Regulation of *Nkx2.2* Gene Expression in the Vertebrate Neural Tube; A Target of Graded Sonic Hedgehog Signalling

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Statement of Declaration

I, Katy Hill, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: Date: 29/4/07

Abstract

Sonic hedgehog (Shh) is a morphogen implicated in the developmental patterning of many vertebrate tissues. One such tissue is the neural tube (NT). In ventral regions of the NT distinct neuronal subtypes emerge in precise spatial order from progenitor cells arrayed along its dorsal-ventral axis. Shh regulates this process by controlling the expression patterns of transcription factors in progenitor cells. In addition, cross-repressive interactions between pairs of transcription factors, expressed in adjacent regions, ensures the generation of defined domains. The regulation of *Nkx2.2* and *Nkx2.9* expression represents an example of this mechanism. The expression of these genes is restricted to a ventral (p3) domain, comprising neural progenitors dorsal to the floor plate. Induction of *Nkx2.2* and *Nkx2.9* requires high levels of Shh signalling. In part this appears to be because the homeodomain protein Pax6 must be repressed to allow *Nkx2.2/2.9* induction. We have analysed the regulatory regions of the Nkx2 genes in order to understand the molecular mechanisms underpinning their expression pattern. The 5' flanking region of *Nkx2.2* and *Nkx2.9* contains a 250bp block of highly conserved DNA (CNCR) that is found in human, mouse, *Fugu* and zebrafish. This region includes a binding site for the transcriptional regulators of the Shh pathway: Gli (GBS). Using a BAC homologous recombination system and assays in zebrafish, we provide evidence that the CNCR is required to direct Nkx2.2a-like gene expression. Mouse *in vivo* reporter assays using fragments containing the CNCR of *zNkx2.2a*, indicate the CNCR is sufficient to direct reporter gene expression in the p3 domain of the NT. Mutational analysis indicates that the GBS is necessary but not sufficient to account for this expression profile. *In vivo* assays further suggest correct *Nkx2.2* expression requires input from additional transcriptional activators as well as a floor plate repressor. All these factors appear to act through regulatory elements within the CNCR.

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Abbreviations

BAC	=	Bacterial Artificial Chromosome
Bcd	=	Bicoid
β-Gal	=	β-Galactosidase
bHLH	=	basic Helix-Loop-Helix
BMP	=	Bone Morphogenetic Protein
BSA	=	Bovine Serum Albumin
Ci	=	Cubitus interruptus
CNCR	=	Conserved Non-Coding Region
CNS	=	Central Nervous System
Cos2	=	Costal 2
DAB	=	3,3-Diaminobenzidine
DAPI	=	4,6-diamidino-2-phenylindole
dhh	=	*desert hedgehog*
DIC	=	Differential Interference Contrast
DIG	=	Digoxigenin
Dl	=	Dorsal (protein)
Dpp	=	Decapentaplegic
E	=	Embryonic Stage
EDTA	=	Ethylenediaminetetraacetic acid
ehh	=	*echidna hedgehog*
EMSA	=	Electrophoretic Mobility Shift Assay
ES	=	Embryonic Stem cells
FGF	=	Fibroblast Growth Factor
Fu	=	Fused

GBS	=	Gli binding site
gDNA	=	genomic DNA
GFP	=	Green Fluorescent Protein
Gro/TLE	=	Groucho/Transducin-Like -Element
GSK3β	=	Glycogen Synthase Kinase 3β
Hb	=	Hunchback
HD	=	Homeodomain
Hh	=	Hedgehog
Hhip1	=	Hedgehog interacting protein 1
hpf	=	hours post fertilisation
HPE	=	Holoprosencephaly
hpe	=	hours post electroporation
HRP	=	Horseradish Peroxidase
ihh	=	*indian hedgehog*
IκB	=	Inhibitor of κB
LB	=	Luria-Bertani Broth
LDA	=	Lipid Dependent Antagonism
m	=	mouse
MN	=	Motorneurons
MW	=	Molecular Weight
Neo	=	Neomycin
NF-κB	=	Nuclear Factor κB
OD	=	Optical Density
omb	=	*optomotor-blind*
otd	=	*orthodenticle*
p	=	progenitors

PAGE	=	PolyAcrylamide Gel Electrophoresis
PB	=	Phosphate Buffer
PBS	=	Phosphate Buffered Saline
PCR	=	Polymerase Chain Reaction
PFA	=	Paraformaldehyde
PKA	=	Protein Kinase A
PNI	=	Pronuclear Injection
Ptc	=	Patched
PVF	=	Perivitelline Fluid
RA	=	Retinoic Acid
rho	=	*rhomboid*
sal	=	*spalt*
SDS	=	Sodium Dodecyl Sulphate
Shh	=	Sonic hedgehog
Smo	=	Smoothened
sna	=	*snail*
sog	=	*short gastrulation*
spz	=	*spätzle*
SSC	=	Salt Sodium Citrate
Su(fu)	=	Suppressor of Fused
TAE	=	Tris Acetate EDTA
TGFβ	=	Transforming Growth Factor β type
TRIS	=	Tris[hydoxymethyl]aminomethane
twhh	=	*tiggy-winkle hedgehog*
twi	=	*twist*
V	=	Interneuron class subtype

V/cm	=	Volts/centimetre
v/v	=	volume/volume
w/v	=	weight/volume
Xbra	=	Xenopus brachyury
Xgsc	=	Xenopus goosecoid
YFP	=	Yellow Fluorescent Protein
z	=	zebrafish
zen	=	*zerknüllt*
zf	=	zebrafish

1 Introduction

1.1 Tissue Patterning - Morphogens

One of the fundamental questions in the development of organisms is how naïve tissue is patterned to become a complex structure. Progenitor cells that make up the structures must receive instruction in order to differentiate into a particular cell type in an ordered and regulated fashion. One common mechanism in embryonic development, is the provision of molecular cues to the tissue. These cues provide positional information and determine the cell type that will form at precise spatial locations. Signals with this characteristic may act at a distance from their source, and several of these signals are morphogens.

1.1.1 Historical Perspective

Our current understanding of tissue patterning has been developed from a series of ideas spanning over a century (reviewed in Wolpert, 1996). In the late 19th century, studies of sea urchin embryos, led Hans Driesch to conclude that the fate of a cell within an embryo was determined by its position. This was the first suggestion that cells were responsive to their environment and received information about their position within the developing embryo. It was many years later that Thomas Hunt Morgan suggested that the source of the positional information could be the presence of a gradient. The gradient, it was hypothesised, provided the tissue with a polarity and ensured that cells at different positions within this gradient would respond accordingly. However, as the idea of gradients fell out of fashion, Morgan's ideas were not further pursued for almost a generation. However, in the 1950s and 1960s, renewed interest from theoreticians reawakened the idea of gradients.

In an influential synthesis, Lewis Wolpert summarised the problem of tissue patterning with the now famous French Flag Problem (Wolpert, 1969). This is the problem of how a French flag-like pattern could be formed from a tissue of totipotent cells; one blue stripe, one white stripe and one red stripe. This can only happen if the

cells all 'know' their position within the tissue. He proposed that the measure of the concentration of a chemical could provide this positional information, whilst the slope of the concentration gradient could determine polarity. The size of the field over which morphogens act are generally small and the length of time required to act is short. These facts led Francis Crick to propose the idea that the patterning relied upon diffusible molecules to provide positional information (Crick, 1970). Although the theoretical basis for a morphogen was firmly established, it was another ~25 years before the first molecular identification of a morphogen.

1.1.2 Molecular Identification of Morphogens

For a molecule to be classed as a morphogen it must fulfil two principle criteria. First it must act at a distance from the source in order to confer long-range pattern. Second it must act at different concentration thresholds to control different cellular outputs. Thus in experiments in which morphogen concentration is altered, predictable changes in cell fate should result (Gurdon and Bourillot, 2001). Experimental evidence from many systems has now identified morphogen candidates that pattern different developing tissues. Some of the most well known examples of morphogens come from studies carried out in *Drosophila*. These include Bicoid (Bcd) that establishes anteroposterior polarity in the *Drosophila* embryo (Driever and Nusslein-Volhard, 1988a; Driever and Nusslein-Volhard, 1988b), Decapentaplegic (Dpp) that patterns the anteroposterior axis of the imaginal wing disc (Podos and Ferguson, 1999) and Dorsal (Dl) that patterns the dorsoventral axis of the early embryo (Roth et al., 1989). Moreover, in vertebrates the mesoderm patterning factor Activin (Smith et al., 1990) has been shown to have morphogen properties, as have the hedgehog family of molecules (Ericson et al., 1997b; Marti et al., 1995; Roelink et al., 1995). In each case, differential regulation of gene expression by these factors has been described. Below I will briefly introduce each of these morphogens.

1.1.3 Morphogen Roles and Mechanisms

Bicoid is a maternal protein laid down in the anterior of the *Drosophila* egg, translation only occurs after fertilisation. It is essential for correct patterning of the

anterior-posterior axis of the developing embryo (reviewed in Ephrussi and St Johnston, 2004). At these stages of development the *Drosophila* embryo is a syncitium: there are no cell walls and all nuclei share the same cytoplasm. The syncitial nature of the embryo allows Bicoid, a transcription factor, to establish an anterior to posterior gradient that provides positional information (Fig. 1.1A: Driever and Nusslein-Volhard, 1988a; Driever and Nusslein-Volhard, 1988b). Therefore, Bicoid is an unusual morphogen, being an intracellular protein instead of a secreted extracellular factor.

Correct anteroposterior patterning of the embryo requires activation of a set of genes called Gap genes. Bicoid activates *orthodenticle* (*otd*) in the most anterior region of the embryo, *otd* responds to high Bicoid concentrations (Fig. 1.1A: Gao and Finkelstein, 1998). A second gene *hunchback* (*hb*) is activated by Bicoid, it responds to lower bicoid concentrations and is therefore expressed throughout the whole anterior half of the embryo, and also in a band at the most posterior of the embryo (Fig. 1.1A: Driever and Nusslein-Volhard, 1989; Struhl et al., 1989). The differential activation of these genes establishes the first gene expression differences along the anterior-posterior axis and thereby initiates patterning by regulating the expression of further anteroposterior patterning genes.

Decapentaplegic (Dpp) is a member of the bone morphogenetic protein (BMP) family of TGFβ signalling molecules. It acts as a long-range signal to pattern several tissues including the ectoderm of the *Drosophila* wing imaginal disc. *dpp* is expressed in a narrow stripe at the anterior-posterior compartment boundary of the wing disc (Fig. 1.1B). The transcription of two downstream genes, expressed within the wing disc, *spalt* (*sal*) and *optomotor-blind* (*omb*), rely upon *dpp* signalling (Lecuit et al., 1996; Nellen et al., 1996). *omb* is expressed in a larger area of the wing disc and further from the source of Dpp than *sal*, which is expressed in a nested region within the *omb* expressing domain (Fig. 1.1B: Lecuit et al., 1996; Nellen et al., 1996). Ectopic expression of *dpp* can recapitulate the pattern of expression of both *omb* and *sal* (Lecuit et al., 1996; Nellen et al., 1996). These results suggest that *omb* induction can occur at low levels of Dpp, while *sal* is only expressed at high concentrations.

Dorsal (Dl) is a DNA binding protein that is related to the mammalian transcription factor NF-κB (Ghosh et al., 1990) and is distributed throughout the dorsoventral axis of the *Drosophila* embryo in a nuclear concentration gradient (Fig. 1.1Ci: Roth et al., 1989). The highest concentration of Dl is located in the most ventral cells, here the mesoderm is established by activation of genes *twist (twi)* and *snail (sna)* (Boulay et al., 1987; Thisse et al., 1991). Lower levels of Dl activate the gene *rhomboid (rho)*, defining a second threshold of Dl activity. *rho* expression determines the ventral presumptive neuroectoderm (Bier et al., 1990). *short gastrulation (sog)* is activated at yet lower levels of Dl in the presumptive neuroectoderm. *sog* therefore occupies a slightly broader domain than *rho* (Francois et al., 1994). Finally the dorsal ectoderm and amnioserosa, the most dorsal structures of the embryo, are controlled by the restriction of *decapentaplegic* (*dpp*) and *zerknüllt (zen)* to these dorsal regions (Doyle et al., 1989; St Johnston and Gelbart, 1987). This is accomplished by low concentrations of Dl. Similar low levels of Dl activate *sog* and repress *zen*.

The activity of Dl protein is controlled by differential subcellular localisation. In the absence of an activation signal, Dl remains in the cytoplasm, bound to an inhibitory protein Cactus, related to mammalian IκB (Wasserman, 1993). Extracellular activation of the pathway leads to a breakdown of this complex and the nuclear translocation of Dl (Fig. 1.1Cii: Bergmann et al., 1996; Reach et al., 1996). The extracellular signal that initiates this process corresponds to the maternal protein Spätzle (Spz). Spz is locally activated at the ventral pole of an embryo by a cascade of proteases located in the perivitelline fluid (PVF), which separates the follicle cells and the oocyte (LeMosy et al., 1999). This locally active Spz ligand migrates laterally and binds to and activates ubiquitous receptor Toll (Morisato and Anderson, 1994; Schneider et al., 1994). At more lateral regions there are less bound Toll receptors and therefore less Dl enters the nucleus. In dorsal regions of the embryo, there are few, if any activated Toll receptors consequently no Dl enters the nucleus. Thus, set up, the nuclear Dl gradient patterns the dorsoventral axis of the embryo by activating the downstream genes discussed above (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989).

Finally, Activin is an example of a morphogen that patterns the mesoderm in *Xenopus* (Fig. 1.1D). It is a member of the TGFβ superfamily, and was the first factor characterised as a mesoderm inducer (Smith et al., 1990). Not only does Activin induce dorsal mesoderm, but it also promotes the cell movements and convergent extension characteristic of dorsal mesoderm (Symes and Smith, 1987). After many years of uncertainty, recent evidence indicates that Activin is the endogenous factor required for normal mesoderm formation in *Xenopus* (Piepenburg et al., 2004).

Various *in vitro* experiments provided evidence to support the idea that Activin acts as a morphogen in the patterning of the mesoderm (Dyson and Gurdon, 1998; Green et al., 1992; Green and Smith, 1990; Gurdon et al., 1994). The addition of low concentrations of Activin to explants of prospective ectoderm tissue induced the lateral mesoderm marker *Xbrachyury* (*Xbra*), whilst a three-fold concentration increase induced the dorsal mesoderm marker *Xgoosecoid* (*Xgsc*) (Gurdon et al., 1994). The same effect was observed with the injection of Activin mRNA, or by the addition of a bead soaked in Activin. In the case of the bead experiments, tissue located close to the source of the Activin received an initial wave of signal that induced *Xbra* expression, this was followed by a subsequent induction of *Xgsc* (Gurdon et al., 1994). So, this suggested a relationship between the distance from the morphogen source and the gene induced; tissue closest to the source expressed *Xgsc* and tissue further away expressed *Xbra*. The induction of gene expression at particular concentration thresholds *in vitro*, mirrors the sequential activation of genes seen in the ventroposterior to dorsoanterior sequence *in vivo* (Green et al., 1992). Subsequent studies of Activin binding to cells, suggested that it is the total number of receptors that are bound by Activin that determines induction of *Xbra* or *Xgsc* (Dyson and Gurdon, 1998). A three-fold increase in Activin concentration, which switches gene expression, results in a three-fold increase in the number of bound receptors.

These examples of morphogens and their embryonic patterning activity, illustrates how a single signalling molecule can differentially regulate several genes at various concentration thresholds. The mechanisms by which the genes downstream of Bcd, Dl, Dpp and Activin interpret the graded signal are as yet not fully understood. However, advances have been made in this field, this will be discussed later.

1.1.4 Duration of Morphogen Signal

Experimental evidence supports the idea that morphogens pattern various developing tissues by setting up concentration gradients. However, recently it has also been proposed that it is not only the concentration of a morphogen, but also the duration of signalling that can determine cell fate. This idea has been suggested to provide an alternate explanation for morphogen activity but has received less consideration in the study of morphogens (Kang et al., 2003; Pages and Kerridge, 2000).

The importance of signal duration has been proposed by two groups to be a key parameter in tissue patterning; Pages and Kerridge proposed a model they termed the sequential cell context model (Pages and Kerridge, 2000), while Kang et al. termed their model the self enabling mechanism (Kang et al., 2003). Both of these models depend on a first wave of signal from the morphogen activating the expression of an early set of genes. These genes code for transcription factors. This first wave sets a context for the cells, which then go on to express a second set of genes after continued signalling by the morphogen. This mechanism is reminiscent of a feed forward loop (see below), in which a signal activates a set of early genes, which then cooperate with the signal to activate downstream target(s). This mechanism therefore implies a time delay while upstream genes are activated in response to initial morphogen exposure.

Pages and Kerridge used this idea to explain Dpp patterning of the imaginal wing disc. Two Dpp target genes, *omb* and *sal*, appear to be expressed at different concentrations of Dpp; low and high respectively and at different times in development. *omb* after 24 hours and *sal* after 72 hours (Lecuit et al., 1996; Nellen et al., 1996). It has also been shown that strong expression of *dpp* can induce both genes at the same time (Lecuit and Cohen, 1998). From these data it was concluded that high Dpp concentration activates *sal* and *omb* but low concentration activates only *omb*. However, the sequential cell context model offers an alternative. In this view Dpp signalling activates *omb* at early time points, and a longer period of Dpp exposure is required for *sal* induction. The induction of *sal* may require Omb or

possibly other targets (Pages and Kerridge, 2000). This model could also explain other examples of tissue patterning by morphogens; for example Activin patterning of the Xenopus mesoderm (Pages and Kerridge, 2000). Nevertheless, the relative importance of morphogen duration compared to concentration remains a poorly understood aspect of tissue patterning.

1.1.5 Sonic Hedgehog

One morphogen not yet discussed, is the vertebrate homologue of *Drosophila* Hedgehog protein; Sonic Hedgehog (Shh). Shh acts as a long-range graded signal and has been shown to be crucial in the patterning of the dorsal-ventral axis of the neural tube (Briscoe et al., 2001; Briscoe and Ericson, 2001; Ericson et al., 1997a; Jessell, 2000; Roelink et al., 1995; Wijgerde et al., 2002) and the anterior-posterior axis of the developing vertebrate limb (Helms et al., 1994; Riddle et al., 1993). Targeted disruption of the Shh gene in mice results in phenotypes similar to those seen in humans in a condition known as holoprosencephaly (HPE: Chiang et al., 1996). These include fusion of the telencephalic vesicle and optic vesicle (cyclopia) and failure to form midline structures such as notochord and floor plate. The action of Shh in patterning the dorsoventral axis of the neural tube and how the gradient is formed and regulated is the basis for this project.

1.2 Formation and Patterning of the Neural Tube

1.2.1 Physical Processes Involved in Neural Tube Formation

The neural tube is the rudimentary structure of the adult central nervous system (CNS) of chordates. The CNS is made up of the brain (forebrain, midbrain and hindbrain) and the spinal cord. The neural tube, which will form the structures found in the CNS, is formed during a process called neurulation. Primary neurulation consists of 4 steps; formation of the neural plate, shaping of the cells that make up the neural plate, bending of the neural plate to form the neural groove and finally closure to form a rod like structure that is the neural tube (reviewed in Colas and Schoenwolf, 2001).

The neural plate is a thickened sheet of ectoderm located in the future dorsal region of the embryo. Bending of the neural plate involves elevation of the neural folds, tissue found at the lateral edges of the plate (Colas and Schoenwolf, 2001). In amniotes these folds then converge towards the midline and form the neural groove. This groove will become the lumen of the primitive neural tube once closure and fusion of the fold has occurred (Colas and Schoenwolf, 2001). The point of tube fusion becomes the roof plate of the neural tube, which is detached from the overlying epithelial ectoderm. Patterning of the naïve homogeneous tissue that comprises the forming neural tube begins during neurulation and continues after neural tube fusion (reviewed in Jessell, 2000). This process will give rise to the multiple neuronal subtypes of the neural tube.

Neurons in the mature spinal cord serve two main purposes; to relay sensory input to higher centres in the brain and to coordinate motor output (reviewed in Jessell, 2000). A key feature of these neurons is that, not only do different neuronal subtypes serve different functions, but functionally distinct neurons are located in spatially segregated domains in the neural tube. The neurons that process sensory input from the periphery and relay information to higher centres in the brain are mainly located in the dorsal neural tube. Neurons that are required for coordination of motor output are restricted to the ventral neural tube.

The process of neurulation in the teleost zebrafish (*Danio rerio*), is slightly different to that of mammals, however there are sufficient similarities to make the system a useful model for analysis (Lowery and Sive, 2004). The thickened sheet of cells, the neural plate, forms from the ectoderm as in amniotes. Cells from the neural plate drop ventrally into the mesenchyme and form the neural keel, which is a solid rod of cells. The midline of the neural keel is always apparent, however cell mixing does occur (Ciruna et al., 2006; Concha and Adams, 1998; Geldmacher-Voss et al., 2003). Cells frequently cross the midline, dividing cells can provide progeny for both sides of the neural tube, this appears to be a characteristic specific to teleosts. The lumen opens up later on within the mass of cells, this occurs in a ventral to dorsal manner (Lowery and Sive, 2004). Nonetheless, the subsequent patterning and specification of the neural tube appears similar in fish and amniotes (Lewis and Eisen, 2003).

1.2.2 Morphogenetic Patterning of Neural Tube by Mesodermal Structures

A major goal of developmental neurobiology is to understand what makes particular neurons form at distinct spatial locations. In the CNS signals emanating from structures adjacent to the neural tube play a central part in this. The neural tube is surrounded on three sides by mesodermal structures: paraxial mesoderm laterally, which will form the somites; and the notochord, which lies ventral to the neural tube along the midline. The notochord is a rod like mesodermal structure, which is an essential structure for developing chordates (reviewed in Stemple, 2005). It plays an important structural role in early embryos, some vertebrates maintain the structure, however, in higher vertebrates it is a transient structure that becomes part of the centre of the intervertebral discs. At the ventral midline of the neural tube itself is a small group of wedge shape cells called the floor plate. Within the ventral neural tube, there are a mixture of interneurons and motor neurons. Motor neurons (MN) are found in the ventral horn of the mature spinal cord and innervate muscles to control motor output. In the developing neural tube, MNs and distinct interneuron groups arise in a precise spatial location within the dorsoventral axis.

Experimental evidence suggests that the notochord is required for the correct patterning of the neural tube (Placzek et al., 1991; Yamada et al., 1991). If the notochord was removed from a developing chick embryo, then the floor plate, a specialised group of cells located at the ventral midline of the neural tube, did not form (Yamada et al., 1991). Conversely, if a second notochord was grafted laterally to the neural tube, a second floor plate formed (Yamada et al., 1991). This second floor plate was accompanied by the formation of additional motor neurons at a distance from the additional floor plate. This led to the conclusion that a signal from the notochord is required for the correct formation of the floor plate and also for providing information about dorsoventral polarity. The floor plate itself has similar inductive properties for inducing further floor plate cells and motor neurons, suggesting it releases signals sufficient to induce floor plate and motor neuron fates (Yamada et al., 1991).

1.2.3 Patterning of Neural Tube by Shh

In the grafting experiment, the position of the motorneurons in relation to the graft suggested that the signal emanating from the notochord and floor plate was capable of inducing particular cell identities at different distances from the signal source (Yamada et al., 1991). Subsequent experiments confirmed this observation and showed that the type of neuronal cell formed was in part determined by the distance from the notochord and floor plate (Placzek et al., 1991; Yamada et al., 1991). These studies also provided evidence that a secreted, diffusible signal was responsible for the patterning activity of the notochord and floor plate (Yamada et al., 1993).

In the search for possible signalling molecules that could be responsible for neuronal patterning, the secreted protein Sonic Hedgehog (Shh) was identified (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). More precisely, the amino terminal peptide of processed Shh (Shh-N) was demonstrated to be responsible for the activity of the notochord and floor plate (Marti et al., 1995). Shh appears to fulfil the criteria required to be classed as a morphogen and evidence supports the hypothesis that it acts to pattern the neural tube.

First, *shh* is expressed in the right place (notochord and floor plate; Fig. 1.2A) and at the times these structures have patterning activity. It also appears to act on tissue at a distance from the source and direct various outputs for these cells (Ericson et al., 1996; Marigo and Tabin, 1996). Shh establishes a ventral to dorsal concentration gradient (Fig 1.2B), which controls the patterning of the tissue via long-range graded signalling (Briscoe et al., 2001; Ericson et al., 1997b; Gritli-Linde et al., 2001; Lewis et al., 2001). Thus the level of Shh along the dorsal ventral axis appears to determine the progenitor domain identity, specifying the neuronal subtype that will develop (Briscoe and Ericson, 2001; Jessell, 2000; Wijgerde et al., 2002). *In vitro* experiments showed that changes in applied Shh concentration, resulted in predictable changes in neuronal cell fates, neurons in more ventral regions required higher concentrations of Shh for induction (Marti et al., 1995; Roelink et al., 1995). Further evidence from loss of function analysis showed that Shh was essential for the formation of the

ventral structures of the neural tube, these did not form in mice lacking functional Shh (Chiang et al., 1996). This patterning action of Shh is a direct effect (Hynes et al., 2000; Wijgerde et al., 2002). Smoothened (Smo) is the signal transducing unit of the Shh receptor, constitutively active forms of Smo ectopically expressed led to cell autonomously induced ventral cell types (Hynes et al., 2000). Conversely, inhibition of Shh signalling cell autonomously (Briscoe et al., 2001) or inactivation of Smo cell autonomously blocked Hh signal transduction (Wijgerde et al., 2002).

1.2.4 Transcription Factor Code

The ability of graded Shh signalling to directly control dorsoventral patterning in the ventral neural tube raised the question of how positional identity is imposed on progenitor cells and how this determines neuronal subtype identity. The progenitor cells interpret the gradient of Shh in the neural tube, by regulation of several transcription factors. These transcription factors, with the exception of the basic helix-loop-helix (bHLH) protein Olig2, are members of the homeodomain (HD) protein family, including Pax7, Pax3, Dbx1, Dbx2, Pax6, Nkx2.2, Nkx2.9, Nkx6.1, Nkx6.2 and Irx3. The proteins are split into 2 classes; Class I proteins, the expression of which are inhibited by Shh e.g. Pax6 and Class II proteins, which are either directly or indirectly activated by Shh e.g. Nkx2.2 (Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997b). Within the same class, genes respond to Shh in a concentration dependent manner. For example the repression of Class I genes with more ventral limits of expression require higher levels of Shh signalling compared to more dorsally restricted Class I genes. Conversely, Class II proteins that have a broad domain of expression in the ventral neural tube are induced by lower concentrations of Shh than Class II genes that are limited to more ventral regions. Combinatorial expression of these Class I and Class II proteins define distinct progenitor domains, giving rise to the 5 distinct groups of post-mitotic neurons: V0, V1, V2, V3 interneurons and motor neurons (MN; Fig. 1.3).

Evidence that graded Shh signalling establishes the transcription factor code emerged from studies of the expression patterns of *Nkx2.2* and *Pax6* (Ericson et al., 1997b). Similar findings have been documented for other Class I and Class II proteins (see

below). In the developing ventral neural tube *Pax6* (Class I) and *Nkx2.2* (Class II), have a mutually exclusive expression pattern with the most ventral progenitors expressing *Nkx2.2* (p3, Fig. 1.3A) while *Pax6* is expressed dorsal to the boundary of *Nkx2.2* (Fig. 1.3A). *In vitro* experiments demonstrated that the expression of both of these proteins is regulated by Shh signalling in a concentration-dependent manner (Ericson et al., 1997b). Cells within chick neural explants, grown in the absence of Shh, express *Pax6*. Exposure to concentrations of Shh above a given threshold leads to repression of *Pax6* and induction of *Nkx2.2*.

Shh regulation of Class I and Class II proteins is not sufficient to explain how the sharply delineated changes in gene expression are established. Cross-repressive interactions between select pairs of Class I and Class II proteins may help to explain this. Class I and II genes can be grouped in pairs, based on two prerequisites; i) the ventral limit of expression of a Class I protein coincides with the dorsal limit of the paired Class II protein and, ii) the pair of Class I and Class II factors display mutual cross-repression (Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997b). With the exception of Pax6, the Class I and Class II proteins act as transcriptional repressors in neural progenitors by recruiting Gro/TLE corepressors (Muhr et al., 2001). This evidence raises the possibility of direct interactions between the proteins and promoters of Class I and Class II genes, and supports the theory of repressive interactions. The interplay between pairs of transcription factors could explain the discrete changes of gene expression between progenitor domains in the ventral neural tube.

Gain- and loss-of-function studies, suggest that Pax6 and Nkx2.2 cross-repression is essential for establishing the progenitor domains and the correct gene expression boundaries (Briscoe et al., 1999; Ericson et al., 1997b). Expression of *Nkx2.2* expanded dorsally in mutant mice lacking functional Pax6 (Ericson et al., 1997b). This resulted in a decrease in motorneuron (MN) production and an expansion in Nkx2.2 produced V3 neurons. Pax6 is therefore required for precise positioning of the boundary between MN and V3 progenitors, and correct production of the neurons from these progenitors. The absence of Pax6 also results in defects in the generation of other ventral neuronal subtypes, in particular V1 and V2 neurons (Ericson et al.,

1997a), suggesting that Pax6 has further roles in the control of neuronal subtype identity in the neural tube. Additional studies indicated that in embryos lacking Nkx2.2, there was a loss of V3 interneurons, and a ventral shift in the MN progenitor marker Olig2 and subsequently in the generation of MNs (Briscoe et al., 1999). Expression of *Pax6* was unchanged, possibly due to the presence of Nkx2.9, another Nkx family member, which is expressed in a similar domain to *Nkx2.2* (Pabst et al., 1998).

Further evidence from other pairs of Class I and Class II proteins supports the idea of reciprocal cross-repressive interactions (Fig. 1.3). The Class II proteins Nkx6.1 and Nkx6.2 adjoin the ventral boundaries of the Class I proteins Dbx2 and Dbx1, respectively (Fig. 1.3B). In double knock-out embryos lacking both Nkx6 proteins, a ventral expansion of *Dbx2* expression was observed (Vallstedt et al., 2001). Moreover, a forced expression of *Nkx6.1* cell autonomously repressed *Dbx2*, and misexpression of *Dbx2* resulted in the downregulation of *Nkx6.1* (Vallstedt et al., 2001).

1.2.5 Neuronal Subtype Identity from Progenitors

The expression of the Class I and Class II proteins defines a series of progenitor domains p0, p1, p2, pMN and p3 (Fig. 1.3B). Each domain occupies a unique dorsoventral territory of neural progenitors and is identified by the combinatorial expression of transcription factors. Each of these progenitor domains will subsequently generate the five distinct neuronal subtypes found in the ventral neural tube; V0, V1, V2, MN, V3.

The specification of the neurons by specific transcription factor profiles is supported by gain- and loss-of-function experiments. The forced expression of a Class I or Class II protein in the neural tube and targeted inactivation of individual Class I or Class II proteins changes the fate and position of individual neuronal subtypes in a manner predicted by the normal profile of transcription factor expression.

The model for patterning the neural tube we can therefore summarise as follows. The initial activation or repression of the Class I and Class II proteins by graded Shh signalling provides positional identity to progenitor cells. Reciprocal repression between pairs of Class I and Class II proteins (e.g. Nkx2.2 and Pax6) enables the conversion of the gradient of Shh protein into discrete all-or-none changes in gene expression. The cross repression may therefore account for the differences in transcription factor expression profiles between adjacent progenitor domains. Each progenitor domain is defined by the combination of transcription factors it expresses. The progenitor cells in each domain then exit the cell cycle to form post mitotic neurons, the cells in each domain differentiate into a specific neuronal subtype; V0, V1, V2, MN or V3.

1.2.6 Additional Neural Tube Patterning Signals

In addition to Shh other extracellular signals have been shown to contribute to correct patterning of the ventral neural tube. During neural induction, progenitors are exposed to fibroblast growth factors (FGFs), originating from the presomitic mesoderm, the regressing node and the neural plate. FGF signalling acts as an inhibitor of neural differentiation ensuring cells remain progenitors. FGFs also inhibit the expression of many of the progenitor transcription factors and it is not until cells have emerged from the influence of FGFs that they mature and begin to express Class I and Class II proteins (Diez del Corral et al., 2002; Novitch et al., 2003).

Conversely, retinoic acid (RA) is expressed anteriorly to FGF, in paraxial mesoderm adjacent to the neural tube. RA and FGF signalling pathways have been shown to have mutually inhibitory effects. RA is necessary for neuronal differentiation and progenitor transcription factor expression (Diez del Corral et al., 2003; Novitch et al., 2003). RA is required for the specification of interneron and motorneuron progenitors (Diez del Corral et al., 2003; Novitch et al., 2003) and promotes Class I protein expression, therefore counteracting the ventralising effects of Shh.

Two additional signalling pathways implicated in dorsoventral patterning are bone morphogenetic proteins (BMPs) and Wnts. Several members of the BMP family are expressed in the dorsal pole of the neural tube where they play a role in specifying dorsal neuronal fates (reviewed in Lee and Jessell, 1999). In addition, BMP proteins appear to oppose Shh mediated ventralisation of the neural tube limiting the dorsal extent of the ventral neural tube (reviewed in Lee and Jessell, 1999). Wnt proteins have been implicated in dorsal neural tube patterning (reviewed in Lee and Jessell, 1999), both *Wnt1* and *Wnt3a* are expressed in the dorsal neural tube at all anterior posterior levels. Overexpression studies of these Wnt proteins suggest a role in control and coordination of progenitor proliferation in the dorsal neural tube. These data suggest that the precise co-ordination of ventral neural patterning depends on interactions between several signalling pathways.

1.3 Hedgehog Signalling

1.3.1 *Drosophila* Hedgehog Signalling

The Hedgehog (Hh) signalling pathway is best understood in *Drosophila* (reviewed in Kalderon, 2005). The transmembrane receptor to which Hh binds is Patched (Ptc) (Fuse et al., 1999; Marigo et al., 1996a; Stone et al., 1996). In the absence of Hh, activity of another transmembrane protein Smoothened (Smo) is repressed by Ptc (Chen and Struhl, 1998; Taipale et al., 2002). If Ptc is deleted or mutated, the Hh pathway is active whether the Hh ligand is present or not (Chen and Struhl, 1996; Ingham et al., 1991). The role of Smo is to transduce the Hh signal intracellularly. Therefore in the most simplified view, binding of Hh to Ptc relieves the repression of Smo, which then transduces the intracellular Hh signal (Murone et al., 1999; Stone et al., 1996). Although initial findings suggested a physical interaction between Ptc and Smo, recent findings indicate this model is not correct (Zhu et al., 2003). In the absence of Hh, Ptc inhibits the level of Smo post-transcriptionally and reduces the proportion of Smo protein at the plasma membrane (Denef et al., 2000; Nakano et al., 2004). How this is achieved remains to be determined.

The transcriptional effector of the Hh pathway is Cubitus interruptus (Ci), in its absence Hh signalling cannot occur (Methot and Basler, 2001). Ci acts as both transcriptional activator and repressor. In the absence of Hh, Ci is proteolytically processed into a truncated repressor form, Ci-75, which inhibits the transcription of Hh target genes (Aza-Blanc et al., 1997). Ci-75 consists of the N-terminal domain and the zinc finger DNA binding domain, but not the C-terminal activator domain of Ci. Formation of Ci-75 requires protein kinase A (PKA: Chen et al., 1998b), which initiates a cascade of phosphorylation by glycogen synthase kinase 3β (GSK3β) and a member of the CK1 family of kinases (Jia et al., 2002; Lum et al., 2003a; Price and Kalderon, 2002). Hh signalling inhibits processing of Ci, this results in the accumulation of the full length Ci, Ci-155 (Methot and Basler, 1999; Ohlmeyer and Kalderon, 1998). Not only does Hh inhibit the cleavage of Ci, it also increases its activation potential, possibly by enhancing nuclear accumulation (Chen et al., 1999; Wang and Holmgren, 2000).

Between Smo and Ci, several components of an intracellular signal transduction pathway have been identified. Costal-2 (Cos2) a kinesin-like protein, stably associates with Ci, and is believed to restrict the movement of Ci in the cytoplasm and promotes cleavage to Ci-75 (Chen et al., 1999; Lum et al., 2003b; Robbins et al., 1997; Sisson et al., 1997). Upon Hh binding, Smo recruits Cos2 thereby reversing the effects and increasing the amount of free un-cleaved Ci (Jia et al., 2003). Another protein, Supressor of Fused, Su(fu), also negatively regulates Ci by physical interaction, which promotes cytoplasmic localisation (Lum et al., 2003b; Methot and Basler, 2000). This negative regulation is relieved by Fused (Fu), which inactivates Su(fu) (Lum et al., 2003b). However, Su(fu) *Drosophila* mutants are viable and Hh signalling appears normal in these embryos (Preat, 1992). Cos2 is necessary for the stabilisation of Fu, thereby having a positive effect on the Hh pathway as well as its negative regulation of the pathway (Lum et al., 2003b).

1.3.2 Vertebrate Shh Signalling

In jawed vertebrates, various homologues of the *Drosophila Hh* gene have been identified. In mice there are 3 homologues, *sonic hedgehog* (*shh*), *desert hedgehog*

(*dhh*) and *indian hedgehog* (*ihh*) (Echelard et al., 1993). Similarly, in zebrafish, there are currently 5 known homologues; *shha* (previously known as *shh*), *shhb* (previously known as *tiggy-winkle hedgehog (twhh)), ihha, ihhb* (previously known as *echidna hedgehog (ehh)*) and *dhh* (Avaron et al., 2006; Currie and Ingham, 1996; Ekker et al., 1995; Krauss et al., 1993). All of these homologues appear to have different expression patterns and play different roles in development. However they appear to share the same signalling pathway. Due to its involvement in the dorsoventral patterning of the neural tube, here I will focus specifically on the role of Shh.

Homologues of several components of the *Drosophila* Hh pathway have been identified. In mammals, two homologues of Ptc have been identified; *Ptc1* and *Ptc2* (Carpenter et al., 1998; Motoyama et al., 1998). As with Ptc in *Drosophila*, Ptc1 acts to stop Hh pathway activity in the absence of ligand. Loss of *Ptc1* in mouse mutants led to constitutive activation of all downstream targets of the Shh pathway resulting in the overgrowth of neural tissue (Goodrich et al., 1997). In addition, Shh target genes, normally expressed in the ventral neural tube, were expressed in dorsal regions in the absence of Ptc. *Ptc2* is expressed in many tissue types, overlapping with the expression of both *shh* and *dhh* (Carpenter et al., 1998; Motoyama et al., 1998). However, *Ptc2* mouse mutants showed no obvious defects in Shh signalling in the neural tube, suggesting it does not play a role in patterning the neural tube (Nieuwenhuis et al., 2006). Only one orthologue of the *Smo* gene has been identified in mouse (Akiyama et al., 1997). Experimental evidence suggests that, as in *Drosophila,* Smo acts as a positive transducer of Shh signalling. *Smo* null mutant progenitor cells in the ventral neural tube were unable to transduce a Shh signal and consequently did not acquire a ventral identity but a dorsal one (Wijgerde et al., 2002).

At the cytoplasmic level, it appears that Mouse and *Drosophila* Hh signalling pathways have diverged, at least to some extent. The absence of the Cos2 orthologues Kif7 and Kif27 in mouse cell lines had no effect on Shh signalling (Varjosalo et al., 2006). However, in zebrafish, inactivation of the Cos2 orthologue by morpholino injection led to ectopic Hh signalling (Tay et al., 2005), while loss of this gene in Drosophila was lethal (Grau and Simpson, 1987). Conversely, while mutations in

Drosophila Su(fu) had no effect on the Hh signalling pathway (Preat, 1992), loss of Su(fu) in mouse led to an increase in Shh activity with a phenotype similar to that of *Ptc1* mutant mice (Cooper et al., 2005; Svard et al., 2006; Varjosalo et al., 2006).

As in *Drosophila*, vertebrate Hh signal culminates in the regulation of Ci-like transcriptional effectors of the pathway. In vertebrates these are known as Gli proteins. The Gli proteins are zinc finger containing transcription factors, and are the homologues of *Drosophila* Ci. The Gli transcription factors bind to identified consensus sequence (Kinzler and Vogelstein, 1990), which has been found in several Shh-responsive genes (see below).

In vertebrates there are 3 Gli proteins; *Gli1, Gli2* and *Gli3* (Ruppert et al., 1988), all are expressed in the neural tube (Brewster et al., 1998; Hui et al., 1994; Lee et al., 1997). The different regulation and roles of these Gli proteins are slowly being understood (reviewed in Jacob and Briscoe, 2003). *Gli1* is expressed in the ventral neural tube and its expression is dependent upon Shh (Bai et al., 2002); in the presence of Shh *Gli1* is activated (Lee et al., 1997; Marigo et al., 1996b). The Gli1 protein contains a zinc finger DNA binding motif and an activator domain at the carboxy terminal, however, it does not contain a repressor domain (Dai et al., 1999; Sasaki et al., 1999).

Like *Drosophila* Ci, Gli2 and Gli3 can be proteolytically processed and are able to act as both activators and repressors (Dai et al., 1999; Ruiz i Altaba, 1999; Sasaki et al., 1999). *Gli2* and *Gli3* are expressed in neural tissue prior to neural tube closure. After that, *Gli2* expression remains uniform, with *Gli3* expression becoming confined to intermediate and dorsal regions of the neural tube (Hui et al., 1994; Lee et al., 1997). Gli2 is an activator in the presence of Shh. A repressor form of Gli2 can be formed (Dai et al., 1999), this processing is believed to be inhibited by Shh signalling (Pan et al., 2006). *In vivo*, however most Gli2 is present in the full length form because the partial processing appears to be very inefficient (Pan et al., 2006). Instead, Shh appears to regulate the stability of Gli2 (Pan et al., 2006). Gli2 is readily degraded by ubiquitination due to hyperphosphorylation by PKA and GSK3. This process is

suppressed by Shh signalling (Pan et al., 2006), resulting in the accumulation of full length protein able to activate transcription.

Like *Gli2*, the activity of *Gli3* is dependent on Shh signalling. In the absence of Shh signalling, Gli3 is processed to a C-terminally deleted form to act as a repressor (Aza-Blanc et al., 2000; Dai et al., 1999; Wang et al., 2000a). In the presence of Shh, the generation of Gli3 repressor is blocked (Litingtung et al., 2002; Wang et al., 2000a). Therefore, Shh acts to stabilise Gli2 and 3, and to block their processing to transcriptional repressors. In the absence of Shh, Gli2 appears to be degraded while partial processing of Gli3 results in an increase in inhibitory forms of Gli3. Notably, even though Gli3 appears to act primarily as a repressor, there is evidence to suggest that it is able to act as an activator. In mice Gli1/Gli2 homozygous double mutants, there was evidence of Shh signalling, suggesting Gli3 was able to act as an activator in the pathway (Park et al., 2000).

Embryos from mouse *Gli3* knock-out lines, displayed a dorsal expansion of intermediate neuronal subtypes, suggesting a dorsal shift in Gli activator activity in the neural tube (Persson et al., 2002). However, the wild type expression pattern of progenitor markers in the intermediate neural tube was rescued in embryos carrying a *Gli3* allele that encoded a protein equivalent to the repressor form (Persson et al., 2002). This indicates that the Gli3 repressor form (Gli3R) is required for correct patterning of the intermediate neural tube. Conversely, Gli2 appears necessary for patterning the ventral neural tube. Mouse embryos lacking *Gli2* failed to develop a floor plate and there was a dramatic reduction or absence of the most ventral neuronal subtypes including V3 interneurons, dopaminergic and serotonergic neurons (Ding et al., 1998; Matise et al., 1998; Park et al., 2000).

Gene knock-out studies of Gli proteins in mice have not only provided evidence that Gli proteins are required for neural tube patterning, but have also provided evidence of a model for their activity. This model suggests that there is a gradient of both Gli activator and repressor forms acting in opposing directions, regulated by Shh. Gli1, 2 and 3 activators form a gradient from ventral to dorsal and the Gli2 and 3 repressors

form a gradient from dorsal to ventral (reviewed in Jacob and Briscoe, 2003). Recent evidence has demonstrated that a gradient of Gli activator activity in the neural tube is sufficient to control the patterning of the neural tube (Stamataki et al., 2005). Different levels of Gli activity mimicked the effects of a Shh gradient by inducing transcriptional markers characteristic of the spatial location along the dorsoventral axis of the neural tube (Stamataki et al., 2005).

1.3.3 Control and Maintenance of Shh Gradient

In order for a morphogen to correctly pattern a tissue, regulating the distribution of the protein is crucial. One way that the Shh gradient is believed to be regulated is by negative feedback (reviewed in Perrimon and McMahon, 1999). This mechanism works by morphogen signalling up-regulating negative inhibitors of the pathway. The attenuation of signal is achieved through proteins that bind and sequester Hh. *Drosophila* studies have identified Ptc as one such protein (Chen and Struhl, 1996). Therefore, Ptc inhibits the Hh pathway in two ways by sequestering Hh and by preventing Smo signal transduction (see above). Mouse *Ptc1*, like *Drosophila Ptc*, has been shown to contain a Gli binding site in its promoter (Agren et al., 2004) and is positively regulated by the Shh signalling pathway (Goodrich et al., 1996). Another protein that has been found to be transcriptionally regulated by the pathway and acts as a negative regulator is Hh-interacting protein 1 (Hhip1: Chuang and McMahon, 1999). A *Drosophila* homologue of this gene has not been identified. There is now experimental evidence to suggest that both Ptc1 and Hhip1 mediate a process called ligand-dependent antagonism (LDA), which controls the range of signalling. Sequestration of Shh is important in this model (Jeong and McMahon, 2005). Experiments using mice lacking both *Ptc* and *Hhip*, provided evidence that the process of LDA was essential for the correct patterning of the ventral neural tube and the positioning of the various neuronal subtypes.

Further factors have been identified that are thought to be involved in the tight regulation of the Shh gradient. Two cell surface fibronectin containing proteins Cdo and Boc were identified, in a microarray screen, as being downregulated in response to Shh (Kang et al., 1997; Kang et al., 2002; Tenzen et al., 2006). Both *Cdo* and *Boc*

are expressed in the dorsal spinal cord, where levels of *Ptc1* and *Hhip1* gene expression are low, *Cdo* is also expressed in the floor plate (Tenzen et al., 2006). Gain- and loss-of-function experiments suggest that Cdo and Boc have complex roles in the maintenance of the gradient. Cdo and Boc, cell autonomously enhance Hh signalling, Cdo increases Shh signalling in the floor plate where the highest Shh signalling levels are required. At more dorsal positions in the neural tube, where Shh, Ptc1 and Hhip1 levels are low, Cdo and Boc are proposed to sensitise cells to low levels of Shh protein. By ectopically expressing Cdo and Boc a non-cell autonomous expansion of dorsal genes was seen in cells dorsal to those containing the transgene (Tenzen et al., 2006). This was consistent with the idea that these proteins bind Shh impeding its ability to spread further through tissue.

1.4 Interpreting Graded Signals

In order for a tissue to be patterned by a morphogen, the signal needs to be interpreted by the receiving cells. Subtle differences in signal are translated into discrete responses by the cell, elaborated as discrete changes in gene expression. A key point is therefore to understand how a cell differentially regulates gene expression in response to small changes in extracellular morphogen concentration. The possible mechanisms by which cells achieve this and examples will be briefly discussed here (reviewed in Ashe and Briscoe, 2006).

1.4.1 Binding Site Affinity and Combinatorial Inputs

The presence of binding sites with different binding affinity for the morphogen activated transcription factors in promoters of target genes, is one way in which differential gene regulation could be achieved by a gradient (Fig. 1.4A). For this mechanism, target genes containing low affinity binding sites would require higher concentrations of the transcriptional regulator in order to be activated. Those promoters containing higher affinity binding sites would be activated by lower concentrations of transcription factor. Thus different groups of genes would respond to the different concentrations of morphogen in one tissue. Often viewed as the canonical mechanism, there are several examples of systems in which this is proposed

to aid the interpretation of a morphogen gradient; notably for genes responding to the Dorsal (Dl) gradient and Bicoid (Bcd) gradient (Driever et al., 1989; Ip et al., 1992; Jiang and Levine, 1993; Struhl et al., 1989).

In the dorsoventral axis of the *Drosophila*, target genes expressed where Dl proteins are present at high concentrations (presumptive mesoderm), contain low affinity binding sites, for example *twist* (*twi*). This ensures *twi* and other type I genes can only be expressed at positions of high Dl concentration (Jiang and Levine, 1993). Conversely, genes that contain high affinity binding sites in their promoters, for example *rhomboid* (*rho*), respond to lower levels of Dl protein and are expressed at a further distance from the source of Dl (ventral neuroectoderm: Ip et al., 1992). However, this view of gradient interpretation is not sufficient to explain all of the Dl dependent outcomes. Recent studies of Dl responsive genes in the dorsoventral axis suggest that it is not only the affinity of Dl binding site but the combination of this and other transcription factors that lead to the correct gene activation (Fig. 1.4B: reviewed in Stathopoulos and Levine, 2004).

A similar observation has been made for genes responsive to Bicoid (Bcd), the factor that patterns the anteroposterior axis of the *Drosophila* embryo (see above). A Bcd responsive gene, *hunchback (hb),* is expressed at low concentrations of Bcd, and contains high affinity sites in the *hb* enhancer (Driever et al., 1989; Struhl et al., 1989). This allows expression even in posterior regions of the embryo where Bcd levels are low (Driever et al., 1989; Struhl et al., 1989). However, in other cases, Bcd responsive genes activated at more posterior regions tend to be activated by other genes and require inputs from transcription factors such as Caudal and Krüppel (Ochoa-Espinosa et al., 2005).

1.4.2 Feed Forward Loops

A second way in which differential gene expression can be regulated in response to a morphogen is with gene networks, such as a feed forward loop (Fig. 1.4C: Mangan and Alon, 2003). A feed forward loop allows the integration of responding genes in a

single pathway to regulate the response to a morphogen. This mechanism involves the sequential activation of two transcription factors, which together activate a third. The responsive gene in the pathway can only be activated if both of the first two factors are present.

Examples of such pathways have been reported in targets of the *Drosophila* gene *dpp,* a ligand for the bone morphogenetic protein (BMP) pathway, which is involved in patterning the dorsoventral axis of the embryo (Lin et al., 2006; Xu et al., 2005). The activation of the Dpp signalling target genes *Race* (Xu et al., 2005) and *C15* (Lin et al., 2006) require induction by both Smad and Zen in order to be expressed. Smad and Zen bind to the enhancer of *Race* at adjacent binding sites, direct interaction between the two transcription factors is required for gene activation (Xu et al., 2005). Both Smad and Zen are targets of Dpp signalling, peak levels of signalling activate Smad and Zen which then activate *Race* (Xu et al., 2005). The target gene *C15* is activated in a similar fashion, however it responds to lower levels of Dpp signalling via Smad and Zen activation (Lin et al., 2006).

1.4.3 Other Mechanisms

In addition to those discussed, there are many other mechanisms that can contribute to how a set of target genes interpret gradients. For example, a positive feedback mechanism, this occurs when a target gene regulates its own expression once activated by the morphogen. A cross repression mechanism, as previously described, between Class I and Class II genes in the dorsoventral patterning of the neural tube could also contribute to the interpretation of morphogen gradients. Finally, patterning could be achieved by the production of reciprocal repressor gradients, which together could determine the threshold responses of target genes. All of these potential mechanisms are unlikely to function alone but in tandem to precisely pattern a naïve tissue and to ensure discrete all or nothing responses by target genes.

1.5 Promoter Analysis: Methods Utilised

In order to understand how the neural tube is patterned by a gradient of Shh we need to understand how extracellular changes in Shh concentration regulate differential gene responses in cells. Second, how are the cross repressive interactions between the homeodomain proteins integrated at the level of individual genes? Finally, how is neural specificity achieved? Shh is expressed in many tissues of the developing embryo, so why are the homeodomain proteins utilised in neural tube patterning only expressed in the CNS? In order to answer these questions, a comprehensive promoter analysis needs to be performed.

Promoter analyses of this type have been initiated for Shh-responsive genes: floor plate marker *FoxA2* (Sasaki and Hogan, 1996; Sasaki et al., 1997); motor neuron progenitor marker *Olig2* (Sun et al., 2006; Xian et al., 2005); as well as *Shh* itself (Epstein et al., 1999; Jeong et al., 2006; Jeong and Epstein, 2003; Muller et al., 1999).

1.5.1 *Shh* and *FoxA2* Promoter Analysis and Protein Interactions

Extensive analysis of the *shh* locus in mouse has identified the enhancer elements needed for correct spatial expression of the *shh* gene in all regions of the mouse neural tube (Epstein et al., 1999; Jeong et al., 2006; Jeong and Epstein, 2003). Initial analysis used reporter constructs, driving *LacZ,* covering 35kb of DNA that included the *shh* locus (Epstein et al., 1999). These enhancers directed precise Shh-like expression of the reporter in distinct locations within the CNS of the transgenic mice, including the ventral midline of the neural tube and notochord (Epstein et al., 1999).

This analysis revealed the presence of several enhancers all driving different expression patterns within the CNS. Enhancer SFPE1, located upstream of the *shh* gene, is a FoxA2 independent enhancer of expression and directed floor plate expression in the hindbrain and spinal cord (Epstein et al., 1999). 2 enhancers (SFPE2 and SBE1) were located within intron 2. SFPE2 directed similar expression to SFPE1 whilst SBE1 directed expression in the ventral midbrain and posterior diencephalon (Epstein et al., 1999). Cross species analysis of human, mouse, chicken

and zebrafish using intron 2 narrowed the SFPE2 down to an 88bp fragment, containing FoxA and Tbx consensus binding sites (Jeong and Epstein, 2003). This fragment can direct Shh-like expression in the notochord and ventral midline. Further analysis using an enhancer trap method identified three further enhancers covering a 400kb region that directed expression in more anterior regions not covered by the previous three enhancers (Jeong et al., 2006). A similar analysis was carried out on the zebrafish *shh* gene (Muller et al., 1999), this identified intronic enhancers that directed notochord and floor plate expression in transgenic mice and zebrafish, suggesting conservation of regulatory mechanisms.

Using a similar method to that used for the analysis of the *shh* promoter, independent notochord and floor plate enhancers in the *FoxA2* promoter were identified (Sasaki and Hogan, 1996). Identification of a Gli binding site within the floor plate enhancer suggested Shh involvement in the activation of floor plate *FoxA2* expression (Sasaki et al., 1997). Targeted mutation of the Shh-responsive Gli binding site within the *FoxA2* floor plate enhancer, led to a loss of reporter expression within the floor plate (Sasaki et al., 1997). Gli1 but not Gli3 can activate the Gli binding site, therefore Gli1 is a likely positive regulator of *FoxA2*.

Together, therefore, the data suggest that FoxA2 acts both upstream and downstream of *shh*. The *shh* gene in zebrafish has been shown to contain 2 FoxA2 binding sites (Chang et al., 1997). Mice homozygous for a mutation in *FoxA2* fail to develop node or notochord (Ang and Rossant, 1994). *Gli1* has been shown to positively regulate *FoxA2* (Sasaki et al., 1997), which in turn activates *shh* expression (Chang et al., 1997; Ruiz i Altaba et al., 1995). This cascade initiated by Shh is essential for induction of the floor plate gene expression profile and for its own expression. However, maintenance of floor plate expression is not Gli dependent (Lee et al., 1997), expression of *Glis* are downregulated in the floor plate once established.

1.5.2 Additional Promoter Analysis Methods

The methods described above for analysis of gene promoters have several limitations: transgenes insert randomly in the genome and the expression is influenced by position of insertion; producing transgenic animals is expensive and time consuming; biochemical studies usually require more material than available from embryo experiments. To overcome these limitations a newly developed method can be used; ES cell analysis. This system has been successfully carried out to analyse the *Olig2* promoter, which identified a region that when deleted led to loss of *Olig2* expression (Xian et al., 2005). This method involves electroporating a BAC containing the DNA of interest into ES cells and then multiple individual clones are allowed to proliferate. Each culture of stable clones is then grown in a media, which causes differentiation into neural cells (ES cell-derived neural cells; ESNCs). This enables transgenic expression to be analysed at each step of the pathway from a totipotent cell to a differentiated neural cell.

Further study of the *Olig2* regulatory regions utilised another method that has successfully identified regulatory regions in the promoters of other genes; BAC transgenesis (Sun et al., 2006). This method targeted a BAC containing *Olig2* with a traceable marker and transgenic animals were generated. This method identified a region of DNA that is believed to drive *Olig2* expression in the motor neuron lineage only, not in oligodendrocytes (Sun et al., 2006).

