ventral horn and ventral interneurons and motor neurons remain in the ventral horn (Reviewed in Caspary and Anderson, 2003; Lupo et al., 2006).

GFP expression overlapping with oligodendrocyte markers *Olig2*, *APC*, and *Sox10* was observed in the ventral funiculus while the *GFP* that co-localized with the astrocytic marker *GFAP* was found in the pial surface. Since previous studies have demonstrated that *Neurogenin 1* functions as an inhibitor of gliogenesis in cortical progenitors, I decided to determine if Neurog1 expression was present in a neural progenitor or in lineage restricted precursors (Nieto et al., 2001; Sun et al., 2001). Tamoxifen-inducible data show that Neurog1 is expressed in lineage restricted

precursors, as seen in *N1-Cre*^{T2} x *R26RYFP* E18 spinal cords. I show that when induced with tamoxifen at E10.5, during neurogenesis, Neurog1 is strictly expressed in precursors to neurons. However, when induced at E12.5 and E15.5, during gliogenesis, Neurog1 is expressed in precursors to astrocytes, as seen by the overlap of *YFP* and *GFAP*. Nonetheless, *Olig2* co-localization with *YFP*, when induced at these stages during gliogenesis, was not observed. Although Neurog1 expression during gliogenesis might be suggestive of an additional function for Neurog1 in this process, I believe that this could be explained a number of ways. A previous model illustrating the role of proneural genes during the neurogenic and gliogenic phases of development describes these as constantly competing with proglial genes to induce their respective differentiation and/or specification (Reviewed in Bertrand et al., 2002). Hence, if Neurog1 is transiently expressed and immediately suppressed in glial restricted precursors I would see reporter expression in glial cells while no functional significance is necessarily attributed to Neurog1 in these cell types. Another explanation for the observation that Neurog1 is expressed in cells that later differentiate into *GFAP* (+) cells is looking closely at radial glia. Radial glia have been shown to have properties of embryonic stem cells and have multipotency in mature neural tissues, allowing them to differentiate into neurons, oligodendrocytes and astrocytes (Barres, 1999; Gaiano et al., 2000; Laywell et al., 2000; Malatesta et al., 2000; Noctor et al., 2001). Furthermore these cells in the adult vertebrate brain express the same molecular markers and have the same ultrastructure characteristics as astrocytes (Barres, 1999; Doetsch et al., 1999; Laywell et al., 2000; Noctor et al., 2001). Radial glia have their soma in the VZ and possess a long process that extends towards the pial surface (Gadisseux et al., 1989; Schmechel and Rakic, 1979) and *GFAP* co-localization is mainly observed in the pial surface. Furthermore, at E18 there are no *YFP* (+) oligodendrocytes, while there are at P30. This could also be explained if those *GFAP/YFP* double

positive cells are multipotent radial glia as they could still give rise to neurons, oligodendrocytes or astrocytes. To test this premise I could look at GFP reporter expression in the spinal cord at E18.5. If this expression co-localizes with markers to Radial glial cells, then Neurog1 is expressed in these adult neural stem cells. Another way is to induce tamoxifen induce at E15.5, during gliogenesis, and look for GFP overlap with Radial glia and glial cell markers in P30 spinal cords.

