# The Role of *Gbx2* in Murine Embryonic Development

A thesis submitted to the University of Adelaide

for the degree of Doctor of Philosophy

by

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# Table of Contents

# Abstract

*Gbx2* (Gastrulation and Brain specific Homeobox 2) is a murine orphan homeobox gene which has been isolated from embryonic brain and also from embryonic stem (ES) cells and embryonal carcinoma (EC) cells. The temporal and spatially restricted expression patterns of *Gbx2* in the developing embryo suggest possible functions for this gene in the pluripotent cells of the inner cell mass (ICM), in the primitive streak at gastrulation, in the developing CNS in the forebrain and the region posterior to the midbrain-hindbrain boundary, in the inner ear, and in the hematopoietic system. Characterization of *Gbx2* expression in embryoid bodies and retinoic acid (RA) induced ES cell aggregates revealed that, while ICM and neural expression patterns were recapitulated in vitro, primitive streak expression was not, indicating that the control of *Gbx2* expression in the primitive streak may be different than at other stages.

Gene targeting of the *Gbx2* locus in ES cells was performed and heterozygous targeted ES cells were introduced into mouse blastocysts. Chimeras were bred, however no germline ES cell contribution was achieved. Homozygous mutant ES cells were created to study the effects of *Gbx2* ablation on cellular differentiation decisions in vitro.

*Gbx2*$^{-/-}$ ES cells proliferate in an undifferentiated state in culture and can be induced to differentiate suggesting that *Gbx2* is not essential for survival of pluripotent cells in the ICM. Wild type and *Gbx2*$^{-/-}$ ES cells cultured as embryoid bodies under various conditions showed no differences in the ability to produce neurons, demonstrating that *Gbx2* is not absolutely required for neural formation, however *Gbx2*$^{-/-}$ RA aggregates produced fewer neurons than wild type, suggesting that *Gbx2* may enhance the responsiveness of ES cells to RA induction, or that *Gbx2*$^{-/-}$ ES cells are impaired in their ability to support the neural subtypes formed by exposure to RA.

Chicken *GBX2* has been reported to be expressed in hematopoietic tissues and has been implicated as a target of Myb activation in myelomonocytic precursors. Experiments described in this thesis demonstrated that *Gbx2* was undetectable in murine hematopoietic tissues, that *Gbx2* was not activated by Myb, and that ablation of *Gbx2* did not inhibit the formation of macrophages in culture, suggesting that *Gbx2* is not required for normal murine hematopoiesis. Thus, although chicken GBX2 and mouse Gbx2 share amino acid identity within the homeodomain, they do not appear to share identical functions.

These experiments suggest that *Gbx2* is more likely to function in providing positional information along the antero-posterior embryonic axis than to control survival, proliferation, or differentiation of neural or hematopoietic progenitor cells. A model is presented which proposes that *Gbx2*, along with *Otx2*, is responsible for early definition of posterior and anterior regions of the developing embryonic axis, thereby establishing the midbrain-hindbrain organizer region.

# Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed

Date 21/12/99

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# Chapter 1

# Introduction

# 1 Introduction

The investigation of molecules controlling the events of embryonic development, mammalian embryonic development in particular, is a complex yet fascinating task. Much progress has been made in this field in recent years due to advancements in recombinant DNA techniques and the fact that many molecular mechanisms have been conserved through evolution, allowing discoveries made about the development of less complex organisms such as the fly to be applied to our understanding of mammalian development. The work described in this thesis was undertaken to investigate the developmental role of *Gbx2*, a murine member of the homeobox gene family which was first identified in Drosophila. *Gbx2* expression had been demonstrated in pluripotent cells of the preimplantation embryo, in the embryonic tissues at gastrulation, and in the developing nervous system, suggesting possible functions in pluripotent cell biology, specification of cellular fate, or patterning of the embryo. This chapter will review the stages of mouse development relevant to *Gbx2* expression patterns, the molecular biology of homeobox genes, and molecular mechanisms controlling developmental events, thereby providing a framework for the understanding of *Gbx2* action.

## 1.1 Early embryogenesis of the mouse

### *1.1.1 Pre-implantation development*

Mouse development begins with the fertilised egg which divides approximately every 20 hours through to the 8 cell morula, or blastomere, stage. Single blastomeres removed from embryos at the 2 cell (Tarkowski, 1959) or 4 cell (Hogan et al., 1994) stage have been shown to be totipotent, giving rise to an entire mouse. At the 8 cell stage, approximately 2.5 days post coitum (d.p.c.), compaction begins, in which the individual blastomeres begin to flatten against each other, increasing cell-cell contact. Compaction results in the establishment of cellular polarisation, producing an inside (basal) and an outside (apical) face. This polarisation is thought to be the first indication of partitioning of cellular potential (Fig. 1.1a,b, Johnson and Maro, 1988).

As the cells of the morula continue to divide, the first differentiation event occurs, resulting in two developmentally restricted cell lineages. Outer cells form the trophectoderm which is committed to forming extraembryonic tissues. Inner cells of the morula give rise to the pluripotent cells of the inner cell mass (ICM) which will go on to form the actual embryo and some extraembryonic tissues such as visceral and parietal endoderm (Gardner, 1983). A fluid filled cavity termed the blastocoel is formed by ion transport via a sodium pump located in the plasma membrane of the

**Figure 1.1 Mouse embryogenesis**

a. Interference contrast micrograph of an 8 cell stage embryo showing compaction. The outer layer is the zona pellucida (**6**).

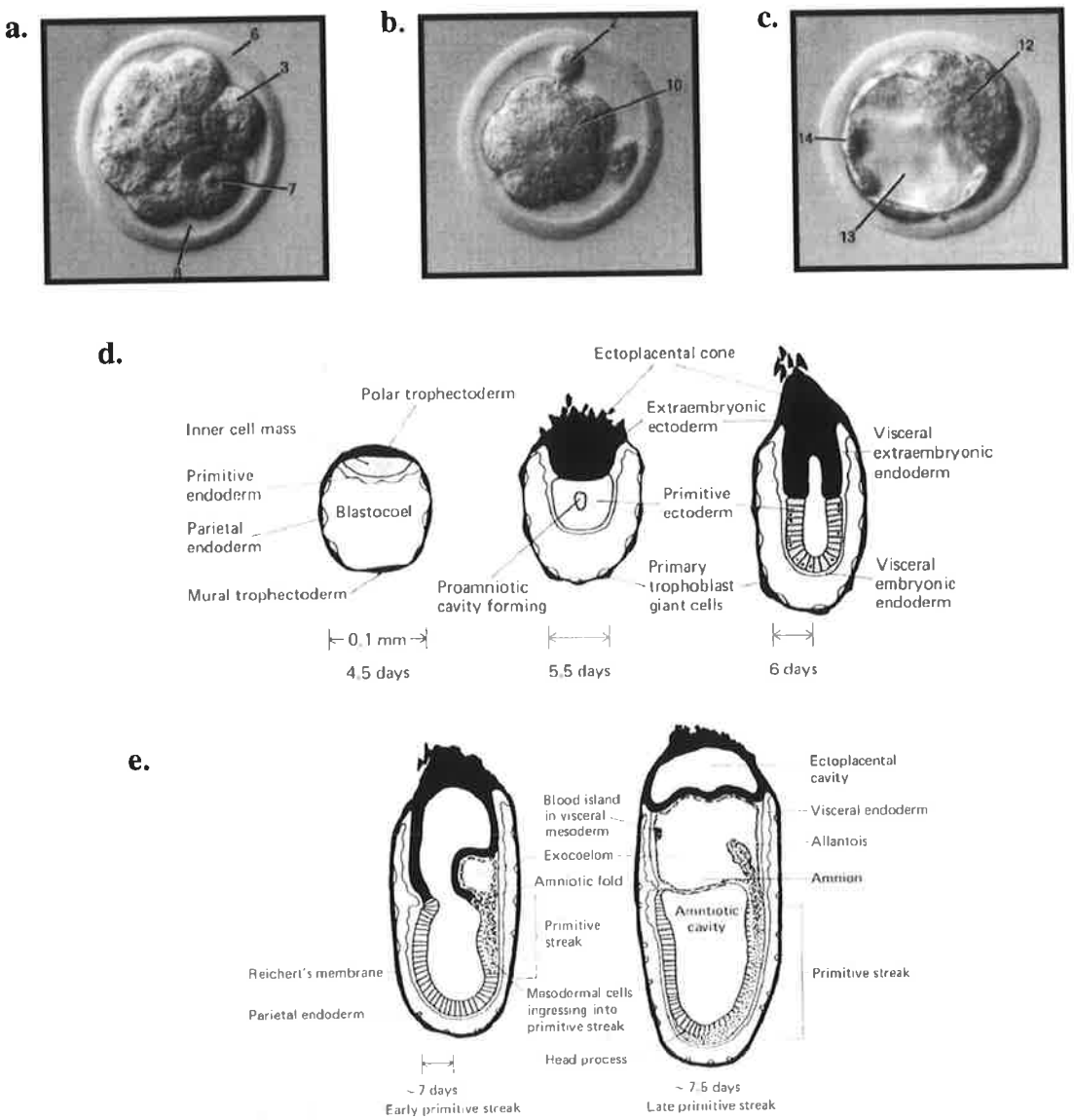
b. Micrograph of a fully compacted morula stage embryo. Cells facing the zona pellucida are trophectoderm, inner cells are the inner cell mass.

c. Micrograph of a blastocyst stage embryo with inner cell mass (**12**), blastocoelic cavity (**13**), and trophectoderm (**14**).

d. Schematic representation of early post implantation embryogenesis showing a fully compacted blastocyst, cavitation in the inner cell mass and formation of primitive ectoderm.

e. Schematic representation of gastrulation showing the initiation and extension of the primitive streak from the posterior toward the anterior aspect of the embryo.

a-c. Reproduced from Kaufman (1992).
d-e. Reproduced from Hogan et al. (1986).

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trophectoderm, resulting in the localisation of the ICM to one side of the embryo which at this stage is termed the blastocyst (Fig. 1.1c, Watson and Kidder, 1988; Watson, et al, 1990). Shortly before implantation, at around 4.0 d.p.c., a second round of differentiation occurs. The layer of cells in the ICM in contact with the blastocoel differentiates into an epithelial cell type termed primitive endoderm, which is fated to give rise to extraembryonic parietal and visceral endoderm. The remaining ICM cells form the epiblast, a population of pluripotent cells which will give rise to all of the ectodermal, mesodermal, and endodermal tissues of the fetus as well as extraembryonic mesoderm (Hogan et al., 1994). Prior to implantation, at 5 d.p.c., the blastocyst hatches from the glycoprotein matrix of the zona pellucida which has surrounded the egg and developing embryo.

### *1.1.2 Peri-implantation development*

Implantation occurs when the mural trophectoderm, which is farthest away from the ICM, contacts the uterine wall and proceeds to invade the epithelial layer and endometrium. After implantation, the cells of the trophectoderm proliferate and differentiate further, forming polyploid giant cells, extra-embryonic ectoderm, chorion, and placental cells (Hogan et al., 1994). The extra-embryonic ectoderm pushes the epiblast into the blastocoel, causing the epiblast to compact (Fig. 1.1d). The primitive endoderm, meanwhile gives rise to two differentiated populations: visceral endoderm, which remains in contact with the epiblast, and parietal endoderm, which differentiated from cells which migrate along the blastocoelic surface of the trophectoderm. Shortly after implantation, around 5.5 d.p.c., the proamniotic cavity forms in the center of the epiblast and the cells of the epiblast organize into a columnar epithelial sheet of primitive ectoderm, thus forming the cup-like structure of the egg cylinder (Fig. 1.1d). A mechanistic explanation for this cavitation invokes a diffusible signal emanating from the primitive endoderm which initiates programmed cell death in all epiblast cells with the exception of those associated with a localized survival signal associated with the basement membrane located between the primitive endoderm and epiblast. This over-rides the death signal in the layer of cells in contact with the basement membrane, resulting in the survival of an epithelial monolayer (Coucouvanis and Martin, 1995).

### *1.1.3 Gastrulation*

Gastrulation begins at 6.5-7 d.p.c., when the primitive ectoderm contains about 1000 cells. This complex event results in the establishment of the basic body plan of the embryo through cellular proliferation and migration, the formation of the ectodermal, mesodermal and endodermal germ layers, and antero-posterior axial patterning. Fate mapping, performed by tracing the movements and descendants of individual labelled cells or transplanted cell populations has shown the recruitment of cell lineages from

discrete regions of the pre-streak epiblast (Fig. 1.2) however these fates are not determined at this stage and can be reprogrammed by transplantation of primitive ectoderm cells to a different location (Beddington, 1982; Tam and Beddington, 1987, 1992; Tam, 1989; Lawson et al, 1991). Epiblast cells from pre- or early primitive streak stage embryos transplanted heterotopically from anterior to posterior, proximal to distal, or the reverse positions will take on the fate of cells in the region to which they have been transplanted (Paramaswaran and Tam, 1995; Tam and Zhou, 1996), however by late primitive streak stage, transplanted ectodermal cells show limited differentiation potential (Beddington, 1982; Paramaswaran and Tam, 1995).

Gastrulation is initially marked morphologically by the appearance of the primitive streak in the posterior region of the embryo. Primitive ectoderm cells of the epiblast lose contact with the endodermal layer, possibly due to breakdown of the extracellular matrix in this region, invaginate, and differentiate, forming a layer of mesoderm between the epiblast and visceral endoderm. The primitive streak elongates both laterally and axially toward the anterior tip (Fig. 1.1e). Mesoderm accumulating in the streak migrates both posteriorly to form extraembryonic mesoderm and anteriorly and laterally to form embryonic mesoderm which contributes to trunk structure (Fig. 1.2). Mesoderm emanating from the anterior primitive streak forms the head process and notochord, which underlie the future brain and spinal cord, and paraxial mesoderm, which condenses into the somites which will give rise to the vertebrae, muscles and dermis. Definitive endoderm, formed by the migration of primitive ectoderm cells into the visceral endoderm layer (Tam and Beddington, 1992), ultimately gives rise to the gut, liver, and lung. Embryonic ectoderm arises from the anterior region of the epiblast and differentiates into the nervous system, skin, and skeleton.

By midgastrulation, a specialized structure called the node is present at the anteriormost tip of the primitive streak. It is thought to be equivalent to the dorsal blastopore lip of *Xenopus* and Henson's node in birds in that it produces signals responsible for the specification of anterior-posterior identity, thus demonstrating organizer activity. Transplantation of the node from late primitive streak stage embryos to a more postero-lateral location in a host embryo from the equivalent stage results in induction of a second axis containing both neural and somitic cell types, but not anterior head structures (Beddington, 1994; Tam et al., 1997), suggesting that these structures are induced by a different organizer. This hypothesis has been validated by recent experiments where chimeras were created in which the embryo was predominantly wild type and the extraembryonic tissues were homozygous mutant for either *nodal* (Varlet et al., 1997) or *Otx2* (Rhinn et al., 1998), genes which are first expressed in anterior visceral endoderm. In either case, the forebrain was deleted and midbrain structures were truncated, whereas in the reverse experiment with wild type

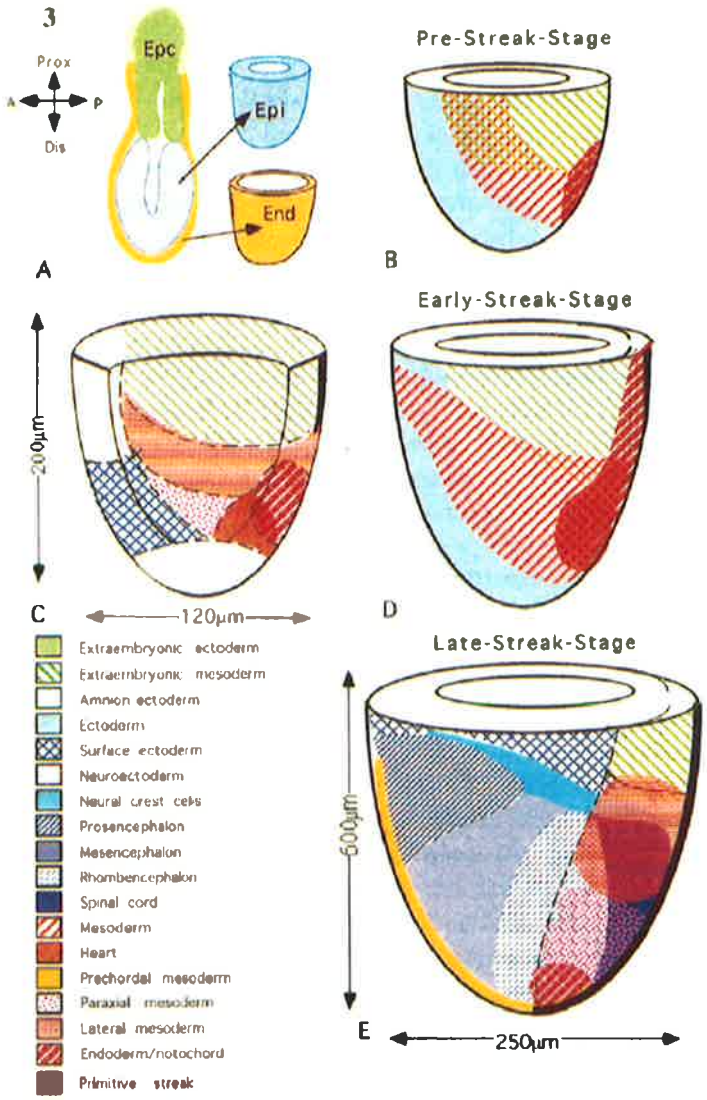



**Figure 1.2 Origins of embryonic lineages**

Fate maps of embryonic lineages in pre-streak through late streak stage embryos. Reproduced from Tam and Behringer (1997).

extraembryonic tissues and mutant embryos anterior patterning was normal. This demonstrates that anterior visceral endoderm signalling is required for the specification of anterior structures.

### *1.1.4 Neurogenesis*

Development of neural tissue and organization of neural structures begins during late gastrulation. Signals emanating from the notochord cause the overlying ectodermal cells to undergo morphological changes, becoming elongated and rising up into a thickened strip along the midline of the ectodermal sheet (Fig. 1.3) (Smith and Schoenwolf, 1989; Doniach, 1993). This region of ectoderm, known as the neural plate, becomes organized into a central strip of columnar cells bordered by flattened cells with an epidermal fate. The edges of this region thicken and rise, forming neural folds and creating a U-shaped neural groove. The neural folds then fuse at their dorsal aspect, creating the neural tube. Gene expression patterns demonstrate that regionalization of the neural tube along an antero-posterior axis occurs very early (section 1.2.2). This early patterning is subsequently refined, leading to the formation of a series of segment-like swellings and constrictions which define various chambers of the brain and spinal cord. Within each region, controlled by a multitude of specific signalling events, neurons with differing morphological and functional characteristics form the structures and neural pathways of the mature central nervous system. The segment-like restrictions of the hindbrain (rhombomeres) demonstrate lineage restriction in the identity of nerves emanating from each rhombomere (Lumsden and Keynes, 1989; Simon and Lumsden, 1993). Neural crest cells, which arise from neurectoderm of the dorsal portion of the neural tube, migrate to a wide variety of tissues, contributing to the peripheral nervous system, epidermal pigment cells, ectomesenchymal cells, and connective tissue (Le Dourain, 1982; Noden, 1983; Lumsden et al., 1991).

## 1.2 Genetic control of early mammalian development

Cell determination and differentiation in the mammalian embryo are driven by signals expressed by the embryo itself (Jackson, 1989). These signals provide positional information and regulate cellular events such as proliferation, determination, differentiation, apoptosis, cell adhesion, and migration. Developmental control factors can be grouped into two broad categories: extracellular factors and intracellular factors. Extracellular factors include diffusible signalling molecules such as *Wnt-1* and *sonic hedgehog*, cell adhesion molecules such as the cadherins, vitamin A metabolites such as retinoic acid, and peptide growth factors which include the Fibroblast Growth Factor (Fgf) family, the Transforming Growth Factor β (Tgfb) family, the Bone Morphogenic protein (BMP) family, and cytokines such as LIF and Ciliary

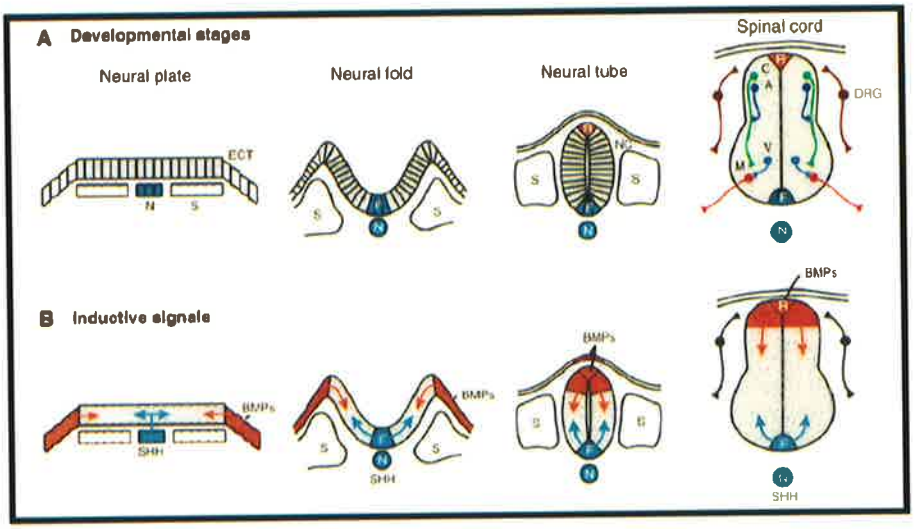



**Figure 1.3 Neural tube formation**

a. Developmental stages of neural tube formation.

b. Inductive signals in neural tube formation. Sonic hedgehog (**SHH**) and other signals arising from paraxial mesoderm and later notochord (**N**) induce ventral identity and floorplate (**F**) formation. BMP's and other signals arising from adjacent ectoderm induce dorsal identity and roofplate (**R**) formation. Neural crest (**NC**) cells arise from dorsal neurectoderm and migrate throughout the embryo.

Reproduced from Tanake and Jessel, 1996.

Neurotropic Factor (CNTF). Extracellular factors generally act through binding receptors on the cell surface, and in many cases activate intracellular signal transduction molecules such as members of the tyrosine kinase or seronine/threonine kinase families which transmit a signal to the nucleus through molecules such as the Smad proteins, resulting in the activation or repression of the target genes. In the nucleus, transcription factors such as the homeobox genes, *brachyury*, the *Sox* family and the *Myo-d* family encode proteins which act to either enhance or repress transcription of other genes through direct or indirect interaction with their control elements.

The genes encoding developmental regulatory factors and the mechanisms by which they act have been the focus of intense investigation in developmental biology in recent years. A complete discussion of all the genes that have been shown to be involved in embryo development is beyond the scope of this review. The following sections describe genes involved in developmental events in the mouse which relate to sites of *Gbx2* expression.

### *1.2.1 Pluripotent cells of the ICM and epiblast*

A number of genes have been identified as markers of pluripotent cell populations of the ICM and epiblast. The *Oct4* homeobox gene is transcribed in the fertilized egg, blastomeres, the ICM, epiblast and primitive ectoderm, and primordial germ cell lineages, but is down regulated in differentiated embryonic and extra-embryonic cell types in vitro and in vivo. (Schöler et al., 1989; Schöler et al., 1990; Rosner et al., 1990; Palmieri et al., 1994). *Oct4* has been demonstrated to be required for survival of pluripotent ICM cells (Nichols et al., 1998). The zinc finger gene *Rex1* is expressed throughout cells of the ICM, but by 5.25 d.p.c. expression is found in the extraembryonic ectoderm and not in the primitive ectoderm (Rogers et al., 1991; T. Pelton, pers. comm.). The mutant phenotype for *Rex1* has not yet been reported. *Fgf5* is not expressed in the ICM, but first appears in the primitive ectoderm by 5.25 d.p.c. and is expressed in primitive ectoderm up to 7.5 d.p.c., and then is expressed in multiple sites, including mesodermal and ectodermal derivatives (Haub and Goldfarb, 1991; Hébert et al., 1991). Ablation of *Fgf5* expression results in mice which are phenotypically normal except for abnormally long hair, demonstrating that FGF5 is not essential in early development (Hébert et al., 1994). Leukemia Inhibitory Factor (LIF), an IL-6 family member, is expressed in extraembryonic tissues of the blastocyst stage embryo and later in development in various tissues, including the skin, lung, intestine, and uterus (Conquet and Brûlet, 1990; Rathjen et al., 1990; Robertson et al., 1993; Nichols et al., 1996). The IL-6 cytokine family has been shown to maintain pluripotent cells in vitro (1.4.1) by signalling through the gp130 pathway, however ablation of LIF, the LIF receptor, or gp130 does not appear to

adversely affect the formation or maintenance of pluripotent cells in mutant embryos (Stewart et al., 1992; Escary et al., 1993; Li et al., 1995; Ware et al., 1995; Yoshida et al., 1996). *Hnf4*, a winged helix transcription factor, is first expressed in primitive and visceral endoderm at 4.5 d.p.c. and has been shown to be required for maintenance of primitive ectoderm (Duncan et al., 1994; Chen et al, 1994; Duncan et al., 1997).

### *1.2.2 Early antero-posterior patterning*

The earliest evidence of asymmetrical antero-posterior (A-P) gene expression is expression of *Hex*, a homeobox gene which is first detected at 4.5 d.p.c. in the primitive endoderm at the interface between the inner cell mass and the blastocoelic cavity. By 5.5 d.p.c. *Hex* expression is restricted to a small population of visceral endoderm cells at the distal tip of the embryo and, due to the anterior-ward migration of this visceral endoderm, is localized to anterior visceral endoderm by 6.0 d.p.c. (Thomas et al., 1997; Thomas et al., 1998) (Fig. 1.4). Other genes expressed in overlapping, but distinct domains of the anterior visceral endoderm prior to gastrulation include *Lim1* (Belo et al., 1997), *Hesx1* (Thomas and Beddington, 1996), and *cer-1* (Belo et al., 1997; Thomas et al., 1997). *Otx2* is expressed throughout the visceral endoderm and primitive ectoderm at this stage, but is restricted to anterior visceral endoderm and anterior primitive ectoderm during gastrulation (Ang and Rossant, 1994; Acampora et al., 1995). *Goosecoid* (*gsc*) is expressed both in the anterior visceral endoderm and in the posterior epiblast (Blum et al., 1992; Conlon et al., 1994). Analysis of *Otx2*, *Hesx1*, and *Lim1* null mutants has confirmed a functional requirement for these genes in anterior head formation, corresponding to their anterior expression pattern (Acampora et al., 1995; Dattani et al., 1998; Shawlot and Behringer, 1995) which is consistent with the previously described role of anterior visceral endoderm as an organizer (1.1.3). *Gsc* mutants, however, die neonatally with cranio-facial and rib defects, which corresponds to *gsc* expression patterns post-gastrulation (Rivera-Pérez, et al., 1995; Yamada et al., 1995).

Posteriorly restricted gene expression is first seen shortly before gastrulation. *Evx1*, *brachyury*, and *Fgf8* are expressed in the posterior epiblast at 6.0 d.p.c. (Fig 1.4, Dush and Martin, 1992; Crossley and Martin, 1995). *Nodal* is expressed in the posterior epiblast prior to primitive streak formation (Fig 1.4, Varlet et al., 1997) and has been implicated in the induction and/or maintenance of the primitive streak (Conlon et al., 1994).

### *1.2.3 Gastrulation*

The morphological changes and cell migration which characterize gastrulation are accompanied by alterations in gene expression patterns (Fig 1.5). *Otx2* expression

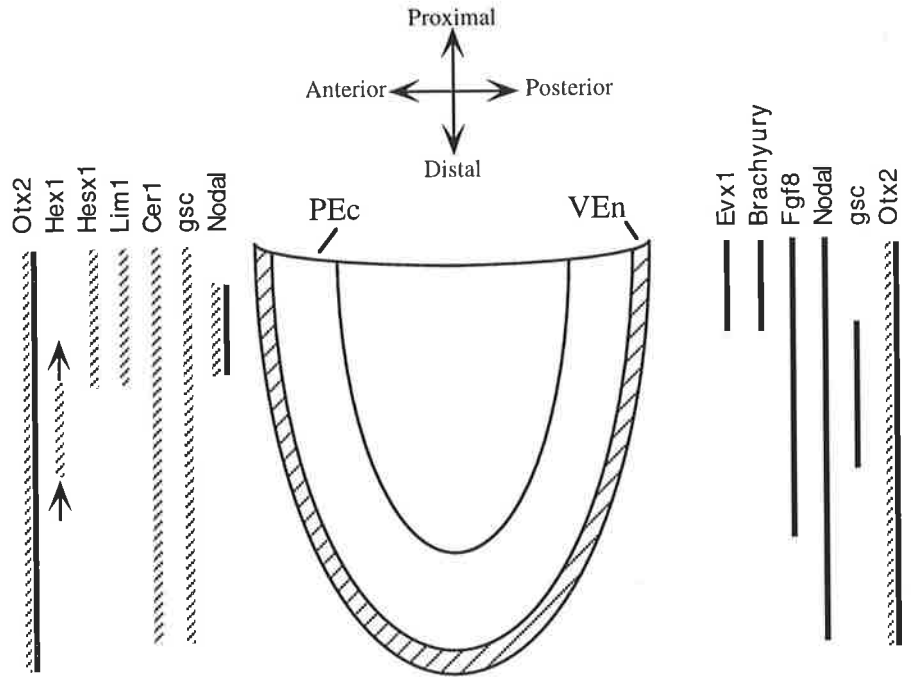



**Figure 1.4 Genes expressed in the pre-gastrulation embryo (5.0-6.0 d.p.c.).**

Hatched line = region of expression in visceral endoderm (VEn), Black line = region of expression in primitive ectoderm (PEc). Hex1 expression is detected in a patch of cells located at a progressively more anterior region over this period of time, as indicated by arrows.

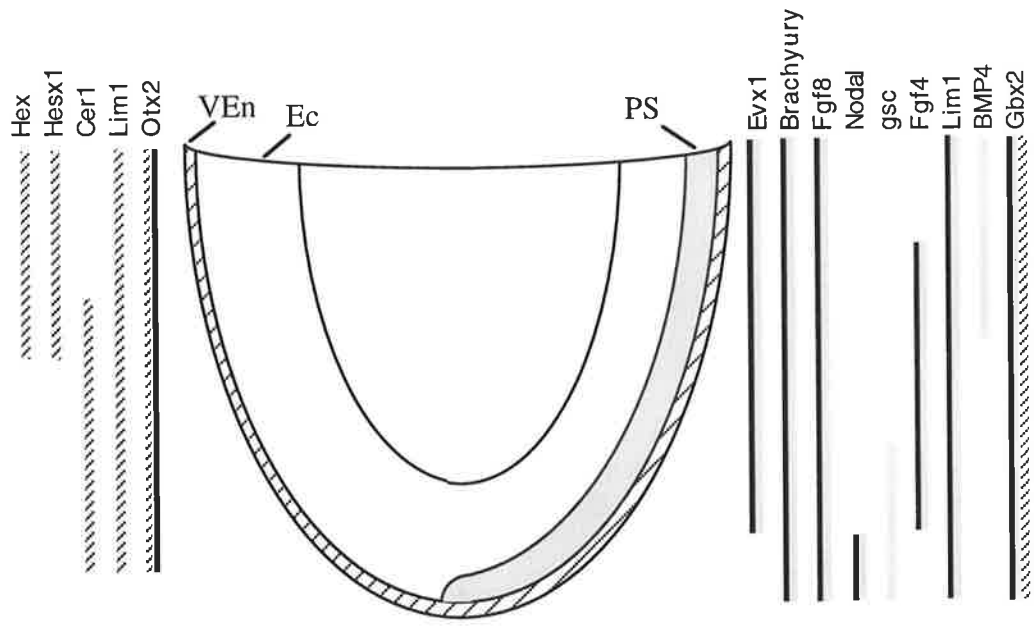



**Figure 1.5 Genes expressed in the late gastrulation stage embryo (7.5 d.p.c.).**

Hatched line = region of expression in visceral endoderm (VEn), Black line = region of expression in ectoderm (Ec), Grey line = region of expression in primitive streak (PS).

becomes restricted to primitive ectoderm and visceral endoderm in regions anterior to the developing primitive streak (Simeone et al., 1993). *Gsc* and *nodal* expression become limited to small region at the anterior of the primitive streak, corresponding to the future node (Blum et al., 1992; Varlet et al., 1997), while *Fgf4*, *Fgf8*, *Bmp4*, *Evx1*, and *brachyury* are expressed in overlapping, but distinct regions of the primitive streak (Niswander and Martin, 1992; Crossley and Martin, 1995; Winnier et al., 1995; Dush and Martin, 1992; Herrmann, 1991). A number of these genes have been shown to be essential for gastrulation to proceed normally. *Bmp4* and *Fgf8* mutant embryos are not able to form mesoderm (Winnier et al., 1995; Meyers et al., 1998), while *brachyury* mutants demonstrate retarded migration of mesodermal cells and defects of the posterior mesoderm and notochord (Yanagisawa et al., 1981; Wilson et al., 1995).

### *1.2.4 Neural induction and axial patterning*

Transplantation of tissue from the node region results in the formation of a secondary axis containing tissues displaying well organized antero-posterior and dorsal-ventral axes (Beddington, 1994; Tam et al., 1997). This demonstrates that the node is an organizer tissue, capable of initiating a signalling cascade leading to the specification and patterning of posterior neurectoderm. Nieuwkoop (1952) suggested a 2-step model of neural patterning in *Xenopus*. The first phase is characterized by induction, in which presumptive neurectoderm is 'activated' or 'induced' to an anterior fate. The second phase involves a 'transforming' signal from the posterior mesoderm which converts the induced cells to progressively more posterior fates along the axis. The identity of these signals in the mouse is still unclear, however three candidate factors which are secreted by the organizer have been described in *Xenopus*. When mRNA encoding Follistatin (Hemmati-Brivanlau et al., 1994), Noggin (Lamb et al., 1993), or Chordin (Sasai et al., 1995) is injected into animal caps, neural tissue is induced which expresses anterior neural plate markers such as *Otx2* and *Xanf-1*, but not more posterior markers such as *krox-20* and *X1Hbox-6*. These factors are thought to act by inhibiting BMP signalling which would otherwise direct an epidermal fate to ectodermal tissues (Wilson and Hemmati-Brivanlau, 1995).

Retinoic acid and the FGF family of signalling molecules have also been implicated as transforming signals. Both molecules show posteriorizing effects when exogenously applied to ectodermal cells (Doniach, 1995; Lamb and Harland, 1995; Durston et al., 1989; Ruiz i Altaba and Jessel, 1991). RA has been shown to down regulate the expression of anterior genes such as *Otx2*, which is expressed in the anterior visceral endoderm and neurectoderm (Pannese et al., 1995, Kolm et al., 1997), and to up regulate the expression of posterior genes, including the *Hox* cluster genes and *Xgbx2* (Simeone, et al., 1990, 1991; Papalopulu et al., 1991; Marshall et al., 1992; Kolm et

al., 1997). FGF also can up regulate the expression of posterior genes such as *Hox* genes, *Xhox7* (*evx1*), *Xgbx2*, and *cad3* (*cdx1*) (Green et al., 1992; Isaacs et al., 1992, King and Moore, 1994; Kolm et al., 1997), however does not down regulate anterior genes such as *Otx2* (Kolm et al., 1997). The simple A-P pattern that is established prior to and during gastrulation is further defined and elaborated in progressive stages of development by signals generated within the neural tube in specific regions along the A-P axis (Figure 1.6, section 1.2.5).

Dorsal-ventral patterning within the neural plate and subsequently in the neural tube is controlled by signals generated by the notochord and the epidermal ectoderm flanking the neural plate. Sonic hedgehog (*shh*) is a diffusible factor produced by the notochord and later the floor plate of the neural tube and its involvement in specifying ventral identity has been demonstrated by misexpression, inhibition, and ablation experiments. Dorsal and lateral *Xenopus* neural tube explants cultured in the presence of *shh*-over-expressing COS cells are induced to form ventral structures such as floorplate and motor neurons (Roelink et al., 1994). Transgenic mice expressing chicken *shh* under the control of *Wnt1* regulatory domains displayed expression of floorplate genes *HNF3β* and mouse *shh* in the midbrain neurectoderm (Echelard et al., 1993). Motor neurons failed to form when neural explants were treated with blocking antibodies to *shh* (Marti et al., 1995; Ericson et al., 1996) and ablation of *shh* expression by gene targeting resulted in severe mutations including degeneration of the notochord and failure to form a floorplate (Chiang et al., 1996). Dorsalizing signals produced by the epidermal ectoderm are likely to include BMP family members such as BMP-2, -4, -7, and dorsalin-1 based on their expression patterns and ability to induce dorsal markers in neural plate cells (Liem et al, 1995, 1996; Basler et al., 1993).

### *1.2.5 Hindbrain and spinal cord patterning*

Hindbrain and spinal cord patterning is characterized by morphologically distinct compartments or segments. Segmental identity is conferred, in the hindbrain at least, by the expression of a limited repertoire of transcription factors. These have been most thoroughly studied in the hindbrain and are dominated by the *Hox* family, which are expressed in overlapping domains along the A-P axis and have been shown by misexpression and ablation studies to specify positional identity in the hindbrain (Fig. 1.6, section 1.3.3) (McGinnis and Krumlauf, 1992). It has been proposed that retinoic acid which is present in the node regulates the nested expression of the *Hox* genes through a concentration gradient (Simeone et al., 1991; Papalopulu et al., 1991, Marshall et al., 1992; Godsave, et al., 1998). In addition, rhombomere-restricted expression has been observed for other transcription factors, receptors, and signalling

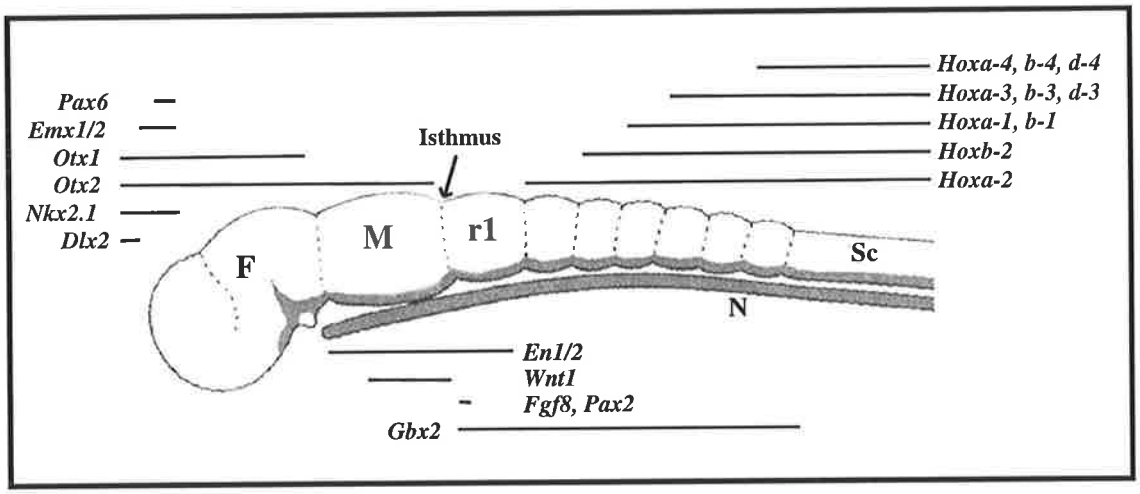



**Figure 1.6 Gene expression in the developing central nervous system**

Schematic representation of expression domains of some of the genes expressed in the developing forebrain (**F**), midbrain (**M**), hindbrain (**r1**-7), and spinal cord (**Sc**). Lines represent limits of gene expression at 9.5 d.p.c. (*Hox* genes, *En1/2*, *Wnt1*, *Fgf8*, *Pax2*, *Gbx2*) or 10.5 d.p.c. (*Pax6*, *Emx1/2*, *Otx1/2*, *Nkx2.1*, *Dlx2*).

Adapted from Lumsden and Krumlauf (1996).

molecules such as *krox-20, kreisler*, Eph-like receptor tyrosine kinases and their ligands, retinoic acid receptors, FGF3 and follistatin (reviewed in Lumsden and Krumlauf, 1996). One of these, *krox-20*, has been shown to function in specifying segmental identity. *Krox-20* is a zinc finger gene expressed prior to segmentation in the regions fated to become rhombomeres 3 and 5 (Wilkinson et al., 1989). When *krox-20* expression is disrupted by gene targeting these segments are deleted from the hindbrain (Schneider-Maunoury et al., 1993), demonstrating its importance in establishing these compartments.

### *1.2.6 Midbrain-hindbrain boundary and midbrain patterning*

The anteriormost region of the hindbrain, rhombomere 1, does not express *Hox* genes, but a number of other genes expressed in this segment and at its anterior border appear to reinforce the boundary between the hindbrain and the midbrain (Fig. 1.6). This region, known as the isthmus, functions as a regional organizer, producing long range signals which specify A-P identity within the midbrain (reviewed in Bally-Cuif and Wassef, 1995; Lumsden and Krumlauf, 1996). The earliest genes demarcating the future isthmus are *Otx2* in the anterior neurectoderm and *Gbx2* in the posterior neurectoderm, by 7.5 d.p.c. (Simeone, et al., 1993; Bouillet et al., 1995). In the presomite stage (7.5-8.0 d.p.c.), *Pax2*, *Wnt1*, and *En1* are expressed broadly in this region, overlapping the *Otx2/Gbx2* border (Rowitch and McMahon, 1995), and *Fgf8* is broadly expressed in the posterior primitive streak and overlying ectoderm (Crossley and Martin, 1995). Expression of these genes is gradually restricted and by 9.5 d.p.c. *Wnt1*, *Fgf8* , *Pax2* and *Gbx2* are expressed in discrete bands either anterior (*Wnt1*) or posterior (*Fgf8*, *Pax2*, *Gbx2*) to the isthmus (Wilkinson et al., 1987; Crossley and Martin, 1995; Rowitch and McMahon, 1995; Bouillet et al., 1995). *En1* and *En2* expression patterns narrow, but continue to straddle the isthmic region (Davis and Joyner, 1988) (Fig. 1.6). FGF8 has been shown to be a critical molecule for establishing this region, through the repression of *Otx2* and the induction of *En1* and *Wnt1* expression. When beads containing FGF8 were implanted in the forebrain region of chick embryos, ectopic expression of *En1*, *Fgf8*, and *Wnt1* was found in concentric rings around the bead while *Otx2*, normally expressed in the forebrain and midbrain, was repressed. Upon further culture, ectopic midbrain structures formed around the bead, suggesting the bead acted as an ectopic isthmus region (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999).

In vivo mutational analysis has revealed a number of genes responsible for patterning this region. Embryos carrying homozygous mutations in *Otx2* fail to develop forebrain, midbrain, or anterior hindbrain structures (Acampora et al., 1995). Null mutation of *En1* has shown that *En1* is essential for specification of the midbrain (Wurst et al., 1994), although *En2* can substitute for *En1* when expressed in the

context of *En1* regulatory regions (Hanks et al., 1995). *Wnt1* mutant mice express *En1* normally at the presomite stage (8.0 d.p.c.), but expression is lost by the 6-7 somite stage (8.5 d.p.c.) demonstrating that *Wnt1* is necessary for maintenance of *En* expression in the midbrain region (McMahon et al., 1992), while FGF8 has been shown to induce *En* expression ectopically, suggesting it may be responsible for the initiation of *En* expression (Crossley et al, 1996).

### *1.2.7 Forebrain patterning*

Patterning in the forebrain is not well understood. Due to its morphological complexity there has been some debate as to whether the regions of the forebrain represent segmentation or not (Puelles et al., 1987; Puelles and Rubenstein, 1993). As was noted in section 1.1.3, the inductive signals specifying the anterior head region differ from those specifying the posterior region, suggesting that different mechanisms may be involved in the patterning of the forebrain. Many homeobox genes, including *Emx*, *Dlx*, *Nkx*, *Pax*, and *Otx* family members, have been mapped to restricted regions of the forebrain (Fig. 1.6) and mutational analysis has demonstrated homeobox gene involvement in specification and differentiation of the forebrain (reviewed in Rubenstein and Shimamura, 1997).

### *1.2.8 Mechanisms of genetic control of development*

Gene function is often identified by analysis of the expression pattern followed by gain-of-function or loss-of-function studies. Together these provide an understanding of how a particular gene influences the process of embryonic development. Analysis of the genetic control of early mammalian development has resulted in the elucidation of functional categories for genes involved in embryogenesis. Some genes appear to control specific cellular function, such as the control of cell proliferation or differentiation. An example of this type of developmental control gene is *Oct4*, which is expressed in all pluripotent cells and is required for their survival in the ICM (Nichols et al., 1998). Genes expressed in the organizer regions of the node, such as *gsc* and *nodal*, and the anterior visceral endoderm, such as *Hex*, and *Hesx1*, are expressed prior to the onset of patterning in these regions and loss-of-function mutations result in the deletion of trunk and anterior structures, respectively. Other genes expressed as a result of organizer activity are involved in morphological patterning. These genes, often transcription factors such as the *Hox* genes, provide positional information along the body axes and direct the expression of other genes which result in the formation of specific structures along those axes.

## 1.3 Homeobox genes

Homeobox genes are a family of transcription factors which share a highly conserved DNA binding region called the homeodomain (Scott et al., 1989). These genes were first identified as the cause of homeotic mutations in *Drosophila* in which structures from one segment of the body are transformed into those of another segment. For example, a mutation of *Antennapedia* causes the formation of legs in place of the antennae (Frischer et al., 1986). Cloning and comparison of several of these genes revealed a highly conserved region termed the homeobox which encodes the protein region known as the homeodomain. Homeobox specific probes have been used to identify and isolate many other homeobox genes in a large number of eukaryotic species.