1.5.3 Identification of Conserved Non-Coding Regions

In order to identify enhancer regions in genes of interest, *in silico* approaches are increasingly exploited. Identifying conserved regions of regulatory DNA by comparing genomic sequences, is a process called phylogenetic footprinting. This method has been very successful, and is based on the idea that regulatory regions that have been conserved are under more selective pressure than non-regulatory regions and mutation rates will be slower. Pair-wise comparison between closely related species, for example human and chimpanzee, provide little information due to the high proportion of conservation in the genome. Comparisons between highly

diverged genomes, on the other hand, such as those of tetrapods and teleosts are more likely to identify regions important in gene regulation.

Once two or more genomes have been chosen, an alignment between the sequences needs to be performed. There are two commonly used methods for alignment; a local alignment that identifies short segments of similarity, and a global alignment that finds similarity across the full length of a sequence. For local alignments, the BLASTZ algorithm (Schwartz et al., 2000) is used, this is a modification on the original Gapped BLAST algorithm (Altschul et al., 1990). This algorithm uses the following strategy; it identifies short near exact matches in sequences and then extends them allowing no gaps. Finally it extends the gap free matches with less sensitive thresholds of matches.

For global alignments the Needleman-Wunsch algorithm is used (Needleman and Wunsch, 1970), this is often carried out by obtaining short local alignments to identify sub segments, subsequently a global alignment is carried out. This algorithm finds the alignment between 2 sequences (DNA bases or amino acids) that provides the maximum score. The scoring is allocated using a similarity matrix, which increases the score (+1) for similar matches and decreases the score for dissimilar matches (-1). There is also a linear gap penalty applied that affects the score of an alignment. This means that any gap, large or small, carries the same penalty and is therefore biased towards alignments with few gaps. There are various implementations of these algorithms available many of which produce visualisations of the alignments.

Cross species comparisons have proven to be effective for identification of regulatory regions in both *Saccharomyces* (Kellis et al., 2003) and in higher eukaryotes (Wasserman et al., 2000). Genes with high upstream conservation appear to be predominantly transcription factors (Iwama and Gojobori, 2004), especially those involved in developmental processes. However, this only identifies the regions of homology, the function and role of these regions then needs to be identified experimentally. One way to proceed is to try to identify putative transcription factor binding sites (reviewed in Tompa et al., 2005). The methods currently available have

an important limitation, they assume that transcription factors bind independently and therefore ignore the proximity of other putative binding sites. The other main limitation to the currently available methods is that they assume all of the identified transcription factor binding sites are functional. This can only be concluded after experimental analysis.

To overcome this obstacle, new approaches are being developed that identify motifs that are common to genes regulated in a similar manner (Donaldson and Gottgens, 2006; Wang and Stormo, 2005). This allows the identification of gene networks without needing prior knowledge about any genes identified. It is hoped this will provide a better understanding of the regulatory transcriptional controls that lead to differential gene expression. These systems can be applied to large eukaryotic genomes, however the regulatory motifs may be positioned further away from the gene of interest compared to the genomes of lower eukaryotes.

1.5.4 Aims

The gene chosen for promoter analysis in this study was *Nkx2.2*. It codes for a homeodomain containing protein, which requires a high concentration of Shh for expression. It is expressed in the developing spinal cord, hindbrain, forebrain (Price et al., 1992) and in the most ventral domain of progenitors and is repressed by Pax6 (Fig. 1.3). *Nkx2.2* mutant mice show a loss of V3 interneurons and a concomitant increase in MNs - a ventral to dorsal transformation (Briscoe et al., 1999).

Recent experimental evidence has provided further clues to how *Nkx2.2* is regulated. Chick neural explant experiments showed that induction of *Nkx2.2* was delayed in response to Shh signalling, and its expression was only consolidated by prolonged exposure to Shh (E. Dessaud personal communication). These data raise further questions that can potentially be answered with a promoter analysis.

The aim of this project is to analyse the promoter regions of the Nkx2 genes that respond to Shh and to isolate regulatory regions that direct specific neural expression and/or confer ventral patterning. The questions to be answered are:

- Is the requirement for Shh direct for *Nkx2.2* expression?
- How does Pax6 repress *Nkx2.2* expression?
- Why is *Nkx2.2* induction delayed?
- Why does *Nkx2.2* expression require prolonged exposure to Shh?

To answer these questions, a combination of *in silico* analysis and *in vivo* analysis in mouse, chick and zebrafish were performed. *In vivo* analysis was carried out through the production of both large (BAC) and small (plasmid) reporter constructs.

Figure 1.1 Diagrams of morphogen gradients and their patterning of developing tissues.

A Bicoid (Bcd) is a maternal protein laid down in the developing *Drosophila* embryo, which sets up an anterior to posterior concentration gradient. It is responsible for determining the expression patterns of the downstream Gap genes, required for the correct patterning of the anteroposterior axis. *orthodenticle* is expressed at high Bcd concentrations and *hunchback* at low levels. **B** A concentration gradient of Decapentaplegic (Dpp) is set up at the anterior posterior compartment border of the *Drosophila* wing disc. Target gene *spalt (sal)* is expressed at high concentrations of Dpp, with *optomotor blind (omb)* expressed at both high and low levels of Dpp. **Ci** A ventral to dorsal gradient of the protein Dorsal (Dl) is set up in the *Drosophila* embryo. This determines the formation of different tissue types in the dorsoventral axis by activating target genes at different concentrations; *dpp* (*decapentaplegic*), *zen* (*zerknüllt*), *sog* (*short gastrulation*), *rho* (*rhomboid*), *sna* (*snail*) and *twi* (*twist*). **Cii** The gradient is intracellular, binding of the ligand Spatzle to the ubiquitous receptor Toll, leads to degradation of the Cactus/Dorsal complex and Dorsal translocates to the nucleus to activate downstream genes. **D** A gradient of Activin is set up in the *Xenopus* embryo from a region named the Nieuwkoop centre. This gradient activates the gene *Xgsc* (*Xenopus goosecoid*) at a higher concentration than *Xbra* (*Xenopus brachyury*), which are required for formation of the dorsal and lateral mesoderm respectively. Diagrams are based on published figures (Ashe and Briscoe, 2006; Gurdon and Bourillot, 2001).

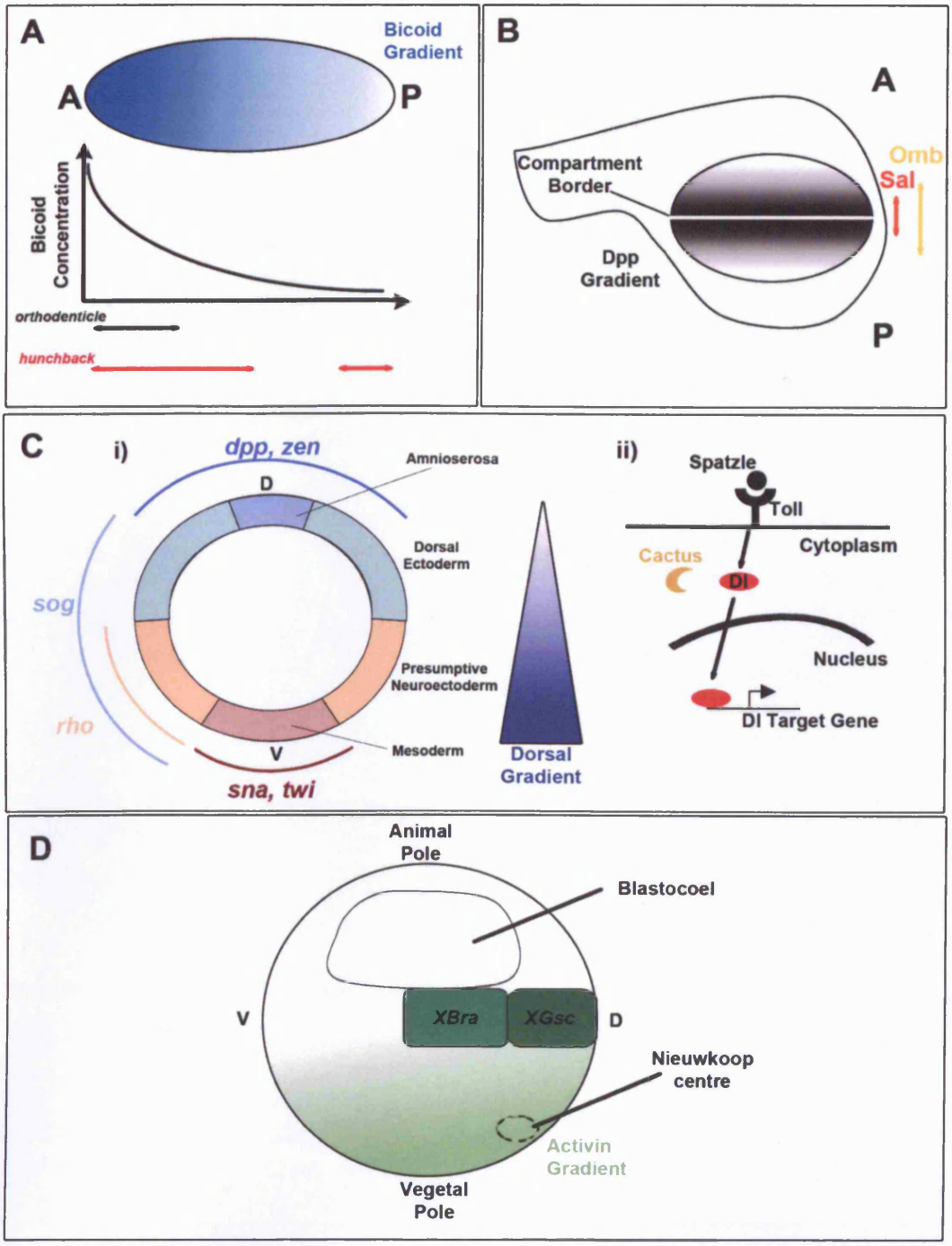

A
Bicoid Gradient
A
P
Bicoid Concentration
orthodenticle
hunchback
B
A
Compartment Border
Omb
Sal
Dpp Gradient
P
C
i)
dpp, zen
Amnioserosa
D
Dorsal Ectoderm
sog
Presumptive Neuroectoderm
rho
V
Mesoderm
sna, twi
Dorsal Gradient
ii)
Spatzle
Toll
Cytoplasm
Cactus
Dl
Nucleus
Dl Target Gene
D
Animal Pole
Blastocoel
V
XBra
XGsc
D
Nieuwkoop centre
Activin Gradient
Vegetal Pole

Figure 1.2 Expression pattern of Shh in vertebrate neural tube and its mechanism of patterning the ventral neural tube via a concentration gradient.

A Transverse section of a chick neural tube at stage 18, immunostained for Shh. Shh protein was observed in the notochord and floor plate. **B** The expression of *Shh* in the notochord (N) and floor plate sets up a protein concentration gradient in the ventral neural tube (light blue, left). At particular thresholds of Shh *in vivo*, 5 distinct neuronal subtypes differentiate (V0, V1, V2, MN and V3, left). This differentiation can be recreated *in vitro* in neural explants, with 2-3 fold increases in Shh concentrations (right). Each of the 5 neuronal subtypes is identified by specific molecular markers (Fig. 1.3). [Images **A** and **B** kindly contributed by James Briscoe].

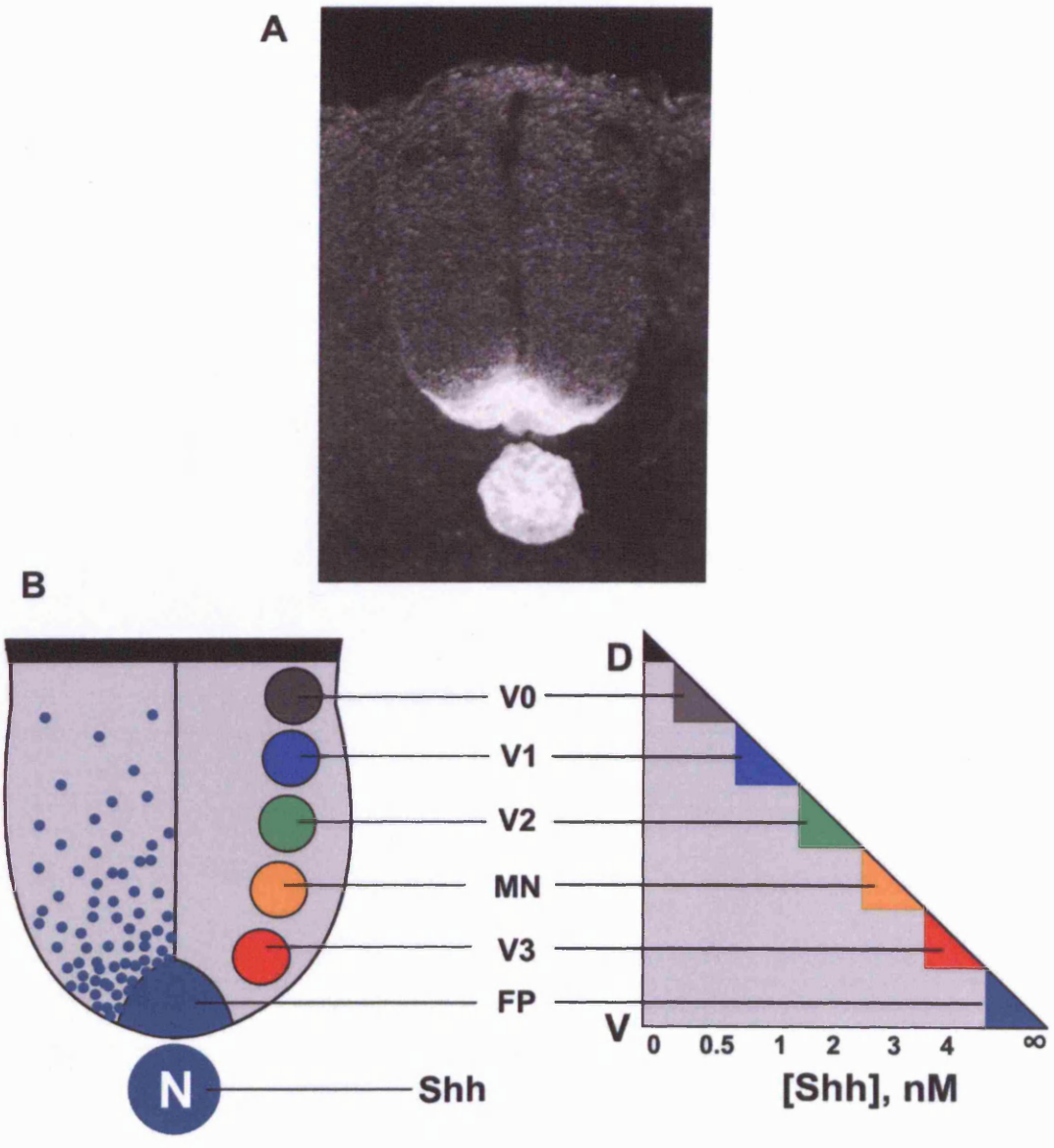

A
B
D
V0
V1
V2
MN
V3
FP
V
0
0.5
1
2
3
4
∞
N
Shh
[Shh], nM

Figure 1.3 Transcription factor code that patterns the ventral neural tube.

A Downstream of Shh, transcription factors are grouped into two classes depending on their response to Shh; Class I genes are inhibited by Shh and Class II genes are activated. Cross repression between these genes leads to a sharpening of borders of expression. The combinatorial expression of transcription factors determines the progenitor domain formed, which then dictates the neuronal fate of the cell. **B** A summary of the expression patterns of transcription factors expressed in the mouse neural tube in progenitor cells (left). Molecular markers indicative of specific post-mitotic neurons in the ventral neural tube (right). [Image **A** kindly contributed by James Briscoe, **B** based on published figure (Persson et al., 2002)].

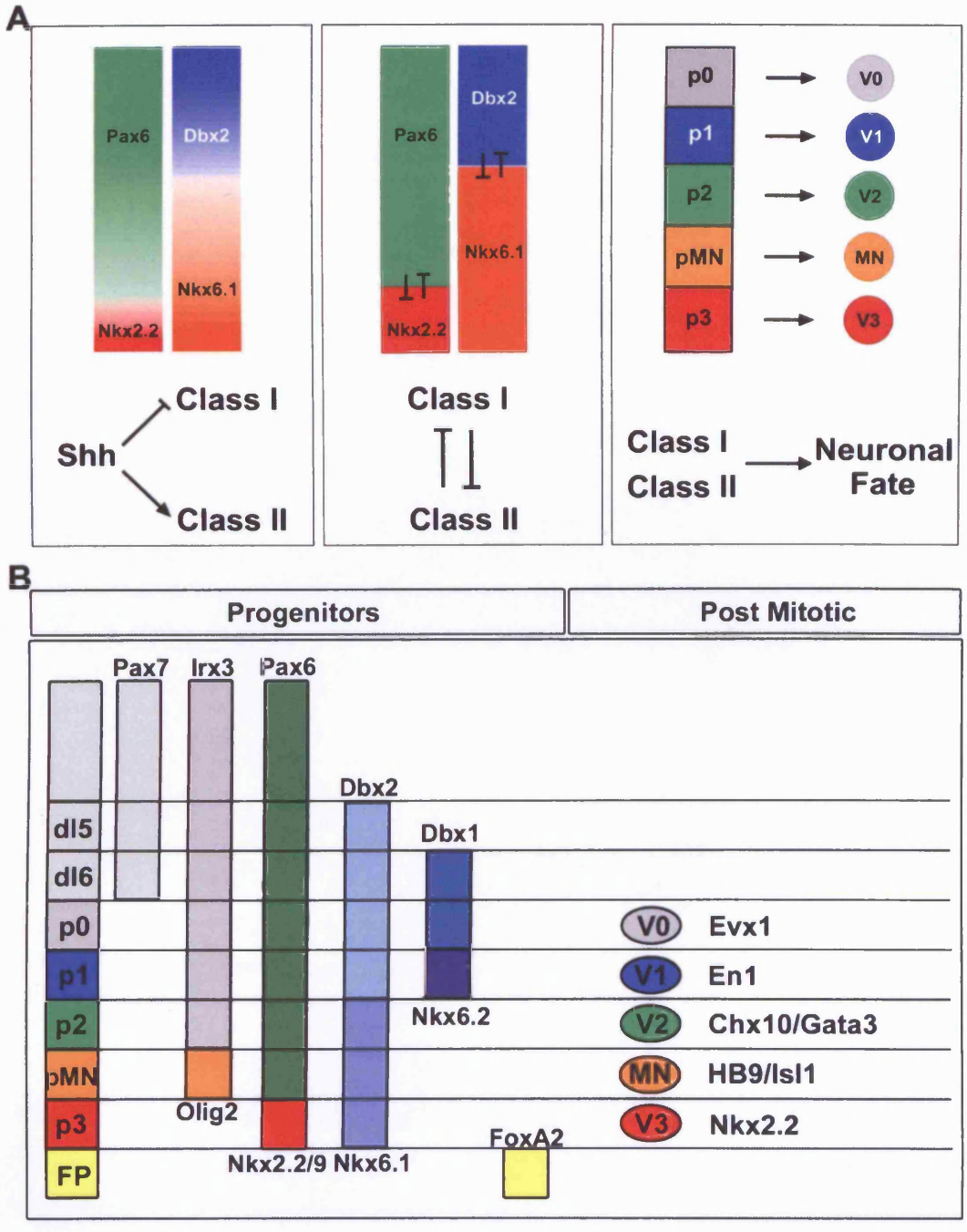
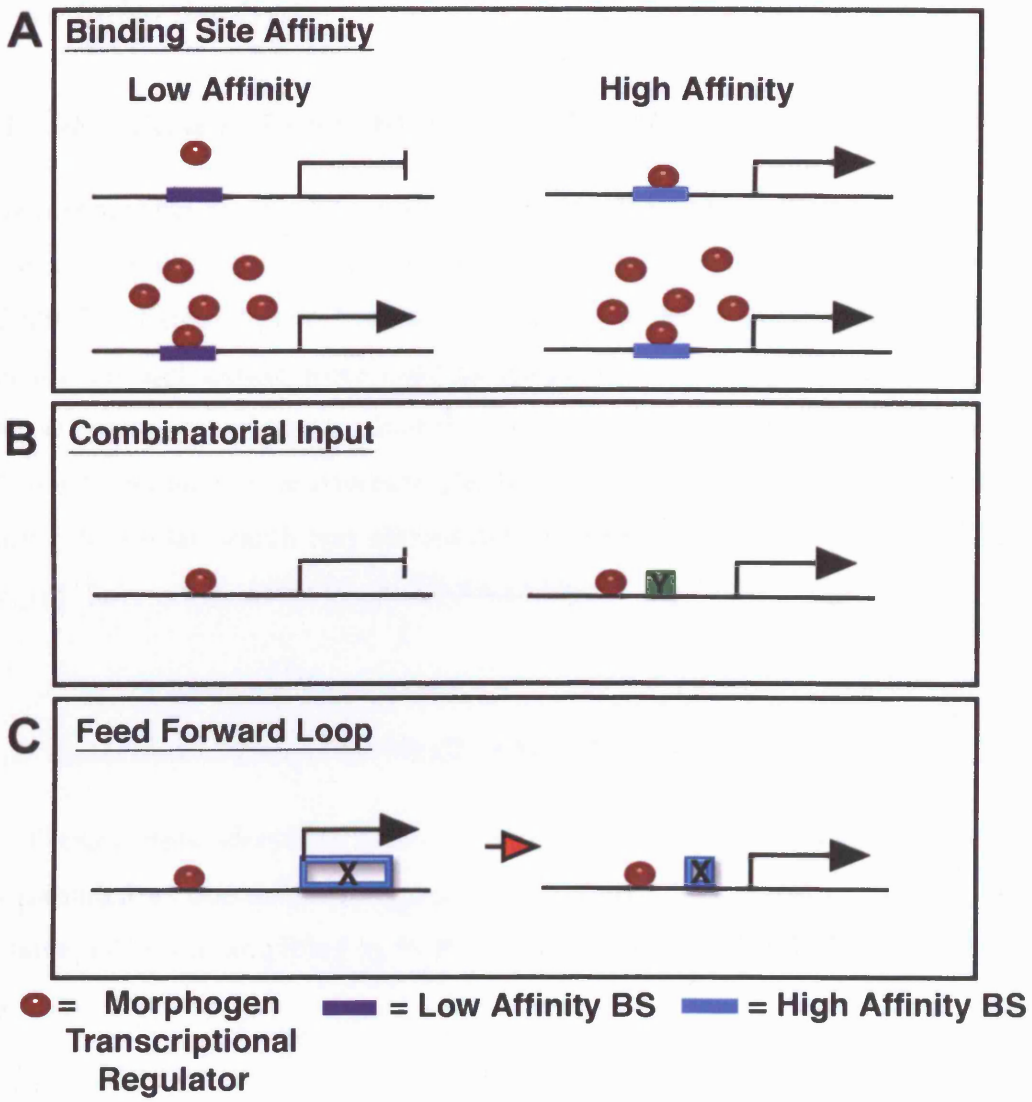

A
Pax6
Dbx2
Nkx6.1
Nkx2.2
Shh
Class I
Class II
Class I
Class II
p0
p1
p2
pMN
p3
V0
V1
V2
MN
V3
Class I
Class II
Neuronal Fate
B
Progenitors
Post Mitotic
Pax7
Irx3
Pax6
Dbx2
Dbx1
dI5
dI6
p0
p1
p2
pMN
p3
FP
Olig2
Nkx2.2/9
Nkx6.1
Nkx6.2
FoxA2
V0 Evx1
V1 En1
V2 Chx10/Gata3
MN HB9/Isl1
V3 Nkx2.2

Figure 1.4 Mechanisms used to interpret graded signals.

Three possible mechanisms used by cells to control differential gene expression in response to extracellular graded signals. **A** Binding site affinity. Genes containing high affinity binding sites (BS) can be activated by low concentrations of the morphogen activated transcription factor. However, enhancers containing low affinity binding sites require higher concentrations of the transcription factor for gene activation. **B** Combinatorial input. The activation of a target gene may require the morphogen activated transcription factor, however in addition, a second transcription factor (Y) may be required. **C** Feed forward loop. The activation of a target gene may require the interaction of two transcription factors, both activated by the same morphogen. The morphogen transcriptional regulator may activate gene X. Then transcription factor X may work in concert with the transcriptional regulator to activate another gene. Figure based on published diagrams (Ashe and Briscoe, 2006).


A
Binding Site Affinity
Low Affinity
High Affinity
B
Combinatorial Input
Y
C
Feed Forward Loop
X
X
= Morphogen Transcriptional Regulator
= Low Affinity BS
= High Affinity BS

2 Materials and Methods

2.1 In Silico Analysis

2.1.1 Identification of Zebrafish and *Fugu* Nkx2 Genes

Known sequences of *Nkx2.1* (Accession numbers; BC006221, BC057607), *Nkx2.2* (Accession Numbers; BC075093, NM_010919), *Nkx2.4* (Accession Numbers; AF202037, AF202038) and *Nkx2.9* (Accession Numbers; BC041090, NM_008701) from human and mouse were used as templates to BLAST search the Ensembl database (www.ensembl.org) zebrafish genome, assembly Zv6. Reciprocal searches with the Ensembl mouse database (NCBI 36 Assembly) further confirmed these results. A similar search was carried out to identify genes in the *Fugu* (*Takifugu rubripes*) Ensembl database (Fugu 4.0 Assembly).

2.1.2 Selection of Zebrafish Nkx2 *In Situ* Probes

EST Clones were identified from the Ensembl Zebrafish Assembly Zv6, which corresponded to untranslated regions (UTR) of the Nkx2 genes. If no EST was available, DNA was amplified by PCR from genomic Zebrafish DNA (see Table 1 for details).

2.1.3 Identification of Zebrafish BAC for Homologous Recombination

BAC ends that mapped to chromosome 17, close to *Nkx2.2a* were identified. From these BACs, any which encompassed the entire *Nkx2.2a* gene and contained a large fragment of 5' (upstream) DNA were isolated. zK257G4 was chosen, which maps to position 41192800-41285800 in genome assembly Zv4 (release date October 2004).

2.1.4 Extracting Non-Coding DNA and Alignment

Nkx2.2 and *Nkx2.9* genes from human, mouse, zebrafish and *Fugu* genomes were identified. 9kb of DNA 5' of genes was exported in FASTA format. DNA was uploaded into Multipipmaker (http://pipmaker.bx.psu.edu/cgi-bin/multipipmaker), which aligned the DNA using a BLASTZ algorithm. To identify putative transcription factor binding sites, MatInspector (http://www.genomatix.de/products/MatInspector/index.html), which identifies possible consensus sites using a library of matrices, was used.

2.2 General Molecular Biology Techniques

2.2.1 Transformation of Chemically Competent Bacteria

Plasmid DNA prepared from ligations was transformed into chemically competent DH5-α *E.coli* bacteria. The bacteria were made competent using the protocol available at the following site: http://bioprotocol.bio.com/protocolstools/protocol.jhtml?id=p386. Up to 500ng of DNA was added to a 100µl aliquot of competent bacteria. This was incubated on ice for 30 mins followed by a heat shock at 42°C for 1 minute and returned to the ice for 2 minutes. 1 ml of LB (Luria-Bertani Broth; 10g/l Tryptone, 5g/l Yeast Extract, 10g/l NaCl, pH 7.0) was added and the mixture was incubated at 37°C for 1 hour. The bacteria was plated onto LB agar plates (LB broth + 15g/l agar) containing a selective antibiotic (0.1mg/ml ampicillin, 50µg/ml kanamycin). Plates were incubated overnight at 37°C.

2.2.2 Plasmid DNA Preparation

For small-scale plasmid DNA preparation the Quantum Prep Plasmid Miniprep Kit (Biorad) was used. For large-scale preparations, the HiSpeed Plasmid Maxi Kit (Qiagen) was used, according to manufacturers' guidelines.

2.2.3 DNA Quantification

DNA quantification was performed by spectrophotometry using the ND-1000 Nanodrop® (LabTech). This takes readings at $\lambda = 260$nm at which an optical density (OD) reading of 1 corresponds to 50μg/ml of double stranded DNA. The purity of the DNA was also measured by calculating the OD_{260}/OD_{280} ratio. A ratio of 1.8 indicated a pure preparation.

2.2.4 Gel Electrophoresis

Gel electrophoresis was carried out to determine nucleic acid size and for separation and purification of nucleic acid products. Gels were prepared by dissolving 1-2% (w/v) agarose, depending on size of DNA to be resolved, in 1X TAE (20mM TRIS acetate, 1mM $Na_2EDTA.2H_20$, pH 8.5) with 0.5mg/ml ethidium bromide. Samples were mixed with 6X Buffer (6X TAE, 50% v/v Glycerol, 0.2% w/v bromophenol blue) and loaded onto the gel alongside a 1kb ladder (Invitrogen) and run at 5-20V/cm gel length. Nucleic acids stained by ethidium bromide were visualised with a UV lamp ($\lambda \approx 302$nm).

2.2.5 PCR and Gel Band Purification

DNA purification from PCR reactions and after band isolation from an agarose gel was carried out using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to manufacturers' guidelines.

2.2.6 DNA Modification

For the following DNA modifications, the enzymes used are listed:

- Digestion of DNA - Restriction enzymes (Roche and NEB)
- Blunting of dsDNA- DNA Polymerase I Large (Promega)
- Ligation of DNA - T4 DNA Ligase (NEB)
- De-phosphorylation - Shrimp alkaline phosphatase (Roche)

Conditions for use of enzymes were according to manufacturers' guidelines.

2.2.7 Southern Blotting

DNA digestion with appropriate restriction enzymes was carried out overnight. Digested DNA was run on a 1% agarose gel until the DNA had run the full length of the gel. The gel was visualised using a UV lamp and photographed with a ruler to ensure the positions of the MW marker could be located on the blot. The agarose gel was washed with distilled water and then washed with a depurination solution (0.125M HCl) until the bromophenol blue in the loading buffer had turned from blue to yellow. The gel was then washed in a denaturation solution (0.4M NaOH) until the bromophenol blue had returned to its original colour.

A capillary blotting technique was used to transfer the nucleic acid to an N^+ Hybond membrane (Amersham Biosciences), the denaturation solution was used as the transfer buffer. The transfer occurred overnight and the membrane was then washed twice for 5 minutes in 5X SSC to remove any excess agarose. The membrane was then washed in Church's Buffer (7% SDS, 1% BSA, 1mM EDTA, 0.25M Phosphate buffer) at 65°C to pre-hybridise the membrane.

Radiolabelled probe was made using the Rediprime™ II DNA Labelling system (Amersham Biosciences) and Redivue α-^{32}P dCTP (Amersham Biosciences) according to manufacturers' guidelines and purified using Microspin S-200 HR Columns (Amersham Biosciences). Purified probe was added to an appropriate volume of Church's buffer, in which the membrane was left to hybridise overnight at 65°C. The membrane was washed in a solution of 0.2X SSC and 0.2% SDS and monitored until background levels were almost undetectable. Membranes were stored at -80°C with X-ray film in autoradiography cassettes with intensifying screens before developing the films. This step was repeated until correct exposure time was identified.

2.2.8 Isolation and Purification of Wild Type Zebrafish Genomic DNA

Embryos were incubated to 24hpf and pooled, 20-30 embryos per extraction. Embryos were digested in 1ml extraction buffer (0.5% SDS, 0.1M EDTA, 10mM TRIS-HCl pH 8.0, 0.1mg/ml Proteinase K) for 4-5 hours at 55°C with occasional agitation. To extract genomic DNA, 1ml phenol/chloroform/isopropyl alcohol (25:24:1) mixture was added, the sample was vortexed and centrifuged for 5 mins at 13000rpm. The aqueous layer was removed and mixed with 1ml phenol/chloroform/isopropyl alcohol (25:24:1) mixture. This extraction step was repeated 3 times. The final aqueous layer was mixed with 1.5ml 100% ethanol and stored at -70°C for 1 hour to precipitate the DNA. Centrifugation at 13000rpm for 30 mins at 4°C pelleted the DNA. The pellet was washed with 70% ethanol before being resuspended in dH_2O and stored at -20°C.

2.2.9 Isolation and Purification of Mouse Genomic DNA

Tail or ear clippings from mice were digested and genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen) following manufacturers' guidelines.

2.2.10 Synthesis of Riboprobes for *In Situ* Hybridisation

RNA probes were constructed from plasmid DNA (Table 1) that was linearised using the appropriate enzyme for at least 2 hours. DNA was run on an agarose gel and the appropriate DNA band was cut and DNA extracted using purification kit (see above). *In vitro* RNA transcription was performed on linearised DNA at 37°C for 2 hours using; 1X DIG-RNA labelling mix (Roche), 1x transcription buffer (Roche), 60 Units RNase inhibitor (Roche), 40 units RNA (T3, T7 or SP6) polymerase (Roche). Probes were then treated with 20 units DNase I (Roche) at 37°C for 15 minutes to remove DNA template and were purified by size-exclusion chromatography through a DEPC water column (Clontech Chroma Spin-100). A list of the RNA probes used can be found in Table 1.

2.3 BAC Manipulation

2.3.1 BAC DNA Preparation for Electroporation into Recombinant *E.coli*

For large-scale preparations of BAC DNA, the Large-Construct Kit (Qiagen) was used according to manufacturers' guidelines with the following modifications to increase DNA yield. Elution from column was carried out with elution buffer heated to 65°C and final precipitation of DNA was performed using glass centrifuge tubes instead of plastic. BAC zK257G4 contains a chloramphenicol resistance gene, BAC was grown in LB + 12.5μg/ml chloramphenicol.

To electroporate the BAC into the EL250 recombinant *E.coli* strain, a single colony was used to inoculate 50ml LB (+ 12.5μg/ml chloramphenicol), which was grown at 32°C overnight shaking at 200rpm. The overnight culture was cooled on ice for 10 minutes and the cells were collected by centrifugation at 4000rpm for 5 minutes, supernatant was removed and cells were washed with ice-cold sterile dH_2O. Centrifugation was repeated and 3 further washes were performed. The cells were resuspended in 200μl ice-cold sterile dH_2O. 50μl aliquots of cells were mixed in 0.1cm gap electroporation cuvettes with 100ng BAC DNA in a volume of 2μl. DNA was electroporated in the following conditions: 1.75kV, 25μF and 200Ω with an expected time constant of 4.0 secs. 1ml of LB was added immediately after pulse was applied and the mixture was incubated at 32°C for 1.5 hours before plating onto LB + 12.5μg/μl chloramphenicol plates. Plates were incubated at 32°C overnight for up to 24 hours for colonies to grow.

2.3.2 Generation of Targeting Constructs

Targeting Vector 1 : used to insert Venus into exon 1 of *Nkx2.2a*.

Venus DNA was amplified from pVenus-N1 (Nagai et al., 2002) using primers 7 and 8 (Table 2) and PCR conditions 2 (Table 3). Product was ligated into pCRII-TOPO Blunt (Invitrogen) and digested EcoRI x SmaI. This fragment (approx. 1kb) was ligated directionally into pIGCN21 (Lee et al., 2001) digested with EcoRI and SmaI which replaces eGFPcre fusion protein. Homology arms were amplified from

zK257G4 BAC DNA to ligate into the targeting vector. The upstream homology arm was amplified using primers 9 and 10 (Table 2) under PCR conditions number 3 (Table 3). The ~600bp product was ligated into pCRII-TOPO Blunt, digested with SalI and ligated into the SalI site upstream of Venus in pIGCN21-Venus. The downstream homology arm was amplified using primers 11 and 12 (Table 2) under PCR conditions number 2 (Table 3). The ~500bp product was ligated into pCRII-TOPO Blunt, digested with SfiI and ligated into the SfiI site downstream of Venus in pIGCN21-Venus.

Targeting Vector 2: Used to delete CNCR from zNkx2.2aVenus BAC

The FRT_5-Neo-FRT_5 (Schlake and Bode, 1994) cassette was amplified from pIGCN21, introducing the change in spacer sequence from FRT_0 to FRT_5 with the PCR primers. This fragment was amplified using primers 13 and 14 (Table 2) with PCR conditions Number 4 (Table 3). The product was ligated into pCRII-TOPO Blunt. Upstream homology arm was amplified from zK257G4 BAC DNA using primers 15 and 16 (Table 2), using PCR conditions number 2 (Table 3). The PCR product was ligated into pCRII-TOPO Blunt and digested with KpnI and SacI and ligated into the pCRII-TOPO Blunt containing FRT_5-Neo-FRT_5, digested with KpnI and SacI. The downstream arm was treated in the same manner, but was amplified using primers 17 and 18 (Table 2) and was digested with NotI and XhoI.

2.3.3 BAC Recombination

Electroporation of targeting cassettes for recombination was carried out using linear fragments of DNA. Targeting vector 1 was digested with XhoI and SacII, targeting vector 2 was digested with KpnI and XhoI.

A colony of EL250 bacteria containing BAC of interest was grown overnight at 32°C in LB (+ 12.5μg/ml chloramphenicol) shaking at 200rpm, this culture was diluted into 25ml LB at 1:50 and grown for a further 2-3 hours until the OD_{600} reached 0.5-0.7. Culture was then transferred to 42°C and shaken for a further 15 minutes.

Culture was transferred to ice, cells were collected, washed and electroporated as previously described (Section 2.3.1). However, only 10ng of linear DNA in a volume of 2μl was electroporated.

To recombine FRT sites, a single colony of bacteria containing correctly targeted BAC DNA was cultured in 5ml LB (+ 12.5μg/ml Chloramphenicol and 50μg/ml Kanamycin) at 32°C shaking at 200rpm. Overnight culture was diluted 1:10 with 10ml LB (+ 12.5μg/ml chloramphenicol only) until OD_{600} = 0.5. 0.1% L-(+)-Arabinose was added to the culture and was incubated for a further hour at 32°C. The culture was then diluted to 10^{-5} with LB and then plated onto LB plates (+ 12.5μg/ml chloramphenicol) and grown overnight at 32°C.

2.3.4 Assaying BAC Recombination

For small-scale preparations to test for correct recombination, 6ml LB (+ 12.5μg/ml chloramphenicol for non recombined BACs or 12.5μg/ml chloramphenicol + 50μg/ml kanamycin) was inoculated with a single colony. The culture was grown overnight at 37°C (for DH10B cells) or 32°C (for EL250 cells). Cells were collected by centrifugation at 13000rpm for 1 minute (a small volume of culture was saved for inoculation of further cultures). The pellet was resuspended in 100μl Resuspension buffer P1 (Qiagen). 200μl of Lysis buffer P2 (Qiagen) was added and solution was mixed by inversion. 150μl of ice-cold Neutralisation buffer P3 (Qiagen) was added and again solution was mixed by inversion. The whole mixture was centrifuged at 13000rpm for 10 minutes at room temperature. The supernatant was collected and mixed with an equal volume of phenol/chloroform/isopropyl alcohol (25:24:1) by inversion. The solution was centrifuged for 15 minutes at 13000rpm and the aqueous layer was removed. This was mixed with 1ml 100% ethanol and incubated at -70°C for 1 hour. The DNA was collected by centrifugation at 13000rpm for 20 minutes at 4°C, and the pellet was washed with 70% ethanol and resuspended in dH_2O.

To test for correct targeting and recombination, both southern blot analysis and PCR were utilised. For the first recombination, the 3' homology arm was used as a probe,

this was digested from the pCRII TOPO Blunt plasmid using EcoRI. For the second homologous recombination step, Venus was used as a probe, this was amplified by PCR as previously described. For the PCR to check the second homologous recombination step, primers 19 + 20 were used (Table 2) with PCR conditions number 1 (Table 3).

2.4 Creating Plasmid Promoter Reporter Constructs

2.4.1 Amplification of Genomic DNA and Plasmid Construction

Zebrafish promoter reporter plasmids were prepared by PCR amplification using condition number 2 (Table 3) from BAC DNA, ligated into pCRII-TOPO Blunt in any orientation, the SalI hsp68LacZ reporter fragment (Kothary et al., 1989; Logan et al., 1993) was ligated into the XhoI site (5' to 3'). Mouse constructs were made in a similar manner, however the DNA template used was genomic DNA, the PCR conditions were number 1 (Table 3) and the plasmid was pCRII-TOPO.

Details of primers (Table 2) for amplification are as follows.

Mouse promoter constructs:

mNkx2.9$^{CNCR+Prom}$LacZ	primers 21 and 22
mNkx2.2$^{CNCR+Prom}$LacZ	primers 23 and 24

Zebrafish reporter constructs:

zNkx2.2$^{CNCR+Prom}$LacZ	primers 25 and 26
zNkx2.2$^{CNCR+Prom}$LacZ-2	primers 27 and 28

Zebrafish deletion constructs based on zNkx2.2$^{CNCR+Prom}$LacZ:

zNkx2.2^{CNCR}LacZ	primers 25 and 29
zNkx2.2$^{\Delta CNCR+Prom}$LacZ	primers 30 and 26

zNkx2.2Δ1$^{CNCR+Prom}$LacZ	primers 31 and 26
zNkx2.2Δ2$^{CNCR+Prom}$LacZ	primers 32 and 26
zNkx2.2Δ3$^{CNCR+Prom}$LacZ	primers 33 and 26
zNkx2.2Δ4$^{CNCR+Prom}$LacZ	primers 34 and 26
zNkx2.2Δ5$^{CNCR+Prom}$LacZ	primers 35 and 26
zNkx2.2Δ6$^{CNCR+Prom}$LacZ	primers 36 and 26

2.4.2 Site Directed Mutagenesis

Construct zNkx2.2$^{CNCR+Prom}$LacZ was subjected to site-directed mutagenesis using the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) to mutate the Gli binding site. The manufacturers' guidelines were adhered to with the following alterations to the PCR; 100ng template DNA was used, annealing temperature was increased to 60°C, elongation time was increased to 8 minutes per cycle and 25 cycles were performed. PCR primers were PAGE purified, primers 37 and 38 (Table 2) were used to introduce the mutation.

2.4.3 Purifying DNA for Mouse Pronuclear Injection

Linear DNA used for pronuclear injection was isolated from the plasmids using NsiI, which isolated the promoter and the reporter on one fragment of DNA. The digested DNA was run on an agarose gel, the correct band was excised and DNA purified, it was resuspended into an injection buffer (10mM TRIS, pH 7.4, + 0.1mM EDTA) and injected at 2ng/μl.

2.4.4 Mouse Genotyping

Purified genomic DNA from mice resulting from pronuclear injection was tested by PCR for the presence of *LacZ*. This was performed using primers 39 and 40 (Table 2) and PCR conditions number 5 (Table 3). A band of approximately 300bp was observed if a *LacZ* transgene was present in the genomic DNA.

2.5 Embryo Manipulation

2.5.1 Zebrafish Embryo Incubation and Harvesting

Zebrafish embryos were collected within 15 minutes of fertilisation and incubated at 28°C in embryo water (red sea salt 0.03g/l, methylene blue 2mg/l). Embryos were staged according to published criteria (Kimmel et al., 1995).

Zebrafish embryos collected for *in situ* hybridisation were fixed in 4% PFA in PB [0.1M Phosphate Buffer] for a minimum period of 24 hours to a maximum of 72 hours at 4°C. Following fixation, embryos were dehydrated with 100% methanol. Embryos were stored in 100% methanol at -20°C until required. For immunohistochemistry, embryos were fixed for 2 hours in 4% PFA at room temperature and either stored in PBST (PBS + 0.1% Triton X-100) at 4°C for up to 2 days or were dehydrated with methanol as previously described, and stored at -20°C. Embryos for X-Gal staining were dechorionated and fixed in 4% PFA with 0.5% glutaraldehyde for 15 minutes at room temperature and stained immediately.

2.5.2 Cyclopamine Treatment of Zebrafish Embryos

Embryos (with chorion intact) were placed in 10μM cyclopamine (dissolved in 100% ethanol) in embryo water at between the 1 and 4 cell stage of development. The embryos were grown up until 24 hours of development at 28°C. In negative control experiments, the same volume of 100% Ethanol was added to embryo water and the embryos were incubated for the same length of time.

2.5.3 Zebrafish Embryo Injection

Embryos were collected in embryo water within 15 minutes of fertilisation and embryos still at the 1 cell stage were isolated. These embryos were lined up against a microscope slide and as much liquid as possible was removed, ensuring the embryos

were fixed in place. Needles were pulled from glass capillaries containing a filament (1mm outside diameter 10cm long). These needles were used to inject 1nl of DNA (diluted to required concentration with Fish Injection Buffer: 0.2M KCl and 5mM HEPES pH 7.3) directly into the single cell. Once the embryos were injected, they were incubated in embryo water at 28°C until required.

2.5.4 Chick Electroporation and Embryo Harvesting

Electroporation of chick embryos was carried out at stages HH 10-12 (Hamburger and Hamilton, 1953) following a published protocol (Briscoe et al., 2000). DNA was diluted accordingly and 5% Fast Green was included in the DNA mixture. Embryos were incubated at 37°C for 24-48 hours. Embryos were then dissected and fixed in ice cold 4% PFA for 30 minutes for X-Gal staining and 1 hour for immunohistochemistry. Fixed embryos for immunohistochemistry and *in situ* hybridisation were equilibrated with 30% sucrose (in 0.1M PB) before mounting in O.C.T compound (BDH) and frozen on dry ice. Embryos were subsequently stored at -80°C. Chick electroporations were kindly performed by Anita Mynett.

2.5.5 Mouse Pronuclear Injection and Embryo Harvesting

Mouse embryos were harvested from and received by (CBA/Ca x C57BL/10) F1 at E0.5 dpc. Injections were carried out as previously described (Hogan et al., 1994). Transient embryos were harvested at stage required. Embryos were fixed with ice cold 4% PFA for 30 minutes for LacZ staining and for 1 hour for immunohistochemistry and *in situ* hybridisation. All mouse pronuclear injections were kindly performed by Sophie Wood (Procedural Services).

2.6 Visualising and Analysing Embryos

2.6.1 Zebrafish Wholemount *In situ* Hybridisation

Whole-mount *in situ* hybridisations were performed as previously described (Thisse et al., 1993) with some modifications. Zebrafish embryos were rehydrated in decreasing

concentrations of methanol (75%, 50% and 25%) in PBT (PBS with 0.1% Tween 20) and then transferred to PBT (2 x 5min). At this point the embryos were dechorionated in PBT. The embryos were then re-fixed in 4% PFA (in 0.1M Phosphate Buffer) for 20 minutes at room temperature and then washed in PBT. After fixation, embryos were transferred to hybridisation buffer (HB: 50% formamide, 5xSSC (pH 7.0), 0.1% Tween-20, 50µg/ml heparin, 500µg/ml type VI torula RNA, 9mM citric acid to pH 6.0-6.5) for 2-5 hours at 68°C. The buffer was then replaced with new HB containing 1µg/ml of DIG-labelled RNA probe and the embryos were incubated at 68°C overnight.

The following washes were performed at 68°C with preheated solutions; 50% HB/2xSSC (5min), 100% 2xSSC (15min) and 100% 0.2xSSC (30min). Washes of 10 minutes each in 50% 0.2xSSC/PBT (twice) and 100% PBT were carried out at room temperature. Embryos were blocked for several hours at room temperature in 2mg/ml BSA and 2% goat/sheep serum in PBT and then incubated overnight at 4°C with alkaline-phosphatase-conjugated anti-DIG antibody Fab fragments diluted 1:2500 in blocking buffer.

On Day 3 embryos were washed with PBT a minimum of 8 times for 15 minutes. The embryos were then rinsed twice (5min) in NTMT (0.1MTris-HCl pH 9.5, 50mM $MgCl_2$, 0.1M NaCl and 0.1% Tween 20). Colour development was performed using NBT/BCIP (Roche ready made tablets; 1 tablet dissolved in 10ml of distilled H_2O). The reaction was stopped with 2mM EDTA in PBS (pH 5.5) and embryos were re-fixed in 4% PFA in PB for 20 minutes at room temperature or overnight at 4°C. Embryos were then taken through a glycerol series (25%, 50%, 75% and 100% glycerol in PBT) and stored at 4°C in 100% glycerol. The yolk cell was mechanically removed from the embryos before photographing. In Figs. 3.4A, 3.4B, 3.10C and 3.10D *in situ* hybridisation was kindly performed by Vanessa Ribes.

2.6.2 Zebrafish Wholemount Immunohistochemistry

If embryos were dehydrated, then a progressive rehydration in 75%, 50%, 25% MeOH/PBST (PBS + 0.1% Tween-20) was performed (5 minutes per wash). Then embryos were washed in PBST (PBS + 0.1% Triton X-100, 3 x 5 mins). If embryos were not dehydrated, these first two steps were ignored.

Embryos were dechorionated in PBST. Embryos were then blocked for 2 hours are room temperature (PBS + 0.1% Triton X-100 + 5% goat serum – heat inactivated). Embryos were incubated overnight with the primary antibody at 4°C (in PBS + 0.1% Triton X-100 + 2% goat serum). Embryos were then washed in PBST (8 x 5 mins) and then incubated with the secondary antibody (in PBS + 0.1% Triton X-100 + 2% goat serum) at room temperature for 2 hours. If required, Phalloidin stain (rhodamine conjugated; Molecular Probes) was diluted (1 in 250) into the secondary antibody mix. Embryos were once again washed with PBST (8 x 5 mins) and then stored in 70% glycerol (in water) at 4°C until required. Yolks cells were mechanically removed before mounting onto slides using Vectashield (Vector Labs) mounting media. If sections were required, embryos were prepared for vibratome sectioning by mounting in molten 10% sucrose and 5% agarose (in water) and allowing mixture to cool and set.

2.6.3 Zebrafish β-Galactosidase Staining

Fixed and dechorionated embryos were washed with PBT at room temperature (4 x 10 minutes). Embryos were washed in staining buffer (2mM $MgCl_2$, 15mM $K_3Fe(CN)_6$, 15mM $K_4Fe(CN)_6$ in PBS) at room temperature for 5 minutes. Then embryos were transferred to staining buffer containing 0.8mg/ml X-gal (dissolved in DMF), then placed at 37°C in the dark until colour developed. The reaction was stopped by washing embryos in PBT at room temperature (3 x 10 mins). Embryos were re-fixed in 4% PFA for 30 minutes at room temperature, then washed in PBT (3 x 5 minutes) at room temperature and stored in 70% glycerol (in PBT) at 4°C. Yolk cells were mechanically removed before photography.

2.6.4 Chick and Mouse β-Galactosidase Staining

Fixed embryos were washed with PBS containing 0.02% NP-40 at room temperature (3 x 20 minutes). Embryos were then transferred to staining solution (5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 5mM EGTA, 0.01% deoxycolate, 2mM $MgCl_2$, 0.1% X-gal in PBS with 0.02% NP-40) and incubated at 37°C in the dark until the colour developed. To stop the reaction the embryos were washed at room temperature in PBS containing 0.02% NP-40 (2 x 5 minutes and 2 x 15 minutes). Embryos were then fixed in 4% PFA overnight at 4°C, followed by washes in PBS at room temperature (3 x 5 minutes). Embryos were prepared for vibratome sectioning by mounting in molten 10% sucrose and 5% agarose (in water) and allowing mixture to cool and set.

2.6.5 Chick and Mouse Immunohistochemistry

Frozen fixed chick and mouse embryos were sectioned at 12μM. Fresh or frozen slides were washed in PBS (3 x 5 mins) and then placed into blocking solution (PBST [PBS + Triton X-100] with 1% BSA) for 15 minutes. Slides were incubated with primary antibodies (Table 4) in blocking solution overnight at 4°C. The following day slides were washed with PBS (3 x 5 mins) and incubated with FITC or Cy3 conjugated secondary antibodies, diluted according to manufacturers' guidelines (Jackson ImmunoResearch Labs) for 2-3 hours in the dark at room temperature. Slides were washed with PBS (3 x 5 mins) and mounted with coverslips using Vectashield with DAPI (Vector Labs).

Immunohistochemistry was also performed on embryos stained for β-galactosidase activity and cryosectioned at 12μM. Slides were bleached (100% EtOH + 0.5% H_2O_2) for 15 mins at 4°C. Slides were washed in PBST (PBS + 0.1% Triton X-100) 3 x 5 mins and blocked (PBST + 1% BSA) for 1 hour at room temperature. Slides were incubated overnight at 4°C with primary antibody in blocking solution. Slides were washed as before and incubated with biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories) diluted in blocking solution for 2-3 hours at room temperature. Slides were washed and incubated with ABC complex (Vector Labs) for 1 hour to bind secondary antibody with avidin-conjugated HRP. Finally slides were

washed and staining was revealed with Fast DAB tablets (Sigma), reaction was stopped with PBST. Slides were post fixed with 4% PFA (in 0.1M PB) and washed with water before mounting with Aquamount (BDH).

2.6.6 Mouse *In Situ* Hybridisation on Sections

Frozen embryos were cryosectioned at 12μM, frozen or fresh slides were refixed at room temperature for 10 minutes in 4% PFA (in 0.1M PB). Slides were washed with PBS (3 x 3 mins) and then acetylated for 10 minutes [1.3% (v/v) triethanolamine, 0.2% (v/v) Conc. HCl, 0.25% (v/v) acetic anhydride (added just before slides) in water]. Slides were washed with PBS (3 x 5 mins) and then prehybridised for 2-5 hours at room temperature with hybridisation buffer [50% (v/v) deionised formamide, 5X SSC, 5X Denhardts, 10mg/ml herring sperm DNA, 10mg/ml bakers yeast RNA] enough to cover the slide. The prehybridisation buffer was replaced with hybridisation mix (200ng of DIG labelled probe/ml hybridisation buffer). The hybridisation mix was heated at 80°C for 5 minutes followed by 5 minutes on ice to denature the probe before applying to the slides. The slides were covered with coverslips and incubated at 70°C overnight in a humidified chamber (5X SSC, 50% formamide).

The following day, slides were washed in 5X SSC at room temperature to wash off the coverslips. The slides were then washed in 0.2X SSC at 70°C for 1 hour, followed by a wash in 0.2X SSC for 5 minutes at room temperature to cool slides down. Slides were transferred to buffer B1 (0.1M Tris pH 7.5, 0.15M NaCl) for 5 minutes at room temperature. Slides were then covered with blocking buffer (B1 + 10% goat serum) and incubated in a humidified chamber (dH_2O) at room temperature for 1 hour. Blocking buffer was then replaced with the antibody solution [1:5000 alkaline phosphatase conjugated anti-DIG antibody (Roche) in buffer B1 + 1% goat serum] and slides were incubated overnight at 4°C in a humidified chamber.

On the final day, slides were washed with buffer B1 (3 x 5 mins) at room temperature. Slides were then transferred to buffer B3 (0.1M Tris.HCl pH9.5, 0.1M NaCl, 50mM

$MgCl_2$) for 5 minutes at room temperature. The stain was developed in the dark using 1ml NBT/BCIP solution [4µl of 100mg/ml NBT in 70% dimethylformamide (Roche) and 4µl of 50mg/ml BCIP in 70% dimethylformamide (Roche) in 1X B3 + 5% PVA]. The colour reaction was stopped by washing slides with dH_2O. Slides were washed (3 x 5 mins) with PBS and then post-fixed (10 minutes) with 4% PFA (in 0.1M PB), followed by further washes with dH_2O (3 x 5 mins). Slides were mounted with coverslips using Aquamount (BDH).

2.6.7 Mouse Wax Sectioning

Mouse embryos stained for β-galactosidase activity were post-fixed and washed in PBS (3x5 mins). Embryos were dehydrated in graded alcohols and cleared in Xylene before embedding in Fibrowax (BDH). Wax blocks were sectioned at 6µM using a rotary microtome. Sections were rehydrated using graded alcohols and washed in distilled water. The slides were subsequently counterstained using haematoxylin and eosin, before a final dehydration and clearing step (using Histoclear) followed by mounting with coverslips using DPX. All wax sectioning and subsequent staining was kindly performed by Elena Grigorieva.

2.6.8 Microscopy and Analysis

Images (using DIC; differential interference contrast) of wholemount embryos or sections were obtained using a Zeiss Axiophot microscope (Axioplan 2 imaging) and an Axiocam HRC Zeiss digital camera using Axiovision software. Fluorescent samples were photographed using the above setup + FluoArc UV lamp. Alternatively a Leica confocal microscope (True confocal scanner Leica TCS SP II) with Leica confocal software was used. All images were subsequently processed with Adobe Photoshop.

2.7 Tables

cDNA	Linearisation	RNA Polymerase	Origin
z*Nkx2.1*	EcoRV	Sp6	Accession Number AF321112 Amplification (zf gDNA): Primers 1 & 2 (Table 2) PCR conditions: No. 1 (Table 3) Cloned into pCRII-TOPO (Invitrogen)
z*Nkx2.2a*	BamHI	T7	Accession Number: BC115166 (Barth and Wilson, 1995)
z*Nkx2.2b*	SalI	Sp6	cDNA clone (Accession Number: AI722890) IMAGE 3722576
z*Nkx2.4a*	HindIII	T3	CDNA clone (Accession Number: AW343688) IMAGE 2640862
z*Nkx2.4b*	EcoRV	Sp6	Accession Number AF253054 Amplification (zf gDNA): Primers 3 & 4 (Table 2) PCR conditions: No. 1 (Table 3) Cloned into pCRII-TOPO (Invitrogen)
z*Nkx2.9*	EcoRV	Sp6	Accession Number DG924560 Amplification (zf gDNA): Primers 5 & 6 (Table 2) PCR conditions: No. 1 (Table 3) Cloned into pCRII-TOPO (Invitrogen)
E.coli LacZ	HindIII	T3	(Huber et al., 2005)

Table 1 Templates for antisense RNA probes used for *in situ* hybridisation.