APPENDIX A

Neurog1 Reporter Plasmids

Plasmid Name: pGEM-5Zf-NLS-eGFP-Ngn1

Inserts and Sizes: 5' Homology: 1.2kb
3' Homology: 1.2k

Reporter: NLS eGFP

Resistance: Amp

Plasmid Size: 6.5kb

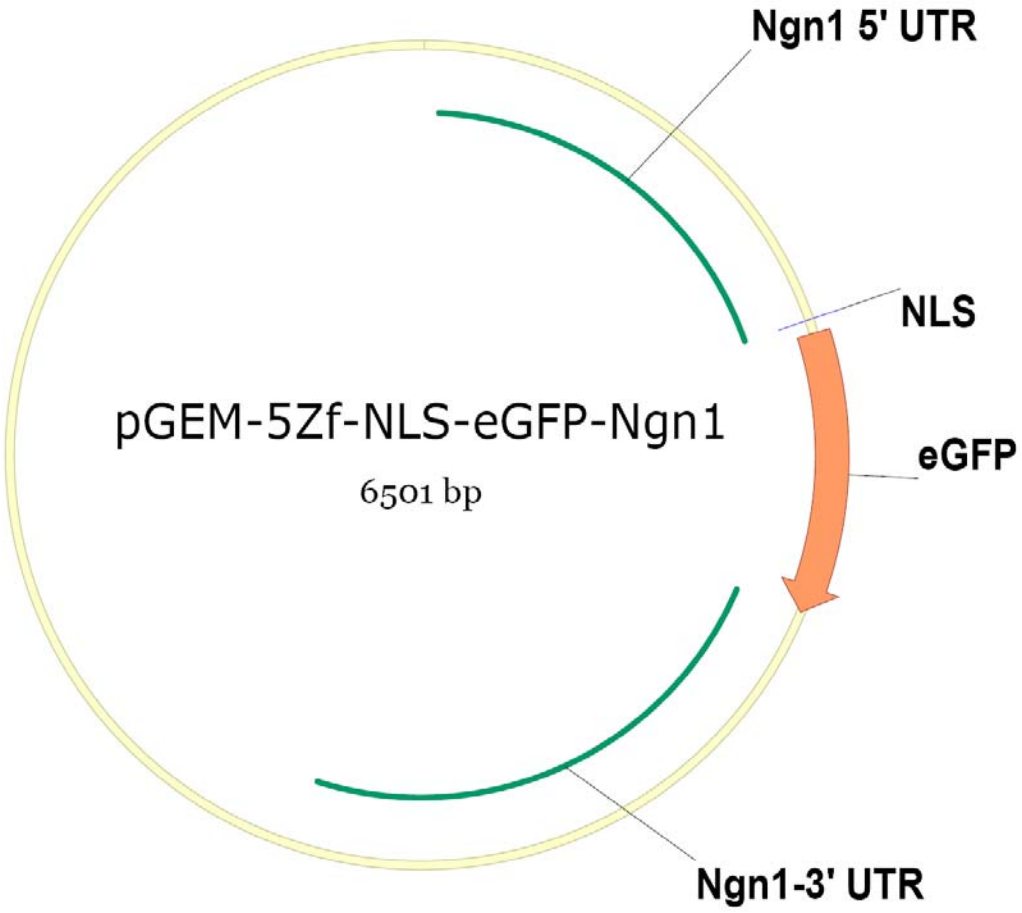
Cloning Sites: 5' Homology: XbaI/NcoI
3' Homology: BglII/SacI

Primers: 5' Homology: TCTAGACGGCAAACAGGAAAAT
CCATGGTGCAGTGTGCAGGACCGA
3' Homology: TCCTGGGGTTCCGGGGCCGCTG
ATCAGGCCATCATGGCCTGTTTACAAATCAATTTCCTC

Template: RP23 457 E22 BAC

Constructed by: Herson I. Quiñones, James Battiste

Plasmid Use: Build Vector (BV) to construct Recombination Cassette (RC) to isolate the RC and insert it into the Shuttle Vector (SV)