### *1.3.1 Homeodomain structure and function*

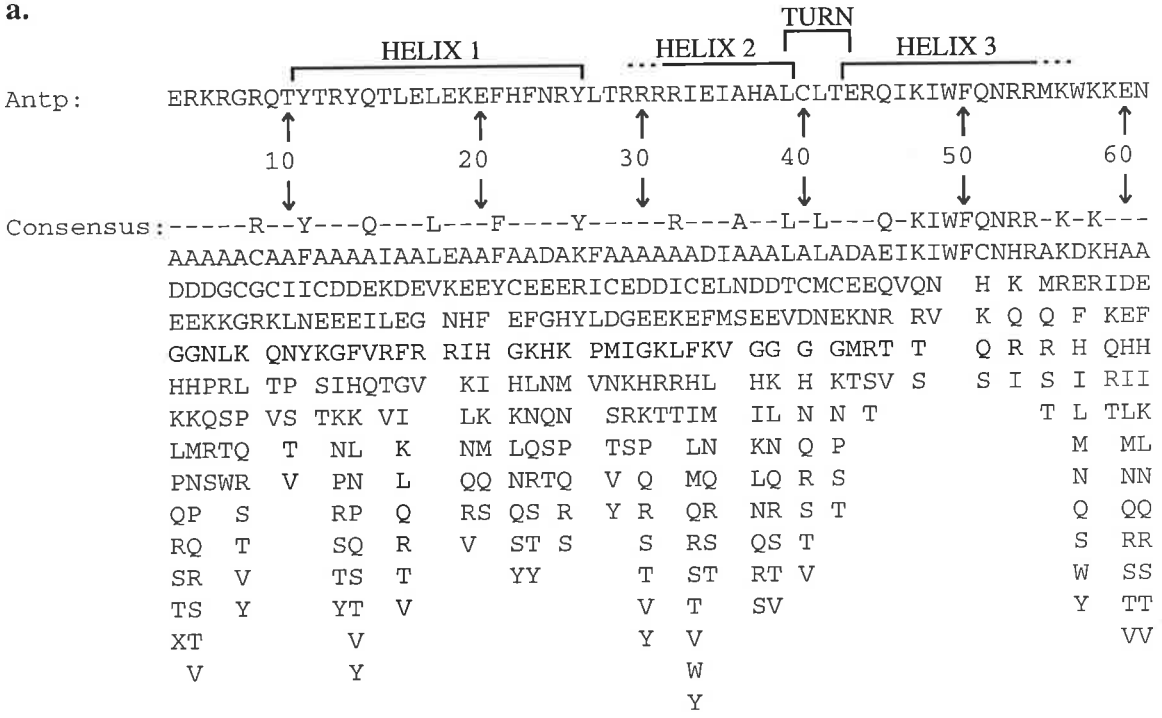
The conserved homeodomain region consists of approximately 60 amino acids folded into 3 α helices in a helix-turn-helix configuration (Fig. 1.7a, Scott et al., 1989). Helix 3, termed the recognition helix, is the most highly conserved region. The 3 dimensional structures of a number of homeodomains, including the *Antennapedia, Engrailed, fushi tarazu*, yeast MATα2, and human *Oct2* homeodomains, have been determined by NMR and X-ray crystallography (Kissinger et al., 1990; Otting et al., 1990; Wolberger et al., 1991; Qian et al., 1994; Sivaraja et al., 1994). The structure of *Engrailed* in the context of its DNA binding site is shown in Figure 1.7b and c. Helix 3 sits in the major groove and is responsible for most of the DNA interactions. Helices 1 and 2 lie on top of helix 3, perpendicular to it and anti-parallel to each other. The N-terminal arm interacts with the minor groove.

Homeodomain proteins have been shown to function by binding to specific sites in the regulatory regions of genes and either activating or repressing gene transcription. This was first demonstrated by the addition of purified homeodomain proteins to in vitro transcription reactions ( Fletcher et al., 1987; Scheidereit et al., 1987) and later confirmed by reporter gene transfection assays in *Drosophila* cell culture (Jaynes and O'Farrell, 1988; Han et al., 1989; Krasnow et al., 1989). DNA binding studies of many homeodomain proteins have determined that TAAT is the core consensus binding site for the homeodomain (Laughon, 1991; Kalionis and O'Farrell, 1993; Gehring et al., 1994).

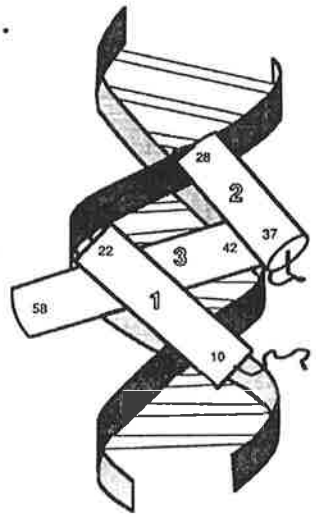
### *1.3.2 Homeodomain Classification*

Homeobox genes are grouped into classes based on their amino acid sequence homology. Conservation of the homeodomain sequence between members of a class

a.




b.




c.

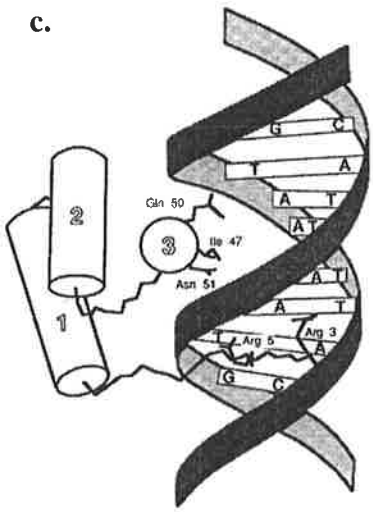



**Figure 1.7 Homeodomain sequence and structure**

a. The *Antennapedia* amino acid sequence was compared with 83 homeodomain sequences. Dashes indicate non-conserved sequence. Alternative amino acid residues which have been identified at each position are shown below the consensus sequence.

b. Schematic representation of the *Engrailed* homeodomain showing the orientation of the alpha helices (**1-3**) in relation to the double stranded DNA helix.

c. Lateral view of the *Engrailed* homeodomain/DNA complex showing base contacts.

a. Reproduced from Scott et al. (1989).
b-c. Reproduced from Kissinger et al. (1990).

is generally greater than 75%, while the homology between classes is less than 50% (Bürglin, 1994). Some homeobox genes are categorized by common conserved motifs present outside the homeodomain such as the paired box, zinc finger, POU domain, or LIM domain (reviewed in Bürglin, 1994). These conserved regions may contribute to DNA binding specificity (Treisman et al., 1991; Schöler, 1991). Finally, homeobox genes may be broadly partitioned into 2 groups based on chromosomal location. In *Drosophila*, a group of 8 homeobox genes of the *Anntenaepedia* or *Bithorax* class were found to be clustered in a region of the genome called the HOM-C complex. In the mouse, human, and other mammals there are 39 similar genes organized into 4 related clusters, Hox A-D (Fig. 1.8). It is thought that these represent duplication and diversification of an ancient gene cluster common to invertebrates and vertebrates (Pendleton, et al., 1993). In addition to the clustered HOM-C or *Hox* genes, there are many homeobox genes which are found scattered throughout the genome and are termed dispersed or orphan homeobox genes.

### *1.3.3 Hox genes*

As described above, the *Hox* genes are organized in 4 clusters in the mouse genome. Paralogous genes are located at the same relative position in different clusters and tend to share a greater degree of homology than genes at adjacent positions within the same cluster (Fig. 1.8) (Scott, et al., 1989). This suggests an evolutionary relationship between clusters. Paralogous genes are found in the same consecutive order along the chromosome and are all translated in the same direction. The timing and pattern of *Hox* gene expression along the anterior-posterior axis of the embryo corresponds to the order of the genes along the chromosome (Fig. 1.8), with 3'-most genes appearing earlier and more rostrally than the next gene in the 5' direction (Murphy et al., 1989; Hunt et al., 1991; Izpisúa-Belmonte et al., 1991). This colinear organization and expression suggests a coordinated set of positional instructions. *Hox* gene expression along the antero-posterior axis is characterized by tightly defined, overlapping anterior boundaries which correspond with segmental boundaries in the hindbrain and spinal cord. Kessel and Gruss (1990) proposed that the combination of *Hox* genes expressed in each somite comprises a 'code' which specifies the morphological identity of that somite.

Retinoic acid has been implicated as a candidate signal for mediating *Hox* gene expression in the posterior neuraxis, based on its concentration-dependent posteriorizing effects (Godsave, et al., 1998) and demonstrated ability to regulate *Hox* gene expression in vitro (Simeone et al., 1991; Papalopulu et al., 1991, Marshall et al., 1992). Hox proteins regulate a limited subset of target genes, indicating a high degree of binding specificity despite the frequent occurrence in the genome of the consensus binding site, TAAT. This specificity is achieved by cooperative DNA

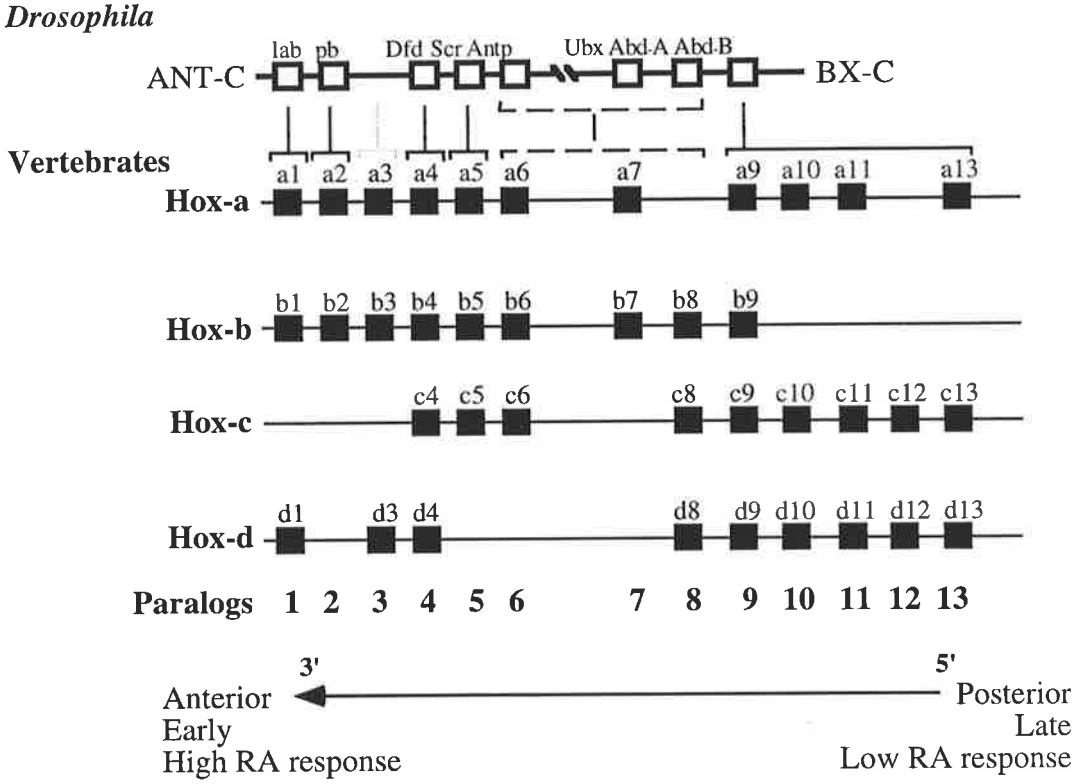



**Figure 1.8 The *Hox* cluster**

Alignment of the four vertebrate *Hox* complexes with the *Drosophila* HOM-C complex. Brackets and shadowed boxes indicate paralogous genes which are related to a specific Drosophila HOM-C homeotic genes. The arrow at the bottom indicates the direction of transcription and colinear expression of the genes with respect to antero-posterior position, time of expression, and retinoic acid response.

Reproduced from Krumlauf (1993).

binding with cofactors which recognize and bind to regions flanking the Hox binding site (reviewed in Mann and Chan, 1996). A number of Hox cofactor proteins have been identified, including the *Drosophila* homeobox gene *Exd* (Chan et al., 1994; Pöpperl et al., 1995) and *Pbx* in mammals (Chang et al., 1995; Phelan et al., 1995).

Many of the mouse *Hox* genes have been analyzed by misexpression or ablation and been shown to function in vivo to provide signals of positional identity, resulting in antero-posterior axial patterning of the trunk and limbs (McGinnis and Krumlauf, 1992, Capecchi, 1997). Homeotic transformations have been observed in *Hoxc-8* null mutant mice where the L1 lumbar vertebra is transformed in appearance to T13, the last thoracic vertebra (Le Mouellic, et al., 1992). However, null mutant phenotypes are not always homeotic as seen in mice lacking *Hoxa-1* which show a deletion of rhombomere 5 and a reduction in rhombomere 4 (Lufkin, 1991; Chisaka et al., 1992), while ectopic expression of *Hoxa-1* results in the posterior transformation of rhombomere 2 to a rhombomere 4 identity (Zhang et al., 1994). Murine *Hox* genes can function through interaction with paralogous or even non-paralogous genes. For example, double mutations of paralogous genes *Hoxa3*, *Hoxb3*, and *Hoxd3* reveal more severe phenotypes than single mutations of any one of the genes, suggesting they act in combination (Capecchi, 1997). These experiments and others provide evidence that *Hox* genes are key regulators of antero-posterior regional identity in the axial skeleton, central nervous system, neural crest cells (Hunt et al., 1991, Guthrie et al., 1992), the developing limb (Dollé, et al., 1993, Small and Potter, 1993) and the heart (Patterson et al., 1998) as well as participating in hematopoiesis (reviewed in Thorsteindottir et al., 1997b), constituting a "code" which determines positional identity .

### *1.3.4 Orphan genes*

Scattered throughout the genome are many homeobox genes termed dispersed or orphan homeobox genes, which contain homeodomains divergent from the *Antp* class. More than 30 orphan homeobox genes have been identified in the mouse (Kappen et al., 1993). Unlike the *Hox* clusters, there is no apparent relationship between genomic localization and expression or function. Orphan genes are often expressed in spatially and temporally restricted patterns in the developing embryo. For example, *Hesx1* is restricted to the anterior visceral endoderm and forebrain (Thomas and Beddington, 1996) and *Hox-11* is expressed in tightly defined regions of the branchial arches, hindbrain and the developing spleen (Roberts et al., 1994). A number of orphan homeobox genes are expressed in the forebrain, midbrain, and rhombomere 1 of the hindbrain, regions where the *Hox* genes are not expressed, including *En*, *Otx*, *Emx*, *Dlx*, and *Pax* family members (reviewed in Rubenstein and Puelles, 1994; Rubenstein and Shimaura, 1997). The functions of orphan homeobox genes are less

well defined than for *Hox* genes, although some appear to be critical for the establishment of pattern formation in the anterior brain and limbs, such as *Otx2*, *Emx2*, and *En1* (Acampora et al., 1995; Pellegrini et al., 1996; Wurst et al., 1994), while others, such as *Hox-11* and *Nkx2.5*, appear to be involved in organogenesis, although this may also be the result of antero-posterior patterning (Roberts et al., 1994; Lyons et al., 1995).

## 1.4 Isolation and manipulation of pluripotent cells in vitro

### *1.4.1 Embryonic stem cells*

An important experimental feature of the mouse is the ability to isolate and maintain pluripotent cells in culture. These cells, termed embryonic stem (ES) cells, are derived from the ICM of pre-implantation blastocysts and can be maintained in culture in an undifferentiated state when grown in the presence of the cytokine leukemia inhibitory factor (LIF) or other gp130 agonists (Evans and Kaufman, 1981; Martin, 1981; Gearing et al., 1987). Even after prolonged periods in culture, ES cells are able to contribute differentiated progeny to all tissues, including the germline of the mouse, upon reintroduction into the developing embryo (Bradley et al., 1984; Robertson, 1987). ES cells can be induced to differentiate in vitro by a number of methods, including withdrawal of LIF or exposure to chemical inducers such as retinoic acid or 3-methoxybenzamide (Smith and Hooper, 1987; Heath and Smith, 1988; Smith, 1991). In addition, ES cells carrying genetic alterations can be used to study gene function both in vitro, in models of differentiation, and in vivo by reintroducing altered ES cells to the germline.

### *1.4.2 Genetic alterations in ES cells and the creation of mutant animals*

Genetically altered ES cells have been used to produce mice with either gain of function or loss of function mutations (reviewed in Capecchi, 1989). Loss of function mutants are created through the technique of gene targeting. This technique, which allows the precise manipulation of a specific locus, relies on the infrequent phenomenon of homologous recombination between introduced DNA fragments and their genomic target site (Smithies et al., 1985). In conventional gene targeting, vectors are designed in which homologous regions of the genomic sequence are interrupted by a mutation which will prevent transcription or translation. The mutation generally includes a selectable marker gene such as $Neo^r$ which allows for the selection of cells incorporating the vector. ES cell clones isolated by selection are screened for the desired mutation and correctly targeted clones are then microinjected into a host blastocyst strain which carries a different coat color marker, enabling easy identification of chimeras. These chimeras are bred and heterozygote offspring

carrying the mutation are then crossed to produce homozygous null mutants which can be analyzed for phenotypic alterations. A large number of loss of function mutant mice have now been produced using gene targeting in ES cells (Brandon et al., 1995 a, b, c), and roles have assigned to many genes based on the mutant phenotype.

A number of refinements to gene targeting methods have broadened its applications. Subtle alterations of small regions of DNA, including single base pair substitutions can be made by several methods using positive-negative selection. These methods involve the introduction of a selection cassette into the region of interest (positive selection) and then, in a second round of targeting, a the replacement of the selection cassette with a construct containing the subtle alteration. Negative selection is used to identify cells in which this replacement has occurred (reviewed in Shearwin-Whyatt et al., 1999). Another refinement is conditional gene targeting in which the site and/or timing of the mutation is controlled. Conditional gene targeting methods (eg. the *Cre/lox* system) generally employ yeast or bacteriophage DNA recombinases which catalyze recombination between specific sequences. These sequences are engineered into the targeting vector such that they flank the region to be deleted. ES cells carrying this alteration are used to create homozygous mice which can then be crossed with mice expressing the appropriate recombinase at a specific time or site, resulting in recombination between the flanking sites and deletion of the intervening sequence only in the cells expressing the recombinase. This technique is particularly useful for studying gene function in cases where the null mutation is lethal to the embryo (reviewed in Shearwin-Whyatt et al., 1999).

### *1.4.3 In vitro differentiation of ES cells*

Spontaneous differentiation of ES cells, achieved by withdrawal of LIF, leads to the formation of a range of poorly defined terminally differentiated cell types. A remanent population of ES cells is probably maintained via feedback expression of LIF by differentiated cell types (Rathjen et al., 1990). More homogeneous differentiation of ES cells can be achieved with the chemical inducers retinoic acid (Smith and Hooper, 1987; Heath and Smith, 1988) and 3-methoxybenzamide (MBA) (Smith, 1991), but the developmental relevance of this differentiation is unclear.

ES cells can also be differentiated by growth in suspension cultures in the absence of LIF. The ES cells aggregate to form spheres known as embryoid bodies which share similarities with the early murine blastocyst (Robertson, 1987). After two to four days of culture the bodies consist of a central mass of undifferentiated cells displaying gene expression pattern similar to that of the ICM, and an outer layer of endoderm similar to the primitive endoderm (Doetschman et al., 1985; Shen and Leder, 1992). Culture for longer periods of time results in formation of cystic embryoid bodies which contain

one or more internal cavities and an ectoderm-like layer with similarities to the proamniotic cavity and primitive ectoderm respectively (Doetschman et al., 1985; Shen and Leder, 1992). This is surrounded by an outer layer comprising visceral or parietal endoderm. With further culture, a variety of tissues form in the embryoid bodies including neurons, muscle, blood and cartilage (Evans and Kaufman, 1981; Martin, 1981; Doetschman et al., 1985). Gene expression and cell type analysis demonstrate that formation of these cell types represents the normal events of embryogenesis and thus is an in vitro model of the progressive stages of embryonic development.

## 1.5 The *Gbx2* homeobox gene

### *1.5.1* Gbx2 *isolation and structure*

A partial length cDNA fragment of the *Gbx2* (Gastrulation and Brain specific Homeobox 2) gene was first isolated by RT-PCR of 13.5 d.p.c. telencephalon (Murtha, et al., 1991, originally termed MmoxA) along with the other member of the GBX family, *Gbx1* (MmoxB). *Gbx1* and *Gbx2* are orphan homeobox genes and have been localized to mouse chromosomes 5 and 1, respectively (Frohman, et al., 1993, Chapman, et al., 1997). Full length *Gbx2* cDNA clones have been isolated from embryonic stem (ES) cells (Chapman and Rathjen, 1995) and embryonal carcinoma (EC) cells (Bouillet et al., 1995). *Gbx1* and *Gbx2* exhibit 96% homology over the homeodomain, differing by only 2 amino acids (Fig. 1.9a), while outside the homeodomain there are additional differences (Chapman and Rathjen, 1995). Sequences related to *Gbx1* and *Gbx2* have been found in other species including human (Lin et al., 1996), chicken (Fainsod and Gruenbaum, 1989, Kowenz-Leutz et al, 1997), *Xenopus* (von Bubnoff et al., 1995), and *Drosophila* (Chiang et al., 1995). These sequences show a strong conservation across the homeodomain (Fig. 1.9a).

The *Gbx2* cDNA encodes a protein predicted to be 348 amino acids in length. It contains a conserved, 60 amino acid proline-rich region near the 5' end, similar to other homeobox proteins such as *Hoxd-4*, *Oct3*, *Dbx2*, *Gsh2*, and *S8* (Chapman et al., 1997). Proline-rich regions have been shown in some cases to have structural roles, such as the hydroxyproline rich extracellular matrix protein and collagen protein, or they can be involved in protein-protein interactions, such as the RNA polymerase II complex and SH3 domain binding proteins (reviewed in Williamson, 1994). They have also been shown to act as transcriptional regulators, presumably by binding to proteins of the initiation complex (Mermod et al., 1989; Williams and Tijan, 1991). *Gbx2* related sequences share a conserved, slightly hydrophobic region near the C-terminus (Chapman et al., 1997), while there tends to be a divergence in sequence in the region between position 70 and the homeodomain at position 247 (Fig. 1.9b).

a.

```
mGbx1   SRRRRTAFTSEQLLELEKEFHCKKYLSLTERSQIAHALKLSEVQVKIWFQNRRAKWKRIK
hGBX1   ------------------------------------------------------------
cGBX1   ------------------------------------------------------------

unp     ----------------R---A----------TS--------------------V-

mGbx2   N-----------------------------------------------------------V-
hGBX2   N-----------------------------------------------------------V-
cGBX2   N-----------------------------------------------------------V-
xgbx2   N-------------------------------V---------------------------V-
```

b.

```
mGbx2   MSAAFPPSLMMMQRPLGSSTAFSIDSLIGSPPQPSPGHFVYTGYPMFMPYRPVVLPPPPPPPPALPQAAL   70
hGBX2   MSAAFPPSLMMMQRPLGSSTAFSIDSLIGSPPQPSPGHFVYTGYPMFMPYRPVVLPPPPPPPPALPQAAL
cGBX2   MSAAFQPSLMMMQRPLGSSTAFSIDSLIGSPPPPAPGHFVYTGYPMFMPYRPVVLPPPPP---ALPQAAL
xgbx2   MSAAFQPPLMMMQRPLGSSTAFSIDSLIGNPPQPSPGHFVYTGYPMFMPYRPVVLPPPPPPPPSLSQATL

mGbx2   QPALPPAHPHHQIPSLPTGFCSSLAQGMALTSTLMATLPGGFSASPQHQEAAAARKFAPQPLPGGGNFDK  140
hGBX2   QPACRPHTLTTRSPACPQASAPAWRRAWRSPLRSWPRSPAASPRRPSTRRRQRPASSRRSRCPAAVTSTR
cGBX2   QPPLPPAPPPPL-PALPGAFCPGLAQGMALTSTLMAALPGSFPASPPRPEAA--RKFAP-----PGNFDK
xgbx2   QSTLSSAHHHHPIPSLPGGFCSSLAQGMALTSTLMATLPGGFSASTQHQEAA--RKFGAQSLHGA--FEK

mGbx2   AEALQA-DAEDGKAFLAKEGS-LLAFSAAEAVQASLVGAVRGQGKDESKVEDDPKGKEESFSLESDVDYS  208
hGBX2   RRRCRL-TRRTAKASWPKRAR-CSPSPRPRRCRLRSSGLSEGKGKTSQRWKTTRS-KEESFSLESDVDYS
cGBX2   ADGLPPPDGGGGGGDDGKTGGLLPFPAADAVHASLAGALRGGPKDDPKAEEEAKGREENFSMDSDLDYS
xgbx2   SDGSQSDGEEGNKTYTTKEGT-LLPFSASE---ASL-GPVRGQGKEESGKEAEGKGKEDSYLMDSDLDYS

mGbx2   SDDNLPGQTAHKEEDPGHALEETPQ-SGG-AAGSTTSTGKNRRRRTAFTSEQLLELEKEFHCKKYLSLTE  276
hGBX2   SDDNLTGQAAHKEEDPGHALEETPP-SSG-AAGSTTSTGKNRRRRTAFTSEQLLELEKEFHCKKYLSLTE
cGBX2   SDENGPAPAAPREEDCGTALEENPP-SAANAAANAAATGKNRRRRTAFTSEQLLELEKEFHCKKYLSLTE
xgbx2   SDDNISCQTAHKEED--TP-EESPQNSNPSNNSNTSSTGKNRRRRTAFTSEQLLELEKEFHCKKYLSLTE

mGbx2   RSQIAHALKLSEVQVKIWFQNRRAKWKRVKAGNANSKTGEPSRNPKIVVPIPVHVSRFAIRSQHQQLEQARP  348
hGBX2   RSQIAHALKLSEVQVKIWFQNRRAKWKRVKAGNANSKTGEPSRNPKIVVPIPVHVSRFAIRSQHQQLEQARP
cGBX2   RSQIAHALKLSEVQVKIWFQNRRAKWKRVKAGNASSKAGEPSRNPKIVVPIPVHVSRFAIRSQHQQLEQARP
xgbx2   RSQIAHVLKLSEVQVKIWFQNRRAKWKRVKAGNTNSKTGEPSRNPKIVVPIPVHVNRFAIRSQHQQLEQARP
```

**Figure 1.9 GBX genes in multiple species**

**a. *Gbx1* and *Gbx2* homeodomain sequences**

Alignment of the amino acid sequence of GBX homeodomain regions from mouse (**mGbx1**, **mGbx2**), human (**hGBX1**, **hGBX2**), chicken (**cGBX1**, **cGBX2**), *Xenopus* (**Xgbx2**),and *Drosophila* (**unp**). Dashes indicate conserved sequences.

**b. *Gbx2* full length amino acid sequences**

Alignment of the amino acid sequence of *Gbx2* genes from mouse (**mGbx2**), human (**hGBX2**), chicken (**cGBX2**), and *Xenopus* (**Xgbx2**). The proline-rich region (31-87), homeodomain (259-306), and c-terminal conserved region (322-344) are shaded.

Genomic DNA digested with a variety of restriction enzymes, southern blotted, and probed with a *Gbx2* specific probe revealed a single band, suggesting the presence of a single copy of the gene (Chapman et al., 1997).

### *1.5.2* Gbx2 *expression in vitro*

*Gbx2* expression has been analyzed by ribonuclease protection assays in undifferentiated and differentiated ES cells and embryoid bodies. A time course study of *Gbx2* expression in differentiating ES cells following exposure to retinoic acid demonstrated expression which increased between 0 and 48 hours (Bouillet et al., 1995). Differentiation of ES cells for 6 days in the presence of either retinoic acid, dimethylsulfoxide, or methoxybenzamide showed a decrease in *Gbx2* expression as compared to undifferentiated ES cells (Chapman et al., 1997). These observations could represent differences in the experimental protocols or indicate the existence of a transient subpopulation of cells which up regulates the gene as the cells progress toward differentiation. Embryoid bodies formed by the culture of ES cells in suspension in the absence of LIF were collected for 12 days and analyzed for *Gbx2* expression. *Gbx2* expression was observed in embryoid bodies after 1 day in culture, consistent with its expression in ES cells, but it was then down regulated from day 2 until day 5. *Gbx2* was re-expressed from day 5 through day 12, although not at the high levels seen in undifferentiated ES cells (Chapman et al., 1997).

### *1.5.3* Gbx2 *expression in vivo*

Embryonic and adult tissues examined by ribonuclease protection analysis demonstrated *Gbx2* expression at high levels in the brain and at low levels in the spleen and female genital tract. Expression was not detected in heart, liver, lung, kidney, testes, or placenta (Bouillet et al., 1995; Chapman et al., 1997).

In situ hybridization analysis of early embryos (Fig. 1.10) detected *Gbx2* expression in the pluripotent cells of the ICM, but not in mural trophectoderm cells at 4.0 d.p.c. (Chapman et al., 1997). Expression is down regulated and not detected in the primitive ectoderm or other cells of the egg cylinder stage embryo (6.0 d.p.c.) (Chapman, 1994; P.Q. Thomas and R. Beddington, unpublished). *Gbx2* is re-expressed in the primitive streak at 6.5 d.p.c. (P.Q.Thomas and R. Beddington, unpublished; Chapman, 1994). Sectioning and in situ hybridization of a 7.5 d.p.c. embryo showed expression in all 3 germ layers of the primitive streak (Bouillet et al., 1995; Wassarman et al., 1997). By 8.5 d.p.c. *Gbx2* expression is restricted to the open neural tube, in the hindbrain with strong expression at the midbrain/hindbrain boundary, and in the pharyngeal pouch region. At 11.5 d.p.c. and beyond *Gbx2* expression is restricted to the inner ear, the CNS and in the brain in the presumptive dorsal thalamus, the mantle part of the basal striatum, the cerebellum, and the anterior

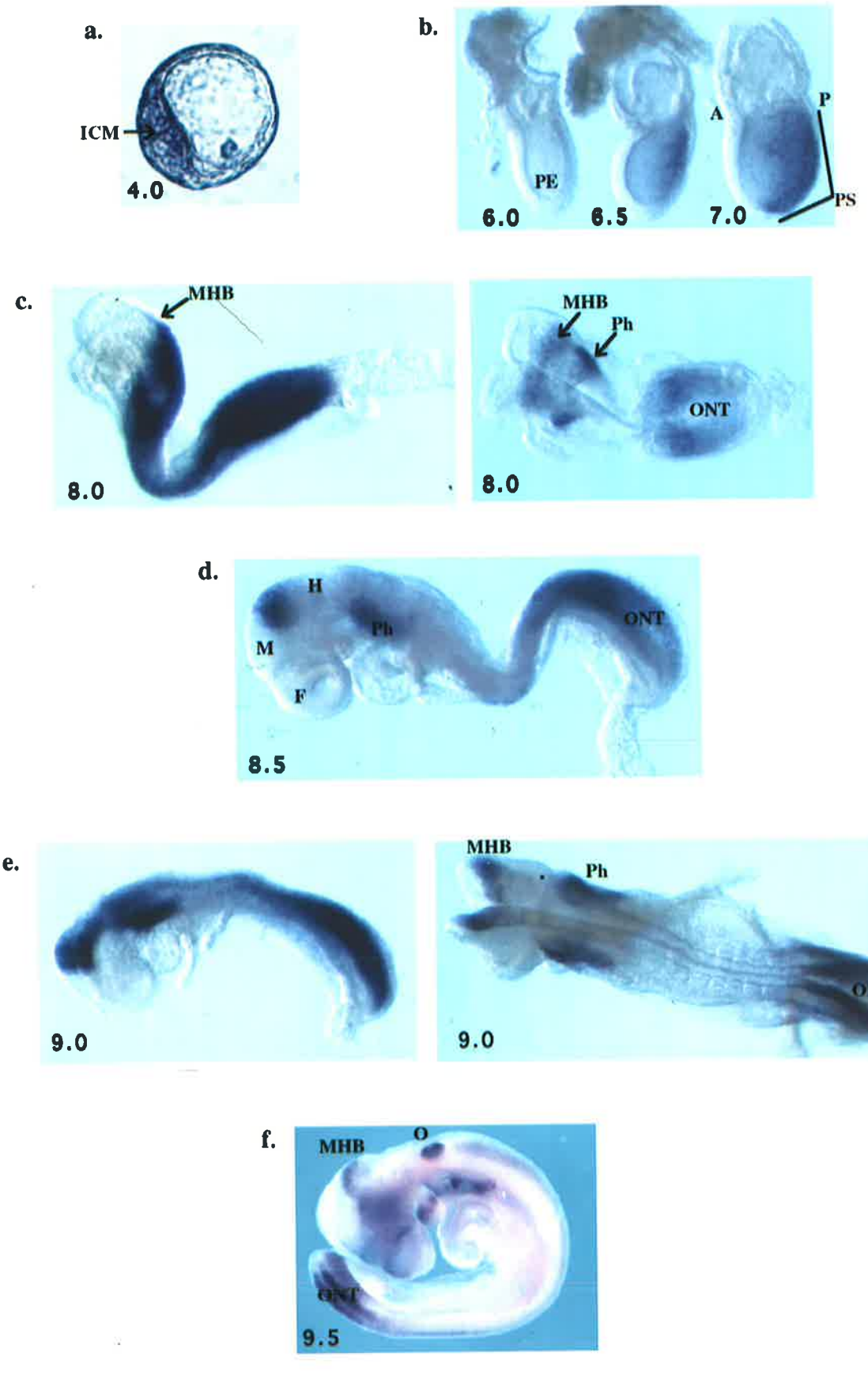
**Figure 1.10 *Gbx2* expression in vivo**

Whole mount in situ hybridization using a *Gbx2* antisense digoxigenin labelled probe of a) 4.0 d.p.c., b) 6.0 d.p.c., c) 8.0 d.p.c., d) 8.5 d.p.c., e) 9.0 d.p.c., and f) 9.5 d.p.c. embryos.

Abreviations:
A = Anterior
ICM = Inner cell mass
MHB = Midbrain/hindbrain boundary
O = Otocyst (inner ear)
ONT = Open neural tube
P = Posterior
PE = Primitive ectoderm
Ph = Pharyngeal pouch
PS = Primitive streak

Photomicrographs courtesy of T. Schultz (a) and P.Q. Thomas and R. Beddington (b-f).

**Figure 1.10**


a.
ICM
4.0
b.
PE
A
P
PS
6.0
6.5
7.0
c.
MHB
8.0
MHB
Ph
ONT
8.0
d.
H
Ph
ONT
M
F
8.5
e.
9.0
MHB
Ph
ONT
9.0
f.
MHB
O
ONT
9.5

hindbrain (Bouillet et al., 1995). This expression pattern suggests three possible stages during which *Gbx2* might play a role in either specifying cellular function or in embryonic patterning: in the pluripotent cells of the preimplantation embryo, during gastrulation in the primitive streak, and during neurulation in the developing neurectoderm and anterior hindbrain.

*Gbx2* expression patterns are only somewhat conserved across species. In *Xenopus*, *Xgbx2* is expressed during gastrulation in the dorsolateral ectoderm, then in neurectoderm and surrounding epidermis with a sharp anterior boundary at the prospective midbrain-hindbrain boundary. Expression is also seen at later stages in the forebrain, visceral arches, and otic vesicle (von Bubnoff et al., 1995). In the chicken, *GBX2* is expressed during gastrulation in the central epiblast outside the primitive streak and Henson's node, then during primitive streak regression in the presumptive neural plate and subsequently in the neural tube with an anterior limit at the midbrain hindbrain boundary. Expression is also detected in the forebrain at later stages, the foregut, branchial and pharyngeal arches, and the otic vesicle (Niss and Leutz, 1998, Shamim and Mason, 1998). In the adult, *GBX2* transcripts have been detected in the brain, bone marrow, spleen, and bursa of fabricius (Kowenz-Leutz et al., 1997). The *Drosophila* homologue, *unplugged*, is expressed in the midline of the CNS in a subset of neuroblasts which generally correspond to *engrailed* expressing cells in the CNS. Expression is also seen outside the CNS in two clusters of ectodermal cells in the labial and first thoracic segments. Later expression is limited to a few cells at the dorsal midline which go on to form the cerebral branches of the tracheal system (Chiang et al, 1995). *Unplugged* has been shown by mutational analysis to be required for development of the cerebral ganglionic branch, which is derived from the first thoracic segment (Chiang et al., 1995).

*Gbx2* expression patterns differ most dramatically during gastrulation in the frog, chicken and mouse, with expression in the frog limited to dorsolateral ectoderm, expression in the chicken limited to regions outside the primitive streak, while expression in the mouse is observed all 3 germ layers of the primitive streak, but not outside the streak. This could reflect differences in the mechanisms of gastrulation or the role of *Gbx2* among these species. In contrast, *Gbx2* expression patterns in the CNS appear similar, with broad expression in early neurectoderm later narrowed to the anterior hindbrain, suggesting a conserved function for *Gbx2* in patterning the anteroposterior neuraxis.

## 1.6 Aims and Approaches

Homeobox genes are known to have critical body and organ patterning functions in embryo development. They appear in temporally and spatially restricted patterns which generally correspond with the timing and site of gene function. *Gbx2* displays an intriguing expression pattern, appearing first in the ICM and then at gastrulation in all 3 germ layers of the primitive streak and finally in specific regions of the developing nervous system, including the significant midbrain/hindbrain boundary, suggesting possible roles for *Gbx2* in the control of pluripotent cell differentiation, gastrulation, or neuraxis formation and specification. The signals controlling proliferation and differentiation of pluripotent cells in early development are of great interest to this laboratory. In addition, gastrulation is still a poorly understood process and the possibility of characterizing the role of *Gbx2* at this developmental stage may lead to a greater understanding of the control mechanisms underlying this event. The midbrain-hindbrain boundary has been the focus of much research in recent years because of its function as a regional organizer. Determining the function of *Gbx2* in this region might increase the understanding of how this boundary is specified. The aim of this project was to investigate the function of *Gbx2* in murine embryonic development. This was to be achieved in two ways. Firstly, a loss of function mutation was to be created using homologous recombination in ES cells in order to investigate *Gbx2* function in vivo. These cells would be used to create mice bearing the mutation which could then be bred to homozygosity and analysed for phenotypic changes. Secondly, the function of *Gbx2* at the cellular level, notably in cell differentiation was investigated by comparative analysis of cellular events in cells in which both *Gbx2* alleles had been interrupted.

# Chapter 2

# Materials and Methods

# 2 Materials and Methods

## 2.1 Abbreviations

A..... amps
ATP..... adenosine triphosphate
BCIP..... 5-bromo-4-chloro-3-indolyl phosphate
B-Me..... ß-mercaptoethanol
bp..... base pair
Ci..... Curie
CIP..... calf intestinal phosphatase
c.p.m..... counts per minute
DAB..... diaminobenzidine
DMEM..... Dulbecco's Modified Eagle Medium
DMF..... dimethylformamide
DNA..... deoxyribonucleic acid
dNTP..... deoxynucleotide
d.p.c..... days post coitum
DTE..... dithioerythritol
DTT..... dithiothreitol
*E. coli*..... Escherichia coli
EDTA..... ethylene diamine tetra acetic acid
EGTA..... ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetra acetic acid
FCS..... fetal calf serum
FLB..... formamide loading buffer
g..... gram
GLB..... gel loading buffer
G418..... G418 sulphate
Hepes..... N-2-hydroxyethyl piperazine-N-ethane sulphonic acid
IMDM..... Iscove's Modified Dulbecco's Medium
IPTG..... isopropyl-β-D-thiogalactopyranoside
I.U..... international units
kb..... kilobase pairs
l..... liter
LB..... luria broth
M..... molar
M-CSF..... macrophage-colony stimulating factor
MBA..... 3-methoxybenzamide
MOPS..... 3-([N-Morpholino]propane sulphonic acid)
MQ $H_2O$..... reverse osmosis filtered water using a Milli-Q™ ion-exchange matrix
min..... minute
MTG..... monothioglycerol
NBT..... nitro blue tetrazolium
O.D..... optical density
O/N..... overnight
PBS..... phosphate buffered saline
PFA..... paraformaldehyde
PCR..... polymerase chain reaction
PEG..... polyethylene glycol
RA..... retinoic acid
RNA..... ribonucleic acid
RNase..... ribonuclease
r.p.m..... revolutions per minute

RT.....room temperature
SDS.....sodium dodecyl sulphate
sec.....second
TE.....Tris-EDTA
TEMED.....N, N, N', N'-teramethyl-ethenediamine
6-TG.....6-thioguanine
u.....units
UTP.....uridine triphosphate
UV.....ultra violet
v.....volume
V.....volts
w.....weight
X-gal.....5-bromo-4-chloro-3-indol-β-D-galactopyranoside

## 2.2 Materials

### *2.2.1 General chemicals and reagents*

All chemicals and reagents used were of analytical grade and important reagents were obtained from the following suppliers:

acetyl Co-A.....Sigma Chemical Co.
agarose.....Sigma Chemical Co.
ampicillin.....Sigma Chemical Co.
ATP.....Boehringer Mannheim Biochemicals
bacto-agar.....Difco Labs Ltd.
bacto-tryptone.....Difco Labs Ltd.
BCIP.....Sigma Chemical Co.
BES.....Calbiochem
bromophenol blue.....Sigma Chemical Co.
chloroform.....BDH Chemicals Ltd.
deoxyribonucleotide triphosphate solutions.....Boehringer Mannheim Biochemicals
DIG RNA Labelling mix.....Boehringer Mannheim Biochemicals
DAB.....Sigma Chemical Co.
DTE.....Sigma Chemical Co.
DTT.....Boehringer Mannheim Biochemicals
ethidium bromide.....Sigma Chemical Co.
IPTG.....Boehringer Mannheim Biochemicals
magnesium acetate.....Sigma Chemical Co.
NBT.....Diagnostic Chemicals Ltd.
ribonucleotide triphosphate solutions.....Boehringer Mannheim Biochemicals
salmon sperm DNA.....Sigma Chemical Co.
Sepharose CL6B.....Pharmacia LKB Biotechnology Inc.
Sequagel-6.....National Diagnostics
Sodium Deoxycholic acid.....Sigma Chemical Co.
spermidine.....Sigma Chemical Co.
Synperonic OP 8.....BDH Lab Supplies
Transcription buffer.....Boehringer Mannheim Biochemicals
tRNA (yeast).....Boehringer Mannheim Biochemicals
Tween 20.....Sigma Chemical Co.
X-gal.....Scimar
yeast extract.....Difco Labs. Ltd.

### 2.2.2 *Tissue culture chemicals and reagents*

ascorbic acid..... Sigma Chemical Co.
CIP buffer ..... Boehringer Mannheim Biochemicals
colcemid..... Sigma Chemical Co.
DMEM..... Gibco BRL
DMSO..... BDH Chemicals Ltd.
FCS..... Commonwealth Serum Labortaories
G418 sulphate..... Gibco BRL
gelatin ..... Sigma Chemical Co.
gentamycin..... Schering Corporation
holo-transferrin..... Sigma Chemical Co.
L-glutamine..... Sigma Chemical Co.
IMDM..... Sigma Chemical Co.
M-CSF..... R & D Systems
methyl cellulose ..... Fluka
β-mercaptoethanol..... Sigma Chemical Co.
mouse IL-3..... gift of T.Gonda, Hanson Center for Cancer Research
MTG..... Sigma Chemical Co.
β-nerve growth factor..... ICN Pharmaceuticals
retinoic acid ..... Sigma Chemical Co.
trypsin..... Difco

### 2.2.3 *Enzymes*

Enzymes were obtained from the following suppliers :

AMV reverse transcriptase ..... Molecular Genetic Resources
CIP..... Boehringer Mannheim Biochemicals
DNase I (RNase free) ..... Boehringer Mannheim Biochemicals
*E. coli* DNA polymerase KF-1 ..... Bresatec Ltd.
proteinase K..... Boehringer Mannheim Biochemicals
restriction endonucleases..... Bresatec Ltd. and New England Biolabs
RNase A ..... Sigma Chemical Co.
RNase T1 ..... Boehringer Mannheim Biochemicals
RNase Inhibitor..... Bresatec Ltd.
SP-6 RNA polymerase..... Bresatec Ltd.
Taq polymerase..... Bresatec Ltd.
T4 DNA ligase ..... Bresatec Ltd.
T7 DNA polymerase..... Pharmacia LKB Biotechnology Inc.
T3 RNA polymerase..... Boehringer Mannheim Biochemicals
T7 RNA polymerase..... Boehringer Mannheim Biochemicals
T4 polynucleotide kinase..... Bresatec Ltd.

### 2.2.4 *Nucleic acid molecular weight standards*

Hpa I digested pUC19 markers were obtained from Bresatec Ltd..Band sizes (bp): 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 26.

Eco RI digested SPP-1 markers were obtained from Bresatec Ltd.. Band sizes (kb): 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, 0.36.

### 2.2.5 *Radionucleotides*

[alpha-$^{32}$P] dATP, [alpha-$^{33}$P] dATP, and [alpha-$^{32}$P] rUTP (3000 Ci/mmol) were supplied by Bresatec Ltd.

### 2.2.6 *Kits*

| | |
|---|---|
| BresaClean | Bresatec Ltd. |
| BTQ kit | Bresatec Ltd. |
| Giemsa staining kit | Sigma Chemical Co. |
| Gigaprime kit | Bresatec Ltd. |
| Megaprime kit | Amersham Corp. |
| T7 Sequenase kit | Pharmacia LKB Biotechnology Inc. |
| Super Z Applicator kit | Helena Laboratories |

### 2.2.7 *Miscellaneous materials*

| | |
|---|---|
| bacterial grade 35mm petri dishes | Crown Scientific |
| bacterial grade 100mm petri dishes | Technoplast |
| 3MM chromatography paper | Whatman Inc. |
| electroporation cuvettes (Gene pulser 4mm) | Biorad |
| filter (0.2 µM) | Whatman Inc. |
| freezing vials | Nunc Inc. |
| Hybond-N$^+$ nylon membrane (0.45 µM) | Amersham Corporation |
| Nytran nylon membrane (0.45 µM) | Schleicher and Schuell |
| phosphorimager screens | Molecular Dynamics |
| tissue culture grade plasticware | Falcon |
| X-ray film (Biomax) | Kodak |

### 2.2.8 *Plasmid vectors*

Cloning and expression vectors were obtained from the following:

| | |
|---|---|
| pBluescript SK+ | Stratagene |
| pGemT | Promega Corp. |
| pGemT EZ | Promega Corp. |
| pCH110 | Pharmacia LKB Biotechnology Inc. |

Recombinant DNA Clones were obtained from the following:

| | |
|---|---|
| pPGKhygro/deltaLT20 (Gassmann et al, 1995) | Gavin Chapman |
| pMGD20neo (Gassmann et al, 1995) | Gavin Chapman |
| λ5.1 (Chapman, et al, 1997) | Gavin Chapman |
| mMoxc7.1 (Chapman and Rathjen, 1995) | Gavin Chapman |
| pMC1TK2 (Mario Capecchi) | Dr. Linda Shearwin-Whyatt |
| mGAP (Rathjen et al., 1990) | Gavin Chapman |
| pR1X | Dr. Joy Rathjen |

Gavin Chapman Dr. Joy Rathjen are currently at the Department of Biochemistry, University of Adelaide, Adelaide, Australia. Linda Shearwin-Whyatt, formerly at the Department of Biochemistry, University of Adelaide, is currently at the Hanson Centre for Cancer Research, Adelaide, Australia.

### *2.2.9 Antibodies*

rabbit anti-rat polyclonal ..... Dako Corporation
goat anti-rabbit-HP conjugated..... Sigma Corporation
anti-digoxigenin-AP Fab fragments ..... Boeringer Mannheim Corp.

### *2.2.10 Antibodies and cytokines not obtained from commercial sources*

recombinant murine IL-3 ..... Dr. T. Gonda
F4/80 (Austin and Gordon, 1981)..... Dr. T. Gonda

Dr. T. Gonda is currently at the Hanson Centre for Cancer Research, Adelaide, Australia.

### *2.2.11 Oligonucleotides*

DNA oligonucleotides were synthesized by Bresatec Ltd. All primer sequences are 5'-3'.