Primer Number	Primer Sequence 5' to 3'
1	GGCTCCTCGTCTGGTATGAA
2	GGGGACGTCTTCATTGTTGA
3	GGAATGGACGCCAGTAAATC
4	ACTAGGCGACATTTCCTCCA
5	ATTGAAGCTTCGCCAGACTC
6	AGTGCTGGTATGTGGGGAAA
7	GATCGAATTCCACCATGGTGAGCAAGGGCG
8	GATCCCCGGGGCAGTGAAAAAAATGCTTTATTTG
9	GATCGTCGACGTCTGCGACAATAGATAAATGACACC
10	GCTGCACCAGTTTGACAATCCTC
11	GATCGGCCGAGGCGGCCAACGCCTGGAGTGTTAGTGC
12	GATCGGCCGAGGCGGCCCGTAGCCTAGCCGATTCAAC
13	GAAGTTCCTATACCTTTTGAAGAATAGGAACTTCGGAATAGGAACTT CAAGATCCCCCTGGCGAAAGGG
14	GAAGTTACTATTCCGAAGTTCCTATTCTTCAAAAGGTATAGGAACTTCAG AGCGCTTTTGAAGCTCGGAT
15	ACTGGGTACCCTTGGCCCGTCCAGTTCAATG
16	ACTGGAGCTCTTTCAATGGACTATCTCTTCATTCATTTCTG
17	ACTGGCGGCCGCGAAACGTGCAACATTGTCACC
18	ACTGCTCGAGCAATTTATGTAGGTCAATATTTTGG
19	GAAGATTGAGTGAATGACCTAGTGG
20	GGTGTCATTTATCTATTGTCGCAG
21	CATTTTGCCAGAGGCAGAGG
22	AAGGGACAGTGAGCGGTCTG
23	AGAGGCAACAGGCTCTAACG
24	CAGGCTTCCAGTTGGCTTTA
25	GCTAGGTACCGCAGTGAATGCCATGAC
26	GCTAGCTAGCTGGATATTGCGCTACTGCTG
27	CAATTCACTCGCACAAATGG
28	TTTTGGTTCGGGATTAAGGA
29	TAGCCAGTCTTCTGCTCATCC
30	GAAACGTGCAACATTGTCACC
31	GTCTGCAGCTTAGCAATCGG
32	GGAGCCGGACATTTGTCTAC
33	CATTTCCCCCATTGTCTGCAG
34	CGTATTGTACAGGGGCGTC
35	TTGGCCCTTAAATAAATGC
36	GCCGGACAAAAGCTTCC
37	GTGTTTGCCCGGGTTCGAAGTGGGAGGATGAGCAGAAGACTGGC
38	GCCAGTCTTCTGCTCATCCTCCCACTTCGAACCCGGGCAAACAC
39	GCACATCCCCCTTTCGCCAGCTGGCGTAAT
40	CGCGTCTGGCCTTCCTGTAGCCAGCTTTCA

Table 2 Primers used for PCR.

Condition No.	Reaction Mixture	Thermal Cycles
1	1X Mastermix (AB Gene) 150ng/ul Primers 100ng Template DNA	x1 - 94°C 2 mins x35 – [94°C 30 secs] [60°C 30 secs] [72°C 30 secs] x1 - 72°C 30 secs
2	1X High Fidelity PCR Mastermix (Roche) 150ng/µl Primers 100ng Template DNA	x1 - 94°C 2 mins x10 – [94°C 10 secs] [60°C 30 secs] [72°C 2 mins] x20 – [94°C 30 secs] [60°C 30 secs] [72°C 2 mins +5secs/cycle] x1 - 72°C 7 mins
3	1X High Fidelity PCR Mastermix (Roche) 150ng/µl Primers 100ng Template DNA	x1 - 94°C 2 mins x10 – [94°C 10 secs] [60°C 30 secs - 0.5°C/cycle] [72°C 2 mins] x20 – [94°C 30 secs] [60°C 30 secs -0.5°C/cycle] [72°C 2 mins +5secs/cycle] x1 - 72°C 7 mins
4	1X High Fidelity PCR Mastermix (Roche) 150ng/µl Primers 100ng Template DNA	x1 - 94°C 2 mins x10 – [94°C 10 secs] [60°C 30 secs] [72°C 90 secs] x15 – [94°C 15 secs] [60°C 30 secs] [72°C 90 secs +5secs/cycle] x1 - 72°C 7 mins
5	1X Mastermix (AB Gene) 330ng/ul Primers 1µl purified gDNA	x1 - 94°C 4 mins x35 – [94°C 30 secs] [60°C 30 secs] [72°C 40 secs] x1 - 72°C 10 mins

Table 3 PCR conditions.

Epitope/Antigen	Species	Origin
β-Galactosidase	Goat	Biogenesis
β-Galactosidase	Mouse	Sigma
Chx10	Rabbit	(Liu et al., 1994)
FoxA2	Mouse	(Ericson et al., 1996)
Gata3	Mouse	Santa Cruz
GFP	Rabbit	Invitrogen, Molecular Probes
Lim1/2	Mouse	(Tsuchida et al., 1994)
Lim3	Mouse	(Ericson et al., 1997b)
Mash1	Mouse	(Lo et al., 1991)
Nkx2.2	Mouse	(Ericson et al., 1997b)
Olig2	Rabbit Guinea Pig	(Ligon et al., 2004) (Novitch et al., 2001)
Pax6	Mouse	(Ericson et al., 1997b)
Pax7	Mouse	(Ericson et al., 1996)

Table 4 Primary antibodies used for immunohistochemistry.

Solution	Formulation
1M Phosphate Buffer (PB)	0.6M $Na_2HPO_4.7H_2O$, 0.2M $NaH_2PO_4.H_2O$
1X Phosphate Buffered Saline (PBS)	137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.7H_2O$, 1.4mM KH_2PO_4, pH 7.4
20X Salt Sodium Citrate (SSC)	3M NaCl, 0.3M Na_2citrate.$2H_2O$, adjust pH to 7.0 with 1M HCl
1 x TAE	40mM Tris Acetate, 2mM $Na_2EDTA.2H_2O$, pH 8.5
LB - Luria-Bertani Broth	10g/l Tryptone, 5g/l Yeast Extract, 10g/l NaCl, pH 7.0

Table 5 Formulation of solutions.

3 Results: Nkx2 Gene Clusters and Expression Patterns

3.1 Nkx2 Gene Clustering in Human, Mouse and Fish Genomes

3.1.1 Expression Patterns of Genes Located in Nkx2 Clusters

To study the interpretation of the Shh gradient in the ventral neural tube, the Nkx2 family of homeodomain proteins was chosen. Within the mammalian genome, the *Nkx2.2* and *Nkx2.4* genes are located close to one another in a cluster linked to the *Pax1* and *FoxA2* genes. A paralogous cluster containing *Nkx2.1* and *Nkx2.9* linked to *Pax9* and *FoxA1* is located on a different chromosome (Santagati et al., 2003; Wang et al., 2000b). The close proximity of these genes and the conservation of their physical order and transcriptional direction in human and mouse (Fig. 3.1A, 3.1B), raises the possibility of a shared mechanism of transcriptional regulation.

Nkx2 genes are expressed in ventral neural tissue, suggesting they may be regulated by Shh signalling. The expression of *Nkx2.1* and *Nkx2.4* is anteriorly restricted in the mouse neural tube. *Nkx2.1* is expressed from E9 in the ventral forebrain; diencephalon and ventral telencephalon (Lazzaro et al., 1991; Price, 1993). *Nkx2.4* expression is less well characterised, nevertheless, published data suggests it is expressed more posterior to *Nkx2.1* in the posterior hypothalamus (Price, 1993). The transcription factor *Nkx2.2* is also detected by *in situ* hybridisation from E9 in developing mouse embryos (Price et al., 1992). It is expressed anteriorly in ventral forebrain and floor plate, partially overlapping *Nkx2.1* expression (Price et al., 1992). At spinal cord levels, *Nkx2.2* is expressed in the most ventral neural progenitors of the neural tube (Price et al., 1992). At early developmental time points, *Nkx2.2* expression is detected in the floor plate (Briscoe et al., 1999), but by E10.5 expression shifts dorsally and is restricted to the progenitor domain for V3 interneurons (Fig. 3.1E: Ericson et al., 1997b). Later it appears to be expressed in a small number of post mitotic V3 interneurons. *Nkx2.9* is expressed in a similar domain to *Nkx2.2* (Pabst et al., 1998), however it is down-regulated in the mouse spinal cord at approximately E10.5 (Briscoe et al., 1999).

The other notable genes found close to the Nkx2 genes are members of the Pax (Paired Box) gene family and the Fox (Forkhead Box) gene family. *Pax1* and *Pax9* are expressed in mesodermal tissue in overlapping domains. Expression of *Pax1* begins at E9 in the ventromedial compartment of the somite, the sclerotome (Deutsch et al., 1988). *Pax9* is expressed in the same region of the somite however its expression is restricted to the posterior sclerotome (Neubuser et al., 1995).

The two Fox genes are located at a greater distance from the Nkx2 genes than *Pax1* and *Pax9* (Fig. 3.1A, 3.1B). They share similar expression patterns, *FoxA1* and *FoxA2* are expressed at earlier stages than the Pax genes and can be detected during gastrulation (Ruiz i Altaba et al., 1993). Both FoxA2 and FoxA1 are detected in cells along the ventral midline of the neural tube (floor plate) at hindbrain and spinal cord levels. *FoxA2* is expressed at E10 and *FoxA1* slightly later, by E13 the floor plate co-expresses both genes. Both are also expressed in the notochord from E10 (Ang et al., 1993; Ruiz i Altaba et al., 1993).

3.1.2 Identification of Nkx2 Gene Clusters in Zebrafish and *Fugu* Genomes

To identify the *Fugu* and zebrafish homologues of these Nkx2 genes, the Ensembl genome assemblies Version 4.0 and Zv6 respectively were searched using BLAST with known human/mouse sequences for *Nkx2.1*, *Nkx2.2*, *Nkx2.4* and *Nkx2.9*. The results (Fig. 3.1C, 3.1D) suggested that a similar clustering has been maintained in both fish species. In zebrafish there appeared to be 3 clusters of Nkx2 genes compared to the 2 clusters identified in human and mouse. The presence of an additional *Nkx2.2/2.4* cluster could be the consequence of the predicted additional whole genome duplication in zebrafish (Amores et al., 1998). The identification of a second *Nkx2.2* gene is supported by published reports of an *Nkx2.2b* gene expressed in the zebrafish lateral floor plate (Schafer et al., 2005).

Nkx2.1 and *Nkx2.4* are paralogous genes and have relatively similar gene sequences. Using the human *Nkx2.4* sequence, two *Nkx2.4* genes were isolated from the zebrafish genome by BLASTZ searching; *Nkx2.4a* and *Nkx2.4b* (Fig. 3.1D). The sequence we have termed *Nkx2.4b* is the gene published as *Nk2.1a* (Fig. 3.1D: Rohr and Concha, 2000). Analysis of the relationships between Nkx2.1 and Nkx2.4 protein sequences from mouse, human and zebrafish (Fig. 3.2), suggests that the zebrafish Nkx2.4b protein is more similar to Nkx2.1 than Nkx2.4. However, regardless of protein sequence, due to gene expression patterns (see below and Discussion) we have suggested the zebrafish *Nkx2.4b* gene is more functionally similar to Nkx2.4 than Nkx2.1. BLASTZ searching using human *Nkx2.1* identified a gene previously termed *Nk2.1b* (Rohr et al., 2001), however, the name *Nkx2.1* will be used for this project (Fig. 3.1D).

Clustering of Nkx2 genes was also apparent in *Fugu* (Fig. 3.1C), a small genome often exploited for identifying enhancers and gene regulatory domains (Aparicio et al., 1995; Brenner et al., 1993). *Nkx2.1* appeared to map to a position on Scaffold_227 of the *Fugu* assembly within the region of the expected cluster. However there are no recognised transcripts, which could therefore indicate that *Nkx2.1* is not expressed in *Fugu*. The *Nkx2.1/2.9* and *Nkx2.2/2.4* arrangements seen in human and mouse have been maintained in both *Fugu* and zebrafish. Thus, we can suggest that this gene arrangement may be significant for the regulation of the genes located in these clusters.

3.2 In Situ Expression Patterns of Nkx2 Genes in Zebrafish

To characterise the expression patterns of the zebrafish Nkx2 genes and to test whether they have similar expression patterns to the mouse homologues, RNA probes for the identified zebrafish Nkx2 genes identified were synthesised and *in situ* hybridisation performed. Analysis was carried out at 11.5hpf (5 somite stage), 16hpf (14 somite stage), 21hpf (24 somite stage), 24hpf (prim-5 stage) and 30hpf (prim-15 stage). Staging was according to Kimmel et al. (1995). As in mouse, *Nkx2.2* and *Nkx2.9* were expressed along the length of the neural tube at 24hpf, a stage equivalent

to E10.5 in mouse (Figs. 3.4G, 3.5G, 3.8G). *Nkx2.1* and *Nkx2.4* were expressed in anterior regions of the neural tube (Figs. 3.3G, 3.6G, 3.7G).

Nkx2.1 (also known as *Nk2.1b*: Rohr et al., 2001) expression was detected at 11.5hpf anteriorly and ventrally in the region of the future brain (Fig. 3.3A, 3.3B), however, published results show expression was observed as early as 10 hpf (Rohr et al., 2001). At 16 hpf the brain has not become morphologically distinct (Kimmel et al., 1995), but it has by 21 hpf. At 21 hpf strong expression of *Nkx2.1* was observed in the anterior ventral forebrain and slightly weaker staining was seen at more posterior positions (Fig. 3.3E, 3.3F). The ventral staining was clearly detected in 24hpf embryos (Fig. 3.3G), more posterior staining was almost undetectable (Fig. 3.3H). Expression was still maintained, albeit more weakly at 30 hpf (Fig. 3.3I, 3.3J). The expression pattern observed for this gene supports previously published results that describes *Nkx2.1* (*Nk2.1b*) as a marker for ventral telencephalon (Rohr et al., 2001). This expression pattern is also equivalent to that previously described in the mouse (Price, 1993).

Nkx2.2a was also expressed as early as 11.5 hpf (Fig. 3.4A, 3.4B), and published reports indicated that the expression of this gene can be detected as early as 9.5 hpf (Barth and Wilson, 1995). Expression was initially seen in anterior regions of the neural tube, the presumptive forebrain. However by 16 hpf a weaker level of expression could be seen extending into more posterior ventral neural tube (Fig. 3.4C, 3.4D). At later stages, ventral neural expression was observed extending the length of the neural tube to spinal cord levels (Fig. 3.4E-3.4J). Consistent with previous findings, expression in the spinal cord and hindbrain was much weaker than at more anterior levels (Barth and Wilson, 1995). *Nkx2.2b* displayed an almost identical expression pattern (Fig. 3.5), the RNA probe appeared to be weaker than *Nkx2.2a*. Published results of *Nkx2.2b* expression suggest that it is expressed at much higher levels than *Nkx2.2a* (Schafer et al., 2005), this difference may be due to probe efficiency. *Nkx2.2b* was expressed as early as ~8hpf (Schafer et al., 2005), and appeared to be expressed until 30 hpf, however the staining was very weak by this stage (Fig. 3.5I, 3.5J).

The expression of both *Nkx2.4a* and *Nkx2.4b* were restricted to the ventral anterior neural tube at all stages characterised (Figs. 3.6, 3.7). Very weak expression was observed at 11.5 hpf in the future head (Figs. 3.6A, 3.6B, 3.7A, 3.7B). By 16 hpf expression was much stronger, but uniform (Figs. 3.6C, 3.6D, 3.7C, 3.7D), at 21 hpf expression of both *Nkx2.4* genes was throughout the forebrain (Figs. 3.6E, 3.6F, 3.7E, 3.7F). By 24 hpf, expression was stronger in more posterior regions, the hypothalamus (Figs. 3.6G-3.6J, 3.7G-3.7J), expression in the more anterior forebrain was present but weaker. In mouse, *Nkx2.4* expression has been identified as being localised to the posterior hypothalamus (Price, 1993). The expression patterns of *Nkx2.1* and *Nkx2.4* in the zebrafish forebrain appear complementary, with little overlap, as noted for the mouse expression patterns (Price, 1993). The expression of zebrafish *Nkx2.4b* (also known as *Nk2.1a*) has been published and is described as a marker for the ventral diencephalon or hypothalamus (Rohr et al., 2001; Rohr and Concha, 2000). This supports the expression pattern seen in Figure 3.7. The similarity in expression of *Nkx2.4a* (Fig. 3.6) and *Nkx2.4b* (Fig. 3.7) further supports the theory that the *Nkx2.4b* is more closely related to *Nkx2.4* than *Nkx2.1*.

Nkx2.9 in mouse has a very similar expression pattern to *Nkx2.2* until E10.5, at which point *Nkx2.9* expression in the spinal cord begins to be down-regulated (Briscoe et al., 1999). A similar expression profile also appears to characterise zebrafish *Nkx2.9*. *Nkx2.9* expression was observed at 11.5 hpf in zebrafish in the future head (Fig. 3.8A, 3.8B), by 16 hpf it was strongly expressed along the entire length of the ventral neural tube (Fig. 3.8C, 3.8D). At 21 hpf and 24 hpf, expression was observed at high levels in ventral forebrain, floor plate, ventral hindbrain and spinal cord (Fig. 3.8E-3.8H). By 30 hpf the expression had reduced (Fig. 3.8I, 3.8J), although was still present, it is possible therefore that, as in mice, expression is down-regulated over time.

3.3 Response of Zebrafish Nkx2 Genes to Shh

Previous studies using cyclopamine have suggested that zebrafish *Nkx2.2a* is under the control of Shh (Stamataki et al., 2005). To test if the other family members

identified are also regulated by Shh, cyclopamine was used. Cyclopamine was added to 1 cell zebrafish embryos and *in situ* analysis carried out at 24 hpf and compared to embryos exposed to vehicle (ethanol) alone. Results indicate that the expression of *Nkx2.1*, *Nkx2.2a*, *Nkx2.2b*, and *Nkx2.9* were dependent on Shh (Figs. 3.9B, 3.9D, 3.9F, 3.10B). Expression of these 4 genes was lost when embryos were treated with 10μM cyclopamine, compared to wild type expression patterns at 24 hpf (Figs. 3.3G, 3.4G, 3.5G, 3.8G). Expression was not lost when embryos from the same clutch were treated with the ethanol control (Figs. 3.9A, 3.9C, 3.9E, 3.10A). The loss of expression of *Nkx2.1*, *Nkx2.2* and *Nkx2.9* is consistent with published results (Pabst et al., 2000; Rohr et al., 2001; Stamataki et al., 2005), which suggests that these genes require Shh signalling in order to be expressed. This is also consistent with experiments in zebrafish *Gli1* mutants in which a direct requirement for Gli1 in *Nkx2.9* expression was identified (Xu et al., 2006). However, loss of *Nkx2.1* expression may be caused by the absence of ventral forebrain tissue due to loss of prechordal mesoderm in embryos in which Shh has been blocked (Chiang et al., 1996). Cyclopamine treatment resulted in expected morphogenetic defects such as fusion of the optic vesicle (Fig. 3.10B, 3.10D, 3.10F: Chiang et al., 1996) and rounded somites instead of the normal 'V' shape (Fig. 3.9B, 3.9D, 3.9F: Wolff et al., 2003).

Intriguingly, *Nkx2.4a* and *Nkx2.4b* expression was maintained in the cyclopamine treated fish (Fig. 3.10C-3.10F), suggesting that its expression is not dependent on Shh signalling. Previous studies have suggested that *Nkx2.4b* expression in the zebrafish diencephalon does not require Shh signalling (Rohr et al., 2001). These data therefore suggest that in contrast to *Nkx2.1*, *Nkx2.2* and *Nkx2.9*, the expression of *Nkx2.4* is not dependent on Shh. This may be due to the differences in anterior posterior differences in CNS midline fates (see Discussion).

Together, these data indicate the *Nkx2* genes found in the zebrafish genome are clustered in a similar manner to those in mouse and also appear to share a similar expression pattern and regulation.

Figure 3.1 Genomic arrangement of *Nkx2.2* and *Nkx2.9* genes and their expression in vertebrate species.

Diagrammatic representation of Human (**A**), Mouse (**B**), *Fugu* (*Takifugu rubripes*, **C**) and Zebrafish (*Danio rerio*, **D**) genomic regions harbouring *Nkx2.2* and *Nkx2.9* orthologues. Solid lines represent chromosomes, the arrows represent direction of gene transcription and slanted parallel lines represent a large gap between genes. The scale bar represents 40kb. Name discrepancies; Human gene *Nkx2.9* is annotated as *Nkx2.8* in Ensembl database, Human and Mouse *Nkx2.1* are both annotated as *TITF1*, zebrafish *Nkx2.1* is *Nk2.1b* or *titf1b* and zebrafish *Nkx2.4b* is *titf1a* or *Nk2.1a*. Data is based on Human NCBI 36 Assembly, Mouse NCBI 36 Assembly, Fugu 4.0 Assembly and Zebrafish Zv6 Assembly. **E-G** *In situ* hybridisation for *Nkx2.2* and *Nkx2.9* in mouse and zebrafish wild type embryos. **E** Mouse E10.5 *in situ* hybridisation for *Nkx2.2* (image kindly contributed by James Briscoe). **F** Mouse E10.5 *in situ* hybridisation for *Nkx2.9*. **G** Zebrafish at 24 hpf, *in situ* hybridisation for *Nkx2.2a* (images **F** and **G** kindly contributed by Vicky Tsoni).

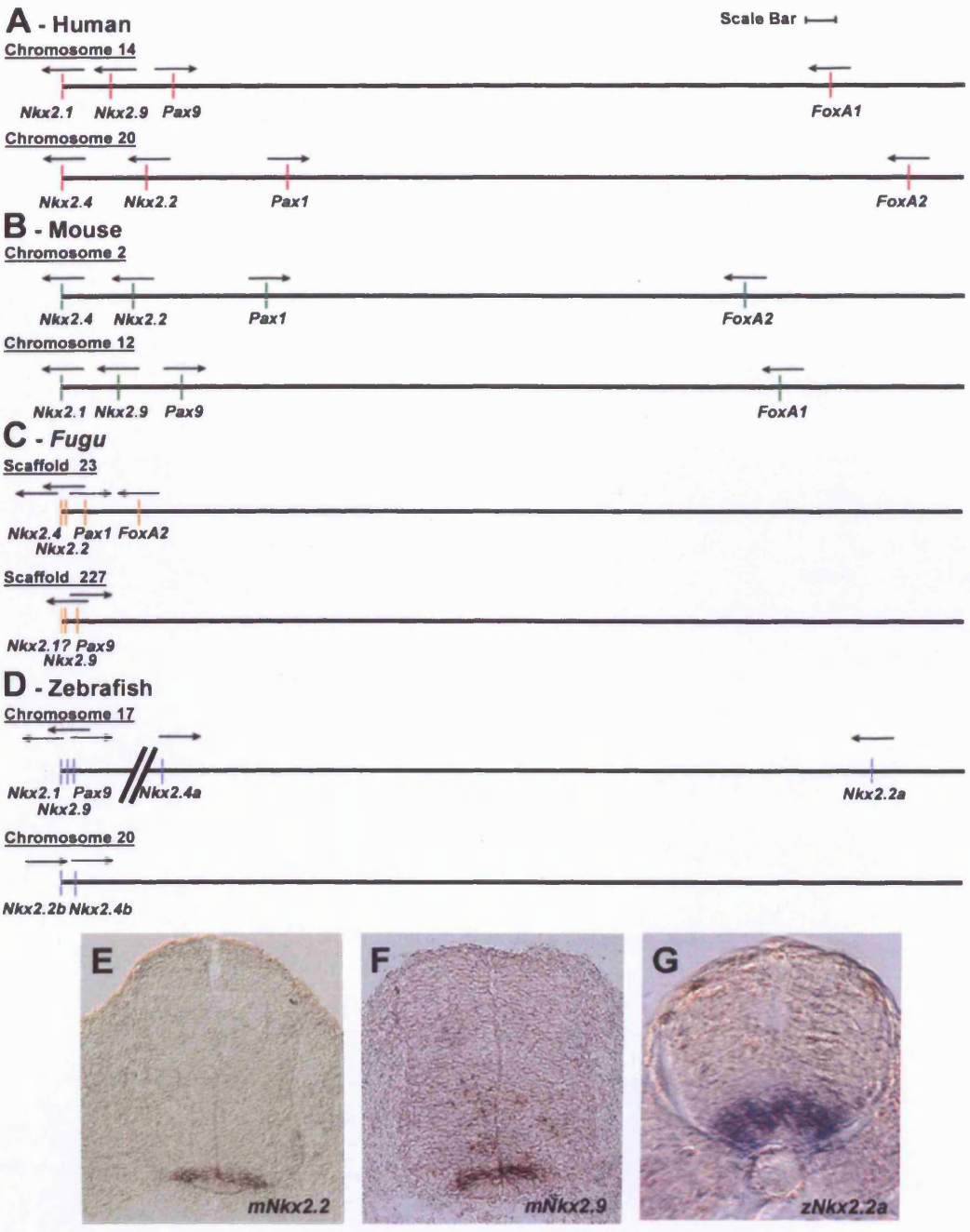

A - Human
Scale Bar
Chromosome 14
Nkx2.1
Nkx2.9
Pax9
FoxA1
Chromosome 20
Nkx2.4
Nkx2.2
Pax1
FoxA2
B - Mouse
Chromosome 2
Nkx2.4
Nkx2.2
Pax1
FoxA2
Chromosome 12
Nkx2.1
Nkx2.9
Pax9
FoxA1
C - Fugu
Scaffold_23
Nkx2.4
Pax1
FoxA2
Nkx2.2
Scaffold_227
Nkx2.1? Pax9
Nkx2.9
D - Zebrafish
Chromosome 17
Nkx2.1
Pax9
Nkx2.9
Nkx2.4a
Nkx2.2a
Chromosome 20
Nkx2.2b
Nkx2.4b
E
mNkx2.2
F
mNkx2.9
G
zNkx2.2a

Figure 3.2 Similarity tree showing relationship between human, mouse and zebrafish Nkx2 proteins.

Full length protein sequences provided by the Genbank database. Nkx2.1, Nkx2.2 and Nkx2.4 proteins from mouse, human and zebrafish were aligned with CLUSTALW. A similarity tree was formed with a Neighbour Joining Method, which makes no assumptions of constant divergence rates and with Uncorrected Distance, which estimates the proportional differences between sequences. Zebrafish Nkx2.1 is the same as the published protein Nk2.1b. The protein sequence of zebrafish Nkx2.4b (published name Nk2.1a) is more similar to zebrafish Nkx2.1. However, due to its observed gene expression pattern (Fig. 3.7) and response to Shh signalling (Fig. 3.10), it appears more functionally similar to Nkx2.4.

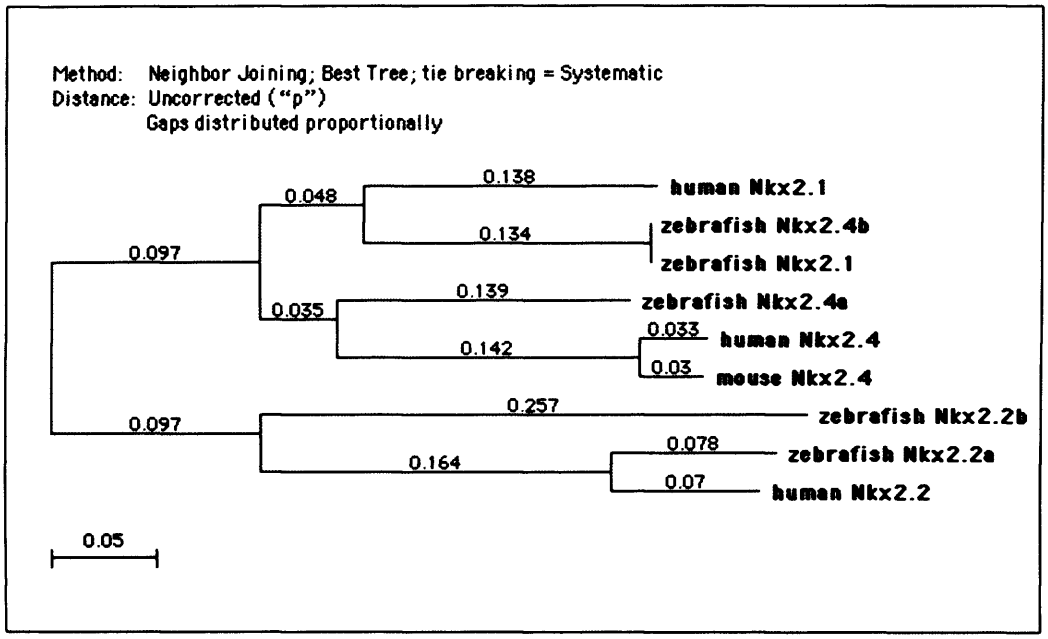

Method: Neighbor Joining; Best Tree; tie breaking = Systematic
Distance: Uncorrected ("p")
Gaps distributed proportionally
0.138
human Nkx2.1
0.048
0.134
zebrafish Nkx2.4b
0.097
zebrafish Nkx2.1
0.139
zebrafish Nkx2.4a
0.035
0.033
human Nkx2.4
0.142
0.03
mouse Nkx2.4
0.257
zebrafish Nkx2.2b
0.097
0.078
zebrafish Nkx2.2a
0.164
0.07
human Nkx2.2
0.05

Figure 3.3 Expression pattern of zebrafish *Nkx2.1* in Wild Type embryos.

Wild type embryos were harvested at various time points and hybridised with RNA probe transcribed from a PCR product for the zebrafish gene *Nkx2.1*, Ensembl Gene ID ENSDARG00000019835. All views of the embryos are lateral with anterior to the left. At 21hpf, 24hpf and 30hpf heads were turned to be seen from a dorsal view and yolk cells were removed. **A, B** show expression at 11.5 hpf, **C, D** at 16hpf, **E, F** at 21hpf, **G, H** at 24hpf and **I, J** at 30hpf. **A, C, E, G, I** were taken at 10X magnification and **B, D, F, H, J** at 20X. Expression was observed as early as 11.5 hpf (**A, B**) in the ventral future head, similarly at 16 hpf (**C, D**). By 21hpf, morphologically distinct brain structures have formed and strong staining was observed in the anterior forebrain (**E, F**). Similar expression patterns were observed at 24 hpf (**G, H**) and 30 hpf (**I, J**), by which point staining appeared to have weakened.

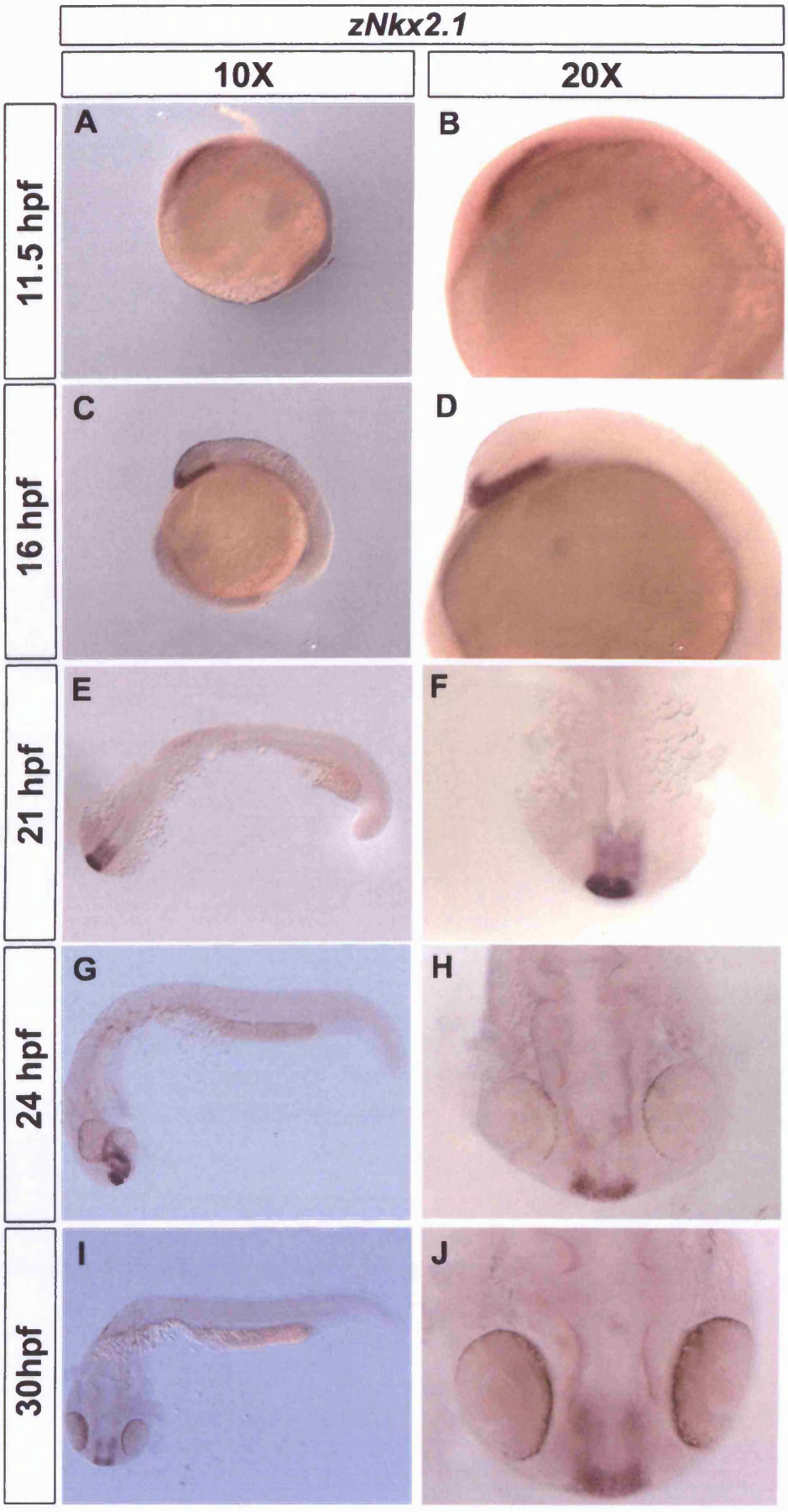

zNkx2.1
10X
20X
11.5 hpf
16 hpf
21 hpf
24 hpf
30hpf
A
B
C
D
E
F
G
H
I
J

Figure 3.4 Expression pattern of zebrafish *Nkx2.2a* in Wild Type embryos.

Wild type embryos were harvested at various time points and hybridised with RNA probe for the zebrafish gene *Nkx2.2a* (Barth and Wilson, 1995), Ensembl Gene ID ENSDARG00000053298. All views of the embryos are lateral with anterior to the left. At 21hpf, 24hpf and 30hpf heads were turned showing a dorsal view and yolk cells were removed. **A, B** show expression at 11.5 hpf, **C, D** at 16hpf, **E, F** at 21hpf, **G, H** at 24hpf and **I, J** at 30hpf. **A, C, E, G, I** were taken at 10X magnification and **B, D, F, H, J** at 20X. Expression in the ventral forebrain at 11.5 hpf was evident (**A, B**), this extended to more posterior parts of the ventral neural tube by 16 hpf (**C, D**). At 21 hpf (**E, F**) the brain has morphologically segmented and expression was observed in the ventral forebrain, hindbrain and spinal cord. Expression patterns were maintained at 24 hpf (**G, H**) and up to 30 hpf (**I, J**) with weaker expression in the hindbrain and spinal cord.

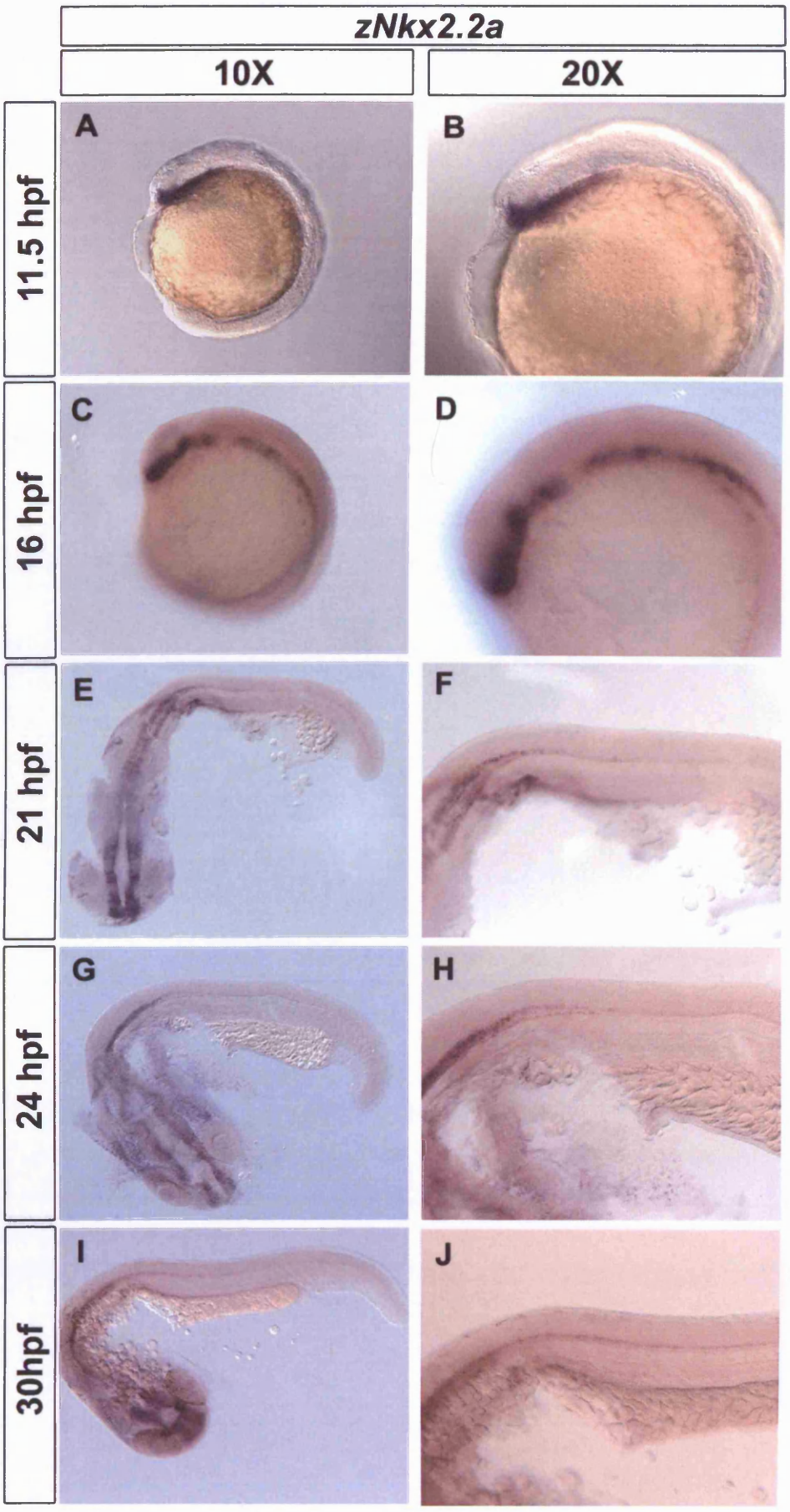

zNkx2.2a
10X
20X
11.5 hpf
16 hpf
21 hpf
24 hpf
30hpf
A
B
C
D
E
F
G
H
I
J

Figure 3.5 Expression pattern of zebrafish *Nkx2.2b* in Wild Type embryos.

Wild type embryos were harvested at various time points and hybridised with RNA probe transcribed from IMAGE clone 3722576 for the zebrafish gene *Nkx2.2b*, Ensembl Gene ID ENSDARG00000052550. All views of the embryos are lateral with anterior to the left. At 21hpf, 24hpf and 30hpf heads were turned to be seen from a dorsal view and yolk cells were removed. **A, B** show expression at 11.5 hpf, **C, D** at 16hpf, **E, F** at 21hpf, **G, H** at 24hpf and **I, J** at 30hpf. **A, C, E, G, I** were taken at 10X magnification and **B, D, F, H, J** at 20X. Expression in the ventral forebrain at 11.5 hpf was evident (**A, B**), which extended to more posterior parts of the ventral neural tube by 16 hpf (**C, D**). At 21 hpf (**E, F**) the brain has morphologically segmented and expression was observed in the ventral forebrain, hindbrain and spinal cord. Expression patterns were maintained up to 24 hpf (**G, H**) with weaker expression in the hindbrain and spinal cord. By 30 hpf, *Nkx2.2b* was almost undetectable in the spinal cord with this probe (**I, J**), but was still present in the anterior parts of the neural tube.

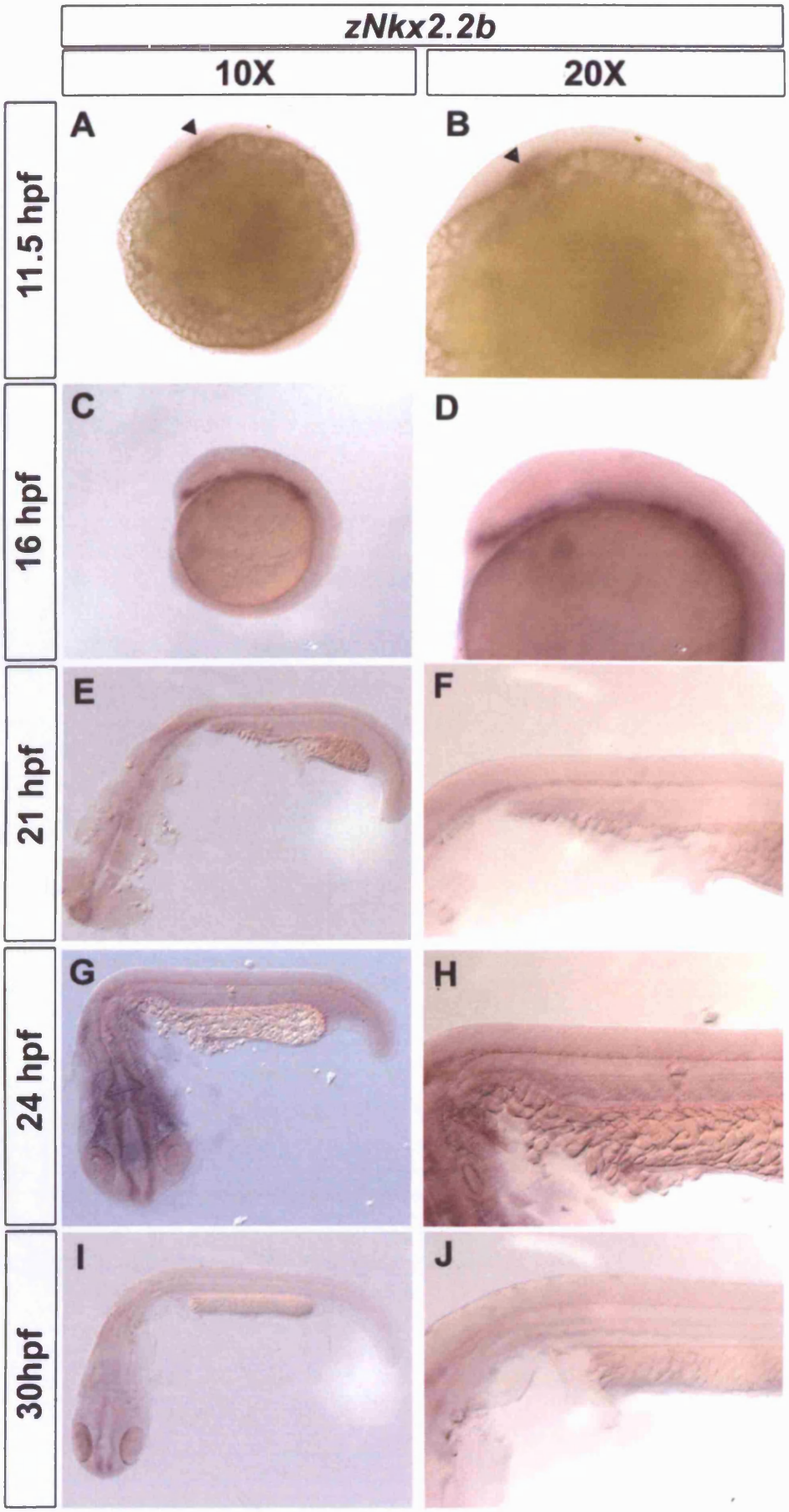

zNkx2.2b
10X
20X
11.5 hpf
16 hpf
21 hpf
24 hpf
30hpf
A
B
C
D
E
F
G
H
I
J

Figure 3.6 Expression pattern of zebrafish *Nkx2.4a* in Wild Type embryos.

Wild type embryos were harvested at various time points and hybridised with RNA probe transcribed from IMAGE Clone 2640862 for the zebrafish gene *Nkx2.4a*, Ensembl Gene ID ENSDARG00000012693. All views of the embryos are lateral with anterior to the left. At 21hpf, 24hpf and 30hpf heads were turned to be seen from a dorsal view and yolk cells have been removed. **A, B** show expression at 11.5 hpf, **C, D** at 16hpf, **E, F** at 21hpf, **G, H** at 24hpf and **I, J** at 30hpf. **A, C, E, G, I** were taken at 10X magnification and **B, D, F, H, J** at 20X. Expression in the ventral forebrain at 11.5 hpf was present but very weak (**A, B**). By 16 hpf expression was much stronger and uniform within the ventral anterior neural tube (**C, D**). At 21 hpf (**E, F**) expression was observed in the ventral forebrain. Expression patterns were maintained to 24 hpf (**G, H**) and 30 hpf (**I, J**) with stronger expression in the more posterior forebrain.

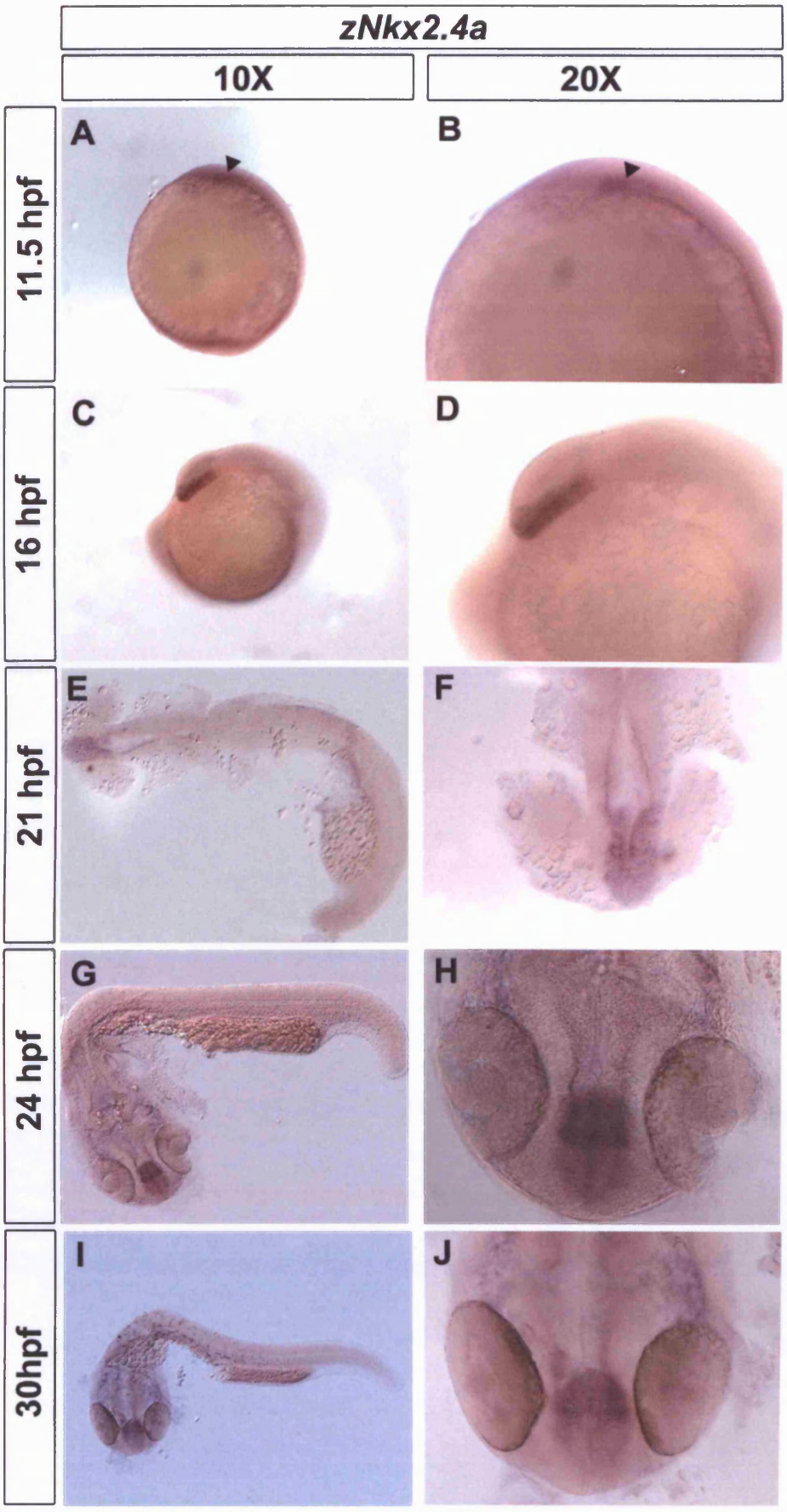

zNkx2.4a
10X
20X
11.5 hpf
16 hpf
21 hpf
24 hpf
30hpf
A
B
C
D
E
F
G
H
I
J

Figure 3.7 Expression pattern of zebrafish *Nkx2.4b* in Wild Type embryos.

Wild type embryos were harvested at various time points and hybridised with RNA probe transcribed from a PCR product for the zebrafish gene *Nkx2.4b*, Ensembl Gene ID ENSDARG00000010461. All views of the embryos are lateral with anterior to the left. At 21hpf, 24hpf and 30 hpf heads were turned to be seen from a dorsal view and yolk cells were removed. **A, B** show expression at 11.5 hpf, **C, D** at 16hpf, **E, F** at 21hpf, **G, H** at 24hpf and **I, J** at 30hpf. **A, C, E, G, I** were taken at 10X magnification and **B, D, F, H, J** at 20X. Expression in the ventral forebrain at 11.5 hpf was weak but detectable (**A, B**), by 16 hpf expression was much stronger and uniform within the ventral anterior neural tube (**C, D**). At 21 hpf (**E, F**) expression was observed in the ventral forebrain. Expression patterns were maintained to 24 hpf (**G, H**) and 30 hpf (**I, J**) with stronger expression in the more posterior forebrain.

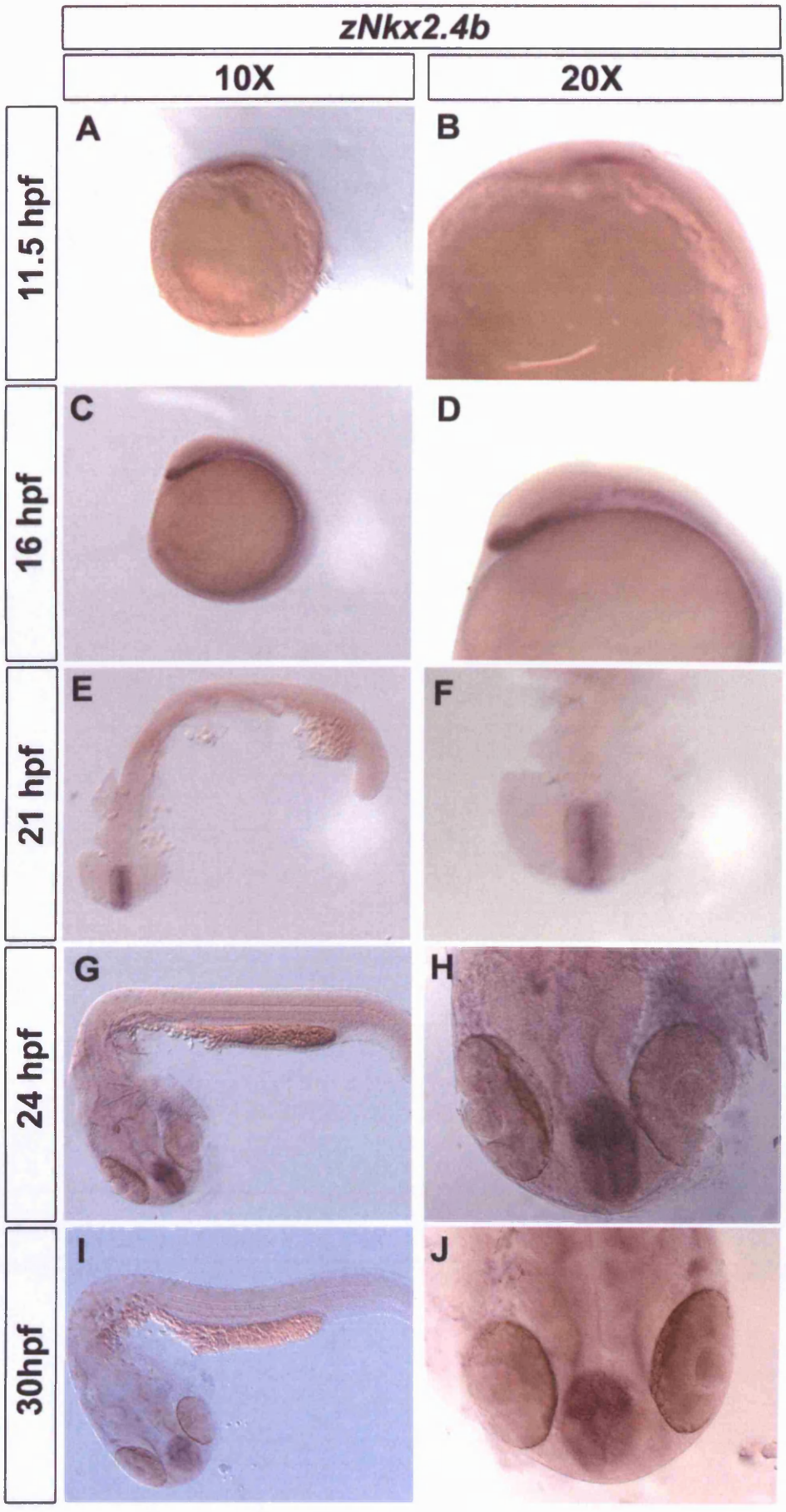

zNkx2.4b
10X
20X
11.5 hpf
16 hpf
21 hpf
24 hpf
30hpf
A
B
C
D
E
F
G
H
I
J

Figure 3.8 Expression pattern of zebrafish *Nkx2.9* in Wild Type embryos.

Wild type embryos were harvested at various time points and hybridised with RNA probe transcribed from a PCR product for the zebrafish gene *Nkx2.9*, Ensembl Gene ID ENSDARG00000020332. All views of the embryos are lateral with anterior to the left. At 21hpf, 24hpf and 30hpf heads were turned to be seen from a dorsal view and yolk cells were removed. **A, B** show expression at 11.5 hpf, **C, D** at 16hpf, **E, F** at 21hpf, **G, H** at 24hpf and **I, J** at 30hpf. **A, C, E, G, I** were taken at 10X magnification and **B, D, F, H, J** at 20X. Expression was seen at 11.5 hpf in the ventral anterior neural tube and was already a strong signal extending posteriorly (**A, B**). By 16 hpf strong expression was observed along the length of the neural tube (**C, D**). At 21 hpf (**E, F**) the brain has morphologically segmented and expression was seen in the ventral forebrain, floor plate and throughout the hindbrain to the very tip of the spinal cord (**E, F**). This was the same expression pattern as at 24 hpf (**G, H**). Expression patterns were maintained to 30 hpf (**I, J**) but the expression had weakened.