Plasmid Name: pGEM-5Zf- Cre-Ngn1

Inserts and Sizes: 5' Homology: 1.2kb
3' Homology: 1.2kb

Reporter: Cre Recombinase

Resistance: Amp

Plasmid Size: 6.5kb

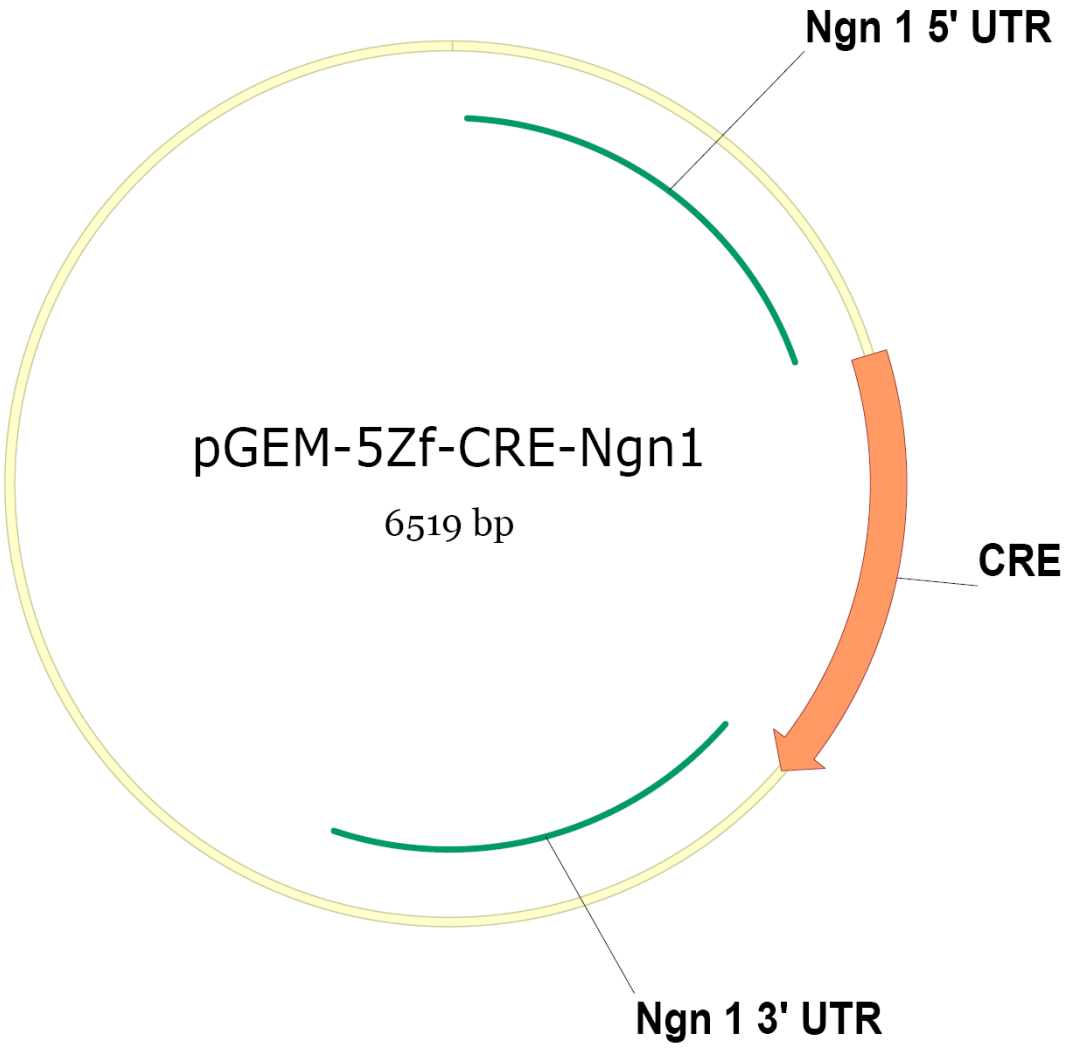
Cloning Sites: 5' Homology: XbaI/NcoI
3' Homology: BglII/SacI

Primers: 5' Homology: TCTAGACGGCAAACAGGAAAAT
CCATGGTGCAGTGTGCAGGACCGA
3' Homology: TCCTGGGGTTCCGGGGCCGCTG
ATCAGGCCATCATGGCCTGTTTACAAATCAATTTCCTC

Template: RP23 457 E22 BAC

Constructed by: Herson I. Quiñones, James Battiste

Plasmid Use: Build Vector (BV) to construct Recombination Cassette (RC) to isolate the RC and insert it into the Shuttle Vector (SV)