Sequencing Primers:

Intron:

| | |
|---|---|
| Pr-3 (c780): | TTC GCT CCA CAG CCA CT |
| Pr-5 (c1057): | CGG GGT CTT CTT CCT TA |
| Pr-6 (g3600): | CCC AGC AGA TTG TCT TT |
| Pr-7 (g2800): | CTT TCC AAT GGG GCA TT |
| Pr-9 | CAT CTG GTG GCG TGT TT |
| Pr-10 | GTG AAA TTG AGG CTC AC |
| Pr-11 | TGA GGC CCA AGA GAA TA |
| Pr-12 | AAA CCA GCC CAA TAA GG |
| Pr-14 | CTG TTC ATC CAA GAG AA |

5' region:

| | |
|---|---|
| Pr-2 (c174): | GCC GGG GAC CTC GCG TT |
| Pr-8 (g1700): | CGC TCC TCT TTC TCA TT |
| Pr-15 | ACT TAA GAG GGA CCC TT |
| Pr-16 | CCG TGT CGT GTG AGC AA |
| Pr-17 | GAC TCT AGG CTC TGA GT |

Primer Extension:

| | |
|---|---|
| PE-1: | AGT GCG TGC GTC CGT CCG TCT GTC C |

PCR amplification of p464 fragment:

| | |
|---|---|
| PC-1: | AGG GTT CTG CTG TAG TA |

PCR amplification of pG290 fragment:

Pr-3 (c780): TTC GCT CCA CAG CCA CT

Pr-5 (c1057): CGG GGT CTT CTT CCT TA

### *2.2.12 Bacterial strains*

The *E. coli* bacterial strain DH5alpha, obtained from Dr. J. Dibbens and stored as glycerol stocks was used as the host for all recombinant plasmids.

DH5alpha: *sup*E44 delta*lac* U169 (phi80 *lac*ZdeltaM15) *hsd*R17 *rec*A1 *end*A1 *gyrr*A96 *thi*-1*rel*A1

### *2.2.13 Bacterial growth media*

Growth media were prepared in double distilled water and sterilised by autoclaving. Antibiotics and other labile chemicals were added after the media solution had cooled to 50°C.

FTB:

glycerol .....0.4% (w/v)

bacto-tryptone.....1.2% (w/v)

yeast extract.....2.4% (w/v)

17 mM $KH_2PO_4$, 72 mM $K_2HPO_4$ added after cooling.

LB:

NaCl.....1% (w/v)

bacto-tryptone.....1% (w/v)

yeast extract.....0.5% (w/v)

The pH was adjusted to 7.0 with NaOH.

Solid Media: Agar plates were prepared by supplementing Luria broth with 1.5% Bacto-agar.

Ampicillin bacterial selection was achieved using 100 µg/ml ampicillin.

## 2.3 Recombinant DNA techniques

### *2.3.1 Restriction endonuclease digestions*

Plasmid DNA suspended in water was digested with 1-2 units of restriction enzyme per µg of DNA at 37°C for 1-24 hours in the presence of 0.1 volume of the appropriate New England Biolabs buffer or Super Duper buffer.

10X Super Duper Buffer:

Tris-HCl (pH 7.8)..... 300 mM

| | | |
|---|---|---|
| KAc | 625 | mM |
| MgAc | 100 | mM |
| Spermidine | 40 | mM |
| DTE | 5 | mM |

### *2.3.2 Endfill reaction*

Up to 10 µg of DNA with 5' overhangs, to be used in blunt end ligations, was endfilled in the following reaction. DNA was heated to 70°C to dissociate sticky ends then combined wih the following and incubated at 37°C for 30 min.

| | | |
|---|---|---|
| dATP, dCTP, dGTP, dTTP | 0.5 | mM |
| 10 x Endfill buffer | 0.1 | v |
| DTT | 100 | mM |
| DNA polymerase (KF-1) | 10 | u |

10 x Endfill Buffer:

| | | |
|---|---|---|
| Tris-HCl (pH 7.5) | 100 | mM |
| $MgCl_2$ | 100 | mM |
| NaCl | 100 | mM |

### *2.3.3 Endchew reaction*

Up to 5 µg of DNA with 3' overhangs to be used in blunt ligations, had 3' overhangs removed by exonuclease digestion in the following reaction allowed to proceed at 37°C for 15 minutes.

| | | |
|---|---|---|
| 10 x T4 Polymerase Buffer | 0.1 | v |
| dATP, dCTP, dGTP, dTTP | 0.5 | mM |
| T4 DNA polymerase. | 5 | u |

10 x T4 DNA polymerase buffer:

| | | |
|---|---|---|
| Tris-HCl (pH 7.4) | 700 | mM |
| $MgCl_2$ | 100 | mM |
| DTT | 50 | mM |

T4 DNA polymerase was inactivated by incubation at 70°C for 10 minutes prior to use of the DNA in ligation reactions.

### 2.3.4 CIP reaction

To the precipitated and dried product of restriction endonuclease digestions 1 u of CIP, 4.0 µl 10 x CIP buffer and water to a final volume of 40 µl were added and incubated at 37°C for 30 minutes.

### 2.3.5 Agarose gel electrophoresis

Horizontal agarose gels (0.8, 1.0, 2.0, or 3.0% agarose in TBE or TAE) submerged in TBE or TAE were used. DNA samples (containing 1 x GLB) were electrophoresed at 100 mA. Nucleic acid was visualised by staining gels with 5 µg/ml ethidium bromide and photographed under short wavelength UV light, unless it was a preparative gel, in which case long wavelenghth UV light was used. Appropriate bands were isolated from preparative gels using sterile scapel blades.

10 x GLB:

| | | |
|---|---|---|
| glycerol | 50% | (v/v) |
| SDS | 0.1% | (w/v) |
| bromophenyl blue | 0.05% | (w/v) |
| xylene cyanol | 0.05% | (w/v) |

TAE:

| | | |
|---|---|---|
| Tris-acetate | 40 | mM |
| Sodium acetate | 20 | mM |
| $Na_2EDTA$ | 1 | mM |

The pH of TAE was adjusted to 8.2.

TBE:

| | | |
|---|---|---|
| Tris-borate | 100 | mM |
| $Na_2EDTA$ | 2.5 | mM |

The pH of TBE was adjusted to 8.3.

### 2.3.6 Isolation of DNA from agarose gels

Appropriate DNA bands were excised from agarose gels using sterile scalpel blades and DNA was eluted with the BresaClean kit using the protocol of the supplier.

### 2.3.7 Ligation reactions

Vector (50 ng), insert DNA (3 fold excess), 1 x ligation buffer and 1 u T4 DNA ligase (cohesive end ligation) or 10 u T4 DNA ligase (blunt end ligation) were mixed in a volume of 20 µl. Reactions were allowed to proceed at 15°C for 1-16 hours.

10 x Ligation Buffer:

| | | |
|---|---|---|
| Tris-HCl (pH 7.5) | 500 | mM |
| $MgCl_2$ | 100 | mM |
| DTT | 100 | mM |
| rATP | 5 | mM |

### *2.3.8 Preparation of competent bacterial cells*

*E. coli* cells were streaked out on L plates and grown at 37°C overnight. A single colony was used to inoculate 5 ml Psi broth (grown with shaking, 37°C, O/N). A 1:30 subculture was made in 15 ml Psi broth (grown for 90 min.,with shaking, 37°C or until O.D. 0.6 was obtained). A 1:20 subculture was made in 100 ml Psi broth (grown for 90 min., with shaking, 37°C or until O.D. 0.5-0.6 was obtained). The bacterial cells were chilled on ice for five minutes and harvested by centrifugation (6000 r.p.m., 4°C, 5 min., SS34 rotor in Sorvall RC-5 centrifuge). After resuspension in 0.4 volume of Tfb 1 the cells were chilled on ice for a further 5 minutes. The cells were harvested by centrifugation (as above) and resuspended in a 0.04 volume of Tfb 2. After incubation on ice for 15 minutes 100 µl aliquots of cell suspension were transfered to eppendorf tubes and snap frozen in a dry ice/ethanol bath. The cells were stored at -80°C until required.

Psi broth:

| | | |
|---|---|---|
| Bacto yeast extract | 0.5% | (w/v) |
| Bacto tryptone | 2% | (w/v) |
| $MgSO_4$ | 0.5 % | (w/v) |

The pH was adjusted to 7.6 with KOH (1 M).

Tfb 1:

| | | |
|---|---|---|
| KAc | 30 | mM |
| RbCl | 100 | mM |
| $CaCl_2$ | 10 | mM |
| Glycerol | 15 % | (v/v) |

The pH was adjusted to 5.8 with Actetic Acid (0.2 M).

Tfb 2:

| | | |
|---|---|---|
| MOPS | 10 | mM |
| $CaCl_2$ | 75 | mM |
| RbCl | 10 | mM |
| glycerol | 15% | |

The pH was adjusted to 6.5 with KOH (1 M).

### *2.3.9 Transformation of competent bacterial cells*

Approximately 10 ng of circular, plasmid DNA or one half volume of ligation reactions was mixed with 50 µl of competent cells and left on ice for 30 minutes. The cells were heat shocked at 42°C for 2 minutes and the cells incubated in 1 ml of LB (with shaking, 37°C 60 min.). The cells were harvested by centrifugation (1400 r.p.m., 30 sec., in an Eppendorf 5415 microfuge), resuspended in 50 µl LB and spread on an L plate supplemented with 100 µg/ml ampicillin and 30 µg/ml chloramphenicol where required. Transformant colonies were grown at 37°C overnight . Where blue/white color selection was required plates were spread with 50 µl IPTG (50 µg/ml) and 20 µl X-gal (50 µg/ml) before transformant cells were plated.

### *2.3.10 Small scale isolation of plasmid DNA*

2 ml LB containing 100µg/ml ampicillin was inoculated with a single transformant colony and incubated shaking overnight at 37°C. 1.5 ml of this culture was transferred to an Eppendorf tube and spun for 1 min at 8000 x g. The cells were resuspended in 50 - 100µl of remaining supernatant, and lysed by the addition of 300µl TENS lysis buffer and briefly vortexing. 200µl 3M sodium acetate pH 5.2 was added, and again mixed by vortexing. The mixture was then spun at 15000 x g in a benchtop centrifuge for 5 min. and the supernatant transferred to a fresh tube, to which 1 ml of cold (-20°C) ethanol was added. This mixture was vortexed and spun at 15000 x g in a benchtop centrifuge for 3 min. and the pellet washed in 70% ethanol, dried, and resuspended in 20-22µl TE. Restriction analysis of 2-4µl of the resuspended DNA was performed in a 20 µl digest containg 1 x Super Duper Buffer, 5 U of the appropriate restriction enzyme(s) and 50 µg / ml RNase A.

TENS Lysis buffer:

| | | |
|---|---|---|
| Tris-HCl (pH 8) | 10 | mM |
| EDTA | 1.0 | mM |
| NaOH | 0.1 | mM |
| SDS | 0.5% | (v/v) |

### *2.3.11 Polymerase Chain Reaction technique*

Polymerase chain reactions were set up in a 20 µl volume in capillary tubes as follows. Circular plasmid template DNA (1 µl of either 20 or 100 ng/µl) was mixed in the capillary tube with the appropriate primers (5 µl each of 10 ng/µl), 1x reaction buffer (BTQ kit), 0.2mM dNTP mix, $MgCl_2$ (1µl of either 1mM or 2.5mM and 1.0 u Taq Polymerase.

Reactions were carried out in a FTS-1 Thermal Cycler (Corbett Research). A typical thermal profile for PCR was:

(1) ..... 94°C, 2 min.

(2-33) ..... 94°C, 10 sec.; 55°C, 30 sec.; 72°C , 60 sec.

(34) ..... 72°C, 5 min.; 25°C.

PCR products were visualised on 2.0 % TAE gels, bands cut out and cleaned using Bresaclean.

### *2.3.12 Non-denaturing polyacrylamide gel electrophoresis*

20% polyacrylamide gels were prepared from a 40% acrylamide stock (38:2 acrylamide:bis-acrylamide). To 0.75 ml 20 x TBE, 6.5 ml of acrylamide stock and 6.75 ml of water were added. 10% ammonium persulphate (250 µl) and 15 µl TEMED were added just prior to pouring between glass plates of the appropriate size separated by 1 mm spacers. Gels were allowed to set for 30 minutes and pre-electrophoresed at 400V in 1 x TBE for 15 min. DNA samples containing 1x GLB were electrophoresed at 400V.

### *2.3.13 Denaturing polyacrylamide gel electrophoresis*

6% polyacrylamide denaturing gels were prepared as follows. 10% ammonium persulphate (400 µl) and 20 µl TEMED were added to 40 ml. of Sequagel just prior to pouring between glass plates (20 X 40 cm) separated by 0.4 mm spacers. The gels were allowed to set for 30 minutes before removal of the comb. Gels were pre-electrophoresed in 1x TBE for 30 min at 1400V and the wells flushed with 1 x TBE before use. DNA in 1 x FLB was electrophoresed at 1300V (or 50°C).

10 x FLB:

| | | |
|---|---|---|
| formamide | 80% | (v/v) |
| EDTA | 10 | mM |
| bromophenyl blue | 1.0 | mg/ml |
| xylene cyanol | 1.0 | mg/ml |

### *2.3.14 Isolation of radiolabelled DNA or RNA fragments from polyacrylamide gels*

After electrophoresis, the glass plates were separated and the gel was covered with plastic wrap. The gel was exposed to X-ray film for 1-2 min. and the DNA or RNA band excised using a sterile scapel blade. DNA or RNA was eluted in gel elution buffer (450 µl, 37°C, O/N) or TE (400 µl, RT, O/N), and ethanol precipitated in the presence of tRNA (20 µg) or glycogen (40 µg).

Gel Elution Buffer:

| | | |
|---|---|---|
| SDS | 0.1% | (v/v) |
| Tris-HCL, pH 7.5 | 10 | mM |
| EDTA | 1 | mM |

*2.3.15 Denaturation of double stranded plasmid DNA for sequencing*

Double stranded plasmid DNA (5-6 µg) in a volume of 15 µl was treated with RNase A (1.5 µl, 10 mg/ml, 37°C, 15 min). The DNA was denatured by incubation in the presence of 3.5 µls of denaturing solution (1 M NaOH, 1mM EDTA, 37°C, 15 min). Denatured plasmid DNA was purified by centrifugation at 1800 r.p.m., on a Sepharose CL-6B column for 3 minutes.

*2.3.16 Sequencing of double stranded plasmid DNA*

Dideoxy sequencing of denatured DNA was carried out using the Pharmacia LKB Biotechnology Inc. T7 polymerase seqencing kit using the protocol of the supplier. The reaction products were separated on 6% denaturing polyacrylamide gels (section 2.3.13). The gels were transferred to dry 3MM filter paper before drying 65°C in vacuo. Radioactivity was detected by exposure to X-ray film at -80°C with intensifying screens, or by exposure to phosphorimager screens.

*2.3.17 Large scale isolation of pure plasmid DNA*

Ultra pure plasmid DNA was prepared by the following method for DNA manipulation and transfection of embryonic stem cells.

FTB (500 ml, 100 µg/ml ampicillin) was inoculated with a single transformant colony and incubated overnight with shaking at 37°C. The cells were harvested by centrifugation (7000 r.p.m., 4°C, 5 minutes, GSA rotor in a Beckman J-21 centrifuge), and the pellet resuspended in 13 ml GTE. To each half of the resulting suspension, 13 ml of lysis solution (0.2 M NaOH/ 1% SDS), was added and swirled on ice for 10 minutes. The lysis suspension was neutralized by the addition of 6.5 ml 3M NaAc pH 4.6. After 5 more minutes on ice, the cell debris was pelleted by centrifugation (12000 r.p.m., 4°C, 10 minutes, SS34 rotor in a Sorvall RC5 centrifuge). The supernatant was retained and nucleic acid precipitated by the addition of 0.6 v of cold iso-propanol. After mixing the nucleic acid was pelleted by centrifugation (10000 r.p.m., 4°C, 10 minutes, SS34 rotor as above). The pellet was drained well and resuspended in 7 ml TE, 7 g CsCl and 400 µl ethidium bromide (10 mg/ml). Debris was removed by centrifugation (3000 r.p.m., 4°C, 10 minutes, SS34 rotor in a Sorvall RC5 centrifuge). The supernatant was transferred to an unused Oakridge tube (polycarbonate, 10 ml) and the tubes balanced with paraffin. A cesium chloride density gradient was formed by centrifugation (45000 r.p.m., 19 hours, 20°C, Ti50 rotor in a Beckman L5-50 ultrcentrifuge). Approximately 1 ml of

circular, plasmid DNA was harvested, under long wavelength UV light into an equal volume of iso-propanol (equilibrated against NaCl saturated TE). The DNA was extracted with equal volumes of iso-propanol until the ethidium bromide was removed. The DNA was diluted 1 in 4 with MQ $H_20$ and ethanol precipitated (0.1 v NaAc, 2.5 v ethanol, -20°C, O/N in a glass Corex tube). The DNA was pelleted by centrifugation (9000 r.p.m., 4°C, 15 min. SS34 rotor, with rubber sleeves) and dried. The DNA was finally resuspended in 400 µl MQ $H_20$ and transferred to an eppendorf tube. The DNA was ethanol precipitated again, pelleted (14000 x g, 15 min, Eppendorf centrifuge 5415C) and resuspended in 400 µl MQ $H_20$. The concentration of plasmid DNA was determined by spectrophotometric analysis (O.D. 260 nm).

GTE:

| | | |
|---|---|---|
| Glucose | 50 | mM |
| Tris-HCl (pH 7.6) | 25 | mM |
| EDTA | 10 | mM |

### *2.3.18 Small scale preparation of high molecular weight DNA from ES cells*

To ES cells grown in 24-well plates 300 µl of lysis solution was added and incubated at 37°C overnight . The cell lysate was transferred to an eppendorf tube and the nucleic acid drawn out of solution with an equal volume of isopropanol and shaking (the preparation was stored at -20°C until required). The nucleic acid was transfered into 500 µl 70% ethanol and precipitated by centrifugation (1400 r.p.m., 10 mins in an Eppendorf 5415 centrifuge). The pellet was air dried and resuspended in 20-50 µl 1 x TE (O/N, 37°C). The DNA (0.4 v) was analysed by Southern Blot.

Lysis Solution:

| | | |
|---|---|---|
| Tris-HCl (ph 8.5) | 100 | mM |
| EDTA | 50 | mM |
| SDS | 0.2% | (v/v) |
| NaCl | 200 | mM |
| Proteinase K | 0.1 | mg/ml |

### *2.3.19 Southern blot analysis of high molecular weight DNA*

Approximately 20 µg of high molecular weight DNA was digested in a volume of 25 µl using a two fold excess of restriction enzyme (37°C, O/N). The DNA in 1 x GLB was run on horizontal 0.8% TAE agarose gels in 1x TAE at 250 mA, 4 hours in an Owl Scientific horizontal tank (A2 model). Gels were photographed under longwave UV light, depurinated (0.25M HCl, 15 min.), denatured (1.5M NaCl/0.5M NaOH, 15 min.), neutralized (3M NaAc pH5.5, 30 min.) and transferred to Hybond-

$N^+$ nylon membranes in 0.2M NaOH or to Nytran nylon membrane in 20x SSC, overnight. The DNA was immobilized by UV crosslinking (Stratagene Inc., UV Stratalinker™ 1800, 1200 kμJ).

SSC:

| | | |
|---|---|---|
| NaCl | 150 | mM |
| sodium citrate | 15 | mM |

### *2.3.20 $\alpha P^{32}$-labelled DNA probes*

Single stranded DNA probes were prepared using the Gigaprime or Megaprime labelling kit and the protocol of the supplier with 50 μCi $\alpha$-$^{32}$PdATP. Unincorporated label was removed from the probe after dilution to a final volume of 100 μl with MQ $H_20$ by centrifugation on a Sepharose CL-6B column (1800 r.p.m., 4 min.).

### *2.3.21 Hybridization of radioactive probes to Hybond-$N^+$or Nytran membranes*

Hybridization reactions at 65°C were carried out in a Hybaid midi hybridization oven. Filters were prehybrized 4 hours-overnight at 65°C in 10 ml of prehybridisation solution/filter. Radiolabelled probes were heated to 100°C for 5 min. with sonicated salmon sperm DNA (1 mg) and snap-cooled on ice before addition to hybridization cylinders. Filters were probed overnight at 65°C before washing for 30 minutes in 2 x SSC/ 1% SDS and 0.2 x SSC/1%SDS at 65°C. Washed filters were sealed in plastic bags and exposed to phosphorimager screens.

Prehybrization Solution:

| | | |
|---|---|---|
| $NaPO_4$ (pH 7.2) | 250 | mM |
| SDS | 7 % | (v/v) |
| PEG 6000 | 10 % | (w/v) |
| EDTA | 1 | mM |
| sonicated DNA | 0.1 | mg |
| $H_2O$ | to 100 ml. | |

### *2.3.22 Total RNA preparation*

Total RNA was isolated from embryoid bodies and ES cell monolayers as follows. Embryoid bodies (EBs) were harvested and media discarded. ES cells were harvested by trypsinization. EBs or cells were washed once in PBS, centrifuged at 1000rpm, 5 min. and the pellet stored at -20°C. One ml. of homogenization buffer was added to the pellet and mixture transferred to a 10 ml phenol-compatible tube. Sterile acid washed glass beads were added to just below the meniscus and the tube was vortexed

for 30-60 sec. A further 3 ml. of homogenization buffer was added and the mixture was incubated 1 hour at 37°C, phenol-chloroform extracted once and then precipitated in 4 ml. iso-propanol and 400µl of 3M NaAc overnight. The nucleic acids were pelleted by centrifugation (3000rpm, 5 min) and the pellet was resuspended in 400µl of DNaseI buffer. 1.5-2.0µl of DNaseI was added and mixture was incubated at 37°C for one hour, then phenol-chloroform extracted and ethanol precipitated.

Homogenization buffer:

| | | |
|---|---|---|
| Tris-HCl pH7.5 | 50 | mM |
| NaCl | 50 | mM |
| EDTA | 5 | mM |
| SDS | 0.5% | (v/v) |
| Proteinase K (added last) | 200 | µg/ml |

DNaseI buffer:

| | | |
|---|---|---|
| Tris-HCl pH8.0 | 50 | mM |
| MgCl | 10 | mM |
| EDTA pH8.0 | 1 | mM |
| DTT | 0.1 | mM |

### *2.3.23 Primer extension*

The extension primer oligonucleotide was end-labelled in the following reaction which was allowed to proceed at 37°C for 1 hour. The labelled primer was purified on a 20% polyacrylamide gel (section 2.3.12) and eluted O/N in TE at room temperature (section 2.3.14).

| | | |
|---|---|---|
| oligonucleotide primer | 100 | ng |
| One-Phor-All buffer (Pharmacia) | 0.1 | v |
| DTT | 10 | mM |
| spermidine | 100 | nM |
| $\alpha$-$^{32}$PdATP | 30 | µCi |
| T4 polynucleotide kinase | 30 | u |

10pmoles of labelled primer was hybridized to 80 µg total RNA in 1x hybridization buffer by incubation at 65°c for 90 min. followed by slow cooling to RT. The primer extension reaction was carried out at 42°C for 1 hour in the following reaction mix.

| | | |
|---|---|---|
| Tris-HCl pH8.3 | 100 | mM |
| $MgCl_2$ | 15 | mM |
| DTT | 8 | mM |

| | | |
|---|---|---|
| dNTP | 220 | nM |
| AMV reverse transcriptase | 5 | u |

The reaction was RNase A (20 mg/ml) digested for 15 min. at 37°C with 10 µg salmon sperm DNA, then phenol/chloroform extracted and ethanol precipitated. Samples were resuspended in 5 µl FLB and run on 7M urea/6% polyacrylamide gels (2.3.13). The gels were transferred to dry 3MM filter paper before drying 65°C in vacuo. Radioactivity was detected by exposure to X-ray film at -80°C with intensifying screens, or by exposure to phosphorimager screens.

10x Hybridization buffer:

| | | |
|---|---|---|
| KCl | 1.5 | M |
| Tris-HCl pH8.3 | 100 | mM |
| EDTA | 10 | mM |

### *2.3.24 RNase protection mapping*

Probe template plasmids were linearized using appropriate restriction endonucleases, ethanol precipitated and resuspended in MQ $H_2O$. Templates were transcribed as in Kreig and Melton (1987) using appropriate RNA polymerases in the presence of 40-240 µCi of $\alpha$-$^{32}$PrUTP. The labelled probe was purified on a 6% polyacrylamide gel (section 2.3.13) and eluted O/N in gel elution buffer at 37°C (section 2.3.14) or by centrifugation on a Sephadex G50 column (3000 r.p.m., 5 min.). Hybridizations and RNase digestions were carried out as described in Kreig and Melton (1987) except that 150,000 c.p.m. of antisense *Gbx2* and 3700 c.p.m. of antisense *mGAP* riboprobes were added to 20 µg total RNA and digested with RNase T1 and RNaseA for 60 min. at 37°C. Products were run on 6% polyacrylamide gels (section 2.3.13) and the gels were transferred to dry 3MM filter paper before drying 65°C in vacuo. Radioactivity was detected by exposure to phosphorimager screens.

### *2.3.25 Northern blot analysis*

Forty µg of total RNA in a volume of 11.25µl was prepared for electrophoresis by the addition of 5µl 10x MOPS, 8.75 µl formaldehyde (37%, pH4.5) and 25 µl deionized formamide. Samples were denatured at 65°C, 15 min., snap cooled on ice and 5 µl of NLB added. The samples were electrophoresed in a 1.2% agarose gel (1x MOPS, 1% formaldehyde)in 1x MOPS at 150V. The RNA in the gel was then transferred to Hybond-N$^+$ nylon membranes in 20x SSC, overnight. The RNA was immobilized by UV crosslinking (Stratagene Inc., UV Stratalinker™ 1800, 1200 kµJ) and pre-hybridized in a Hybaid cylinder at 65°C for 4-6 hours in Pre-hybridization buffer.

Pre-hybridization buffer:

| | | |
|---|---|---|
| Deionized formamide | 6.0 | ml |
| 20x SSC | 2.5 | ml |
| 50x Denhardts solution | 1.0 | ml |
| 1M NaPO4 (pH 6.8) | 0.2 | ml |
| 10% SDS | 1.0 | ml |
| denatured Salmon sperm DNA | 1.0 | mg |
| denatured yeast tRNA | 1.0 | mg |

Riboprobes were prepared as in Kreig and Melton (1987) using appropriate RNA polymerases in the presence of 60 µCi of $\alpha$-$^{32}$PrUTP. The labelled probe was purified by centrifugation on a Sephadex G50 column (3000 r.p.m., 5 min.), denatured at 100°C for 5 min. and added to the cylinder containing the membrane. Membranes were probed overnight at 65°C before washing for 30 min. in 2 x SSC/ 1% SDS at 50°C and for 40 min. in 0.2 x SSC/1%SDS at 70°C. Washed membranes were sealed in plastic bags and exposed to phosphorimager screens.

### *2.3.26 Digoxigenin labelled RNA probes*

The appropriate DNA template plasmid was linearized by restriction enzyme digest, ethanol precipitated, and then resuspended in MQ H2O. RNA transcription was carried out in the following reaction mix for 3 hours at 37°C.

| | | |
|---|---|---|
| DNA template | 1 | µg |
| DTT | 10 | mM |
| RNase Inhibitor | 40 | u |
| Transcription buffer | 0.1 | v |
| DIG RNA Labelling Mix | 0.1 | v |
| RNA polymerase | 50 | u |

Ten units DNase I were added to the mixture and the reaction was allowed to proceed for 15 min. at 37°C. The reaction was ethanol precipitated and resuspended in 100 µl MQ H2O and 40 u RNase Inhibitor. The probe was checked by denaturing 5 µl and then run on an agarose gel (section 2.3.5).

### *2.3.27 Whole mount in situ hybridization of embryoid bodies*

Embryoid bodies were harvested by removal of the medium and washing with cool PBS, then were fixed in 4% PFA in PBT (PBS, 0.1% Tween 20) overnight at 4°C The fixed embryoid bodies were washed twice with PBT and dehydrated through an increasing methanol in PBT series (25%, 50%, 75%, 100% methanol) on ice and stored at -20°C in 100% methanol. Embryoid bodies were rehydrated in a decreasing methanol in PBT series on ice and then rinsed 3 times in PBT. The bodies were washed 3 times for 20 min. each, at room temperature, in RIPA buffer.

RIPA buffer:

| | | |
|---|---|---|
| NaCl | 150 | mM |
| Synperonic OP 8 | 1 | % |
| Sodium Deoxycholic acid | 0.5 | % |
| SDS | 0.1 | % |
| EDTA | 1 | mM |
| Tris pH 8.0 | 50 | mM |

Embryoid bodies were fixed for 20 min. in 4% PFA/PBT, 0.2% glutaraldehyde, then washed 3 times with PBT for 5 min each. Cells were washed with 1:1 Hybridization buffer:PBT, then with Hybridization buffer and placed into wells of a 24-well tissue culture tray. Denatured salmon sperm DNA (10 µg/ml) and tRNA (100 µg/ml) were added to the Hybridization buffer, half of this mixture was set aside and the remainder used to replace the buffer in each well. The trays were placed in a box containing paper toweling soaked in 1:1 formamide:H2O and the box was sealed with tape and incubated at 65-70°C for 1-5 hours.

Hybridization buffer:

| | | |
|---|---|---|
| Formamide (deionized) | 50 | % |
| 20x SSC pH 4.5 | 1 | v |
| heparin | 50 | µg/ml |
| Tween 20 | 0.1 | % |

Denatured digoxigenin labelled probes (2.3.24) were added to the remaining Hybridization buffer mix to give a probe concentration of 1:100 to 1:200 and this was used to replace the buffer in the wells. The trays were returned to the box, sealed and incubated overnight at 65-70°C. The embryoid bodies were washed with wash buffer once, then 3 times for 30 min. each at 65°C. The embryoid bodies were then washed with 1x TBST 3 times at room temperature.

Wash buffer:

| | | |
|---|---|---|
| Formamide (deionized) | 50 | % |
| 20x SSC pH 4.5 | 1 | v |
| Tween 20 | 0.1 | % |

10x TBST:

| | | |
|---|---|---|
| NaCl | 8 | g |
| KCl | 0.2 | g |
| Tris pH 7.5 | 250 | mM |

Tween 20.....1.0 %

Embryoid bodies were washed with 10% FCS in 1x TBST at room temperature for 1 hour. Anti-digoxigenin-AP Fab fragment was added to 1% FCS in 1x TBST to give a concentration of 1:2000 and was used to replace the solution in the wells. The trays were placed in a box with paper soaked in H2O and incubated at 4°C overnight. The wells were washed with 1x TBST at room temperature 3 times for 5 min. each. The embryoid bodies were washed with at least a further 3 changes of 1x TBST over the course of a day and left overnight at 4° C. The embryoid bodies were washed with Alkaline Phosphatase buffer at room temperature 3 times for 10 min. each. Stain was made in a foil wrapped tube by adding 4.5 µl of NBT (75 mg/ml in 70% DMF) and 3.5 µl of BCIP (50mg/ml in DMF) to each ml of Alkaline Phosphatase buffer and was used to replace the buffer in the wells. The trays were covered in foil and developed at room temperature until color developed. The reaction was stopped by rinsing 3 times with PBT containing 1mM EDTA.

Alkaline Phosphatase buffer:

NaCl..... 100 mM

MgCl..... 50 mM

Tris pH 9.5 ..... 100 mM

Tween 20.....0.1 %

## 2.4 Tissue Culture Techniques

### *2.4.1 Cell lines*

The ES cell lines used throughout the course of this work were obtained from: E14TG2a (Austin Smith, CGR Edinburgh, UK) and feeder independent D3 (Lindsay Williams, Ludwig Institute, Melbourne, Australia).

The isolation of E14TG2a and D3 is described in Hooper et al., (1987) and Doetschman et al., (1985) respectively.

### *2.4.2 Solutions*

1 x PBS:

NaCl..... 80 g

KCl .....0.2 g

$KH_2PO_4$.....0.2 g

$Na_2HPO_4$ .....1.15 g

PBS was made up to 1 litre with MQ $H_2O$ and sterilised by autoclaving (20 psi at 140°C , 25 min.).

### Trypsin EDTA:

trypsin (1:250)..... 1 g
10 x Versene..... 100 ml
Trypsin EDTA was filter sterilised.

### 10 x Versene:

EDTA ..... 2 g
NaCl..... 80 g
KCl ..... 2 g
$KH_2HPO_4$ ..... 2 g
$Na_2HPO_4$ ..... 11.5 g
Versene was made up to 1 litre with MQ $H_2O$, filter sterilised and stored at 4°C.

### β-mercaptoethanol/PBS:

β-mercaptoethanol..... 100 mM
PBS..... 14.1 ml

### LIF conditioned medium:

COS cell medium conditioned with LIF was prepared as described by Smith (1991) with the exception that COS-1 cells were used.

### MED II conditioned medium:

DMEM supplimented wtih 10% FCS, 40 mg.ml gentamycin, and 1mM L-glutamine was conditioned with a human hepatocarcinoma cell line (HepG2) was prepared as described by Rathjen, et al (1999).

## *2.4.3 Media*

### Incomplete ES cell medium:

Incomplete ES cell medium was made with Dulbecco's modified Eagle's medium (DMEM) powder (high glucose, with L-glutamine, without sodium bicarbonate and sodium pyruvate).

DMEM..... 67.4 g
$NaHCO_3$..... 18.5 g
Hepes..... 23.8 g
gentamycin..... 6.25 ml
The pH of incomplete ES cell medium was adjusted to 7.4 and made up to 5 litres with MQ $H_2O$, filter sterilized and stored at 4°C.

### Complete ES (CES) cell medium:

incomplete ES cell medium..... 85% (v/v)

| | | |
|---|---|---|
| FCS | 10-15% | (v/v) |
| LIFconditoned medium | 100 | U/ml |
| β-mercaptoethanol | 0.1 | mM |
| L-glutamine | 1 | mM |

EPL cell medium:

| | | |
|---|---|---|
| incomplete ES cell medium | 42.5% | (v/v) |
| FCS | 10-15% | (v/v) |
| Med II conditoned medium | 42.5% | (v/v) |
| β-mercaptoethanol | 0.1 | mM |
| L-glutamine | 1 | mM |

Embryoid body (EB) medium:

| | | |
|---|---|---|
| incomplete ES cell medium | 85% | (v/v) |
| FCS | 10-15% | (v/v) |
| β-mercaptoethanol | 0.1 | mM |
| L-glutamine | 1 | mM |

Methyl Cellulose (MC) medium:

| | | |
|---|---|---|
| IMDM | 84% | (v/v) |
| FCS | 15% | (v/v) |
| methyl cellulose | 0.9% | (v/v) |
| MTG | $4.5 \times 10^{-4}$ | M |
| ascorbic acid | 50 | µg/ml |

MC medium was supplemented with 400U/ml mouse IL-3 and 10ng/ml recombinant human M-CSF for the enhancement of macrophage formation.

### *2.4.4 ES cell maintenance*

ES cells were routinely maintained as described in (Smith, 1991) with the exception that ES cells were grown in complete ES cell medium containing 15% FCS and under 10 % $CO_2$. ES cells were passaged at approximately 80% confluence.

### *2.4.5 Stable transfection of ES cells with targeting vector DNA by electroporation*

The vector DNA (150 µg) was linearized by restriction enzyme digestion (37°C, O/N) and ethanol precipitataed. After three washes in 70 % ethanol the DNA was resuspended in sterile PBS (150 µl). ES cells were harvested in ES cell medium (150 $cm^2$ flasks, 1200 r.p.m., 5 min) rinsed in CES cell medium (20 ml) and resuspended in PBS (20 ml). After a cell count was done the cells were harvested by centrifugation (as above) and resuspended in PBS at a concentration of $1.54 \times 10^8$ cells/ml. To 650 µl of cell suspension in a cuvette (Bio-Rad, 4 mm disposable

electroporation cuvette) the resuspended DNA (150 µl) was added and imediately transfected (Bio-Rad Genepulser, 800 mV, 3 µF).

ES cells transfected with pGNE, pGHT, or pCH110 (Chapter 4) were plated into complete ES cell medium (20 ml, 37°C) at 1 x $10^5$/ml in 10 cm diam. plates and grown at 37°C in 10% $CO_2$. Selection with G418 (200 µg/ml) and/or hygromycin (110 µg/ml)/G418 was applied 24 hours after transfection. Transfected cells were also plated into complete ES cell medium (10 ml, 37°C) at 1 x $10^5$/ml in a 10 cm diam. plate to determine the number of cells surviving electroporation and to determine the transfection efficiency. The plate of cells transfected with pCH110 was fixed and histochemical staining for β-galactosidase activity (section 2.4.6) carried out to determine the transfection efficiency.

### 2.4.6 *Histochemical staining for β-Galactosidase activity*

β-Galactosidase activity expressed by transfected ES cells was detected by the following procedure. Cells were rinsed three times in PBS and fixed in 0.2% glutaraldehyde for 5 minutes. After a further three rinses in PBS cells were incubated in β-galactosidase stain solution containing 400 µg/ml X-gal at 30°C for 16 hours. Cells were viewed under phase contrast and bright field optics using the Nikon Diaphot or Zeiss Axioplan Universal microscopes.

β-galactosidase stain solution:

| | | |
|---|---|---|
| $K_3Fe(CN)_6$ | 0.45 | mM |
| $K_4Fe(CN)_6$ | 0.45 | mM |
| $MgCl_2$ | 1 | mM |

### 2.4.7 *Isolation, expansion and storage of targeted clones*

After 6-8 days of selection in the appropriate medium, single ES cell colonies were picked by gentle scraping and suction into a drawn out pasteur pipette and transferred to 96 well trays containing 50 µl PBS per well. 100 µl/well of trypsin was added and the trays incubated at RT for 2 min. 100 µl/well of CES was added and the colonies dispersed by pipetting up and down. One half of the cell suspension was transferred to each of 2 wells in 2 separate 24 well trays containing 1 ml CES. Cells were cultured further until near confluence then either harvested for DNA (2.3.18) or frozen. Each well was rinsed with PBS, 50 µl/well trypsin added and incubated at 37°C 30 sec. 150 µl/well CES was added and cells dispersed by pipetting up and down. 200 µl/well of 2x Freeze mix (80% FCS, 20% DMSO) was added and the trays sealed with clingwrap. The trays were stored at -80°C.

### 2.4.8 Karyotypic analysis of ES cell lines

All ES cell lines which screened positively for homologous recombination events by southern blot were karyotyped by the method of (Robertson, 1987). Briefly, rapidly growing ES cells were arrested in mitosis by treatment with colcemid, chromosome spreads were prepared and were solid stained. Chromosome spreads were viewed under phase contrast optics on the Zeiss Axioplan Universal microscope.

### 2.4.9 RA Differentiation of ES cells

Retinoic acid (RA) differentiation was carried out by plating cells in bacteriological dishes, at a density of $1x10^5$ cells/ml, in EB medium supplemented with 1 μM RA. After 48 hours, the medium was replaced. After 2 more days the aggregates were seeded individually or collectively into tissue culture dishes in EB medium. The presence of neurons was assessed by microscopic examination two days after seeding.

### 2.4.10 EPL cell formation

EPL cells were formed by plating ES cells onto tissue culture plates in the presence of EPL cell medium. The cells were harvested for further use after 2-3 days of culture.

### 2.4.11 Embryoid body formation

ES or EPL cells were plated at a cell density of $10^5$ cells/ml in 10 ml of EB medium in non-adherant petri dishes. After 2 days of culture the cell aggregates were split into two dishes and the medium replaced. The medium was replaced thereafter every 2 days.

### 2.4.12 Formation of hematopoetic lineages in MC culture

To assess the formation of macrophages, aggregates were formed from ES cells and EPL cells, by plating 1 x $10^5$ cells/ml in 10 ml EB medium in bacteriological grade 10cm petri dishes. After 48 hours aggregates were transferred into 1.25ml MC media supplemented with 400U/ml IL-3 and 10ng/ml recombinant human M-CSF. On selected days, colonies were scored for the presence or absence of macrophages by microscopic examination.

Macrophage-like cells were collected from MC cultures, cystospun, air-dried and stained with May-Grünwald-Giemsa stain or with the macrophage-specific antibody F4/80 (Austyn and Gordon, 1981).

### 2.4.13 Immunohistochemical staining with monoclonal antibody F4/80

Macrophage-like cells were collected from MC cultures, cystospun onto glass slides and air-dried. Cells were fixed in acetone:methanol:formaldehyde (47.5:47.5:5) for 90 sec. and then rinsed three times in PBS. Slides were blocked in 1% BSA/PBS for 2 min. and then incubated with the monoclonal antibody F4/80 (90% in mouse serum) for 2 hours at 4°C. After three rinses in PBS the slides were blocked again with 1% BSA/PBS for 2 min and then incubated with the secondary antibody, rabbit anti-rat (1:200 in PBS/10% mouse serum) for 1 hour at 4°C. After a further three rinses in PBS the slides were incubated with the tertiary antibody, goat anti-rabbit-Horseradish Peroxidase conjugated (1:200 in PBS) for 30 min. at 4°C. Slides were rinsed 3 times in PBS and then color was developed by adding 3-500µl/slide of prepared DAB cells and incubating at RT. Color development was stopped with PBS. Cells were viewed under phase contrast and bright field optics using the Nikon Diaphot or Zeiss Axioplan Universal microscopes.

### 2.4.14 Formation of neuronal lineages in embryoid bodies grown in Med II and FGF-4

ES cells were plated at a cell density of $10^5$ cells/ml in 10 ml of EB or EPL medium in non-adherant petri dishes. After 2 days of culture the cell aggregates were split into two dishes and the medium replaced. The medium was replaced on days 4, 5, and 6 of culture. On day 7, individual embryoid bodies were seeded in wells of a 24-well tissue culture tray in medium containing 45% DMEM, 45% F12, and 10% FCS. On day 8, the medium was changed to 50% DMEM, 50% F12, .01% ITSS, and 20ng/ml FGF 4.

## 2.5 Mouse manipulations and analysis

### 2.5.1 Chimera production

Chimeric mice were produced by blastocyst microinjection by the methods described in (Hogan et al., 1994). Targeted ES cells were introduced into CBA x C57BL blastocysts. Chimeras were identified by coat color assessment. E14TG2a ES cells are homozygous for the chinchilla allele ($c^{ch}/c^{ch}$) and chimerism was readily detected by the presence of sandy coat color pigmentation.

### 2.5.2 GPI analysis of sperm

GPI analysis of sperm obtained from the epididymis of male chimeras was carried out using the Super Z applicator kit using the protocol of the supplier.

### *2.5.3 Breeding*

Male chimeras produced with E14TG2a ES cells and targeted derivatives were mated with Balb/c female mice, where transmission of the ES cell genotype through the germline is revealed by the production of grey offspring.

## 2.6 Phosphorimager analysis and autoradiograph scanning

Gels and filters were exposed to Storage Phosphor Screens (Molecular Dynamics) and processed using a Molecular Dynamics PhosphorImager running the ImageQuant software package. Autoradiographs were scanned using a Hewlett Packard *ScanJet IIcx* scanner running the DeskScan II 2.0 software package. PhosphorImager and DeskScan files were manipulated using the AdobePhotoshop™, MacDraw Pro and Canvas programs, and printed using a Hewlett Packard LaserJet4 printer.

## 2.7 Containment facilities

### *2.7.1 Recombinant DNA manipulations*

All manipulations involving viable organisms which contained recombinant DNA were carried out in accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

### *2.7.2 Animal manipulations*

All procedures involving animals were carried out with the approval of the University of Adelaide Animal Ethics Committee.

# Chapter 3

## Isolation and characterization of the *Gbx2* gene

# 3 Isolation and characterization of the *Gbx2* gene

## 3.1 Introduction

The first full length *Gbx2* cDNA was isolated from a D3 ES cell cDNA library with a partial *Gbx2* homeodomain fragment, isolated by RT-PCR using degenerate primers from the *En-2* homeodomain (Chapman,1994 ; Chapman and Rathjen, 1995). The full length *Gbx2* cDNA clone is 2.12 kb in length with a 1.02 kb open reading frame flanked by 423 bp and 677 bp 5' and 3' untranslated regions. The *Gbx2* homeodomain is located near the 3' end of the coding region, as is a slightly hydrophobic, 23 amino acid region which is conserved in Gbx2 sequences from different species, suggesting it may be functionally important. A proline rich region, found near the 5' end of the transcript (Chapman and Rathjen, 1995) may act as a transcriptional regulator (Mermod et al, 1989; Williams and Tijan, 1991).

*Gbx1* and *Gbx2* have been mapped to mouse chromosomes 5 and 1, respectively, based on the absence of recombination between *En2* and *Gbx1* and *En1* and *Gbx2* in a panel of interspecies back cross progeny (Frohman et al., 1993). This study suggested tight linkages between *En2* and *Gbx1* and *En1* and *Gbx2* and the possibility of evolutionary conservation between the two loci which could signify a functionally important organization of *Gbx* and *En* genes similar to that of the *Hox* genes. Physical mapping of the human genes *GBX2* and *EN1* (Matsui, et al., 1993; Lin et al., 1996) has shown that these genes are not closely linked in humans. Chapman et al. (1997) demonstrated that *Gbx2* probes localize to murine chromosome 1 C5-E1, a distance between of 4.1 - 19.1 cM from the location of *En1*, suggesting that these genes are not closely linked in the mouse either.

Molecular genetic investigation of potential roles of *Gbx2* required the isolation and characterization of the genomic locus in order to facilitate the construction of gene targeting vectors for the creation of null mutant mice and also to uncover potential regulatory elements within the proximal 5' region. This chapter describes the initial mapping by restriction enzyme digest of several clones isolated by Chapman from an E14TG2a genomic library (Chapman et al., 1997) and the subsequent isolation and characterization of a 5kb fragment containing the entire genomic locus.

## 3.2 Isolation and mapping of a *Gbx2* genomic clone

*Gbx2* genomic clones were isolated by screening a λ2001 library (Karn et al, 1984) constructed using E14TG2a genomic DNA (Boehm et al., 1991) with a 270 bp Hinc II/Pst I fragment of *Gbx2* cDNA (Chapman et al., 1997). Thirty positive plaques were

**Figure 3.1 Restriction enzyme mapping of genomic Lambda clones**

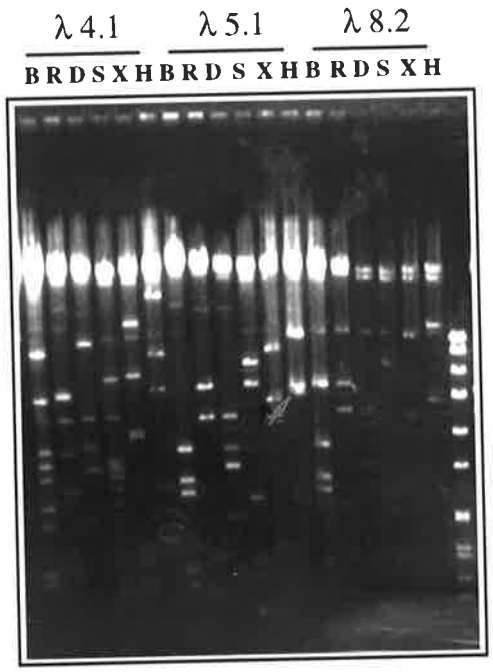
a. Photograph of agarose gel showing various restriction enzyme digests of 3 Lambda clones (λ 4.1, λ 5.1, λ 8.2). B= BamH I, R= Eco RI, D= Hind III, S= Sac I, X= Xba I, H= Xho I. Arrow shows 5 kb Xho I fragment containing *Gbx2*. Molecular size marker is Eco RI digested SPP-1 (BresaGen Ltd.)

b. Results of Southern blot analysis of restriction enzyme digests from (A), probed with a 190 bp Eco RI/ Sma I fragment from the 5' untranslated region of *Gbx2* cDNA. Arrow shows 5 kb Xho I fragment containing *Gbx2*.