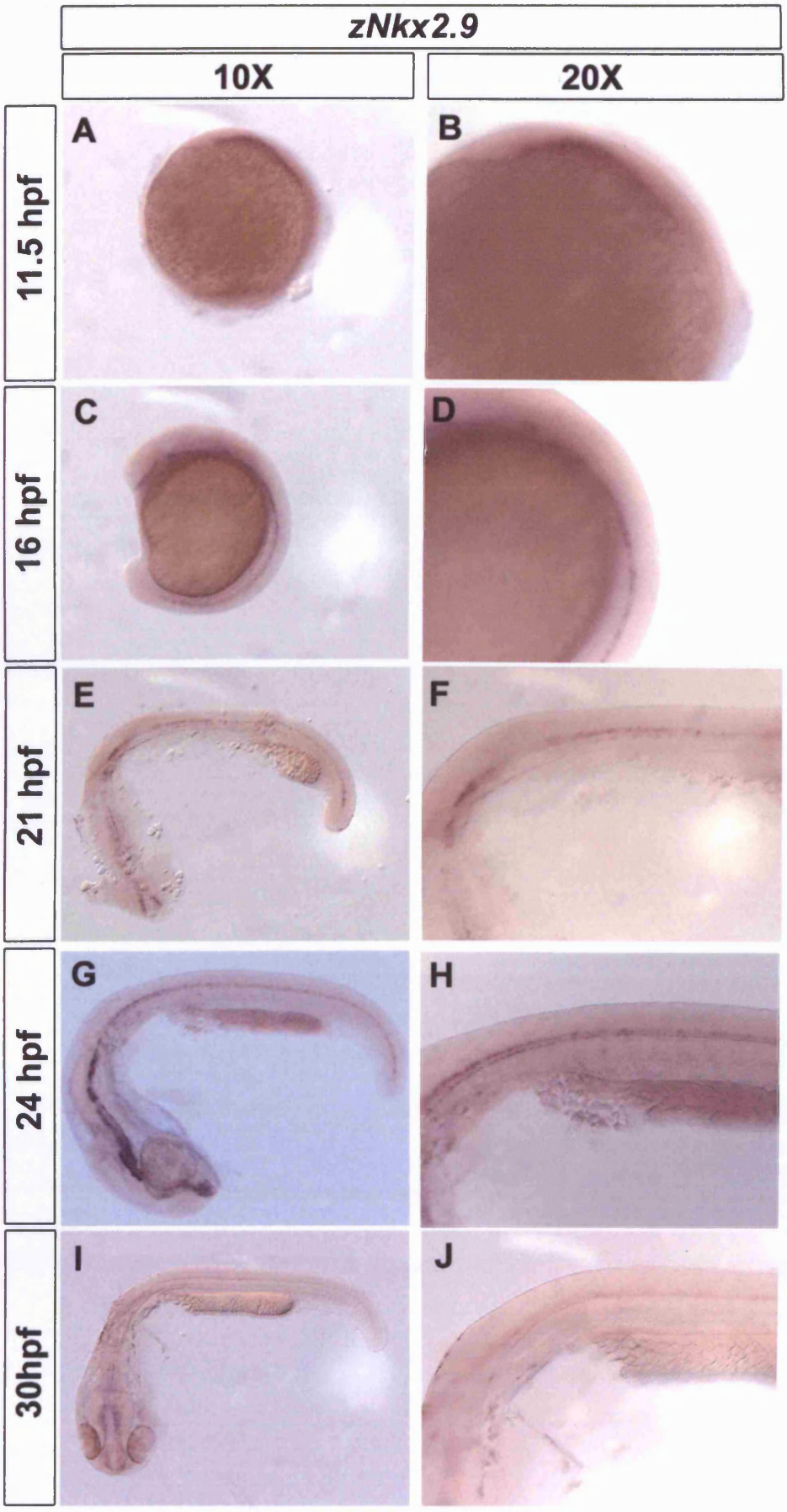

zNkx2.9
10X
20X
11.5 hpf
16 hpf
21 hpf
24 hpf
30hpf
A
B
C
D
E
F
G
H
I
J

Figure 3.9 Expression patterns of *Nkx2.2* and *Nkx2.9* in cyclopamine treated embryos.

All views of the embryos are lateral with anterior to the left, yolk cells have been removed from embryos, which are 24 hpf. Pictures were taken at 20X magnification. Embryos were either placed in ethanol control (**A, C, E**) or were placed in 10μM of the Shh inhibitor cyclopamine (**B, D, F**) at the 1 cell stage and grown to 24 hpf. *In situ* hybridisation for *Nkx2.2a* (**A, B**), *Nkx2.2b* (**C, D**) and *Nkx2.9* (**E, F**) revealed that cyclopamine treatment blocks expression of all three genes. Ethanol treatment did not affect expression, compare **A, C, E** with Figs. 3.4G, 3.5G, 3.8G.

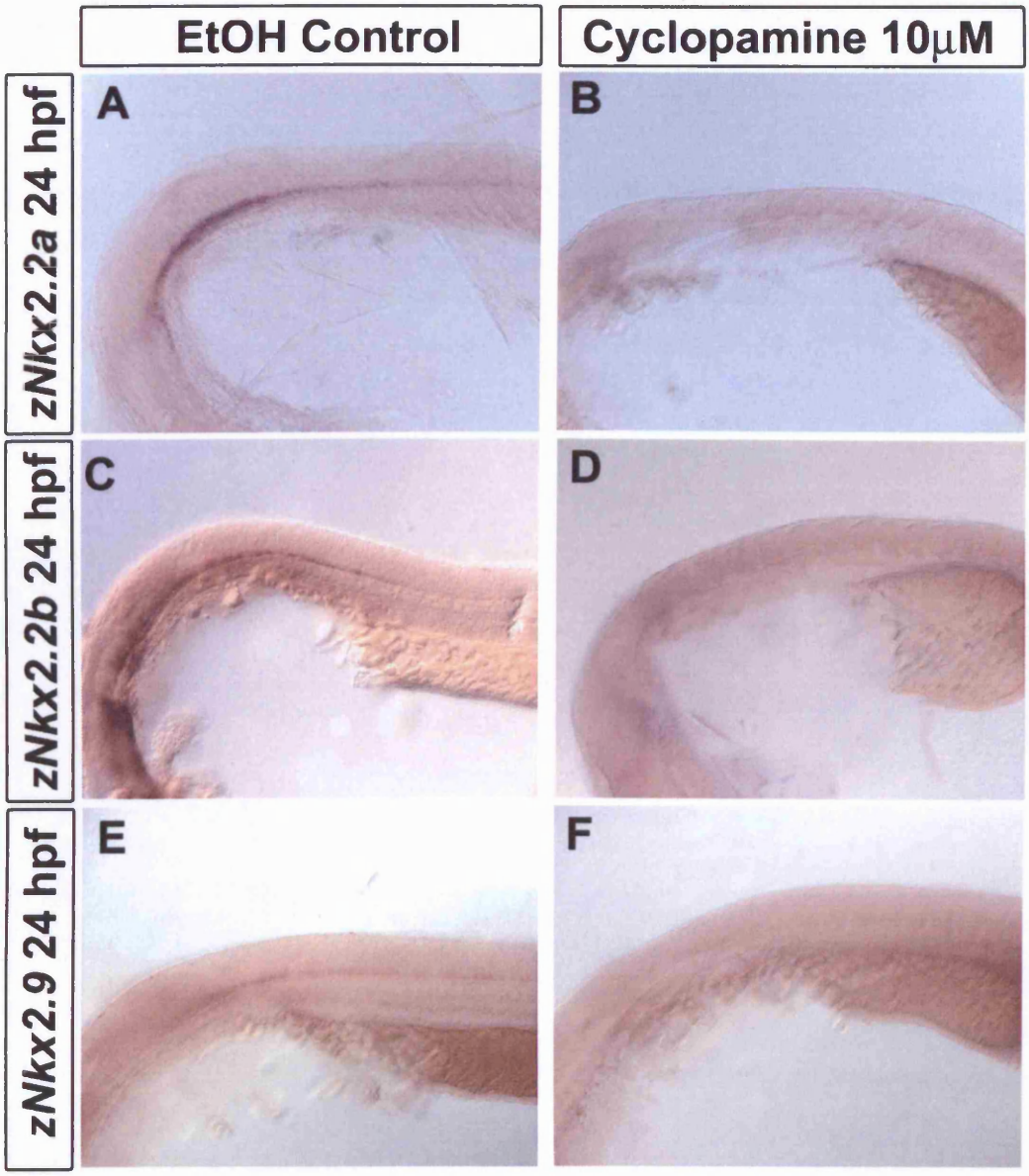
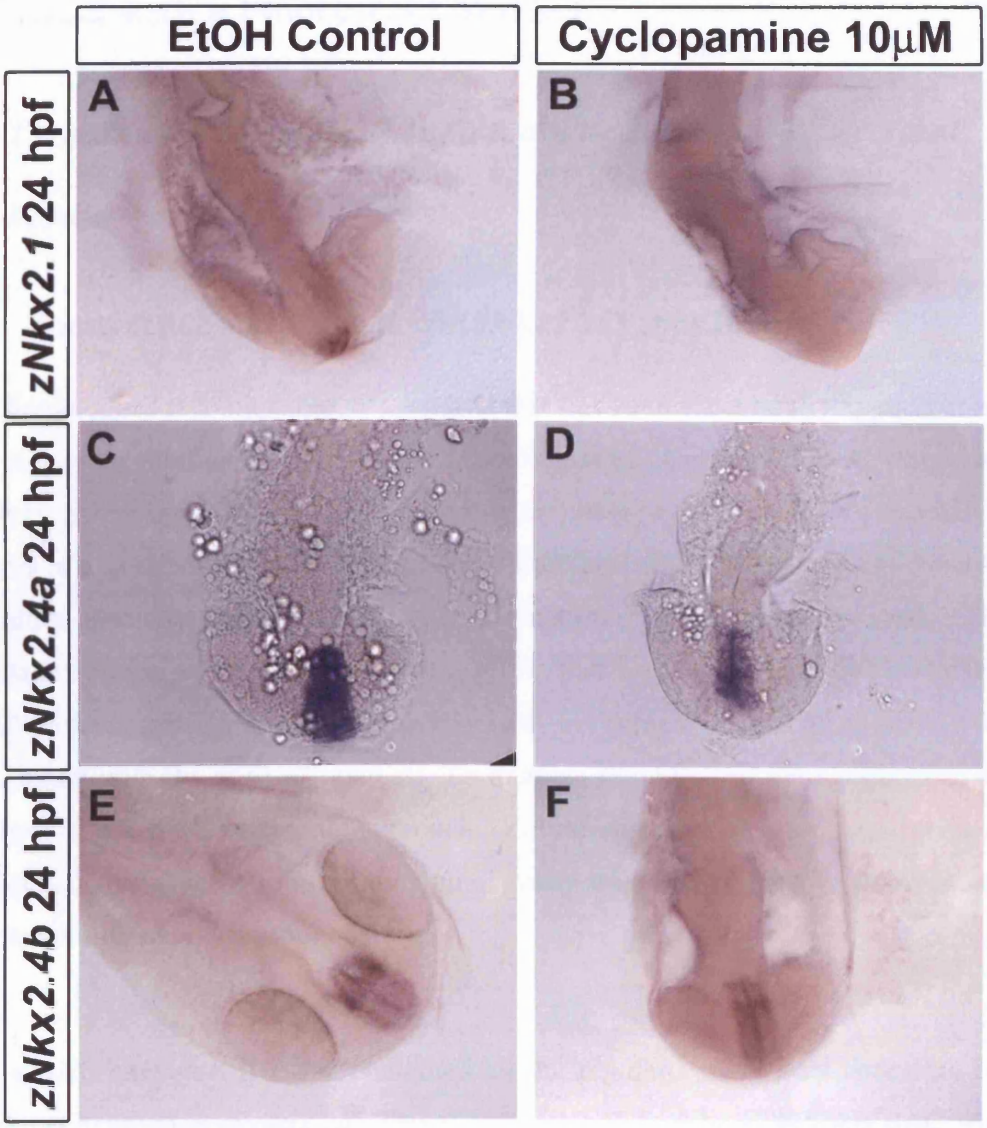

EtOH Control
Cyclopamine 10μM
zNkx2.2a 24 hpf
zNkx2.2b 24 hpf
zNkx2.9 24 hpf
A
B
C
D
E
F

Figure 3.10 Expression patterns of *Nkx2.1* and *Nkx2.4* in cyclopamine treated embryos.

All views of the embryos are dorsal, yolk cells have been removed from embryos. Pictures were taken at 20X magnification. Embryos were either placed in ethanol (**A, C, E**) or were placed in 10μM of the Shh inhibitor cyclopamine (**B, D, F**) at the 1 cell stage and grown to 24 hpf. *In situ* hybridisation for *Nkx2.1* (**A, B**) revealed that cyclopamine treatment blocks expression compared to the ethanol control where expression was the same as in wild type embryos (Fig. 3.3G). This loss of expression may be due to loss of prechordal mesoderm in embryos that have Shh signalling blocked. However, *Nkx2.4a* (**C, D**) and *Nkx2.4b* (**E, F**) show that expression in cyclopamine treated embryos was similar to ethanol control and wild type embryos (Figs 3.6G, 3.7G) suggesting that neither *Nkx2.4a* nor *Nkx2.4b* expression requires Hh signalling.


EtOH Control
Cyclopamine 10μM
zNkx2.1 24 hpf
zNkx2.4a 24 hpf
zNkx2.4b 24 hpf
A
B
C
D
E
F

4 Results: BAC Homologous Recombination; Targeting *Nkx2.2* with a Fluorescent Marker

4.1 Targeting Zebrafish Nkx2.2a Containing BAC with Fluorescent Marker Venus

4.1.1 Construction and Validation of zNkx2.2aVenus BAC

BAC homologous recombination is a method that has been successfully applied to the study of gene regulation. BACs contain large fragments of genomic DNA, which due to advances in homologous recombination techniques, can easily be modified. Markers of expression such as GFP can be inserted into coding sequence and putative regulatory elements can be modified and changes in expression assayed. The advantage of using a BAC transgenic approach, is that because of their size, it is likely that all of the regulatory elements necessary to direct expression will be present in the BAC, along with the gene of interest. To analyse the *Nkx2.2a* gene regulation we therefore took a BAC transgenic approach. Zebrafish embryos were used for these experiments due to the ease of production of many transient transgenic embryos and the accessibility of embryos for analysis.

First, a BAC was identified that contained all the regulatory elements necessary for the correct expression of *Nkx2.2a*. Targeting the *Nkx2.2a* gene within this BAC with a reporter recapitulated the endogenous *Nkx2.2a* expression pattern in zebrafish. Then removal of presumptive regulatory non-coding DNA confirmed the necessity of these elements for correct expression of *Nkx2.2a*.

A BAC containing 93kb of zebrafish genomic DNA (zK257G4), including the *Nkx2.2a* gene, was chosen for targeting. The BAC covered approximately 74kb of genomic DNA upstream (5') and 17kb of DNA downstream (3') of the *Nkx2.2a* gene. We therefore thought it likely that all the necessary regulatory elements for expression of *Nkx2.2a* were present (Fig. 4.1A). To perform the targeting, the BAC

recombination system developed by Neil Copeland and colleagues was used (Lee et al., 2001; Yu et al., 2000).

This homologous recombination system uses a modified DH10B *E.coli* strain, EL250 (Lee et al., 2001). Two *Red* genes encoded by the bacteriophage λ are required for recombination: *exo* and *bet* (Poteete, 2001; Stahl, 1998). A third λ phage gene is also needed, *gam*, this inhibits RecBCD exonuclease to ensure linear DNA is not degraded by the bacteria (Poteete, 2001; Stahl, 1998). The genes required are expressed from a defective prophage, which is integrated into the *E.coli* chromosome of the modified strains (Lee et al., 2001; Yu et al., 2000). The EL250 strain contains the defective prophage, with the recombination genes expressed by a strong λ promoter P_L. This promoter is repressed by temperature sensitive λ cI857 repressor within the strain (Lee et al., 2001; Yu et al., 2000). Consequently, only very low levels of expression occur at 32 °C, however upon increasing the temperature to 42°C for 15 minutes, *exo*, *bet* and *gam* are expressed at high levels and allow recombination. EL250 also contains an arabinose inducible *Flpe recombinase* gene (Buchholz et al., 1998; Lee et al., 2001), allowing recombination between *FRT* sequences upon introduction of arabinose into the growth media.

EL250 was transfected by electroporation with BAC zK257G4. To confirm the integrity of the BAC in the clone of EL250s used for subsequent targeting, the DNA digestion pattern with 3 different restriction enzymes was compared with that of the purified DNA used for the transfection (results not shown). These data indicated that the clone of EL250 used contained intact zK257G4.

The strategy for introducing a marker into the *Nkx2.2a* locus is outlined in Fig. 4.1B and 4.1C. The zK257G4 containing EL250 were transfected with a targeting plasmid containing a gene for the fluorescent reporter, *Venus*, flanked by DNA homologous to exon 1 of the *Nkx2.2a* gene in the BAC (Fig. 4.1B). Venus is a modified version of the commercially available marker YFP, that contains a point mutation which enhances its brightness compared to the original fluorescent marker (Nagai et al., 2002). The targeting construct also contained a selectable Neomycin (*Neo*) resistance

gene to allow selection of BACs in which recombination with the plasmid had occurred (Fig. 4.1C). Once the BAC had been targeted, a second recombination, induced by arabinose, was carried out to allow recombination between the FRT_0 sites flanking the *Neo* thereby deleting the marker. The result was the *Nkx2.2a* locus targeted with a traceable marker (Fig. 4.1C).

In order to check that the BAC was correctly targeted, Southern blots were carried out (Fig. 4.2). The *Venus-Neo* cassette was targeted to Exon 1 of the *Nkx2.2a* gene, this introduced an extra MfeI restriction enzyme site to the *Nkx2.2a* Locus (Fig. 4.2Ai, 4.2Aii). Accordingly, digestion of BAC DNA with MfeI and probing a Southern Blot with a radiolabelled probe from the 3' of exon 1, corresponding to the 3' arm of the targeting construct, identified clones, in which the locus had been correctly targeted. In the original, untargeted BAC the 3' probe identifies a 4.8kb fragment (Fig. 4.2Ai); the correct insertion of the cassette into exon 1 results in a 3.7kb fragment being identified with the 3' probe (Fig. 4.2Aii). Upon deletion of the *Neo* marker, by *FRT* site recombination, a further decrease in the size of the fragment to 2.4kb is predicted (Fig. 4.2Aiii).

Results of the Southern blot with the 3' arm probe are shown in Fig. 4.2B. The digestion and probing of the original, untargeted BAC identified a fragment of approximately 4.8kb (lane 1, Fig. 4.2B). After digestion of targeted BACs the same probe identified two bands of approximately 3.7kb and 2.4kb (lanes 2 and 3, Fig. 4.2B). Finally 8 clones, which were targeted and had *Neo* deleted were also digested and labelled, there were 2 different outcomes with these clones. Two clones (clones 2 and 7) contained 2 fragments of approximately 2.4kb and 3.7kb that were labelled by the 3' arm probe (lanes 5 and 10, Fig. 4.2B). The other 6 clones upon digestion contained one labelled fragment of 2.4kb (lanes 4, 6-9 and 11 Fig. 4.2B). The results from the Southern blot suggest that the targeted BACs contained the correct sized insert (3.7kb), which was positioned correctly (lanes 2 and 3 Fig. 4.2B). Upon deletion of the *Neo*, the clones were again of the correct size (2.4kb), suggesting the FRT_0 sites had correctly recombined leaving the expected product (Fig. 4.2Aiii).

The presence of the second labelled fragment of 2.4kb in the recombined BACs (lanes 2 and 3, Fig. 4.2B) could be explained by *FRT* recombination and deletion of *Neo* prior to arabinose induction of *Flpe recombinase*. To test this hypothesis, a second Southern blot was carried out using a *Neo* radiolabelled probe to test for loss of the *Neo* marker (Fig. 4.2C). Consistent with the lack of *Neo*, the digestion and probing of the original BAC did not produce any bands (lane 1, Fig. 4.2C). On the other hand, the digested, targeted BACs contained a single fragment of 3.7kb labelled by the *Neo* probe (lanes 2 and 3, Fig. 4.2C). Clones in which *Neo* had been deleted (lanes 4-11, Fig. 4.2C) were not labelled by the probe in 6 of the 8 clones, but fragments of 3.7kb were labelled in 2 clones (lanes 5 and 10, Fig. 4.2C). Thus this blot confirmed the loss of *Neo* in the clones in which the *FRT* sites had been recombined (Fig. 4.2C lanes 4-11). The exceptions to this were clones 2 and 7, in which *Neo* had not been completely deleted (Fig 4.2C, lanes 5 and 10). Moreover these data are consistent with the idea that the 2.4kb fragments highlighted by probing the targeted BACs using the 3'arm probe (lanes 2 and 3, Fig. 4.2B) resulted from BACs in which *Neo* had been deleted. This was probably due to basal levels of transcription of *Flpe recombinase* in the absence of arabinose.

4.1.2 Analysis of Zebrafish Embryos Injected with zNkx2.2aVenus BAC

The resulting zNkx2.2aVenus BAC (clone 8*, Fig. 4.2B, 4.2C) was chosen for further analysis. To test whether it was able to drive *Venus* expression in the *Nkx2.2a* domains in the neural tube, it was injected into the cytoplasm of early one cell stage zebrafish embryos. Varying concentrations were tested and the embryos were incubated for 24 hpf. The embryos were fixed, immunostained for GFP (recognises Venus protein) and numbers of embryos positive for Nkx2.2-like *Venus* expression were counted (Table 6). Embryos analysed were injected with 3 different concentrations of BAC DNA, an average of 33% of these embryos stained positive for Venus (Table 6). There is a trend of an increased percentage of positive embryos as BAC concentration was increased (Table 6).

Analysis of the distribution of Venus in transgenic embryos indicated that the expression of *Venus* (Fig. 4.3) appeared to recapitulate endogenous expression of

Nkx2.2a (Fig. 3.4). Expression was apparent in the most ventral regions of the neural tube. In a large proportion of embryos strong expression of the fluorescent marker was seen the entire length of the ventral neural tube (Fig. 4.3A, 4.3C) and was particularly strong in anterior regions of the embryo (Fig. 4.3B, 4.3D). In approximately 20% of the embryos positive for Venus, expression was only observed in the ventral anterior neural tube including ventral forebrain and the ventral midline of the midbrain and hindbrain. In these embryos, Venus was not present in the spinal cord (data not shown). Expression in tissue other than the ventral neural tube, consisting of more than 1 or 2 positive cells, was observed in approximately 10% of embryos positive for Venus (as seen in Fig 4.3A). However, this ectopic expression was weak in comparison to the Nkx2.2-like expression and was not consistently observed in a spatially restricted location. Moreover, this ectopic expression was always accompanied by specific Nkx2.2-like expression in the neural tube.

The presence of bilateral pairs of Venus positive cells in the ventral neural tube (Fig. 4.3B, 4.3D) could be explained by the division of cells observed during zebrafish neurulation. It is consistent with previous observations of bilateral distribution of daughter cells across the neural tube midline (see Introduction: Ciruna et al., 2006; Concha and Adams, 1998; Geldmacher-Voss et al., 2003).

To confirm that the Venus expressing cells were located ventrally in the neural tube, vibratome sectioning and confocal microscopy was performed (Fig. 4.3E-4.3G). Embryos were counterstained with Phalloidin (red), which stained the actin cytoskeleton, to visualise neural tube morphology. Spinal cord sections (Fig. 4.3E) showed Venus expression in the most ventral cells of the neural tube, comparable to wild type expression of *Nkx2.2a* (Fig. 3.1G). Sections through the midbrain (Fig. 4.3F) confirmed that the Venus expression in more anterior regions of the neural tube was also restricted to *Nkx2.2a* expressing domains. This is also apparent in the high magnification view of the forebrain and midbrain (Fig. 4.3G).

These data suggest that the *Nkx2.2a* gene was correctly targeted with Venus and that all the regulatory elements required for the endogenous expression pattern are present

in the BAC. Using this *in vivo* assay, further analysis of the *Nkx2.2a* regulatory machinery was carried out.

Concentration of Construct Injected (ng/µl)	Number of Embryos Positive for Nkx2.2-like Venus Expression	Total Number of G0 Embryos Analysed (24 hpf)	% Embryos Positive for Nkx2.2-like Venus Expression
20	83	261	32%
50	11	31	35%
100	17	42	40%

Table 6 Results of injection of zNkx2.2aVenus BAC; a zebrafish *Nkx2.2a* containing BAC targeted with a fluorescent marker.

Table shows the concentration of the BAC injected into the cytoplasm of early one-cell stage zebrafish embryos. Analysis of embryos was carried out at 24 hours post fertilisation (hpf). At each concentration the number of embryos immunostained positive for Venus was recorded; these embryos were positive for Nkx2.2a-like Venus expression in the neural tube. The third column shows the total number of embryos analysed. The final column indicates the percentage of embryos positive for Nkx2.2a-like Venus expression at 24 hpf.

4.2 In silico Alignment and CNCR Identification

Utilising the results obtained in Chapter 3, we analysed the promoter sequences of the four vertebrate *Nkx2.2* and *Nkx2.9* genes. To do this, 9kb of sequence 5' of the *Nkx2.2* and *Nkx2.9* genes was extracted from the Ensembl database and analysed using the Pipmaker BLASTZ alignment (Schwartz et al., 2000). This program aligns large pieces of DNA and identifies small regions of homology. From this alignment (Fig. 4.4A) a region of non-coding DNA of approximately 250bp was identified which contains very high homology between both genes across all four species, we refer to this as the Conserved Non-Coding Region (CNCR). This region is highlighted in green and pink (Fig 4.4A) indicating unbroken regions with 50-70% homology and greater than 70% homology, respectively. Potential transcription factor binding sites within this region were identified using MatInspector from Genomatix (Fig. 4.4B). Several of these may be relevant to the regulation of *Nkx2* genes (see Discussion). Most notable was the presence of a motif identical to a canonical Gli Binding Site (GBS), which was present and conserved in each of the 8 sequences analysed, suggesting the possibility of direct regulation by Shh signalling.

4.3 *Deletion of CNCR from zNkx2.2aVenus BAC Leads to Loss of Reporter Expression*

4.3.1 Construction and Validation of zNkx2.2aVenusΔCNCR BAC

As injection into zebrafish of zNkx2.2aVenus BAC correctly recapitulated endogenous *Nkx2.2a* expression, we wanted to test if this expression was dependent upon the presence of the CNCR. To test this, the CNCR was deleted from the *Venus* targeted BAC. This was achieved using the same homologous recombination system, (Fig. 4.5).

Targeting of the BAC to create zNkx2.2aVenus BAC left an FRT_0 site within exon 1 of *Nkx2.2a*. In order to avoid recombination with this FRT_0 site during the second round of homologous recombination, FRT_5 sites (Schlake and Bode, 1994) were used to flank *Neo* (Fig. 4.5A). The frequency of recombination between FRT_5 sites and other *FRT* sites is <1% (Schlake and Bode, 1994). Targeting Vector 2 (Fig. 4.5A) therefore consisted of *Neo* driven by *β-lactamase* promoter, flanked by FRT_5 sites. This whole cassette was in turn flanked by homology arms that were designed to target *Neo* to the CNCR, thereby deleting it (Fig. 4.5B). *Flpe recombinase* was induced by provision of arabinose into the growth media, FRT_5 sites recombined, thereby deleting *Neo* (Fig. 4.5B). Due to the basal level of *FRT* recombination observed in the first homologous recombination to create zNkx2.2Venus BAC, no intermediate clones (Fig. 4.6Aii) were tested. The *FRT* recombination step was undertaken immediately and resulting BACs tested.

After the homologous recombination was carried out to delete the CNCR and *Neo*, PCR and Southern Blots were carried out to test for correct targeting (Fig. 4.6). PCR using primers that span the region targeted provided an indication that the targeting had occurred correctly (PCR primer locations indicated in Fig. 4.6Ai). The expected PCR product of the zNkx2.2aVenus BAC was 1.4kb (Fig. 4.6Ai). Upon insertion of targeting vector 2 into the BAC and recombining FRT_5 sites, the expected PCR product was 1.2kb (Fig. 4.6Aiii). PCR of the zNkx2.2aVenus BAC (Fig. 4.6B lane 7)

and targeted clones 3 and 6 (Fig 4.6B, lanes 3 and 6) provided products of the expected size. In contrast clone 4 generated a PCR product of ~1.4kb suggesting it was not correctly targeted (Fig. 4.6B lane 4).

To confirm this interpretation, the PCR product was digested with AvaI. Clones in which targeting had not occurred contained an AvaI site, while deletion of the CNCR removed this site (Fig. 4.6A). Therefore, the zNkx2.2aVenus BAC PCR product would be cut into 2 fragments upon digestion, whilst the clones with the CNCR deleted would remain as 1.2kb fragments. The results (Fig. 4.6C) indicated that clones 3 and 6 had lost the AvaI restriction site due to the maintenance of the 1.2kb fragment (Fig. 4.6C lanes 3 and 6). In contrast clone 4 had maintained the AvaI site and was digested producing two fragments, suggesting it had not been correctly targeted (Fig. 4.6C lane 4). The zNkx2.2aVenus BAC PCR product, upon digestion, contained 2 DNA fragments, confirming the presence of the AvaI restriction site (Fig. 4.6C lane 7).

To further confirm that the BAC had been correctly targeted, a Southern blot of the BACs digested with AvaI and PstI was performed using a radiolabelled *Venus* probe (Fig. 4.6D). Upon digestion of the zNkx2.2aVenus BAC with AvaI, the *Venus* probe would identify a fragment of 2.4kb (Fig. 4.6Ai). However, a correctly targeted BAC would remove the AvaI restriction site. Therefore the *Venus* probe would bind to a fragment of 5.1kb (Fig. 4.6Aiii). In addition, digestion of the zNkx2.2aVenus BAC DNA with PstI, followed by probing with *Venus* would label fragments of the same size as digestion of BACs in which the CNCR has been deleted (423bp Fig. 4.6Ai-iii). This is due to the position of the PstI sites in relation to the *Venus* insertion, however, any change in fragment size may indicate an incorrect recombination between FRT_0 and FRT_5 sites.

Digestion of the zNkx2.2aVenus BAC DNA with AvaI and probing with *Venus* provided a band of 2.4kb, as expected, (Fig. 4.6D lane 8). The same DNA digested with PstI and probed with *Venus*, labelled a fragment of approximately 423bp, again as predicted (Fig. 4.6D lane 16). After targeting the BAC and homologous

recombination to delete *Neo*, digestion and probing with *Venus* labelled a fragment of 5.1kb in BAC clones 3 and 6 (Fig. 4.6D lanes 3, 6, and 7). The PstI digest and probing of clones 3 and 6 labelled fragments of 423bp (Fig. 4.6D lanes 11, 14 and 15), the same size as that of the zNkx2.2aVenus BAC. The labelling of fragments of the same size in targeted and untargeted BACs indicated that no recombination between different *FRT* sites had occurred. The PCR and Southern Blot analysis indicated that clones 3 and 6 had been correctly targeted to delete the CNCR without altering the DNA surrounding the target site.

4.3.2 Analysis of Zebrafish Embryos Injected with zNkx2.2aVenus-ΔCNCR BAC

To test whether CNCR is required for expression of *Nkx2.2a,* purified zNkx2.2aVenusΔCNCR BAC DNA (clone 6* Fig. 4.6) was injected into the cytoplasm of early one cell stage zebrafish embryos at 20ng/μl and embryos were incubated for 24 hours. Embryos were fixed before immunostaining for GFP and numbers of positive embryos recorded (Table 7). In >99% of the embryos, no specific Nkx2.2-like staining in the neural tube was observed (Fig. 4.7A-4.7D). Transverse sections of embryos counterstained with Phalloidin showed there was no Venus in the neural tube (Fig. 4.7C'). Non-specific Venus was observed in ~10% of embryos. This non-specific expression was comparable to that observed on injection of zNkx2.2aVenus BAC (Fig. 4.3) and was present in a variety of tissues. In 4 out of 591 embryos analysed, staining was observed in the neural tube (Fig. 4.7E-4.7F) and appeared to be restricted to the *Nkx2.2a* domain. However the level of expression and number of expressing cells was low compared to zNkx2.2aVenus BAC.

Together these results provide evidence that the CNCR in the context of the whole gene is required for correct endogenous *Nkx2.2a* expression.

Concentration of Construct Injected (ng/µl)	Number of Embryos Positive for Nkx2.2-like Venus Expression	Total Number of G0 Embryos Analysed (24 hpf)	% Embryos Positive for Nkx2.2-like Venus Expression
20	2	413	0.5%
40	2	178	1%

Table 7 Results of injection of zNkx2.2aVenusΔCNCR BAC; a zebrafish *Nkx2.2a* containing BAC targeted with a fluorescent marker with the CNCR deleted.

The BAC contains a *Venus* marker targeted to Exon 1 of zebrafish *Nkx2.2a* and the identified 250bp CNCR has been deleted. Table shows the concentration of the BAC injected into the cytoplasm of early one-cell stage zebrafish embryos. Analysis of embryos was carried out at 24 hours post fertilisation (hpf). For each round of injections the number of embryos immunostained positive for GFP was recorded; these embryos were positive in an Nkx2.2a-like domain. The third column shows the total number of embryos analysed. The final column records the percentage of embryos positive for *Venus* in an Nkx2.2-like domain.

Figure 4.1 Schematic for the construction of zNkx2.2aVenus BAC.

A Diagram showing the position of the zK257G4 BAC with respect to the *Nkx2.2a* gene within the zebrafish genome. The gene is located at one end of the BAC; with the direction of gene transcription marked by a black arrow and a white cross marks the position of the Conserved Non-Coding Region (CNCR). **B** A diagram of the BAC containing the *zNkx2.2a* locus consisting of two exons and the procedure for targeting *Venus* to the first exon (scale bar is 200bp). This BAC was transfected into EL250 bacterial cells. The targeting vector contained the *Venus* marker (+ SV40 polyA tail) fused to a Neomycin (*Neo*) resistance gene (+ *β-lactamase* promoter) all flanked by FRT_0 sites. This entire cassette was flanked by homology arms of approximately 500bp, which targeted the cassette to the first exon of *Nkx2.2a* by homologous recombination. This was achieved by transfection of the targeting vector into the EL250 bacteria followed by a heat shock that activated the recombination machinery. This first product (**Ci**) then underwent a second recombination, induced by introduction of arabinose into the culture medium, which directed *Flpe recombinase* expression. The FRT_0 sites recombined to delete the DNA between them. The resulting BAC (**Cii**) contained Venus and one FRT_0 site within the first exon of the *Nkx2.2a* gene.

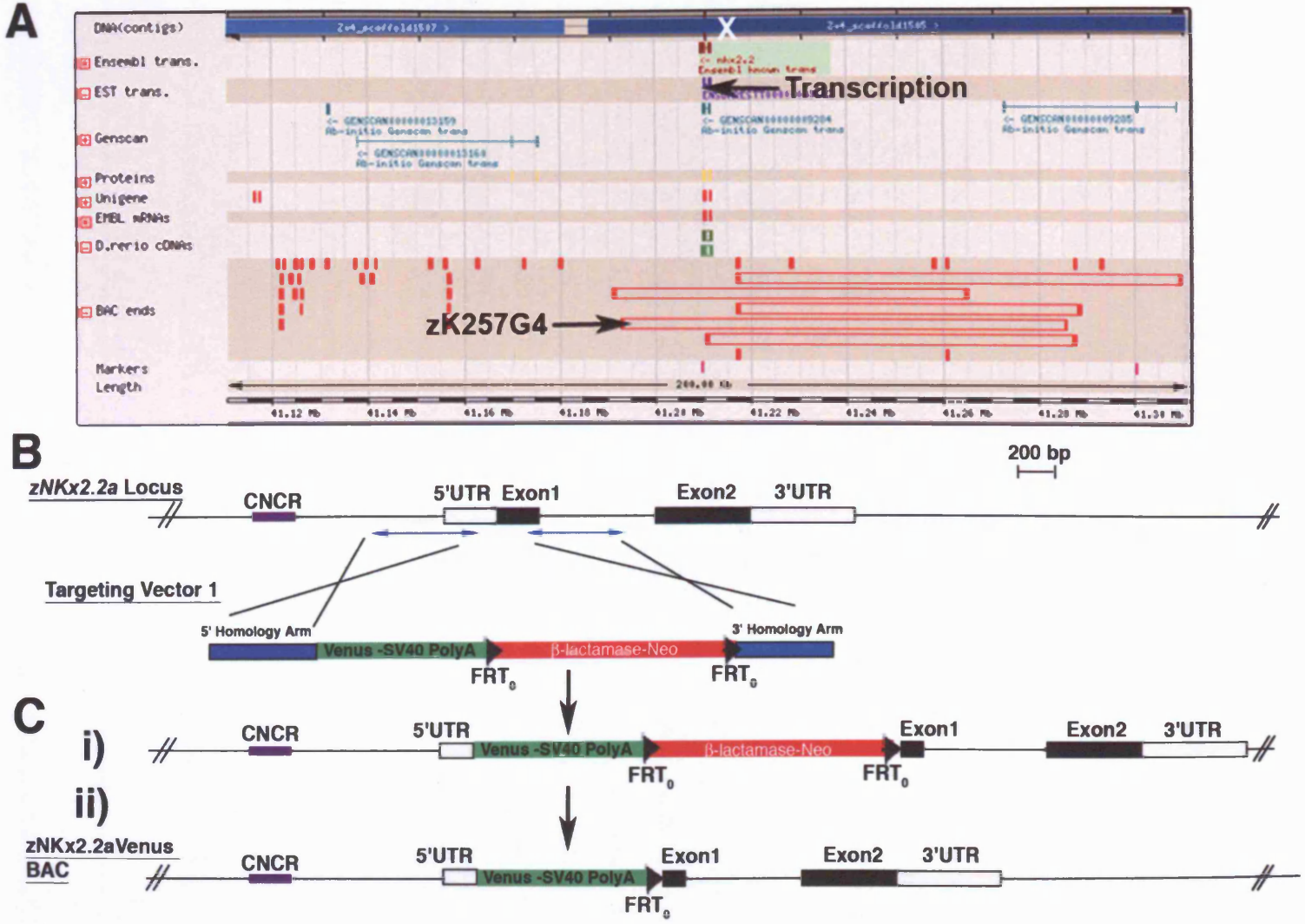

A
DNA(contigs)
Ensembl trans.
EST trans.
Genscan
Proteins
Unigene
EMBL mRNAs
D.rerio cDNAs
BAC ends
Markers
Length
Transcription
zK257G4
B
200 bp
zNKx2.2a Locus
CNCR
5'UTR
Exon1
Exon2
3'UTR
Targeting Vector 1
5' Homology Arm
Venus -SV40 PolyA
β-lactamase-Neo
3' Homology Arm
FRT0
FRT0
C
i)
CNCR
5'UTR
Venus -SV40 PolyA
β-lactamase-Neo
Exon1
Exon2
3'UTR
FRT0
FRT0
ii)
zNKx2.2aVenus
BAC
CNCR
5'UTR
Venus -SV40 PolyA
Exon1
Exon2
3'UTR
FRT0

Figure 4.2 Testing zNkx2.2aVenus BAC for correct targeting.

Upon targeting of the *Nkx2.2a* containing BAC, the correct positioning of the targeting cassette was tested. This was carried out using a Southern blot approach, a schematic of which can be seen in **A**. The 2 radiolabelled probes used were the 3' homology arm and *Neo*, the positions of binding of these probes are marked on the diagrams (**A**) with red arrows. The probes were hybridised to DNA cut with MfeI restriction enzyme (M). The sizes of the bands to which the probe was expected to bind are marked with black arrows. The Southern blots using the 3' arm probe (**B**) and the *Neo* probe (**C**) are shown. **B** and **C:** lane 1 is the original untargeted BAC; lanes 2 and 3 show recombined BACs (clones 1 and 2) targeted with *Venus-Neo*; lanes 4-11 show BACs in which the *FRT* sites have recombined to delete the *Neo* marker (clones 1 to 8, * marks the clone used for further experiments). The probe labelled a band in lane 1 of 4.8kb, as expected. In lanes 2 and 3, recombined BACs show a drop in the size of the band to 3.7kb, upon deletion of *Neo* the band size drops further to 2.4kb (lanes 4-11). The 2.4kb labelled band in the recombined BAC (lanes 2 and 3) represents a recombination and deletion of *Neo*, this was confirmed by probing the digest with a *Neo* probe (**C**). The *Neo* probe labelled a 3.7kb band in the lanes containing the digestion of the recombined BACs and those containing clones in which *Neo* had not been successfully deleted (lanes 2, 7). This experiment provided evidence that the *Venus* has been targeted correctly to the *Nkx2.2a* locus in zNkx2.2aVenus BAC.

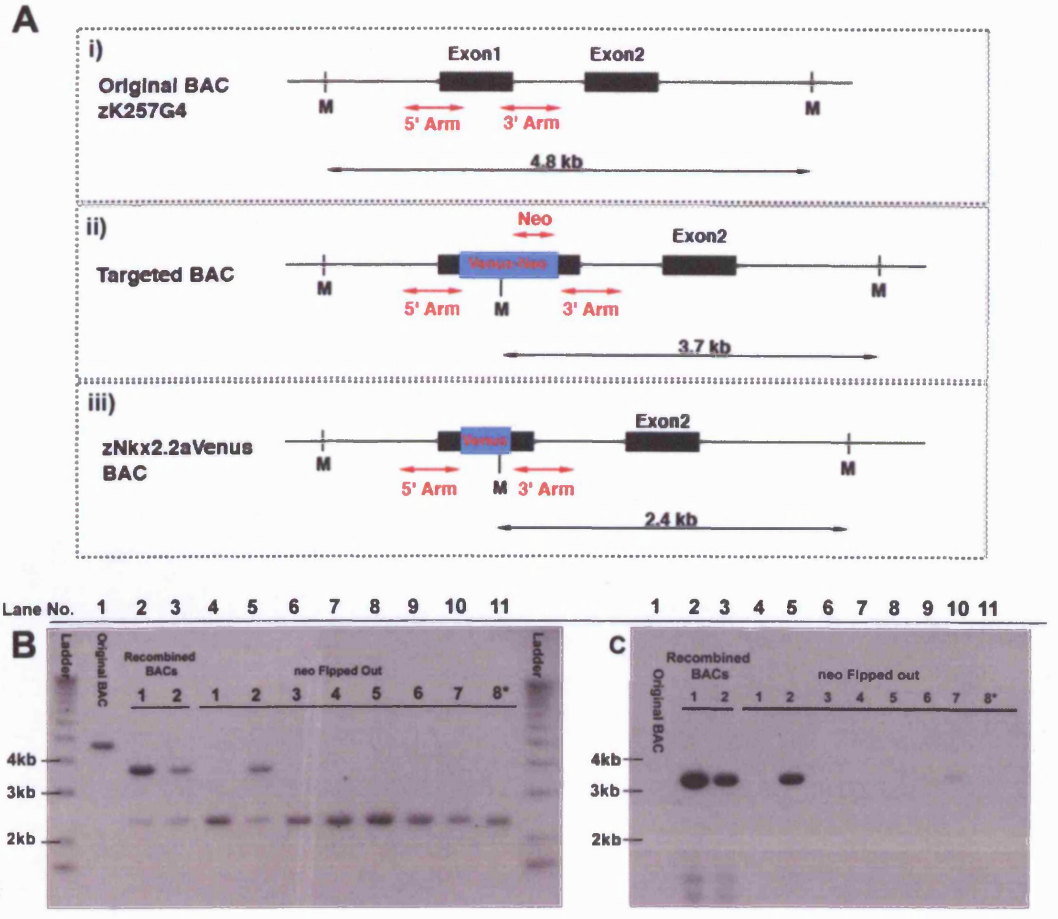

A
i)
Original BAC
zK257G4
Exon1
Exon2
M
5' Arm
3' Arm
M
4.8 kb
ii)
Targeted BAC
Neo
Exon2
M
5' Arm
M
3' Arm
M
3.7 kb
iii)
zNkx2.2aVenus
BAC
Exon2
M
5' Arm
M
3' Arm
M
2.4 kb
Lane No. 1 2 3 4 5 6 7 8 9 10 11
B
Ladder
Original BAC
Recombined BACs
neo Flipped Out
1 2 1 2 3 4 5 6 7 8*
Ladder
4kb
3kb
2kb
1 2 3 4 5 6 7 8 9 10 11
C
Original BAC
Recombined BACs
neo Flipped out
1 2 1 2 3 4 5 6 7 8*
4kb
3kb
2kb

Figure 4.3 Expression patterns of zNkx2.2aVenus BAC in 24hr zebrafish embryos after injection.

A, C 24 hour embryos at 10X magnification. Embryo views are lateral, with the head turned for a dorsal view, **B, D** 20X magnification of anterior regions of the same embryos. Embryos were injected with zNkx2.2aVenus BAC DNA at early 1 cell stage in the cytoplasm at 20ng/μl. Embryos were incubated to 24 hpf, fixed and stained for GFP by whole-mount immunohistochemistry. GFP was observed in the ventral neural tube from anterior to posterior (**A, C**) or in anterior regions alone in 33% of the embryos analysed. A small amount of non-specific expression was seen (**A**), however this was limited. The expression pattern of the Venus marker recapitulates the endogenous *Nkx2.2a* expression (see Fig. 3.4). High magnification confocal microscopy was performed for embryos counterstained with Phalloidin (red, **E-G**). 100μM vibratome transverse sections were obtained of trunk (**E**) and midbrain (**F**), showing Nkx2.2a-like Venus expression in the ventral neural tube. **G** a dorsal view of the fore- and midbrain.

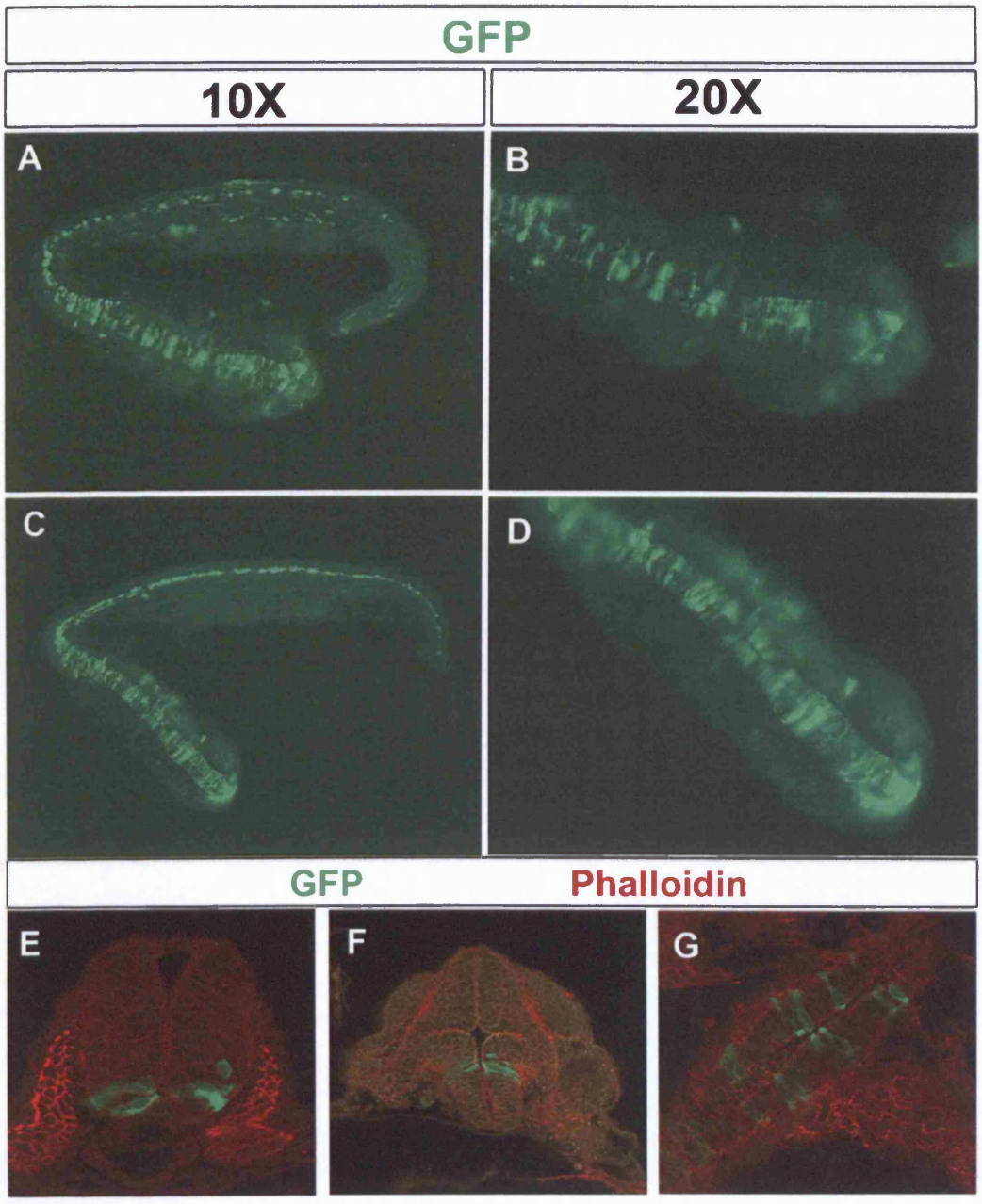

GFP
10X
20X
A
B
C
D
GFP
Phalloidin
E
F
G

Figure 4.4 BLASTZ alignment of human, mouse, zebrafish and *Fugu Nkx2.2* and *Nkx2.9* gene promoters identifying a Conserved Non-Coding Region (CNCR) and putative transcription factor binding sites located within the CNCR.

9kb of DNA 5' of the 8 genes was aligned using a Pipmaker BLASTZ alignment. This alignment, **A**, identified a region of homology when comparing all of the sequences to the human *Nkx2.2* gene. This region, termed the Conserved Non-Coding Region (CNCR), is located approximately 2kb 5' of the transcription start site. Highlighted in green and pink are regions of homology of 50-70% and >70% identity respectively. **B** The *Nkx2.2* genes are more similar to each other than to the *Nkx2.9* genes. Many short motifs of sequence homology are shared between the genes (shaded boxes) these correspond to characterised binding motifs for several transcription factors.

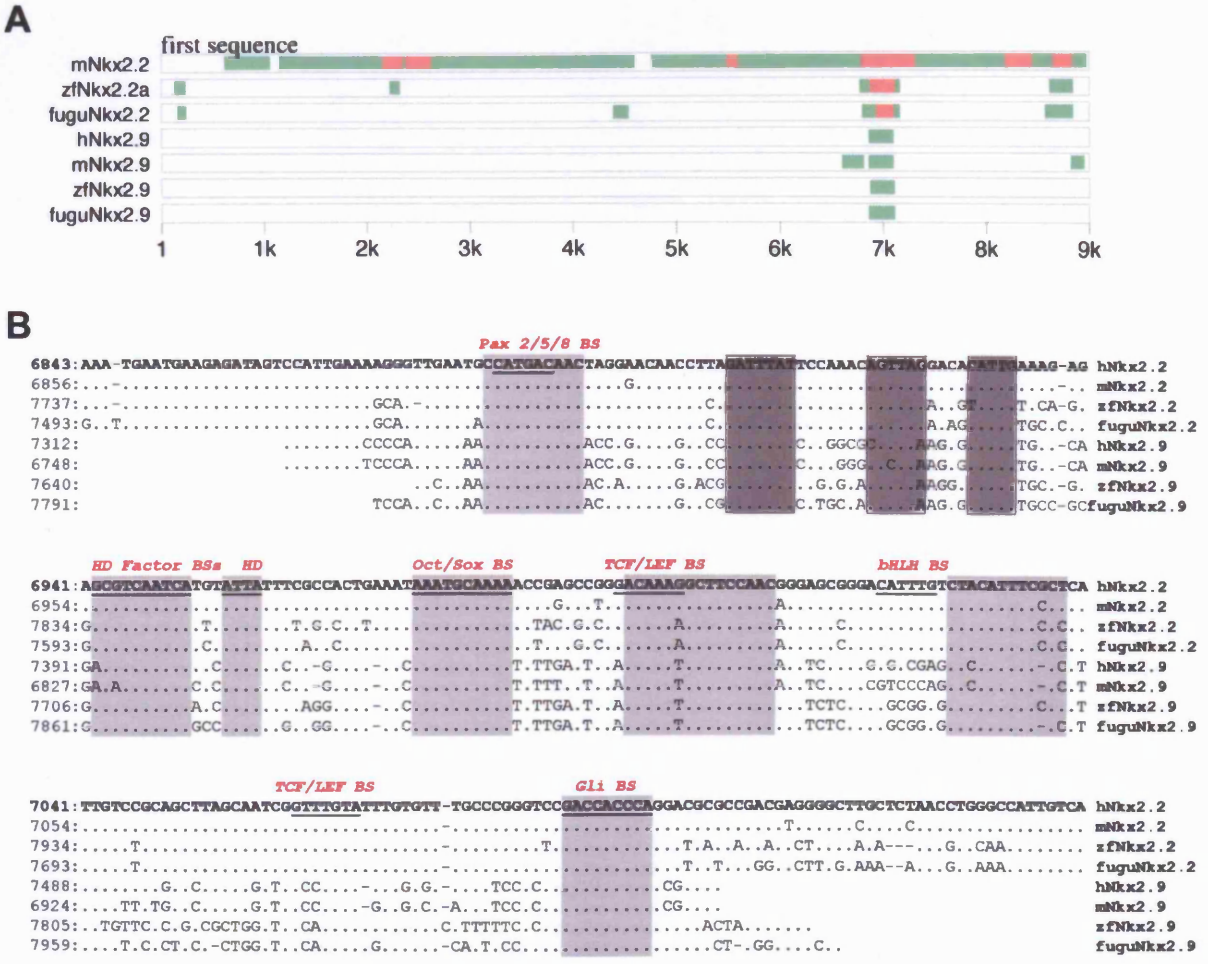

A
first sequence
mNkx2.2
zfNkx2.2a
fuguNkx2.2
hNkx2.9
mNkx2.9
zfNkx2.9
fuguNkx2.9
1
1k
2k
3k
4k
5k
6k
7k
8k
9k
B
Pax 2/5/8 BS
HD Factor BSs
HD
Oct/Sox BS
TCF/LEF BS
bHLH BS
TCF/LEF BS
Gli BS
hNkx2.2
mNkx2.2
zfNkx2.2
fuguNkx2.2
hNkx2.9
mNkx2.9
zfNkx2.9
fuguNkx2.9

Figure 4.5 Schematic for constructing zNkx2.2aVenusΔCNCR BAC.

A second homologous recombination experiment was set up to delete the CNCR from the zNkx2.2aVenus BAC. **A** The *zNkx2.2a* locus in BAC zK257G4 targeted with *Venus* into exon 1 (scale bar 200bp). Targeting Vector 2 consisted of *Neo* driven by a *β-lactamase* promoter flanked by FRT_5 sites, which are in turn flanked by homology arms. The homology arms were designed to target *Neo* precisely to the CNCR, thereby deleting it. The targeting vector was transfected into EL250 cells containing the zNkx2.2aVenus BAC. A heat shock was administered to the cells, which induced recombination between the BAC and the targeting vector. **B** The resulting BAC contained a *β-lactamase Neo* in place of the CNCR. *Flpe recombinase* was under the influence of an arabinose inducible promoter. Addition of arabinose to the growth media induced recombination between the FRT_5 sites, resulting in the deletion of the CNCR.

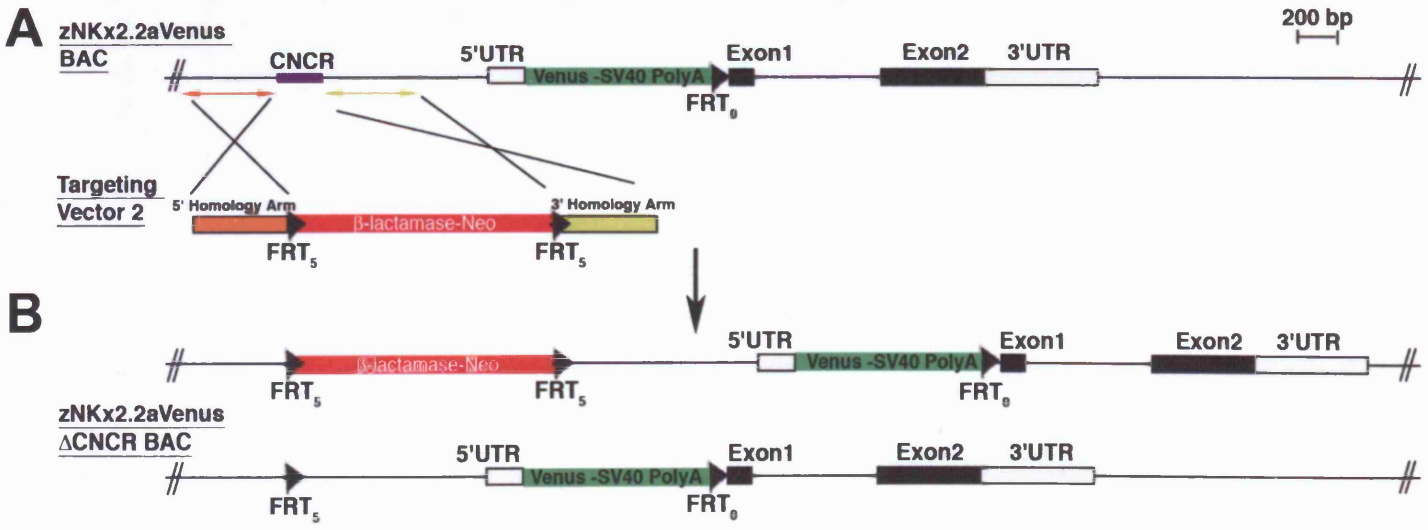

A
zNKx2.2aVenus
BAC
CNCR
5'UTR
Venus -SV40 PolyA
Exon1
Exon2
3'UTR
200 bp
FRT0
Targeting
Vector 2
5' Homology Arm
β-lactamase-Neo
3' Homology Arm
FRT5
FRT5
B
β-lactamase-Neo
FRT5
FRT5
5'UTR
Venus -SV40 PolyA
Exon1
Exon2
3'UTR
FRT0
zNKx2.2aVenus
ΔCNCR BAC
5'UTR
Venus -SV40 PolyA
Exon1
Exon2
3'UTR
FRT5
FRT0

Figure 4.6 Testing zNkx2.2aVenusΔCNCR BAC for correct deletion of CNCR upstream of *zNkx2.2a* gene.