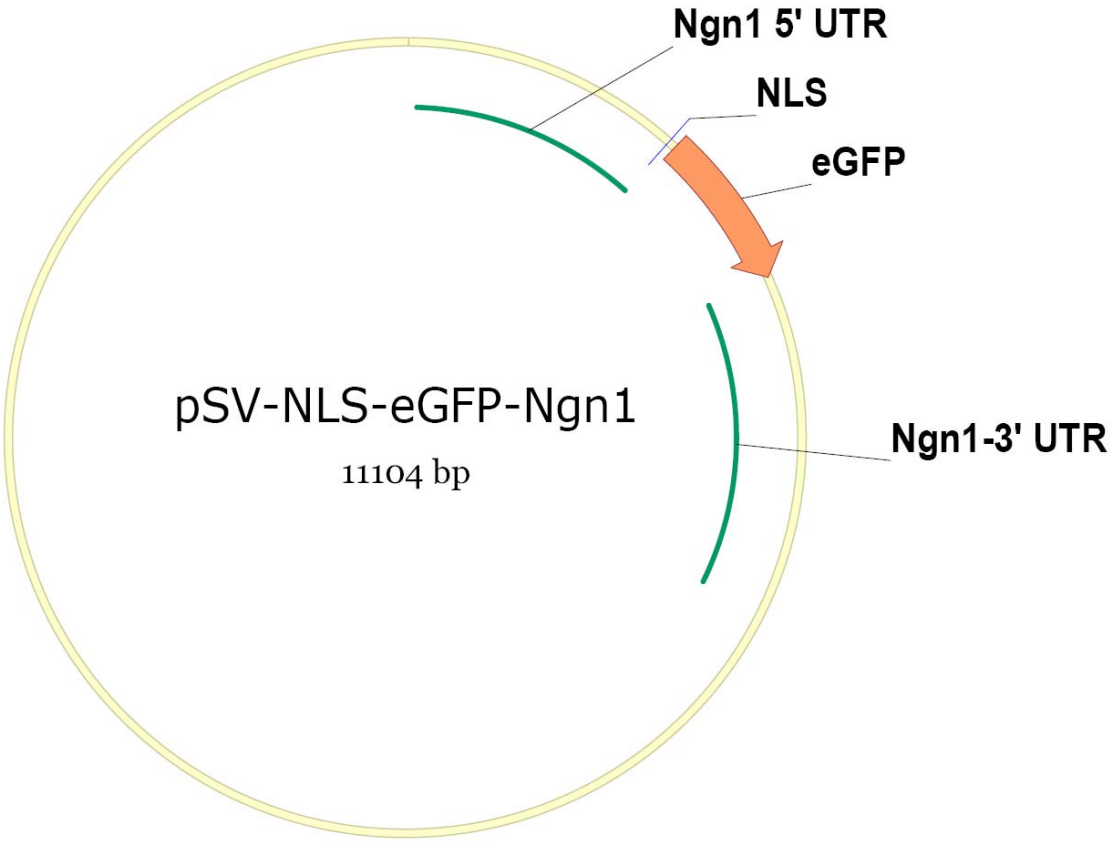
Plasmid Name: pSV-eGFP-Ngn1

Inserts and Sizes: N1-eGFP-RC; 3.5kb **Reporter:** NLS eGFP

Cloning Sites: SalI **Resistance:** Tet

Constructed by: Herson I. Quiñones, James Battiste **Plasmid Size:** 11kb

Plasmid Use: Shuttle Vector (SV) to recombine N1-eGFP RC into RP23 457 E22 BAC.