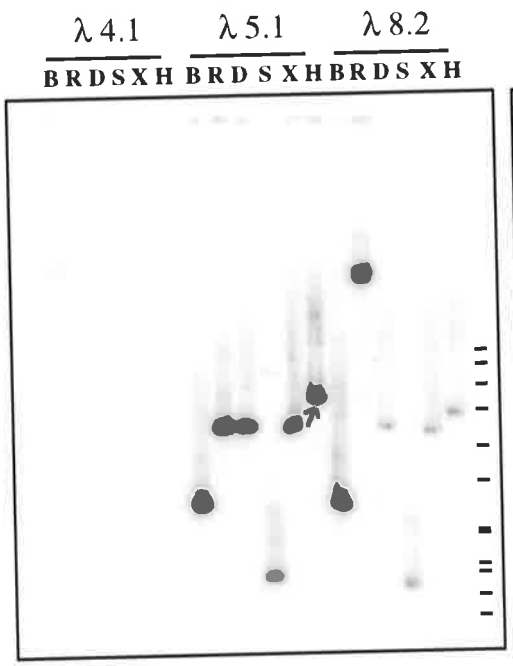
c. Results of Southern blot analysis of restriction enzyme digests from (A), probed with a 450 bp Bgl II/ Eco RI fragment from the 3' untranslated region of *Gbx2* cDNA. Arrow shows 5 kb Xho I fragment containing *Gbx2*.

**Figure 3.1**

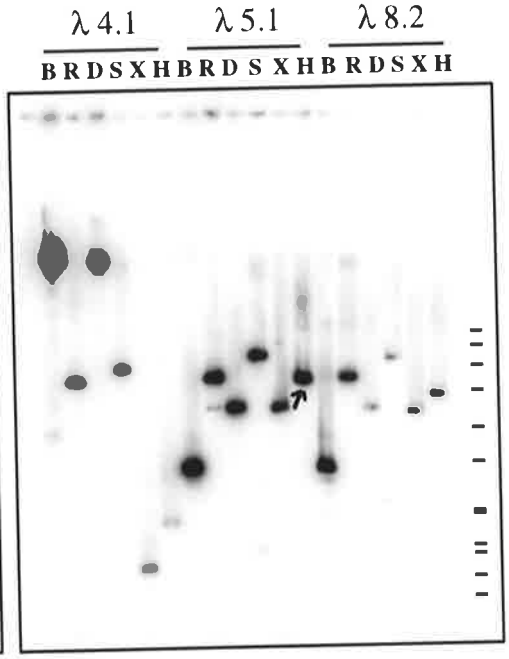
**a.**




**b.**




**c.**



identified in initial screening. Six of these were selected and re-screened at three dilutions. Five positive plaques were picked from the second round screening and three of these were positive when screened by Southern blot. These three λ clones were analyzed by restriction enzyme digestion (Fig. 3.1a) and Southern blot analysis. Filters were hybridized with a 190 bp Eco RI/Sma I fragment from the 5' untranslated region of the *Gbx2* cDNA (Fig. 3.1b) and then with a 450 bp Bgl II/Eco RI fragment from the 3' untranslated region of the *Gbx2* cDNA (Fig. 3.1c). A Xho I fragment, approximately 5kb in length, which hybridized to both the 5' probe and the 3' probe was identified in clone λ5.1, indicating that this genomic fragment contained the entire *Gbx2* coding sequence.

The 5 kb Xho I fragment was isolated from λ5.1 and cloned into the Xho I site in pBluescript II SK (pGXH5). Restriction enzyme mapping (Fig. 3.2b) and comparison of this map with a restriction enzyme map of a *Gbx2* cDNA clone (Chapman and Rathjen, 1995, Fig 3.2a) revealed a gap between the most 3' Bgl I site (position 898) and the most 5' PflM I site (position 1116) of more than 1 kb, suggesting the presence of an intron.

## 3.3 Sequencing of the putative promoter region and intron

Primers were designed against the cDNA regions near the most 3' Bgl I site (5'TTCGCTCCACAGCCACT3') and the most 5' PflM I site (5'CGGGGTCTTCTTCCTTA3'). These were used to sequence across the intron/exon boundaries. Further primers were then designed from intronic sequences to allow sequencing of the entire 1092 bp intron in both directions (Fig. 3.2b, section 2.2.11).

A primer (5'GCCGGGGACCTCGCGTT3') designed from the 5' region of the published cDNA sequence was used to sequence the regulatory region immediately 5' of this region. Additional primers were designed to extend the sequencing in the 5' direction and to complete the sequencing in both directions (Fig. 3.2b, section 2.2.11). A total of 579 bp 5' of the Pst I site (position 139) was sequenced. No difference from the published sequence (Chapman and Rathjen, 1995) was observed. The entire genomic sequence, including the cDNA sequence previously determined by Chapman and Rathjen (1995), is shown in Figure 3.3. Potential promoter elements were identified using the MatInspector program (Quandt et al., 1995) (Fig. 3.3).

The intron was located 215 bp 5' of the homeobox and was flanked by splice acceptor (TTTTGCTTTCAG/T) and splice donor (TCG/GTGAGT) sites that closely fit the consensus splice acceptor($(^{T}/_{C})_{n}N(^{C}/_{T})$AG/G) and donor ($(^{C}/_{A})$AG/GT$(^{A}/_{G})$AGT) sites

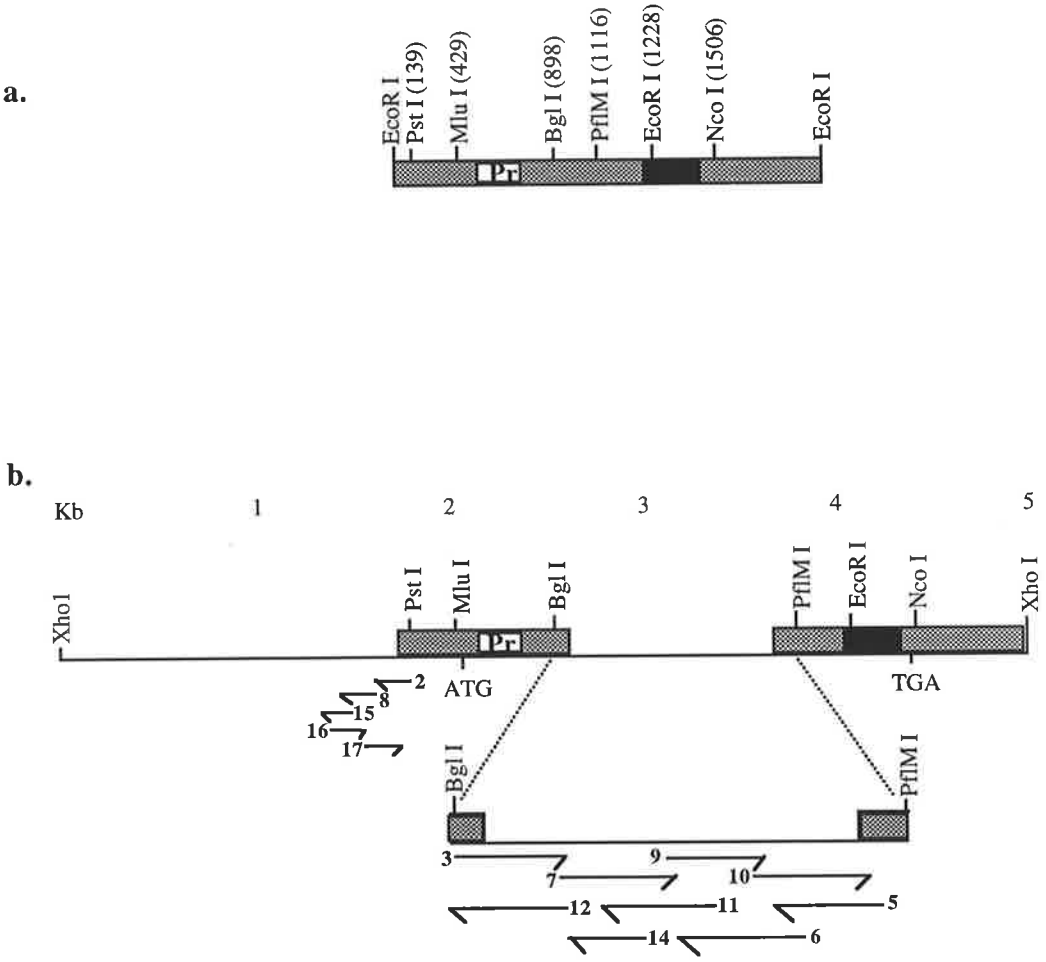



**Figure 3.2 Genomic organization of *Gbx2***

a. Restriction enzyme map of a*Gbx2* cDNA clone showing selected restriction enzyme sites.

b. Intron/exon structure and sequencing strategy (arrows) for the mouse *Gbx2* gene. Promotor and intron sequence was obtained in both directions. The intron (line), exons (shaded boxes), homeobox (**Hb**, black), proline-rich region (**PR**, white), and translation initiation/termination codons are indicated. Sequencing primers (**2**-**17**) are described in section 2.2.11.

**Figure 3.3 Sequence of the *Gbx2* gene**

Position 1 is defined by the longest primer extension product.

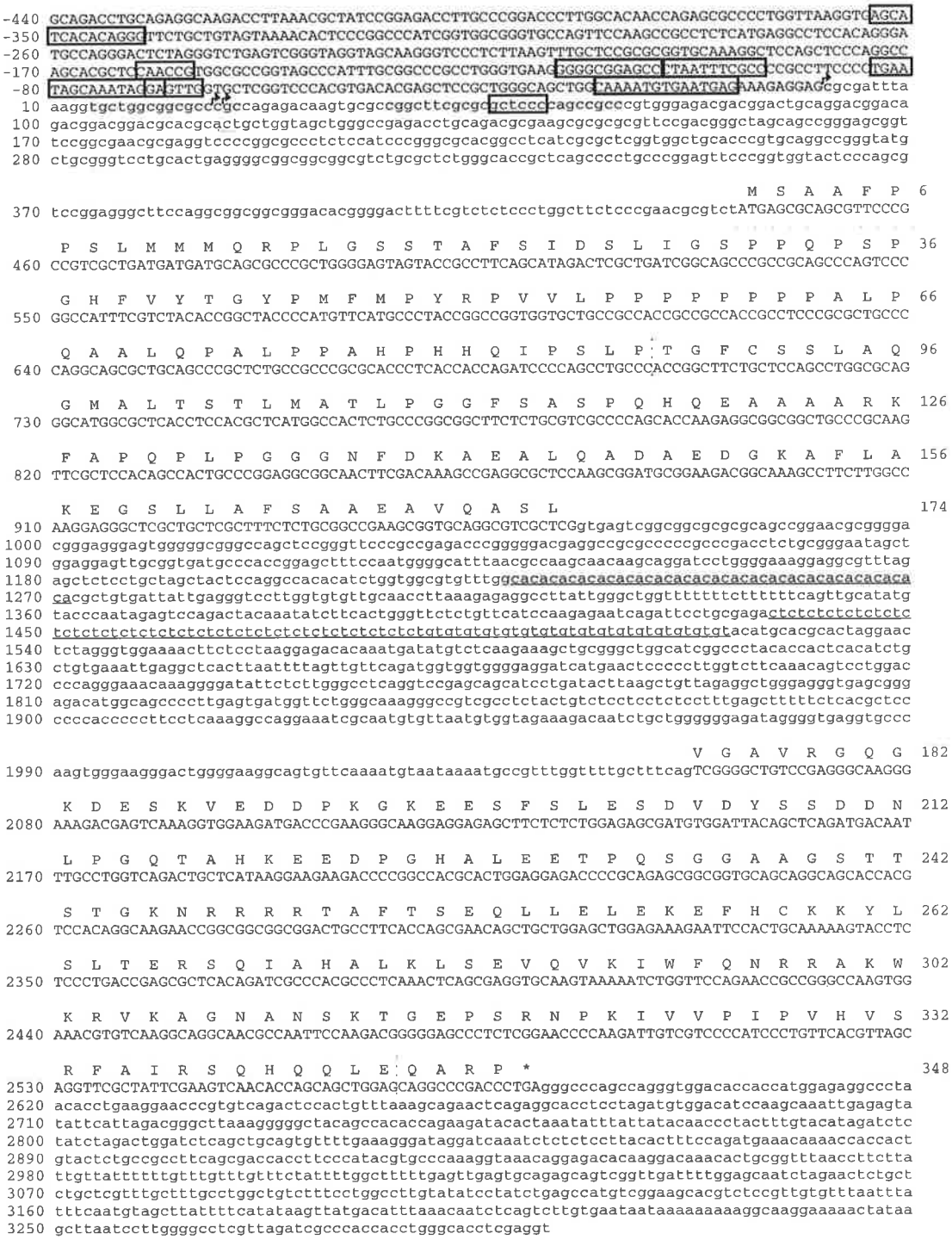
Sites of transcription initiation are indicated by arrows.

Coding regions and proximal promoter are in capitals, while the 5' untranslated region, intron, and 3' untranslated region are in lowercase. Dinucleotide repeats within the intron are underlined.

The proline-rich region (nucleotide position 532-702), homeobox (2272-2451), and C-terminal conserved region (2497-2565) are shaded.

Sequences matching consensus binding sites for Sp1 ($^{G}/_{T}$ GGGCGG$^{G}/_{A}{}^{G}/_{A}{}^{C}/_{T}$) at -117, S8 at -106, Oct 1 (ATGCAAT) at -23 and -354 (reverse orientation), Brn2 ($^{C}/_{A}$ $T_nTAAT$) at -84, and c-myb (PyAAC$^{G}/_{T}$ G) at -69 and -160 (reverse orientation) are boxed. The MED-1 (GCTCC$^{C}/_{G}$) motif at +57 is boxed.

# Figure 3.3



defined by Mount (1982). The intron contained a number of dinucleotide repeats in the form of $(CA)_{22}$ and $(CT)_{27}(GT)_{16}$ (Fig. 3.3). Dinucleotide repeats such as $(CA)_{n>10}$ (Dietrich et al., 1992) and $(TC)_{18}C(TG)_{25}$ (Cornall et al., 1991) are often found to exhibit high degrees of polymorphism within a population and thus are useful for genomic mapping. The position of the intron was identical to the intron position of the human *GBX2* gene (Lin et al., 1996). The *Drosophila* gene, *unplugged*, has two introns, a 3.5 kb intron located 119 bp 5' of the homeobox which bears no similarity to the mouse and human site, and a 62 bp intron located 132 bp 3' of the start of the homeobox. Genomic *Gbx2* sequences from other species have not been published.

The potential transcription activation domain (Williams and Tijan, 1991) of *Gbx2*, a 60 amino acid proline-rich region surrounding a core of 8 successive proline residues was located in exon 1 (Chapman and Rathjen, 1995). The homeobox region and the C-terminal hydrophobic region (Chapman et al., 1997) of *Gbx2* were located in exon 2.

Analysis of the 440 bp 5' of the transcription initiation site revealed a number of potential promoter elements (Fig 3.3), including consensus binding sequences for Sp1 (Dynan et al., 1986) at -117, the mesoderm specific, PRD-like homeodomain protein S8 (de Jong et al., 1993) at -106, and the POU domain transcription factors Oct1 (Kemler and Schaffner, 1990) at -23 and -354 (reverse orientation) and Brn 2 (Li et al., 1993) at -84. Two sites at -69 and -160 (reverse orientation) varied from the hexanucleotide consensus sequence of the proto-oncogene c-Myb transcription factor (Biedenkapp et al., 1988) by one nucleotide each, although the core binding sequence, GTTR, was present in both sites.

No conserved TATA box or initiator element (Inr) sequences were found within 882 nucleotides upstream of the open reading frame, which would suggest that the gene lacks a unique transcriptional start point (reviewed in Dynan, 1986; Novina and Roy, 1996). This could explain the differences in the 5' end of *Gbx2* cDNA transcripts which have been published (Chapman and Rathjen; 1995, Bouillet *et al.*, 1995).

CpG rich regions have been found in TATA-less promoters such as hypoxanthine phosphoribosyl transferase (HPRT) and HMG CoA reductase (Gardiner-Garden and Frommer, 1987). A C+G rich region (67% of nucleotides) was located in the *Gbx2* sequence at position -230 to +440. This region included 83 CpG dinucleotides and is a CpG island. CpG islands are defined by Gardiner-Garden and Frommer (1987) as regions with a G+C content >50% and an Observed/Expected GpC ratio ((#CpG/(#C x #G)) x # nucleotides in region) of >0.6. The G+C rich region of *Gbx2* has an Observed/Expected GpC ratio of 1.03.

MED-1 (Multiple Start Site Element-Downstream) is a conserved element found downstream of the transcription initiation sites in a number of TATA-less promoters with multiple start sites, including HPRT and HMG CoA reductase (Ince and Scotto, 1995). It lies 20-45 bp downstream of the most 3' transcriptional start site and a maximum of 110 bp downstream of the most 5' start site. A sequence corresponding to the MED-1 consensus was identified in the *Gbx2* sequence at +57.

## 3.4 Determination of *Gbx2* transcriptional initiation sites

Primer extension analysis and RNase protection analysis were performed to determine the transcriptional initiation site or sites of *Gbx2*. An extension primer (Ex) designed to hybridize to nucleotides 95 to 119 (5'AGTGCGTGCGTCCGTCCGTCTGTCC3') was hybridized to D3 ES cell total RNA and primer extension carried out using AMV reverse transcriptase. Primer extension analysis revealed 3 distinct transcription start sites (Fig 3.4a). The start site corresponding to the longest transcript was 442 nucleotides upstream of the initiating ATG. Shorter transcripts initiated at +27 and +28.

A 5' primer (5'AGGGTTCTGCTGTAGTA3') and the extension primer were used to PCR amplify a region from –344 to 119 of the pGXH5 plasmid. The 464 bp long amplified product was cloned into the T-tailed vector pGEM-T. This plasmid (p464) was linearized with Sal I and used to generate radiolabelled antisense riboprobes by transcription with T7 RNA polymerase for RNase protection analysis. The riboprobes were hybridized to D3 ES cell total RNA and RNase digestion carried out at various temperatures (section 2.3.24). RNase protection analysis confirmed the positions identified by primer extension analysis as the only start sites of transcription (Fig. 3.4b).

## 3.5 Discussion

### *3.5.1 Gene structure*

Comparison of *Gbx2* related genes in other species, including *Drosophila*, *Xenopus*, and human, reveals conserved sequences including a proline rich region near the N terminus, the homeodomain, and a C-terminal hydrophobic region (Chapman et al., 1997). Initial analysis of genomic structure, based on restriction enzyme mapping and sequencing revealed the presence of a single 1 kb intron, dividing the proline rich region from the homeobox and C-terminal region. The position of the conserved regions and the intron was maintained between the mouse and human *GBX2* sequence despite a frameshift which occurs in the human gene due to the deletion of a thymidine

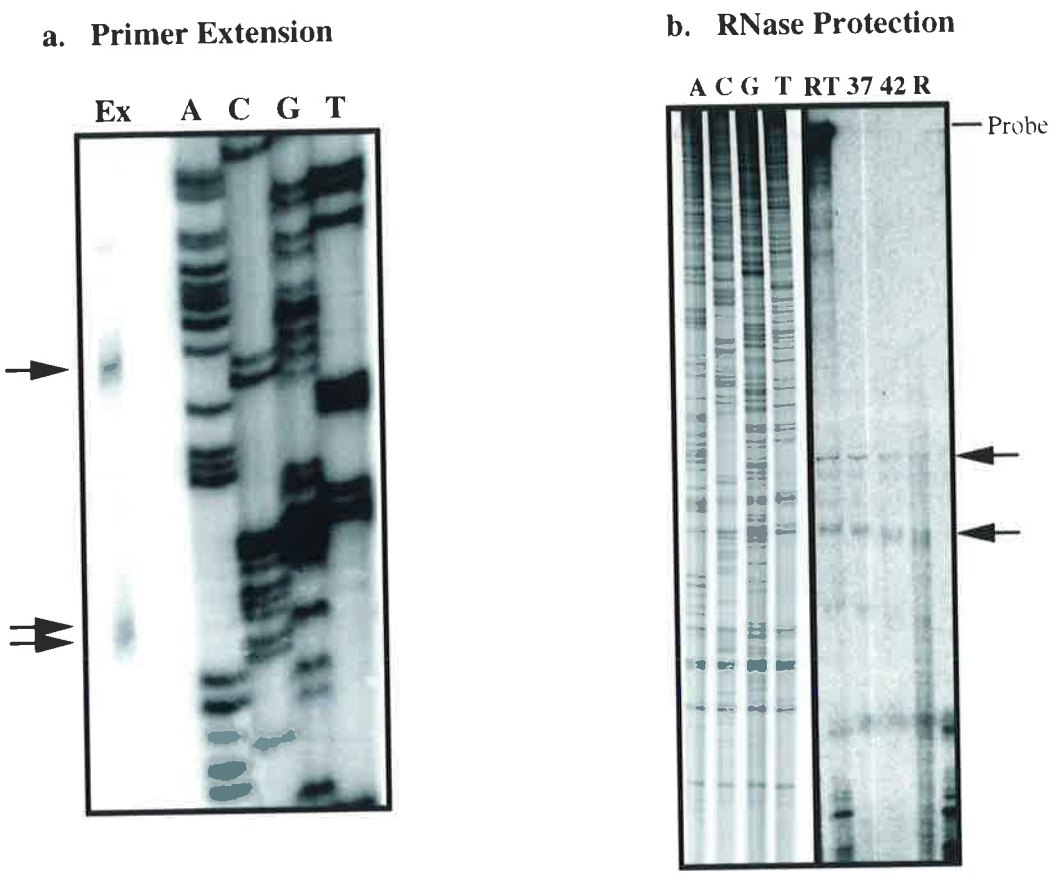



**Figure 3.4 Transcriptional initiation site analysis**

a. Detection of transcript initiation sites within the *Gbx2* gene using primer extension analysis. 80 µg of D3 ES cell RNA was reverse transcribed with extension primer (**Ex**). Transcript start sites (arrows) were determined by comparison to sequencing reactions on GHX5 plasmid template primed using the extension primer (lanes **A**, **C**, **G**, and **T**).

b. RNase protection analysis of the 5' end of the *Gbx2* message. 10 mg of D3 ES cell RNA was hybridized to antisense Gbx2 riboprobe and RNase digested at room temperature (**RT**), 37°C (**37**), and 42°C (**42**) for 30 min. 10 mg of tRNA (**R**) was also hybridized and digested at 4°C for 30 min. Lengths of protected products (arrows) were determined by comparison to sequencing reactions (lanes **A**, **C**, **G**, and **T**) as in the primer extension. Undigested riboprobe (**Probe**) indicated.

at position 660. This frameshift results in an altered amino acid sequence until a second frameshift, caused by the deletion of two guanine nucleotides at positions 2113 and 2114 restores the reading frame to a conserved sequence, including the homeodomain and the C-terminal region (Lin et al., 1996; Chapman et al., 1997). Human *GBX2* contains a 1kb intron in exactly the same position as mouse Gbx2 and the intronic sequence which has been published (42 bp) is identical to the corresponding mouse sequence (Lin et al., 1996). A single intron upstream of the homeobox is also found in other homeobox genes, including the *En* and *Dlx* family genes (Logan et al., 1992), *HoxA5* (Fibi et al., 1988), and *HoxB4* (Graham, et al., 1988), however whether this indicates an evolutionary relationship between these genes or if the position of the intron is functionally significant is unknown. The *Drosophila* gene, *unplugged*, has two introns located 119 bp 5' of the homeobox and 132 bp 3' of the start of the homeobox, in the region which codes for the third helix of the homeodomain. The intron position in the homeobox is conserved in a number of homeobox genes including *Drosophila Abd*, *pb*, *NK-1* and *lab*, and mouse *Dlx* (Chiang et al., 1995). The intron positions of other *Gbx1* or *Gbx2* sequences are unknown.

### *3.5.2 Transcription Control*

Transcription initiates when basal transcription factors recognize and bind to core promoter elements contained in the region near to the initiation site. The TATA box (TAAa/tAa/t), normally located approximately 25 to 30 bp 5' of the transcription start site, and the initiator element (Inr) (YYANt/aYY) which encompasses the start site are the most common core promoter elements. Core promoters may contain both elements, either element or neither (Novina and Roy, 1996). No homology for either of these elements was identified at the appropriate positions suggesting that *Gbx2* belongs to the $TATA^-/Inr^-$ class of promoters which includes such housekeeping genes as HPRT and HMG CoA reductase and which often have multiple start sites (Ince and Scotto, 1995; Novina and Roy, 1996). Ince and Scotto (1995) have investigated whether these multiple start sites might be specifically regulated by an alternate initiator-like element rather than by a random response due to the lack of core promoter elements. They found a conserved sequence (GCTCCC/G), termed MED-1 (Multiple Start Site Element-Downstream) in the region 20-45 bp downstream of the most 3' start sites, but no more than 110 bp downstream of the most 5' start site in 14 of the 15 gene sequences examined. When this site was mutated transcription was reduced by the decreased utilization of more 3' start sites. Ince and Scotto proposed that Med-1 and its as yet unknown cognate binding proteins may act in the activation of multiple start sites. The *Gbx2* sequence is consistent with this observation as it contains a

MED-1 consensus sequence 57 bp downstream of the most 5' initiation site and 29 bp downstream of the most 3' initiation site.

The most 5' transcriptional initiation site as determined by primer extension and RNase protection analyses was 2 nucleotides 5' of a reported human *GBX2* cDNA (Lin et al., 1996), while Bouillet et al. (1995) reported a mouse *Gbx2* cDNA sequence initiating 21 bp 3' of the longest transcript identified in these experiments. The single published chicken *GBX2* cDNA sequence has only 55 nucleotides in a 5' untranslated region which bears little resemblance to mouse *Gbx2* (Kowenz-Leutz et al., 1997). Xenopus cDNA has 318 bp and the *Drosophila* gene *unplugged* has 125 bp of 5' untranslated sequence, both of which are greatly divergent from the mouse sequence and do not appear to correlate with the transcription initiation sites of the mouse gene (von Bubnoff et al., 1995; Chiang et al., 1995).

Analysis of the upstream region revealed a number of candidates for potential control elements of *Gbx2* transcription. Sp-1, of which there are several consensus sites in the *Gbx2* regulatory region, has been implicated in transcriptional activation of a number of TATA-less genes, including mouse dihydrofolate reductase (DHFR) (Dynan et al., 1986) and the human Ha-ras promoter (Lu et al., 1994). *Gbx2* also contains a CpG island spanning the region from –230 to +440. CpG islands have been found in TATA-less promoters such as HPRT and HMG CoA reductase and, although the function of these dinucleotide repeats in transcriptional or post-transcriptional regulation of gene expression is not well understood, it has been suggested that they may enhance transcriptional control of a gene by involving additional transcription factors or by preventing methylation of control sequences (Gardiner-Garden and Frommer, 1987). Thus, *Gbx2* has been shown to demonstrate features typical of a TATA-less promoter with multiple start sites.

### *3.5.3 Potential regulatory networks*

Homeobox genes are known to regulate the expression of other homeobox genes (Struhl and White, 1985; Sasaki et al., 1990; Arcioni, et al., 1992). It is interesting, then, that the *Gbx2* promoter region contained consensus binding sites for S8, Oct1, and Brn2 homeodomain proteins. *Gbx2* and *S8* expression at 7.0-7.5 d.p.c. appear to be complementary in that *Gbx2* is expressed in the primitive streak (Bouillet et al., 1995) while *S8* is expressed in all mesoderm tissues outside the primitive streak (deJong and Meijlink, 1993). S8 (recently renamed prx-2) has been shown to function cooperatively with the homeobox gene prx-1 (formerly Mhox) to maintain cell fate within the craniofacial mesenchyme (Lu et al., 1999). It could be useful to determine whether the S8 protein is involved in the repression of *Gbx2* expression during

gastrulation or vice versa. Oct1 is a ubiquitously expressed protein and Brn2 is required for the formation of a subset of neurons in the hypothalamous (Nakai et al., 1995). The Oct1 site in the *Gbx2* promoter region at -23 and the Brn2 site at -84 differ by only one nucleotide from the defined octamer motif and may bind other octamer-binding proteins such as the pluripotent cell-specific Oct4 protein (reviewed in Schöler, 1991). Oct4 has been shown to regulate the expression of *Rex1*, a zinc finger gene expressed in the ICM, but not in later pluripotent cell populations. Oct4 can either activate or repress *Rex1* expression depending on the cellular environment, by recruiting auxilliary adapter proteins such as the adenovirus E1A protein (Ben-Sushan et al, 1998). This is an example of the complexity of gene regulation by homeodomain proteins. Given the overlap between *Oct4* and *Gbx2* expression in pluripotent cells *in vitro* and *in vivo*, it would be interesting to investigate whether the Oct4 protein can regulate *Gbx2* expression. The chicken homologue, GBX2, has been shown to be a target of the *myb* oncogene (Kowenz-Leutz et al., 1997), although whether it is a direct target or whether activation is initiated by an intermediate transcription factor was not determined. The identification of two consensus binding sequences for Myb binding in the *Gbx2* promoter region which deviate from the Myb consensus binding site by one nucleotide suggests that *Gbx2* may be a direct target of Myb.

# Chapter 4

# Targeted disruption of the *Gbx2* locus

# 4 Targeted disruption of the *Gbx2* locus

## 4.1 Introduction

Gene targeting via homologous recombination in ES cells has become a widely practiced technique for introducing alterations into predetermined sites in the mouse genome (Brandon, et al., 1995a). These alterations may result in the loss of gene expression or altered gene function and provide a means to study the function and control of gene products, either in vitro or in vivo. Homologous recombination is used most frequently to create loss of function mutations (Brandon et al., 1995a,b,c) by replacing or disrupting a functional region of the coding sequence of a gene with a selectable marker gene. Marker genes are often used to confer antibiotic resistance and can be used to select for integration of the vector into the host genome by culture in the appropriate antibiotic enriched medium. A refinement of this method, termed positive-negative selection (Mansour, 1988), exploits the observation that non-homologous ends are integrated during non-homologous, but not homologous recombination. A selection marker gene which confers sensitivity to a reagent (negative selection), such as the herpes simplex virus thymidine kinase (HSV-*tk*) gene, is placed outside the region of homology on the targeting vector. Selection for vector integration and against HSV-*tk* expression is used to enrich for homologous integrants. This chapter describes the creation of a null mutation at the mouse *Gbx2* locus by homologous recombination in ES cells.

Recombination between homologous regions of DNA is a rare event and in order to maximize the frequency of targeting a number of variables must be considered in the design of the targeting vector. The total amount of homologous DNA has proved crucial to the efficiency of targeting, with efficiency increasing exponentially from 2 to 10 kb of homology (Deng and Capecchi, 1992). Isogenic DNA, that is, of the same mouse strain from which the ES cells were derived, also increases targeting efficiency by minimizing the base pair mismatching between the incoming targeting vector and the native DNA (te Riele et al., 1992, van Deursen and Wieringa, 1992).

Embryonic stem cells are used to create null mutations because they have been shown to retain germline competence after long periods of in vitro culture and after genetic manipulation, although the accumulation of mutations, particularly those resulting in karyotypic abnormalities may result in the loss of germline competence (Hogan et al., 1994). Germline competence of the ES cells can be assessed by injecting the cells into blastocysts of a strain of mice with a different coat color and then crossing the resulting chimeras such that ES cell contribution in the offspring can be identified via

coat color. Alternatively, potential germline competence can be assessed by the presence of strain-specific isozyme markers, such as glucosephosphate isomerase (GPI), in the sperm of male chimeras, where the ES cells and the blastocyst are derived from strains expressing different isoforms of GPI. These isozymes can be identified by differences in electrophoretic mobility.

## 4.2 Construction of *Gbx2* targeting vectors

### *4.2.1 Vector design*

Isogenic DNA from an E14TG2a genomic library (Chapman et al., 1997; section 3.2) was used for preparing the targeting vector to minimize base pair mismatching during homologous recombination. A 5 kb Xho I fragment containing the entire *Gbx2* sequence was isolated from a λ2001 genomic library and cloned into pBluescript II SK (clone pGXH5, section 3.2, Fig. 4.1). In order to ensure creation of a *Gbx2* null mutation, targeting vectors were designed to remove the entire protein coding region, from the Mlu I site 29 bp upstream of the ATG site to the Nco I site 24 bp downstream of the stop codon, or to the Mlu I site approximately 900 bp downstream of the stop codon. This region was replaced with a selection cassette comprising either a neomycin resistance or a hygromycin resistance gene positioned between a phosphoglycerate kinase (PGK) promotor and a polyA termination sequence (Gassmann et al., 1995).

### *4.2.2 Neomycin resistant vector construction*

The 5kb genomic clone (pGXH5) was digested with Mlu I and Nco I, treated with Calf Intestinal Alkaline Phosphatase (CIP) and endfilled (2.3.2). A 5.8 kb band containing the Bluescript vector backbone, the 5' end and the 3' end of the *Gbx2* gene was purified by agarose gel electrophoresis. The selection marker plasmid pMGD20neo (Gassmann et al., 1995) was digested with Eco RI and BamH I and endfilled and a 1.4 kb fragment was purified. The two fragments were ligated and used to transform E. coli. Resulting clones were screened by restriction enzyme mapping for integration of the neo cassette in a 5'-3' direction (pGbN) (Fig. 4.2) which contained 2.15 kb of *Gbx2* homology 5' of the insert and 0.7 kb of *Gbx2* homology 3' of the insert.

Total amounts of homology greater than 2 kb surrounding the replacement site are required to maximize the frequency of homologous recombination (Deng and Capecchi, 1992). To increase the *Gbx2* homology in the targeting vector and to provide external probes for screening it was necessary to clone the genomic regions

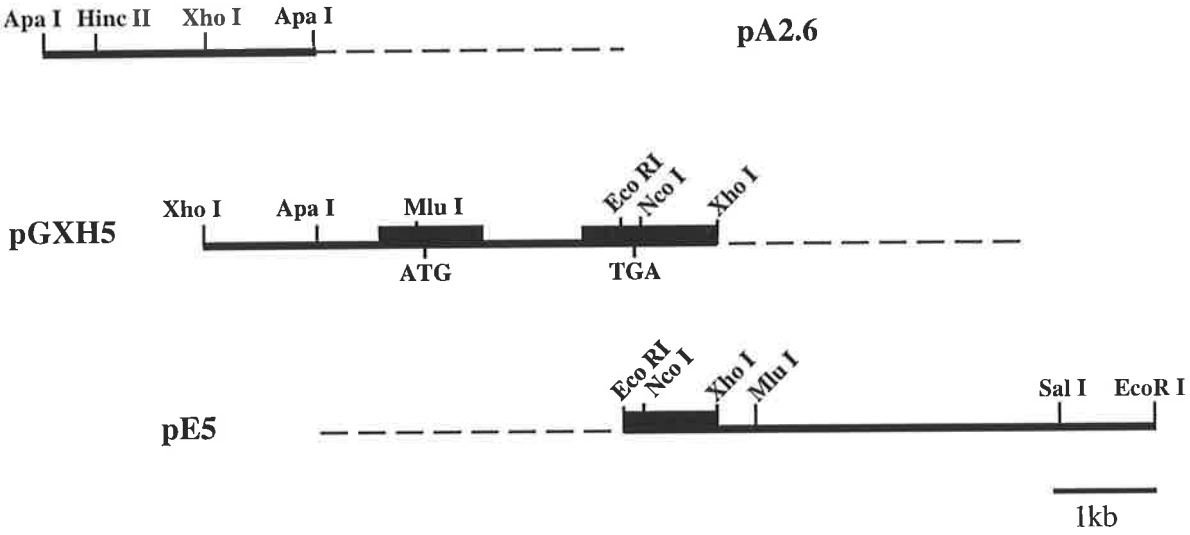



**Figure 4.1** ***Gbx2*** **genomic clones**

pA2.6: A 2.6 kb Apa I fragment from the region 5' of *Gbx2*. The 3' end overlaps pGXH5 between the Xho I and Apa I sites. The Hinc II/Xho I fragment was used as a 5' external probe for Southern blot analysis.

pGXH5: A 5.0 kb Xho I fragment containing the *Gbx2* coding region.

pE5: A 5.2 kb Eco RI fragment from the region 3' of *Gbx2*. The 5' end overlaps pGXH5 between the Eco RI and Xho I sites. The Sal I/Eco RI fragment was used as a 3' external probe for Southern blot analysis.

— — = pBluescript II SK sequence, ▬ = *Gbx2* genomic sequence,

▬ = *Gbx2* exon sequence,

**Figure 4.2 Targeting vector construction - neomycin containing vector**

Schematic drawing of the construction of a neomycin containing targeting vector:

a. pMGD20neo was digested with Eco RI and BamH I, endfilled, and the 1.4 kb fragment containing the PGK driven, neomycin-polyA cassette was purified.

b. pGXH5 was digested with Mlu I and Nco I, CIP treated, endfilled and the 5.8 kb band containing the Bluescript vector and 5' and 3' ends of *Gbx2* gene was purified.

c. The 2 fragments from a. and b. were ligated, creating pGbN, a plasmid containing 2.15 kb of 5' *Gbx2* homology and 0.7 kb of 3' *Gbx2* homology.

d. pGbN was digested with Xho I and the 4 kb fragment containing the *Gbx2* homology and neo insert was purified.

e. pE5 was digested with Xho I, CIP treated and the 7.2 kb fragment containing an additional 3.4 kb of 3' *Gbx2* homology and the Bluescript vector was purified.

f. The 2 fragments from c. and d. were ligated, creating pGNE.11, the targeting vector consisting of 2.15 kb of 5' homology and 4.1 kb of 3' homology surrounding the neo selection cassette. The remaining 0.86 kb Sal I/ EcoR I fragment at the 3' end of the vector was used as an external probe for Southern analysis of targeted lines.

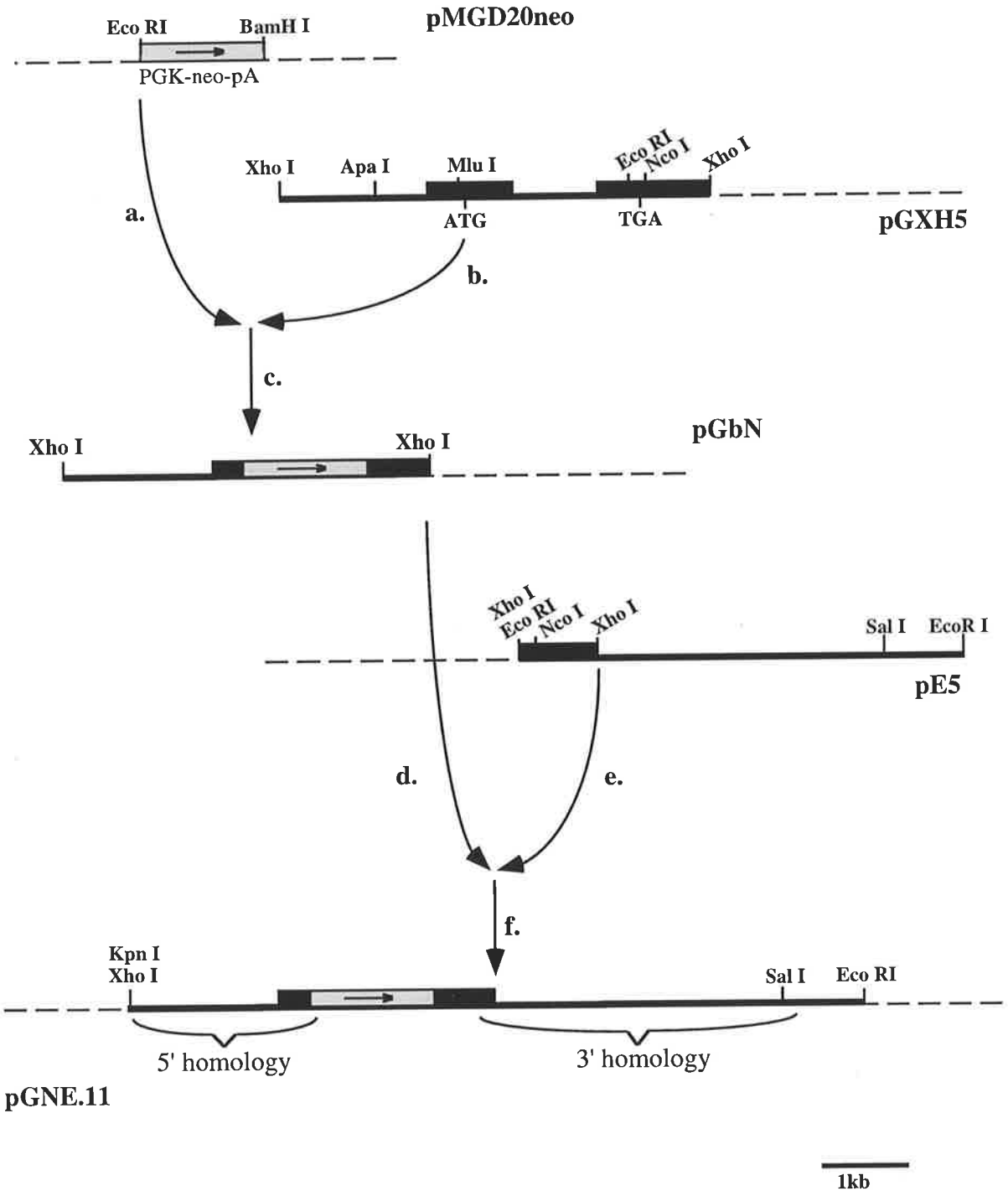
— — — = pBluescript II SK sequence

▬▬ = *Gbx2* genomic sequence

▮▮ = *Gbx2* exon sequence

⊏⟹⊐ = PGKneo cassette (arrow indicates direction of transcription)

**Figure 4.2**


pMGD20neo
Eco RI
BamH I
PGK-neo-pA
a.
Xho I
Apa I
Mlu I
Eco RI
Nco I
Xho I
ATG
TGA
pGXH5
b.
c.
Xho I
Xho I
pGbN
Xho I
Eco RI
Nco I
Xho I
Sal I
EcoR I
pE5
d.
e.
f.
Kpn I
Xho I
Sal I
Eco RI
5' homology
3' homology
pGNE.11
1kb

immediately 5' and 3' of the 5 kb Xho I fragment. A 2.6 kb Apa I fragment of the genomic lambda clone λ5.1 which hybridized to a probe from the 5' untranslated region of *Gbx2* cDNA (section 3.2), was subcloned into the Apa I site of Bluescript II SK (pA2.6, Fig. 4.1). This clone contained a 1.1 kb overlap with the 5' end of pGXH5. A 5.2 kb Eco RI fragment of the genomic lambda clone λ5.1 which hybridized to a probe from the 3' untranslated region of *Gbx2* cDNA (section 3.2, Fig. 3.2c), was subcloned into the Eco RI site of Bluescript II SK (pE5, Fig. 4.1). This clone contained a 970 bp overlap with the 3' end of pGXH5.

pE5 was digested with Xho I and treated with CIP. The 7.2 kb fragment containing the additional *Gbx2* homologous sequence and the Bluescript II vector backbone was purified and ligated to a 4.25 kb Xho I fragment of pGbN containing the *Gbx2* sequence and neo insert. The resulting targeting vector (pGNE.11, Fig. 4.2) was 11.4 kb. When linearized with Sal I and Kpn I, it yielded an 8.5 kb fragment with 2.15 kb of *Gbx2* homology at the 5' end and 4.1 kb of *Gbx2* homology at the 3' end.

### *4.2.3 Hygromycin resistant vector construction*

The 5 kb genomic clone pGXH5 was digested with Mlu I and Xho I and a 2.2 kb band containing 5' *Gbx2* sequences was purified by gel electrophoresis. pE5 was digested with Mlu I and Xho I, CIP treated, and the 7.0 kb band containing the vector and 4.0 kb of 3' *Gbx2* homology was purified by gel electrophoresis. These 2 fragments were ligated to produce pGmE5 (Fig. 4.3).

The selection marker plasmid PGKhygro/deltaLT20 (Gassmann et al., 1995) was digested with Cla I and Sma I, endfilled, and a 1.8 kb fragment containing the hygro cassette was purified by electrophoresis. pGmE5 was digested with Mlu I, endfilled, CIP treated and purified by electrophoresis. The two fragments were ligated to produce pGmHgE5, in which the hygro cassette was integrated in a 5'-3' direction (Fig 4.3).

It was necessary to remove 0.9 kb from the 3' end of the *Gbx2* homologous region for use as an external probe for screening targeted lines. In addition, a thymidine kinase selection marker was added at the 3' end of the hygromycin vector in order to facilitate selection of homologous recombinants over random integrants in the targeting process by the use of negative selection (Mansour et al., 1988). pGmHgE5 was digested with Not I, endfilled, digested with Sal I, CIP treated, and the 11 kb fragment was purified by gel electrophoresis. A thymidine kinase selection marker (pMC1TK2, obtained from M. Capecchi, Howard Hughes Medical Institute, Salt Lake City, Utah), consisting of the HSV-*tk* gene driven by the promotor from pMC1-Neo (Thomas and

**Figure 4.3 Targeting vector construction - hygromycin containing vector**

Schematic drawing of the construction of a hygromycin containing targeting vector:

a. pGXH5 was digested with Mlu I and Nco I, CIP treated, endfilled and the 2.2 kb band containing 5' *Gbx2* homology was purified.

b. pE5 was digested with Mlu I and Xho I, CIP treated and the 7.0 kb fragment containing 4.0 kb of 3' *Gbx2* homology and the Bluescript vector was purified.

c. The 2 fragments from a. and b. were ligated, creating pGmE5.

d. pGmE5 was digested with Mlu I, endfilled, CIP treated and the 9.2 kb linearized plasmid was purified.

e. PGKhygro/deltaLT20 was digested with Cla I and Sma I, endfilled, and the 1.8 kb fragment containing the PGK driven, hygromycin-polyA cassette was purified.

f. The 2 fragments from c. and d. were ligated, creating pGmHgE5, a plasmid containing 2.15 kb of 5' *Gbx2* homology and 4.0 kb of 3' *Gbx2* homology surrounding the hygro selection cassette.

g. pGmHgE5 was digested with Not I, endfilled, digested with Sal I, CIP treated, and the 10.1kb linearized plasmid was purified.

h. pMC1TK2 was digested with Sac I, endchewed, digested with Sal I, and the 2.1 kb fragment containing the MC1 driven HSV-tk2 cassette was purified.

i. The 2 fragments from e. and f. were ligated, creating pGHT, the 12.2 kb targeting vector consisting of 2.15 kb of 5' *Gbx2* homology and 3.1 kb of 3' *Gbx2* homology surrounding the hygro selection cassette with the TK cassette and vector at the 3' end.