Upon targeting the *Nkx2.2a*-containing BAC to delete the CNCR, correct positioning of the *FRT_5-Neo* cassette had to be verified. This was performed using a combination of Southern blots and PCR, a schematic of which can be seen in **A**. The DNA bound by the PCR primers is marked with red arrows 1 and 2, the size of the expected product is marked (red arrows) above the BAC (represented by a solid black line). The Southern blot probe recognised the inserted fluorescent marker *Venus*, the position of binding is marked (black arrows) above the BAC. The probes were hybridised to DNA cut with restriction enzymes AvaI (A) and PstI (P). The sizes of the bands to which the probe was expected to bind are marked (black arrows) below the BAC. **B, C,** show the agarose gels of uncut and AvaI cut PCR products respectively. The PCR was performed on targeted zNkx2.2aVenusΔCNCR BACs (lanes 1-6, * denotes clone used for further experiments) and zNkx2.2aVenus BAC (lane 7). Expected PCR product size of 1.2kb was observed in lanes 1, 3 and 6 (**B**), in which CNCR and *Neo* had been deleted. Digested products were the same size as uncut due to the loss of AvaI site (**C**). Uncut product size of 1.4kb for the zNkx2.2aVenus BAC (**B**), and digested band sizes of 0.6kb and 0.8kb were observed as expected (**C**). **D** BAC DNA digested with both AvaI (lanes 1-8) and PstI (lanes 9-16) and probed for the presence of *Venus* sequence. Lanes 1-7 and 9-15 are BACs, which lack the CNCR and *Neo*. Lanes 8 and 16 contain DNA from zNkx2.2aVenus BAC. Upon AvaI digestion (**D**, lanes 6 and 7), the *Venus* probe binds to expected band of 5.1kb in BACs, which have had the CNCR and *Neo* deleted. The labelled band was 2.4kb in the lane containing digested zNkx2.2aVenus BAC (**D**, lane 8). Upon PstI digestion, there was no change in labelled band size (423bp) between zNkx2.2aVenus BAC and the BACs in which the CNCR and *Neo* had been deleted (**D**, lanes 14 and 15).

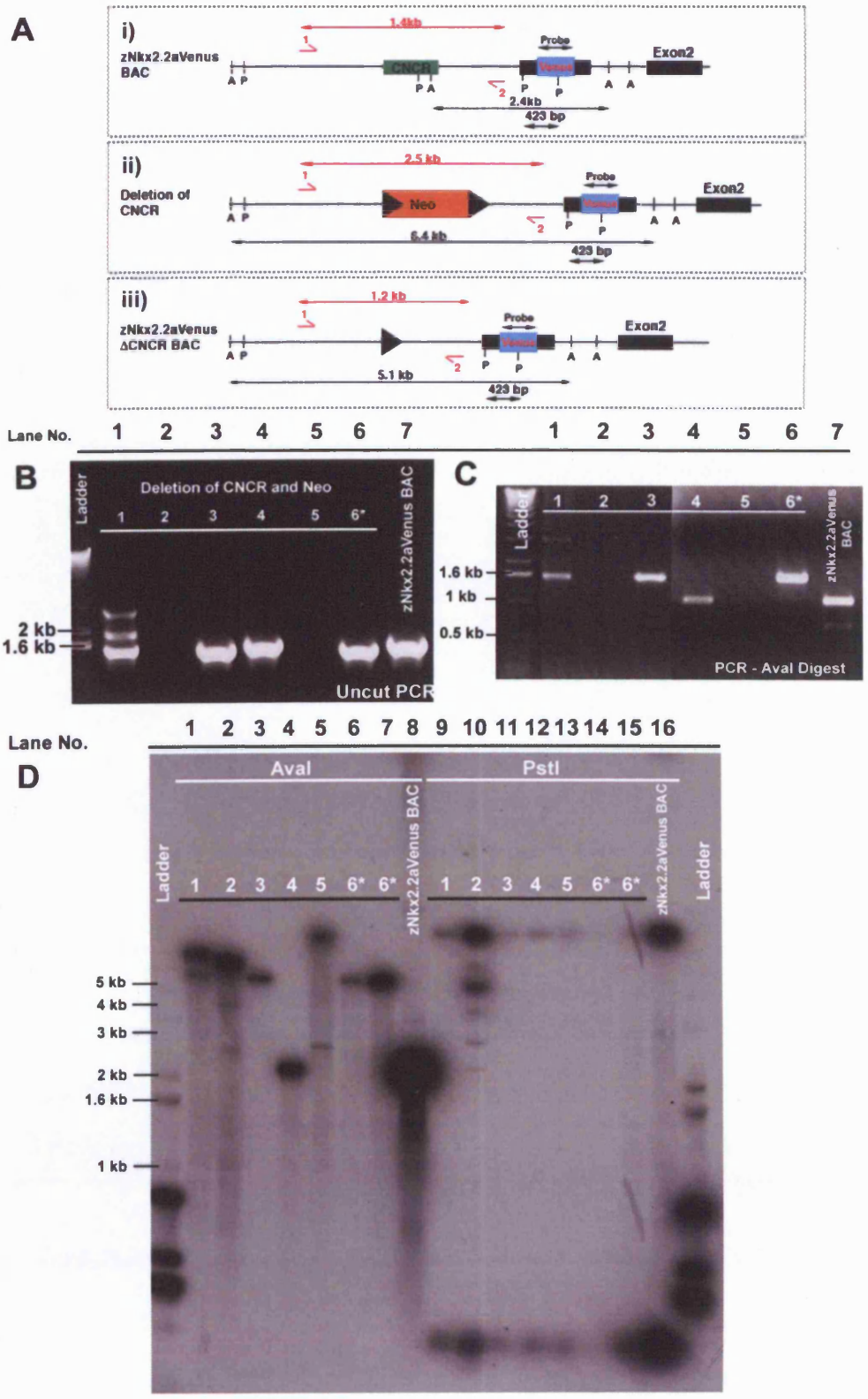
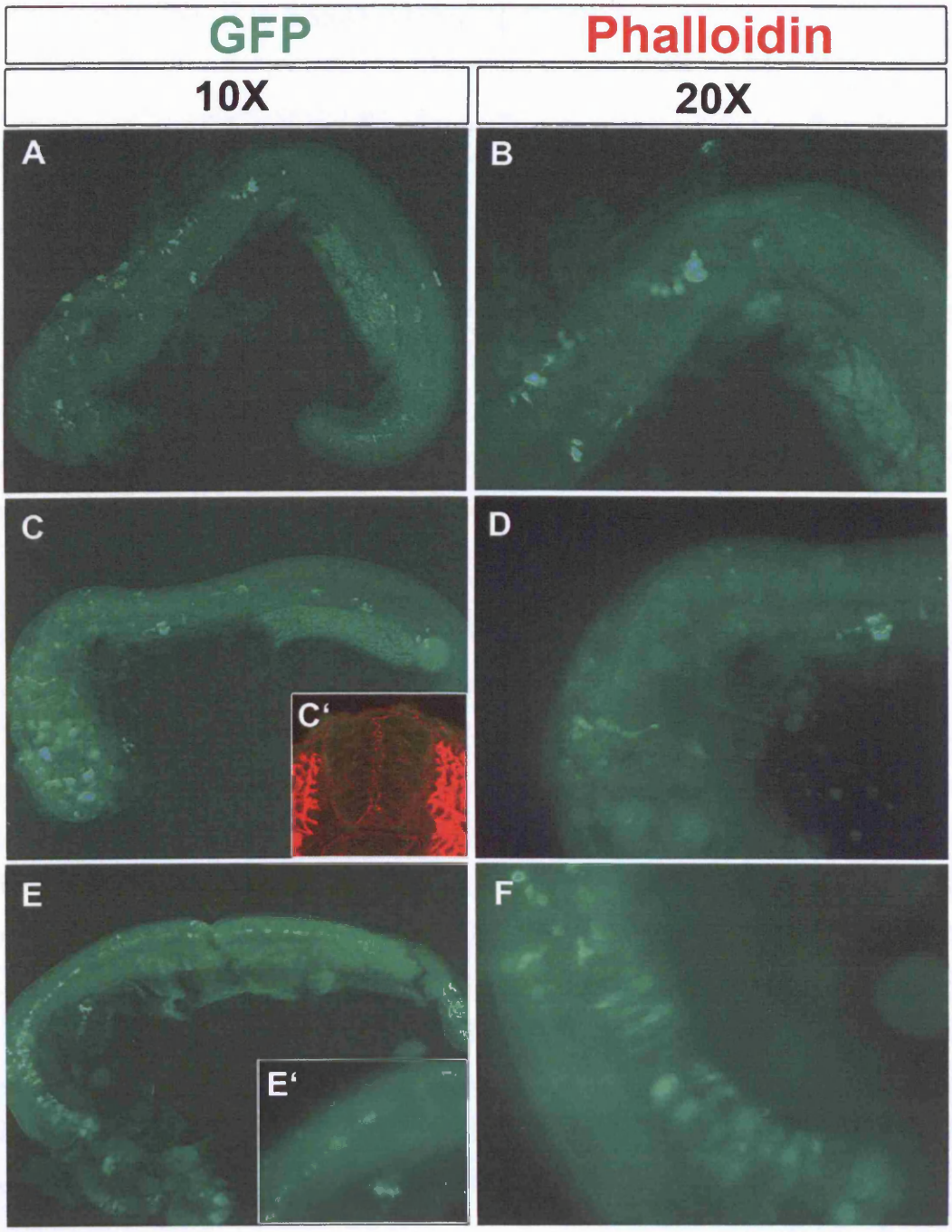

A
i)
zNkx2.2aVenus BAC
1.4kb
Probe
Exon2
CNCR
2.4kb
423 bp
ii)
Deletion of CNCR
2.5 kb
Neo
6.4 kb
423 bp
iii)
zNkx2.2aVenus ΔCNCR BAC
1.2 kb
5.1 kb
423 bp
Lane No. 1 2 3 4 5 6 7 1 2 3 4 5 6 7
B
Deletion of CNCR and Neo
Ladder
zNkx2.2aVenus BAC
2 kb
1.6 kb
Uncut PCR
C
1.6 kb
1 kb
0.5 kb
PCR - AvaI Digest
Lane No. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
D
AvaI
PstI
5 kb
4 kb
3 kb
2 kb
1.6 kb
1 kb

Figure 4.7 Expression patterns of zNkx2.2aVenusΔCNCR BAC in 24hr zebrafish embryos after injection.

A, C, E 24 hour embryos at 10X magnification. Embryo views are lateral, with the head turned for a dorsal view. **B, D, F** 20X magnification of anterior regions of the same embryos. Embryos were injected with zNkx2.2aVenusΔCNCR BAC DNA at early one cell stage in the cytoplasm at 20ng/μl. Embryos were incubated to 24 hpf, fixed and stained for GFP by whole-mount immunohistochemistry. In >99% of the injected embryos (587/591) either no expression was seen at all or non-specific expression was observed in many different tissue types (**A-D**). Suggesting that CNCR deletion caused loss of specific Nkx2.2-like expression, therefore it is necessary for its expression. **C'** To confirm there was no ventral neural tube expression of Venus, embryos were counterstained with Phalloidin (red), vibratome sectioned (100μM) and viewed with confocal microscopy. **E, F** embryos in which Nkx2.2-like ventral neural tube staining was observed, cells positive for Venus were observed in the ventral spinal cord (**E'**) and ventral hindbrain (**F**). However, expression was weaker and observed in fewer cells than upon injection of zNkx2.2aVenus BAC.


GFP
Phalloidin
10X
20X
A
B
C
C'
D
E
E'
F

5 Results: Analysing the Role of Conserved Non-Coding Region (CNCR); Assay Development

5.1 Generation of Promoter Reporter Constructs and Assay Results

5.1.1 Generation of Promoter Reporter Constructs

In silico analysis identified *Nkx2.2* conserved non-coding region (CNCR) a 250bp region close to the transcriptional start site of the *Nkx2.2* and *Nkx2.9* genes (Chapter 4), that is highly conserved across 4 diverse genomes examined. Moreover, transient assays in zebrafish embryos using a BAC containing an *Nkx2.2a* reporter indicated the necessity for the CNCR to direct correct *Nkx2.2* expression.

We therefore sought to test if the CNCR was sufficient to direct expression of a reporter gene in the ventral neural tube. Regions of promoter sequence, containing the CNCR, from mouse and zebrafish *Nkx2.2* and *Nkx2.9* of between 1kb and 1.3kb were cloned upstream of a hsp68lacZ reporter construct (Kothary et al., 1989; Logan et al., 1993). The orientation of these regions were all 3'-5' with respect to the hsp68lacZ, to test for the orientation independent enhancer activity. mNkx2.9$^{CNCR+Prom}$LacZ consisted of 1kb DNA from the mouse *Nkx2.9* promoter sequence fused to hsp68lacZ (Fig. 5.1A), mNkx2.2$^{CNCR+Prom}$LacZ included an equivalent region from mouse *Nkx2.2* (Fig. 5.1B). In addition, two zebrafish constructs, containing different regions of the *Nkx2.2a* promoter; zNkx2.2$^{CNCR+Prom}$LacZ and zNkx2.2$^{CNCR+Prom}$LacZ-2 containing 1.3kb and 1kb of DNA respectively (Fig. 5.1C, 5.1D) were generated.

These four constructs were then tested *in vivo* to determine where in the embryo they directed *LacZ* expression. Various *in vivo* assays were performed to find the method that provided the most reliable, consistent results. These included chick electroporation, zebrafish injection and mouse pronuclear injection (PNI). The different techniques and results are discussed below.

5.1.2 Chick Electroporation Assay Development

The first assay relied on chick electroporation. The zebrafish *Nkx2.2a* construct, zNkx2.2$^{CNCR+Prom}$LacZ, was electroporated at a variety of dilutions at HH stage 10-12 (Hamburger and Hamilton, 1953). Embryos were harvested and stained for β-galactosidase activity. At high concentrations (500ng/μl) there was considerable, almost ubiquitous, expression in the neural tube (Fig. 5.2B), indicated by the blue staining. At lower concentrations (100ng/μl; Fig. 5.2A), β-galactosidase activity appeared more restricted to ventral regions of the neural tube corresponding to regions where *Nkx2.2* is expressed.

To confirm these data, further electroporations at 100ng/μl were carried out. Promoter construct plasmid DNA was co-electroporated with a control vector (pCAGGS: Niwa et al., 1991) expressing *GFP* at high levels. Therefore, any cell that was electroporated, expressed *GFP*, enabling identification of transfected tissue. Electroporated embryos were immunostained for both GFP and β-galactosidase (Fig. 5.2C-5.2H). Analysis of a series of transfected embryos indicated that while some embryos demonstrated ventrally restricted expression of zNkx2.2$^{CNCR+Prom}$LacZ, there was little consistency in the electroporations. Sometimes specific ventral staining was detected (Fig. 5.2C-5.2E) however in other embryos, a more ubiquitous expression pattern was seen using the same concentration and preparations of DNA. Performing the analysis of the embryos 48 hours post electroporation (hpe; Fig. 5.2F-5.2H) did not increase specificity, neither did lowering the concentration of DNA further (data not shown). Therefore, while this approach shows some promise, it will require further development before offering a reliable assay.

5.1.3 Zebrafish Injection Assay Development

We next tested transient analysis in zebrafish embryos as an assay for enhancer activity. The same construct (zNkx2.2$^{CNCR+Prom}$LacZ) was injected into early 1-cell zebrafish embryos. Fish were fixed and stained for β-galactosidase activity at 24 hours post fertilisation (hpf). This technique resulted in a high level of mosaic, non-

specific expression in injected zebrafish (Fig. 5.3A-5.3C). Small numbers of cells expressing the reporter were observed in what appeared to be the Nkx2.2 domain, in approximately 10% of injected embryos (black arrowheads in Fig. 5.3D). There was also *LacZ* expression in the notochord in many embryos (Fig. 5.3D). The specific Nkx2.2-like expression was observed in a higher proportion of embryos compared to injection of the control plasmid: lacZhsp68 reporter plasmid, containing no promoter fragment (results not shown).

In an attempt to improve this assay, a second method of injection was tested. This involved injecting the reporter DNA (i.e. hsp68lacZ) and the promoter sequence ($zNkx2.2^{CNCR+Prom}$) as separate pieces of DNA (Muller et al., 1997). This has been used as a rapid and efficient reporter assay for identifying expression patterns of zebrafish enhancer elements (Muller et al., 1999; Muller et al., 1997; Woolfe et al., 2004). This method relies upon integration of both DNA fragments into chromosomal DNA. DNA injected into zebrafish embryos integrates randomly into the fish genome early in development. Concatamerisation of the reporter and enhancer fragments occurs, allowing the enhancer to exert its regulatory effects upon the reporter in a cis- or trans- manner (reviewed in Muller et al., 2002). Expression of the reporter will only be seen in transient embryos when both fragments have been inserted together. Thus, this method negates the problem of episomal expression from injected plasmids.

This method of injection resulted in much more tissue specific expression, compared to injection of a single reporter plasmid (Fig. 5.4). Less ectopic expression was observed, however expression remained highly mosaic. Activity of β-galactosidase was detected in ventral neural tube cells (as indicated by black arrowheads in the high magnification images Figs. 5.4B, 5.4D, 5.4F). On close inspection, the cells positive for β-galactosidase seemed to be located adjacent to the floor plate and therefore in the correct location to be reporting Nkx2.2 activity. Once again, using this method of injection, specific expression was detected in a higher percentage of injected embryos (30%) compared to those injected with the reporter alone (10%; results not shown). Nevertheless, the mosaicism and ectopic nature of expression in both of the zebrafish assays tested did not make either method suitable for obtaining clear and precise results.

5.1.4 Mouse Pronuclear Injection Assay Development

Finally mouse pronuclear injection (PNI) was tested. This involves injection of linear DNA into mouse pronuclei, which are then transferred into female hosts. Development of embryos was allowed to proceed until the required stages, at which point they were harvested for analysis. E10.5 embryos were recovered and stained for β-galactosidase activity (Fig. 5.5). All three constructs analysed, resulted in ventral Nkx2.2/2.9-like *LacZ* expression along the length of the neural tube.

In the initial experiment using mNkx2.9$^{CNCR+Prom}$LacZ construct; 3 out of 13 embryos demonstrated β-galactosidase activity. Expression was observed along the entire length of the neural tube including the midbrain and the spinal accessory nerve (Fig. 5.5A, 5.5B). This is consistent with data from Santagati et al. (2003) who used a similar construct. The expression was restricted to neural tissue, suggesting there are regulatory regions within the 1kb sequence limiting expression to the neural tube. From transverse cryo-sections (Fig. 5.5B), *LacZ* expression was observed in 3 bands of the neural tube: ventrally; in an intermediate domain; and in the most dorsal tip of the neural tube. The ventral domain appears to correspond to the endogenous *Nkx2.2/2.9* expression (Fig. 3.1E, 3.1F). The other regions of β-galactosidase expression do not coincide with endogenous domains of *Nkx2.9* expression (Fig. 3.1F). This suggests that mNkx2.9$^{CNCR+Prom}$LacZ lacks regulatory elements necessary to block dorsal or intermediate expression. The expression of *Nkx2.9* in the trunk in mice is normally down-regulated at E10.5 (Briscoe et al., 1999), however, in E10.5 mice there was still *LacZ* expression from mNkx2.9$^{CNCR+Prom}$LacZ. This may be due to the persistence of LacZ, which is recognised as having a long half-life. Alternatively, the loss of elements that would normally down-regulate expression at this point in development might account for persistent expression. This assay generated a significant proportion of injected embryos positive for the *LacZ* reporter, with little or no ectopic expression, compared to chick and zebrafish assays discussed above.

We next tested mNkx2.2$^{CNCR+Prom}$LacZ. This construct comprises a region of the mouse *Nkx2.2* promoter equivalent to that of the mouse *Nkx2.9* (Fig. 5.1B).

Alignment of the mouse *Nkx2.2* and *Nkx2.9* promoters was used to identify this region (alignment not shown). Mouse pronuclear microinjection was performed and E10.5 transient embryos were analysed, 1 out of 13 embryos were positive for β-galactosidase activity. Wholemount staining of E10.5 embryos showed β-galactosidase enzyme activity along the length of the neural tube (Fig. 5.5C). Transverse cryo-sections of the embryo revealed *LacZ* expression in ventral regions of the spinal cord (Fig. 5.5D).

In a further experiment, zNkx2.2$^{CNCR+Prom}$LacZ was injected, 1 out of 10 recovered embryos expressed β-galactosidase at E10.5 (Fig. 5.5E). Expression of *LacZ* was confined to the most ventral domain of the neural tube (Fig. 5.5F), the position of which was equivalent to the endogenous expression of *Nkx2.2/2.9* (Fig. 3.1E, 3.1F). This construct therefore appears sufficient to reproduce the endogenous expression of *Nkx2.2*.

Together these data suggest that the mouse PNI provides a reliable assay for testing reporter activity. The analysis indicated a high degree of specificity with low levels of ectopic expression. Further analysis of reporter constructs was carried out using this method.

5.2 Analysis of Stable Mouse Transgenic Lines for Nkx2 Reporter Constructs

5.2.1 Analysis of mNkx2.9$^{CNCR+Prom}$LacZ Stable Line

A stable mouse line containing the mNkx2.9$^{CNCR+Prom}$LacZ construct was obtained and the expression pattern of *LacZ* from E9.5 to E13.5 was analysed (Fig. 5.6). Wholemount analysis of reporter expression revealed strong expression at E9.5 in the neural tube (Fig. 5.6A), which continued at both E10.5 (Fig. 5.6D) and E11.5 (Fig. 5.6G). In wild type embryos, *Nkx2.9* is down-regulated in the trunk by E10.5 (Briscoe et al., 1999), in the transgenic line this was not the case. This may reflect the stability of the β-galactosidase, which would lead to a perdurance of enzyme activity

within embryos. At E12.5 (Fig. 5.6J) and E13.5 (Fig. 5.6M) the *LacZ* expression had decreased in the majority of the spinal cord but could still be detected in more anterior neural tube and in younger, posterior spinal cord.

Wax sections of embryos stained for β-galactosidase activity and counterstained with eosin, show clearly the down-regulation of expression in the neural tube at later time points. At E9.5 and E10.5 (Fig. 5.6B, 5.6E), expression at hindbrain level of the neural tube showed reporter expression in both lateral ventral portions of the neural tube and in intermediate regions as previously seen in transient embryos. Expression in the migrating accessory nerve could also be seen at E10.5 (Fig. 5.6E). At the forelimb level of the spinal cord, at E9.5 and E10.5 (Fig. 5.6C, 5.6F) expression was seen in an Nkx2.9-like manner in ventral neural tube and in an ectopic intermediate region. By E11.5 (Fig. 5.6H, 5.6I) expression in the ventral neural tube at spinal cord levels had been lost as would be expected for wild type *Nkx2.9*. At later time points the neural tube expression of the reporter construct had been lost, however, a small amount of expression persisted at E12.5 at hindbrain levels of the neural tube (Fig. 5.6K).

During patterning of the ventral neural tube, Nkx2 and Pax6 cross repress each others expression (Briscoe et al., 2000; Briscoe et al., 1999; Ericson et al., 1997b). To test to see if the mNkx2.9$^{CNCR+Prom}$LacZ reporter was sensitive to repression by Pax6 the mNkx2.9$^{CNCR+Prom}$LacZ line was crossed with mice that lack functional Pax6 (*Sey*) (Hill et al., 1991). These mice produce a truncated form of the Pax6 protein, resulting in *Nkx2.2* and *Nkx2.9* expression expanding dorsally in the neural tube (Briscoe et al., 1999; Ericson et al., 1997b). The expansion of endogenous *Nkx2.2* expression in embryos lacking Pax6 was evident (Fig. 5.7F; black arrow heads) compared to heterozygous littermates (Fig. 5.7E). Moreover, in homozygous mice lacking Pax6, there was a dorsal expansion of the reporter (Fig. 5.7B, 5.7D) compared to heterozygous littermates (Fig. 5.7A, 5.7C). This was most apparent at anterior levels of the spinal cord (Fig. 5.7A, 5.7B) compared to posterior levels (Fig. 5.7C, 5.7D). This finding is consistent with a greater expansion of Nkx2 at anterior levels compared to posterior levels in *Sey* mice (Ericson et al., 1997b). Strikingly, the dorsal limit of the reporter expansion appeared to coincide with the ventral limit of Pax7

expression (Fig. 5.7H). These results indicate that the regulatory elements necessary for repression of Nkx2 genes by Pax6 are present in mNkx2.9$^{CNCR+Prom}$LacZ. The absence of Pax6 also appeared to de-repress reporter expression in the floor plate (Fig. 5.7B, 5.7D, 5.7F, 5.7H), however this may be due to a slight timing difference in the two embryos compared. Alternatively, it may be due to the long half-life of LacZ causing perdurance of the reporter. In order to establish if there really was floor plate expression, further analysis needs to be carried out.

Stable lines of mice containing both of the zebrafish reporter constructs were also generated. The expression patterns of the promoter constructs from E9.5 to E13.5 were analysed for these lines (Fig. 5.8, 5.12). Immunohistochemistry for the two lines at E10.5 (Fig. 5.9, 5.10, 5.13, 5.14) and E11.5 (Fig. 5.11, 5.15) was also performed to determine the population of neurons positive for LacZ in an intermediate position in the neural tube.

5.2.2 Analysis of zNkx2.2$^{CNCR+Prom}$LacZ Stable Line

A stable mouse line containing the zNkx2.2$^{CNCR+Prom}$LacZ construct was obtained and the expression pattern of *LacZ* from E9.5 to E13.5 was analysed (Fig. 5.8). Analysis of wholemount embryos assayed for β-galactosidase activity showed very strong expression at E9.5 (Fig. 5.8A) along the length of the neural tube. This strong expression persisted to E10.5 (Fig. 5.8D) and E11.5 (Fig. 5.8G). At E12.5 (Fig. 5.8J) and E13.5 (Fig. 5.8M), β-Gal activity was still apparent but staining was much weaker in the neural tube, especially in the anterior neural tube. The *LacZ* expression pattern was similar to that of endogenous *Nkx2.2* in mouse embryos, where there are many progenitor cells expressing *Nkx2.2* at earlier developmental stages (Briscoe et al., 1999; Ericson et al., 1997b). However, at later time points, E12.5 and E13.5, fewer progenitor cells are present, only a very few post mitotic cells may still be expressing *Nkx2.2*.

Embryos expressing zNkx2.2$^{CNCR+Prom}$LacZ transgene were vibratome sectioned and hindbrain and forelimb sections were analysed from E9.5 to E13.5 (Fig. 5.8).

Hindbrain sections at E9.5 (Fig. 5.8B) and E10.5 (Fig. 5.8E) showed *LacZ* expression in the most ventral neural tube and floor plate. This was a broader pattern of *LacZ* expression than that of wild type *Nkx2.2* (Briscoe et al., 1999). At spinal cord levels at E9.5 (Fig. 5.8C), the expression of *LacZ* was very strong in the ventral neural tube and floor plate, a pattern similar to wild type expression patterns (Briscoe et al., 1999; Jeong and McMahon, 2005). However, expression of *LacZ* at E10.5 at forelimb level (Fig. 5.8F) was slightly broader than *Nkx2.2* wild type expression (Briscoe et al., 1999). Weak *LacZ* expression at hindbrain level at E11.5 and E12.5 was located ventrally (Figs. 5.8H, 5.8K), and the pattern of expression was not dissimilar to *Nkx2.2* wild type expression patterns at E12.5 (Vernay et al., 2005). Expression of *LacZ* in the spinal cord at E11.5 (Fig. 5.8I) and E12.5 (Fig. 5.8L) was similar to that of *Nkx2.2* in the wild type (Briscoe et al., 1999), both progenitor and post mitotic cells were positive for β-galactosidase activity. By E13.5, the *LacZ* expression at both hindbrain (Fig. 5.8N) and forelimb levels (Fig. 5.8O) was more restricted to the midline. These data suggest that the zNkx2.2$^{CNCR+Prom}$LacZ reporter is able to direct Nkx2.2-like expression at equivalent embryonic stages to the expression of wild type *Nkx2.2*.

To identify the precise location of the *LacZ* expression in the ventral neural tube, immunohistochemistry of mice containing the transgene zNkx2.2$^{CNCR+Prom}$LacZ was performed. Embryos were harvested at E10.5 (Figs. 5.9, 5.10) and E11.5 (Fig. 5.11) and cryosectioned before co-immunostaining with antibodies against β-galactosidase and a variety of progenitor and post-mitotic neuronal markers. At E10.5, the ventral LacZ seemed to be restricted to the Nkx2.2 p3 domain (Fig. 5.9A-5.9I). Almost all Nkx2.2 positive cells were also positive for LacZ (Fig. 5.9A). Moreover, no co-expression of LacZ and Olig2 was observed, suggesting there was no reporter expression in the pMN domain (Fig. 5.9D). There were also no observed cells expressing LacZ and FoxA2 (Fig. 5.9G), therefore the reporter was repressed from the floor plate by E10.5. These data suggest that the reporter expression observed in the ventral neural tube is restricted to the Nkx2.2 p3 domain at the same point in development that this is observed endogenously.

At E10.5, there were some LacZ expressing cells in a more intermediate domain, seen both upon enzymatic staining (Fig. 5.8F) and immunostaining (Fig. 5.9A). In order to identify the intermediate population of cells expressing LacZ, further immunostaining was performed. Double positive cells for LacZ with Pax6 (Fig. 5.9J) and with Mash1 (Fig. 5.9M) were identified (marked by white arrowheads in the figures). Both of these progenitor markers identify populations of cells in the p2 progenitor domain. In order to further confirm this finding, co-immunostaining of post mitotic markers with LacZ was performed. LacZ was observed in cells positive for Lim1/2 (V0, V1 and V2 interneuron marker; Fig. 5.10A, 5.10A') and Lim3 (MN and V2 interneuron marker; Fig. 5.10D, 5.10D'). LacZ positive cells were also observed co-expressing V2 interneuron specific markers Gata3 (V2b interneuron marker; Fig. 5.10G, 5.10G') and Chx10 (V2a interneuron marker; Fig. 5.10J, 5.10J'). This further confirmed that the expression of LacZ in intermediate domain was confined to V2 interneurons, subtypes a and b, both as progenitor and post mitotic cells.

The expression of the LacZ was assessed at E11.5 to see if the reporter construct zNkx2.2$^{CNCR+Prom}$LacZ was able to direct specific expression at later developmental stages (Fig. 5.11). Ventral restriction of LacZ to the p3 domain was observed, the ventral LacZ positive cells were located in the Nkx2.2 positive domain (Fig. 5.11A), but not present in the FoxA2 positive floor plate (Fig. 5.11D). As observed at E10.5, almost all Nkx2.2 positive cells were also positive for LacZ, suggesting that the zNkx2.2$^{CNCR+Prom}$LacZ construct directs Nkx2.2-like expression precisely at E11.5 as well as E10.5.

Expression of LacZ in intermediate domains of the neural tube seen at E10.5 was seen in fewer cells at E11.5 (Fig. 5.11G-5.11R). There were more post-mitotic cells at E11.5 than E10.5 and were therefore more cells expressing markers Lim1/2 (Fig. 5.11G-5.11I), Lim3 (Fig. 5.11J-5.11L), Gata3 (Fig. 5.11M-5.11O) and Chx10 (Fig. 5.11P-5.11R). However, co-expression with LacZ was only observed with Chx10 (Fig. 5.11P) in a slightly more posterior region of the neural tube. Therefore, we can conclude that the construct is able to direct correct ventral expression in the ventral tube at E11.5. However, by this developmental stage, there is less ectopic expression of the reporter, therefore very little expression was observed in the V2 interneurons.

5.2.3 Analysis of zNkx2.2$^{CNCR+Prom}$LacZ-2 Stable Line

A stable mouse line containing the zNkx2.2$^{CNCR+Prom}$LacZ-2 construct was obtained and the activity of β-Galactosidase from E9.5 to E13.5 was analysed (Fig. 5.12). The general pattern of expression observed was very similar to that of the line containing transgene zNkx2.2$^{CNCR+Prom}$LacZ (Fig. 5.8) but slightly weaker. Expression of *LacZ* at E9.5 (Fig. 5.12A), E10.5 (Fig. 5.12D) and E11.5 (Fig. 5.12G) in wholemount stained embryos was observed the entire length of the neural tube. At E12.5 (Fig. 5.12J), expression occupied the same domain, but was only faintly detectable. At E13.5 (Fig. 5.12M) expression was barely visible in wholemount embryos.

In order to analyse the precise location of the β-Galactosidase activity in the neural tube, embryos were vibratome sectioned, hindbrain and spinal cord level sections were analysed (Fig. 5.12). Expression of *LacZ* at both hindbrain and forelimb levels of E9.5 embryos was identified in the most ventral domain of the neural tube, including the floor plate (Fig. 5.12B, 5.12C). By E10.5, the expression was maintained in the ventral domain, but was no longer present in the floor plate (Fig. 5.12E, 5.12F). This dorsal shift of expression and repression from the floor plate is comparable to wild type *Nkx2.2* expression (Briscoe et al., 1999). At E10.5, *LacZ* expression was also observed in a few cells in the intermediate domain of the neural tube at forelimb level (Fig. 5.12F). By E11.5 *LacZ* expression was restricted to the ventral domain at both hindbrain (Fig. 5.12H) and forelimb level (Fig. 5.12I). At E12.5 and E13.5 expression of *LacZ* was restricted to very few cells in the ventral midline in the hindbrain (Figs. 5.12K, 5.12N) and spinal cord (Figs. 5.12L, 5.12O).

Enzymatic staining of the embryos harbouring the zNkx2.2$^{CNCR+Prom}$LacZ-2 (Fig. 5.12) reporter construct showed that the expression of the reporter was similar to that in embryos containing zNkx2.2$^{CNCR+Prom}$LacZ (Fig. 5.8). Both constructs were capable of directing ventral neural tube expression similar Nkx2.2 expression observed in wild type embryos. However, the zNkx2.2$^{CNCR+Prom}$LacZ-2 construct appeared to direct a weaker expression that that of zNkx2.2$^{CNCR+Prom}$LacZ. This was also observed when E10.5 and E11.5 embryos were immunostained for LacZ. At E10.5, LacZ expression

was fairly strong in the ventral p3 domain of the neural tube (Figs. 5.13, 5.14), however by E11.5, the number of LacZ positive cells had dramatically reduced (Fig. 5.15).

Immunohistochemistry revealed LacZ expression from zNkx2.2$^{CNCR+Prom}$LacZ-2 in cells positive for Nkx2.2 (Fig. 5.13A). The cells positive for the reporter were positioned ventral to the Olig2 positive pMN domain (Fig. 5.13D) and dorsal to the FoxA2 expressing floor plate cells (Fig. 5.13G). This confirmed that the reporter construct was directing LacZ expression in the p3 domain at E10.5.

LacZ was detected by immunohistochemistry in a few cells in a more intermediate domain of the neural tube of mice harbouring construct zNkx2.2$^{CNCR+Prom}$LacZ-2 (Fig. 5.13A). In order to determine the location of these cells, further immunohistochemistry was performed. Construct zNkx2.2$^{CNCR+Prom}$LacZ directed expression of LacZ in V2 interneurons at both the progenitor and post-mitotic stages. To test if the same population of neurons was expressing the reporter, similar staining with V2 markers was performed (Figs. 5.13, 5.14). Co-expression of the reporter with progenitor markers Pax6 (Fig. 5.13J) and Mash1 (Fig. 5.13M) was not observed at E10.5. However this may be due to the anterior-posterior level of the sections, at more posterior positions there appear to be fewer intermediate cells expressing LacZ. At more anterior positions of E10.5 embryos, the zNkx2.2$^{CNCR+Prom}$LacZ-2 construct appeared to direct expression of LacZ in a greater number of cells in more intermediate domains (Fig. 5.14). Immunohistochemistry for post mitotic markers revealed co-expression of LacZ with Lim1/2 (Fig. 5.14A), Lim3 (Fig. 5.14D), Gata3 (Fig. 5.14G, 5.14G') and Chx10 (Fig. 5.14J, 5.14J'). These data suggest that at E10.5 the zNkx2.2$^{CNCR+Prom}$LacZ-2 reporter construct directed LacZ expression in post-mitotic V2 interneurons, subtypes a and b. At E11.5, the intermediate population of LacZ positive cells were also positive for Lim3 (Fig. 5.15J, 5.15J'), Gata3 (Fig. 5.15M) and Chx10 (Fig. 5.15P). However, in the sections analysed, no cells were observed positive for LacZ and Lim1/2 (Fig. 5.15G). These data suggest that LacZ expression is directed in V2 post-mitotic interneurons at E11.5 by reporter construct zNkx2.2$^{CNCR+Prom}$LacZ-2.

To see if the zNkx2.2$^{CNCR+Prom}$LacZ-2 construct was able to maintain Nkx2.2-like ventral reporter expression, E11.5 embryos were analysed by immunohistochemistry. At E11.5, the expression of ventral LacZ directed by zNkx2.2$^{CNCR+Prom}$LacZ-2 appeared to be weaker than that observed at E10.5, there were fewer cells expressing LacZ in the p3 domain (Fig. 5.15). zNkx2.2$^{CNCR+Prom}$LacZ directed expression in all cells of the ventral p3 domain at both E10.5 and E11.5 (Figs. 5.9, 5.11). However, E11.5 mouse embryos harbouring zNkx2.2$^{CNCR+Prom}$LacZ-2 did not appear to express LacZ in all cells of the Nkx2.2 positive p3 domain (Fig. 5.15A). LacZ expression was not detected in the FoxA2 positive floor plate cells (Fig. 5.15D).

Combining all of the results from enzymatic- and immunostaining of transgenic mice, we can conclude that both of the zebrafish reporter constructs zNkx2.2$^{CNCR+Prom}$LacZ and zNkx2.2$^{CNCR+Prom}$LacZ-2 direct reporter expression in both the ventral neural tube and in a more intermediate region of the neural tube. The ventral expression of *LacZ* corresponds to the *Nkx2.2* expressing p3 domain, the more intermediate region corresponds to the p2 domain. The zNkx2.2$^{CNCR+Prom}$LacZ-2 reporter directs much weaker expression and also directs apparent down-regulation of expression prematurely at E11.5 in the p3 domain compared to endogenous *Nkx2.2* expression.

5.3 The CNCR is Sufficient to Direct LacZ Expression in the Ventral Neural Tube

The data indicated that the reporter constructs containing the Conserved Non-Coding Region (CNCR) were capable of driving Nkx2.2/2.9-like expression. The constructs used contained the 250bp CNCR together with approximately 1kb of additional DNA. To address whether the CNCR alone was sufficient for this activity, two constructs based on zNkx2.2$^{CNCR+Prom}$LacZ were made (Fig. 5.16). One consisted of just the CNCR, zNkx2.2^{CNCR}LacZ (Fig. 5.16B) and one was the reciprocal region, zNkx2.2$^{\Delta CNCR+Prom}$LacZ (Fig. 5.16C) of the original construct (Fig. 5.16A). Mouse PNI was used to test the expression of *LacZ* directed by these reporter constructs.

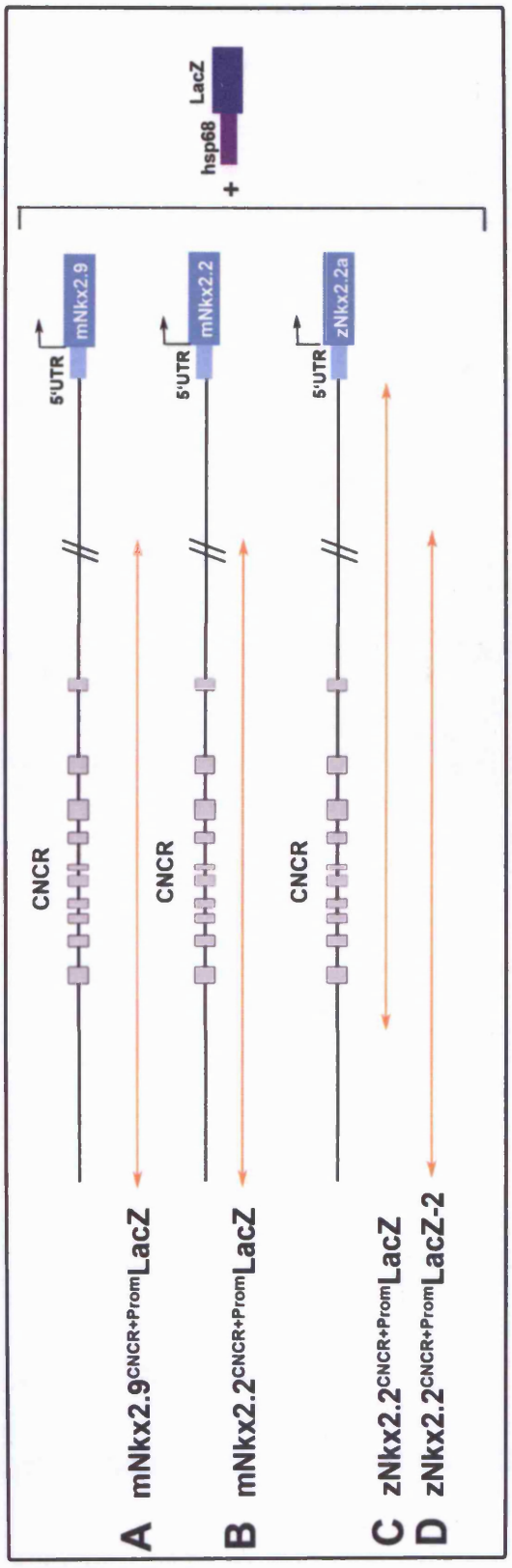
A representation of the transient results of these injections at E10.5 can be seen in Fig. 5.16. Injection of the construct containing only the CNCR, resulted in ventral Nkx2.2-like expression in the neural tube (Fig. 5.16D, 5.16E) albeit at apparently lower levels than zNkx2.2$^{CNCR+Prom}$LacZ. Intermediate expression was also detected in similar domains to those seen with PNI of the full-length construct, zNkx2.2$^{CNCR+Prom}$LacZ, and also the other zebrafish construct zNkx2.2$^{CNCR+Prom}$LacZ-2. Expression in the ventral neural tube driven by this promoter construct was seen in 6 out of 36 injected embryos at E10.5 (Table 8).

In contrast, zNkx2.2$^{\Delta CNCR+Prom}$LacZ did not result in any β-galactosidase activity (Fig. 5.16F). This result was confirmed by multiple injection events, the results of this can be seen in Table 8. Out of 29 injected embryos analysed at E10.5, 0 were positive for β-galactosidase activity.

Construct for PNI	**Number of Injected Embryos Stained Positive for β-galactosidase in ventral NT**
zNkx2.2^{CNCR}LacZ	6/36
zNkx2.2$^{\Delta CNCR+Prom}$LacZ	0/29

Table 8 Results from mouse pronuclear injection of zebrafish reporter constructs; zNkx2.2^{CNCR}LacZ and zNkx2.2$^{\Delta CNCR+Prom}$LacZ.

The numbers in the right hand column indicate the fraction of embryos that at E10.5 were positive for β-galactosidase activity using X-Gal substrate. These embryos were injected with constructs containing a *LacZ* gene driven by a minimal promoter (hsp68) and a region of the zebrafish *Nkx2.2a* gene promoter. The construct containing an identified conserved non-coding region (CNCR) led to β-galactosidase expression in the ventral neural tube (NT) in 6 out of 36 embryos. The construct lacking this region led to 0 out of 29 positive embryos.


LacZ
hsp68
+
5'UTR
mNkx2.9
CNCR
A mNkx2.9CNCR+PromLacZ
5'UTR
mNkx2.2
CNCR
B mNkx2.2CNCR+PromLacZ
5'UTR
zNkx2.2a
CNCR
C zNkx2.2CNCR+PromLacZ
D zNkx2.2CNCR+PromLacZ-2

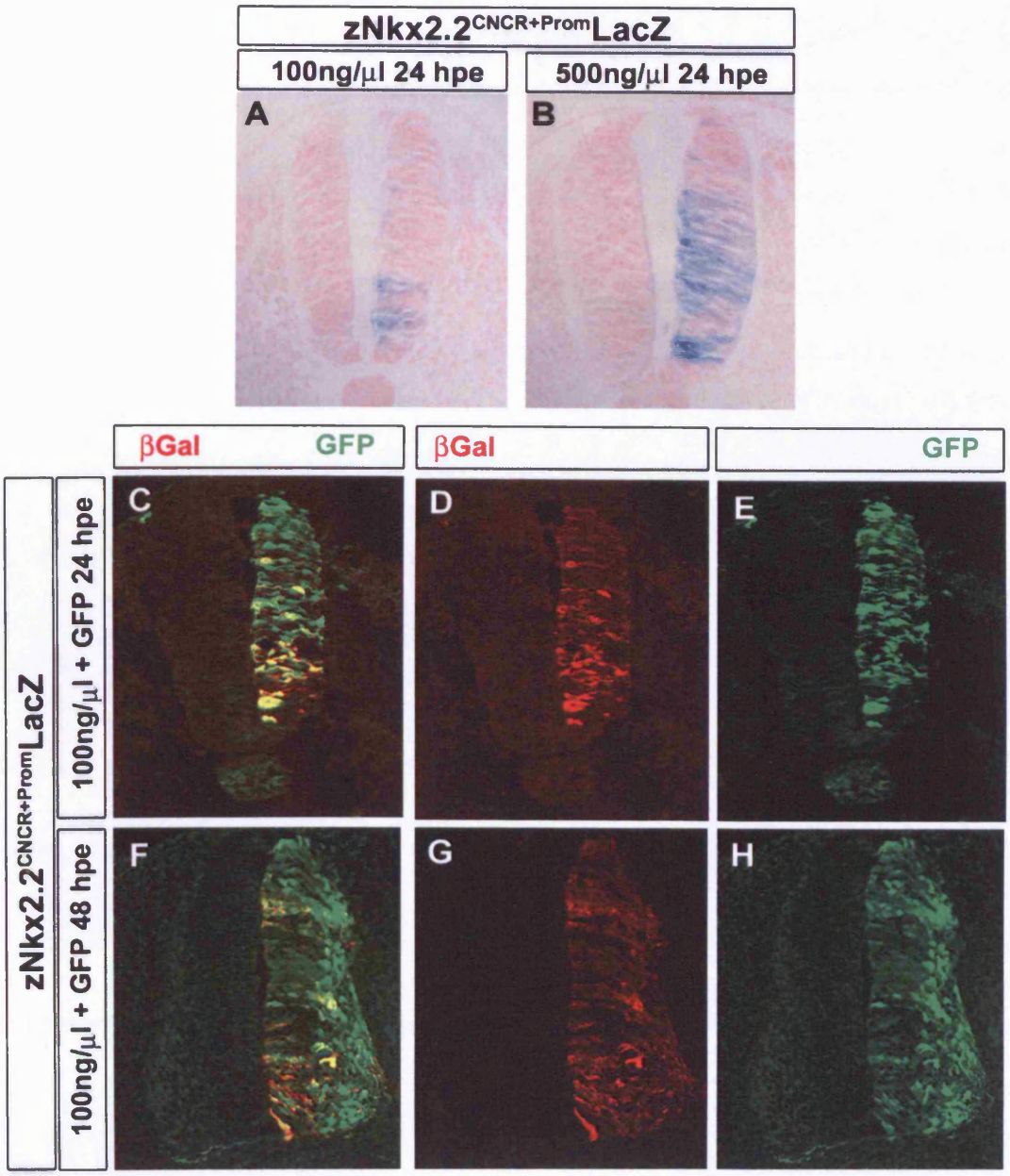

zNkx2.2CNCR+PromLacZ
100ng/µl 24 hpe
500ng/µl 24 hpe
A
B
βGal
GFP
βGal
GFP
zNkx2.2CNCR+PromLacZ
100ng/µl + GFP 24 hpe
100ng/µl + GFP 48 hpe
C
D
E
F
G
H

Figure 5.3 Zebrafish embryos stained for β–Galactosidase activity 24 hours post fertilisation (hpf) after injection of zNkx2.2$^{CNCR+Prom}$LacZ reporter construct.

Uncut plasmid DNA (40ng/μl) was injected into the cytoplasm of 1-cell stage zebrafish embryos. Embryos were incubated until 24 hpf, fixed and stained for β–Galactosidase activity. Representative images of embryos photographed at 10X (**A-C**), and at 20X (**D**). Lateral views of embryos are shown, with anterior to the left, yolk cells have been removed. Stained cells (black arrowheads) were located in the *Nkx2.2* domain in the ventral neural tube, the large cells (black arrows) positive for β-gal are notochord cells. The expression of the reporter was very mosaic and the promoter construct was also expressed in ectopic locations throughout the embryo.

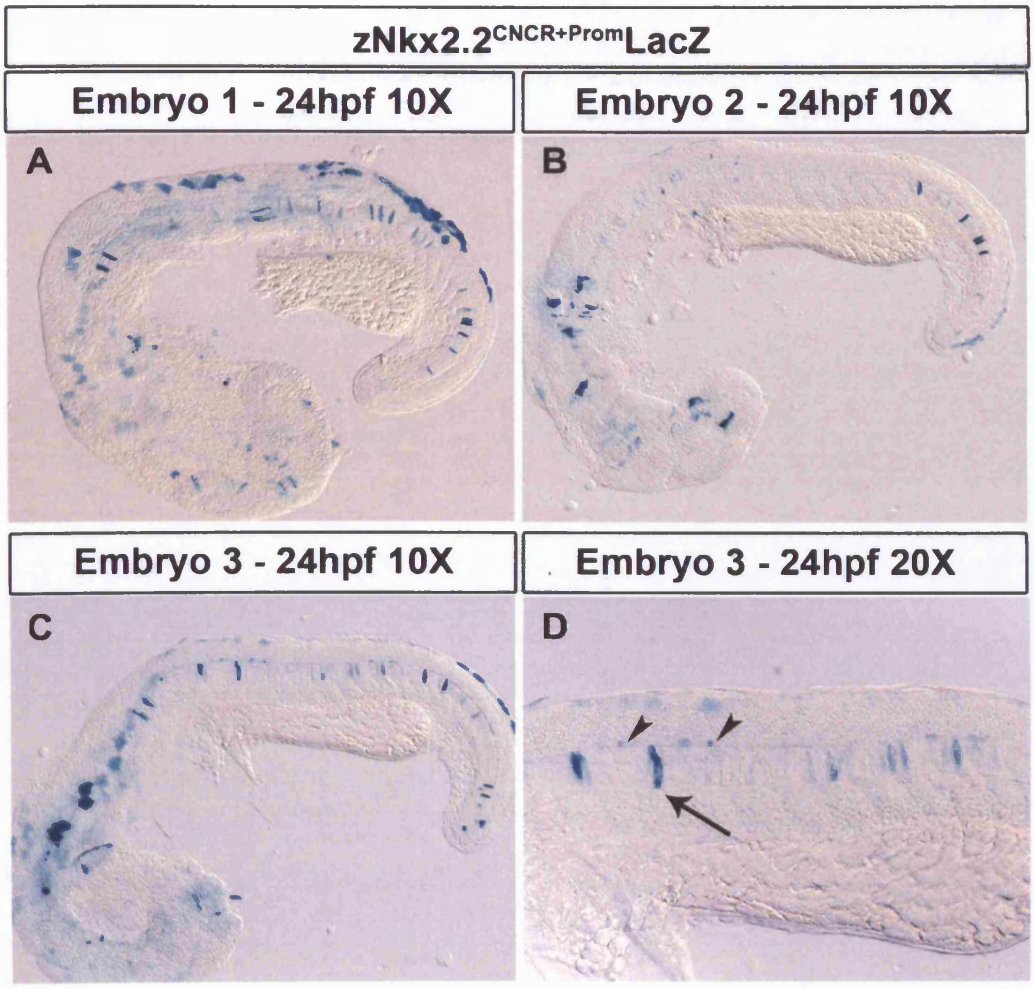

zNkx2.2CNCR+PromLacZ
Embryo 1 - 24hpf 10X
Embryo 2 - 24hpf 10X
A
B
Embryo 3 - 24hpf 10X
Embryo 3 - 24hpf 20X
C
D

Figure 5.4 Zebrafish embryos stained for β-Galactosidase activity 24 hpf after injection of separate promoter (zNkx2.2$^{CNCR+Prom}$) and reporter (hsp68LacZ) fragments.

Independent fragments of the promoter and reporter were co-injected at 60ng/μl and 12.5ng/μl respectively into the cytoplasm of 1-cell stage embryos. Embryos were incubated until 24 hours post fertilisation (hpf) and then fixed and stained for β-Galactosidase activity. The embryos are lateral with anterior to the left, heads have been turned for a dorsal view, and yolk cells were removed. Embryos were photographed at 10 X (**A, C, E**) and 20X (**B, D, F**). The amount of ectopic *LacZ* expression was reduced compared to the conventional injection method (Fig. 5.3), however, the amount of mosaicism of expression was not reduced. Cells positive for *LacZ* in the ventral neural tube are marked by black arrowheads (high magnification (**B, D, F**).

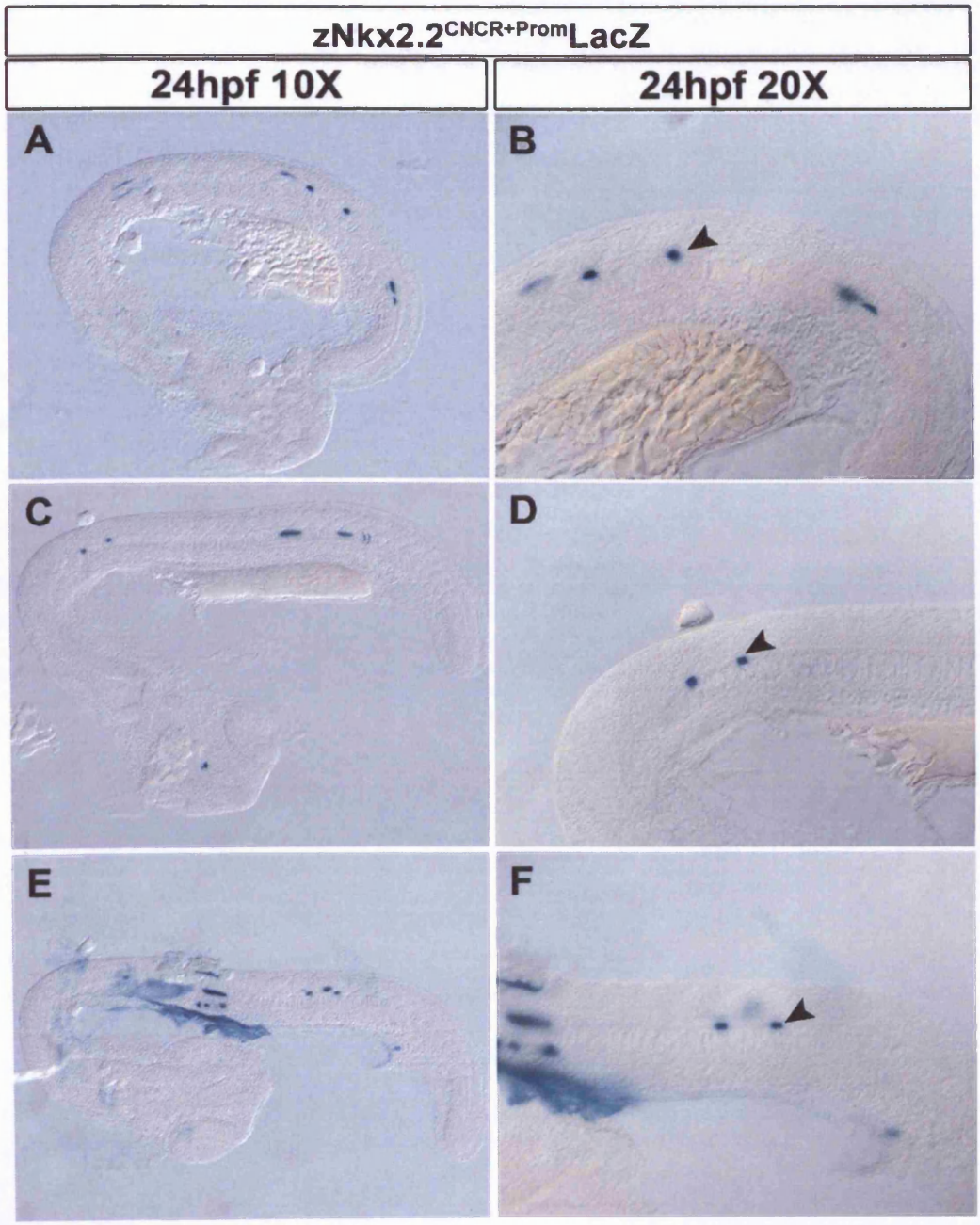

zNkx2.2CNCR+PromLacZ
24hpf 10X
24hpf 20X
A
B
C
D
E
F

Figure 5.5 Transient results of mouse pronuclear injection at E10.5 of mouse (mNkx2.9$^{CNCR+Prom}$LacZ, mNkx2.2$^{CNCR+Prom}$LacZ) and zebrafish (zNkx2.2$^{CNCR+Prom}$LacZ) promoter constructs.

mNkx2.9$^{CNCR+Prom}$LacZ (**A, B**), mNkx2.2$^{CNCR+Prom}$LacZ (**C, D**) and zNkx2.2$^{CNCR+Prom}$LacZ (**E, F**) were microinjected at 2ng/μl into mouse pronuclei, which were transferred to host females and left until E10.5. After harvesting, embryos were fixed and stained for *LacZ* expression. mNkx2.9$^{CNCR+Prom}$LacZ directed expression along the length of the ventral neural tube in 3 out of 13 transient embryos (**A**). The expression in the spinal accessory nerve (**A**, black arrowhead) is consistent with the observation that in *Nkx2.9* mutant mice there are defects in this nerve (Pabst et al., 2003). Expression was also observed in an intermediate domain of the neural tube (**B**). A low level of expression in the most dorsal cells of the neural tube was observed when the neural tube was cryo-sectioned (**B**). Injection of mNkx2.2$^{CNCR+Prom}$LacZ led to 1 out of 13 embryos staining positive for *LacZ* expression in ventral neural tube, in a domain similar to endogenous *Nkx2.2* expression (**C, D**). Some ectopic expression within the more intermediate neural tube was observed. zNkx2.2$^{CNCR+Prom}$LacZ directed expression of *LacZ* in 1 out of 10 transient embryos in the ventral neural tube (**E**), the expression appeared specific to the mouse *Nkx2.2* domain (**F**), with no ectopic expression.