Plasmid Name: pSV-Cre-Ngn1

Inserts and Sizes: N1-Cre-RC; 3.5kb

Reporter: Cre Recombinase

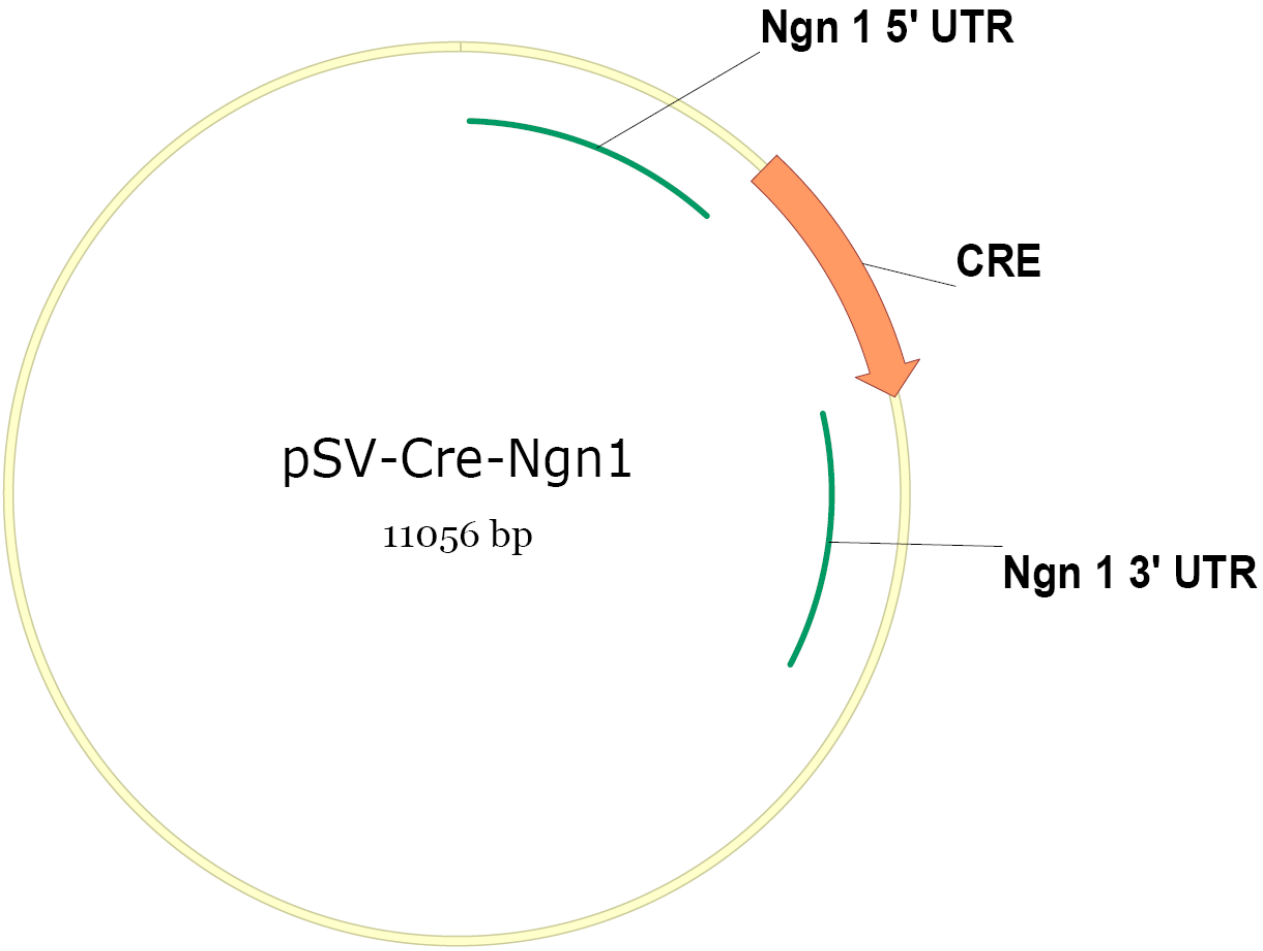
Cloning Sites: SalI

Resistance: Tet

Constructed by: Herson I. Quiñones, James Battiste

Plasmid Size: 11kb

Plasmid Use: Shuttle Vector (SV) to recombine Cre recombinase RC into RP23 457 E22 BAC.