= pBluescript II SK seqeuence



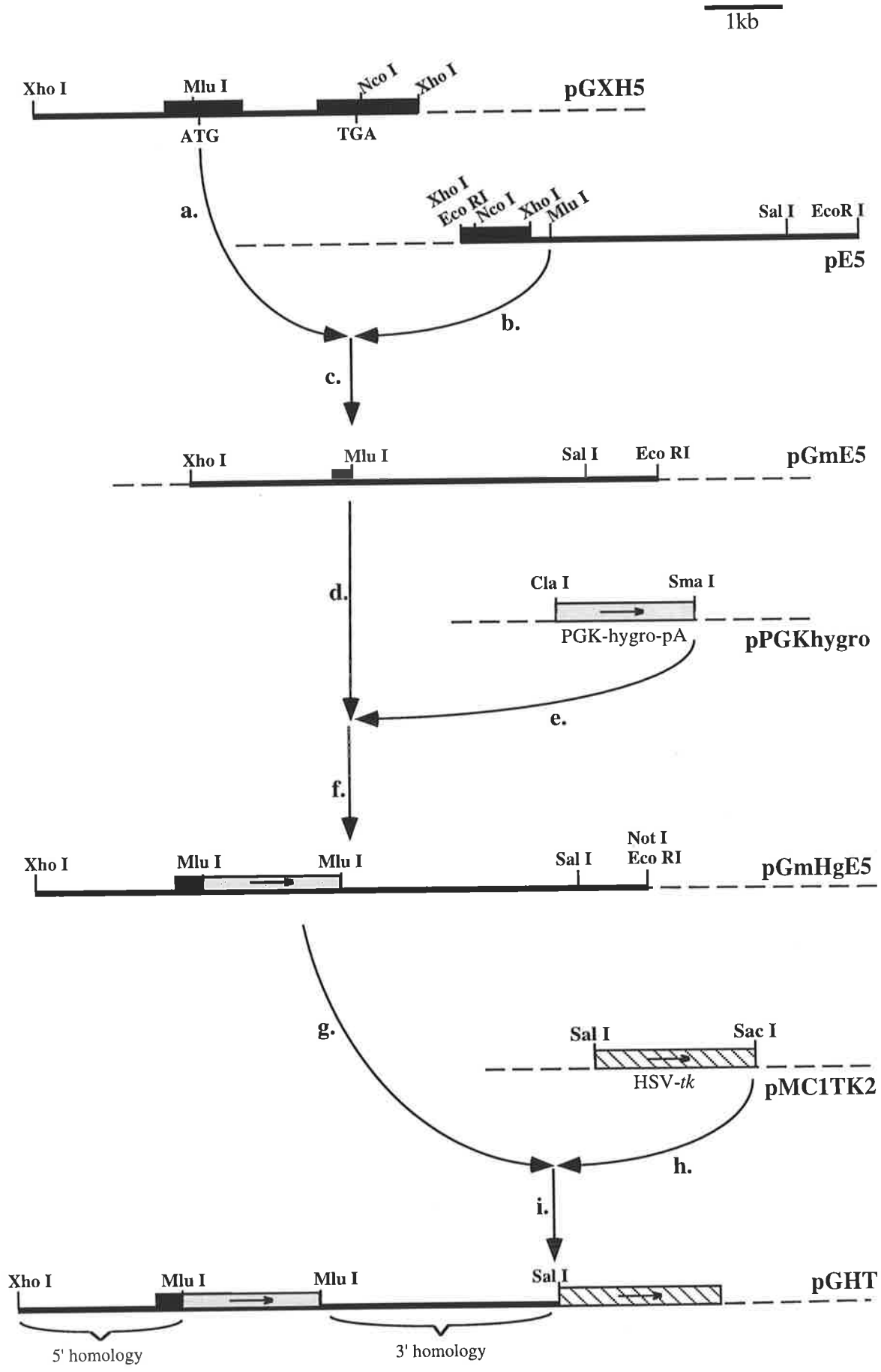
= *Gbx2* genomic sequence

= *Gbx2* exon sequence

= PGKhygro cassette (arrow indicates direction of transcription)

= TK cassette (arrow indicates direction of transcription)

**Figure 4.3**


1kb
Xho I
Mlu I
Nco I
Xho I
pGXH5
ATG
TGA
a.
Xho I
Eco RI
Nco I
Xho I
Mlu I
Sal I
EcoR I
pE5
b.
c.
Xho I
Mlu I
Sal I
Eco RI
pGmE5
d.
Cla I
Sma I
PGK-hygro-pA
pPGKhygro
e.
f.
Xho I
Mlu I
Mlu I
Sal I
Not I
Eco RI
pGmHgE5
g.
Sal I
Sac I
HSV-tk
pMC1TK2
h.
i.
Xho I
Mlu I
Mlu I
Sal I
pGHT
5' homology
3' homology

Capecchi, 1987) was digested with Sac I, blunt ended by the endchew method (2.3.3), digested with Sal I and a 2.1 kb band containing the thymidine kinase cassette was purified by electrophoresis. The two fragments were ligated to produce pGHT which contains the selection cassette integrated in the same transcriptional orientation as *Gbx2* (Fig 4.3).

The complete targeting vector, pGHT, was 12.2 kb and contained 2.15 kb of *Gbx2* homology 5' of the hygromycin resistance insert and 3.1 kb of *Gbx2* homology 3' of the insert, followed by the thymidine kinase selection cassette and the Bluescript vector backbone linearized with Xho I (Fig 4.3).

## 4.3 Creation of ES cell lines heterozygous for a *Gbx2* null mutation

### *4.3.1 Determination of gene targeting selection conditions*

The E14 ES cell line derivative E14TG2a (Hooper et al., 1987) was used for gene targeting at the *Gbx2* locus as it had been previously shown to be germline competent following multiple rounds of gene targeting in vitro in this laboratory (Whyatt and Rathjen, 1997).

The levels of G418 and hygromycin required for effective selection against unmodified E14TG2a ES cells were determined by culture of E14TG2a cells in the presence of varying concentrations of G418 (175-250μg/ml) or hygromycin (50-300μg/ml). Cell death was assessed by microscopic examination compared to cultures grown in the absence of antibiotic. A G418 concentration of 200 μg/ml was sufficient to cause greater than 99 percent cell death (Table 4.1) after 5 days. A hygromycin concentration of 100 μg/ml was sufficient to cause greater than 99 percent cell death (Table 4.1) after 3 days. Concentrations of 200 μg/ml G418 or 110 μg/ml hygromycin were used in the gene targeting procedures as these were the minimal concentrations required to achieve effective selection against non-transfected cells.

### *4.3.2 ES cell targeting*

$1x10^8$ E14TG2a ES cells, passage number 22, were transfected by electroporation with 150 μg pGNE.11 DNA linearized by digestion with Sal I and Kpn I (Fig 4.2) or pGHT DNA linearized with Xho I (Fig. 4.3). The electroporation conditions (2.4.5) have been shown to minimise cell death caused by electroporation and to prevent the integration of multiple copies of the targeting vector into the genome (Mountford et al., 1994). The electroporated cells were plated at a density of $1x10^5$ cells/ml in complete ES (CES) medium and after 24 hours the medium was changed to G418 or

**a. G418**

| G418 Conc. | % cell death - day 4 | % cell death - day 5 |
|---|---|---|
| 175 µg/ml | 50-60 | >95 |
| 200 µg/ml | >95 | >99 |
| 250 µg/ml | >99 | >99 |

**b. Hygromycin**

| Hygro Conc. | % cell death - day 3 |
|---|---|
| 50 µg/ml | 80 |
| 100 µg/ml | >99 |
| 150 µg/ml | >99 |
| 200 µg/ml | >99 |
| 300 µg/ml | >99 |

**Table 4.1 Results of selection media toxicity assays**

a. E14TG2a ES cells were grown in the presence of G418 selective medium at indicated concentrations and examined at days 4 and 5 for cell death.

b. E14TG2a ES cells were grown in the presence of hygromycin selective medium at various concentrations and examined at day 3 for cell death.

hygromycin selection medium. After 6-8 days of selection, single colonies were isolated, trypsinized, and replated into 2 wells of a 24 well plate in CES medium and expanded (2.4.7). Cells from one well were frozen (2.4.7) and cells from the other were used to prepare genomic DNA (2.3.18) for Southern blot analysis.

### *4.3.3 Southern blot analysis of resistant lines*

20 µg genomic DNA isolated from G418 or hygromycin resistant cell lines was digested with Hinc II, Eco RI, or Eco RI and Eco RV and subjected to Southern analysis (2.3.19). Probes which hybridized to regions external to the targeted region (Fig. 4.4a) were used to identify lines with a correctly inserted targeting vector.

**5' external probe:** This probe was a 950 bp Hinc II/ Xho I fragment of pA2.6 (4.2.2), encompassing the *Gbx2* genomic region immediately 5' of the targeting vectors. It hybridizes to a 5.2 kb Hinc II band from untargeted alleles, an 8.65 kb band from correctly targeted neo$^r$ containing alleles (Fig. 4.4b), and a 4.4 kb band from correctly targeted hygro$^r$ containing alleles (Fig 4.4c).

**3' external probe:** This probe was an 860 bp Sal I/ Eco RI fragment of pE5 (4.2.2), encompassing the *Gbx2* genomic region immediately 3' of the targeting vectors. It hybridizes to a 5.2 kb Eco RI band from untargeted alleles, a 6.4 kb Eco RI band from correctly targeted neo$^r$ containing alleles (Fig. 4.4b), and a 4.1 kb Eco RI/ Eco RV band from correctly targeted hygro$^r$ containing alleles (Fig 4.4c).

**Neo probe:** A 685 bp Pst I fragment from the neo gene in pMGD20 was used to detect the neo selection cassette in transfected lines selected in G418 medium to confirm that correctly targeted ES cell lines contained a single vector insert. Filters containing a correctly targeted clone as determined by 5' or 3' external probes were stripped by boiling in 0.1% SDS for 10 min. and reprobed with the neo probe. It hybridizes to an 8.65 kb Hinc II kb band and a 6.4 kb Eco RI band from correctly targeted neo$^r$ containing alleles (Fig. 4.4b).

**Hygro probe:** A 474 bp Hinc II/Sma I fragment from the hygro gene in PGKhygro/deltaLT20 was used to detect the hygro selection cassette in transfected lines selected in hygromycin medium. Filters containing a correctly targeted clone as determined by 5' or 3' external probes were stripped by boiling in 0.1% SDS for 10 minutes and reprobed with the hygro probe. It hybridizes to a 4.4 kb Hinc II band and a 4.1 kb Eco RI/ Eco RV band from correctly targeted hygro$^r$ containing alleles (Fig 4.4c).

Three independent transfections, selections, and screenings were performed. A total of 487 clonal lines were screened by Southern blot analysis and 8 correctly targeted lines were identified, representing an average targeting frequency of 1.6% (Table 4.2, Fig. 4.4b and c). These cell lines maintained normal ES cell-like morphology through

**Figure 4.4 First round targeting**

a. Representation of the general targeting strategy using either the neomycin containing targeting vector or the hygromycin containing targeting vector by homologous recombination. 1) The vector is linearized by restriction enzyme digest and electroporated into 2) ES cells with 2 wildtype alleles. 3) Following selection in antibiotic medium, clones are screened for correctly targeted integration events using probes from outside the regions of homology. Positions of restriction enzyme sites are shown.

b. Southern blot analysis of neomycin resistant clones. Genomic DNA was digested with either Hinc II or EcoR I. The digests were electrophoresed and then blotted onto nylon membranes. The membranes were probed with either 5' or 3' external probes. Membranes containing positive clones (shown here as 132, 159, and 185) were stripped and reprobed with a neomycin probe to confirm a single integration event.

c. Southern blot analysis of hygromycin resistant clones. Genomic DNA was prepared digested with either Hinc II or EcoR I/ EcoR V. The digests were electrophoresed and then blotted onto nylon membranes. The membranes were probed with either 5' or 3' external probes. Membranes containing positive clones (shown here as 92) were stripped and reprobed with a hygromycin probe to confirm a single integration event.

## Figure 4.4

**a. General targeting strategy**

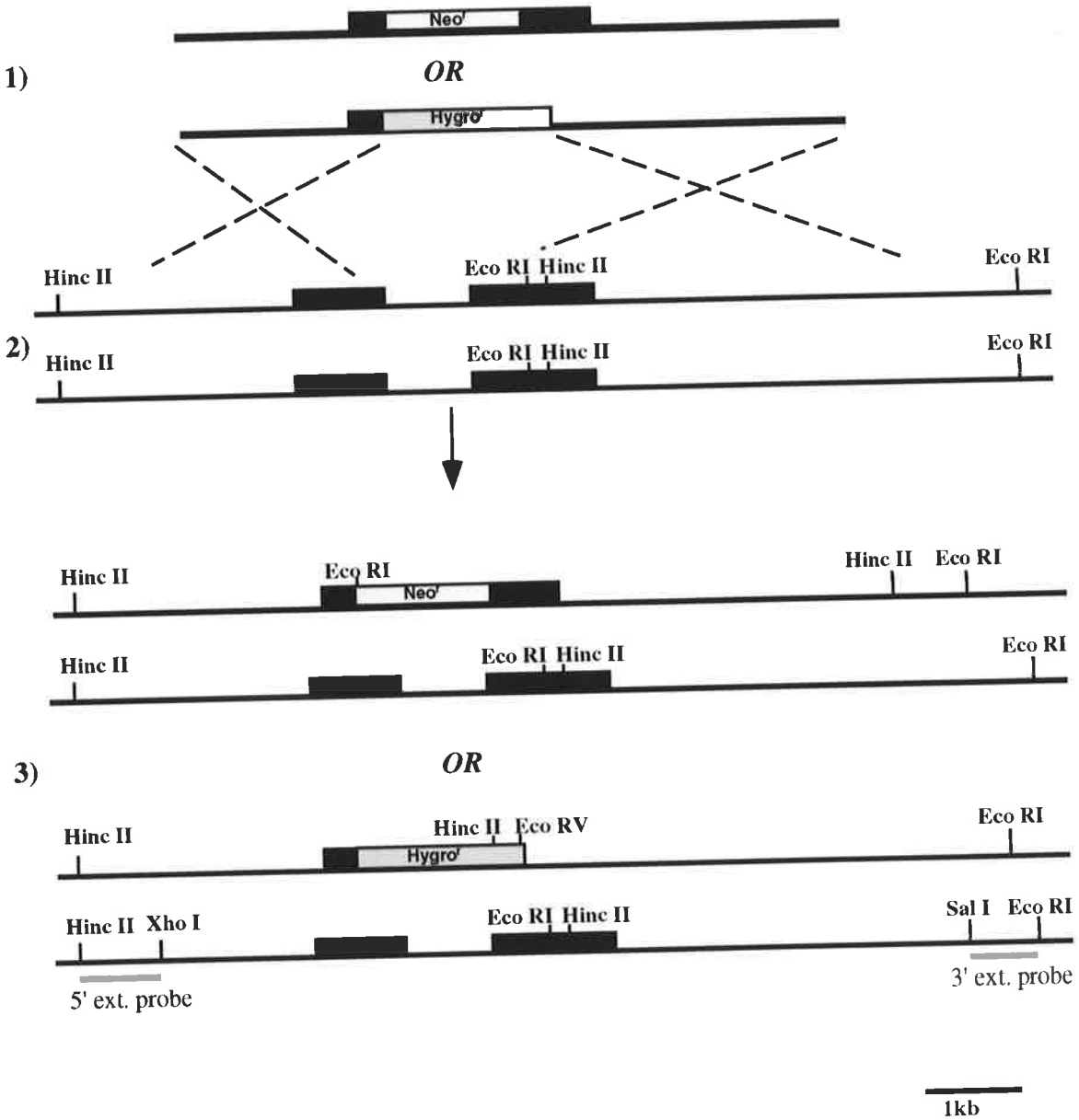
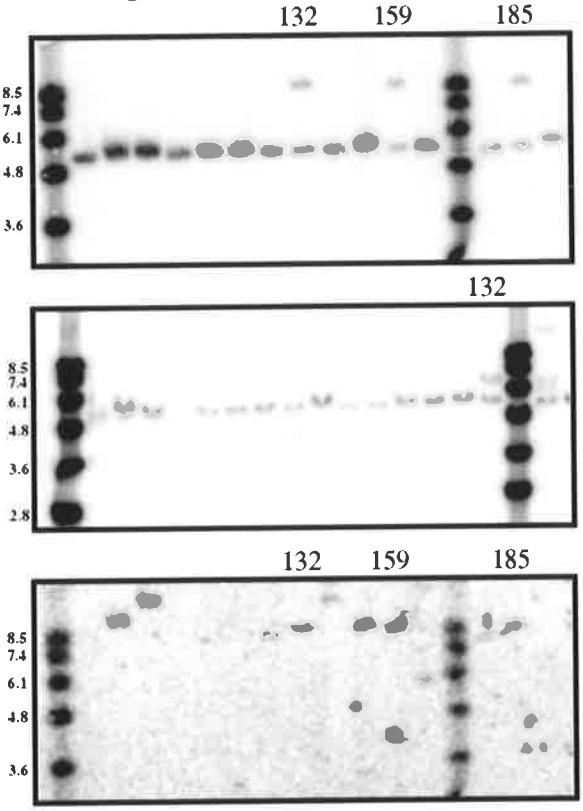


**Figure 4.4 (Continued)**

**b. Screening of neomycin resistant clones**




**Expected band size:**

**5' probe** (Hinc II digest):

WT: 5.2 kb

Neo: 8.6 kb

**3' probe** (Eco RI digest):

WT: 5.2 kb

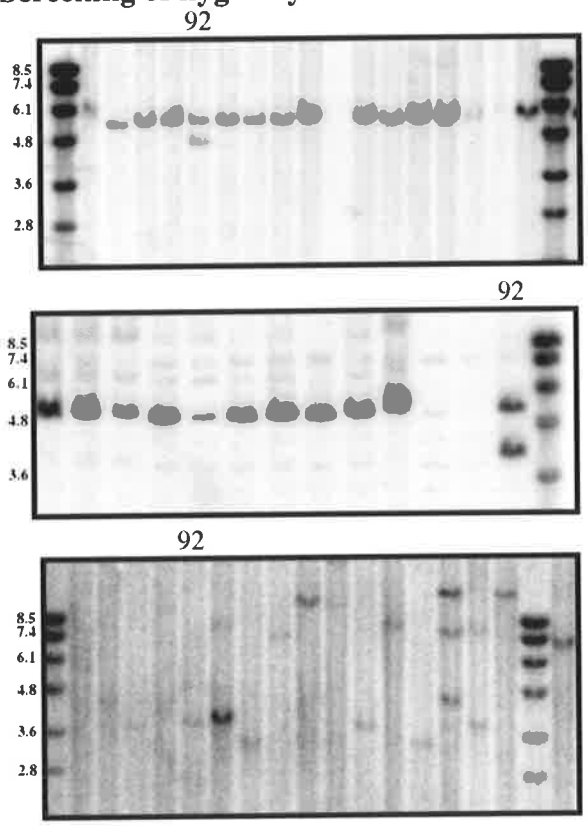
Neo: 6.4 kb

**Neo probe** (Hinc II digest):

Reprobed Hinc II digest membrane with neo probe

Neo: single band at 8.6 kb

**c. Screening of hygromycin resistant clones**




**Expected band size:**

**5' probe** (Hinc II digest):

WT: 5.2 kb

Hygro: 4.4 kb

**3' probe** (Eco RI/Eco RV digest):

WT: 5.2 kb

Hygro: 4.1 kb

**Hygro probe** (Hinc II digest):

Reprobed Hinc II digest membrane with hygro probe

Hygro: single band at 4.4 kb

repeated passages. One cell line, GNE.1076, was thought to possibly contain cells of both untargeted and targeted origin based on the fainter intensity of targeted bands as compared to the untargeted bands. This cell line was thawed, expanded, and plated at low density in G418 selection medium. Single colonies were selected and grown as described in 4.3.2. DNA from these lines was analyzed by Southern blot and 14/19 clones were shown to be correctly targeted.

| Transfection # | Construct | Correctly Targeted Clones | # Screened | % Targeting Efficiency |
|---|---|---|---|---|
| 1 | GNE.11 | 3 | 162 | 1.85 |
| 2 | GNE.11 | 1 | 96 | 1.04 |
| 3 | GHT | 4 | 229 | 1.75 |
| **Total** | | **8** | **487** | **1.6** |

**Table 4.2 Results from first round targeting**

Results of three separate targeting events using either neomycin (GNE.11) or hygromycin (GHT) containing vectors and E14TG2a ES cells.

Six correctly targeted lines (GNE.132, GNE.159, GNE.185, GNE.1076.8, H1.32, and H1.183) were microinjected into to mouse blastocysts in order to assess their ability to contribute to chimeric mice and their germline competence (section 4.5).

## 4.4 Creation of ES cell lines containing a *Gbx2* null mutation

### *4.4.1 Null mutations in vitro*

ES cell lines containing null mutations in both alleles of the *Gbx2* gene were required for in vitro differentiation assays (chapter 6). ES cells with homozygous mutations can be isolated directly from mutant embryos (Nagy and Rossant, 1996) or by genetic manipulation of heterozygous mutant lines. The latter can be achieved either by sequential targeting using different selection markers (te Riele et al., 1990) or by selection for higher levels of expression of the neomycin resistance gene by elevating the concentration of G418 (Mortensen et al., 1992), thereby selecting for rare cells in which gene conversion has replaced the wildtype allele with a second copy of the targeted allele. Sequential targeting with different selection markers was used to achieve homozygous mutations of *Gbx2*.

### *4.4.2 ES cell targeting*

ES cell lines, GNE.159, H1.32, and GNE.1076.8, containing either the neo$^r$ or hygro$^r$ genes inserted into one allele at the *Gbx2* locus (4.3), were chosen for a second round of gene targeting based on their normal growth characteristics and morphology. These cell lines also had normal karyotypes (Table 4.4). $1x10^8$ ES cells were transfected (2.4.5) with a linearized vector, either GHT (cell lines GNE.159 and GNE.1076.8) or GNE.11 (cell line H1.32) (Fig. 4.4a). The electroporated cells were plated at a density of $1x10^5$ cells/ml in complete ES (CES) medium and after 24 hours the medium was changed to selection medium containing G418 (200 µg/ml) and hygromycin (110 µg/ml). The presence of G418 in the medium ensured that only cells which maintained the originally targeted allele would survive. The cells were washed twice with PBS and new medium was added every 1-2 days. After 9-14 days of selection, single colonies were isolated, trypsinized, and replated into 2 wells of a 24 well plate in CES medium and expanded (2.4.7). Cells from one well were frozen (2.4.7) and cells from the other were used to prepare genomic DNA (2.3.18) for Southern blot analysis.

### *4.4.3 Southern blot analysis of resistant lines*

20 µg genomic DNA isolated from G418/hygromycin resistant cell lines was digested with Hinc II, Eco RI, or Eco RI/Eco RV and subjected to Southern analysis (2.3.19). Probes which hybridized to regions external to the targeted region (Fig. 4.5a) were used to identify lines with a correctly inserted targeting vector.

**5' external probe:** This probe was a 950 bp Hinc II/ Xho I fragment of pA2.6 (4.2.2), encompassing the *Gbx2* genomic region immediately 5' of the targeting vectors. It hybridizes to a 5.2 kb Hinc II band from untargeted alleles, an 8.65 kb band from correctly targeted neo$^r$ containing alleles, and a 4.4 kb band from correctly targeted hygro$^r$ containing alleles (Fig 4.5b).

**3' external probe:** This probe was an 860 bp Sal I/ Eco RI fragment of pE5 (4.2.2), encompassing the *Gbx2* genomic region immediately 3' of the targeting vectors. It hybridizes to a 5.2 kb Eco RI band from untargeted alleles, a 6.4 kb Eco RI band from correctly targeted neo$^r$ containing alleles, and a 4.1 kb Eco RI/ Eco RV band from correctly targeted hygro$^r$ containing alleles (Fig 4.5b).

**Neo probe:** A 685 bp Pst I fragment from the neo gene in pMGD20 was used to detect the neo selection cassette in transfected lines selected in G418 medium to confirm that correctly targeted ES cell lines contained a single vector insert. Filters containing a correctly targeted clone as determined by 5' or 3' external probes were stripped by boiling in 0.1% SDS for 10 minutes and reprobed with the neo probe. It hybridizes to an 8.65 kb Hinc II kb band and a 6.4 kb Eco RI band from correctly targeted neo$^r$ containing alleles.

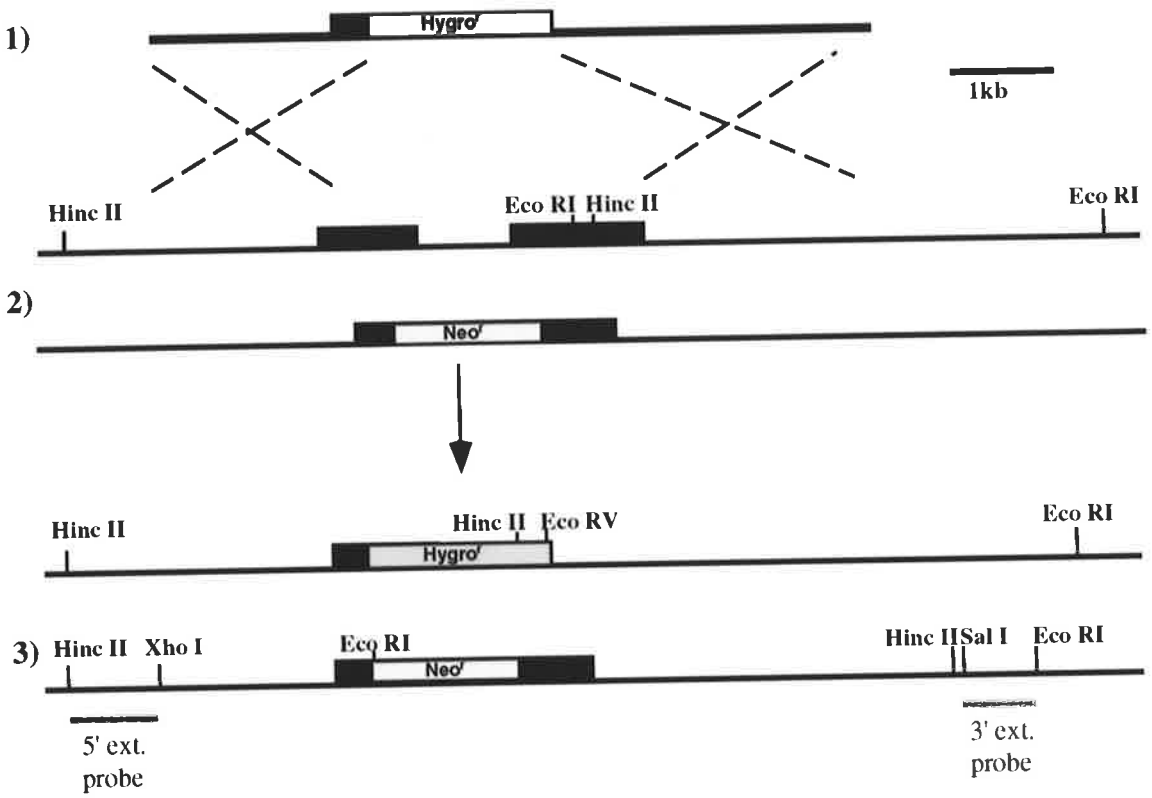
**Figure 4.5 Second round targeting**

a. Schematic of targeting strategy for the replacement of both alleles with targeted*Gbx2* loci by homologous recombination. 1) Linearized vector containing a antibiotic selection cassette, in this case hygromycin resistance (Hygro), is electroporated with 2) ES cells containing a single targeted allele, in this example with a neomycin selection cassette (Neo). 3) Clones are screened for correctly targeted integration events. Positions of restriction enzyme sites are shown.

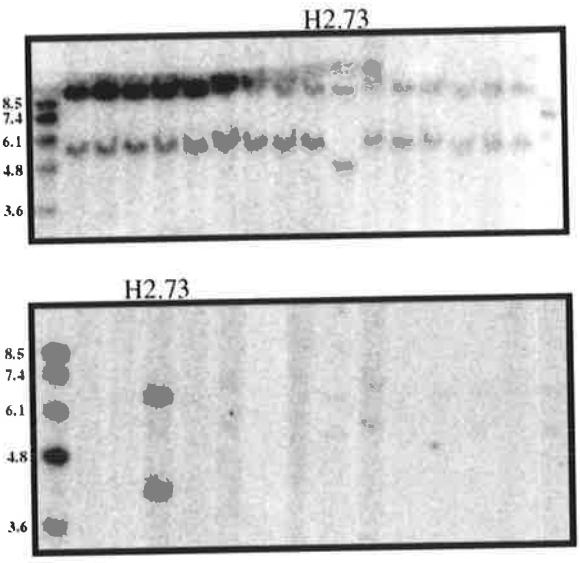
b. Southern blot analysis of neomycin/hygromycin resistant clones. Genomic DNA from clones was digested with either Hinc II or EcoR I/ EcoR V. The digests were electrophoresed and then blotted onto nylon membranes. The membranes were probed with either 5' or 3' external probes. Expected band sizes are shown.

## Figure 4.5

### a. Targeting strategy




### b. Screening neomycin/hygromycin resistant clones




**Expected band size:**

**5' probe** (Hinc II digest):

WT: 5.2 kb
Neo: 8.6 kb
Hygro: 4.4 kb

**3' probe** (Eco RI/Eco RV digest):

WT: 5.2 kb
Neo: 6.4 kb
Hygro: 4.1 kb

Three independent transfections, selections, and screenings were performed. 1,490 clonal lines were screened and one line, H2.73, was correctly targeted (Table 4.3, Fig. 4.5b). H2.73 was subjected to karyotypic analysis of chromosomal spreads (2.4.8, Fig. 4.6) and was found to contain 93% karyotypically normal cells. The morphology of this cell line was ES cell-like following extended passage in vitro (Fig. 4.7).

| Transfection # | Construct | ES cell line | Correctly Targeted Clones | # Screened | % Targeting Efficiency |
|---|---|---|---|---|---|
| 1 | GHT | GNE.159 | 1 | 197 | 0.5 |
| 2 | GNE.11 | H1.32 | 0 | 503 | |
| 3 | GHT | GNE.1076.8 | 0 | 790 | |
| **Total** | | | **1** | **1490** | |

**Table 4.3 Results from second round targeting**
Results of three separate targeting events using either neomycin (GNE.11) or hygromycin (GHT) containing vectors and ES cell lines from first round targeting containing either a neomycin (GNE.159, GNE.1076) or hygromycin (H1.32) targeted *Gbx2* allele.

## 4.5 Assessment of germline competence of ES cell lines heterozygous at the *Gbx2* locus

### *4.5.1 Karyotype analysis and creation of chimeras*

Karyotypic analysis of heterozygous *Gbx2* targeted ES cell lines was carried out by examination of chromosomal spreads (2.4.8). Five out of the eight correctly targeted ES cell lines (GNE.132, GNE.159, GNE.1076.8, H1.32, and H1.183) had normal karyotypes in greater than 50% of the smears examined (Table 4.4).

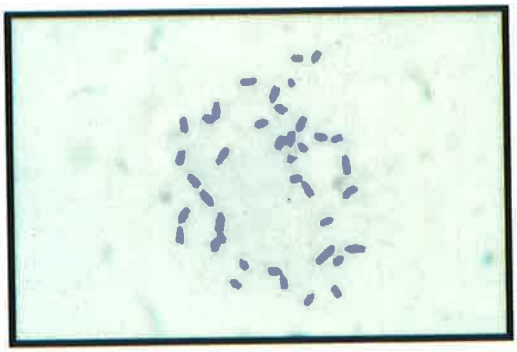


**Figure 4.6 Karyotype of homozygous *Gbx2* mutant ES cells**

Photograph of a chromosomal spread of H2.73 (homozygous mutant) ES cells. Magnified 1000x.

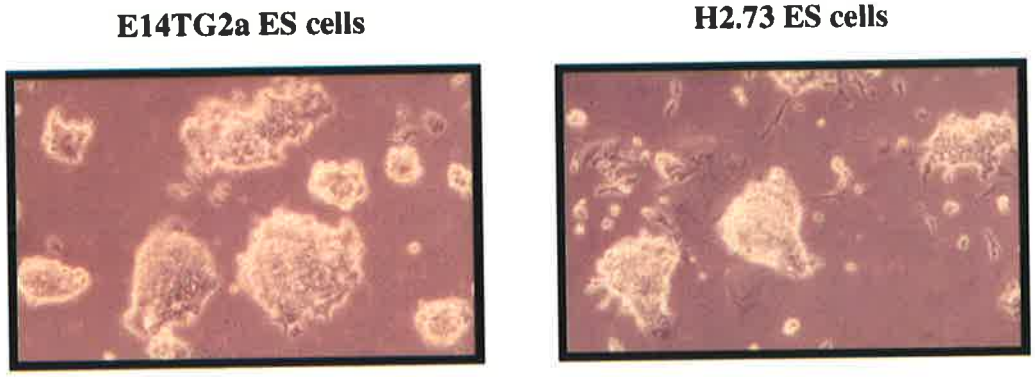



**Figure 4.7 ES cell phenotype**

Photograph of E14TG2a (wildtype), passage 29, and H2.73 (homozygous mutant), passage 6, ES cells growing on tissue culture plastic in ES cell medium. Magnified 125x.

| ES cell line | 39 chromosomes | 40 chromosomes | 41 chromosomes |
|---|---|---|---|
| GNE132 | 20 % | 75 % | 5 % |
| GNE159 | 5 % | 80 % | 15 % |
| GNE185 | 5 % | 45 % | 50 % |
| GNE1076.8 | 6.5 % | 87 % | 6.5 % |
| H1.32 | 13 % | 87 % | 0 % |
| H1.37 | 0 % | 20 % | 80 % |
| H1.92 | 90 % | 10 % | 0 % |
| H1.183 | 0 % | 100 % | 0 % |

**Table 4.4 Karyotypic analysis of heterozygous targeted ES cell lines**

Results of karyotypic analysis showing the number of chromosomes counted in chromosome spreads prepared from targeted ES cell lines, expressed as percentages.

These five targeted lines were microinjected into 3.5 d.p.c. CBA F2 host blastocysts. Injected blastocysts were placed in the uteri of pseudopregnant females. Microinjection of ES cells was carried out in collaboration with Steve McIlfatrick (BresaGen Ltd., Adelaide) and Joe Wrin (Department of Biochemistry, Adelaide University). Offspring were assessed for chimerism by coat color examination (Fig. 4.8) and 112 chimeras were identified out of a total of 216 pups (52%) with the percentage chimerism ranging from one to 85 percent (Table 4.5). The chimeras appeared mostly to be healthy, although three highly chimeric pups were runted at birth and did not survive beyond the first week. Also, there were rare abnormalities in the eyes and one chimera had a deformed foreleg. Highly chimeric animals often show a bias toward the male phenotype as the ES cells generally used are male. Six of the chimeras with ≥50% chimerism by coat color were female (50%, 50%, 50%, 60%, 80%, and 85% chimeric) and three were male (55%, 60%, and 75% chimeric). This could indicate a failure of ES cell derived sperm to survive, however the number of animals and percent chimerism were too low to support this conclusion and this conclusion is inconsistent with the mutant phenotype observed by Wassarman et al. (1997).

| Cell line | # µinj. | # embryos transferred | # pups | # chimeras | Range of % chimerism |
|---|---|---|---|---|---|
| GNE 132 | 11 | 266 | 35 | 12 | 1-5% |
| GNE 159 | 9 | 228 | 60 | 32 | 1-60% |
| GNE 1076.8 | 5 | 155 | 52 | 35 | 1-60% |
| H1.183 | 4 | 167 | 45 | 13 | 3-20% |
| H1.32 | 4 | 127 | 24 | 20 | 1-85% |
| **TOTAL** | | **943** | **216** | **112** | |

**Table 4.5 Analysis of chimeric mice produced from targeted ES cell lines**

Summarized results of chimeric mice produced by microinjection of 6 targeted ES cell lines into host blastocysts. Each ES cell line was injected on a number of days (# µinj.). The number of chimeric pups and the range of coat color chimerism is listed.

### *4.5.2 Germline competence of targeted ES cell lines*

Chimeric mice produced from ES cell lines GNE.159, GNE.185, GNE.1076.8, H1.32, and H1.183 were mated in order to assess the ability of the ES cells to contribute to the germ line. Mice from ES cell line GNE.132 were not mated due to low levels of chimerism. Thirty-three male chimeras, ranging in percentage chimerism from 4 to 75 percent were mated to BALB/c females. Five chimeric females (25%, 25%, 30%, 80% and 85% chimeric) were mated to BALB/c males. Most of the chimeric matings produced between one and nine litters, however 6 of the 33 males were unable to sire any pups. There was no ES cell contribution, as determined by coat color (Fig. 4.8), in any of the 1,748 offspring produced. These results are summarized in Table 4.6.

### *4.5.3 GPI analysis of chimeric males*

GPI analysis was carried out in order to determine if the observation that none of the offspring were generated from ES cell derived germ cells was due to an inability of the targeted ES cells to contribute to the germ cell population. Mouse strains differ in the expression of glucosephosphate isomerase (GPI) enzyme variants and this can be used to trace cells or their derivatives following introduction into a mouse of a different strain (Lyon and Searle, 1989). GPI is encoded by a single locus with two major

| Chimera | % Chimerism | # of litters | # of pups (agouti) |
|---|---|---|---|
| **GNE.1076.8:** | | | |
| BI86 #3 (F) | 30 | 3 | 24 |
| BI87 #1 | 5 | 6 | 72 |
| #2 | 5 | 7 | 71 |
| #3 | 5 | 7 | 89 |
| #4 | 4 | 5 | 66 |
| #5 | 4 | 5 | 46 |
| BI88 #1 | 40 | 7 | 94 |
| #2 | 10 | 6 | 56 |
| #9 (F) | 25 | 2 | 17 |
| SM9 #1 | 7 | 4 | 46 |
| SM10 #1 | 25 | 1 | 7 |
| #2 | 8 | 7 | 74 |
| **TOTAL** | | | **662** |
| **H1.183:** | | | |
| SM2 #2 | 60A | 6 | 61 |
| SM3 #1 | 6 | 4 | 42 |
| SM4 #1 | 20 | 2 | 16 |
| #2 | 18 | 7 | 82 |
| #3 | 8 | 4 | 42 |
| **TOTAL** | | | **243** |
| **H1.32:** | | | |
| SM6 #1 | 45 | 6 | 90 |
| #2 | 25 | 9 | 79 |
| #4 | 3 | 6 | 68 |
| #6 (F) | 80 | 6 | 47 |
| SM8 #1 | 75 | 0 | 0 |
| #2 | 55 | 8 | 72 |
| #3 | 30 | 6 | 73 |
| #4 | 20 | 0 | 0 |
| #5 (F) | 85 | 4 | 21 |
| **TOTAL** | | | **450** |
| **GNE.159:** | | | |
| BI42 #1 | 10 | 0 | 0 |
| #2 | 5 | 7 | 71 |
| BI47 #1 | 5 | 4 | 35 |
| #2 | 5 | 0 | 0 |
| #9 (F) | 25 | 3 | 25 |
| SI4 #3 | 5 | 0 | 0 |
| BI58 #1 | 15 | 0 | 0 |
| BI60 #1 | 10 | 7 | 61 |
| #2 | 5 | 6 | 58 |
| #3 | 5 | 6 | 65 |
| SI6 #1 | 40 | 9 | 78 |
| #2 | 15 | 0 | 0 |
| **TOTAL** | | | **393** |
| **GNE.132:** | none bred | | |

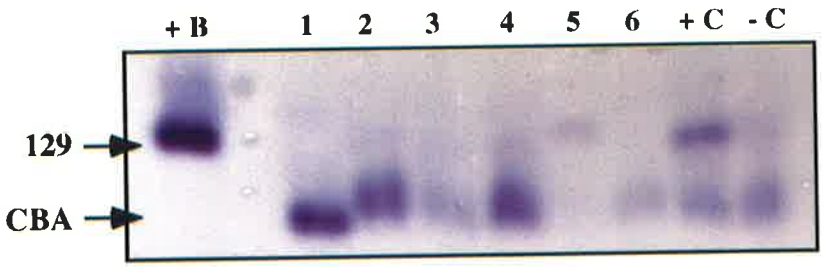
**Table 4.6 Analysis of germline transmission targeted ES of cell lines**

Results of cross breeding of 38 chimeric mice with BALB/c mates. All of the offspring displayed agouti coat color, reflecting host blastocyst rather than ES cell contribution to the germline.

SM8 # 1 2 3 4

**Figure 4.8 Chimeric mice**

Photograph of chimeric littermates produced with ES cell line H1.32.
Pale coat color (chinchilla) indicates ES cell contribution.
Dark coat color (agouti) indicates blastocyst contribution.




| Lane | Chimera | % Coat Chimerism | # offspring |
|---|---|---|---|
| 1 | BI88 #1 | 40% | 94 |
| 2 | SM6 #1 | 45% | 90 |
| 3 | SM8 #2 | 55% | 72 |
| 4 | SM8 #3 | 30% | 73 |
| 5 | SM8 #4 | 20% | 0 |
| 6 | SM10 #1 | 25% | 7 |

**Figure 4.9 GPI analysis of chimeric mice**

GPI analysis of tissue from epididymis of chimeric male mice (lanes 1-6).
129 mouse blood (lane +B), sperm of a chimera with 129 ES cell contribution
(lane +C), the sperm of a chimera with no detectable ES cell contribution (lane -C)

alleles, *Gpi-1s*$^a$ and *Gpi-1s*$^b$. The products of these alleles differ in electrophoretic ability and can be detected by starch gel electrophoresis followed by a visualization reaction based on the enzyme's catalytic activity (DeLorenzo and Ruddle, 1969). The *Gpi-1s*$^a$ allele occurs in strains which include 129 (Hooper et al., 1987) and BALB/c (DeLorenzo and Ruddle, 1969), while the *Gpi-1s*$^b$ allele is present in the wild and in inbred strains such as C57BL and CBA (DeLorenzo and Ruddle, 1969). E14TG2a derived ES cells were isolated from a 129 strain mouse (Hooper et al., 1987) and their derivatives in the tissues of a chimeric mouse can therefore be distinguished from host blastocyst-derived cells, in this case CBA strain.

Seven chimeric male mice displaying high coat color chimerism were sacrificed and sperm was collected from the epididymis of five of the animals. No sperm was able to be obtained from two animals (SM8 #1 and SM8 #4) which were sterile. One of these animals (SM8 #1) was found to have a small (approximately 1 cm diameter) tumor in the scrotal area, although the testes appeared intact. While the epididymis of SM8 #4 did not contain sperm, a number of other cell types were released when the epididymis was squeezed, including fat and blood cells. These were included in the GPI analysis. The cells were lyzed by repeated freeze-thaw cycles and the relative contribution of ES cell derived germ cells was determined by GPI isozyme analysis (Fig. 4.9). Samples SM6#1, SM8 #2, and SM8 #3 showed faint bands migrating to the same distance as the 129 (*Gpi-1s*$^a$) control, indicating low level contribution of ES cells to the sperm. In contrast, the SM8 #4 cells analyzed appeared to be entirely ES cell derived.

## 4.6 Discussion

### *4.6.1 Gene targeting and analysis of germline transmission of targeted ES cells*

This chapter describes the use of homologous recombination to replace the coding region of the *Gbx2* locus with a selectable marker in murine ES cells. Large regions of homologous DNA were placed on either side of the selection cassette and isogenic DNA was used to create the 5' and 3' homologous regions in order to minimize base pair mismatching during recombination and to maximize the frequency of homologous recombination. Electroporation conditions were used which have been previously demonstrated to minimize cell death and prevent integration of multiple copies of the targeting vector (Mountford et al., 1994; Whyatt and Rathjen, 1997).

Targeting of a single allele using a single selection medium resulted in 8 correctly targeted ES cell lines out of 487 clones screened, representing an average targeting efficiency of 1.6%. Targeting of the second allele by a second round of

electroporation and screening of heterozygous ES cell lines was much less efficient. Of the 1490 ES cell clones screened only one cell line was found to be correctly targeted in the second allele, suggesting a targeting efficiency of $7x10^{-4}$ or less. A decrease in efficiency would be expected as homologous recombination occurs randomly on either allele, and cells in which recombination had replaced the first round selection cassette would not survive selection in medium containing both antibiotics. The expected 50% decrease in targeting efficiency does not fully explain the low rate of homologous recombination observed in the second round targeting.

The five cell lines used to create chimeras retained a normal karyotype as did the single homozygous mutant cell line. Chimeras of up to 85%, as assessed by coat color, were produced with heterozygous mutant cell lines. The chimeras produced were largely normal in appearance, although several highly chimeric pups appeared runted at birth and did not survive beyond the first week. Also, there were rare abnormalities in the eyes, a defect also noted by others working with E14TG2a cells and E14TG2a derived cell lines (Whyatt, 1996; Smith, 1991).

Thirty-eight chimeric mice, generated from 5 independently targeted lines, ranging in coat color chimerism from 4 to 85%, were mated for the purpose of assessing germline transmission. None produced any offspring with an ES cell derived haplotype. The 5 chimeric sperm samples tested by GPI analysis (coat color chimerism ranging from 25 to 55%) showed low ES cell contribution to the sperm, suggesting that germline transmission would be a rare event. Despite the fact that E14TGa ES cells have been used successfully in this laboratory to create germline transmitting chimeras (Whyatt and Rathjen, 1997), it is possible that a mutation or mutations arose during culture and passaging of the ES cells which affected the efficiency with which the ES cells contribute to the germline. In addition, difficulties and inexperience in the blastocyst injection technique could account for the relatively low level of chimerism in general. The possibility that *Gbx2* could be required for sperm viability appears unlikely given that, while germline transmission was being analyzed, the laboratory of G. Martin reported the phenotype of *Gbx2* mutant mice created by gene targeting (Wassarman et al., 1997). Heterozygous offspring bearing this mutation were phenotypically normal, while homozygous mice died neonatally with severe defects in the anterior hindbrain, forebrain, and inner ear. Although the presence of ES cell-derived sperm in three chimeras suggested that repeated breeding might eventually produce germline transmission of the null allele, given the existence of *Gbx2* mutant mice in another laboratory it was decided at that time not to pursue further the production of *Gbx2* mutant mice in this laboratory.

### *4.6.2 ES cell lines containing a homozygous* Gbx2 *mutation*

Deletion of the region encoding *Gbx2* in both alleles resulted in a viable ES cell line. The growth pattern of the homozygous *Gbx2* mutant ES cell line indicated that *Gbx2* was not required for maintenance of an ES cell-like morphology or proliferation. Thus, *Gbx2* does not appear to be essential for the survival of pluripotent cells in the in vitro equivalent of the inner cell mass. This is consistent with the observations of Wassarman et al. (1997) that embryonic inner cell mass does not appear to be affected by *Gbx2* mutation.

ES cell lines in which both alleles of a gene are mutated provide unique opportunities for biochemical and developmental analysis of gene function. The developmental potential of mutated ES cells can be assessed by differentiation of the cells in vitro, or by examining the contribution of the cells to tissues in chimeric mice following injection of the cells into host blastocysts and development in vivo. This can permit analysis of cell decisions that are otherwise obscured by embryonic lethality, and in some cases can be coupled with detailed analysis of cell differentiation by the measurement of marker gene expression. Subsequent chapters describe the studies undertaken using heterozygous and homozygous *Gbx2* mutant ES cell lines for in vitro differentiation.