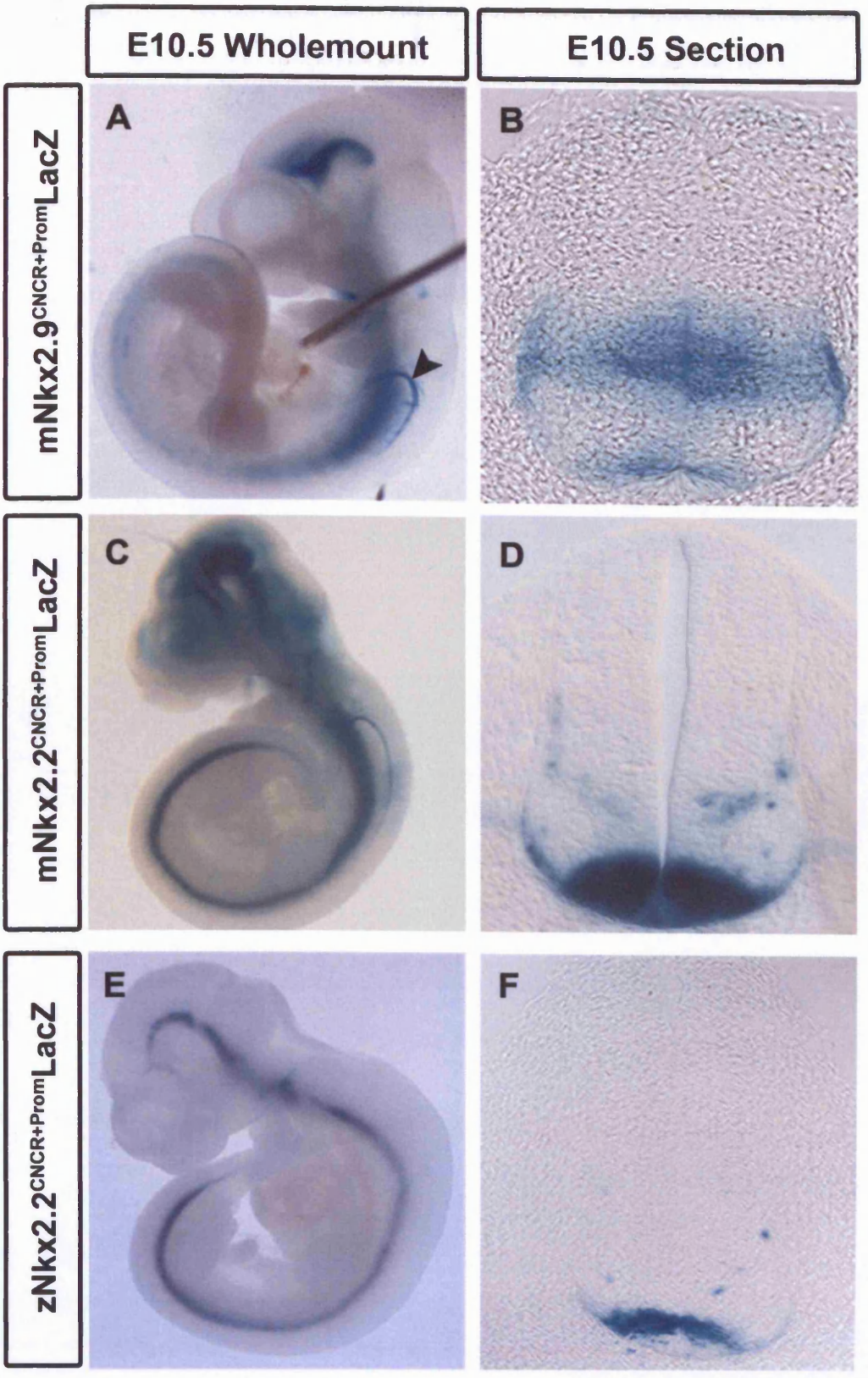

E10.5 Wholemount
E10.5 Section
mNkx2.9CNCR+PromLacZ
mNkx2.2CNCR+PromLacZ
zNkx2.2CNCR+PromLacZ
A
B
C
D
E
F

Figure 5.6 Expression patterns of LacZ from E9.5 to E13.5 in a stable mouse line containing reporter construct mNkx2.9$^{CNCR+Prom}$LacZ.

Embryos were collected from a stable mouse line containing reporter construct mNkx2.9$^{CNCR+Prom}$LacZ from E9.5 to E13.5 and embryos were stained for β-galactosidase activity. Wholemount embryos were photographed (**A, D, G, J, M**). Embryos were also sectioned and counterstained with eosin (**B, C, E, F, H, I, K, L, N, O**). Expression at E9.5 was observed throughout the length of the neural tube (**A**), when sectioned this was identified as expression in both ventral and intermediate positions within the neural tube (**B, C**). At E10.5 (**D**), expression was still seen throughout the neural tube in an Nkx2.9-like pattern, however it was weaker than at E9.5. Expression of *Nkx2.9* in wild type embryos would at this time point be down-regulated in spinal cord. This was not the case for the transgene, which was still expressed in both ventral and intermediate positions of the neural tube (**E, F**). At E11.5, expression in the neural tube was still strong anteriorly, however was weaker posteriorly (**G**). This was also observed in the sections, expression was almost undetectable at posterior levels (**I**), but was still expressed ventrally in the hindbrain (**H**). At E12.5, there was still neural tube expression in the brain (**J**), however expression at hindbrain level was very weak (**K**) and has been lost in the spinal cord at forelimb level (**L**). Expression at E13.5 has been lost in all neural tube tissue (**M, N, O**).

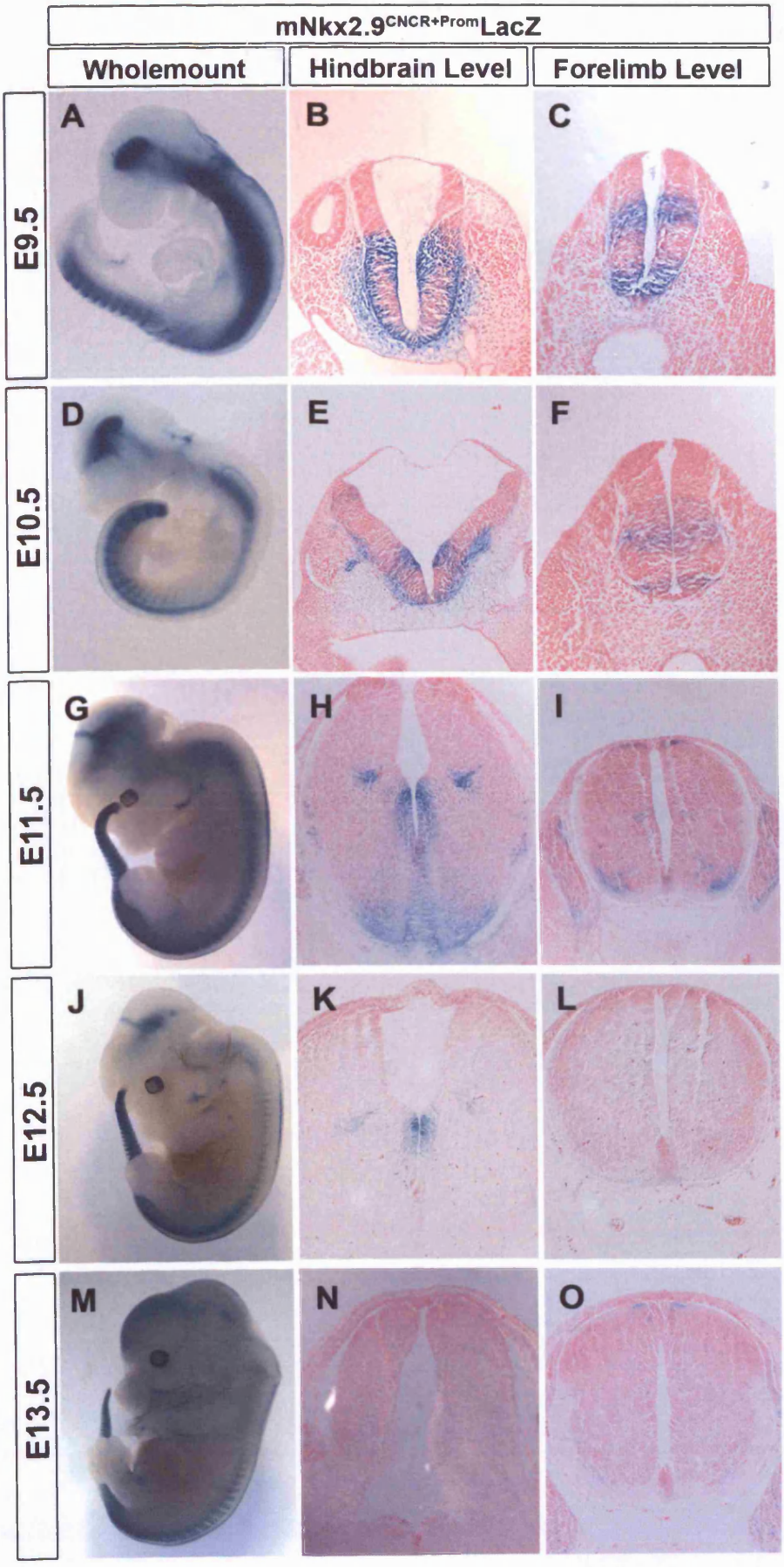

mNkx2.9CNCR+PromLacZ
Wholemount
Hindbrain Level
Forelimb Level
E9.5
E10.5
E11.5
E12.5
E13.5
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 5.7 Results of crossing mNkx2.9$^{CNCR+Prom}$LacZ containing mouse line with *Pax6* mutant line *Sey*.

To test if the promoter region in the reporter construct mNkx2.9$^{CNCR+Prom}$LacZ was regulated by Pax6, the stable mouse line containing this transgene was crossed with a mouse containing a mutation in the *Pax6* gene, *Sey*. Cryo-sections of stained heterozygous littermates (**A, C, E, G**) were compared to littermates homozygous for the *Pax6* mutation (**B, D, F, H**) at the same level of the anterior-posterior axis. Expansion in LacZ staining was seen at anterior levels in mNkx2.9$^{CNCR+Prom}$LacZ/*Sey*$^{-/-}$ embryos (**B**) compared to the heterozygote (**A**). This dorsal expansion of ventral *LacZ* expression was also observed in more posterior sections, but not as evident (**C, D**). Immunohistochemistry for Nkx2.2 (brown; **E, F**) suggested that endogenous *Nkx2.2* expression expanded more dorsally in homozygotes (**F**) compared to heterozygotes (**E**), marked by the black arrowheads. This dorsal expansion was due to the lack of repression by Pax6 in its absence. Immunohistochemistry for Pax7 (**G, H**) revealed that the dorsal limit of expansion of the LacZ reporter coincided with the ventral limit of endogenous *Pax7* expression (brown).

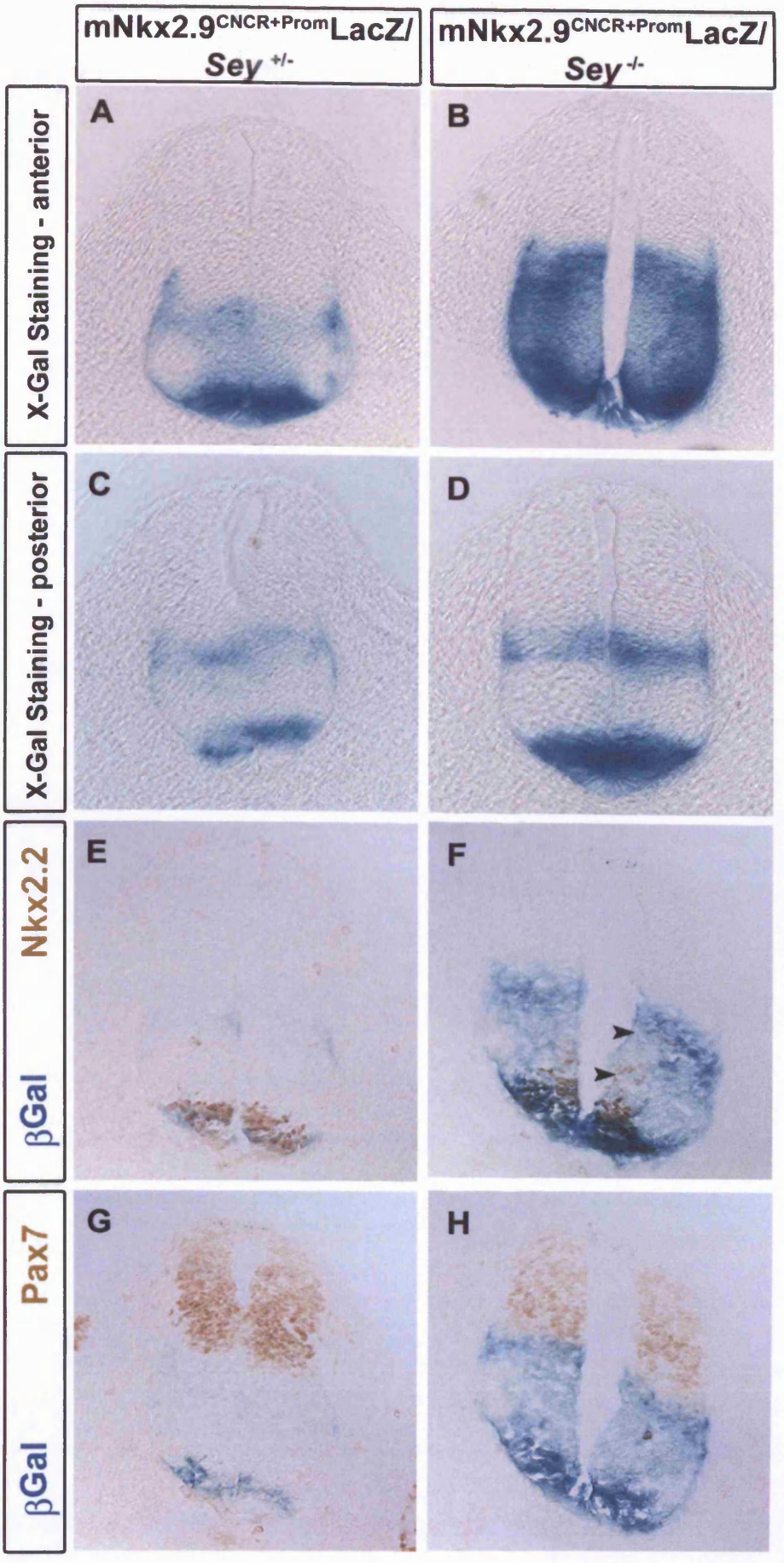

mNkx2.9CNCR+PromLacZ/ Sey +/-
mNkx2.9CNCR+PromLacZ/ Sey -/-
X-Gal Staining - anterior
X-Gal Staining - posterior
βGal
Nkx2.2
βGal
Pax7
A
B
C
D
E
F
G
H

Figure 5.8 Expression patterns of LacZ from E9.5 to E13.5 in stable mouse line containing reporter construct zNkx2.2$^{CNCR+Prom}$LacZ.

Embryos were collected from a stable mouse line containing reporter construct zNkx2.2$^{CNCR+Prom}$LacZ from E9.5 to E13.5 and embryos were stained for β-galactosidase activity. Wholemount embryos were photographed (**A, D, G, J, M**). Embryos were also vibratome sectioned (50 μM) (**B, C, E, F, H, I, K, L, N, O**). Expression at E9.5 was identified the whole length of the neural tube (**A**), when sectioned, expression of the transgene was evident in the ventral neural tube (**B, C**) at both hindbrain and forelimb levels, in a broader domain than *Nkx2.2* in wild type embryos. At E10.5 (**D**), expression was observed throughout the neural tube in an Nkx2.2-like pattern, albeit weaker than at E9.5. The transgene directed expression in the ventral neural tube again at both hindbrain and forelimb levels (**E, F**). At E11.5, *LacZ* expression in the neural tube was still strong (**G**). This was also observed in sections, expression of *LacZ* was still found ventrally in the spinal cord (**H**) and hindbrain (**I**). At E12.5, there was still neural tube expression the length of the neural tube (**J**), however expression was weak. This is obvious at hindbrain level (**K**) and in the spinal cord at forelimb level (**L**). Expression at E13.5 had been reduced further (**M**), in both the hindbrain (**N**) and spinal cord (**O**) it was restricted to the midline.

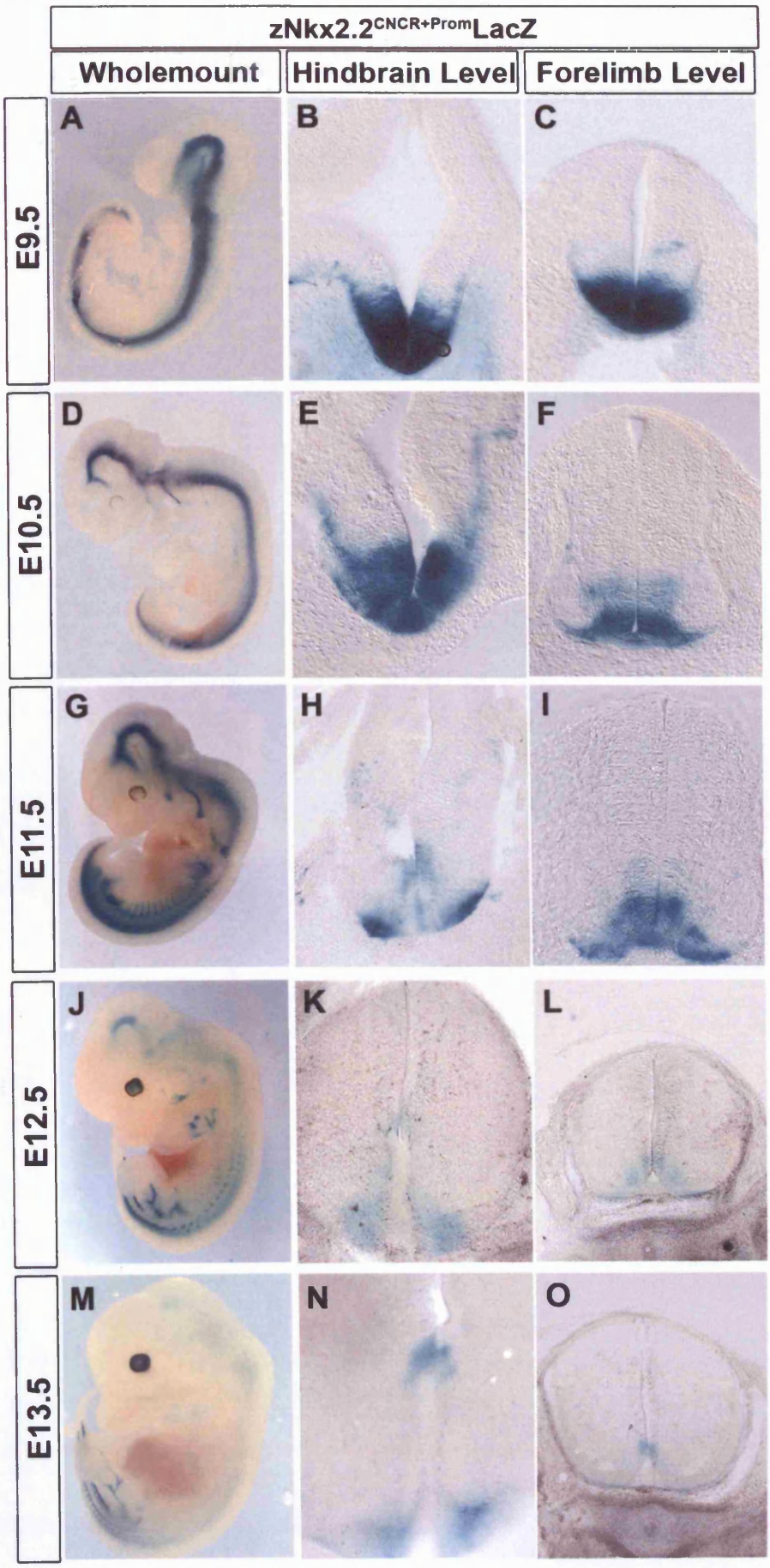

zNkx2.2CNCR+PromLacZ
Wholemount
Hindbrain Level
Forelimb Level
E9.5
E10.5
E11.5
E12.5
E13.5
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 5.9 Immunohistochemistry for neural tube progenitor markers in zNkx2.2$^{CNCR+Prom}$LacZ mouse lines at E10.5.

Embryos were collected from a stable mouse line containing reporter construct zNkx2.2$^{CNCR+Prom}$LacZ at E10.5. Co-immunostaining for neuronal progenitor markers and β-galactosidase was performed on 12μM cryosections. **A-C** Nkx2.2 and β-gal staining, **D-F** Olig2 and β-gal staining, **G-I** FoxA2 and β-gal staining, **J-L** Pax6 and β-gal staining, **M-O** Mash1 and β-gal staining. **A** Nkx2.2 staining shows that almost all Nkx2.2 positive cells appeared to co-express the reporter β-gal suggesting that the zNkx2.2$^{CNCR+Prom}$LacZ transgene was able to correctly recapitulate *Nkx2.2* expression at E10.5. **D** Olig2 marks the motor neuron progenitor domain (pMN), the β-gal positive cells were located ventral to the Olig2 domain with no co-expression. **G** By E10.5 the expression of the transgene appeared to have receded from the floor plate and there was very little co-expression with the floor plate marker FoxA2. The zNkx2.2$^{CNCR+Prom}$LacZ transgene directed β-gal expression in a few neurons in a more intermediate domain than those in the Nkx2.2 p3 domain. The intermediate β-gal expressing neurons co-expressed progenitor markers Pax6 (**J**) and Mash1 (**M**), marked by white arrows. Mash1 and Pax6 are markers for V2 interneuron progenitors.

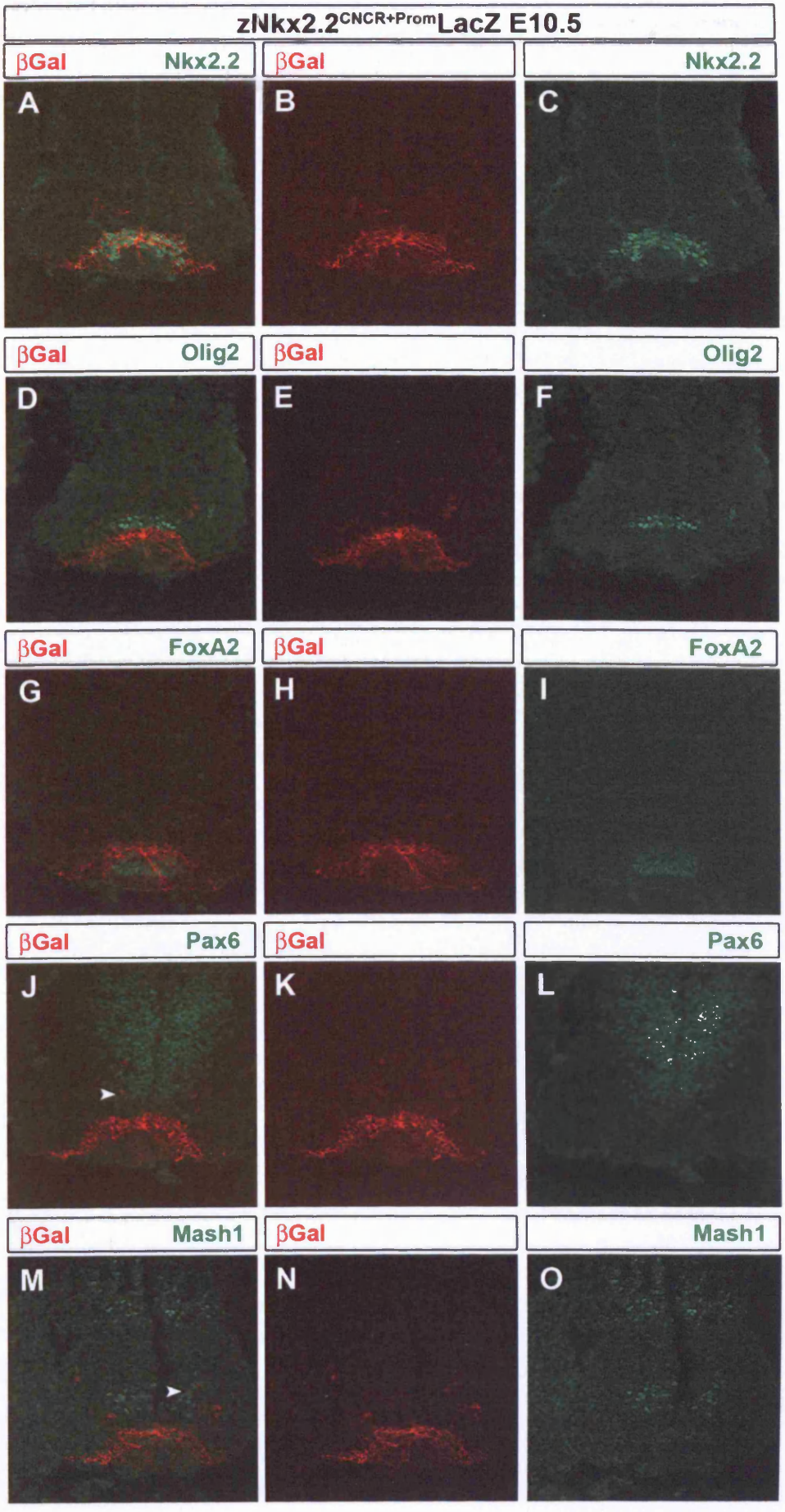

zNkx2.2CNCR+PromLacZ E10.5
βGal Nkx2.2
βGal
Nkx2.2
A
B
C
βGal Olig2
βGal
Olig2
D
E
F
βGal FoxA2
βGal
FoxA2
G
H
I
βGal Pax6
βGal
Pax6
J
K
L
βGal Mash1
βGal
Mash1
M
N
O

Figure 5.10 Immunohistochemistry for neural tube post mitotic neuronal markers in zNkx2.2$^{CNCR+Prom}$LacZ mouse lines at E10.5.

Embryos were collected from a stable mouse line containing reporter construct zNkx2.2$^{CNCR+Prom}$LacZ at E10.5. Co-immunostaining for neuronal post-mitotic markers and β-galactosidase was performed on 12μM cryosections. **A-C** Lim1/2 and β-gal staining, **D-F** Lim3 and β-gal staining, **G-I** Gata3 and β-gal staining, **J-L** Chx10 and β-gal staining. Immunostaining for post-mitotic markers suggests that the transgene directed intermediate expression of β-gal in both progenitor (Fig. 5.9) and post-mitotic neurons. Co-expression of β-gal with Lim1/2 (**A, A'**), Lim3 (**D, D'**), Gata3 (**G, G'**) and Chx10 (**J, J'**) suggests these intermediate cells were V2 interneuron post mitotic cells of both a and b subtype.

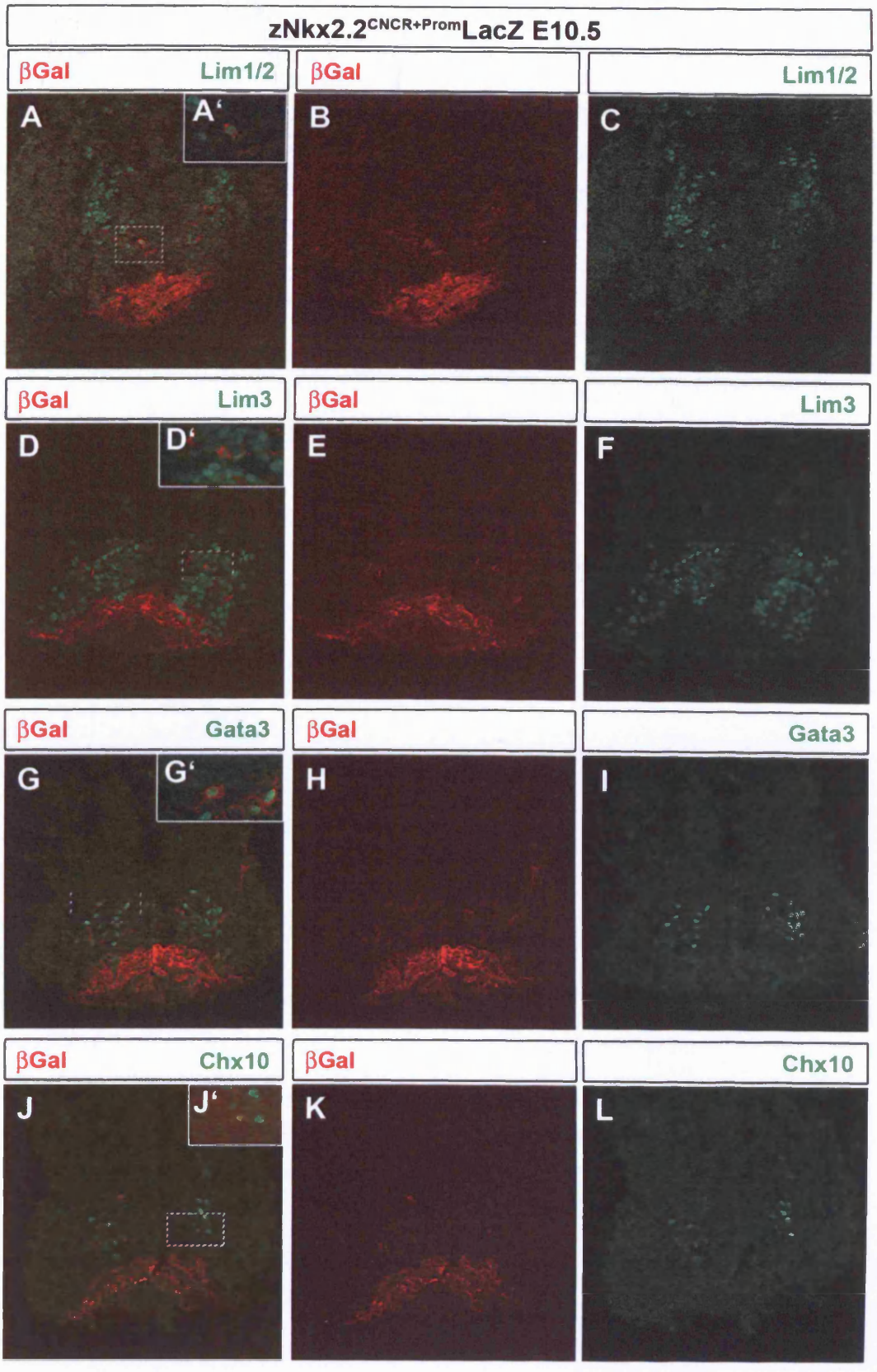

zNkx2.2CNCR+PromLacZ E10.5
βGal
Lim1/2
A
A'
B
C
βGal
Lim3
D
D'
E
F
Gata3
G
G'
H
I
Chx10
J
J'
K
L

Figure 5.11 Immunohistochemistry for neural tube progenitor and post mitotic neuronal markers in zNkx2.2$^{CNCR+Prom}$LacZ mouse lines at E11.5.

Embryos were collected from a stable mouse line containing reporter construct zNkx2.2$^{CNCR+Prom}$LacZ at E11.5. Co-immunostaining for both neuronal progenitor and post mitotic markers with β-galactosidase was performed on 12μM cryosections. **A-C** Nkx2.2 and β-gal staining, **D-F** FoxA2 and β-gal staining, **G-I** Lim1/2 and β-gal staining, **J-L** Lim3 and β-gal staining, **M-O** Gata3 and β-gal staining, **P-R** Chx10 and β-gal staining. By E11.5, the β-gal expressing cells were restricted to the V3 interneuron progenitor domain, there were very few if any in the V2 interneuron domain. Almost all of the Nkx2.2 positive cells also expressed β-gal (**A**), however, there were no cells co-expressing FoxA2 and β-gal (**B**). The number of post-mitotic neurons had increased compared to staining at E10.5. Due to the reduced number of β-gal positive intermediate cells, no co-expression with V2 interneuron post-mitotic markers Lim1/2 (**G**), Lim3 (**J**), Gata3 (**M**) with β-gal was identified. However, a few cells were identified co-expressing β-gal and Chx10 (**P**). This suggests that those few intermediate β-gal positive cells, were located in the V2 domain.

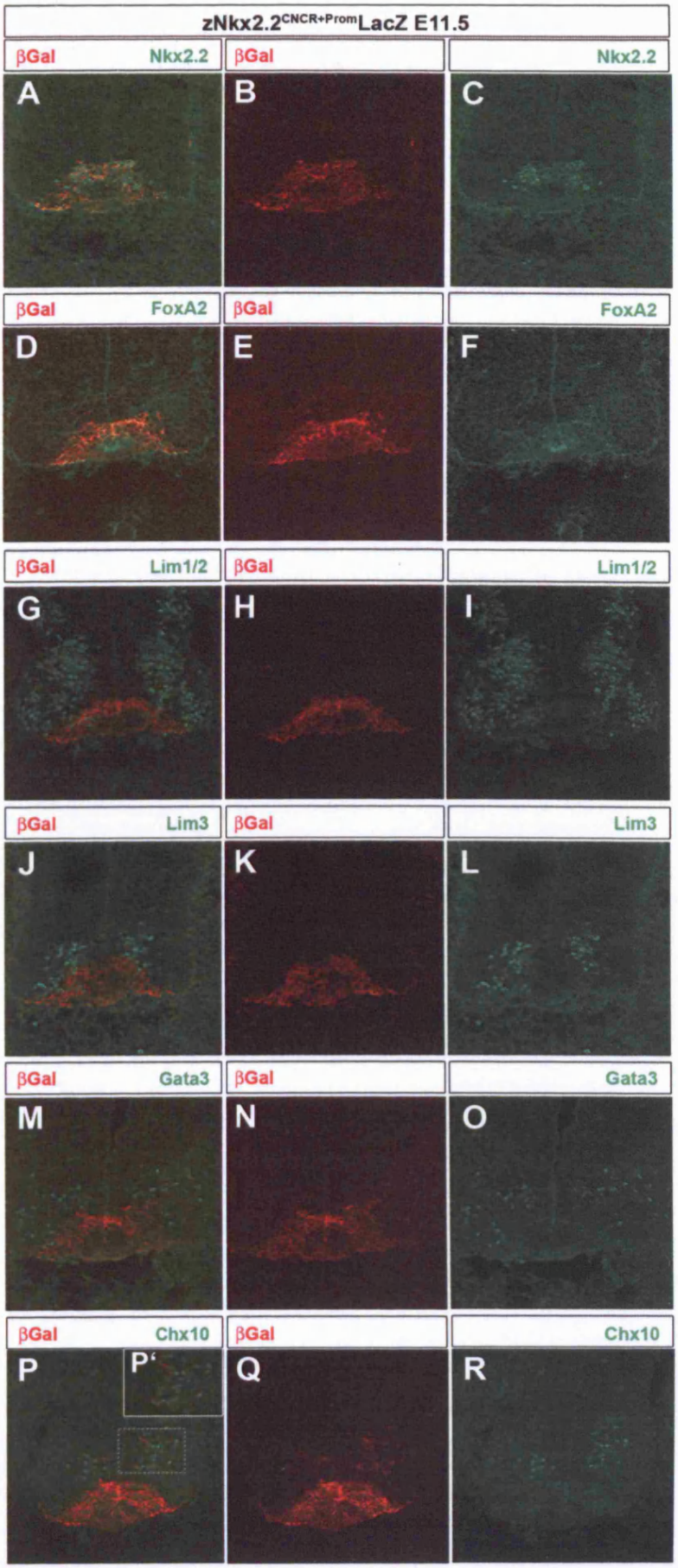

zNkx2.2CNCR+PromLacZ E11.5
βGal
Nkx2.2
A
B
C
βGal
FoxA2
D
E
F
βGal
Lim1/2
G
H
I
βGal
Lim3
J
K
L
βGal
Gata3
M
N
O
βGal
Chx10
P
P'
Q
R

Figure 5.12 Expression patterns of *LacZ* from E9.5 to E13.5 in stable mouse line containing reporter construct zNkx2.2$^{CNCR+Prom}$LacZ-2.

Embryos were collected from a stable mouse line containing reporter construct zNkx2.2$^{CNCR+Prom}$LacZ-2 from E9.5 to E13.5 and embryos were stained for β-galactosidase activity. Wholemount embryos were photographed (**A, D, G, J, M**) and vibratome sectioned (50 μM) (**B, C, E, F, H, I, K, L, N, O**). *LacZ* expression at E9.5 was identified the length of the neural tube (**A**), upon sectioning weak expression of the transgene was evident in the ventral neural tube (**B, C**) in a domain similar to *Nkx2.2* expression in wild type embryos. At E10.5 (**D**), expression was still present throughout the length of the neural tube in an Nkx2.2-like pattern. The transgene was expressed in the ventral neural tube (**E, F**), a few cells expressed *LacZ* in an intermediate domain of the neural tube, at forelimb level (**F**). At E11.5 expression of *LacZ* was also present the length of the neural tube (**G**). This was observed in transverse sections, expression of *LacZ* was located ventrally in the hindbrain (**H**) and spinal cord (**I**). By E12.5, there was very weak neural tube expression of *LacZ* the length of the neural tube (**J**), which was barely visible at E13.5 (**M**). At E12.5 and E13.5, *LacZ* expression was restricted to a few cells at the most ventral midline at hindbrain level (**K, N**) and forelimb level (**L, O**).

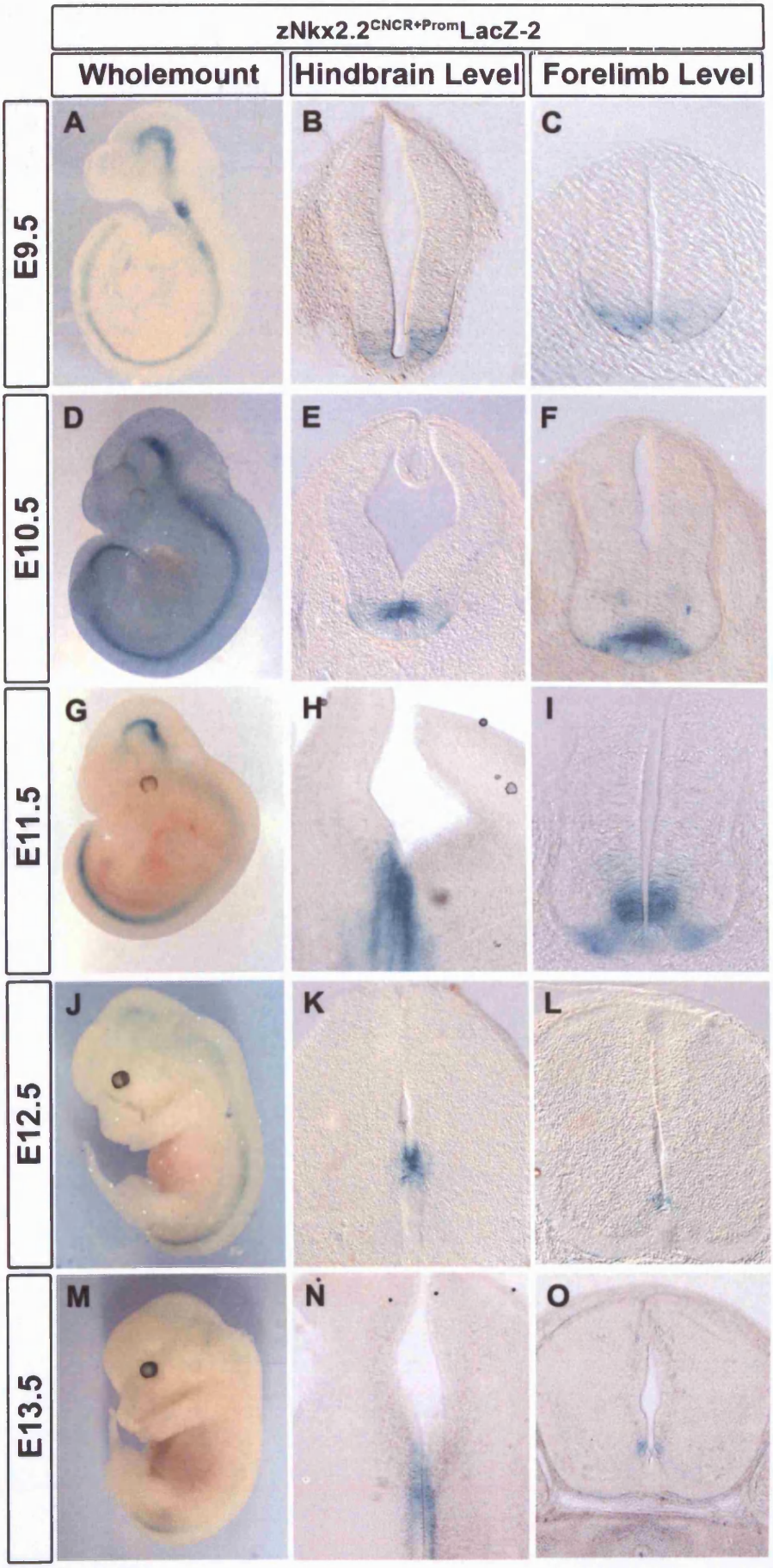

zNkx2.2CNCR+PromLacZ-2
Wholemount
Hindbrain Level
Forelimb Level
E9.5
E10.5
E11.5
E12.5
E13.5
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 5.13 Immunohistochemistry for neural tube progenitor markers in zNkx2.2$^{CNCR+Prom}$LacZ-2 mouse lines at E10.5.

Embryos were collected from a stable mouse line containing reporter construct zNkx2.2$^{CNCR+Prom}$LacZ-2 at E10.5. Co-immunostaining for neuronal progenitor markers and β-galactosidase was performed on 12μM cryosections. **A-C** Nkx2.2 and β-gal staining, **D-F** Olig2 and β-gal staining, **G-I** FoxA2 and β-gal staining, **J-L** Pax6 and β-gal staining, **M-O** Mash1 and β-gal staining. **A** Nkx2.2 staining shows that most of the Nkx2.2 positive cells appeared to co-express the reporter β-gal suggesting that the zNkx2.2$^{CNCR+Prom}$LacZ-2 transgene directs expression in the Nkx2.2 positive p3 domain at E10.5. **D** Olig2 marks the motor neuron progenitor domain (pMN), the β-gal positive cells were observed in a position ventral to the Olig2 domain with no co-expression. **G** By E10.5 the expression of the transgene appeared to have receded from the floor plate and there was very little co-expression of β-gal with the floor plate marker FoxA2. The zNkx2.2$^{CNCR+Prom}$LacZ-2 transgene directed β-gal expression in a few neurons in a more intermediate domain than those in the Nkx2.2 p3 domain. However, this was only observed in very few embryos, not enabling identification of neuronal subtype in this set of immunostaining with progenitor markers Pax6 (**J**) and Mash1 (**M**). Mash1 and Pax6 mark V2 interneuron progenitors.

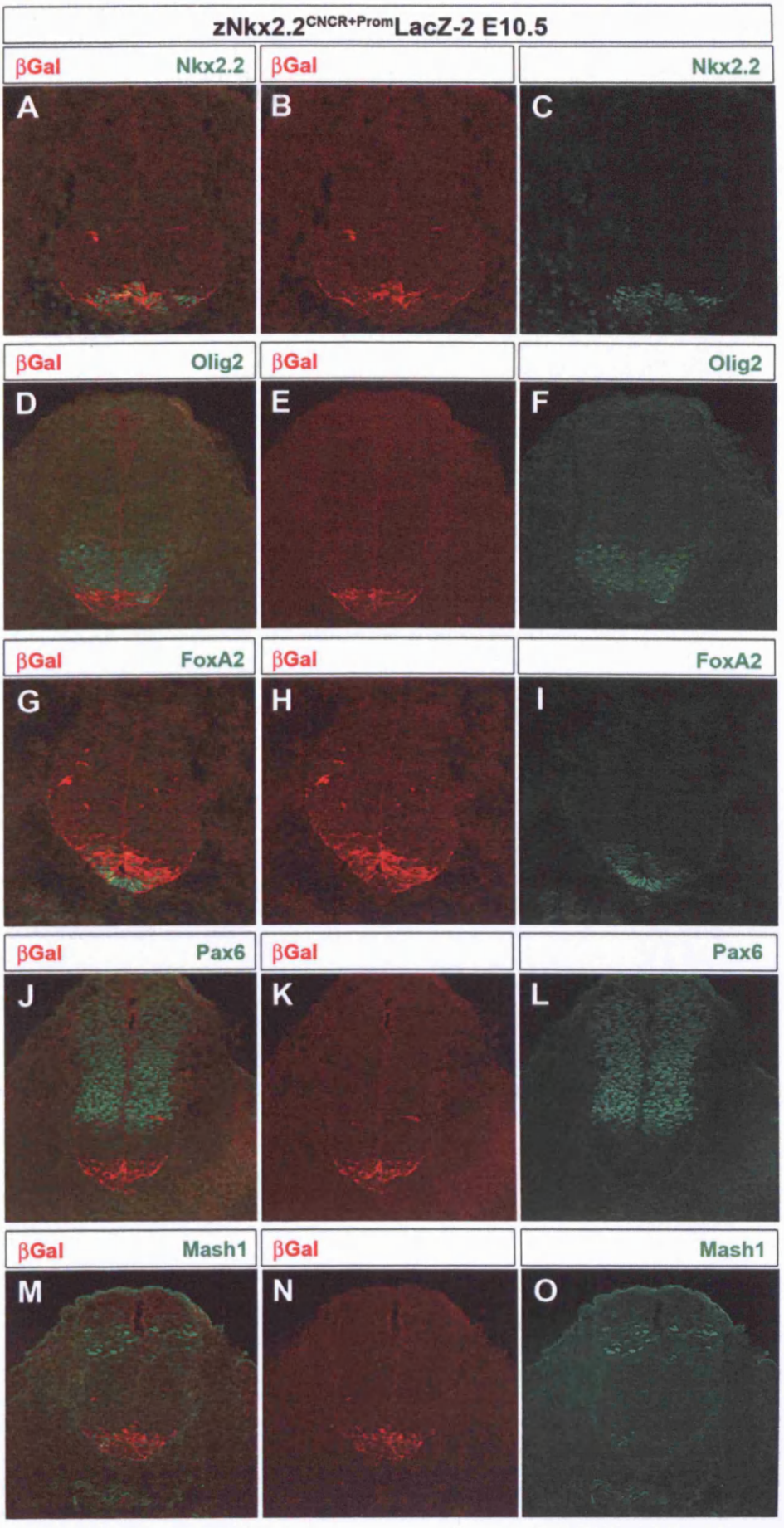

zNkx2.2CNCR+PromLacZ-2 E10.5
βGal
Nkx2.2
A
βGal
B
Nkx2.2
C
βGal
Olig2
D
βGal
E
Olig2
F
βGal
FoxA2
G
βGal
H
FoxA2
I
βGal
Pax6
J
βGal
K
Pax6
L
βGal
Mash1
M
βGal
N
Mash1
O

Figure 5.14 Immunohistochemistry for neural tube post mitotic neuronal markers in zNkx2.2$^{CNCR+Prom}$LacZ-2 mouse lines at E10.5.

Embryos were collected from a stable mouse line containing reporter construct zNkx2.2$^{CNCR+Prom}$LacZ-2 at E10.5. Co-immunostaining for neuronal post mitotic markers and β-galactosidase was performed on 12μM cryosections. **A-C** Lim1/2 and β-gal staining, **D-F** Lim3 and β-gal staining, **G-I** Gata3 and β-gal staining, **J-L** Chx10 and β-gal staining. As was observed with reporter construct zNkx2.2$^{CNCR+Prom}$LacZ, immunostaining for post-mitotic markers suggests that the transgene zNkx2.2$^{CNCR+Prom}$LacZ-2 directed intermediate expression of β-gal in post mitotic neurons. Co-expression of β-gal with Lim1/2 (**A**), Lim3 (**D**), Gata3 (**G, G'**) and Chx10 (**J, J'**) suggests these intermediate cells were V2 interneuron post mitotic cells of both a and b subtype.

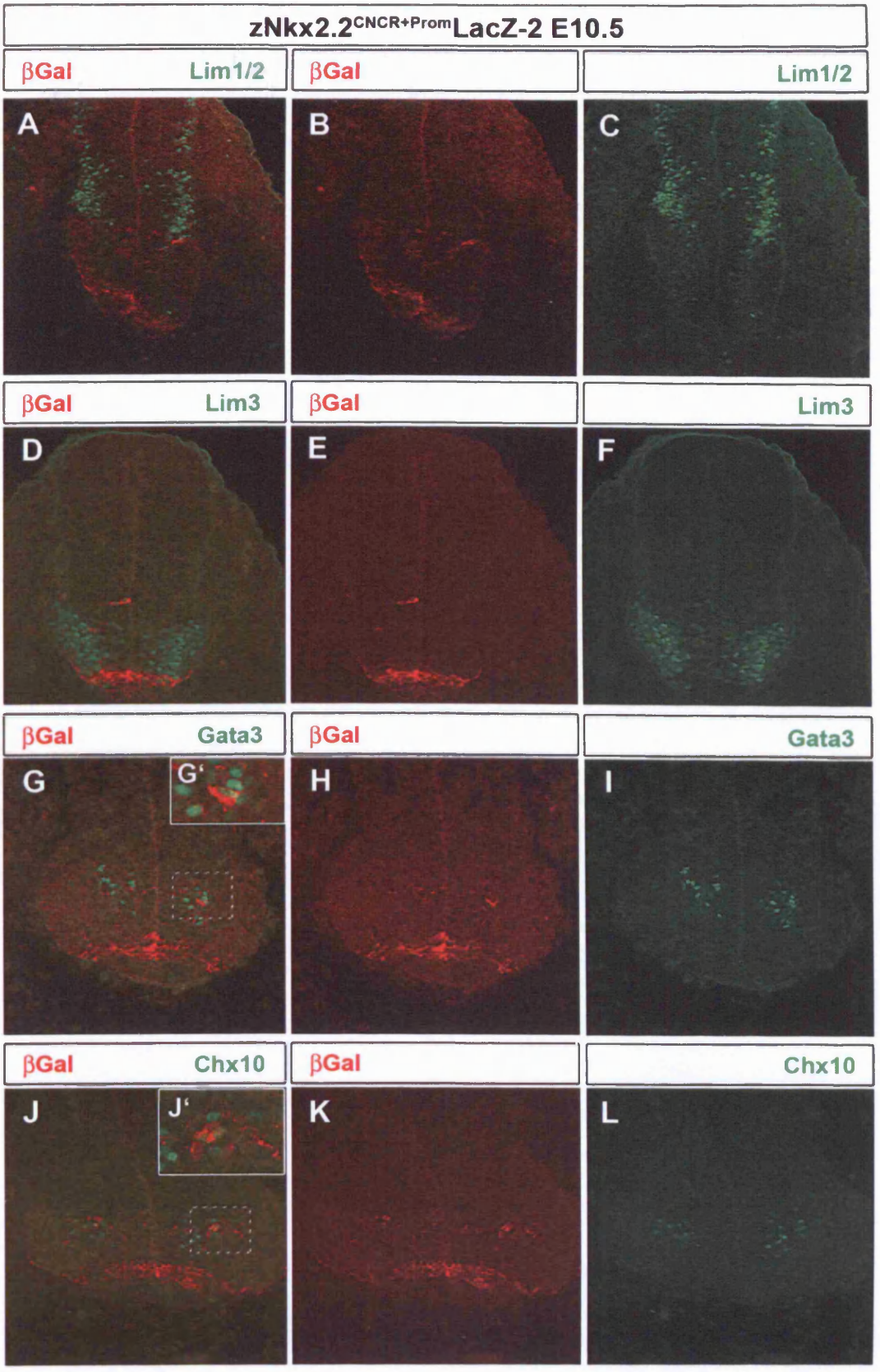

zNkx2.2CNCR+PromLacZ-2 E10.5
βGal
Lim1/2
βGal
Lim1/2
A
B
C
βGal
Lim3
βGal
Lim3
D
E
F
βGal
Gata3
βGal
Gata3
G
G'
H
I
βGal
Chx10
βGal
Chx10
J
J'
K
L

Figure 5.15 Immunohistochemistry for neural tube progenitor and post mitotic neuronal markers in zNkx2.2$^{CNCR+Prom}$LacZ-2 mouse lines at E11.5.

Embryos were collected from a stable mouse line containing reporter construct zNkx2.2$^{CNCR+Prom}$LacZ-2 at E11.5. Co-immunostaining for neuronal progenitor and post mitotic markers with β-galactosidase was performed on 12μM cryosections. **A-C** Nkx2.2 and β-gal staining, **D-F** FoxA2 and β-gal staining, **G-I** Lim1/2 and β-gal staining, **J-L** Lim3 and β-gal staining, **M-O** Gata3 and β-gal staining, **P-R** Chx10 and β-gal staining. By E11.5, the number of β-gal expressing cells within the V3 interneuron progenitor domain had reduced compared to the number seen at E10.5. There were still a few cells expressing LacZ in the V2 interneuron domain. The ventral LacZ expressing cells were restricted to the Nkx2.2 positive p3 domain, however not all Nkx2.2 positive cells also expressed β-gal (**A**). There were no cells co-expressing FoxA2 and LacZ (**B**), suggesting that the transgene did not direct expression in the floor plate at E11.5. The number of post-mitotic neurons observed in E11.5 embryos had increased compared to E10.5. The β-gal positive intermediate cells were found to co-express several V2 interneuron markers: Lim3 (**J, J'**), Gata3 (**M,** marked with white arrow), Chx10 (**P**, marked with white arrow). However, no cells were identified co-expressing β-gal and Lim1/2 (**G**). These data suggest the intermediate population of LacZ positive cells were V2 interneurons of subtypes a and b.