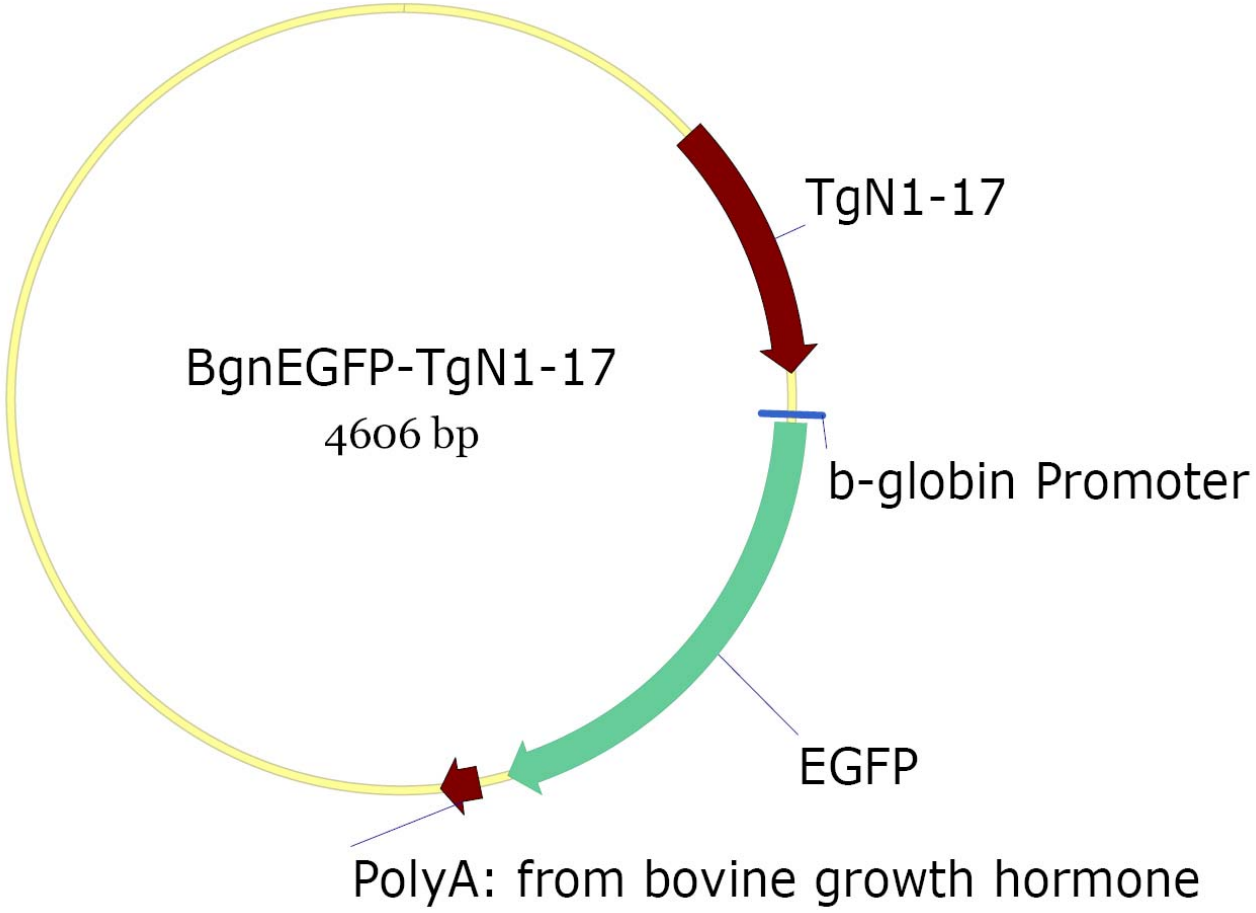


Plasmid Name: BgnEGFP-TgN1-17

Inserts and Sizes:	TgN1-17; 500bp	**Reporter:** NLS EGFP
		Resistance: Amp
Cloning Sites:	BglII/BamHI	**Plasmid Size:** 4.6kb

Primers: AAAAGATCTGTCATTCATGAAATTCTTCCACCCGC,
AAAAGGATCCTGAGCCCTGGAGACAGAAGA

Template: RP23 457 E22 BAC
Constructed by: Herson I. Quiñones
Plasmid Use: Reporter plasmid used to determine if a 500bp DNA sequence located 18.6kb 5' of the Neurog1 coding region is sufficient to drive GFP reporter expression. After cloning TgN1-17 into the plasmid, I then isolate the fragment by using SalI and XhoI. This fragment is the purified and used for pronuclear injection.