# Chapter 5

## *Gbx2* expression patterns in vitro models of early development

# 5 *Gbx2* expression patterns in vitro models of early development

## 5.1 Introduction

The expression pattern of *Gbx2* was described in section 1.5.3. In the course of this project, *Gbx2* function was investigated in mutant mice created by gene targeting by another group (Wassarman et al., 1997). Heterozygous mutants were phenotypically normal while homozygous mutants died within one day of birth. The major defect revealed by morphological analysis was the absence of a normal cerebellum. In addition, there were abnormalities in the forebrain in some of the mutants, the supraoccipital bone was smaller than normal or absent, and the vestibular organ of the inner ear was abnormal. Further study of hindbrain defects in embryos harvested at various time points revealed a truncation in the anterior hindbrain and the absence of derivatives of the isthmus (midbrain-hindbrain boundary) and rhombomeres 1-3, including IV and V motor nuclei. Structures arising from neural crest cells derived from this region appeared to be unaffected, suggesting that either early development in the region is sufficient to form functional neural crest cells or that neural crest cells from adjacent regions are able to substitute for cells from the affected region. Expression of genes normally localized to tightly restricted regions around the midbrain-hindbrain boundary was altered, resulting in a region between the midbrain and rhombomere 4 in which genes from either side of the midbrain-hindbrain boundary (*Otx2*, *Wnt1*, *Fgf8*) were expressed in a diffuse, patchy pattern.

While Wassarman's work demonstrates that *Gbx2* plays an important role in the formation of these structures, it provides no understanding of the mechanisms resulting in the defects. It is not known, for example, whether the defects are due to loss of *Gbx2* function in neural differentiation, gastrulation or inner cell mass. It is also unknown whether *Gbx2* acts primarily to provide positional identity along the antero-posterior axis or whether it specifies a particular cellular differentiation pathway. The reported involvement of the chicken homologue, *GBX2*, in hematopoietic differentiation (Kowenz-Leutz, et al., 1997) further suggested that *Gbx2* may have a role in cell type specification. Therefore it was decided to investigate possible *Gbx2* functions using a system which would allow patterning and cell type specification functions to be disassociated. In vitro differentiation of ES cells was chosen as a model because, while cell lineage differentiation occurs in an ordered sequential manner resembling that of the embryo, axial pattern formation is not recapitulated in vitro.

### *5.1.1 Embryoid body differentiation*

In vitro differentiation of pluripotent cells offers a model of early differentiation events in a system which lends itself to simple manipulation and observational techniques. It circumvents experimental difficulties associated with early stage embryos, namely their small size and the inaccessibility of the developmental environment of the uterus, and enables the isolation of large numbers of cells at a selected stage of development which can then be subjected to biochemical analysis. Pluripotent cells (embryonic stem (ES) cells) can be isolated from the inner cell mass of the preimplantation embryo and maintained for long periods in culture in an undifferentiated state in the presence of gp130 agonists such as Leukemia Inhibitory Factor (LIF) or cytokines of the IL-6 family (Evans and Kaufman, 1981; Martin, 1981; Smith et al., 1988; Williams et al., 1988; Piquet-Pellorce et al., 1994). These cell lines are characterized by gene expression patterns and differentiation potentials which correlate with those of the embryonic inner cell mass (Beddington and Robertson, 1989; Rathjen et al., 1999) and can contribute to all adult tissues of the mouse, including the germ line, when reintroduced into blastocysts (Bradley et al., 1984; Robertson, 1987). ES cells differentiate spontaneously following withdrawal of LIF into a limited number of embryonic cell types in an uncontrolled and non-reproducible manner.

A preferred method of differentiation which results in a controlled, reproducible process reminiscent of embryogenesis is obtained by culturing ES cells in suspension (Martin, 1981; Doetschman et al., 1985; Shen and Leder, 1992). ES cells cultured under these conditions aggregate to form compact spheres called embryoid bodies which go on to differentiate in an ordered, progressive manner. Differentiation of embryoid bodies begins with the formation of an outer primitive endoderm layer and an inner primitive ectoderm layer, followed by the generation of embryonic germ layers, and then progresses further to form terminally differentiated derivatives of all three germ layers, including muscle, hematopoietic tissues, endothelial tissue, and neurons (Martin, 1981; Doetschman et al., 1985; Wiles and Keller, 1991). This sequence of events appears to be analogous to the events of early embryo development as demonstrated by temporal gene expression patterns and the ordered appearance of differentiated cell types (Doetschman et al., 1985; Shen and Leder, 1992; Rohwedel et al., 1994) and thus is a useful model for studying the signals regulating pluripotent cell biology and differentiation in early development. Embryoid bodies have been used to screen ES cells containing gene trap inserted genes for interesting expression patterns during differentiation (Baker et al, 1997, Stanford et al, 1998, Gajovic et al., 1998), to analyze transcriptional regulation of gene expression during differentiation following mutagenesis of binding sites in the promotor region (Gemel et al., 1999), and to investigate gene function during cell differentiation by the use of homozygous mutant

ES cells (Weiss et al., 1994; Robb et al., 1996; Duncan et al., 1997; Shalaby et al., 1997; Wilder et al., 1997; Metzler et al., 1999).

### *5.1.2 Neural differentiation*

Neurectoderm and differentiated neurons normally comprise only a small percentage of the total cells in a differentiated embryoid body, making it difficult to detect genes expressed in neural cells or a subset of neural cells (Wobus et al., 1988; Bain et al., 1995). ES cell differentiation can be directed by the application or withdrawal of exogenous factors (Johannson and Wiles, 1995; Keller et al., 1993; Shen and Leder, 1992), allowing selective enrichment of a target cell type, such as neurons.

Retinoic acid (RA) has been used to increase the proportion of cells which progress through the neuronal pathway in the differentiation of EC and ES cells (Jones-Villaneuve et al., 1982; Bain et al., 1995). The mechanism by which RA induces neuron formation is not well understood. In vitro differentiation of ES cells in the presence of RA results in the up regulation of the secreted signalling molecule sonic hedgehog (*shh*) which is normally expressed in the ventral neural tube (floor plate) and notochord and influences formation of certain motor neurons and ventral interneurons in the neural tube (Renoncourt et al., 1998). Neural progenitors formed from ES cells in the presence of RA have been characterized as primarily ventral (*Pax6* expressing), suggesting that *shh* is expressed by the ES cells or differentiating cells in response to RA and may be influencing neuronal fate (Renoncourt et al., 1998). Neural differentiation directed by RA has been shown to result in up regulation of neural genes, such as *Wnt1*, MASH1, and Neurofilament-L, and repression of mesodermal genes, such as *brachyury* and α-cardiac actin, in a sequential order (Bain et al., 1995; Bain et al., 1996). RA differentiation results in the formation of a variety of neural derivatives including glial cells and functional neurons (Strübing et al., 1995; Fraichard et al., 1995;), although these derivatives appear to be primarily ventral in character (Renoncourt et al., 1998).

In vivo, RA is found in the node and has been reported to have effects on hindbrain development (Lumsden and Krumlauf, 1996; Blumberg et al., 1997), causing the posteriorization of anterior hindbrain structures and gene expression. This suggests that RA may also have a role in patterning the anteroposterior axis. As RA causes posteriorization in a region overlapping that which is affected by *Gbx2* ablation, it is of interest to assess whether *Gbx2* is responsive to RA induction as it may shed light on the roles of both *Gbx2* and RA.

### *5.1.3 EPL cells and mesodermal differentiation*

Work done by others in this laboratory has resulted in a system in which ES cells, when cultured in the presence of a hepatocarcinoma cell conditioned medium (MED II), are consistently and uniformly transformed/differentiated into a novel cell type termed Early Primitive Ectoderm-like (EPL) cells (Rathjen et al., 1999). EPL cells are morphologically different from ES cells, growing as a monolayer in which individual cells with one or more nucleoli are visible. EPL cells also display a unique gene expression pattern which is consistent with differentiation potential in vitro and in vivo and responsiveness of primitive ectoderm cells in vivo to cytokines (Rathjen et al., 1999). Embryoid bodies formed from EPL cells show striking differences from those formed from ES cells in both morphology and differentiation (Lake, 1996; Lake et al., in press). EPL cell embryoid bodies appear more irregular and disorganized than ES cell bodies. The expression pattern of the pluripotent cell marker *Oct4*, the primitive ectoderm marker *FGF5* and the nascent mesoderm marker *brachyury* suggest an acceleration of pluripotent cell differentiation in EPL cell bodies. Furthermore, EPL cell bodies are unable to form visceral endoderm, a tissue which has been implicated as a source of signals critical for development of the embryo (Spyropoulos and Capecchi, 1994; Coucouvanis and Martin, 1995; Beddington and Robertson, 1998). EPL cell embryoid bodies form mesoderm, as assessed by early mesodermal markers *brachyury* and *goosecoid*, by day 2-3 of culture as compared to day 4 in ES cell embryoid bodies, and the levels of expression of these genes is much higher in EPL cell bodies (Lake 1996; Lake et al., in press). Terminally differentiated mesoderm is also formed both earlier and at a higher frequency in EPL cell bodies as demonstrated by cardiac muscle formation and the expression of cardiac gene marker *Nkx2.5*. EPL cell bodies have a markedly lower capacity for neuron formation as compared to ES cell bodies, however RA induced EPL cell aggregates are capable of forming neurons at similar frequencies to ES cells, suggesting that this is not an inherent restriction in differentiation capacity, but is due to the altered embryoid body differentiation environment. Thus, EPL cells offer a preferred in vitro system for the analysis of mesoderm and mesodermal derivatives.

### *5.1.4* Gbx2 *expression in vitro*

Chapman et al. (1997) reported *Gbx2* expression by RNase protection analysis in MBL-5 ES cells and embryoid bodies. *Gbx2* was expressed most highly in ES cells, followed by a rapid down regulation of expression by day 2 of culture until day 5 when mRNA was detected, although at a low level, through day 12. This appears to correlate with embryonic expression in the inner cell mass, followed by a down regulation in the primitive ectoderm. Re-expression at day 5 may correlate with primitive streak expression during gastrulation, however, markers of primitive streak

formation such as *brachyury* were not assessed. This chapter investigates the expression patterns of *Gbx2* in both embryoid bodies and retinoic acid differentiated ES cell aggregates in order to understand the relationship between differentiation events in the models and in embryonic development, and to begin to assess the question of whether *Gbx2* plays a role in axial patterning or in cell type specification.

## 5.2 *Gbx2* expression during embryoid body differentiation

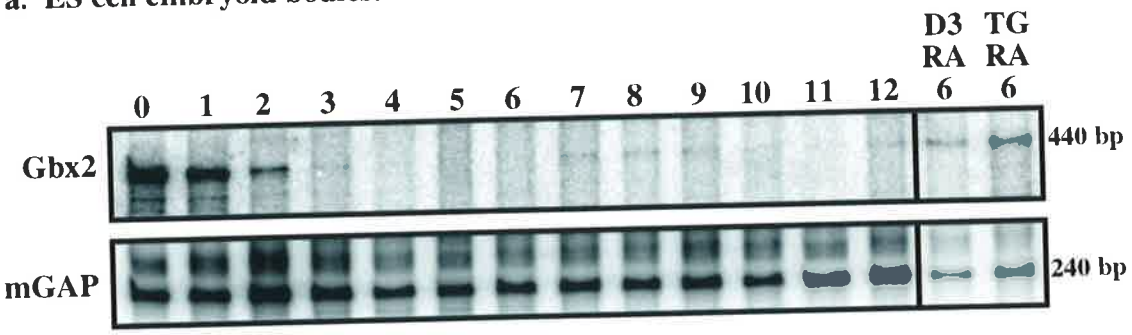
### *5.2.1* Gbx2 *expression in embryoid body mRNA*

D3 ES or EPL cells were plated as single cell suspension in EB medium in non-adherent plates as described in section 2.4.11. Cell aggregates or embryoid bodies were harvested daily on days 1-8 and on day 12 and were fixed and dehydrated for in situ hybridization (section 2.3.27). Alternatively, cells, aggregates, or embryoid bodies were harvested on days 0-12 for total RNA preparation (section 2.3.22). RNase protections were carried out using 20µg total RNA from D3 ES and EPL cell embryoid bodies using a *Gbx2* cDNA probe which hybridizes to and protects a 440 bp region of *Gbx2* mRNA (Chapman and Rathjen 1995), and a *mGAP* probe (Rathjen et al., 1990) as a loading control which hybridizes to and protects a major band of 240 bp.

*Gbx2* was expressed at moderate levels in ES cells (day 0) and was down regulated by day 3 (Fig. 5.1a) in ES cell embryoid bodies. Transient mRNA expression was detected from around day 7 through day 9. While it would appear from these results that inner cell mass expression is recapitulated in vitro in the early expression on days 0-3, the late re-expression of *Gbx2* on day 7 could represent either gastrulation-stage expression or neural specific expression. The formation of primitive germ layers which characterizes gastrulation normally occurs around days 4-5 in ES cell embryoid bodies (Keller et al., 1993; Lake, 1996; Lake et al., in press), suggesting that the *Gbx2* re-expression seen here was not primitive streak equivalent, but neural in origin.

EPL cell embryoid bodies showed a similar down regulation pattern from day 0-3 (Fig. 5.1b). Although EPL cells represent early primitive ectoderm, there is evidence that *Gbx2* expression is not completely down-regulated in EPL cells until day 6 of culture in Med II (Rathjen et al., 1999), therefore it is not inconsistent to see a gradual down regulation in EPL embryoid bodies. No re-expression of *Gbx2* was seen at any later time points (Fig. 5.1b). Given that EPL cell embryoid bodies have been shown to have minimal ability to form neurons (Lake, 1996; Lake et al., in press), this result is consistent with the hypothesis that the transient *Gbx2* expression in ES cell embryoid bodies on days 7-9 was neural in origin rather than streak-associated.

**a. ES cell embryoid bodies:**




**b. EPL cell embryoid bodies:**


**Figure 5.1 *Gbx2* expression – RNase protection analysis**

a. RNase protection analysis of *Gbx2* in ES embryoid bodies and RA induced ES cell aggregates. 20 µg of total RNA from D3 ES cell embryoid bodies harvested on days **0-12** of culture, and D3 (**D3d6**) and TG2a (**TGd6**) RA induced ES cell aggregates was hybridized to an antisense*Gbx2* probe or an antisense *mGAP* probe (as a loading control) and digested at 37°C for 1 hour.

b. RNase protection analysis of *Gbx2* expression in EPL cell embryoid bodies. 20 µg of total RNA from D3 EPL cell embryoid bodies harvested on days **0-12** of culture was hybridized to an antisense *Gbx2* probe or an antisense *mGAP* probe (as a loading control) and digested at 37°C for 1 hour.

Further support for this hypothesis stems from the observation that EPL cell embryoid bodies produce more mesoderm than ES cell embryoid bodies (Lake, 1996; Lake et al., in press). The lack of *Gbx2* expression in differentiated EPL cell embryoid bodies indicates that *Gbx2* is not expressed in forming mesoderm.

### 5.2.2 *Formation of primitive germ layers in embryoid bodies*

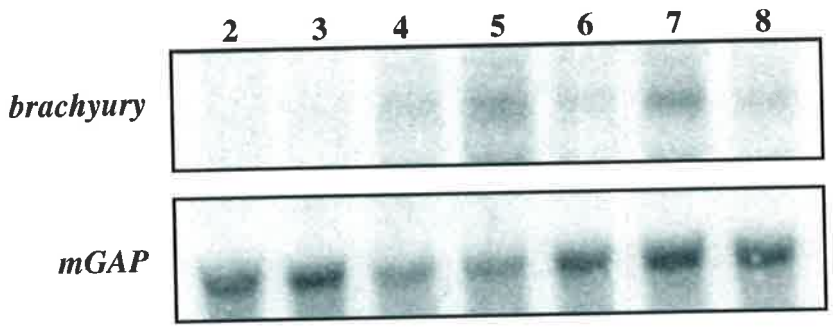
Expression of *brachyury* was analyzed in order to determine the onset of gastrulation-like differentiation events in the embryoid bodies used to map *Gbx2* expression. Northern blot analysis (2.3.24) was performed on 40µg total RNA from D3 ES cell embryoid bodies (5.2.1) using a *brachyury* riboprobe transcribed from *brachyury* cDNA linearized with Bam H1. The filter was then stripped and reprobed with a *mGAP* riboprobe as a loading control. *Brachyury* expression was detected as early as day 4, with expression peaking at day 5 in ES cell embryoid bodies and as early as day 3, with expression peaking at day 4 and persisting until day 7 in EPL cell embryoid bodies (Fig. 5.2). This, in conjunction with the results presented in 5.2.1, indicates that embryonic expression of *Gbx2* in the primitive streak is not recapitulated in embryoid bodies and that the transient re-expression of *Gbx2* seen in ES cell, but not in EPL cell embryoid bodies is likely to represent later sites of expression, such as neurectoderm and its derivatives.

### 5.2.3 Sox1 *expression in ES cell embryoid body mRNA*

Based on *Gbx2* expression patterns in vivo, the reported mutant phenotype, and the observation that *Gbx2* did not appear to be expressed during gastrulation-like events in vitro, it was presumed that the likely cell type expressing *Gbx2* in embryoid bodies would be of neural origin. In order to test this hypothesis, expression of a neural-specific marker gene was analyzed in the embryoid bodies. *Sox1* is a transcription factor which is expressed early in neurectoderm formation (Pevny et al., 1998). In vivo, it is first detected at 7.5 d.p.c. in the anterior half of the embryo in all neurepithelial cells of the developing neural plate. Following neural tube closure expression is down regulated until, by day 13.5, expression is restricted to the ventricular zone, which contains proliferating neural precursors. *Sox1* expression has also been demonstrated in vitro in P19 cells induced to differentiate in the presence of retinoic acid, but not in undifferentiated P19 cells (Pevney et al., 1998). *Sox1* is therefore useful as a marker for early neural differentiation in vitro and may provide confirmation of the identity of the differentiated cell type expressing *Gbx2* in embryoid bodies.

RNase protection was performed on 20µg total RNA from D3 ES cell embryoid bodies (5.2.1) using a riboprobe transcribed from a Bam H1 linearized *Sox1* cDNA

**ES cell embryoid bodies**




**EPL cell embryoid bodies**

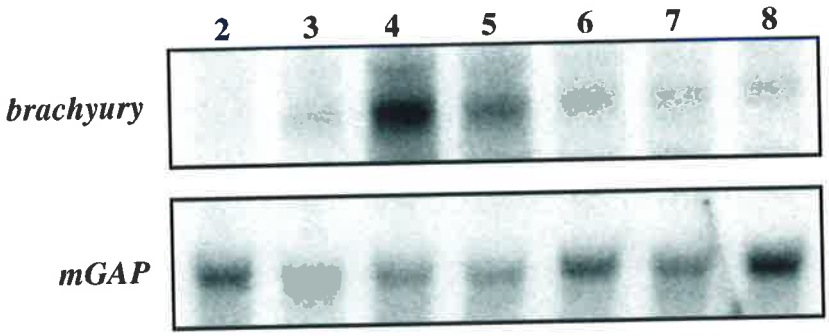



**Figure 5.2** ***Brachyury*** **expression in embryoid bodies**

Northern blot analysis of *brachyury* expression in ES and EPL cell embryoid bodies. 40 µg of total RNA from D3 ES cell or EPL cell embryoid bodies harvested on days **2-8** of culture was electrophoresed and blotted onto a nylon membrane. The membrane was hybridized to a *brachyury* antisense riboprobe, then stripped and reprobed with a *mGAP* antisense riboprobe (as a loading control).

Northern blot provided by M. Bettess.

plasmid (pR1X, J. Rathjen) which hybridizes to and protects a 370 bp region of *Sox1* mRNA, and a *mGAP* probe (Rathjen et al., 1990) as a loading control which hybridizes to and protects a major band of 240 bp. *Sox1* was first detected at a low level at day 5 and increased through day 10 (Fig 5.3), suggesting that formation of neurectoderm in embryoid bodies occurs during this period.

### *5.2.4 Whole mount in situ hybridization of embryoid bodies*

RNase protection cannot delineate whether all cells or only a subset of cells are expressing a transcript. Whole mount in situ hybridization (WMISH) is a relatively sensitive technique which enables spatial patterns of expression to be analysed as well as temporal patterns, however it does not provide a measure of the relative levels of expression. WMISH was performed on ES cell embryoid bodies using a digoxygenin labelled antisense probe transcribed using T3 RNA polymerase from *Gbx2* cDNA, linearized with Mlu I, or with a sense probe transcribed using T7 polymerase from *Gbx2* cDNA, linearized with Mlu I to detect *Gbx2* transcripts, or using a digoxygenin labeled antisense probe transcribed using T3 RNA polymerase from a *Sox1* cDNA plasmid (pR1X, J. Rathjen), linearized with Bam HI, or with a sense probe transcribed using T7 RNA polymerase from *Sox1* cDNA plasmid pR1X, linearized with Hind II (section 2.3.26).

*Gbx2* mRNA was detected in day 1 aggregates in many cells uniformly distributed throughout the aggregates (Fig. 5.4a). These *Gbx2* expressing cells are likely to be undifferentiated ES cells. Expression gradually decreased through day 3 and little or no *Gbx2* staining was detected until day 7 and 8 when small patches and individual cells stained positively. This subset of staining cells is likely to be neurectoderm or neural derivatives. No expression was seen in day 12 embryoid bodies, which is consistent with expression in neurectoderm and down regulation in differentiated neurons. No staining was detected with sense probe (data not shown).

Very little *Sox1* expression was detected in embryoid bodies (Fig 5.4b). Single cells or small clusters of cells stained positively in rare embryoid bodies on days 5-7. Slightly more bodies contained a few positively staining cells on day 8. By day 12 there was only background staining. This is consistent with the observations of *Gbx2* stained embryoid bodies, indicating the formation of small amounts of neurectoderm and neural derivatives in these bodies.

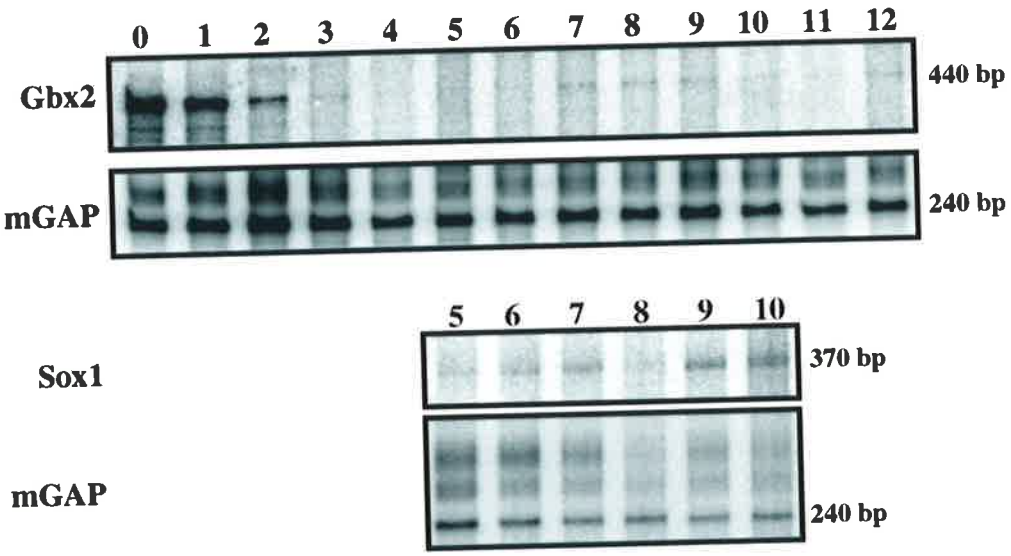
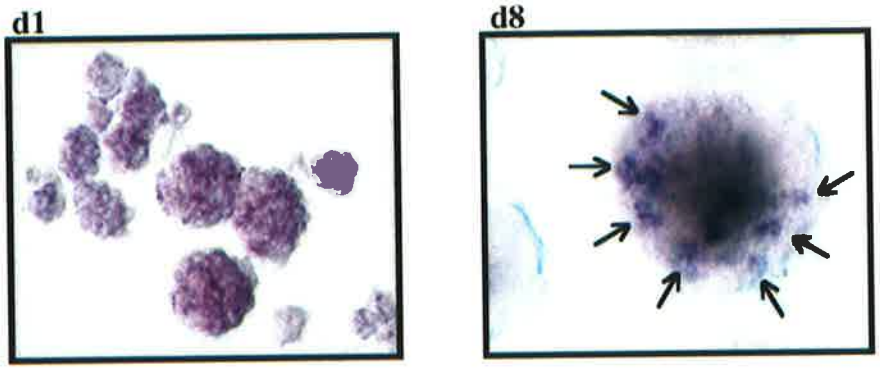



**Figure 5.3** ***Gbx2*** **and** ***Sox1*** **expression – RNase protection analysis**

RNase protection analysis of *Gbx2* and *Sox1* expression in ES cell embryoid bodies. 20 µg of total RNA from D3 ES cell embryoid bodies harvested on days **0-12** of culture was hybridized to an antisense *Gbx2*, *Sox1*, or *mGAP* probe (as a loading control) and digested at 37°C for 1 hour.

*Sox1* RNase protection provided by J. Rathjen.

**a. Gbx2**




**b. Sox1**

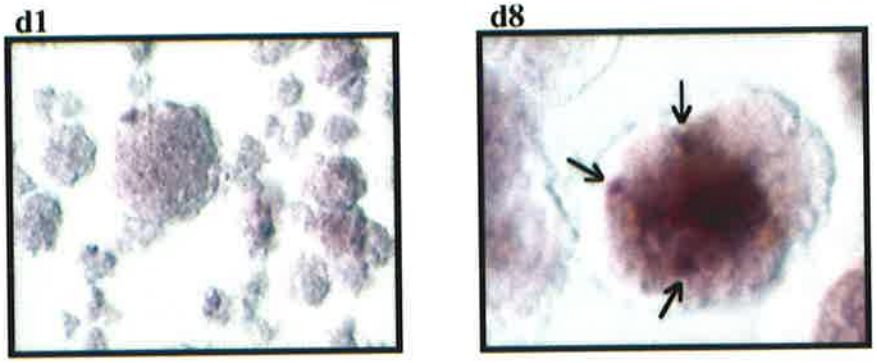



**Figure 5.4 *Gbx2* and *Sox1* expression in embryoid bodies - WMISH**

Whole mount insitu hybridization (WMISH) of D3 ES cell embryoid bodies, harvested on days 1 and 8 of culture. Embryoid bodies were probed with digoxygenin labelled *Gbx2* (**a**) or *Sox1* (**b**) antisense probes. Arrows indicate positively stained regions in day 8 embryoid bodies

## 5.3 *Gbx2* expression during ES cell differentiation in the presence of retinoic acid

### *5.3.1* Gbx2 *expression in retinoic acid differentiated aggregate mRNA*

Retinoic acid was used to enrich for neuron formation in embryoid bodies in order to examine whether *Gbx2* expression responds directly or indirectly to RA induction. E14TG2a or D3 ES cells were plated as a single cell suspension in EB medium containing 1μM RA in non-adherent plates (section 2.4.9). After 4 days the medium was changed to EB medium without RA and was changed every 2 days thereafter. Cell aggregates were harvested daily on days 1-7 and were fixed and dehydrated for in situ hybridization (section 2.3.27). Alternatively, cells or aggregates were harvested on days 0-7 for total RNA preparation (section 2.3.22). RNase protection was performed on 20μg total RNA prepared from retinoic acid differentiated aggregates using the *Gbx2* and mGAP probes described in 5.2.1.

*Gbx2* expression decreased gradually from day 0 through day 4, then increased through day 6 (Fig 5.5). There was a slight decrease in expression on day 7. This expression pattern may reflect the change in the balance of cell populations between undifferentiated ES cells which were decreasing in number, and newly forming neurectoderm which expresses *Gbx2*. These data are not inconsistent with those of Bain et al. (1996), who reported the detection of early neural markers *Wnt1* and MASH1 on days 2 and 4, respectively, following the addition of RA to day 4 ES cell aggregates, while later neural markers NF-M, $GAD_{65}$ and $GAD_{67}$ were detected on days 4-5 of RA culture. Expression of *Gbx2* was much stronger in retinoic acid differentiated aggregates than in spontaneously differentiated embryoid bodies (Fig. 5.1), indicating that *Gbx2* is responsive to RA induction.

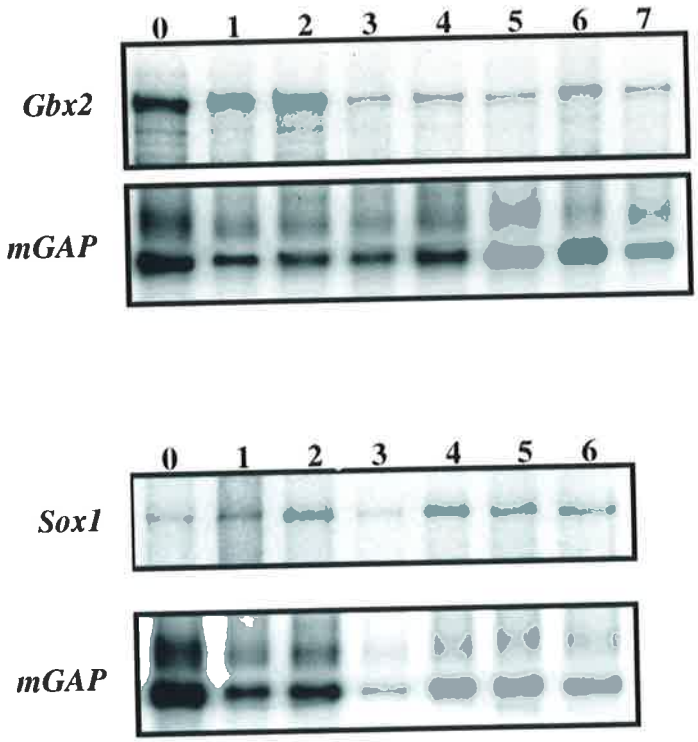
### *5.3.2* Sox1 *expression in retinoic acid differentiated aggregate mRNA*

*Sox1* expression in differentiating RA aggregates was analyzed in order to establish the dynamics of neurectoderm formation in these aggregates. RNase protection was performed on 20μg total RNA from RA differentiated aggregates as described in section 5.2.3. *Sox1* expression first appeared around day 1 and gradually increased through day 6 (Fig. 5.5), demonstrating the gradual formation of neurectoderm in the aggregates.

### *5.3.3 Whole mount in situ hybridization of retinoic acid differentiated aggregates*

Spatial patterns of *Gbx2* and *Sox1* expression in RA treated aggregates were investigated by the use of WMISH technique as in section 5.2.4.

a.




b.

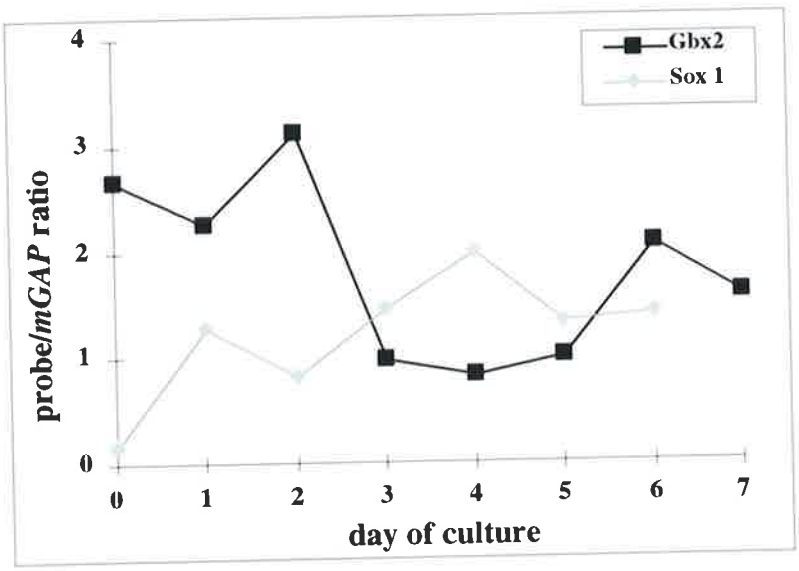



**Figure 5.5 *Gbx2* and *Sox1* expression in RA induced ES cell aggregates**

a. RNase protection analysis of *Gbx2* and *Sox1* expression in RA induced ES cell aggregates. 20 µg of total RNA from D3 RA induced ES cells harvested on days **0-7** was hybridized to antisense *Gbx2*, *Sox1*,or *mGAP* probes and digested at 37°C for 1 hour.

b. Comparison of relative probe/*mGAP* ratios of D3 RA induced ES cell aggregates. Band intensity was measured using ImageQuant software. Ratio= *Gbx2* or *Sox1* value / *mGAP* value * 100.

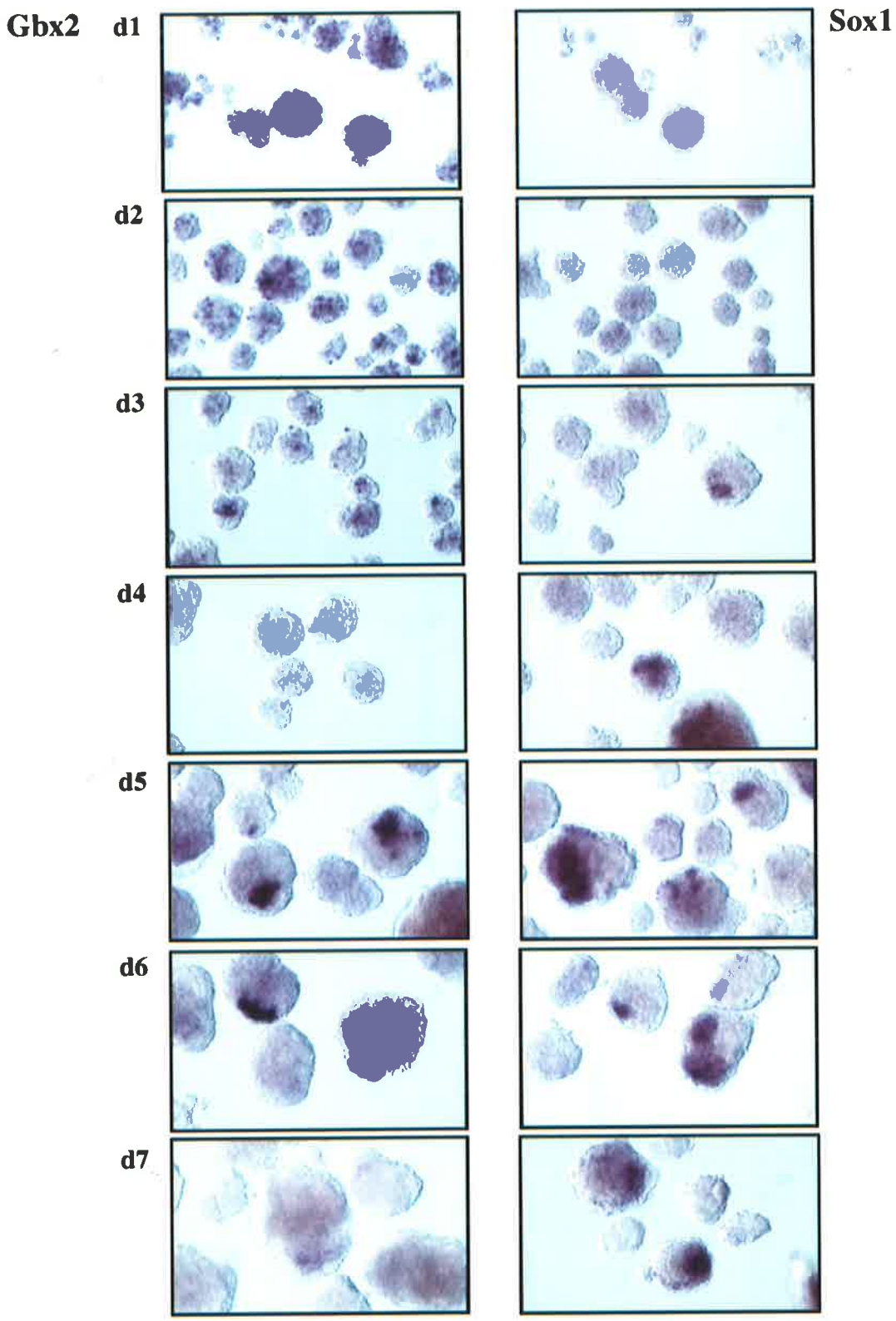



**Figure 5.6 *Gbx2* and *Sox1* expression in RA induced ES cell aggregates -WMISH**

WMISH of D3 RA induced ES cell aggregates, harvested on days 1-7 of culture. Aggregates were probed with a digoxygenin labelled *Gbx2* or *Sox1* antisense probe

Staining with *Gbx2* hybridized probe was more intense in retinoic acid differentiated aggregates than in embryoid bodies at days 5-7 (Figs. 5.4a and 5.6). As was seen by RNase protection analysis, *Gbx2* expression gradually decreased through day 4, then increased on days 5 and 6 (Fig. 5.6). Earlier patterns of expression differed in appearance from later patterns. In early (days 1-3) aggregates, expression was seen in many cells scattered uniformly throughout the aggregates, in the same pattern seen in early embryoid bodies, which suggests expression in undifferentiated ES cells. Later expression, from day 4 onward, tended to be in one or more small to large patches in only a proportion of the aggregates. This pattern is most likely to result from a subset of cells within a region differentiating into neurectoderm and neural derivatives. WMISH showed a decrease in the number of bodies and the proportion of cells within each body staining positively on day 7, suggesting that *Gbx2* may be down regulated in all or a proportion of terminally differentiated neurons. No staining was detected with sense probe (data not shown).

Expression of *Sox1* was much stronger in retinoic acid differentiated cells than in embryoid bodies (Fig 5.6). Expression first appeared at day 2 in single cells or small clusters. Staining increased in intensity and in the size of the patches from day 4 through day 6. The number of bodies with positively staining patches decreased slightly on day 7. No staining was detected with sense probe (data not shown). This pattern appears similar to that seen on days 4-7 in *Gbx2* RA aggregates, supporting the hypothesis that *Gbx2* is expressed in forming neurectoderm.

## 5.4 Discussion

In vitro differentiation of ES cells has been used to investigate genes involved in development. The reproducible and sequential differentiation pathway of embryoid bodies has been mapped by the expression of marker genes and observation of differentiated cell types and has been shown to correlate with early differentiation events in the embryo. The differentiation of ES cells into primitive endoderm and primitive ectoderm which occurs around 4.0-4.5 d.p.c. in the embryo has been demonstrated, by a decrease in *Rex1* (a marker of inner cell mass) and an increase in *Fgf5* (a marker of primitive ectoderm), to occur between days 2-3 of embryoid body culture (Lake, 1996; Lake et al., in press). Gastrulation, which involves the formation of the three primitive germ layers and the initial specification of axial patterning, begins in the embryo at around 6.0 d.p.c. *Brachyury*, a gene expressed transiently in the forming mesoderm at gastrulation, is expressed in ES cell embryoid bodies transiently around days 4 and 5 (Keller et al., 1993; Lake et al., in press). Development of terminally differentiated cells such as muscle, neurons, and hematopoietic lineages

follow specified pathways in the embryo. Embryoid bodies, while a much simpler system than the embryo, recapitulate some of those pathways in a reproducible manner. Primitive erythroid markers such as βH1 globin are seen around day 4-5 in embryoid bodies (Keller et al., 1993; Weiss and Orkin, 1996). Macrophage precursors and β major globin, markers of definitive hematopoiesis, are detected as early as day 6 (Wiles and Keller, 1991; Keller et al., 1993). By day 7, hematopoietic progenitors isolated from embryoid bodies are capable of generating primitive and definitive erythrocytes, myeloid, and mixed lineage progenitors (Ling and Neben, 1997). Cardiac muscle and cardiac muscle marker genes such as *Nkx2.5* and α-cardiac actin are seen by day 8 (Shen and Leder, 1992; Lake, 1996; Lake et al., in press). Neuron formation can be detected microscopically on day 10 and the early neurectoderm marker *Wnt1* has been detected as early as day 8 (Lake, 1996; Lake et al., in press; Rohwedel et al., 1998). This predictable pattern of differentiation is helpful for correlating the embryonic and embryoid body environment at a particular point in time.

*Gbx2* expression in the embryo is detected in the inner cell mass of preimplantation blastocysts, but not in primitive ectoderm or other cell types of egg cylinder stage embryos (Chapman et al., 1997). Expression reappears with the formation of the primitive streak and it is found throughout all three primitive germ layers of the streak (Bulfone et al., 1993; Bouillet et al., 1995; Wassarman et al., 1997). *Gbx2* expression is then gradually restricted to neural lineages, including regions of the forebrain, the hindbrain and spinal cord, and structures of the developing inner ear (Bouillet et al., 1995; Wassarman et al., 1997). Expression has also been reported in the adult mouse spleen and in the hematopoietic tissues of the chicken (Bouillet et al., 1995; Chapman et al., 1997; Kowenz-Leutz et al., 1997).

*Gbx2* expression in vitro was similar to in vivo expression in the mouse, although some differences were evident. *Gbx2* was most strongly expressed in undifferentiated ES cells and expression was down regulated when ES cells were differentiated either as embryoid bodies or by RA induction. This correlates with early in vivo expression in the ICM, but not in the primitive ectoderm. The formation of primitive germ layer cell types in embryoid bodies, which recapitulates the events of gastrulation, occurs around days 4-5 of culture. This was confirmed by examination of *brachyury* expression in the ES and EPL cell embryoid bodies used for *Gbx2* expression analysis. EPL cell embryoid bodies have been shown to be severely limited in their capacity to form neurons, while the formation of mesoderm is enhanced (Lake et al., 1996; Lake et al., in press). *Brachyury* was detected earlier and at higher levels in EPL cell bodies, confirming these results. The observation that *Gbx2* was not

expressed during mesoderm formation and was re-expressed in ES cell embryoid bodies from day 7-9, but not significantly in EPL cell embryoid bodies demonstrates that the expression is most likely to be in neural rather than mesodermal lineages. Comparison of *Sox1* expression in ES cell embryoid bodies from day 5-10 by quantitative and spatial methods demonstrates that neurectoderm is formed at this time. It is possible that *Gbx2* expression during gastrulation is regulated by a signal not present in the differentiating embryoid body, however later embryoid body expression and expression in cells exposed to RA, suggests that the signals regulating *Gbx2* expression in neurectoderm are present in the embryoid body environment. This is consistent with the observation of the conservation of neurectodermal, but not gastrulation-specific, *Gbx2* expression patterns across species (section 1.5.3).

The observation that *Gbx2* expression in embryoid bodies is most likely to be in neural lineages rather than in mesodermal lineages brings into question the significance of *Gbx2* expression in mesoderm and hematopoietic tissues in vivo. This question is explored in detail in Chapter 7.

*Gbx2* was also shown to respond to RA induction. Expression levels were higher in RA treated aggregates than embryoid bodies and WMISH showed large dark staining patches of cells in RA aggregates as opposed to only small patches in a few embryoid bodies. This was not unexpected as retinoic acid induced *Gbx2* expression has been reported previously in both mouse embryonal carcinoma cells (Bouillet, et al, 1995) and frog embryos (von Bubnoff et al., 1995). The expression of *Gbx2* at days 5 and 6 in RA induced aggregates was greater than its expression in embryoid bodies at days 7-9. This supports the hypothesis that *Gbx2* is expressed in neural lineages in vitro.

Further support comes from a comparison of the expression of *Gbx2* and *Sox1* in RA induced aggregates by whole mount in situ hybridization and RNase protection. *Gbx2* expression which was strong in undifferentiated ES cells was gradually lost over days 1-3. *Sox1* was not significantly expressed in undifferentiated ES cells, however *Gbx2* and *Sox1* showed a similar pattern of expression in patches of cells within the embryoid bodies, beginning at day 4 and increasing through day 6. The intensity of staining and size of the patches decreased for both genes on day 7, suggesting that some terminally differentiated neurons do not express these genes. Neural gene expression in RA induced cell cultures has not been extensively mapped, however, using a system where ES cells were aggregated in suspension for 4 days before exposure to RA, Bain et al.(1996) have reported the expression of early neural markers *Wnt1* and MASH1 as early as 2 days after RA exposure and late neural markers NF-M, $GAD_{65}$, and $GAD_{67}$ on days 4-5 of RA culture. This indicates a lag of

several days before the appearance of neural marker genes, thus the expression pattern of *Gbx2* is consistent with a decrease in ES cell expression due to the differentiation of ES cells, followed by an increase in neural lineage expression in response to RA induction.

It is unknown whether the response to RA is an in vitro phenomenon or if *Gbx2* is also regulated by RA in vivo. The role of retinoic acid in the development of the embryo is not clear. It is thought to play a role in the specification of posterior structures (Morriss-Kay and Sokolova, 1996; Maden et al, 1996; reviewed in Means and Gudas, 1995). Blumberg et al. (1997), demonstrated that increased retinoic acid receptor activity suppresses anterior neural structures, while dominant negative receptors caused the anteriorization of posterior structures and loss of posterior gene expression in the hindbrain. RA is present in the node and it has been suggested as a regulator of the nested *Hox* gene expression pattern in CNS through a concentration gradient (Simeone et al., 1991, Papalopulu et al., 1991, Marshall et al., 1992, Godsave et al., 1998). While no retinoic acid responsive element (RARE) binding sites were identified within the 486 bp region 5' of the first transcription initiation site of the *Gbx2* locus (section 3.3), it is possible that *Gbx2* is indirectly regulated by RA or that it is directly activated by RA, but at a site outside the 486 bp region examined.

The results of experiments described in this chapter demonstrate that *Gbx2* expression during in vitro differentiation recapitulates some, but not all of the in vivo expression pattern. While inner cell mass and neural lineage expression was seen in vitro, no expression was observed in gastrulation stage equivalent embryoid bodies or in *brachyury* expressing mesoderm. This is most intriguing as it suggests that the regulatory elements controlling expression during this stage are different which may indicate two distinct mechanisms for *Gbx2* regulation. The absence of axial patterning in embryoid bodies and RA aggregates suggests that the appropriate signals to establish embryonic axes are not delivered in the in vitro environment. It is possible, then, that *Gbx2* expression during gastrulation indicates a function in establishing positional information, while expression in undifferentiated pluripotent cells and in neural lineages suggests a possible regulatory role in the differentiation and specification of these cell types.

The in vitro differentiation system described here may provide a valuable tool for further investigation into the possibility of distinct roles for *Gbx2* at different stages of development by creating an environment in which the expression of *Gbx2* is restricted to stages where it may be playing a role in differentiation and cell type specification. This allows for the disassociation of positional information and cell specification

functions, and thus further investigations into the potential requirement of *Gbx2* for survival and specification of differentiating cell types, as described in Chapter 6 and 7, center on the use of this system.

# Chapter 6

# The role of *Gbx2* in neural cell differentiation and lineage specification

# 6 The role of *Gbx2* in neural cell differentiation and lineage specification

## 6.1 Introduction

The in vivo expression patterns of *Gbx2* described in section 1.5.3 suggested a role in neural cell differentiation. Notably, the broad expression pattern in early neurectoderm indicated the possibility of a "cellular function" type role, in which *Gbx2* could be required for neurectoderm proliferation or differentiation. The experiments described in this chapter were designed to test this hypothesis.