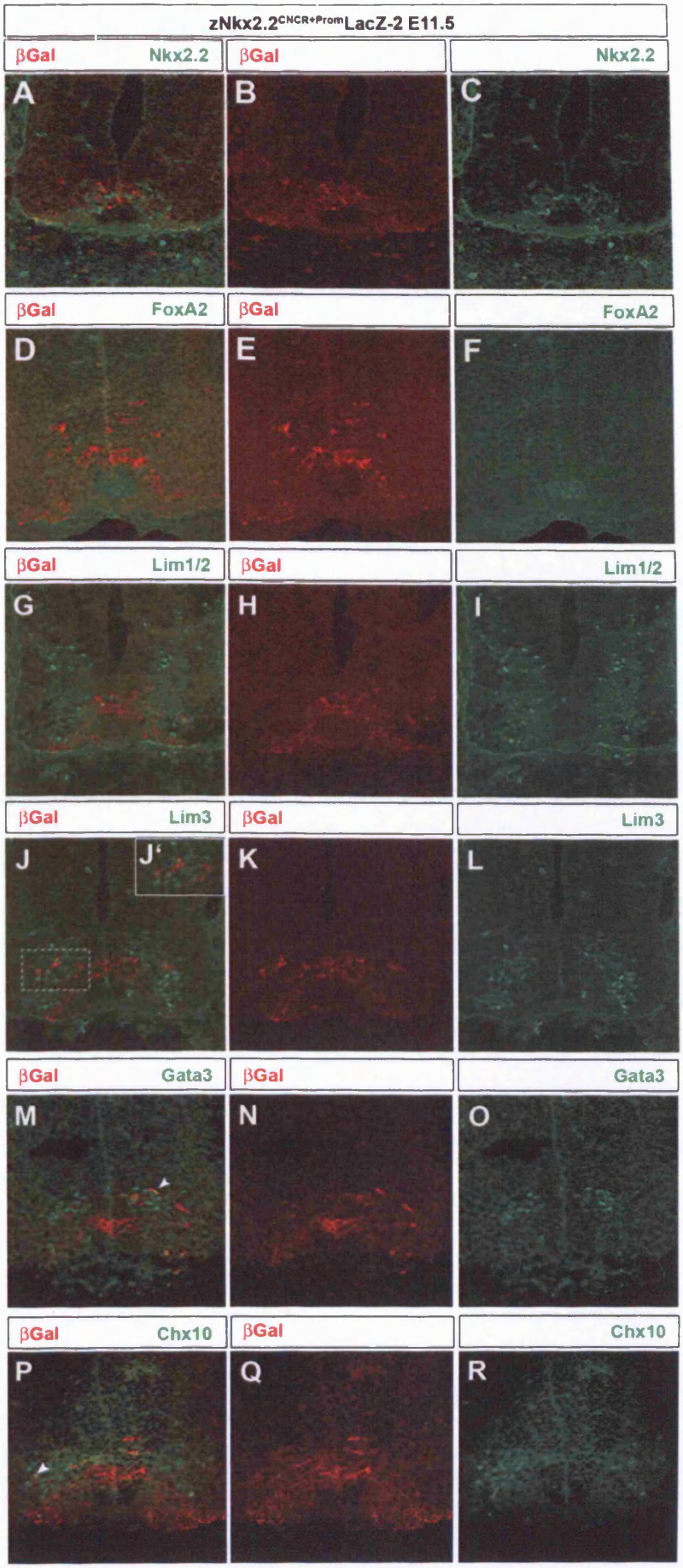

zNkx2.2CNCR+PromLacZ-2 E11.5
βGal Nkx2.2
βGal
Nkx2.2
A
B
C
βGal FoxA2
βGal
FoxA2
D
E
F
βGal Lim1/2
βGal
Lim1/2
G
H
I
βGal Lim3
βGal
Lim3
J
J'
K
L
βGal Gata3
βGal
Gata3
M
N
O
βGal Chx10
βGal
Chx10
P
Q
R

Figure 5.16 CNCR is necessary and sufficient for the activity of zNkx2.2$^{CNCR+Prom}$LacZ.

Reporter construct zNkx2.2$^{CNCR+Prom}$LacZ (**A**) contained 1.3kb of DNA from the zebrafish *Nkx2.2a* gene. Mouse pronuclear injection showed it could direct specific Nkx2.2-like expression of reporter *LacZ* in the ventral neural tube. Constructs were created based on this original plasmid containing the same reporter but either just the 250bp CNCR (**B**) or the reciprocal region, which did not contain the CNCR (**C**). Reporter constructs zNkx2.2^{CNCR}LacZ and zNkx2.2$^{\Delta CNCR+Prom}$LacZ were assayed by mouse pronuclear injection and embryos were stained for β–galactosidase activity at E10.5. Injection of zNkx2.2^{CNCR}LacZ led to reproduction of the expression pattern of the zNkx2.2$^{CNCR+Prom}$LacZ transgene that contained the CNCR and DNA up to the transcription start site of the zebrafish *Nkx2.2a* gene (**D**). The expression pattern in ventral neural tube was comparable to *Nkx2.2* expression. Vibratome sections (50μm) showed reporter expression at intermediate locations in the neural tube (**E**) in addition to the ventral expression. This result suggests the CNCR was sufficient for ventral reporter expression in the neural tube. Injection of the reciprocal region that did not contain the CNCR did not direct reporter expression (**F**) in any of the 29 embryos analysed. The absence of the CNCR in the promoter reporter construct resulted in a loss of *LacZ* expression suggesting the CNCR was necessary for correct ventral neural tube reporter expression.

A zNkx2.2CNCR+PromLacZ
B zNkx2.2CNCR LacZ
C zNkx2.2ΔCNCR+PromLacZ
CNCR
5'UTR
zNkx2.2a
hsp68
LacZ
+
zNkx2.2CNCR LacZ
zNkx2.2ΔCNCR+PromLacZ
E10.5
D
E
F

6 Results: Identification of CNCR Domains Necessary for Ventral Neural Tube Expression

6.1 Mutation of the Gli Binding Site Located Within the CNCR

In silico analysis of the *Nkx2.2* and *Nkx2.9* promoters identified a 250bp conserved regulatory element (CNCR; Chapter 4), which contained many potential transcription factor binding sites. Most notable of these was the Gli binding site (GBS), raising the possibility that there is a direct requirement for Shh signalling in *Nkx2.2* induction.

We next set out to test the hypothesis, to determine if the putative GBS in the CNCR was necessary for the activity of this element. To ascertain this, we mutated the GBS located at the 3' end of the CNCR and assayed the expression pattern *in vivo*. The Gli binding site consensus sequence 5' GACCACCCA 3' was mutated to the sequence 5' GAAGTGGGA 3' using a commercially available site-directed mutagenesis kit (Fig. 6.1A-6.1C). The mutated sequence has previously been shown to be unable to bind to Gli proteins in gel mobility shift assays (Sasaki et al., 1997).

Mouse PNI assayed the reporter activity of the construct containing the mutated GBS, zNkx2.2$^{CNCR+Prom}$GBSMutLacZ. Sections of E10.5-E11.5 transient mouse embryos showed a loss of *LacZ* expression in the ventral domain of the neural tube (Fig. 6.1E-6.1G). Of the 192 embryos analysed, only 5 contained observable β-galactosidase activity. In 3 of the β-galactosidase positive embryos, we observed *LacZ* expression in an intermediate population of neurons in the neural tube, but not in the ventral neural tube (Fig. 6.1E, 6.1F). The remainder of the embryos had no β-galactosidase activity in the neural tube (Fig. 6.1G). This contrasted with PNI of zNkx2.2$^{CNCR+Prom}$LacZ in which 7 out of 48 transient embryos analysed were positive for β-galactosidase, all of which contained activity in the ventral neural tube. From this we concluded that the GBS in the context of zNkx2.2$^{CNCR+Prom}$LacZ is necessary for the ventral expression of *LacZ*. The GBS, however, is not required for the

intermediate neural tube expression observed in a small proportion of injected embryos.

6.2 *Gli Binding Site is not Sufficient to Drive Ventral Neural Tube Expression*

We next wanted to determine which regions of the CNCR were sufficient to direct expression in the ventral neural tube. We first generated a construct containing the GBS but none of the other conserved putative transcription factor binding sites (Fig. 4.4). This construct, zNkx2.2Δ1$^{CNCR+Prom}$LacZ (Fig. 6.2B), was assayed by mouse PNI. E10.5 embryos were stained for β-galactosidase activity. 88 transient embryos were analysed, of these, 7 were positive for β-galactosidase activity. All of these positive transient embryos contained intermediate neural tube staining (Fig. 6.3A-6.3C) similar to that observed with zNkx2.2$^{CNCR+Prom}$GBSMutLacZ, however, none displayed *LacZ* expression in the ventral neural tube. This result indicates that zNkx2.2Δ1$^{CNCR+Prom}$LacZ, which includes the GBS (start position of construct marked on Fig. 6.2E), is not sufficient to drive expression of *LacZ* in the ventral neural tube.

To identify which regions are required to drive ventral expression, a second construct was made: zNkx2.2Δ2$^{CNCR+Prom}$LacZ included an extra 32bp of DNA 5' to the start site of zNkx2.2Δ1$^{CNCR+Prom}$LacZ (Fig. 6.2E). The enhancer activity of this construct was assayed with mouse PNI. The resulting transient embryos were analysed at E10.5-E11.5. Of the 50 transient embryos analysed, 6 stained positive for *LacZ* expression (Fig. 6.3D-6.3F). All of these positive embryos displayed a restoration of the ventral *LacZ* expression in the neural tube, albeit weaker, and in some cases more ventrally restricted than in the original zNkx2.2$^{CNCR+Prom}$LacZ construct (Fig. 5.8). The intermediate expression previously described was also apparent in these transient embryos (Fig. 6.3D-6.3F). A stable mouse line containing the zNkx2.2Δ2$^{CNCR+Prom}$LacZ construct was generated (see below), analysis indicated that the *LacZ* expression in the ventral neural tube was comparable to zNkx2.2$^{CNCR+Prom}$LacZ (Figs. 6.4-6.6). However, one noticeable difference in expression pattern was the expression of *LacZ* in the floor plate not seen in embryos

containing the zNkx2.2$^{CNCR+Prom}$LacZ transgene. The loss of floor plate repression upon deletion of fragments of the CNCR will be discussed later (Chapter 7).

To further define the fragment within the CNCR necessary for ventral neural tube expression, a third construct was made, zNkx2.2Δ3$^{CNCR+Prom}$LacZ (Fig. 6.2D, 6.2E). In this construct the putative bHLH binding site was removed (Fig. 6.2E). zNkx2.2Δ3$^{CNCR+Prom}$LacZ contained the GBS and a further 13 bp 5' to the GBS compared to zNkx2.2Δ1$^{CNCR+Prom}$LacZ. Transient mouse embryos were analysed after PNI at E10.5-E11.5 and stained for β-galactosidase activity. Of the 95 transient embryos analysed, 10 stained positive. Expression of *LacZ* in 9 of these embryos was comparable to that observed with zNkx2.2$^{CNCR+Prom}$LacZ and zNkx2.2Δ2$^{CNCR+Prom}$LacZ constructs; expression was observed in the ventral and intermediate neural tube (Fig. 6.3G-6.3I).

The results obtained from the 3 constructs: zNkx2.2Δ 1$^{CNCR+Prom}$LacZ, zNkx2.2Δ2$^{CNCR+Prom}$LacZ and zNkx2.2Δ3$^{CNCR+Prom}$LacZ suggested that the GBS alone was not sufficient for the ventral neural tube expression of reporter gene *LacZ*. However the addition of an approximately 13bp element restored expression in the ventral neural tube. The putative bHLH binding site present in the CNCR is not necessary (Fig. 6.2). Intermediate expression in the neural tube is maintained in all three constructs.

6.3 Characterisation of the Intermediate Cell Population Expressing LacZ in the Neural Tube of the zNkx2.2Δ2$^{CNCR+Prom}$LacZ Stable Mouse Line

The construct zNkx2.2Δ2$^{CNCR+Prom}$LacZ when expressed transiently in mouse resulted in both ventral expression of reporter LacZ and also expression in a more intermediate population of cells (Fig. 6.3D-6.3F). Our previous analysis of zNkx2.2$^{CNCR+Prom}$LacZ and zNkx2.2$^{CNCR+Prom}$LacZ-2 suggested the intermediate expression comprised a population of V2 interneurons (Chapter 5) while the ventral population labelled V3

interneurons. To characterise the LacZ positive cells in the mouse line expressing zNkx2.2Δ2$^{CNCR+Prom}$LacZ, embryos were harvested at E10.5 and E11.5. These embryos were analysed by immunohistochemistry for molecular markers of progenitor cells and post mitotic neurons in the neural tube.

In E10.5 zNkx2.2Δ2$^{CNCR+Prom}$LacZ embryos, β-galactosidase activity was observed at high levels in the ventral V3 domain of the neural tube and in an intermediate population of cells in the neural tube (Fig. 6.4). The ventral LacZ expression marks a population of cells that also expresses Nkx2.2 (Fig. 6.4A-6.4C) and FoxA2 (Fig. 6.4G-6.4I). However, not all Nkx2.2 expressing cells co-expressed LacZ. The dorsal limit of β-galactosidase activity was more ventral to that of Nkx2.2 (marked by white arrowhead Fig. 6.4A). This difference could be explained by the temporal induction of expression in the p3 domain. The levels of β-galactosidase enzyme at more dorsal positions may not have reached sufficient levels to be detected by the antibody at this time point. In contrast expression of Nkx2.2 may have reached sufficient levels to be detected. To resolve this, the same immunostaining was performed at a later time point (see below, E11.5 analysis). LacZ was also expressed in floor plate cells marked by expression of FoxA2 (Fig. 6.4G-6.4I). At these stages there were a few Nkx2.2 positive cells in the floor plate (Fig. 6.4C: Jeong and McMahon, 2005), but the majority of expression had shifted dorsally.

The intermediate population of cells expressing LacZ at E10.5 in zNkx2.2Δ2$^{CNCR+Prom}$LacZ embryos were observed at a position dorsal to that of Olig2 (Fig. 6.4D-6.4F), which is expressed in the pMN domain. Olig2 expression appeared to mark the ventral limit of the LacZ positive cells in the intermediate neural tube (Fig. 6.4D). Pax6, a marker for the progenitor cells in the pMN domain and more dorsal progenitor populations, was co-expressed with LacZ in a small number of cells (Fig. 6.4J), indicating the zNkx2.2Δ2$^{CNCR+Prom}$LacZ drives LacZ expression in progenitor cells. To determine the identity of the progenitors cells, co-immunostaining with Mash1 was performed on E10.5 sections. Mash1 marks a population of progenitor cells that differentiate into V2b interneurons as well as a dorsal population of cells in the neural tube (Li et al., 2005; Mizuguchi et al., 2001). In E10.5 embryos some of the Mash1 positive cells in the V2 interneuron progenitor

population co-expressed LacZ (Fig. 6.4M-6.4O), suggesting the LacZ positive intermediate population of cells are progenitors for V2 interneurons at E10.5.

To determine if zNkx2.2Δ2$^{CNCR+Prom}$LacZ directed expression of LacZ in post mitotic cells, co-immunostaining for β-galactosidase and post-mitotic markers was performed. In mouse embryos at E10.5 the numbers of post-mitotic neurons were low, however, there were populations of cells expressing Lim1/2 (V0, V1, V2 interneurons), Lim3 (V2 interneuron, MN), Gata3 (V2b interneuron) and Chx10 (V2a interneuron) expressing cells (Fig. 6.5). E10.5 sections from the transgenic embryos were co-stained for these 4 post-mitotic markers and β-galactosidase. Double positive cells were observed with all four markers; Lim 1/2 (Fig. 6.5A-6.5C), Lim3 (Fig. 6.5D-6.5F), Gata3 (Fig. 6.5G-6.5I) and Chx10 (Fig. 6.5J-6.5L). This result confirms that the intermediate β-galactosidase expressing cells in the mouse line containing zNkx2.2Δ2$^{CNCR+Prom}$LacZ reporter are V2 interneuron cells, of both V2a and V2b subtypes. The reporter is expressed in both the progenitor and post-mitotic cells.

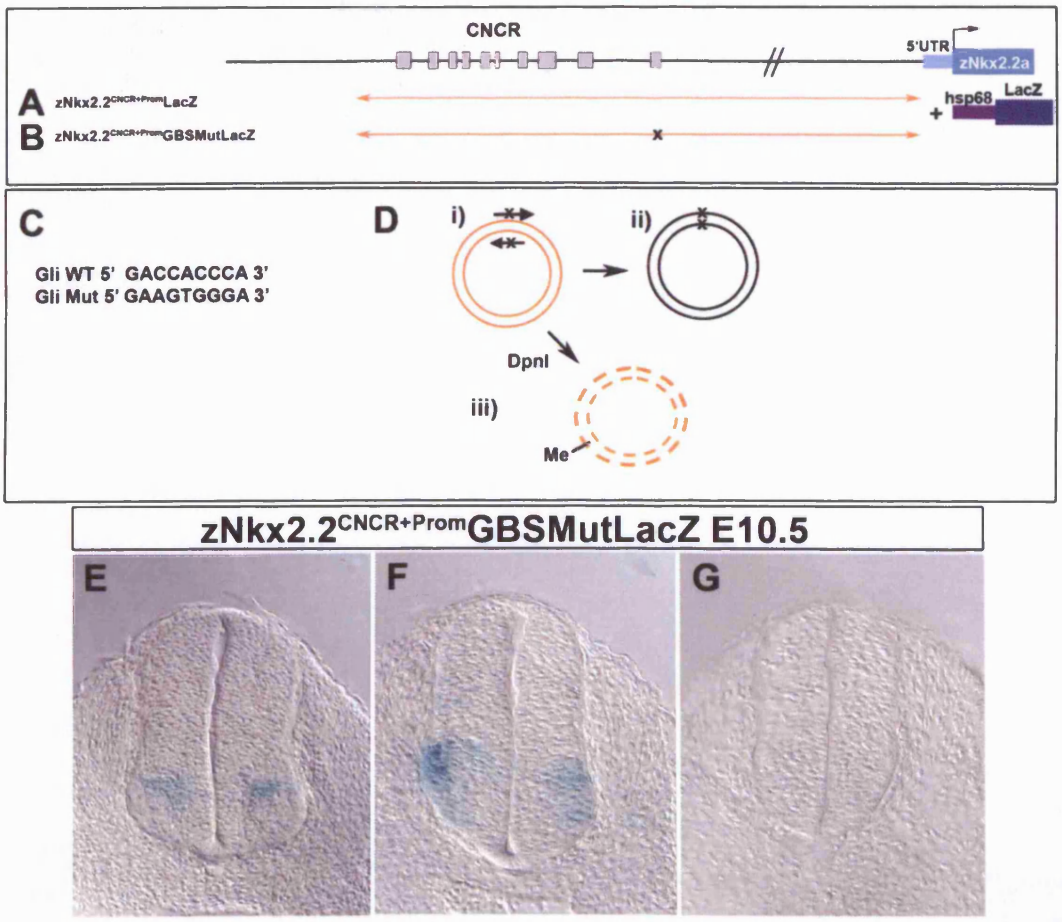
In order to determine the expression of LacZ in the zNkx2.2Δ2$^{CNCR+Prom}$LacZ transgenic mouse at later stages, E11.5 embryos were analysed. The expression of the reporter in the ventral V3 interneuron domain and floor plate cells at these later stages was much reduced (Fig. 6.6), there were very few cells expressing the reporter in the ventral domain of the neural tube. The few ventral LacZ expressing cells observed were restricted to the Nkx2.2 positive p3 domain (Fig. 6.6A), however none were seen in the FoxA2 positive floor plate (Fig. 6.6D) as was observed at E10.5. It would therefore appear that in the reporter construct zNkx2.2Δ2$^{CNCR+Prom}$LacZ, an element that directs correct V3 domain expression of LacZ at E11.5 is missing. At E10.5, the LacZ reporter was not detected in more dorsal Nkx2.2 positive p3 domain progenitor cells. This therefore suggests that the zNkx2.2Δ2$^{CNCR+Prom}$LacZ construct is able to direct expression within the p3 domain, however it cannot recapitulate precise Nkx2.2-like expression.

The LacZ positive cells in the more intermediate domain of the E11.5 mouse embryos harbouring zNkx2.2Δ2$^{CNCR+Prom}$LacZ, did not appear to be reduced in number

compared to E10.5 embryos (Fig. 6.6). At E10.5 this population of cells marked V2 post-mitotic interneurons, to confirm if these were the same cells, immunohistochemistry of β-Gal with the same 4 post-mitotic neuronal markers was performed (Fig. 6.6). Double positive cells were observed (Fig. 6.6 marked with white arrows) with all four markers; Lim1/2 (Fig. 6.6G), Lim3 (Fig. 6.6J), Gata3 (Fig. 6.6M) and Chx10 (Fig. 6.6P). These data suggest that the construct zNkx2.2Δ2$^{CNCR+Prom}$LacZ directs expression of the reporter in V2 post-mitotic interneurons at E11.5 as was observed at E10.5.

Figure 6.1 Transient results of mouse pronuclear injection at E10.5 of promoter construct zNkx2.2$^{CNCR+Prom}$GBSMutLacZ.

Constructs were made from the zebrafish *Nkx2.2a* promoter. zNkx2.2$^{CNCR+Prom}$LacZ contained 1.3kb of promoter DNA fused upstream of hsp68 (minimal promoter) and reporter LacZ (**A**). zNkx2.2$^{CNCR+Prom}$GBSMutLacZ is based on the zNkx2.2$^{CNCR+Prom}$LacZ construct, but contains a mutation in the Gli binding site (GBS) located within the Conserved Non-Coding Region (CNCR: **B**). The mutation was from consensus binding site 5' GACCACCCA 3' to 5' GAAGTGGGA 3' (**C**) known to no longer bind to Gli proteins (Sasaki et al., 1997). The mutagenesis was carried out using a commercially available kit (Stratagene). The method relied upon PCR of the plasmid of zNkx2.2$^{CNCR+Prom}$LacZ using overlapping primers both containing the mutation (**Di**). The resulting plasmid contained mutations (x) on both strands (**Dii**). The template DNA was digested using DpnI, which cuts any methylated or hemi-methylated DNA (**Diii**), the template DNA was therefore destroyed upon transfection into competent bacteria to amplify mutated DNA. The new construct was assayed by mouse pronuclear injection, to test if the mutation of the GBS would lead to a change in reporter expression pattern. Embryos were harvested at E10.5 and stained for β-galactosidase activity before sectioning. Ventral expression of *LacZ* was lost in these embryos (**E-G**), intermediate staining, albeit weak, was observed in 3 out of 192 embryos (**E, F**). However, most embryos were negative for any LacZ expression (**G**), suggesting the GBS was necessary for ventral reporter expression.


CNCR
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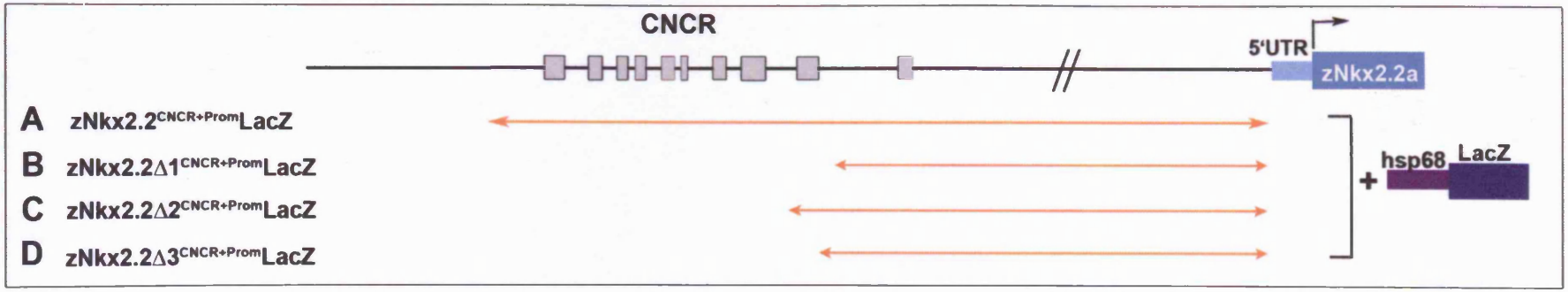
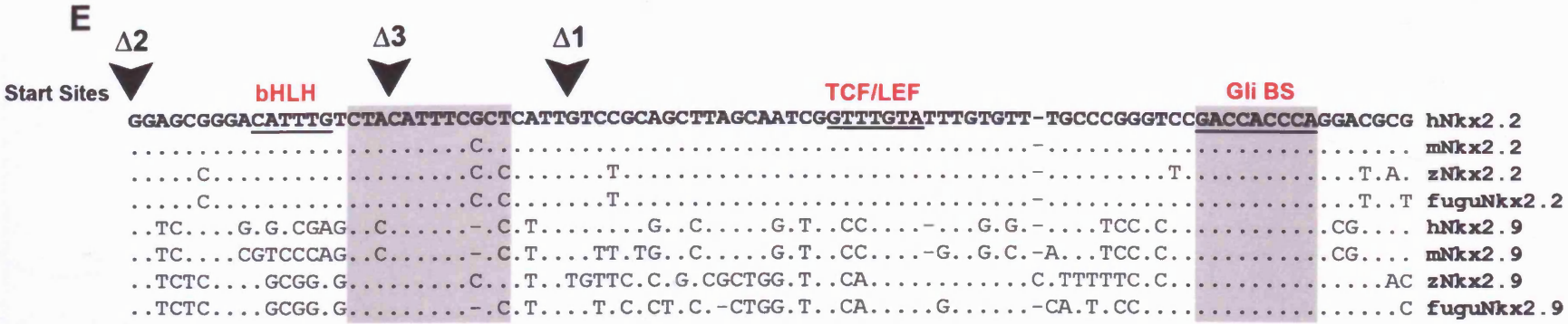
B zNkx2.2CNCR+PromGBSMutLacZ
hsp68
LacZ
C
Gli WT 5' GACCACCCA 3'
Gli Mut 5' GAAGTGGGA 3'
D
i)
ii)
DpnI
iii)
Me
zNkx2.2CNCR+PromGBSMutLacZ E10.5
E
F
G

Figure 6.2 Summary diagrams of the deletion series of zNkx2.2$^{CNCR+Prom}$LacZ constructed to determine CNCR enhancer fragments sufficient to drive LacZ expression in the ventral neural tube.

Enhancer fragments based on zNkx2.2$^{CNCR+Prom}$(**A**) were constructed and placed upstream of a minimal reporter (hsp68) and a reporter (LacZ). These constructs all contained the Gli binding site (GBS) found at the 3' end of the identified CNCR and additional 5' DNA. The first construct, zNkx2.2Δ1$^{CNCR+Prom}$LacZ, contained only the GBS (**B, E**), the second, zNkx2.2Δ2$^{CNCR+Prom}$LacZ, contained an extra 32bp DNA 5' to Δ1 (**C, E**) and the third, zNkx2.2Δ3$^{CNCR+Prom}$LacZ, contained an extra 13bp DNA 5' to Δ1 (**D, E**). **E** The DNA sequence from the CNCR contained in the constructs.


CNCR
5'UTR
zNkx2.2a
A zNkx2.2CNCR+PromLacZ
B zNkx2.2Δ1CNCR+PromLacZ
C zNkx2.2Δ2CNCR+PromLacZ
D zNkx2.2Δ3CNCR+PromLacZ
hsp68 LacZ

E


Δ2
Δ3
Δ1
Start Sites
bHLH
TCF/LEF
Gli BS
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mNkx2.2
zNkx2.2
fuguNkx2.2
hNkx2.9
mNkx2.9
zNkx2.9
fuguNkx2.9

Figure 6.3 Transient results of mouse pronuclear injection at E10.5/E11.5 of zebrafish promoter constructs zNkx2.2Δ1$^{CNCR+Prom}$LacZ, zNkx2.2Δ2$^{CNCR+Prom}$LacZ and zNkx2.2Δ3$^{CNCR+Prom}$LacZ.

Expression patterns of reporter constructs zNkx2.2Δ1$^{CNCR+Prom}$LacZ, zNkx2.2Δ2$^{CNCR+Prom}$LacZ and zNkx2.2Δ3$^{CNCR+Prom}$LacZ were assayed by mouse PNI. These constructs were made from deleting portions of zNkx2.2$^{CNCR+Prom}$LacZ, which in transient mouse embryos at E10.5 led to intermediate and ventral *LacZ* expression in the neural tube. Embryos were harvested at E10.5 and E11.5 and stained for β-galactosidase activity and sectioned before analysis. zNkx2.2Δ1$^{CNCR+Prom}$LacZ transient embryos at E10.5 (**A, B**) and E11.5 (**C**) displayed expression of *LacZ* in an intermediate position within the neural tube, but there was a loss of ventral expression. zNkx2.2Δ2$^{CNCR+Prom}$LacZ was tested in the same manner and expression of LacZ at E10.5 (**D, E**) and E11.5 (**F**) was also observed in an intermediate domain, however ventral expression was restored, albeit weaker than zNkx2.2$^{CNCR+Prom}$LacZ (Fig. 5.8). Finally zNkx2.2Δ3$^{CNCR+Prom}$LacZ was tested, E10.5 embryos were harvested and stained (**G-I**). Expression was similar to results observed with zNkx2.2$^{CNCR+Prom}$LacZ and zNkx2.2Δ2$^{CNCR+Prom}$LacZ; *LacZ* was expressed in both intermediate and ventral positions.

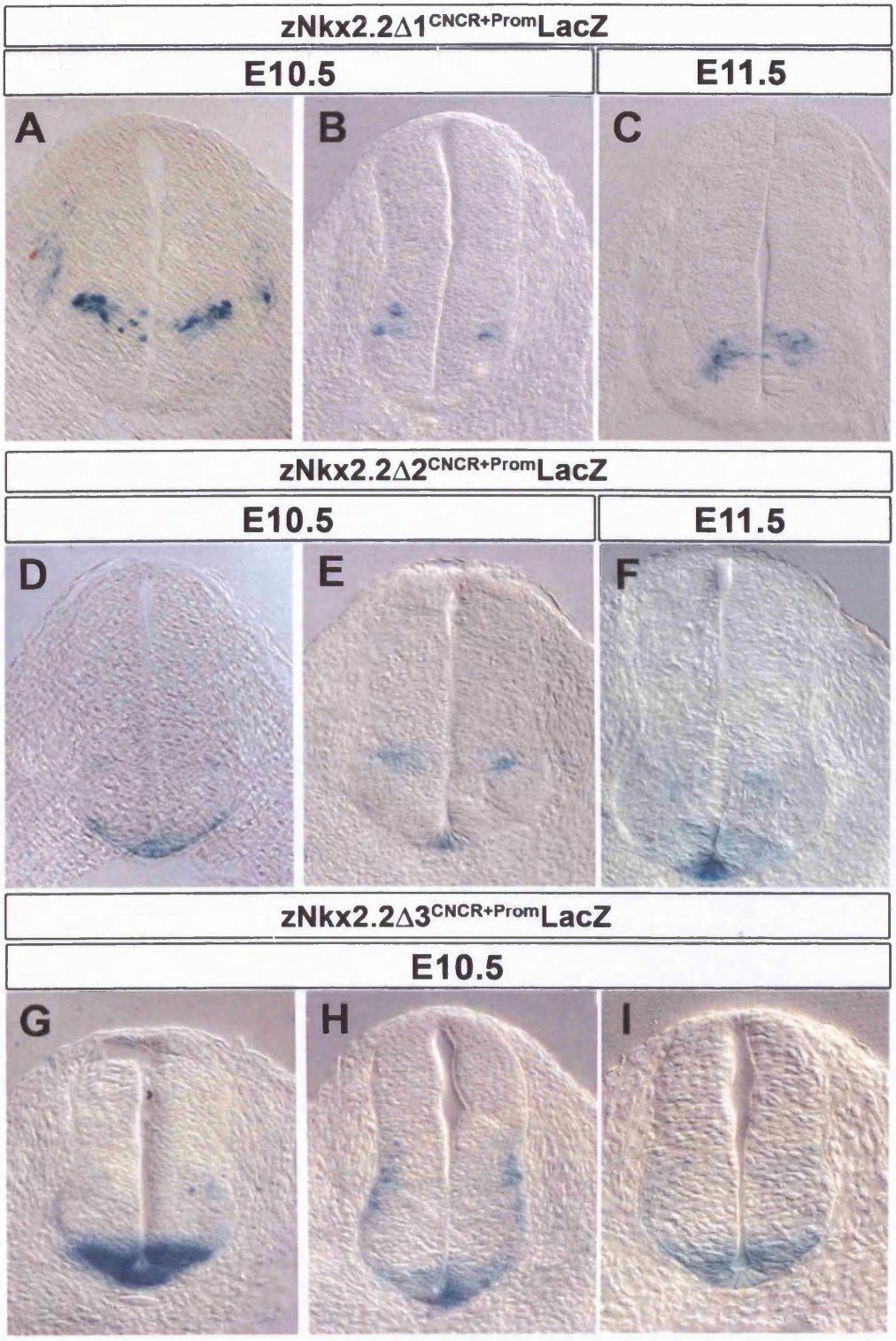

zNkx2.2Δ1CNCR+PromLacZ
E10.5
E11.5
A
B
C
zNkx2.2Δ2CNCR+PromLacZ
E10.5
E11.5
D
E
F
zNkx2.2Δ3CNCR+PromLacZ
E10.5
G
H
I

Figure 6.4 Immunohistochemistry for neural tube progenitor markers in zNkx2.2Δ2$^{CNCR+Prom}$LacZ mouse lines at E10.5.

A stable mouse line containing the reporter transgene zNkx2.2Δ2$^{CNCR+Prom}$LacZ was created. Embryos were harvested at E10.5, cryosectioned and co-immunostained for molecular markers of neural tube progenitor cells and the reporter β-galactosidase. **A-C** Nkx2.2 and β-Gal staining, **D-F** Olig2 and β-Gal staining, **G-I** FoxA2 and β-Gal staining, **J-L** Pax6 and β-Gal staining, **M-O** Mash1 and β-Gal staining. LacZ positive cells located in the ventral neural tube, co-expressed Nkx2.2 (**A**) and FoxA2 (**G**), suggesting expression in both the p3 domain and the floor plate of the neural tube. The dorsal limit of LacZ expression in the ventral domain (**A**, marked by white arrowhead) did not extend as far dorsally as the Nkx2.2 limit. The dorsal limit of ventral LacZ expression marked the ventral limit of MN marker Olig2 (**D**). The intermediate LacZ positive cells were located dorsal to the Olig2 positive cells (**D**) and some cells co-expressed Pax6 (**J**) and Mash1 (**M, M'**), suggesting the intermediate population marked V2 interneuron progenitors.

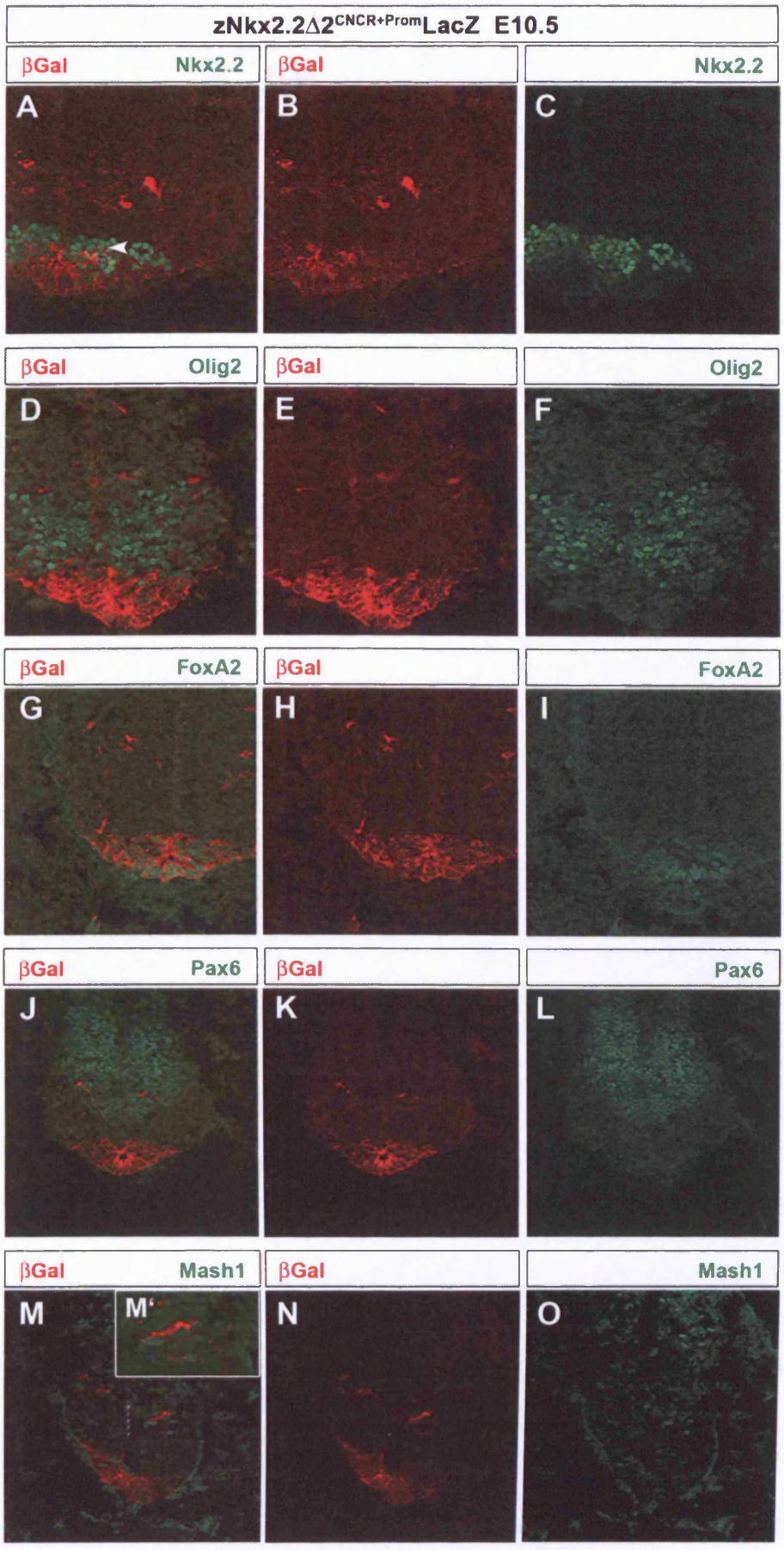

zNkx2.2Δ2CNCR+PromLacZ E10.5
βGal
Nkx2.2
A
βGal
B
Nkx2.2
C
βGal
Olig2
D
βGal
E
Olig2
F
βGal
FoxA2
G
βGal
H
FoxA2
I
βGal
Pax6
J
βGal
K
Pax6
L
βGal
Mash1
M
M'
βGal
N
Mash1
O

Figure 6.5 Immunohistochemistry for neural tube post-mitotic markers in zNkx2.2Δ2$^{CNCR+Prom}$LacZ mouse lines at E10.5.

A stable mouse line containing the reporter transgene zNkx2.2Δ2$^{CNCR+Prom}$LacZ was created. Embryos were harvested at E10.5, cryosectioned and co-immunostained for molecular markers of neural tube post-mitotic cells and the reporter β-galactosidase. **A-C** Lim1/2 and β-Gal staining, **D-F** Lim3 and β-Gal staining, **G-I** Gata3 and β-Gal staining, **J-L** Chx10 and β-Gal staining. Co-expression of the LacZ reporter in intermediate positions of the neural tube with Lim1/2 (**A**), Lim3 (**D, D'**), Gata3 (**G, G'**) and Chx10 (**J, J'**) suggests the cells were V2 interneurons. The co-expression of both Gata3 and Chx10 suggests the reporter was expressed in both V2a and V2b subtypes.

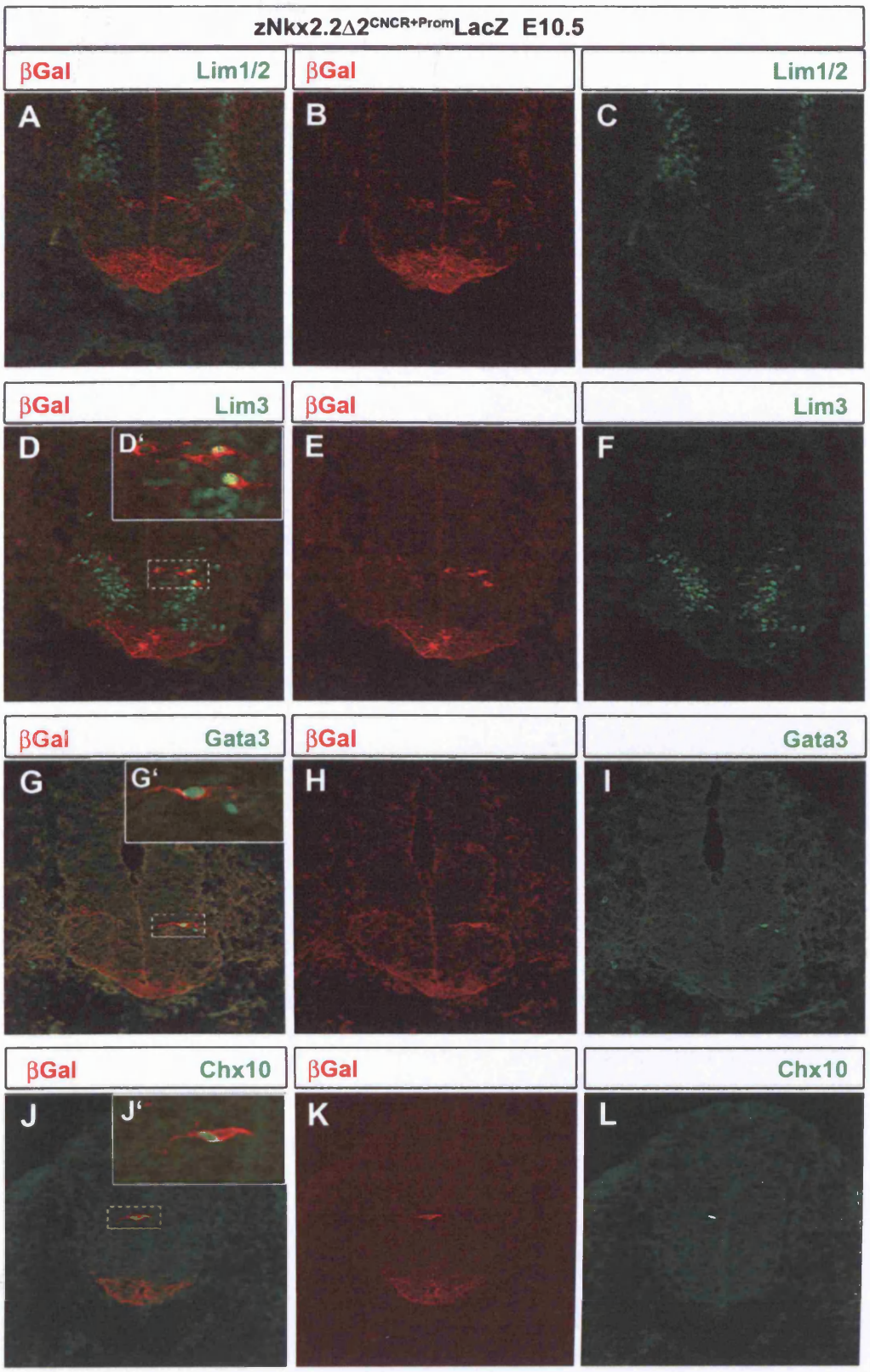
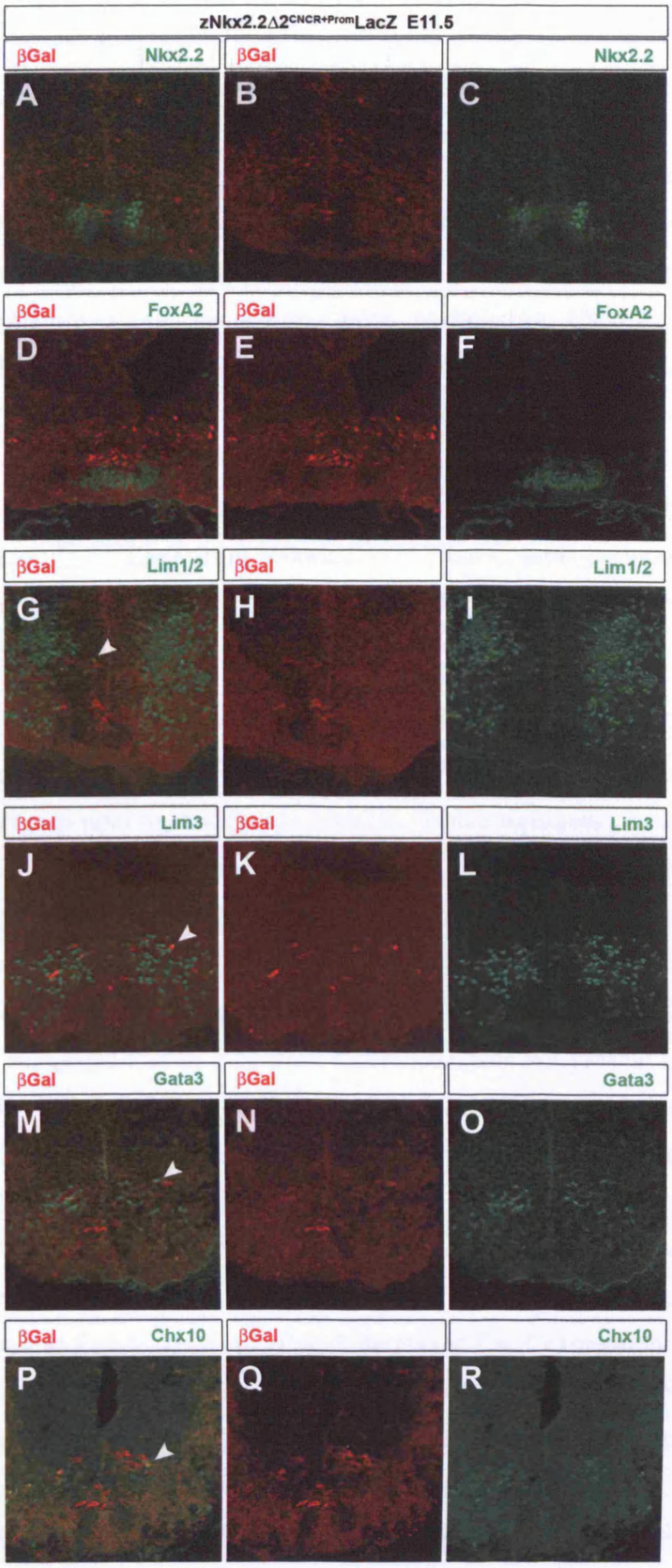

zNkx2.2Δ2CNCR+PromLacZ E10.5
βGal
Lim1/2
βGal
Lim1/2
A
B
C
βGal
Lim3
βGal
Lim3
D
D'
E
F
βGal
Gata3
βGal
Gata3
G
G'
H
I
βGal
Chx10
βGal
Chx10
J
J'
K
L

Figure 6.6 Immunohistochemistry for neural tube progenitor and post-mitotic markers in zNkx2.2Δ2$^{CNCR+Prom}$LacZ mouse lines at E11.5.

Embryos were harvested at E11.5 after mouse pronuclear injection of reporter construct zNkx2.2Δ2$^{CNCR+Prom}$LacZ. Co-immunostaining for neuronal progenitor and post mitotic markers with β-galactosidase was performed on 12μM cryosections. **A-C** Nkx2.2 and β-gal staining, **D-F** FoxA2 and β-gal staining, **G-I** Lim1/2 and β-gal staining, **J-L** Lim3 and β-gal staining, **M-O** Gata3 and β-gal staining, **P-R** Chx10 and β-gal staining. By E11.5, the β-gal expressing cells restricted to the V3 interneuron progenitor domain, were dramatically reduced in number compared to the number observed at E10.5 (Fig. 6.4). Only a few cells expressing Nkx2.2 also expressed β-gal (**A**) and there were no cells co-expressing FoxA2 and LacZ (**B**). The number of post-mitotic neurons present at E11.5 in the neural tube had increased compared to E10.5. Concomitantly there was an increase in the number of β–Gal positive intermediate cells. These intermediate LacZ positive cells were co-expressing Lim1/2 (**G**), Lim3 (**J**), Gata3 (**M**) and Chx10 (**P**). Double positive cells are marked with white arrowheads. This co-expression suggested the more intermediate cells were V2 post-mitotic interneurons of both subtypes a and b.


zNkx2.2Δ2CNCR+PromLacZ E11.5
βGal
Nkx2.2
A
B
C
βGal
FoxA2
D
E
F
βGal
Lim1/2
G
H
I
βGal
Lim3
J
K
L
βGal
Gata3
M
N
O
βGal
Chx10
P
Q
R

7 Results: Nkx2.2 Floor Plate Repression

7.1 Reporter Floor Plate Repression

In both stable and transient transgenic mice containing reporter constructs zNkx2.2Δ2$^{CNCR+Prom}$LacZ and zNkx2.2Δ3$^{CNCR+Prom}$LacZ, *LacZ* expression was apparent in the floor plate at E10.5-E11.5 (Figs. 6.3 - 6.6). In contrast, at the same developmental stage, transgenic mice harbouring zNkx2.2$^{CNCR+Prom}$LacZ and zNkx2.2$^{CNCR+Prom}$LacZ-2 constructs, showed repression of *LacZ* in the floor plate (Figs. 5.8 – 5.15). Importantly, by E11.5, *Nkx2.2* expression had been repressed from the floor plate (Fig. 5.11A: Briscoe et al., 1999). These data suggested that regulatory elements contained within the region of the CNCR that distinguishes zNkx2.2Δ2$^{CNCR+Prom}$LacZ from zNkx2.2$^{CNCR+Prom}$LacZ, must include one or more sites necessary for floor plate repression. To test this hypothesis, further constructs were generated and assayed with mouse PNI.

We first set out to confirm that zNkx2.2$^{CNCR+Prom}$LacZ was repressed in the floor plate in a manner similar to endogenous Nkx2.2. Stable transgenic mouse lines containing zNkx2.2$^{CNCR+Prom}$LacZ reporter construct were generated and immunohistochemistry was performed to identify the precise location of β-galactosidase activity. Cryosections of E10.5 and E11.5 embryos of four independent lines were obtained and immunohistochemistry for β-gal, FoxA2 and Nkx2.2 performed (Fig. 7.1). Results suggested that in 3 of these lines LacZ was not present in the floor plate at either E10.5 or E11.5 (Fig. 7.1E-7.1P). Moreover, β-gal was coincident with Nkx2.2 (Fig. 7.1E-7.1P; stable line D analysed further in Chapter 5). Expression of LacZ at both E10.5 and E11.5 in lines B, C, and D was restricted to the Nkx2.2 positive domain (Fig. 7.1E, 7.1G, 7.1I, 7.1K, 7.1M, 7.1O). No co-expression between LacZ and FoxA2 was observed at E10.5 or E11.5 (Fig. 7.1F, 7.1H, 7.1J, 7.1L, 7.1N, 7.1P). In contrast at E10.5, transgenic line A displayed LacZ expression in both the Nkx2.2 expressing domain (Fig. 7.1A) and the FoxA2 expressing domain (Fig. 7.1B). Analysis at E11.5 showed LacZ expression had not been restricted to the Nkx2.2 domain only and was co-expressed with both Nkx2.2 and FoxA2. The repression of reporter expression in 3 out of 4 lines supports the idea that the zNkx2.2$^{CNCR+Prom}$LacZ

construct directs expression precisely in the Nkx2.2 p3 domain of the ventral neural tube and is sufficient to confer repression in the FoxA2 positive floor plate.

To delineate the region of CNCR necessary for floor plate repression, three further deletion constructs based on zNkx2.2$^{CNCR+Prom}$LacZ were constructed (Fig. 7.2); zNkx2.2Δ4$^{CNCR+Prom}$LacZ, zNkx2.2Δ5$^{CNCR+Prom}$LacZ and zNkx2.2Δ6$^{CNCR+Prom}$LacZ. The ability to direct *LacZ* expression was assayed by mouse PNI. Transient embryos were harvested between E10.5 and E12, and stained for β-galactosidase activity (Fig. 7.3A-7.3E) or analysed for *LacZ* expression by *in situ* hybridisation (Fig. 7.3F-7.3H). *In situ* hybridisation provides a reliable guide to the precise location of gene expression. This is because the long half-life of the β-galactosidase product means that the precise timing and location of the down-regulation of expression is concealed by perdurance of LacZ activity.

Embryos containing the zNkx2.2Δ4$^{CNCR+Prom}$LacZ transgene, stained for β-galactosidase activity at E10.5 displayed *LacZ* expression in the p3 domain, however it showed some repression in the floor plate (2/2; Fig. 7.3A). Embryos, assayed for β-galactosidase activity at E11.5, containing transgene zNkx2.2Δ5$^{CNCR+Prom}$LacZ demonstrated 2 different outcomes (Fig. 7.3B, 7.3C). In 4 of the 9 embryos positive for β-galactosidase activity in the neural tube, expression was restricted to the p3 domain (Fig. 7.3B). In the remaining 5 embryos, expression was in both the p3 domain and the floor plate (Fig. 7.3C). Enzymatic staining of embryos harbouring zNkx2.2Δ4$^{CNCR+Prom}$LacZ and zNkx2.2Δ5$^{CNCR+Prom}$LacZ constructs appear to display high levels of LacZ expression in the ventral neural tube. These constructs also appear to direct floor plate repression of *LacZ*. To confirm that the weak expression observed within the floor plate was due to LacZ perdurance, *in situ* hybridisation for *LacZ* was performed. This analysis revealed that constructs expressing transgenes zNkx2.2Δ4$^{CNCR+Prom}$LacZ and zNkx2.2Δ5$^{CNCR+Prom}$LacZ at E11.5 did not express *LacZ* in the floor plate (Fig. 7.3F, 7.3G). Both of these constructs appeared to express *LacZ* in the p3 domain, in which *Nkx2.2* is normally expressed, but neither appeared to express *LacZ* in intermediate domains (see Chapters 5 and 6).

Analysis of embryos by enzymatic staining at E11.5 containing zNkx2.2Δ6$^{CNCR+Prom}$LacZ, revealed 5 out of 8 positive embryos expressed *LacZ* in the floor plate only and not the p3 domain (Fig. 7.3D). 3 out of 8 embryos were positive for *LacZ* expression in the floor plate and the p3 domain (Fig. 7.3E) albeit in a weaker and more restricted pattern than that observed with zNkx2.2Δ4$^{CNCR+Prom}$LacZ and zNkx2.2Δ5$^{CNCR+Prom}$LacZ. *In situ* analysis of the zNkx2.2Δ6$^{CNCR+Prom}$LacZ construct showed *LacZ* expression in transient mice in the floor plate only (Fig. 7.3H).

We can conclude from these data that the enhancer region present in constructs zNkx2.2Δ4$^{CNCR+Prom}$LacZ and zNkx2.2Δ5$^{CNCR+Prom}$LacZ contain the floor plate repressor element and are sufficient to maintain an Nkx2.2-like expression pattern. This indicates that the floor plate repressor element is positioned between the start of the zNkx2.2Δ5$^{CNCR+Prom}$LacZ construct and the start of the zNkx2.2Δ6$^{CNCR+Prom}$LacZ construct. Analysis of the sequence reveals several putative transcription factor binding sites (see Discussion). The results observed from assaying zNkx2.2Δ6$^{CNCR+Prom}$LacZ are difficult to interpret because they are so different from other constructs. However, floor plate expression was observed in all embryos analysed, suggesting that the floor plate repressor element has been lost.

Figure 7.1 Immunohistochemistry of stable transgenic lines containing transgene zNkx2.2$^{CNCR+Prom}$LacZ at E10.5 and E11.5.

Embryos from 4 independent stable transgenic lines containing transgene zNkx2.2$^{CNCR+Prom}$LacZ. Representative transverse sections from each line, embryos harvested at E10.5 (**A, B, E, F, I, J, M, N**) and E11.5 (**C, D, G, H, K, L, O, P**). Immunohistochemistry for Nkx2.2 (green; **A, E, I, M, C, G, K, O**) and FoxA2 (green; **B, F, J, N, D, H, L, P**) with β-galactosidase (red) was performed. In line A (**A-D**) LacZ was co-expressed at E10.5 and E11.5 with both Nkx2.2 (**A, C**) and FoxA2 (**B, D**). In the other three lines at E10.5 and E11.5, cells double positive for β-Gal and Nkx2.2 (**E, G, I, K, M, O**) were observed but not for β-Gal and FoxA2 (**F, H, J, L, N, P**).