Plasmid Name: BgnEGFP-TgN1-18

Inserts and Sizes: TgN1-18; 1.6Kb

Reporter: NLS EGFP

Resistance: Amp

Cloning Sites: BglII/BamHI

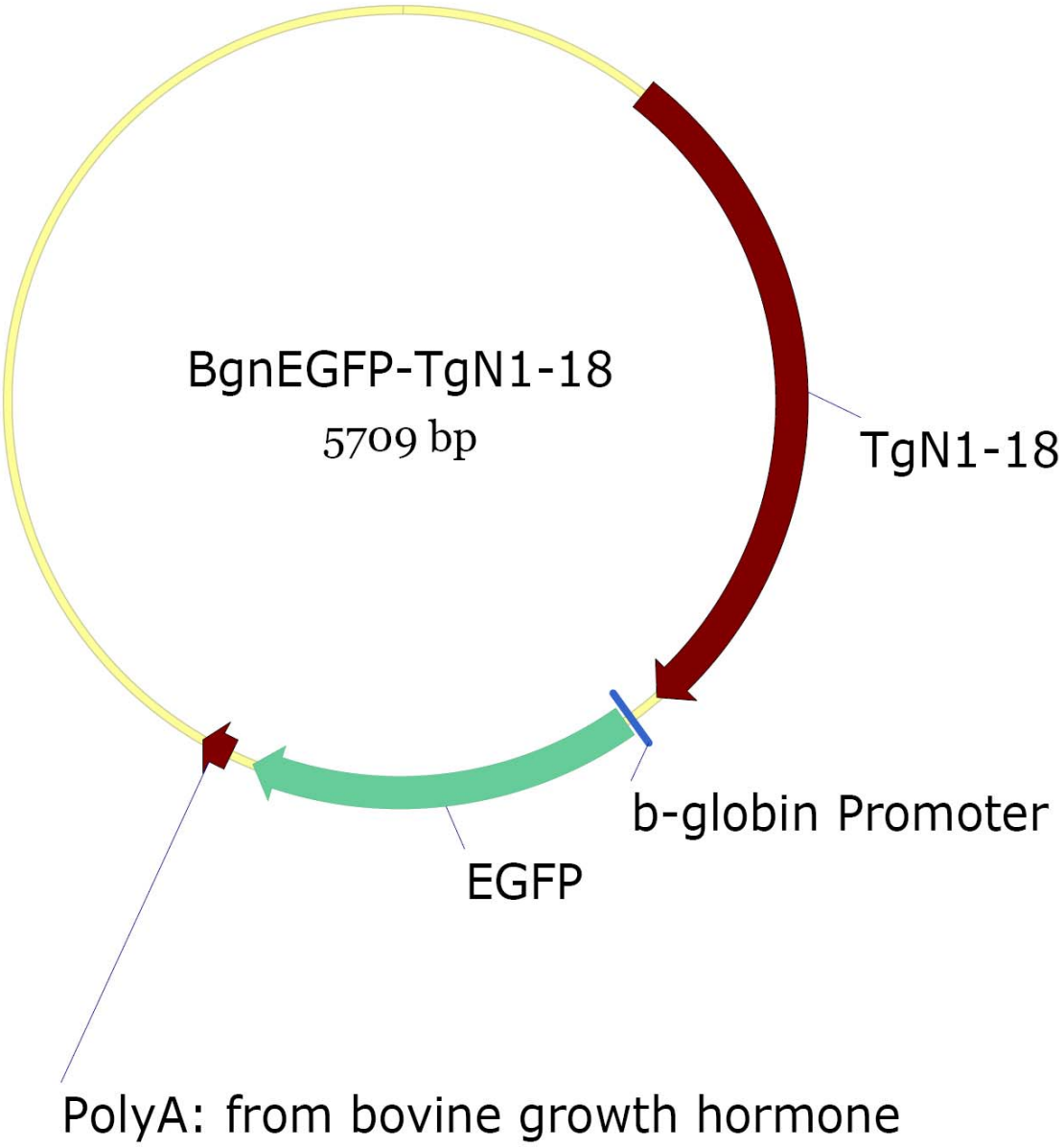
Plasmid Size: 5.7kb

Primers: AAAGGATCCTGACAAATGCCCAAT, CCCTCGAATTCTGTTCTTAAGGGTTC

Template: RP23 457 E22 BAC

Constructed by: Herson I. Quiñones

Plasmid Use: Reporter plasmid used to determine if a 1.6Kb DNA sequence located 5.2kb 3' of the Neurog1 start site is sufficient to drive GFP reporter expression. After cloning TgN1-17 into the plasmid, I then isolate the fragment by using SalI and XhoI. This fragment is the purified and used for pronuclear injection.



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Vitae

Herson Isaac Quiñones was born in the small town of Sabana Grande, Puerto Rico on January 20th, 1978. While attending the Jose A. Castillo Middle School he began a Science Fair project that would earn him a total of 53 national and international awards. He would later obtain a provisional patent for a natural insecticide he created using a methanolic extraction out of the chili peppers. He attended High School at the Colegio San Jose in San German, Puerto Rico and graduated in 1996. He then obtained a full scholarship from the Vice-President for Research at the University of Arizona, in Tucson, Arizona, where he graduated with a Bachelor of Science with a degree in Molecular and Cellular Biology and minors in Chemistry, Mathematics, Physics, and Spanish. During his undergraduate years he performed research in the Arizona Cancer Center, the Mayo Clinic and Foundation, and the School of Medicine at the University of Puerto Rico, and presented his research in various scientific meetings. He also became part of the Undergraduate Biology Research Program and the Minority Access to Research Careers Program at the University of Arizona. In June of 2000 he was accepted to the University of Texas, Southwestern Medical Center in Dallas, where he joined the Neuroscience Graduate Program and became part of Dr. Jane E. Johnson's laboratory. During his graduate career he obtained a Predoctoral Fellowship from the National Institutes of Health and the Minority Access to Research Careers Program, which provided him with financial support for his research for 5 years.

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