### *6.1.1* Gbx2 *mutations*

Wassarman et al. (1997) reported the creation of *Gbx2* mutant mice by homologous recombination. Heterozygous mutants were phenotypically normal, while homozygous mutants died within one day of birth. The major defect revealed by morphological analysis was the absence of a normal cerebellum as described in section 5.1. The analysis of phenotype and gene expression patterns in the homozygous mutants created by Wassarman et al. established that *Gbx2* is required for specification of the cells which give rise to the region encompassing the isthmus through rhombomere 3, however the mechanism by which *Gbx2* performs this function was not determined. In addition, the mutation created in this experiment did not delete the entire gene, resulting in the transcription of a partial RNA message containing the conserved proline rich region in the first exon (Chapman and Rathjen, 1995, Chapman et al., 1997). This message could potentially be translated into a truncated, but functional protein. This aberrant protein, if it is produced in the mutant mice, does not appear to be sufficient to specify the anterior hindbrain region, however it may retain sufficient functionality to support the normal function of *Gbx2* at other stages of development or in other tissues. It could also cause abnormalities by a dominant negative effect, thereby obscuring the null mutant phenotype.

The constructs prepared for gene targeting in this project were designed to delete the entire coding region of *Gbx2* as well as the intron (section 4.2.1), thereby creating a true *Gbx2* null allele. Homozygous mutant ES cells were created (section 4.4) in order to study the effects of *Gbx2* ablation in the simpler, more easily manipulated environment of in vitro differentiation.

### *6.1.2 Effects of null mutations on cell differentiation and proliferation*

Embryoid bodies do not duplicate the spatial organization and axial patterning which occurs from the gastrulation stage onward in embryos, precluding their use in the

analysis of the molecular control of these events, however in vitro differentiation of ES cells has been used extensively in conjunction with in vivo studies to investigate the effect of gene function on the formation of different cell lineages and to explore regulatory hierarchies of transcription factors. The actions of genes involved in hematopoietic differentiation are perhaps the best characterized. For example, ES cells lacking a functional GATA1 gene do not form mature erythrocytes in chimeras, despite the ability to contribute to white blood cell lineages (Pevney et al., 1991). In vitro differentiation of these cell lines determined that the defect was limited to definitive, and not primitive, hematopoiesis (Weiss, et al., 1994). Similarly, in vitro differentiation studies of *scl*$^{-/-}$ ES cells have established that the gene is required for both primitive and definitive hematopoiesis (Robb et al., 1996, Elefanty et al, 1997), while analysis of *Flk1*$^{-/-}$ ES cells in vitro and in vivo indicated a role for the gene in the migration of prehematopoietic mesoderm to the yolk sac environment (Shalaby et al., 1997). In vitro differentiation has also been used to examine the role of genes required for early embryo development. *Fgf4*$^{-/-}$ embryos do not develop beyond the implantation stage and the embryos rapidly degenerate. In vitro differentiation of *Fgf4*$^{-/-}$ ES cells indicated that *Fgf4* is not required to maintain pluripotent cells of the inner cell mass, but is required for survival and/or proliferation of differentiated cells (Wilder et al., 1997).

### *6.1.3 Effects of null mutations on regulatory hierarchies*

In vitro differentiation of ES cells can provide information on downstream targets of specific genes by examining the differences in gene expression patterns and differentiation potential between wildtype and mutant ES cells. *Hnf4*$^{-/-}$ embryos die in utero due to severe defects at the gastrulation stage. *Hnf4* is expressed in visceral endoderm prior to gastrulation, therefore it was hypothesized that *Hnf4* regulates the expression of visceral endoderm specific proteins required to support gastrulation. Examination of candidate gene expression during the in vitro differentiation of mutant ES cells substantiated this hypothesis (Duncan et al., 1997). GATA6$^{-/-}$ embryos display a similar phenotype to *Hnf4*$^{-/-}$ embryos and GATA6$^{-/-}$ embryoid bodies do not express *Hnf4*. Ectopic expression of GATA6 activated the *Hnf4* promoter in non-endodermal cells, demonstrating that GATA6 lies upstream of *Hnf4* in the regulatory cascade controlling visceral endoderm formation (Morrisey et al., 1998).

In vitro differentiation of ES cells has also been used to study gene function in a disease model. Mutation of the Huntingtin (HD) gene is associated with the neurodegenerative disorder Huntington's Disease. Analysis of mutations in vivo has demonstrated that it also plays a crucial role in development. Homozygous null mutant embryos die around 7.5 d.p.c. with severe defects in extraembryonic tissues, while

mice expressing low levels of the mutated protein die perinatally with defects in the forebrain and midbrain. The role of HD in neuron formation was assessed by in vitro differentiation of $HD^{-/-}$ ES cells which were found to be capable of forming functional neurons. Moreover, $HD^{-/-}$ ES cells displayed a dramatic increase in neuron formation in response to RA treatment, demonstrating that HD is not essential for development of neural lineages.

This chapter explores the question of whether *Gbx2* is required for neuron formation or survival and begins to address the regulatory hierarchy of molecules involved in neuron differentiation through the analysis of $Gbx2^{-/-}$ ES cells differentiated as embryoid bodies and RA treated aggregates.

## 6.2 The effect of *Gbx2* ablation on neuron formation in vitro

### *6.2.1 Embryoid body differentiation systems*

Embryoid bodies differentiate into a wide variety of cell types, including muscle, hematopoietic tissues, endothelial tissue, and neurons, however in the absence of a neuron inducing stimulus such as retinoic acid only a small percentage of the differentiating cells will form neurons. A method has recently been developed (section 2.4.14, J. Rathjen, unpublished) to enhance the formation of neurectoderm in embryoid bodies. Embryoid bodies (EBM) cultured in suspension in EB medium containing the hepatocarcinoma conditioned medium Med II (Rathjen et al.,1999) for 7 days form elevated levels of neurectoderm as demonstrated by morphology, the detection of early neural genes *Sox1* and *Gbx2,* and immunohistochemical detection of the pan neural marker, N-Cam, and the neural stem cell marker, nestin. FACS analysis of N-Cam stained populations indicates that up to 96% of the differentiating cells have assumed a neurectodermal fate. Also, formation of mesodermal lineages, as assessed by expression of *brachyury* or differentiation into cardiocytes, is severely reduced or absent (J. Rathjen, unpublished). When EBMs are seeded onto tissue culture plates in serum free medium containing FGF4 they produce large numbers of morphologically identifiable neurons.

Expression patterns of pluripotent cell marker gene *Oct4*, *Sox1*, and *Gbx2* were examined by RNase protection and WMISH on days 4-8 in EBMs. *Oct4* is the earliest gene expressed and is down regulated as the pluripotent cells differentiate. *Gbx2* is detected at low levels at day 4, expression increases through day 7 and decreases on day 8. *Sox1* expression, first seen at day 6, gradually increases through day 8 to around 95% of the population (J. Rathjen, unpublished). These patterns reflect the temporal expression patterns observed in vivo during gastrulation and early

neurulation (Yeom et al., 1991, Schöler 1991; Bouillet et al., 1995; Pevney et al, 1998). The EBMs provide an in vitro differentiation model for neuron formation in which neural progenitors are formed efficiently from pluripotent cells in a manner analogous to the formation of neural cells during embryogenesis, and therefore may be a preferable alternative to chemical induction of neuron formation from ES cells using retinoic acid.

### *6.2.2 Neuron formation in embryoid body differentiation systems*

E14TG2a (*Gbx2*$^{+/+}$), GNE.159 (*Gbx2*$^{+/-p}$), and H2.73 (*Gbx2*$^{-/-}$) ES cells (Chapter 4) were plated in non-adherent culture dishes in either EB medium (10% FCS) or EPL medium (10% FCS) as described in section 2.4.14. On day 7 of culture, embryoid bodies from each plate were seeded into individual tissue culture plate wells in DMEM/F12 medium (10% FCS). The next day, the medium was changed to a serum free medium containing FGF4 and the appearance of neurons was assessed microscopically in all wells on days 8 and 10 of culture. The results, expressed as the percent of embryoid bodies containing 1 or more neurons (Fig. 6.1), showed no difference in the ability of wild type, heterozygous, or homozygous mutant cell lines to form neurons when cultured either as normal embryoid bodies or as EBMs, demonstrating that *Gbx2* is not essential for neuron formation. Approximately 2-fold increase in the number of neuron-forming bodies observed in EBMs in all three cell lines compared to ES cell embryoid bodies, consistent with the demonstrated ability of this culture system to enhance formation of neural progenitors. *Gbx2*$^{-/-}$ ES cells are therefore competent to respond normally to neurectoderm-inducing signals of biological origin.

### *6.2.3 Neuron formation in retinoic acid treated aggregates*

E14TG2a (*Gbx2*$^{+/+}$) and H2.73 (*Gbx2*$^{-/-}$) ES cells were plated at low density in EB medium supplemented with 1μM retinoic acid (RA), in non-adherent plates (section 2.4.9). After 4 days of culture the aggregates were seeded onto a tissue culture plate and RA was removed from the medium. After two more days of culture the plates were assessed microscopically for neuron formation (Fig. 6.2a). The results are expressed as the percentage of aggregates containing one or more neurons or grouped according to the number of neurons in each aggregate (Fig. 6.2b and c). The *Gbx2*$^{-/-}$ cell line was capable of forming neurons, again demonstrating that *Gbx2* is not essential for neuron production, however there was a statistically significant difference between the *Gbx2*$^{+/+}$ and *Gbx2*$^{-/-}$ cell lines in the number of aggregates which formed neurons, decreasing from an average of 33% of *Gbx2*$^{+/+}$ aggregates to only 6% of *Gbx2*$^{-/-}$ aggregates (Fig. 6.2b). This difference was also reflected in the number of neurons produced by each aggregate, for example, 10% of *Gbx2*$^{+/+}$ aggregates had 6-

20 neurons per aggregate as compared to only 1% of *Gbx2*$^{-/-}$ aggregates (Fig. 6.2c). These data suggest that ablation of *Gbx2* reduces the ability of ES cells to respond to retinoic acid induction of neuronal differentiation.

## 6.3 The effect of *Gbx2* ablation on *Sox1* expression

*Sox1* is a transcription factor which is expressed uniformly in the neural plate and developing neural tube beginning at 7.5 d.p.c. and later is restricted to the ventricular zone of the CNS (Pevny et al., 1998). In vivo, *Gbx2* expression is observed at least a day earlier than *Sox1*, during gastrulation in all three germ layers of the primitive streak (Bouillet et al., 1995). *Gbx2* expression then overlaps with *Sox1* expression in the neural plate and neural tube during neurulation. In vitro, *Gbx2* and *Sox1* expression in embryoid bodies overlap, although *Sox1* expression appears by day 5, earlier than *Gbx2*. This is likely due to the lack of gastrulation-specific *Gbx2* expression in embryoid bodies (section 5.2). In RA treated ES cell aggregates, *Gbx2* was expressed in undifferentiated ES cells and in patches of differentiated cells beginning around day 4 and increasing through day 6, while *Sox1* was not significantly expressed in ES cells, but showed a similar expression pattern in patches of cells from day 4 increasing through day 6 (section 5.3). These observations suggested that *Gbx2* may be upstream of *Sox1* expression in early neurectoderm. In order to test this hypothesis, *Sox1* expression in *Gbx2*$^{+/+}$ ES cell RA aggregates was compared to *Gbx2*$^{-/-}$ ES cell RA aggregates.

### *6.3.1* Sox1 *expression in retinoic acid differentiated ES cell aggregates*

E14TG2a (*Gbx2*$^{+/+}$) or H2.73 (*Gbx2*$^{-/-}$) ES cells were plated as single cell suspensions in EB medium containing 1μM RA in non-adherent plates (section 2.4.9). Cells or aggregates were harvested on days 0-7 for total RNA preparation (section 2.3.22). RNase protection was performed on total RNA prepared from RA differentiated aggregates using a riboprobe transcribed from a BamH1 linearized *Sox1* cDNA plasmid and a *mGAP* probe (section 5.2.3). *Sox1* expression in *Gbx2*$^{+/+}$ RA aggregates first appears around day 1 and gradually increases through day 6. (section 5.3.2, Fig. 6.3a). In order to compare relative *Sox1* expression in *Gbx2*$^{+/+}$ and *Gbx2*$^{-/-}$ aggregates which were probed in different experiments, the band intensity was measured using ImageQuant software and the ratio of *Sox1/mGAP* was calculated for each lane. The value for *Gbx2*$^{+/+}$ specimens were then adjusted by the ratio obtained for a *Gbx2*$^{+/+}$ specimen probed at the same time as the *Gbx2*$^{-/-}$ samples (Fig. 6.3b,c). *Sox1* expression in *Gbx2*$^{-/-}$ RA aggregates relative to *mGAP* expression was much lower than *Gbx2*$^{+/+}$ RA treated aggregates at all time points. These results demonstrate that *Gbx2* is not obligatory for *Sox1* expression. The decreased level of expression may be the result of a decrease in the formation of neurectoderm.

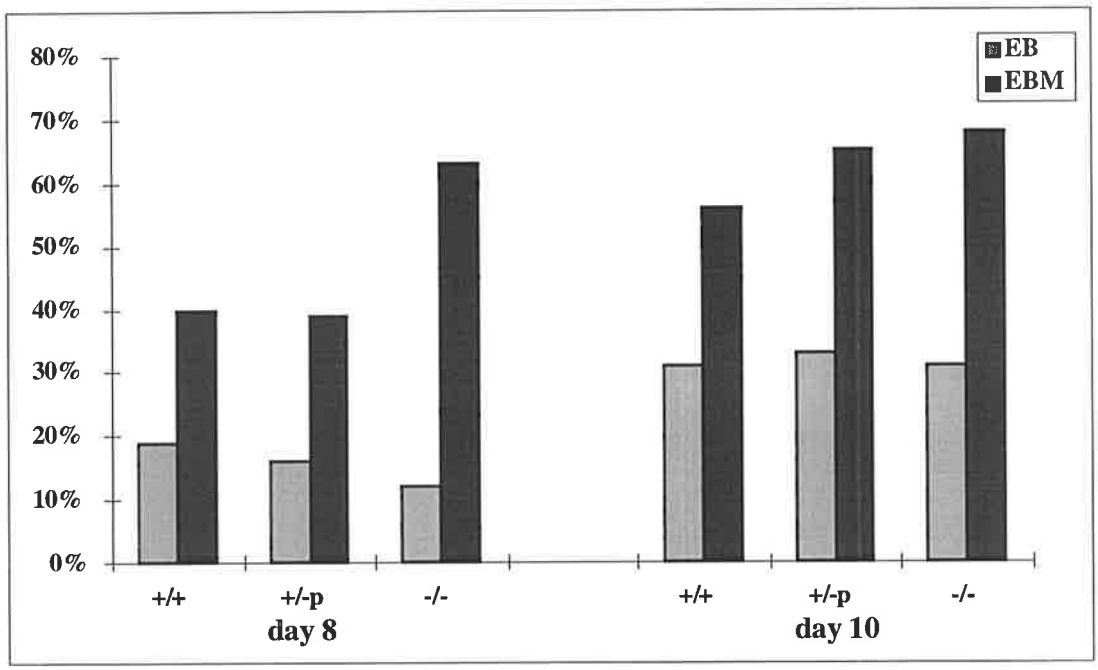
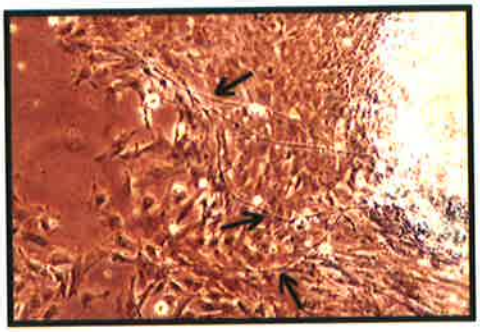


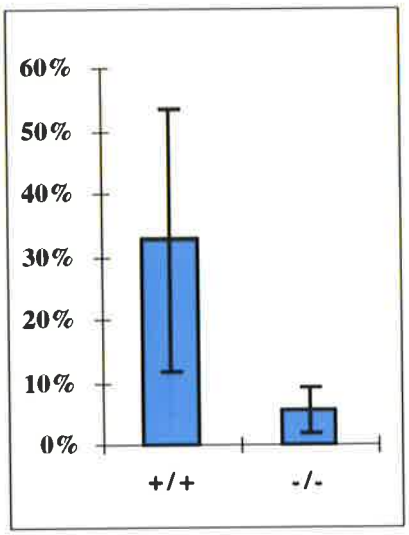

**Figure 6.1 Neuron formation in embryoid bodies cultured in the absence and presence of Med II**

Graph representing the percent of embryoid bodies containing one or more neurons on day 8 and 10 of culture. Embryoid bodies were grown for 7 days in the absence (**EB**) or presence (**EBM**) of Med II. *Gbx2*$^{+/+}$ (**+/+**), *Gbx2*$^{+/-}$ (**+/-p**), and *Gbx2*$^{-/-}$ (**-/-**) ES cell lines were analyzed. The experiment was repeated three times and results shown here are indicative of standard outcomes.

a.



b.




c.

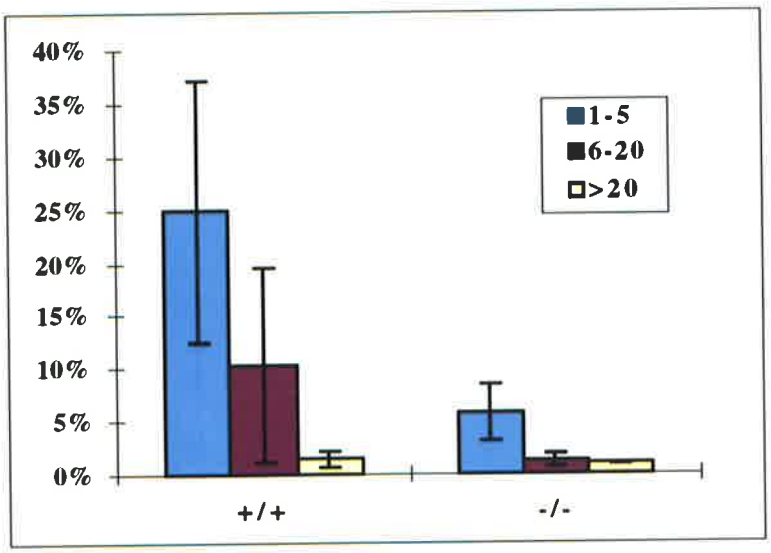



**Figure 6.2 Neuron formation in RA induced aggregates**

a. Photograph of an RA induced ES cell aggregate containing mature neural cells displaying long extended processes (arrows).

b. Graph representing the percent of RA induced ES cell aggregates containing one or more observable neurons on day 6 of culture. *Gbx2*+/+ (+/+)and *Gbx2*-/- (-/-) ES cell lines were analyzed.

c. Graph representing the percent of RA induced ES cell aggregates containing one or more observable neurons on day 6 of culture, divided into subgroups based on the number of neurons per aggregate . *Gbx2*+/+ (+/+)and *Gbx2*-/- (-/-) ES cell lines were analyzed.

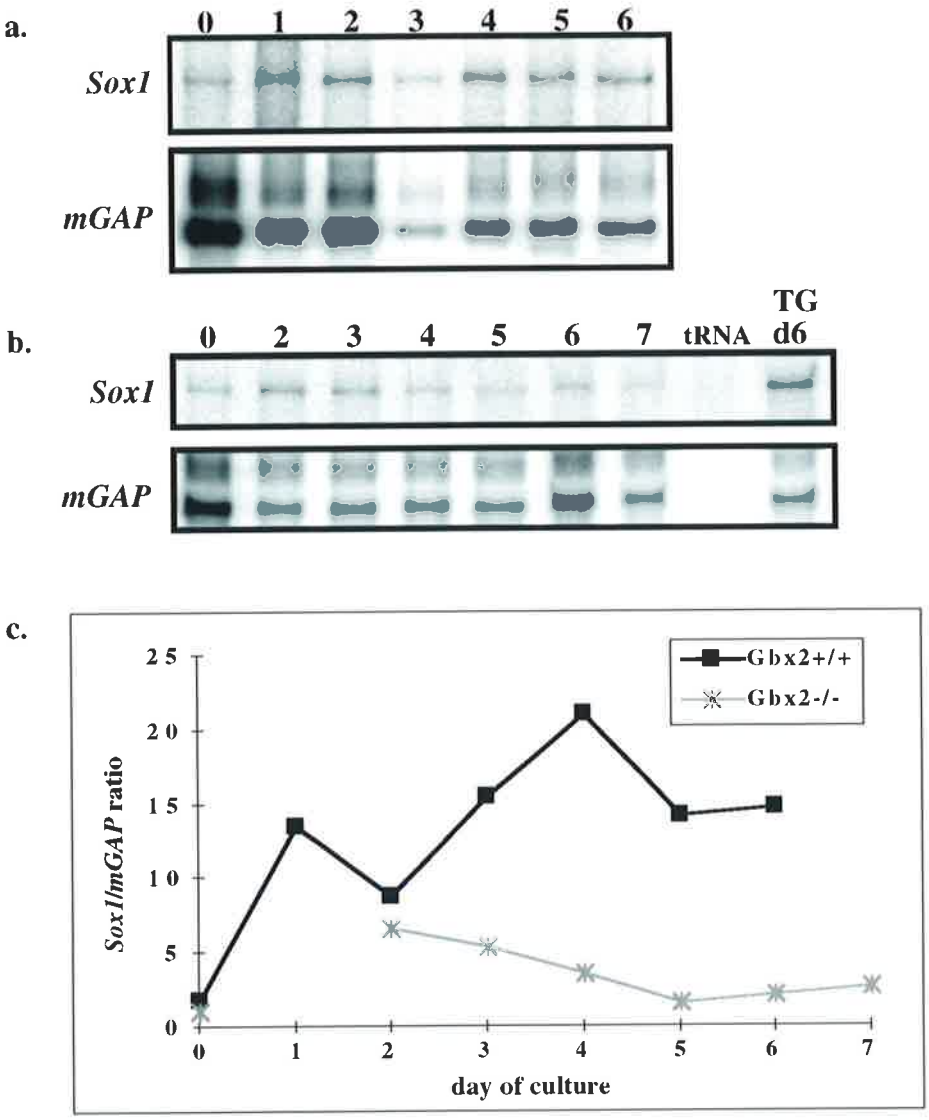



**Figure 6.3** ***Sox1*** **expression in RA treated aggregates**

a. RNase protection analysis of *Sox1* expression in $Gbx2^{+/+}$ RA induced ES cell aggregates. 20 µg of total RNA from (days **0-6**) RA induced ES cell aggregates was hybridized to an antisense *Sox1* probe or an antisense *mGAP* probe (as a loading control) and digested at 37°C for 1 hour.

b. RNase protection analysis of *Sox1* expression in $Gbx2^{-/-}$ RA induced ES cell aggregates. 20 µg of total RNA from $Gbx2^{-/-}$ (days **0, 2-7**) or E14TG2a ($Gbx2^{+/+}$) (**+/+**) RA induced ES cell aggregates or tRNA (**tRNA**) was hybridized to an antisense *Sox1* probe or an antisense *mGAP* probe and digested at 37°C for 1 hour.

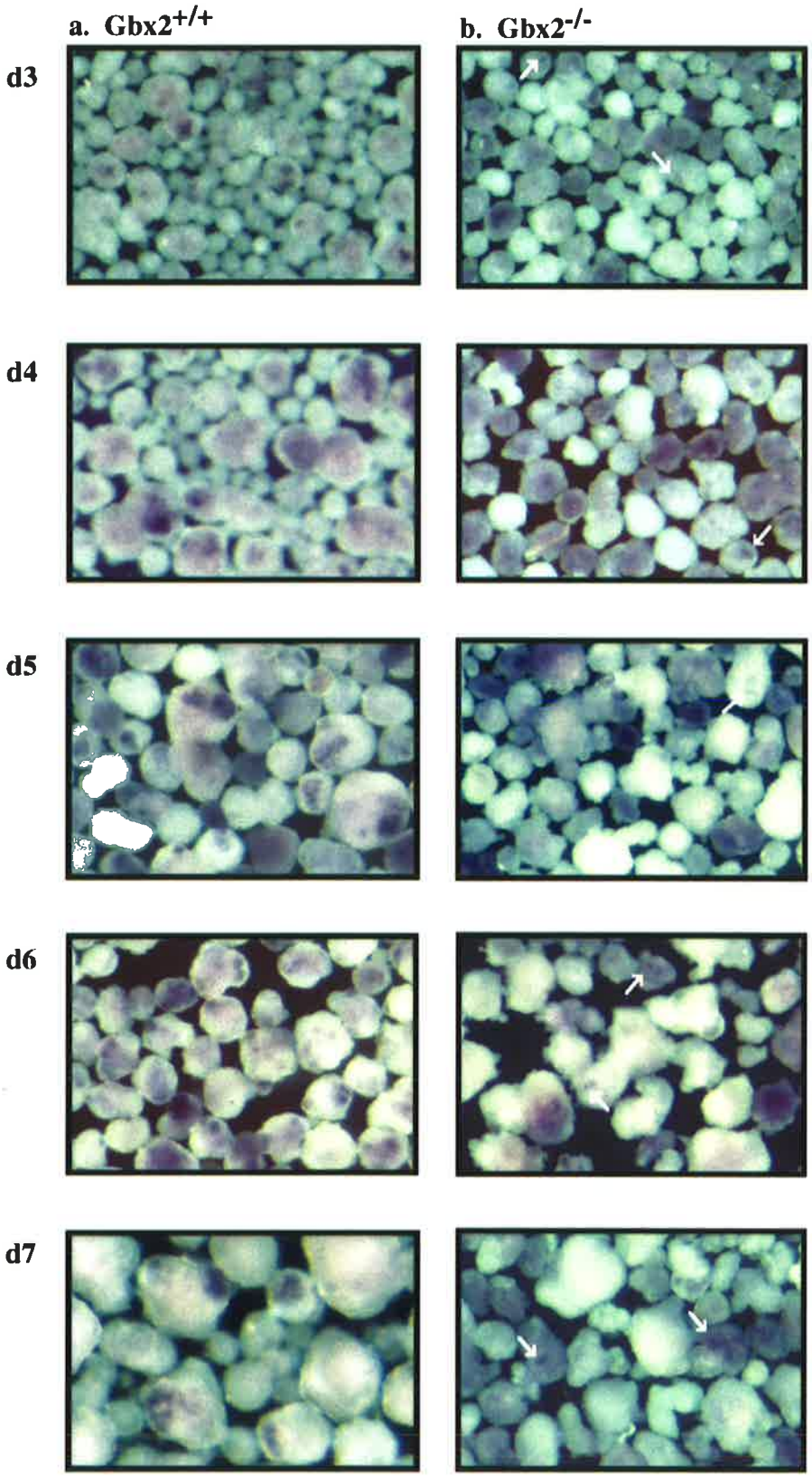
c. Comparison of relative *sox1/mGAP* ratios from $Gbx2^{+/+}$ and $Gbx2^{-/-}$ RA induced ES cell aggregates. Band intensity was measured using ImageQuant software. Ratio=*Sox1* value/*mGAP* value x 100. Values for $Gbx2^{+/+}$ aggregates were adjusted by comparison of ratios from a sample (**+/+**, Fig. 6.3b) run at the same time as $Gbx2^{-/-}$ samples.

**Figure 6.4 *Sox1* expression – Whole mount in situ hybridization (WMISH)**

a. WMISH of RA induced aggregates formed from *Gbx2*$^{+/+}$ ES cells, harvested on days 3-7 of culture. Aggregates were probed with a digoxygenin labelled *Sox1* antisense probe or sense probe (data not shown).

b. WMISH of RA induced aggregates formed from *Gbx2*$^{-/-}$ ES cells, harvested on days 3-7 of culture. Aggregates were probed with a digoxygenin labelled *Sox1* antisense probe or sense probe (data not shown). Arrows indicate examples of positively stained cells.

**Figure 6.4**



### 6.3.2 *Whole mount in situ hybridization of* Sox1 *expression in retinoic acid differentiated ES cell aggregates*

Spatial patterns of *Sox1* expression were investigated by the use of WMISH technique as in section 5.2.4. E14TG2a ($Gbx2^{+/+}$) or H2.73 ($Gbx2^{-/-}$) ES cells were plated as single cell suspensions in EB medium containing 1μM RA in non-adherent plates (section 2.4.9). Cell aggregates were harvested daily on days 1-7 and were fixed and dehydrated for in situ hybridization (section 2.3.26). *Sox1* expression in $Gbx2^{+/+}$ RA aggregates increases from dispersed, low level expression at day 2 to distinct positive staining in a few small clusters of cells in some of the larger aggregates on day 3 and then in strongly staining patches of cells from day 4, increasing in size and number of aggregates through day 6 (Fig 6.4a). *Sox1* expression in $Gbx2^{-/-}$ RA aggregates recapitulated the dispersed, low level expression at day 2. At day 3 a few positively staining cells were seen in small clusters in a small percentage of aggregates. Positively staining patches of cells were seen in fewer than 25% of aggregates on days 4 and 5. The number of aggregates with positively staining patches decreased slightly on days 6-7. Overall, many fewer cells stained positively for *Sox1* in $Gbx2^{-/-}$ than in $Gbx2^{+/+}$ RA aggregates, however the intensity of staining within those cells which were positive was similar in both groups (Fig. 6.4b). These data correlated with the reduced *Sox1* expression in $Gbx2^{-/-}$ aggregates seen by RNase protection (6.3.1).

## 6.4 Discussion

### 6.4.1 *The role of* Gbx2 *in pluripotent cells*

The fact that $Gbx2^{-/-}$ ES cells proliferate and can be maintained in an undifferentiated state in normal ES cell culture demonstrates that *Gbx2* is not essential for survival of pluripotent cells and its expression at the inner cell mass stage of embryo development may not be associated with a critical function. This is consistent with the phenotype observed by Wassarman et al. (1997) in which $Gbx2^{-/-}$ embryos survived early development without obvious defect. Further, $Gbx2^{-/-}$ ES cells differentiated into both ectodermal and mesodermal lineages in an embryologically relevant differentiation assay, demonstrating that *Gbx2* is not required for pluripotent cell differentiation. It was demonstrated in section 5.2 that gastrulation-specific *Gbx2* expression is not recapitulated in embryoid bodies and is not required for differentiation of ES cells into mesodermal or neurectodermal lineages. The lack of conservation of this expression pattern across species suggests that *Gbx2* may not have a critical function in specific germ layers during gastrulation, however the conservation across species of *Gbx2* expression in the developing neurectoderm could be consistent with a role in cell specification or patterning in the CNS.

### *6.4.2 The role of* Gbx2 *in neural lineages*

The major phenotypic defect of *Gbx2* mutant mice is a deletion of the anterior hindbrain (Wassarman et al., 1997). *Gbx2* may be necessary to provide developmental information specifying neural subtype identity in this region. Alternatively, the loss of *Gbx2* may result in an overall failure of survival or differentiation of neurectoderm which is compensated for by other genes expressed in adjacent regions, thus limiting the effects of *Gbx2* ablation. This type of overlapping signalling is seen in the expression of the En family members *En1* and *En2* which are normally expressed in overlapping regions in the midbrain and anterior hindbrain. In knockout experiments, *En1/En2* double mutant mice show a more severe phenotype than either single mutant alone, suggesting an additive contribution of each gene in specifying the midbrain region (Joyner, 1996). Several different in vitro differentiation methods were performed to assess whether the absence of *Gbx2* results in a defect of neural lineage specification.

Embryoid bodies differentiate into a wide variety of cell types, however in the absence of a neuron inducing stimulus only a small percentage of the differentiating cells will form neurons (Wobus, et al., 1988; Bain et al., 1995). A novel method was used to investigate neuron differentiation in which neurectoderm formation is enhanced in the first 7 days of differentiation by culture in the presence of the conditioned medium, Med II, after which terminal differentiation of neurons is directed by withdrawal of serum and the addition of FGF4. This system may be a better model than chemically induced differentiation for investigating the role of *Gbx2* in embryonic development as it appears to reflect the normal embryonic progression of neurectoderm formation based on temporal expression patterns of the marker genes *Oct4*, *Gbx2*, and *Sox1*.

$Gbx2^{+/+}$, $Gbx2^{+/-}$, and $Gbx2^{-/-}$ cell lines showed no difference in their ability to form neurons either in normal embryoid bodies or in embryoid bodies grown in the presence of Med II, demonstrating that *Gbx2* is not required for neuron formation in embryonic-like differentiation models. In addition, $Gbx2^{-/-}$ embryoid bodies were not impaired in their ability to respond to the neural inducing signals in Med II, suggesting that *Gbx2* is unlikely to be required for neurectoderm induction or for differentiation of neural progenitors. While *Gbx2* function may not be obligatory, a role for this gene can not be excluded as differentiating embryoid bodies may express other proteins which are capable of supporting neural precursors in the absence of *Gbx2*. Furthermore, it is possible that *Gbx2* is required for FGF signalling in the neural differentiation pathway and that, in EBM's, FGF4 added to the medium on day 8 compensates for the lack of *Gbx2* expression, however this is not consistent with the

published mutant phenotype in which neurectoderm formation was not completely ablated (Wassarman et al., 1997).

### 6.4.3 *Retinoic acid and* Gbx2 *regulation*

Retinoic acid has been used extensively to enhance neuron formation in vitro (Jones-Villaneuve et al., 1982; Bain et al., 1985; Wobus, 1988; Bain et al., 1995) although the mechanism by which RA induces neuron formation is not clear. *Gbx2*$^{-/-}$ ES cells formed fewer neurons in the presence of retinoic acid, both in the number of aggregates with one or more neurons and in the number of neurons per aggregate, than *Gbx2*$^{+/+}$ ES cells. The differences observed between the wild type and mutant ES cells were unexpected, as the reported mutant phenotype is able to form neurons in most of the brain and spinal cord. Furthermore, in vitro differentiation of embryoid bodies showed that there is no inherent defect in the neuron forming ability of *Gbx2*$^{-/-}$ ES cells.

The fact that fewer neurons were formed in *Gbx2*$^{-/-}$ RA aggregates could indicate that *Gbx2* enhances the response of ES cells and differentiating neural precursors to induction by retinoic acid. An alternative explanation of these differences arises from the observations that neurons formed by RA induction have a primarily ventral identity (*Pax6* expressing) (Renoncourt et al., 1998), whereas ES cells grown in a defined culture medium without RA form both dorsal (*Pax3* expressing) and ventral neurons (Li et al., 1998). Sonic hedgehog (*shh*), a secreted signalling molecule which is normally found in the ventral neural tube and notochord and is responsible for formation of certain motor neurons and ventral interneurons (Ericson et al., 1997), has been shown to be up regulated in RA treated ES cell aggregates (Renoncourt et al., 1998). Thus the mechanism by which RA induces neuron formation in vitro may involve extensive *shh* signalling, resulting in preferential formation of ventral neurons. It is possible that only a small subset of neurons arising from the ventrally restricted population of neural precursors formed in the presence of RA is able to differentiate or survive in the absence of *Gbx2*. It has been suggested, however, that in vitro culture in RA, similar to experiments in which excess RA is administered exogenously to the embryo, may not reflect the role of retinoic acid in normal neural development (Kastner et al., 1995).

Alternatively, this model may be representative of the effects of axial patterning information on neural sub-type specification. Exogenous RA has been shown to cause posteriorization of anterior brain structures and gene expression (Lumsden and Krumlauf, 1996; Blumberg et al., 1997), suggesting that the embryonic role of retinoic acid may include providing positional information along the anteroposterior

axis. It is possible that *Gbx2* may play a role in interpreting the positional information provided by RA in the anterior hindbrain region and the differences observed between $Gbx2^{+/+}$ and $Gbx2^{-/-}$ cell lines in RA differentiation in vitro may reflect a failure to respond to specific positional information specifying neural subpopulations.

The results of RA induced differentiation, together with the in vitro differentiation of embryoid bodies in the absence or presence of Med II, suggest that *Gbx2* is more likely to specify regional identity than to control survival or proliferation of neural progenitors.

### *6.4.4* Gbx2 *ablation and* Sox1 *regulation*

*Sox1* is a transcription factor which is expressed only around the time of neural induction. It is first detected around 7.5 d.p.c. in neurectoderm of the neural plate and developing neural tube and later is restricted to the dividing neural cells of the ventricular zone (Pevney et al., 1998). Induced expression of *Sox1* in P19 embryonal carcinoma cells enhances the formation of neurons to a degree similar to that seen with RA induction (Pevney et al., 1998). Other members of the Sox family have also been shown to be responsible for the specification and development of particular cell lineages, such as *Sox4* which is required for mature B cell formation (Schillam et al., 1996) and *Sry* which is responsible for specifying the male phenotype (reviewed in Lovell-Badge and Hacker, 1995). It appears, then, that *Sox1* is involved in cell fate decisions rather than providing positional information. Targeted mutation of *Sox1* results in mice which are micropthlamic, develop cataracts, and suffer spontaneous seizures (Nishiguchi et al., 1998). Analysis of these mutants revealed that *Sox1* is required for expression of γ-crystallin genes which encode the major structural proteins of lens fiber cells. The lack of obvious early neural defects may be due to the functional redundancy of *Sox2* and *Sox3* which are also expressed in the CNS (Nishiguchi et al., 1998).

In vivo, *Gbx2* expression is observed at least a day earlier than *Sox1*, in all three germ layers of the primitive streak and then expression overlaps that of *Sox1* in the early neurectoderm, suggesting that *Gbx2* might regulate the expression of *Sox1* in this cell type. The *Sox1* promoter region has not yet been characterized and thus it is not known whether it contains Gbx2 binding sites.

In order to determine whether *Gbx2* regulated *Sox1* expression in neurectoderm, *Sox1* expression was compared in $Gbx2^{+/+}$ and $Gbx2^{-/-}$ ES cell RA aggregates. Both RNase protection analysis and WMISH analysis showed a decrease in the amount of *Sox1* expression in the null mutant ES cell line, although expression was not totally ablated,

demonstrating that *Gbx2* is not obligatory for *Sox1* expression. WMISH showed that fewer aggregates and fewer cells within each aggregate expressed *Sox1* in the $Gbx2^{-/-}$ aggregates, however the intensity of staining in those cells was similar to $Gbx2^{+/+}$ aggregates, indicating that this decrease in *Sox1* is likely to reflect decreased neurectoderm formation in the aggregates. Thus, while *Sox1* is not directly downstream of *Gbx2*, its expression appears to be dependant on the formation of neurectoderm.

# Chapter 7

# The role of *Gbx2* in the specification of mesoderm and macrophage lineages

# 7 The role of *Gbx2* in the specification of mesoderm and macrophage lineages

## 7.1 Introduction

### *7.1.1* Gbx2 *and mesoderm formation*

The expression of *Gbx2* in the mesodermal layer of the primitive streak during gastrulation in vivo suggested a possible role in mesoderm formation or differentiation. Examination of Gbx2 expression in embryoid bodies revealed a diminished expression during mesoderm formation (section 5.2.2) which was not expected, however this is not inconsistent with the lack of mesodermal defects observed in *Gbx2* mutant mice (Wassarman et al., 1997), suggesting that *Gbx2* is not likely to be required for mesoderm formation.

### *7.1.2* Gbx2 *and hematopoiesis*

The hypothesis that *Gbx2* might be involved in hematopoiesis arose from experiments in which *GBX2*, the chicken homologue of murine *Gbx2*, was shown to be an activator of chicken myelomonocytic growth factor (cMGF) (Kowenz-Leutz et al., 1997). cMGF is a cytokine which stimulates growth and survival of transformed myeloid cells and which stimulates colony formation and differentiation of normal bone marrow cells (Leutz et al., 1984, 1989; Metz et al., 1991). An electrophoretic mobility shift assay, using nuclear extracts from AMV and E26 transformed myeloblasts, identified a novel protein which bound to sites in the *cMGF* promotor region. Subsequent screening of a cDNA library with an oligonucleotide probe representing one of these sites resulted in isolation of the gene encoding GBX2 and in vitro binding assays and reporter gene assays confirmed that GBX2 is capable of activating transcription of *cMGF* (Kowenz-Leutz et al., 1997). Based on the finding that avian myeloblastosis virus (AMV), which expresses a mutated *v-myb* oncogene, causes the secretion of cMGF in transformed monoblasts (Dini et al., 1995), Kowenz-Leutz et al. (1997) investigated the ability of the Myb protein to activate *GBX2* transcription and, using a conditionally expressed *v-myb* macrophage cell line, demonstrated that *GBX2* is a target gene of the v-Myb protein. Further analysis with *c-myb* expressing cell lines indicated that an activated signalling pathway, such as Ras, is required for activation of *GBX2* transcription by c-Myb while the mutations present in the leukemogenic AMV v-Myb render it independent of signalling and allow constitutive activation of *GBX2* (Kowenz-Leutz et al., 1997). Finally, Kowenz-Leutz's group (1997) showed that ectopic expression of GBX2 in primary chicken bone marrow cells, in the presence of a transforming E26 *v-Myb-ets* gene, resulted in

differentiation of myelomonocytic precursor cells into monoblasts. They concluded from these experiments that GBX2 plays a role in hematopoiesis by specifying the monocytic phenotype in myelomonocytic precursors (Kowenz-Leutz et al., 1997).

### *7.1.3 The role of* c-myb *in hematopoiesis*

The proto-oncogene *c-myb*, which encodes a DNA binding protein, is expressed most abundantly in erythroid, myeloid, and lymphoid progenitor cells and is down regulated upon differentiation (Westin et al., 1982; Craig and Bloch, 1984). Expression has been reported in a number of human malignancies, including some of neurectodermal and hematopoietic origin (Slamon et al., 1986; Thiele et al., 1987), in human embryonic and adult neural tissue and is down regulated in neuroblastoma cells exposed to retinoic acid (Thiele et al., 1988). Expression has also been reported in embryonic stem cell and embryonal carcinoma cell lines (Dyson et al., 1989). Mice deficient in c-Myb have been created through homologous recombination (Mucenski et al., 1991). Homozygous mutant offspring die around 15 d.p.c. due to severe anemia. Further examination of the phenotype revealed that while yolk sac hematopoiesis was unaffected by the mutation, adult-type hematopoiesis, which first occurs in the fetal liver around 9.5 d.p.c., was severely compromised. Fewer cells from all hematopoietic lineages except megakaryocytes were observed in mutant livers. This suggested that c-Myb may be required to maintain proliferation of certain hematopoietic progenitor cells and that without c-Myb these cells proceed to differentiate, resulting in a small population of terminally differentiated hematopoietic cells. A model for hematopoietic differentiation based on these data and the data described by Kowenz-Leutz et al. (1997) is that Myb, while generally promoting proliferation rather than differentiation of progenitor cells, also acts to promote the maturation of myelomonocytic precursors via up regulation of *Gbx2* in conjunction with an activated signalling pathway in these precursors. *Gbx2* then directs the myelomonocytic precursors to mature into monocytes, possibly through the up regulation of growth factors such as cMGF.

There has been no murine homologue of cMGF isolated to date, which raises the question of whether *Gbx2* has a role in monoblast/macrophage formation in the mouse and whether it is activated by murine Myb protein. Two sequences (GAGTTG, CGGTTG (reverse orientation)) have been identified at positions 69 bp and 160 bp 5' of the first *Gbx2* transcriptional start site which deviate from the Myb consensus binding site (CC/AGTTR) by one nucleotide (section 3.3), however it has not been determined whether Myb protein binds to these sites. While *Gbx2* mRNA has been detected at low levels in the adult spleen (Bouillet et al., 1995; Chapman et al., 1997), it is unknown whether *Gbx2* is expressed in the yolk sac, fetal liver or fetal or adult

bone marrow or whether *Gbx2* plays a role in the hematopoietic system either during development or in the adult mouse.

### *7.1.4 Murine hematopoiesis*

Development of the murine hematopoietic system occurs at multiple sites and stages. The first evidence of hematopoiesis is the appearance of blood islands which arise from extraembryonic mesoderm in the yolk sac at around 7.5 d.p.c.. These islands contain primitive erythrocytes and some primitive macrophages (Moore and Metcalf, 1970). By 8.5 d.p.c. circulation has been established and by 9.5 d.p.c. the liver has been colonized with hematopoetic stem cells and begins to produce precursors of all lineages. Stem cells which colonize the liver are thought to have both extra-embryonic and intra-embryonic origins (Robb, 1997; Cumano et al., 1996). The paraaortic splanchnopleura, which encompasses the aortic, gonad, and mesonephros (AGM) region, has been isolated from precirculation stage (7.5 d.p.c.) embryos and differentiated in culture to form lymphoid as well as erythroid and myeloid lineages, while yolk sacs isolated at the same time were only able to form erythroid and myeloid lineages (Cumano et al., 1996), suggesting differences in differentiation potential of the hematopoietic stem cells arising from the two sites. From birth onward the bone marrow and spleen become the primary sites of hematopoiesis, having been colonized by circulating stem cells (Moore and Metcalf, 1970).

Macrophages are formed earlier than other leukocytes as they have an important role to play in the early embryo in tissue remodelling and in forming a defensive barrier to the placenta (reviewed in Brighton and Krummel, 1996). Mature macrophages expressing a surface marker F4/80 have been detected as early as day 10 in the yolk sac and liver (Morris et al., 1991). There is evidence of differences between embryonic macrophage formation, in which mature macrophages develop from a primitive macrophage precursor, and adult macrophage formation where myelomonocytic precursors differentiate into monocytes and then macrophages (reviewed in Naito et al., 1996; Bonifer et al., 1998). Macrophage formation is influenced by a number of growth factors, including IL-6, IL-3, GM-CSF, and M-CSF which is critical for the development and differentiation of a variety of macrophage populations (Naito et al., 1996).

Genes involved in hematopoiesis have often been first identified by their association with translocations occurring in leukemias as well as by their expression in normal or leukemic cell lines. A number of *Hox* genes have been implicated in this way and in vitro overexpression of these genes, including *HoxA10*, *HoxB3*, *HoxB4*, and *HoxB8*, have demonstrated effects on hematopoietic differentiation potential (Perkins

and Cory, 1993; Sauvageau, et al., 1995; Sauvageau, et al., 1997; Thorsteinsdottir et al., 1997a). Other transcription factors involved in hematopoiesis have been investigated in vivo by gene targeting techniques. GATA-1 (Pevney et al., 1991), Flk1 (Shalaby et al., 1995), TGF-β1 (Dickson et al., 1995), and *scl* (Robb et al., 1996) have been shown to be required for both primitive (yolk sac) and definitive hematopoiesis. Other genes such as *c-myb*, *c-kit*, *Cbfa2*, and *Cbfb* are required for definitive hematopoiesis, but not primitive hematopoiesis (reviewed in Dzierak, 1998). The molecular mechanisms regulating macrophage lineage commitment and differentiation, embryonic or adult type, are not well understood. Mice lacking a functional c-fos gene display an increase in the number of tissue macrophages (Grigoriadis et al., 1994) and PU.1 null mice lack both myeloid and lymphoid lineages (Scott et al., 1994), however the complex signalling patterns regulating the survival, proliferation, commitment, and differentiation of the numerous intermediate cell types in the myelomonocytic pathway are still largely undescribed.