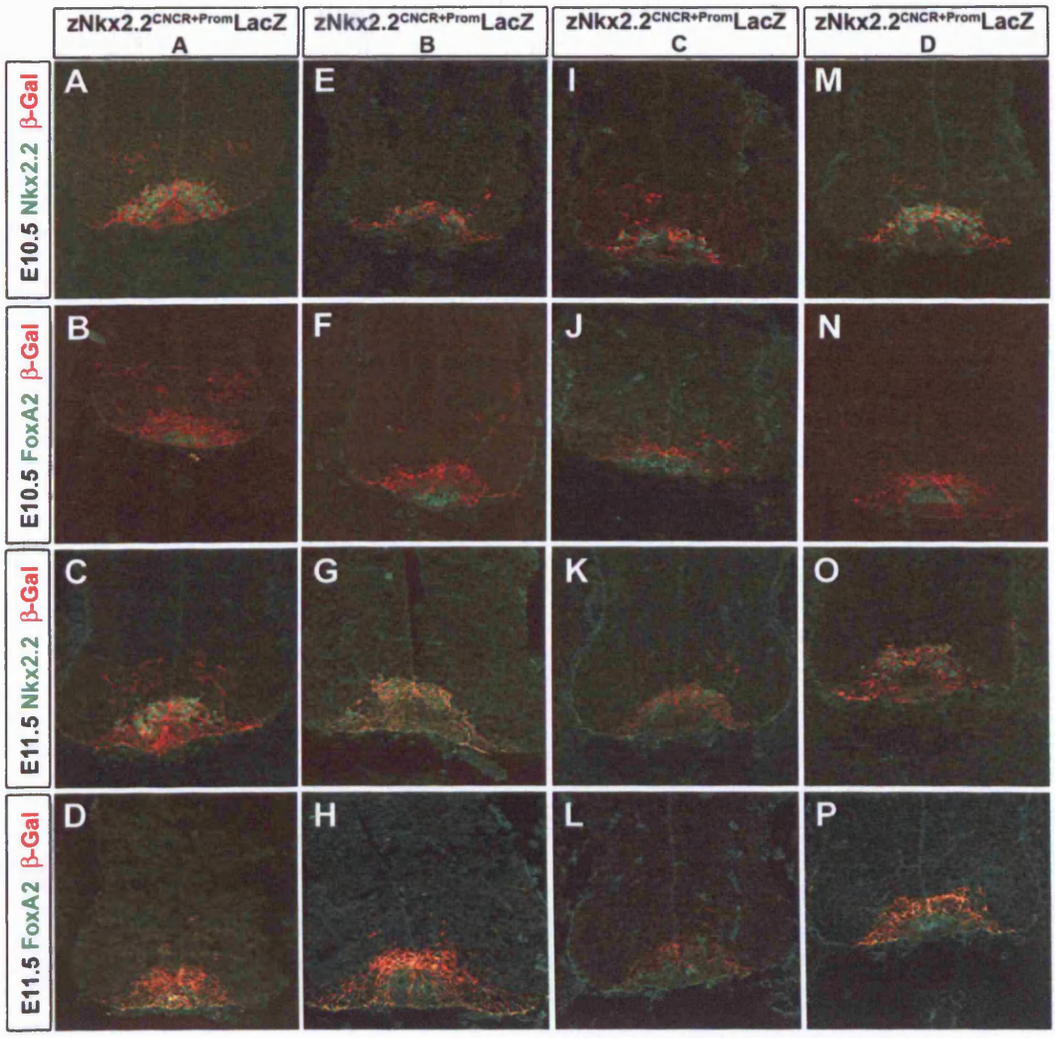
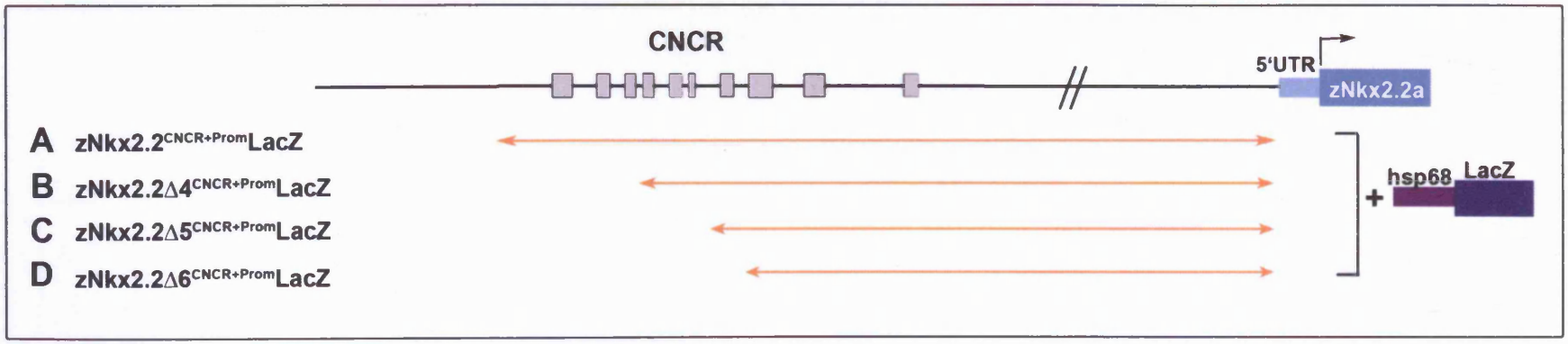

zNkx2.2CNCR+PromLacZ A
zNkx2.2CNCR+PromLacZ B
zNkx2.2CNCR+PromLacZ C
zNkx2.2CNCR+PromLacZ D
E10.5 Nkx2.2 β-Gal
E10.5 FoxA2 β-Gal
E11.5 Nkx2.2 β-Gal
E11.5 FoxA2 β-Gal
A
E
I
M
B
F
J
N
C
G
K
O
D
H
L
P

Figure 7.2 Summary diagrams of the deletion series of zNkx2.2$^{CNCR+Prom}$LacZ constructed to determine CNCR enhancer fragments necessary to repress *LacZ* expression in the floor plate.

Enhancer fragments based on zNkx2.2$^{CNCR+Prom}$(**A**) were constructed and placed upstream of a minimal reporter (hsp68) and a reporter (LacZ). These constructs all contained the Gli binding site found at the 3' end of the identified CNCR and additional 5' DNA. The first construct, zNkx2.2Δ4$^{CNCR+Prom}$LacZ (Δ4), contained 56bp less than the zNkx2.2$^{CNCR+Prom}$LacZ construct at the 5' end (**B, E**). For the second construct, zNkx2.2Δ5$^{CNCR+Prom}$LacZ (Δ5), an additional 32bp of DNA from Δ4 was deleted (**C, E**). The third, zNkx2.2Δ6$^{CNCR+Prom}$LacZ (Δ6), an extra 30bp from Δ5 was deleted (**D, E**). **E** An alignment of the DNA sequence of the CNCR from the human, mouse, *Fugu* and zebrafish *Nkx2.2* and *Nkx2.9* genes contained in the constructs. The black triangles mark the start sites of Δ4, Δ5 and Δ6 constructs.


CNCR
5'UTR
zNkx2.2a
A zNkx2.2CNCR+PromLacZ
B zNkx2.2Δ4CNCR+PromLacZ
C zNkx2.2Δ5CNCR+PromLacZ
D zNkx2.2Δ6CNCR+PromLacZ
hsp68 LacZ

E

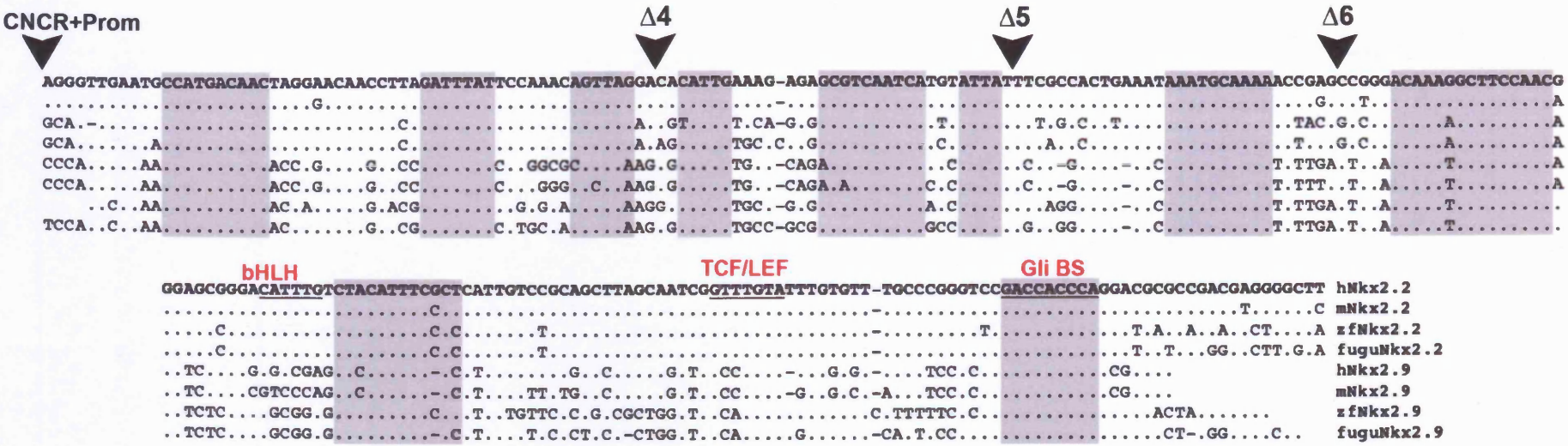
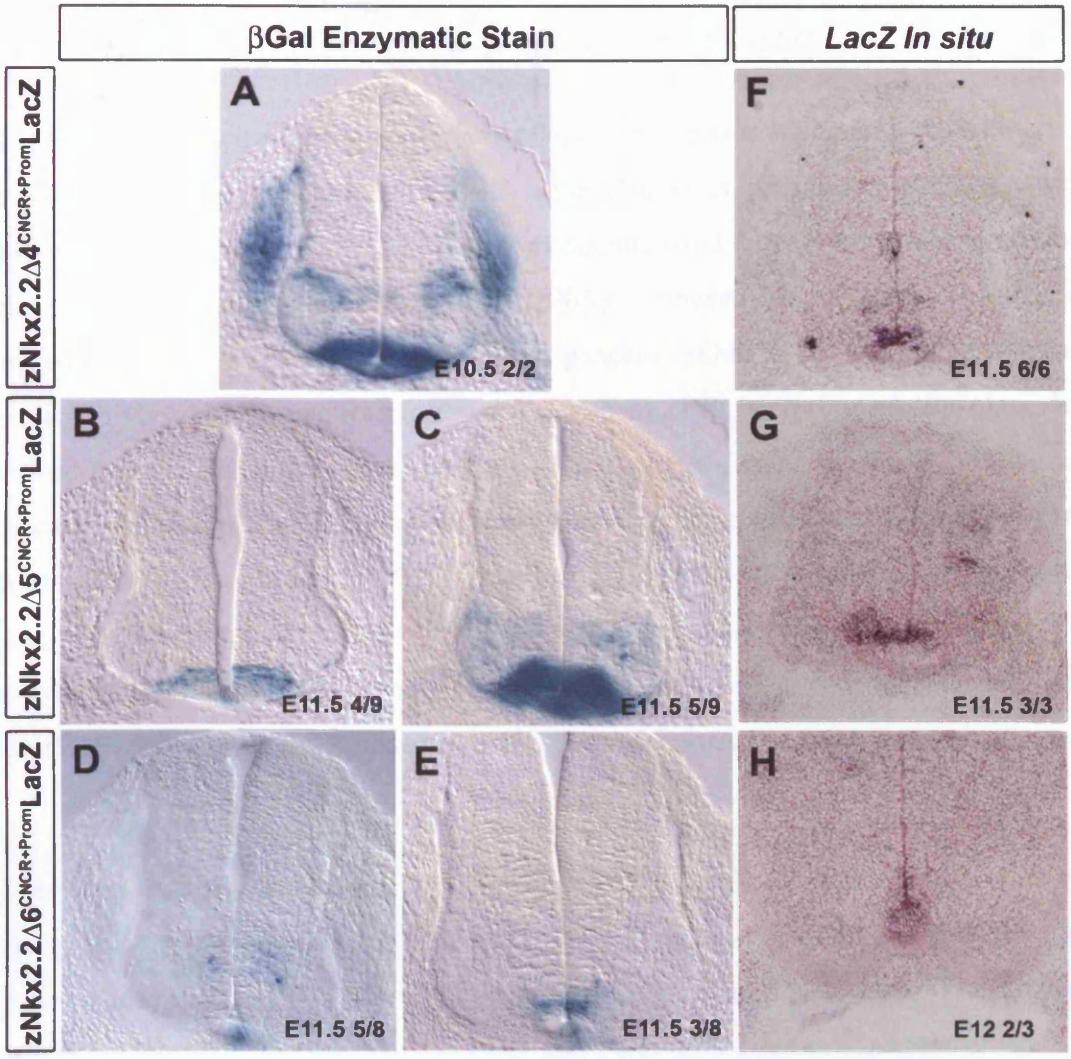

CNCR+Prom
Δ4
Δ5
Δ6
bHLH
TCF/LEF
Gli BS
hNkx2.2
mNkx2.2
zfNkx2.2
fuguNkx2.2
hNkx2.9
mNkx2.9
zfNkx2.9
fuguNkx2.9

Figure 7.3 *LacZ* expression from promoter constructs zNkx2.2Δ4$^{CNCR+Prom}$LacZ, zNkx2.2Δ5$^{CNCR+Prom}$LacZ and zNkx2.2Δ6$^{CNCR+Prom}$LacZ at E10.5-E12 following PNI in mouse.

Expression patterns of reporter constructs zNkx2.2Δ4$^{CNCR+Prom}$LacZ, zNkx2.2Δ5$^{CNCR+Prom}$LacZ and zNkx2.2Δ6$^{CNCR+Prom}$LacZ were assayed by mouse PNI. Two methods of analysis were performed; embryos were stained for β-galactosidase activity (**A-E**) or *in situ* hybridisation (ISH) for *LacZ* (**F-H**) was performed. Embryos were harvested at either E10.5 or E11.5 before staining for β-galactosidase activity, followed by vibratome sectioning at 50μM. Embryos were harvested at E11.5 or E12 and cryosectioned at 12μM before ISH was performed. Any embryos positive for β-Gal activity in the ventral neural tube were further analysed. zNkx2.2Δ4$^{CNCR+Prom}$LacZ transient embryos displayed repression of *LacZ* in the floor plate in 2/2 positive embryos analysed by enzymatic staining at E10.5 (**A**) and 6/6 positive embryos analysed by ISH at E11.5 (**F**). Expression of *LacZ* was observed in the Nkx2.2 p3 domain in these embryos. Embryos injected with zNkx2.2Δ5$^{CNCR+Prom}$LacZ displayed repression of *LacZ* in the floor plate in 4/9 positive embryos analysed by enzymatic staining at E11.5 (**B**), however, 5/9 of the embryos analysed displayed expression of *LacZ* the floor plate (**C**). All of the 9 embryos were positive for β-galactosidase activity in the p3 domain, this expression was very strong in most of the embryos observed (**C**). 3/3 positive embryos analysed by ISH at E11.5 displayed repression of *LacZ* in the floor plate (**B**). Embryos injected with zNkx2.2Δ6$^{CNCR+Prom}$LacZ displayed expression of *LacZ* in the floor plate only in 5/8 positive embryos analysed by enzymatic staining at E11.5 (**D**) and in 2/3 positive embryos analysed by ISH at E12 (**H**). However, 3/8 of the embryos analysed for β-galactosidase activity displayed expression of *LacZ* in the p3 domain and the floor plate (**E**).


βGal Enzymatic Stain
LacZ In situ
zNkx2.2Δ4CNCR+PromLacZ
zNkx2.2Δ5CNCR+PromLacZ
zNkx2.2Δ6CNCR+PromLacZ
A
E10.5 2/2
F
E11.5 6/6
B
E11.5 4/9
C
E11.5 5/9
G
E11.5 3/3
D
E11.5 5/8
E
E11.5 3/8
H
E12 2/3

8 Discussion

8.1 Evolutionary Conservation of Nkx2 Gene Cluster

Nkx genes are the chordate orthologues of the *Drosophila* NK genes. A relative of the more familiar Hox cluster, the NK gene cluster is present in the *Drosophila* genome, and is believed to be derived from the ancestral 'megacluster' of homeobox genes (reviewed in Garcia-Fernandez, 2005). There are several evolutionary conserved clusters of Nkx genes in chordate genomes (Garcia-Fernandez, 2005), each seeming to play a different role in the development of the vertebrate body-plan. It is believed that the last common ancestor of humans and *Drosophila* had 7 NK genes that have since duplicated and split many times to form the clusters in our present genome (Garcia-Fernandez, 2005). There is a good correlation between an increase in cluster duplication and complexity of the systems patterned. The reasons why homeobox gene clustering has been conserved across evolution are not fully understood, however it does raise the possibility of a shared regulatory mechanism.

It was therefore not a surprise to identify Nkx2 gene clusters in the zebrafish and *Fugu* genomes, comparable to those previously identified in human and mouse (Fig. 3.1: Santagati et al., 2003; Santagati et al., 2001; Wang et al., 2000b). The evidence that *Nkx2.1, Nkx2.2, Nkx2.9, FoxA2, Pax1* and *Pax9* genes present in these clusters are Shh responsive and expressed ventrally (*Pax1* and *Pax9* in the ventral somites), supports the idea of co-regulation (Figs. 3.9, 3.10: Neubuser et al., 1995; Pabst et al., 2000; Sasaki et al., 1997). However, not all of the genes in this cluster are regulated by Shh, as demonstrated here by the independence of the *Nkx2.4* gene expression from Shh signalling (Fig. 3.9: Rohr et al., 2001). Therefore, co-regulation by Shh is not sufficient to explain the maintenance of the clustering for all of its members. Perhaps additional features of their regulation may provide a reason for the evolutionary conservation of the gene cluster.

8.2 Conservation of Nkx2 Gene Cluster in Zebrafish

The expression patterns of the Nkx2 genes identified in zebrafish appeared to be comparable to those seen in mice. *Nkx2.2 a/b* and its paralogue *Nkx2.9* were expressed the length of the anterior-posterior axis in the ventral neural tube (Figs. 3.4, 3.5, 3.6), as observed in mouse (Briscoe et al., 1999; Price et al., 1992). The expression pattern of the z*Nkx2.2a/b* genes were comparable to previously published results (Barth and Wilson, 1995; Schafer et al., 2005). Not only were the expression patterns similar, but the timing of expression between mouse and zebrafish also appears to be maintained. In mice *Nkx2.9* is down-regulated in the trunk region of the neural tube from E10.5, whilst expression of *Nkx2.2* is maintained (Briscoe et al., 1999). *In situ* hybridisation performed on zebrafish embryos between 11 hours post fertilisation (hpf) and 30hpf suggested that *Nkx2.9* was also down-regulated at an equivalent developmental stage (after 24hpf; Fig. 3.8I), whilst *Nkx2.2 a/b* expression was maintained (Figs. 3.4I, 3.5I).

Nkx2.1 and *Nkx2.4* in mice are expressed in the ventral telencephalon and posterior hypothalamus respectively (Price, 1993; Wang et al., 2000b). Like *Nkx2.2* and *Nkx2.9* the expression of these genes are ventrally restricted. Zebrafish *Nkx2.1* (also known as *Nk2.1b*) was expressed in the anterior ventral telencephalon at 24 hpf, whilst the two *Nkx2.4* genes were expressed in a more posterior position of the forebrain, the hypothalamus (Figs. 3.3H, 3.6H, 3.7H: Rohr et al., 2001; Rohr and Concha, 2000). Comparison of the expression patterns suggested there was very little overlap in the expression of these Nkx2 genes. Thus zebrafish *Nkx2.1* and *Nkx2.4* appear to have comparable expression profiles to the pattern of expression of the equivalent genes observed in mouse (Price, 1993).

The expression of *Nkx2.1, Nkx2.2 a/b* and *Nkx2.9* are reliant upon the presence of Shh, because inhibition of Shh signalling by cyclopamine leads to a loss of expression (Figs. 3.9, 3.10A, 3.10B). This supports previously published studies in zebrafish (Rohr et al., 2001; Stamataki et al., 2005) and also similar studies in mouse embryos (Pabst et al., 2000). Importantly, expression of *Nkx2.4a/b* does not appear to be dependent upon Shh for its expression (Fig. 3.10C-3.10F: Rohr et al., 2001). No

similar studies have been carried out in mice however we would predict that *Nkx2.4* expression is also independent of Shh in amniotes.

The Nk2.1a (Rohr and Concha, 2000) and Nk2.1b (Rohr et al., 2001) proteins have been published as being members of the Nkx2.1 group of proteins. Comparing their protein sequence with that of human and mouse Nkx2.1 supports this observation (Fig. 3.2). However, analysis of expression patterns and their behaviour in response to Shh signalling suggests that the Nk2.1a gene is functionally more similar to Nkx2.4. Due to this we have termed the proteins zebrafish Nkx2.4b (Nk2.1a) and zebrafish Nkx2.1 (Nk2.1b). Expression of z*Nkx2.1* is restricted to the ventral telencephalon (Fig. 3.3: Rohr et al., 2001), which is also the case for the mouse *Nkx2.1* (Price, 1993). It is also down-regulated in response to cyclopamine treatment, which blocks Shh (Fig. 3.10: Rohr et al., 2001). Expression of *Nkx2.4a* and *Nkx2.4b* is restricted to the hypothalamus (Figs. 3.6, 3.7: Rohr et al., 2001; Rohr and Concha, 2000), as is mouse *Nkx2.4* (Price, 1993). Expression of both of the Nkx2.4 genes is not dependent upon Shh signalling (Fig. 3.10: Rohr et al., 2001). Together these data suggest Nk2.1a is functionally similar to Nkx2.4. In addition to the functional data, the genetic linkage of *Nkx2.4b* to *Nkx2.2b* on zebrafish chromosome 20 (Fig. 3.1D) also suggests it is an Nkx2.4 paralogue. Genes *Nkx2.2* and *Nkx2.4* are closely linked in the *Fugu*, mouse and human genomes (Fig. 3.1A-3.1C), while *Nkx2.1* is linked to *Nkx2.9*.

The variation in response to cyclopamine treatment of the different Nkx2 genes reflects their position of expression along the anterior-posterior axis of the embryo. The most ventral cells in the CNS have one of two fates: floor plate cells; or in the most anterior CNS, hypothalamic cells (reviewed in Kapsimali et al., 2004). The specification of hypothalamic cells is believed to be controlled by members of the Nodal and Wnt families (Kapsimali et al., 2004). Repression of Wnt signalling directs the formation of hypothalamic cells at the expense of floor plate cells (Kapsimali et al., 2004). In addition hypothalamus induction requires Nodal signalling (Kapsimali et al., 2004; Rohr et al., 2001). Therefore, regulation of markers of the hypothalamus, such as *Nkx2.4*, may require input from Nodal and Wnt pathways for correct induction. Shh signalling is required for hypothalamus formation, this structure is

missing in mice lacking Shh, however it is not believed to be necessary for all aspects of its induction (reviewed in Wilson and Houart, 2004).

Together these data provide evidence to suggest that not only have the expression patterns of the Nkx2 genes in zebrafish and mice been conserved, but also the regulation and timing of expression. The presence of a highly conserved non-coding region (CNCR) upstream of both the mouse and zebrafish Nkx2.2/2.9 genes (see below) suggest a possible mechanism to account for the similarity in expression.

8.3 CNCR Drives Nkx2.2/9-Like Expression

Analysis of vertebrate genomic DNA containing the Nkx2 genes had previously identified a regulatory region of approximately 1kb upstream of *Nkx2.9* that was capable of driving Nkx2.2/2.9 like expression in the neural tube of mice (Santagati et al., 2003). We confirmed that this region was present in the promoters of human, mouse and fish *Nkx2.9* genes. Moreover this region was also present in the promoters of the paralogous gene *Nkx2.2* (Fig. 4.4). Once this region had been identified, we set out to test if it was both necessary and sufficient for *Nkx2.2* gene expression.

Using a BAC homologous recombination system, we ascertained that the CNCR was necessary for correct *Nkx2.2* expression (Chapter 4). The targeting of a fluorescent reporter to the first exon of *Nkx2.2a* in zebrafish recapitulated its expression in the ventral neural tube of 24 hr embryos (Fig. 4.3). Deletion of the CNCR from the BAC, led to loss of specific expression in >99% of embryos analysed (Fig. 4.7).

In vivo evidence from this study suggests that the 250bp CNCR identified upstream of both *Nkx2.2* and *Nkx2.9* is sufficient to drive correct expression of the reporter in the ventral neural tube of mice (Chapter 5). In the case of mouse *Nkx2.9*, the mNkx2.9$^{CNCR+Prom}$LacZ reporter construct, containing the CNCR, was able to direct *LacZ* expression in the ventral most domain of the spinal cord in an expression pattern comparable to wild type (Fig. 5.6: Pabst et al., 1998; Santagati et al., 2003). The comparable construct from mouse *Nkx2.2*, mNkx2.2$^{CNCR+Prom}$LacZ, also containing the

CNCR, was able to direct reporter expression, when assayed by mouse pronuclear injection, in a pattern similar to that of wild type expression (Fig. 5.5: Briscoe et al., 1999).

Constructs containing 1-1.3kb of zebrafish *Nkx2.2a* promoter DNA, upon injection into mouse pronuclei, directed *LacZ* expression in a pattern comparable to that of wild type mouse *Nkx2.2* (Figs. 5.5, 5.8, 5.12). These constructs, zNkx2.2$^{CNCR+Prom}$LacZ and zNkx2.2$^{CNCR+Prom}$LacZ-2, contained the CNCR plus other fragments of the promoter. Moreover, assaying further constructs (zNkx2.2^{CNCR}LacZ and zNkx2.2$^{\Delta CNCR+Prom}$LacZ) suggested that the CNCR alone was sufficient to direct *LacZ* expression in an Nkx2.2 like domain. Deletion of the CNCR in this context led to a complete loss of *LacZ* expression (Fig. 5.16).

Together these data suggest that the CNCR region identified upstream of *Nkx2.2* and *Nkx2.9* genes in several vertebrate genomes contains all the required regulatory elements for correct *Nkx2.2* expression in the mouse and zebrafish neural tube. Cyclopamine experiments in zebrafish (see above) indicate that the expression depends on Shh signalling. Thus the CNCR represents a Shh responsive neural enhancer.

The reporter plasmids frequently directed expression of *LacZ* in a more intermediate domain of the neural tube in addition to the ventral Nkx2.2 like expression. This non-specific expression was not observed in the zebrafish embryos harbouring the zNkx2.2aVenus BAC (Fig. 4.3). This may have been caused by the absence of a repressive element in the small plasmids that would normally block this expression. The ectopic *LacZ* expression was observed in the p2 domain in both progenitor and post-mitotic cells. At later stages the zNkx2.2$^{CNCR+Prom}$LacZ construct was unable to direct *LacZ* expression in the intermediate population, suggesting expression in post-mitotic cells may have been due to perdurance of the enzymatic product (Chapters 5 and 6). *Nkx6.1* and *Nkx6.2* are orthologues of the *Drosophila NK6* gene, both are expressed in the ventral neural tube (Fig. 1.3B). *Nkx6.1* is expressed in the p2 domain

in progenitor cells. There may be some homology between the regulatory elements of *Nkx6.1* and *Nkx2.2*, which may explain the p2 domain expression.

8.4 Evolutionary Conservation of Regulatory Elements and Scalability of Development

Importantly, similar activity was observed with the mouse and zebrafish *Nkx2.2* regulatory elements and both recapitulated the endogenous *Nkx2.2* expression in transgenic mice. Thus, the regulatory element is interchangeable between species. This highlights the evolutionary conservation of regulatory mechanisms within vertebrates. This indicates that the same factors direct expression of zebrafish and mouse *Nkx2.2*, supporting the idea that dorsal ventral patterning is equivalent in vertebrate neural tube development.

The scale and timing of neural tube development in zebrafish and mice vary. The process of neurulation in zebrafish takes only a matter of hours for completion, while the same process in mice takes 2 days. In both mouse and zebrafish, *Nkx2.2* in induced at equivalent times during neurulation. In addition, the *Nkx2.2* expressing domain within the neural tube of mouse and zebrafish is different. *Nkx2.2* is expressed in zebrafish in a domain that is approximately 2 cells wide in the dorsoventral axis. In mice *Nkx2.2* expressing cells occupy a region closer to 6 cells wide. The ability of the zebrafish regulatory element to direct mouse Nkx2.2-like expression, suggests that differences in the proportion and timing of the ventral neural tube expressing *Nkx2.2* is not controlled by differences within the regulatory element. Therefore the scalability of expression must reside elsewhere in the system, not in the regulatory architecture. One candidate would be Shh signalling itself, perhaps the regulation of the Shh ligand and its range of activity differs proportionally between species.

8.5 Direct Gli Input Required for CNCR

One of the questions this project aimed to answer was whether the requirement for Shh for *Nkx2.2* expression was direct. Experimental evidence suggested that Shh signalling is required for *Nkx2.2* expression (Chapter 3: Pabst et al., 2000; Stamataki et al., 2005). Identification of a Gli binding site within the CNCR (Fig. 4.4) raised the possibility that the requirement was direct. Analysis of *Gli1* mutant zebrafish identified a loss of *Nkx2.9* expression, suggesting a requirement for Gli and therefore a direct Shh requirement (Xu et al., 2006). Similarly in *Gli2* mutant mice there is a loss of *Nkx2.2* expression, suggesting a Shh/Gli requirement (Ding et al., 1998).

The Gli binding site identified in the CNCR is identical to the previously described "canonical" binding sequence (5' GACCACCCA 3': Kinzler and Vogelstein, 1990). It has been shown to bind to all Gli proteins and to *Drosophila* Ci (Agren et al., 2004; Muller and Basler, 2000; Sasaki et al., 1997). To test the requirement for this site in the CNCR, reporter plasmid zNkx2.2$^{CNCR+Prom}$GBSMutLacZ contained a mutation in the Gli binding site (GBS) present in the CNCR in the *zNkx2.2a* reporter construct. This mutation (5' GAAGTGGGA 3') has been shown not to bind to Gli proteins using electrophoretic mobility shift assays (EMSA: Sasaki et al., 1997). Mutation of the GBS within zNkx2.2$^{CNCR+Prom}$LacZ led to a loss of all ventral *LacZ* expression (Fig. 6.1), suggesting a direct requirement for Shh/Gli for correct *Nkx2.2* expression. These data therefore offer an explanation for the requirement for Shh signalling in the induction of *Nkx2.2*.

The finding that the CNCR is interchangeable between species for correct gene expression, combined with the evidence that all three Gli proteins and Ci are able to bind to the identified GBS, suggests a degree of functional redundancy but divergence of Gli protein function in mice and zebrafish. The activation of the ventral cell types in the neural tube (floor plate and V3) appears to be due to the action of Gli1 in zebrafish (Karlstrom et al., 2003), however this role is performed by Gli2 in mouse (Ding et al., 1998; Matise et al., 1998; Park et al., 2000). It seems likely therefore that the different functions of Gli1/2 proteins in these species are not due to differences in the binding specificity of these proteins. The differences are more likely to be due to

differences in the expression or transcriptional activity of Gli1/2 in the two species. The conservation of the GBS sequence between mouse and zebrafish is consistent with the idea that the activation of the reporter by the CNCR is due to level of Gli activity and not the identity of the Gli protein itself.

8.6 Combinatorial Inputs Required for Nkx2.2 Expression

The loss of *LacZ* expression upon mutating the GBS suggested that this site was necessary for correct *Nkx2.2* expression. However deletion constructs based upon the CNCR suggested that the GBS was not, alone, sufficient to direct Nkx2.2-like expression. Reporter construct zNkx2.2Δ1$^{CNCR+Prom}$LacZ, which contained the GBS but not the majority of the conserved domains within the CNCR, was not sufficient to drive Nkx2.2 like ventral *LacZ* expression (Fig. 6.3). The addition of ~13bp to this construct however restored the ventral expression of *LacZ* (zNkx2.2Δ3$^{CNCR+Prom}$LacZ, Fig. 6.3, summarised Fig.8.1A). These results suggest that in addition to Gli, a second 'X' factor is necessary for the ventral activation of *Nkx2.2*.

Sequence analysis identifies several consensus sites within this 13bp (Fig. 8.1A). One such binding sequence is for *Glial cells missing* genes (*gcm* and *gcm2*). These proteins were originally identified as *Drosophila* glial cell determinant genes in the CNS (reviewed in Van De Bor and Giangrande, 2002). The binding site for this transcription factor has been identified, which is conserved in flies and mammals (Akiyama et al., 1996). Although *gcm* genes have been shown to have a gliogenic role in *Drosophila*, it has been suggested that in addition it has a neurogenic role in the post embryonic development of the visual system (Chotard et al., 2005). This is believed to be in co-operation with the Hh signalling pathway. Recently it has been shown that the chick orthologue of *gcm*, *c-Gcm1*, is expressed in the early developing chick spinal cord including neural progenitors in the region that expresses *Nkx2.2* (Soustelle et al., 2007). Overexpression studies in chick suggest it has a neurogenic rather than a gliogenic role (Soustelle et al., 2007). While in mice the role of this protein (Gcm1) has been associated with placental formation (Schreiber et al., 2000). Mice carrying mutations in the *Gcm1* gene appear to form a normal nervous system, however the presence of a Gcm1 paralogue raises the possibility of functional

redundancy. The expression of mouse *Gcm1* and *Gcm2* was identified by PCR in the CNS, however the levels were low and no *in situ* hybridisation is currently available (Kim et al., 1998). It was proposed that they are mainly expressed in non-neural tissue.

There is also a SOX binding site in the 13bp of CNCR 5' to the GBS (Fig. 8.1A). SOX proteins are a large group of high-mobility-group (HMG) containing transcription factors that play many roles during embryogenesis. The SOX proteins are grouped, based on their structural similarity. SOXB proteins are believed to promote neuronal progenitor identity, and maintain the expression of neural progenitor genes (reviewed in Pevny and Placzek, 2005). Shh is present in many developing tissues other than the CNS, however *Nkx2.2* does not appear to respond to Shh signalling in these other tissues. SOX1, a SOXB protein, has been shown to be crucial for inducing a neural fate in cells (Pevny et al., 1998), so it is possible that the presence of SOX sites in the CNCR is essential for neural specific expression of *Nkx2.2*.

Additional reporter constructs, zNkx2.2Δ4$^{CNCR+Prom}$LacZ, zNkx2.2Δ5$^{CNCR+Prom}$LacZ and zNkx2.2Δ6$^{CNCR+Prom}$LacZ, which contained more 5' DNA to zNkx2.2Δ2$^{CNCR+Prom}$LacZ provided further clues to the regulation of *Nkx2.2* expression (Chapter 7, summarised Fig. 8.1B). The addition of 21bp 5' to zNkx2.2Δ2$^{CNCR+Prom}$LacZ to create zNkx2.2Δ6$^{CNCR+Prom}$LacZ directed reporter expression restricted to the floor plate only with occasional limited expression in the Nkx2.2 p3 domain (Fig. 7.3). This suggests that a repressor element was present leading to loss of reporter expression in an Nkx2.2-like manner between zNkx2.2Δ2$^{CNCR+Prom}$LacZ and zNkx2.2Δ6$^{CNCR+Prom}$LacZ. Moreover, the addition of 29bp 5' to zNkx2.2Δ6$^{CNCR+Prom}$LacZ, to make zNkx2.2Δ5$^{CNCR+Prom}$LacZ restored ventral reporter expression, suggesting an activator element had been added, that led to ventral reporter expression. However, it is possible that the process of deleting DNA to make zNkx2.2Δ6$^{CNCR+Prom}$LacZ, destroyed an enhancer essential for correct ventral Nkx2.2-like expression, producing an anomalous result for zNkx2.2Δ6$^{CNCR+Prom}$LacZ (Fig. 7.3). zNkx2.2Δ4$^{CNCR+Prom}$LacZ also directed ventral Nkx2.2-like expression, suggesting all of the elements required for correct Nkx2.2-like expression were present in this construct. These data together

suggest that the regulation of *Nkx2.2* requires input from multiple positive and negative regulators. Analysis of the region to identify possible factors shows predicted binding sites for several transcription factors, including those for Oct1 and Pou3f2 binding sites (Fig. 8.1B).

Both Oct1 and Pou3f2 are members of the POU family of transcription factors, which contain a POU specific domain and a homeodomain, both are necessary for DNA binding (reviewed in Latchman, 1999). *Oct1* is a ubiquitously expressed transcription factor (Scholer et al., 1989), whilst *Pou3f2* (*Brn-2*) is expressed in the mouse CNS and is important for correct development of the neocortex (Sugitani et al., 2002). *Pou3f2* has been shown to be expressed in the ventral telencephalon (E13.5) and throughout the dorsoventral axis of the spinal cord (E10.5: Castro et al., 2006). Many factors in this family have been implicated in patterning and development of the nervous system. The binding site identified may be for any of the octamer factors, since families of these proteins share similar core recognition sequences.

Together these data suggest that although the Gli binding site is necessary for *Nkx2.2* expression, the site alone is not sufficient. The finding that an additional 13bp is required for correct expression suggests that at least one additional factor is required to bind to the CNCR to drive expression. Gcm1 and SOX could be possible candidates for this process. However, construction of further plasmids suggests that the mechanism for gene induction is more complex than this. Assaying the activity of constructs zNkx2.2Δ5$^{CNCR+Prom}$LacZ and zNkx2.2Δ6$^{CNCR+Prom}$LacZ provided evidence that there may be additional activators and repressors (Fig. 8.1B) located in the CNCR controlling *Nkx2.2* induction.

8.7 Nkx2.2 Floor Plate Repression

Nkx2.2 is initially expressed at ~E8.5 in the most ventral cells of the mouse neural tube, (Briscoe et al., 1999; Jeong and McMahon, 2005). *FoxA2* expression is initiated at a similar time point (Jeong and McMahon, 2005). These ventral cells will go on to form the floor plate, which expresses *FoxA2* but not *Nkx2.2*. As development

proceeds the expression of *Nkx2.2* shifts dorsally to occupy a domain just dorsal to the expression of *FoxA2* (Briscoe et al., 1999). This suggests that a mechanism must repress *Nkx2.2* expression within the floor plate. Is FoxA2 the repression factor? *Nkx2.2* and *FoxA2* are co-expressed in the same cells at both hindbrain (J. Jacob, personal communication) and trunk level (Jeong and McMahon, 2005) which argues against a simple repressive activity of FoxA2 on *Nkx2.2* expression. Moreover, data suggests FoxA2 acts as an activator (Denson et al., 2000; Wang et al., 2000c). However, this does not rule out the involvement of FoxA2 in the repression of Nkx2.2 as there could be sequential induction of these two genes or additional genes (see below).

The analysis of the CNCR has provided evidence to suggest that it contains a repression element, which blocks *Nkx2.2* expression in the floor plate. Transgenic mouse lines harbouring the reporter construct zNkx2.2$^{CNCR+Prom}$LacZ did not express *LacZ* in the floor plate (Fig. 7.1). However, deletion of DNA at the 5' end of the CNCR to create construct zNkx2.2Δ2$^{CNCR+Prom}$LacZ directed *LacZ* expression in the floor plate upon mouse pronuclear injection (Fig. 6.3). In order to narrow down the element that contained the floor plate repression element, further constructs were made. Analysis of mice harbouring these constructs suggested that the floor plate repressor was positioned between the start site of zNkx2.2Δ5$^{CNCR+Prom}$LacZ and zNkx2.2Δ2$^{CNCR+Prom}$LacZ.

To further define the region necessary for floor plate repression, mouse pronuclear injection of construct zNkx2.2Δ6$^{CNCR+Prom}$LacZ was performed. This construct directed expression in the floor plate alone, as discussed in Section 8.6. The loss of ventral expression in this construct was possibly due to the loss of an activator. This expression of *LacZ* in the floor plate suggests that the floor plate repressor is located between the start sites of zNkx2.2Δ5$^{CNCR+Prom}$LacZ and zNkx2.2Δ6$^{CNCR+Prom}$LacZ (summarised Fig. 8.1B). The analysis of this region to identify possible binding sites identified octamer sites (see Section 8.6). However, the identity of the floor plate repressor is unclear since there are no obvious candidates for the floor plate repressor within this region of the CNCR (Fig. 8.1B).

In addition to *FoxA2*, there are several genes expressed in the floor plate that may directly or indirectly repress *Nkx2.2* expression. A microarray screen identified a number of genes up-regulated in response to high levels of Shh signalling, these included the Forkhead protein Foxj1 (C. Cruz personal communication). *In situ* analysis confirmed it is expressed in the floor plate. Mutation of *Foxj1* in mice leads to random left-right body patterning, suggesting it plays a role in regulating asymmetric gene expression (Chen et al., 1998a). It is also believed to be involved in 9+2 motile cilia biogenesis (Chen et al., 1998a) due to their absence in *Foxj1* mutant mice. While there is currently no evidence to suggest that Foxj1 plays any part in repressing *Nkx2.2* expression, it along with other transcription factors represent candidates for mediating the down-regulation of Shh in the floor plate.

8.8 Pax6 Repression of Nkx2.2

Gain-of-function studies indicate that the dorsal limit of *Nkx2.2* expression is determined by Pax6 expression (Briscoe et al., 2000). It is still not known, however, how Pax6 exerts its repressive effects on *Nkx2.2*. The cross-repressive interaction between Nkx2.2 and Pax6 is well documented. The loss of functional Pax6 in *Sey* mice leads to an expansion in *Nkx2.2* expression and a dorsal shift of ventral neuronal subtypes (Ericson et al., 1997b). In *Nkx2.2* mutant mice, there is no change in the ventral boundary of *Pax6* expression (Briscoe et al., 1999), however this may be due to the expression of *Nkx2.9* in the same domain. The evidence that Pax6 functions as a transcriptional activator (Muhr et al., 2001) indicates that the activity of Pax6 on the *Nkx2.2* gene is likely to be indirect.

One recently proposed mechanism for the repression of *Nkx2.2* expression by *Pax6* suggested that the Wnt pathway was involved (Lei et al., 2006). This model suggested that the inhibitory components of the Wnt pathway via TCF/LEF binding sites, are necessary to determine the dorsal *Nkx2.2* expression boundary. The model proposes that Pax6 activates an inhibitor of the Wnt pathway, *SFRP2* (secreted frizzled related protein 2), which is expressed in the ventral neural tube, not including

the p3 domain. The inhibition of Wnt signalling by SFRP2, means that Tcf4 recruits Groucho co-repressors, which in turn negatively regulate *Nkx2.2*, therefore restricting its expression to the p3 domain. However, this explanation suggests that *Pax6* expression is already determined, which then controls the boundary of *Nkx2.2* expression. This fails to explain how the *Pax6* boundary is set up in the first place. Moreover our analysis of the CNCR indicates that the TCF/LEF binding sites do not account for the observed expression pattern of *Nkx2.2* in the neural tube.

It was noticeable that none of the reporter constructs analysed showed a dorsal expansion of *LacZ* (Chapters 5-7). In contrast, in *Sey* mice lacking functional Pax6, expression of the reporter expanded (Fig. 5.7). This suggests that the CNCR responds to the repressive activity of Pax6 and that the repressive activity is not obviously localised to a specific element. Although, we cannot rule out the possibility that we have failed to identify a specific element responsible for Pax6 mediated repression, our data suggest an alternative; that the repressive activity of Pax6 is via a different mechanism.

How then could Pax6 determine the dorsal limit of Nkx2.2 expression? Pax6 could indirectly determine the dorsal *Nkx2.2* expression boundary by regulating Shh signalling. For example, Pax6 could positively regulate an inhibitor of Shh signalling or up-regulate the expression of Gli repressor proteins. One mechanism could be activating and/or increasing the levels of Gli3 protein and inhibiting the levels of Gli2, which would therefore repress Shh signalling.

8.9 Model for Nkx2.2 Regulation

The analysis of the regulatory elements together with the observed characteristics of *Nkx2.2* expression allows us to formulate a model to explain its regulation. Recent evidence suggests that there is a sequential induction of *Olig2* and *Nkx2.2* in ventral neural cells (E. Dessaud personal communication), with both the strength and time of Shh signalling determining *Nkx2.2* induction. There is a significant delay between the provision of Shh protein and induction of *Nkx2.2*. Moreover, Gli activity must be

maintained for *Nkx2.2* induction, as its expression was not consolidated in cells if Shh signalling was removed prematurely (E. Dessaud personal communication). Finally, tissue exposed to Shh shows a down-regulation of Gli activity over time (E. Dessaud personal communication). At the time when significant numbers of cells have induced *Nkx2.2* Gli activity had decreased and stabilised. This temporal desensitisation to the Shh signal is via an up-regulation of ligand inhibitors such as Ptc1 (E. Dessaud personal communication).

Analysis of the regulatory elements necessary for *Nkx2.2* expression has provided evidence to suggest that *Nkx2.2* induction requires Gli activity and an additional factor 'X'. We can therefore suggest that the delay in the induction of *Nkx2.2* expression is due to the need to induce Gli transcriptional activity and for this to induce factor 'X'. In addition the requirement for prolonged Gli activity for *Nkx2.2* induction is accounted for by the requirement for X and Gli to jointly act upon the regulatory element. These data are consistent with a feed forward loop being involved in the regulation of *Nkx2.2* by Shh signalling (Fig. 8.1C). Shh signalling initiates Gli activity, which induces X, then Gli activity must be maintained with X for *Nkx2.2* induction. Hence premature removal of Gli activity does not allow *Nkx2.2* induction. The consolidation of *Nkx2.2* expression requires continued Shh signalling, once established *Olig2* expression is down-regulated. Nkx2.2 then represses *Olig2* expression to ensure it is not re-expressed within the p3 domain.

In addition to the mechanism that accounts for the Shh responsiveness of the CNCR, the evidence suggests that combinations of positive and negative inputs are required for *Nkx2.2* gene expression (Fig. 8.1D). The expression of the *LacZ* reporter in ventral regions after injection of zNkx2.2Δ3$^{CNCR+Prom}$LacZ provided evidence that in addition to the GBS another positive factor binds with the 13bp located 5' to the GBS (Fig. 8.1A). Together these factors are sufficient to confer ventral expression. However, this simple interpretation is further complicated by the analysis of mice harbouring zNkx2.2Δ5$^{CNCR+Prom}$LacZ and zNkx2.2Δ6$^{CNCR+Prom}$LacZ. Analysis of zNkx2.2Δ6$^{CNCR+Prom}$LacZ expression (Fig. 7.3), demonstrated that the reporter directs expression in the floor plate but no ventral neural tube expression. The ventral expression is restored by injection of zNkx2.2Δ5$^{CNCR+Prom}$LacZ. Together these data

suggest the spatial distribution of factors (Fig. 8.1D) that would explain the results observed in Chapters 6 and 7, and also demonstrate a method of fine-tuning the regulation that would ensure precise positioning of *Nkx2.2* expression.

The model predicts that the injection of zNkx2.2Δ1$^{CNCR+Prom}$LacZ, containing only the GBS, was not sufficient for activation of Nkx2.2-like expression. Injection of zNkx2.2Δ3$^{CNCR+Prom}$LacZ, containing both the GBS and an activator recognition site directed ventral expression in both the p3 domain and floor plate. However, injection of zNkx2.2Δ6$^{CNCR+Prom}$LacZ, containing the GBS as well as both an activator and repressor recognition site led to non-specific expression in floor plate and occasionally the p3 domain. However, this construct was not sufficient to direct expression in a precise Nkx2.2-like domain. Finally injection of zNkx2.2Δ5$^{CNCR+Prom}$LacZ, containing the feed forward activator in addition to activator and repressor recognition sites, directed precise expression in the p3 domain only. Therefore this model suggests the presence of binding sites for general activators and repressors acting upon the CNCR as well as Shh regulated factors (Fig. 8.1D). In addition a floor plate repression factor appears to bind in the region present in the zNkx2.2Δ5$^{CNCR+Prom}$LacZ construct. This element ensures down-regulation of *Nkx2.2* in the floor plate. Finally Pax6 sets the dorsal limit of *Nkx2.2* expression either by activating a repressor of *Nkx2.2* or by regulating the level of Shh signalling.

8.10 Conclusions

The results obtained from this project and other experiments have provided evidence for a model, which may explain the mode through which genes are differentially expressed in response to the morphogen Shh. It is a model that could be applied to other morphogens crucial in the development of many eukaryotes. This model differs from the canonical view of morphogen interpretation, in which genes respond to particular thresholds of morphogen by binding affinity. In the canonical view, the morphogen gradient is itself sufficient to regulate the differential gene expression that patterns the tissue. The changes in morphogen concentration are translated into changes in the activity or number of active transcriptional effector molecules. Target genes are then activated based on the presence of numbers of binding sites for these

effectors and the relative binding affinity. Our model suggest that the differential control of gene expression is due to the presence of other factors required for gene induction and also from the duration of morphogen signalling.

Our results support the 'sequential cell context' and 'self enabling mechanism models' (see Introduction) previously proposed (Kang et al., 2003; Pages and Kerridge, 2000). Relating these mechanisms to *Nkx2.2* expression, we can predict that early signalling of Shh could activate an early set of genes that would down-regulate *Pax6* expression. Continued Shh signalling would activate a late set of genes that would then activate *Nkx2.2* expression.

8.11 Future Work

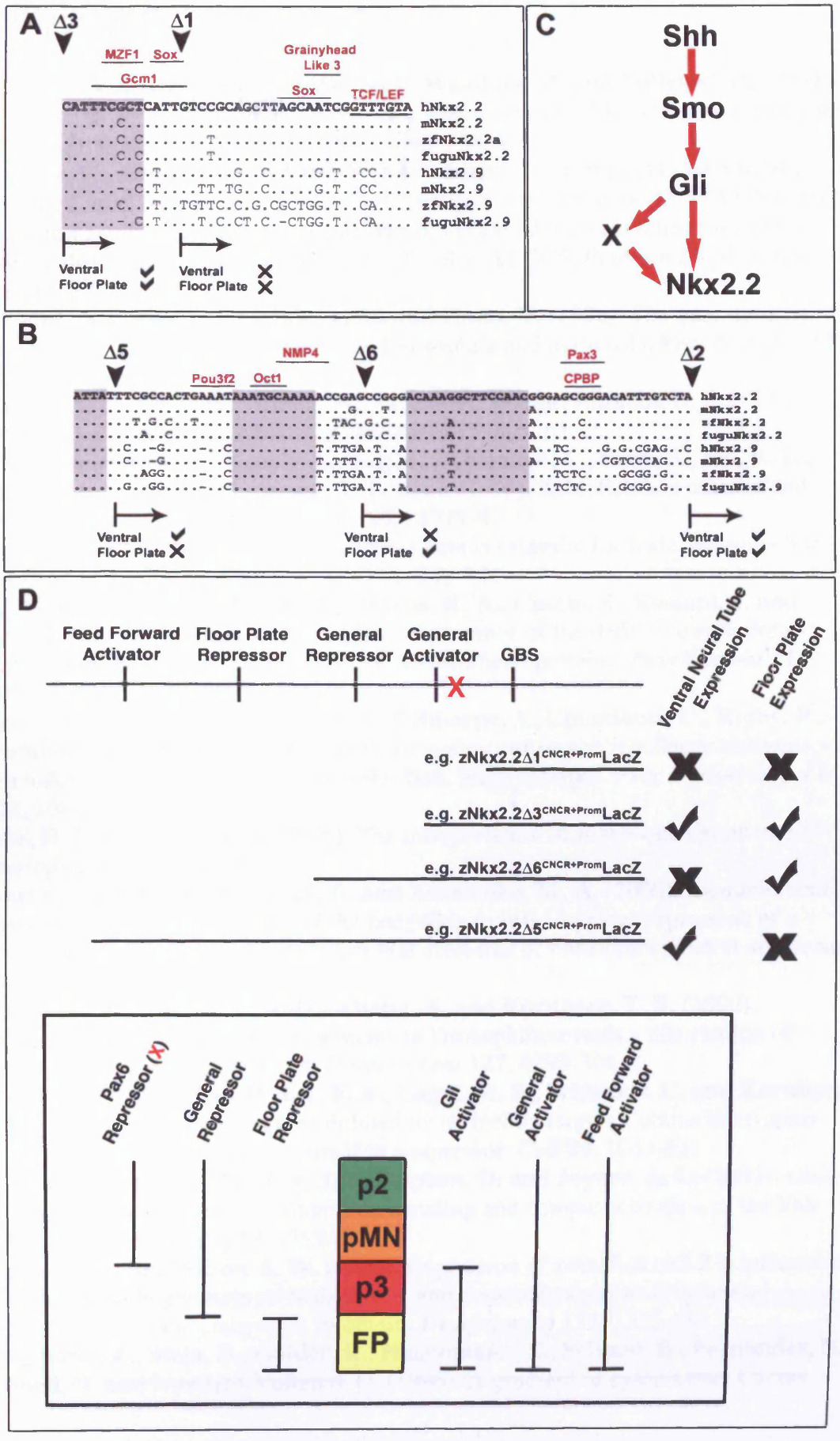
The key issue that arises from this study is the need to find the factors that bind to the identified elements within the CNCR. More specifically, to test individual regions that this project has proposed are important for floor plate repression and for ventral expression of *Nkx2.2*. Whether the candidate factors bind to particular fragments, e.g. binding of the Gcm1 protein to the 13bp fragment isolated in Chapter 6. Also to identify possible activator and repressor proteins that support the proposed model (Fig. 8.1D).

To further examine the importance of the elements required for floor plate repression or for the feed forward activation, these elements could be tested in the context of the BAC. The elements of interest could be reintroduced into the BAC in which the CNCR has been deleted. The change in Venus expression within the neural tube could be analysed after injection of the BAC into zebrafish embryos.

If further regulatory factors were identified for the induction of *Nkx2.2*, it would be interesting to see if the promoters of other genes expressed in the neural tube contain similar binding sites. This would identify other genes that may be regulated by Shh in a similar manner, and may provide confirmation about the nature of the differential gene expression in response to morphogenetic Shh signalling.

Figure 8.1 A model for induction of *Nkx2.2*.

A Alignment of region of CNCR identified to be necessary for ventral Nkx2.2-like expression. The region lies 5' to the identified Gli binding site and is predicted to contain an element bound by a general activator. The start sites for constructs zNkx2.2Δ3$^{CNCR+Prom}$LacZ (Δ3) and zNkx2.2Δ1$^{CNCR+Prom}$LacZ (Δ1) are marked on above the sequence. The ability of the constructs to direct LacZ expression (✔) or repression (✘) in the floor plate or ventral p3 domain, is marked below the sequence. Putative transcription factor binding sites identified by MatInspector have been marked on above the sequence. Gcm1 = Glial Cells Missing Homologue 1, MZF1 = Myeloid Zinc Finger Protein 1. **B** Alignment for a region identified to contain both a general repressor for the expression of *Nkx2.2* and also a floor plate repressor. The start sites for constructs zNkx2.2Δ2$^{CNCR+Prom}$LacZ (Δ2), zNkx2.2Δ5$^{CNCR+Prom}$LacZ (Δ5) and zNkx2.2Δ6$^{CNCR+Prom}$LacZ (Δ6) are marked on above the sequence. The ability of the constructs to direct *LacZ* expression (✔) or repression (✘) in either the floor plate or ventral p3 domain of the neural tube is marked below the sequence. In addition, putative transcription factor binding sites identified by MatInspector are marked above the sequence. NMP4 = Nuclear Matrix Protein 4, Pou3f2 = POU domain, Class 3 factor 2 (also known as N-Oct3, Brn2), Oct1 = Octamer 1, Pax3 = Paired Box Gene 3, CPBP = Core Promoter Binding Protein. **C** A diagram of the proposed feed forward loop for the regulation of Nkx2.2 by Shh signalling. **D** A proposed model that ties in the data from the promoter analysis of *Nkx2.2* to explain the observed expression patterns. Red X marks the suggested site for an element that mediates Pax6 repression if it is directly through the CNCR.


A
Δ3
Δ1
MZF1
Sox
Gcm1
Grainyhead Like 3
Sox
TCF/LEF
CATTTCGCTCATTGTCCGCAGCTTAGCAATCGGTTTGTA hNkx2.2
mNkx2.2
zfNkx2.2a
fuguNkx2.2
hNkx2.9
mNkx2.9
zfNkx2.9
fuguNkx2.9
Ventral Floor Plate
Ventral Floor Plate
C
Shh
Smo
Gli
X
Nkx2.2
B
Δ5
NMP4
Δ6
Pax3
Δ2
Pou3f2
Oct1
CPBP
ATTATTTCGCCACTGAAATAAATGCAAAAACCGAGCCGGGACAAAGGCTTCCAACGGGAGCGGGACATTTGTCTA hNkx2.2
mNkx2.2
zfNkx2.2
fuguNkx2.2
hNkx2.9
mNkx2.9
zfNkx2.9
fuguNkx2.9
Ventral Floor Plate
Ventral Floor Plate
Ventral Floor Plate
D
Feed Forward Activator
Floor Plate Repressor
General Repressor
General Activator
GBS
Ventral Neural Tube Expression
Floor Plate Expression
e.g. zNkx2.2Δ1CNCR+PromLacZ
e.g. zNkx2.2Δ3CNCR+PromLacZ
e.g. zNkx2.2Δ6CNCR+PromLacZ
e.g. zNkx2.2Δ5CNCR+PromLacZ
Pax6 Repressor (X)
General Repressor
Floor Plate Repressor
Gli Activator
General Activator
Feed Forward Activator
p2
pMN
p3
FP

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