### *7.1.5 In vitro models of hematopoiesis*

The differentiation of nascent mesoderm into hematopoietic precursors in embryoid bodies appears to mimic yolk sac hematopoiesis, with the formation of primitive erythrocytes and macrophages being the most prevalent lineages (Wiles and Keller, 1991; Keller et al., 1993). Differentiation can be further directed by the addition of exogenous factors specifically toward erythroid, granulocytic, or monocytic lineages (Wiles and Keller, 1991; Keller et al., 1993). This controlled differentiation system has been used to investigate molecules regulating hematopoiesis. For example, $scl^{-/-}$ mice die in utero around day 9 due to failure of primitive hematopoiesis, preventing analysis of later stages of development (Robb et al., 1996). $scl^{-/-}$ ES cells failed to contribute to hematopoietic lineages in chimeric mice and did not form hematopoietic colonies when differentiated in vitro, suggesting a role in very early hematopoietic development. Examination of gene expression of a number of hematopoietic transcription factors in embryoid bodies demonstrated that hematopoietic lineage restricted genes such as GATA-1, EKLF, and PU.1 were not expressed in $scl^{-/-}$ embryoid bodies, while markers of ventral mesoderm such as *brachyury*, BMP-4 and Flk1 were expressed, supporting a model in which *scl* is required for specification of early hematopoietic precursors (Elefanty et al., 1997). This type of in vitro differentiation system could be useful to test whether *Gbx2* deficient ES cells are capable of forming normal monocyte/macrophage lineages.

Experimental evidence suggests that *GBX2* is involved in hematopoiesis in the chicken as a result of activation by Myb. Myb has been demonstrated in the mouse to be required for the proliferation of hematopoietic precursors in adult-type hematopoiesis,

including myeloid lineages. These data, taken together with the detection of *Gbx2* transcripts in the mouse spleen and the presence of two potential Myb binding sites in the region 5' of the *Gbx2* start site, suggest that *Gbx2* may be involved in murine hematopoiesis by specifying the differentiation of myelomonocytic precursors toward the macrophage phenotype. The aims of this chapter were to investigate the role of *Gbx2* in mesoderm and macrophage formation and its potential regulation by Myb.

## 7.2 *Gbx2* as a target of Myb

Primary murine fetal liver cells transformed with a fusion protein gene consisting of the ligand binding domain of the estrogen receptor and a truncated c-myb (ERMYB cells) exhibit an immature myeloid phenotype when grown in the presence of β-estradiol and differentiate upon removal of β-estradiol into granulocytes and macrophages (Hogg et al., 1997). Upon activation by binding of the β-estradiol ligand to the receptor portion of the fusion protein, the protein is transported to the nucleus where the Myb domain is able to transactivate target genes. When β-estradiol is withdrawn from the culture medium, no further nuclear translocation occurs and the activation of target genes ceases, presumably due to degradation of the Myb protein. The Myb target genes *bcl-2* and *c-kit* have been shown to respond to β-estradiol challenge by decreased expression levels within 24 hours of β-estradiol withdrawal (Hogg et al., 1997).

ERMYB cells (T. Gonda) were maintained without feeder layers in DMEM medium containing antibiotics, 16% FCS, 400 U/ml GM-CSF, and 1μM β-estradiol. ERMYB cells were then washed in PBS and resuspended in medium without β-estradiol and cells were harvested for total RNA preparation at 0, 24, and 48 hours after β-estradiol withdrawal. Alternatively, β-estradiol was reintroduced to the medium after 24 hours and cells were harvested 48 hours after the reintroduction of β-estradiol (Hogg et al., 1997). Total RNA was prepared from these cells and *Gbx2* expression was quantified by RNAse protection assay (2.3.24) using a riboprobe transcribed from a plasmid (pG290) containing a 290 bp fragment of *Gbx2* cDNA from a region 5' of the homeobox, which protects a 290 bp fragment of *Gbx2* RNA, and a riboprobe which protects a major mGAP band at 240 bp (Rathjen et al., 1990) as a loading control. Strong *Gbx2* expression was detected in control (ES cell) RNA, while no *Gbx2* expression was observed in the ERMYB cell RNA at any time point, regardless of whether Myb was activated or not (Fig. 7.1). These data suggest that *Gbx2* is neither positively or negatively regulated by Myb in mouse fetal liver cells.

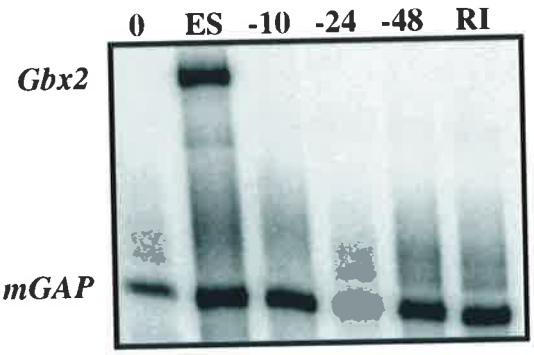



**Figure 7.1** ***Gbx2* expression in ERMYB cells**

RNAse protection assay of 20 µg total RNA from ERMYB cell line at timepoints after β-estradiol removal and E14TG2a ES cells hybridized with antisense *Gbx2* and *mGAP* probes. **0**, 0 hours, **-10**, 10 hours after removal, **-24**, 24 hours after removal, **-48**, 48 hours after removal, **RI**, cells grown for 24 hours without β-estradiol, then in the presence of 1µM β-estradiol for 48 hours.

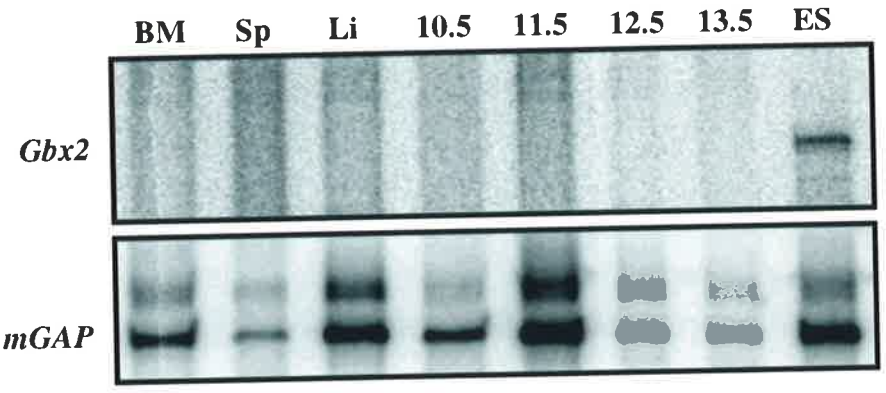



**Figure 7.2** ***Gbx2* expression in hematopoietic tissues**

RNAse protection assay of 20 µg total RNA from various hematopoietic tissues and E14TG2a ES cells hybridized with antisense *Gbx2* and *mGAP* probes. **BM**, bone marrow, **Sp**, spleen, **Li**, fetal liver, **10.5**, yolk sac from 10.5 d.p.c. embryo, **11.5**, yolk sac from 11.5 d.p.c. embryo, **12.5**, yolk sac from 12.5 d.p.c. embryo, **13.5**, yolk sac from 13.5 d.p.c. embryo, **ES**, E14TG2a ES cells.

## 7.3 Expression of *Gbx2* in hematopoietic tissues

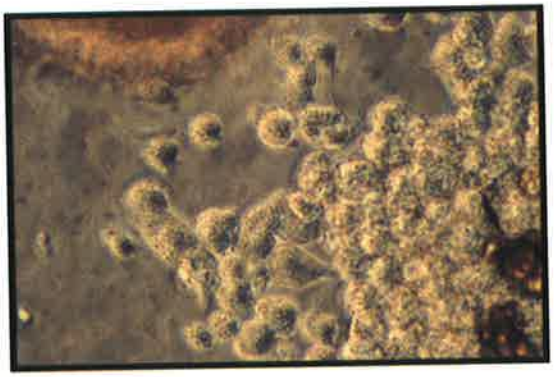
Kowenz-Leutz et al. (1997) report the detection of *GBX2* transcripts in chicken hematopoietic tissues including bone marrow, bursa of fabricius, liver, spleen, and thymus. In order to further define *Gbx2* expression and its potential for regulation of hematopoiesis, total RNA from mouse bone marrow, spleen, fetal liver, and yolk sac from 10.5-13.5 d.p.c. embryos was subjected to RNAse protection analysis using a probe which protects a 440 bp fragment of *Gbx2* RNA (Chapman and Rathjen, 1995) and a probe which protects a major mGAP band at 240 bp as a loading control (Rathjen et al., 1990). The results (Fig. 7.2) show *Gbx2* expression in the positive control (ES cell) RNA, but no detectable *Gbx2* expression in any of the hematopoietic tissues tested, including adult spleen. This is in contrast to other reports of low level *Gbx2* expression in the mouse spleen (Bouillet et al., 1995; Chapman et al., 1997). These data suggest that *Gbx2* expression differs in the mouse and chicken hematopoietic systems and therefore the role of *Gbx2* may be different in the two species.

## 7.4 Effects of ablation of *Gbx2* expression on mesoderm and macrophage formation in embryoid bodies

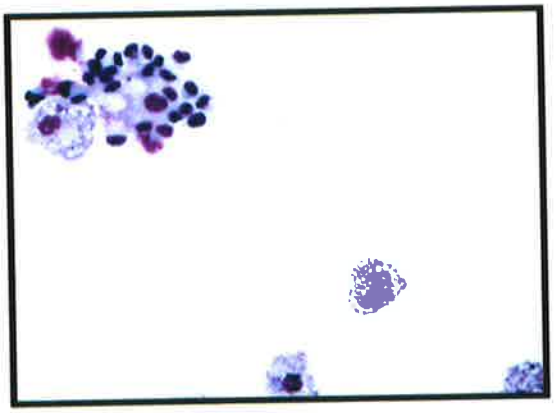
Wild type E14TG2a ($Gbx2^{+/+}$), heterozygous mutant ($Gbx2^{+/-p}$), and homozygous mutant ($Gbx2^{-/-}$) ES cells were differentiated as embryoid bodies in semi-solid methyl cellulose (MC) medium in the presence of exogenous murine IL3 and human M-CSF as described in section 2.4.12. Methyl cellulose provides a supportive structure which prevents differentiating hematopoietic cells from leaving the area surrounding the colony. This may lead to local accumulation of factors necessary for the differentiation of hematopoietic cells (Wiles and Keller, 1991). IL3 and M-CSF are cytokines which have been shown to enhance the development of macrophages in embryoid body cultures (Wiles and Keller, 1991, Keller et al., 1993).

Macrophage-like cell formation was observed in embryoid bodies as early as day 6 of culture (Fig. 7.3a). Macrophage identity was confirmed by morphology when stained with May-Grünwald-Giemsa stain (Fig. 7.3b) and by positive staining with the macrophage specific antibody, F4/80 (Austyn and Gordon, 1981) (Fig. 7.3c). After 15 days the number of colonies forming cardiac (beating) muscle was counted. After 18 days of culture the number of colonies containing greater than 5 macrophages was counted. Approximately 10% of embryoid bodies contained more than 5 macrophages on day 18. This is lower than previously reported frequencies of 30-50% (Wiles and Keller, 1991) and may be due to differences in culture conditions, such as FCS batches. The results, shown in Figure 7.4, a and b, indicated no significant change in the number of macrophage producing colonies or cardiac muscle producing colonies

a.



b.



c.

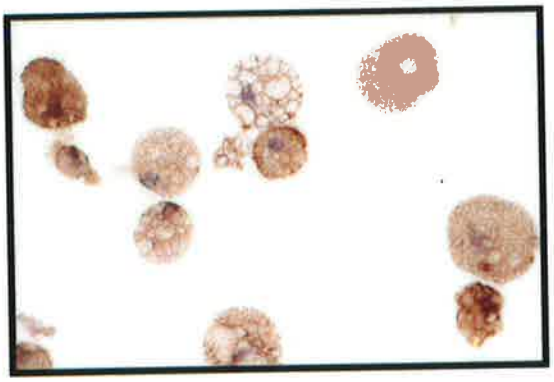


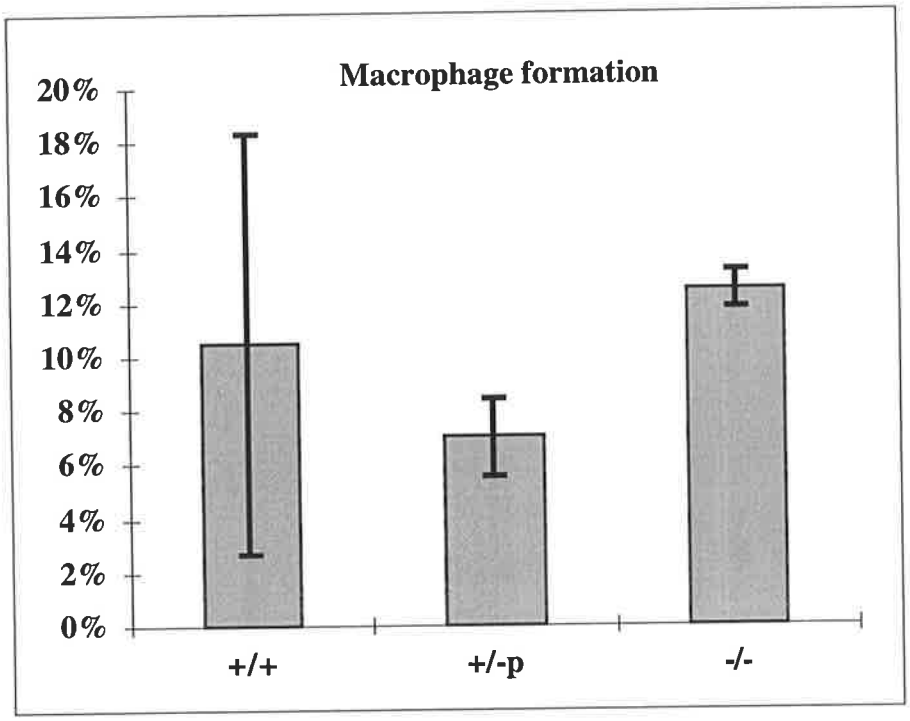
**Figure 7.3 Macrophage formation from ES cells**

a. Photograph of macrophage-like cells surrounding an ES cell embryoid body grown in MC medium supplemented with mIL3 and hM-CSF on day 18 of culture. Magnified 125x.

b. Photograph of cytospun cells from MC culture stained with May-Grünwald-Giemsa stain. Magnified 200x.

c. Photograph of cytospun cells from MC culture stained with monoclonal antibody F4/80 followed by rabbit anti-rat antibody, and detected by goat anti-rabbit antibody conjugated to horseradish peroxidase. Brown color indicates the presence of bound antibody. Counterstained with May-Grünwald-Giemsa stain. Magnified 200x.

a.




b.

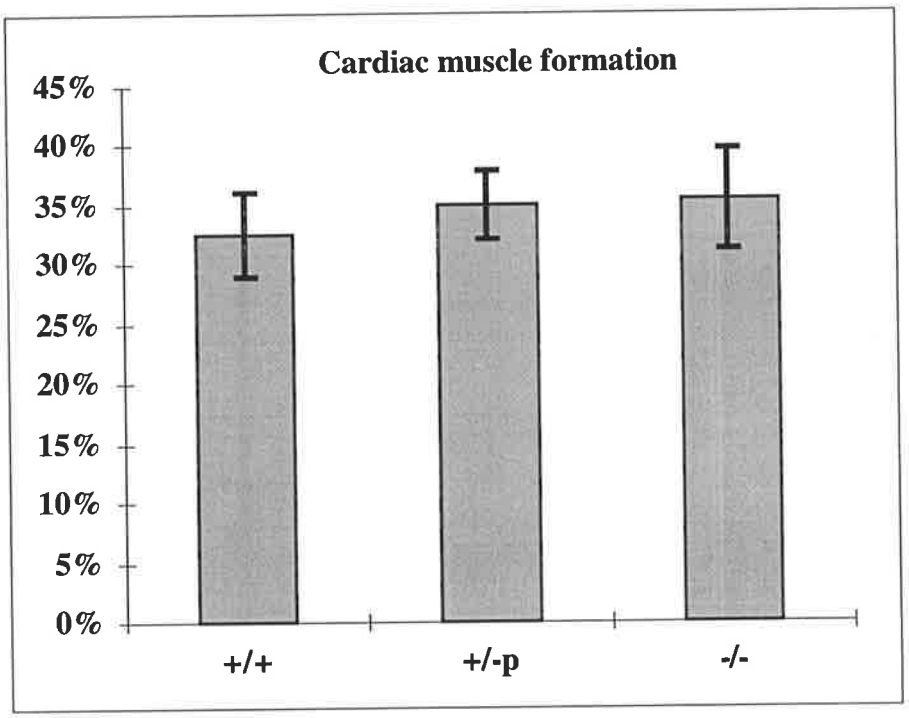



**Figure 7.4 Mesoderm derivatives from ES cells in MC culture**

a. Graph representing the percent of colonies containing 5 or more macrophages after 18 days of culture in MC medium supplemented with mIL-3 and hM-CSF.

b. Graph representing the percent of colonies containing cardiac (beating) muscle after 15 days of culture in MC medium supplemented with mIL-3 and hM-CSF.

**+/+**, *Gbx2*$^{+/+}$ ES cell line, **+/-p**, *Gbx2*$^{+/-}$ ES cell line used for second allele targeting (parental), **-/-**, *Gbx2*$^{-/-}$ mutant ES cell line.

upon partial or complete ablation of *Gbx2* expression, therefore it appears that *Gbx2* is not obligatory for mesoderm or macrophage formation. This is consistent with the reported phenotype of mouse mutants (Wassarman et al., 1997).

## 7.5 Mesoderm and macrophage formation in EPL cell embryoid bodies

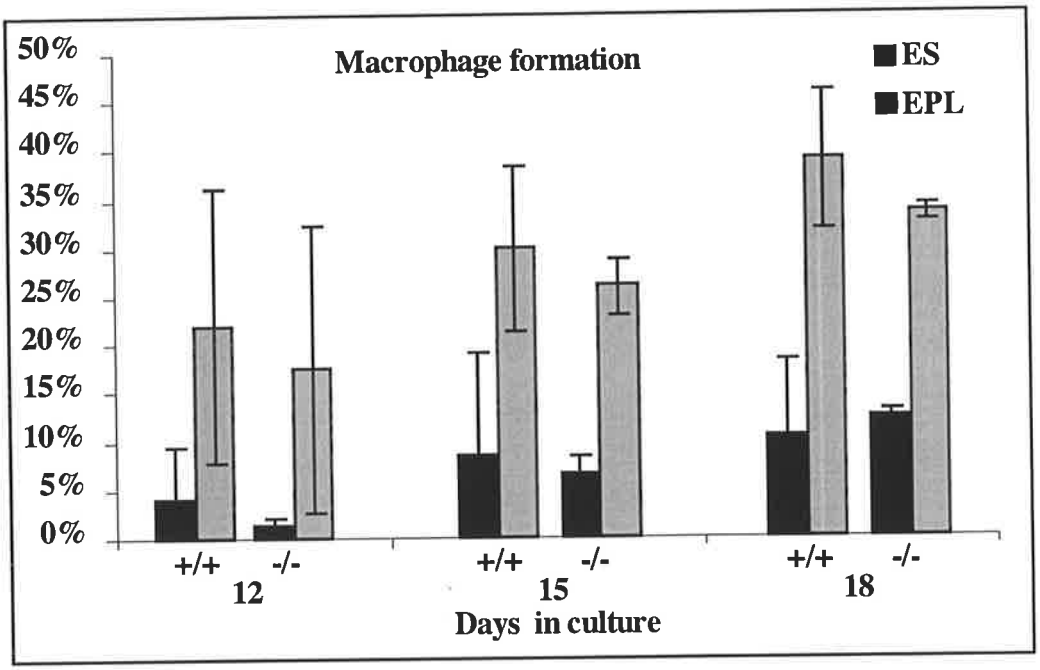
EPL cell embryoid bodies form nascent mesoderm, as evidenced by early mesodermal markers *brachyury* and *goosecoid*, earlier and more extensively than ES cell embryoid bodies (Lake, 1996; Lake et al., in press). Terminally differentiated mesoderm in the form of cardiac muscle, as evidenced by cardiac muscle marker Nkx2.5 and the presence of beating muscle, also forms earlier and more extensively in EPL cell embryoid bodies than in ES cell embryoid bodies (Lake, 1996; Lake et al., in press). These data do not determine whether the elevated levels of nascent mesoderm are committed to a cardiocyte fate or might represent a multipotent or pluripotent mesodermal precursor that can be programmed to alternate mesodermal fates. This was tested by analysis of macrophage formation in EPL cell embryoid bodies in response to the appropriate exogenous factors. *Gbx2*$^{-/-}$ ES cells, when cultured in EPL medium (2.4.10) formed colonies typical of EPL cells in appearance as expected given that they appear to differentiate normally. *Gbx2*$^{+/+}$ and *Gbx2*$^{-/-}$ ES and EPL cells were used to assess the formation of macrophages in order to determine whether the elevated levels of nascent mesoderm in EPL cell embryoid bodies were developmentally restricted to myogenic lineages or could be reprogrammed to alternative developmental fates and whether *Gbx2* ablation had any effect on this differentiation potential.

*Gbx2*$^{+/+}$ and *Gbx2*$^{-/-}$ ES and EPL cell aggregates (day 2 of culture) were differentiated in MC medium supplemented with mIL-3 and hM-CSF (2.4.12) and scored for the presence of macrophages on days 12, 15 and 18 and cardiac (beating) muscle on days 15 and 18 (Fig. 7.5a and b). Macrophages were first observed on day 4 in EPL cell embryoid bodies and day 6 in ES cell embryoid bodies. EPL cell embryoid bodies exhibited an approximately 3-fold increase in macrophage formation and an approximately 3-fold decrease in cardiac muscle formation. There was no difference between *Gbx2*$^{+/+}$ and *Gbx2*$^{-/-}$ cell lines in the differentiation of ES or EPL cells. These data support the conclusions that *Gbx2* is not essential for mesoderm or macrophage formation and that EPL cell embryoid bodies produce elevated levels of nascent mesoderm which can be directed into a hematopoietic fate.

## 7.6 Discussion

The work of Kowenz-Leutz et al. (1997) suggested a possible role for *GBX2* in hematopoiesis in the chicken, specifically in the growth and differentiation of

a.

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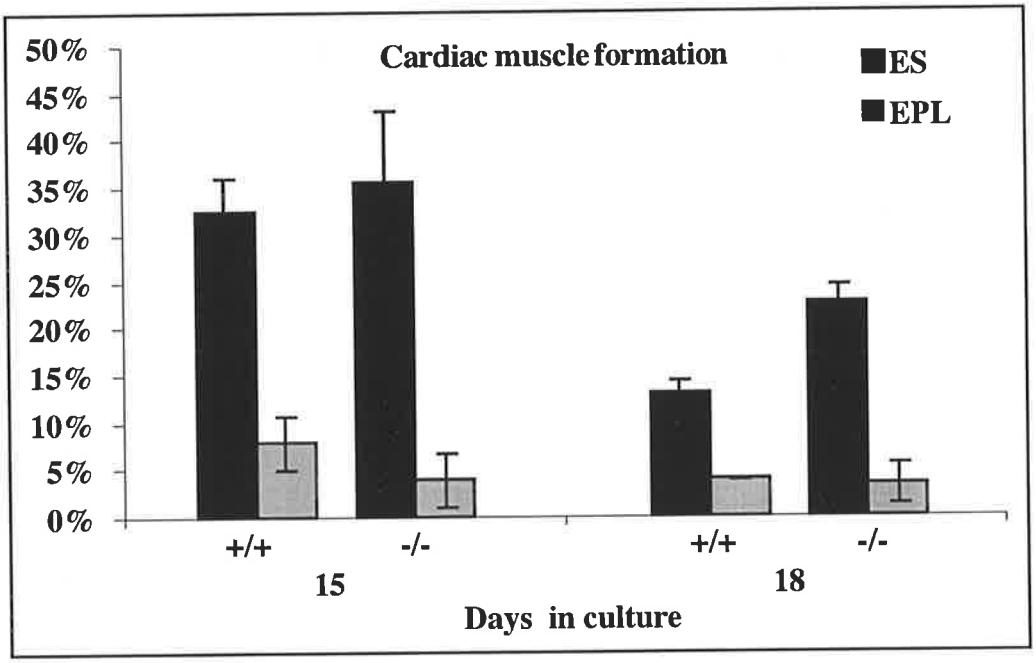


**Figure 7.5 Mesoderm derivatives from ES and EPL cells in MC medium**

a. Graph representing the percent of colonies containing 5 or more macrophages after 12, 15, and 18 days of culture in MC medium supplemented with mIL-3 and hM-CSF.

b. Graph representing the percent of colonies containing cardiac (beating) muscle after 15 and 18 days of culture in MC medium supplemented with mIL-3 and hM-CSF.

**ES**, ES cells, **EPL**, EPL cells, **+/+**, *Gbx2*$^{+/+}$, **-/-**, *Gbx2*$^{-/-}$

myelomonocytic precursors into monocytes/macrophages in response to transcriptional regulation by c-Myb. Evidence that Myb is required for hematopoiesis in the mouse (Mucenski et al., 1991), the presence of c-Myb consensus binding sites in the 5' regulatory region of *Gbx2* (section 3.3), and detection of *Gbx2* transcripts in the spleen (Bouillet et al., 1995; Chapman et al., 1997) were consistent with the possibility that *Gbx2* may influence hematopoiesis in the mouse in response to activation by Myb. The aims of this chapter were to investigate the role of *Gbx2* in mesoderm and macrophage formation and its potential regulation by Myb.

The ERMYB cell line has been used to characterize potential Myb target genes by their response to the decrease in active Myb resulting from a withdrawal of β-estradiol from the culture medium (Hogg et al., 1997). This system differs from that described by Kowenz-Leutz et al. (1997) in several respects. Kowenz-Leutz's group used a MC-29 transformed chicken macrophage-like cell line in which v-Myb is conditionally activated by estradiol (Burk and Klempnauer, 1991), while ERMYB cells are derived from murine fetal liver cells in which c-Myb is conditionally activated by estradiol. The ERMYB system may more closely represent the in vivo gene expression environment as it uses a primary rather than transformed cell line. Kowenz-Leutz, et al. (1997) have shown that an activated intracellular signalling pathway is required for the induction of *GBX2* by c-Myb. In ERBMYB cells the Ras signalling pathway is activated by the presence of GM-CSF in the culture medium (McCormick and Gonda, 1997), thus nuclear localized c-Myb should be capable of inducing target genes in this system. While Kowenz-Leutz et al. detected *GBX2* expression as early as 4 hours after addition of estradiol and increasing expression through 24 hours, no *Gbx2* expression was detected at any time point after withdrawal or reintroduction of β-estradiol in the ERMYB cells. The ERMYB cells were shown by morphology and neutrophil or macrophage specific esterase staining to differentiate into neutrophils and macrophages within 48 hours of β-estradiol withdrawal (data not shown). These results suggest that *Gbx2* is not a target gene of c-Myb in the mouse, indicating that the role of Myb in mouse hematopoiesis may be different from its role in chicken hematopoiesis. In addition, it appears that *Gbx2* is not expressed during the differentiation of macrophage precursors in the ERMYB system, suggesting that *Gbx2* may not be important for specification of this cell type.

Another observation by Kowenz-Leutz et al. (1997) was the detection of *GBX2* transcripts in chicken hematopoietic tissues including bone marrow, bursa of fabricius, liver, spleen, and thymus. Although others (Bouillet et al., 1995; Chapman et al., 1997) have reported low levels of *Gbx2* expression in the adult mouse spleen, no expression was detected in fetal and adult hematopoietic tissues, including yolk sac from 10.5-13.5 d.p.c. embryos, fetal liver, adult bone marrow and spleen. This

supports the concept that *Gbx2* may not be involved in hematopoiesis in the mouse as it is in the chicken.

The ability to direct ES cell differentiation and to manipulate the culture conditions in vitro enables the investigation of cellular interactions and decisions of early development in normal or mutated ES cells. In vitro differentiation methods were used to further investigate a possible role for *Gbx2* in hematopoiesis and in the differentiation of myeloid precursors in particular, as described in sections 7.4 and 7.5. *Gbx2*$^{-/-}$ ES cells were unaltered in the ability to form macrophages, showing that *Gbx2* is not essential for macrophage formation. In vitro differentiation methods have also been used to characterize EPL cell differentiation potential and gene expression patterns (Lake, 1996; Lake et al., in press). While it has been previously established that EPL cells form nascent mesoderm and cardiac muscle more readily than ES cells when cultured as embryoid bodies, the assay described in section 7.5 demonstrated that the nascent mesoderm produced by EPL cells could be directed to differentiate into macrophages as well cardiac muscle. Furthermore, *Gbx2* ablation had no effect on this enhanced macrophage production, confirming that *Gbx2* is not essential for macrophage formation.

In this system, EPL cells formed macrophages more readily than ES cells, while ES cells formed more cardiac muscle than EPL cells. This could be explained by the difference in the cellular environment and the signals being generated from within the early embryoid body during differentiation. EPL cell embryoid bodies do not form visceral endoderm and therefore are not exposed to the signals from visceral endoderm which direct ectoderm formation, resulting in the enhanced formation of mesoderm by default (Lake, 1996; Lake et al., in press). ES cell embryoid bodies, on the other hand, form visceral endoderm and thus are exposed to multiple differentiation signals. During the differentiation process in MC medium, EPL cells may more efficiently respond to cytokine signals in the medium, while ES cells receive additional signals from within the embryoid body which may preferentially direct differentiation toward cardiac muscle.

Several reports suggest that there are differences between embryonic macrophage formation, in which mature macrophages develop from a primitive macrophage precursor, and adult macrophage formation where myelomonocytic precursors differentiate into monocytes and then macrophages (reviewed in Naito et al., 1996; Bonifer et al., 1998). If *Gbx2* does have a function in proliferation of macrophage precursors or their differentiation it is unknown whether that function is restricted to embryonic or adult type differentiation. It is possible that *Gbx2* could be influencing intra-embryonic hematopoiesis in the AGM or at later stages in the liver, bone marrow,

or spleen. The system employed by Kowenz-Leutz et al. (1997) to explore the role of *GBX2* in chicken hematopoiesis used transformed monoblasts and adult bone marrow cells in which macrophages are formed from myelomonocytic precursors, whereas the ERMYB cell line described in section 7.2 is derived from mouse fetal liver cells and is likely to reflect embryonic macrophage formation. It is unclear from currently available data whether ES cell differentiation results in embryonic or adult type macrophage formation. It would be interesting, therefore, to obtain hematopoietic precursors from the bone marrow of neonatal *Gbx2* null mutant mice in order to study the effects of *Gbx2* ablation on adult type macrophage formation.

The conclusions which can be drawn from the experiments described in this chapter are that *Gbx2* does not appear to be a target gene of Myb in hematopoietic precursors and that it is not a critical participant in the differentiation of mesoderm or myelomonocytic precursors in the mouse. This suggests that the mechanisms of hematopoietic differentiation may be different in the chicken and the mouse.

## Addendum: ALAS2 ablation and macrophage formation

The in vitro differentiation of ES cells into hematopoietic lineages was also used to investigate the differentiation potential of another gene targeted ES cell line produced within the Department of Biochemistry. 5-aminolevulinate synthetase (ALAS2) is the rate limiting enzyme in the biosynthesis of heme in red blood cells (Bottomly and Muller-Eberhard, 1982; May, et al., 1995) and point mutations in this gene have been implicated in X-linked sideroblastic anemia (Bottomley, 1988). Dr. Brian May's laboratory in the Department of Biochemistry, University of Adelaide, is investigating the pathophysiology of this disease and have generated ES cells in which the ALAS2 gene is disrupted. As the gene is located on the X chromosome, a single targeting event was required to eliminate expression. Ablation of gene expression resulted in embryonic lethality at 10 d.p.c. with the notable absence of hemoglobinized cells, despite the presence of erythroid precursors (T. Sadlon, personal communication). Due to the early lethality of the mutation it was difficult to determine whether myeloid lineage differentiation was affected in the mutants, and therefore in vitro hematopoietic differentiation was used to study the effects of the mutation on macrophage formation.

An ES cell line (R3) in which the ALAS2 gene had been disrupted by gene targeting (T. Sadlon, unpublished) was provided by Dr. T. Sadlon, Department of Biochemistry, University of Adelaide. E14TG2a ES cells and R3 ES cells were cultured in suspension for 2 days in EB medium and then aggregates were further cultured in MC medium supplemented with mIL3 and hM-CSF for 11 days (section 2.4.12) in order to assess the ability of ALAS2 deficient ES cells to differentiate into macrophages in culture. On day 11 of culture, 49% of R3 embryoid bodies had produced macrophages as compared to 11% of E14TG2a embryoid bodies, indicating that a mutation resulting in the loss of ALAS2 expression does not adversely affect the potential of ES cells to form macrophages. Thus it appears that the stage at which ALAS2 function is critical in the hematopoietic differentiation pathway is likely to be downstream of myelomacrophage precursor formation and may be restricted to erythroid lineages.

# Chapter 8

# General Discussion

# 8 General Discussion

Homeobox genes have repeatedly been demonstrated to be important molecules in mediating the events of embryonic development. They have been implicated in early specification of regional identity, pattern formation, and organogenesis (Chapter 1). Many homeobox genes have been investigated by means of gene targeting in the mouse (reviewed in Brandon et al., 1995a, b, c; St-Jacques and McMahon, 1996; Vollmer and Clerc, 1998), revealing phenotypes related to their expression patterns in vivo. The work presented in this thesis was aimed at expanding understanding of the role of *Gbx2* in murine development.

## 8.1 Investigation of potential functions of *Gbx2* in embryogenesis and hematopoiesis

The temporal and spatially restricted expression patterns of *Gbx2* during embryogenesis suggested possible functions in the pluripotent cells of the inner cell mass, in the primitive streak at gastrulation, in the developing CNS in the forebrain and the region posterior to the midbrain-hindbrain boundary (isthmus), and in the inner ear. In vitro investigations of the regulatory pathway of Myb in chicken hematopoietic cells suggested that *GBX2* might have a role in directing precursor cells to the monocytic phenotype (Kowenz-Leutz et al., 1997). In the course of this work, Wassarman et al. (1997) described the phenotype of mutant mice created by gene targeting. Disruption of gene expression results in neonatal lethality, with observable defects being most pronounced in the cerebellum and also in the forebrain and inner ear. Further study of the hindbrain defects revealed a truncation in the anterior hindbrain and the absence of derivatives of the isthmus and rhombomeres 1-3. While Wassarman's work suggests a role for *Gbx2* as a patterning gene, specifying this region along the antero-posterior axis of the CNS, it provides no understanding of the cellular mechanisms resulting in these defects. It is not known, for example, whether the defects are due to loss of expression during neural differentiation or loss of gastrulation or inner cell mass specific expression. It is also unknown whether *Gbx2* acts primarily to provide positional identity along the antero-posterior axis or whether it specifies a particular cellular differentiation pathway. The possibility of a role in specifying cell fate in the hematopoietic system as suggested by the experiments with chicken *GBX2* was not addressed in these experiments. In addition, as described in Chapter 6, the mutation created by Wassarman et al. was not a complete deletion of the gene and therefore may not represent the phenotype of a true null mutant.

For these reasons it was decided to investigate possible *Gbx2* functions using a system which would allow patterning and cell type specification functions to be disassociated.

In vitro differentiation of ES cells was chosen as a model because, while cell lineage differentiation occurs in an ordered sequential manner resembling that of the embryo, axial pattern formation is not recapitulated in vitro. In addition the mutation created for this project was designed to completely eliminate the translation of any *Gbx2* gene product, thus creating a true null mutant.

### *8.1.1* Gbx2 *function in the inner cell mass*

The observation that *Gbx2* null mutant ES cells were able to survive and proliferate in an undifferentiated state in culture and could be induced to differentiate by standard methods is consistent with the observation of Wassarman et al. (1997) that *Gbx2* expression is not required for survival, proliferation, or early differentiation events of the inner cell mass in vivo. *Gbx2* null mutant ES cells were shown to be competent to form ectodermal and mesodermal lineages in embryoid bodies and to respond to biologically derived factors for the creation of EPL cells which were normal in appearance, marker gene expression and differentiation potential. These findings suggest that the function of *Gbx2* in the ICM is non-essential or that *Gbx1*, which is expressed in ICM and has a high degree of sequence homology with *Gbx2* (Thomas, 1995; Chapman and Rathjen, 1995), or another homeobox gene is able to compensate for *Gbx2* function in the inner cell mass. Non-essential function or functional redundancy has been observed for a number genes expressed in the blastocyst. For example, LIF-receptor and gp130 are also expressed in the ICM and embryos containing deletions of these genes are able to survive beyond gastrulation (Nichols et al., 1996; Li et al., 1995; Ware et al., 1995; Yoshida et al., 1996).

### *8.1.2* Gbx2 *function in gastrulation*

*Gbx2* is re-expressed in the embryo at gastrulation in the developing primitive streak. *Gbx2* first re-appears around 6.5 d.p.c. in the posterior region of the embryo where primitive streak formation is being initiated and progresses anteriorly in all three germ layers of the streak (Bouillet et al., 1995, Wassarman et al., 1997). In vitro expression mapping of *Gbx2* during embryoid body differentiation and RA induced ES cell differentiation demonstrated a discrepancy with the in vivo expression pattern. While expression in the ICM and later in neurectoderm were recapitulated in vitro as demonstrated by ES cell expression and increased expression in RA induced ES cell aggregates, there was no expression detected in gastrulation stage equivalent ES or EPL cell embryoid bodies, as demonstrated by transient expression of the early mesoderm marker *brachyury*. ES cell embryoid bodies differentiate in an ordered and progressive manner, forming the three germ layers seen in the embryonic primitive streak and proceed to form terminally differentiated cell types representative of all three germ layers (Martin, 1981; Doetschman et al., 1985; Wiles and Keller, 1991; Shen

and Leder, 1992). The absence of *Gbx2* expression during the formation of gastrulation stage germ layers in these bodies indicates a difference between in vitro and in vivo expression patterns. EPL cell embryoid bodies demonstrate enhanced nascent mesoderm formation as evidenced by increased and earlier expression of *brachyury* (Rathjen et al., 1999), and the lack of *Gbx2* expression during the differentiation of these bodies supports the finding that the *Gbx2* expression pattern in vitro is inconsistent with its expression during gastrulation in vivo.

This indicates that the signal required for expression of *Gbx2* in the primitive streak may be different from other stages and that it may be an early spatial patterning signal which is not provided in the embryoid body environment. The identity and source of early spatial patterning signals in the mouse embryo is not yet fully understood. *Hex*, the first known gene to express antero-posterior asymmetry, is expressed in a few cells of the visceral endoderm at the distal tip of the egg cylinder which then migrate in one direction, determining the anterior aspect of the embryo (Thomas et al., 1997; Belo et al., 1997). This apparently sets up a cascade of signals which confer anterior identity. It is unknown what triggers this anterior-ward migration, and thus the establishment of asymmetry, or whether there is a corresponding posteriorizing signal. The fact that embryoid bodies do not form an identifiable antero-posterior axis and thus are unable to respond to the complex positionally restricted signals to form highly organized structures suggests that the early asymmetry signal or signals is missing from the embryoid body environment. The results discussed below demonstrate that in vitro recapitulation of the in vivo gastrulation stage *Gbx2* expression pattern is not required for the formation of neurectoderm, neurons, or blood cells in embryoid bodies. This suggests that, while gastrulation stage expression of *Gbx2* may be required for axial patterning, it does not appear to be necessary for specification of these cell types.

### *8.1.3* Gbx2 *function in the CNS*

Another major site of *Gbx2* expression is in the developing CNS. *Gbx2* expression in the CNS at 8.5 d.p.c. is restricted to the open neural tube and in the hindbrain with strong expression at the midbrain/hindbrain boundary. From 11.5 d.p.c. onwards *Gbx2* expression in the CNS becomes further restricted to the presumptive dorsal thalamus, the mantle part of the basal striatum, the cerebellum, and the anterior hindbrain (Bouillet et al., 1995). In vitro expression mapping of *Gbx2* demonstrated transient expression in ES cell embryoid bodies around the time of neurectoderm formation, as determined by expression of the neural marker, *Sox1*. There was no *Gbx2* expression in EPL cell embryoid bodies, which is consistent with the observation that EPL cell embryoid bodies have been shown to be severely restricted

in their ability to form neurons (Lake et al., in press). In addition, *Gbx2* expression was increased in ES cells differentiated in the presence of retinoic acid, which is consistent with expression in neurectoderm and neural derivatives.

When wild type and *Gbx2*$^{-/-}$ ES cells were cultured as embryoid bodies for 7 days and then plated in serum free, FGF-containing medium no differences were observed in neuron forming capacity between wild type and mutant bodies. Furthermore, when ES cells were cultured as embryoid bodies in the presence of a conditioned medium which enhances neurectoderm formation, both wild type and *Gbx2*$^{-/-}$ ES cells were able to respond to the signals in the medium to produce an increased number of neurons. These results show that *Gbx2* was not absolutely required for neural formation. This is consistent with the observation that *Gbx2* mutation in vivo resulted in mice capable of forming a variety of neural subtypes in the forebrain and regions caudal to rhombomere 3 (Wassarman et al., 1997). In contrast to these results, a difference in neural differentiation potential was seen when wild type and *Gbx2*$^{-/-}$ ES cells were differentiated in the presence of retinoic acid. *Gbx2* null mutant ES cells produced fewer neurons than wild type ES cells, suggesting that *Gbx2* may enhance the responsiveness of ES cells and differentiating neural precursors to RA induction, or alternatively, that *Gbx2*$^{-/-}$ ES cells are impaired in their ability to support the predominantly ventral neural subtype which is preferentially formed by exposure to RA (Renoncourt et al., 1998).

### *8.1.4* Gbx2 *function in hematopoiesis*

In addition to expression in the CNS, *Gbx2* expression has been reported at low levels in the mouse spleen (Bouillet et al., 1995, Chapman et al., 1997) and in chicken hematopoietic tissues, including bone marrow, spleen, and the bursa of fabricius (Kowenz-Leutz et al., 1997). Ectopic expression of chicken *GBX2* in vitro results in the commitment of myelomonocytic precursors to the monocytic phenotype (Kowenz-Leutz et al., 1997) suggesting a possible role in hematopoiesis. The evidence that *GBX2* plays a role in chicken hematopoiesis is based on in vitro data that *GBX2* is a target of Myb, a known regulator of hematopoiesis, that GBX2 binds to the regulatory region of the *cMGF* gene, a myelo-monocytic growth factor, and that ectopic expression of *GBX2* in transformed adult bone marrow cells results in the formation of monocytes (Kowenz-Leutz et al., 1997). Experiments described in Chapter 7 were undertaken to determine whether *Gbx2* plays a role in the mouse hematopoietic system. The results demonstrated that *Gbx2* was not a target of Myb in mouse fetal liver cells, that *Gbx2* was not detectable in hematopoietic tissues of the embryonic or adult mouse, and that wild type and *Gbx2*$^{-/-}$ ES cells exhibited equal capacity for formation of macrophages in culture. These data, taken together with the observation

that no murine equivalent of cMGF has been identified, suggest that *Gbx2* does not appear to be required for normal murine hematopoiesis. This is consistent with the observation that there were no obvious hematological defects in *Gbx2* mutant mice, however it is unknown whether myeloid lineages were specifically examined in these mice (Wassarman et al., 1997). Thus, although chicken GBX2 and mouse Gbx2 share amino acid identity within the homeodomain, they do not appear to share identical functions.

### *8.1.5* Gbx2 *and transcriptional regulatory networks*

*Sox1* is a transcription factor which is expressed in neurectoderm around the time of neural induction and then is restricted to the dividing neural cells of the ventricular zone (Pevney et al., 1998). Targeted mutation of *Sox1* revealed that it is required for expression of γ-crystallin genes which encode the major structural proteins of lens fiber cells (Nishiguchi et al., 1998). *Sox1*, like other members of the Sox family (Schillam et al., 1996; Lovell-Badge and Hacker, 1995), appears to be involved in regulating cellular decisions rather than providing positional information. In vivo, *Gbx2* expression is observed at least a day earlier than *Sox1*, in all three germ layers of the primitive streak and then expression overlaps that of *Sox1* in the early neurectoderm, suggesting that *Gbx2* might regulate the expression of *Sox1* in this cell type. *Sox1* expression was compared in $Gbx2^{+/+}$ and $Gbx2^{-/-}$ ES cell RA aggregates and a decrease was seen in the amount of *Sox1* expression in the null mutant ES cell line, although expression was not totally ablated. These results demonstrated that *Gbx2* is not obligatory for *Sox1* expression. The decreased level of expression is likely to be the result of decreased neurectoderm formation.

In the chicken, *GBX2* has been shown by gel shift assay to bind to sites in the *cMGF* promoter region. Ectopically expressed GBX2 was shown to induce expression of a reporter gene under the control of the cMGF promoter (Kowenz-Leutz et al., 1997). Kowenz-Leutz et al. (1997) investigated the ability of the Myb protein to activate *GBX2* transcription and, using a conditionally expressed *v-myb* macrophage cell line, demonstrated that *GBX2* is a direct target gene of the v-Myb protein. A conditionally activated Myb cell line (ERMYB) was used to investigate whether murine *Gbx2* is a Myb target gene. No expression of *Gbx2* was detected at any time point prior to or after removing estradiol from the medium, suggesting that *Gbx2* is not a target gene of c-Myb in the mouse. In addition, it appears that *Gbx2* is not expressed during the differentiation of macrophage precursors in the ERMYB system, suggesting that *Gbx2* may not be important for specification of this cell type.

It appears from the results of the in vitro differentiation experiments described in this thesis that *Gbx2* is more likely to function in providing positional information along the antero-posterior embryonic axis than to control survival, proliferation, or differentiation of neural or hematopoietic progenitor cells. This is consistent with the observation that most homeobox genes function as patterning signals.

## 8.2 A model for *Gbx2* function in early neural patterning

Several recent reviews have implicated *Gbx2*, along with *Otx2*, in the earliest establishment of the midbrain-hindbrain boundary, by 7.5 d.p.c. Based on the early expression of *Gbx2* and the alterations in gene expression patterns in the isthmic region seen in *Gbx2* mutant embryos, Wassef and Joyner (1997) suggest that *Gbx2* may be required to promote anterior hindbrain development and to establish the isthmic organizer, possibly by inducing or stabilizing expression of genes such as *Wnt1*, *Pax2*, and *Fgf8*. Simeone (1998) suggests that the *Otx2* posterior border may result from retinoic acid signalling, based on the observation that exogenous RA at mid-late primitive streak stage represses *Otx2* expression, or from *Gbx2* signalling, based on the caudal expansion of *Otx2* in *Gbx2* mutant mice. Martinez et al. (1999) demonstrated that FGF8 coated beads implanted in the presumptive forebrain of chick embryos induced a secondary isthmus with repression of *Otx2* and induction of *En1*, *Fgf8*, and *Wnt1* around the bead. They concluded that the midbrain-hindbrain boundary is established by a negative feedback loop in which OTX2 represses *Fgf8* in the region anterior to the isthmus and FGF8 represses *Otx2* in the posterior region. They also suggest that *Gbx2* may increase the sensitivity of cells in the posterior region to the repressive effects of FGF8. In a similar experiment, Liu et al. (1999) demonstrated that FGF8 coated beads implanted in midbrain or caudal forebrain explants of mouse embryos repressed *Otx2* expression and induced expression of the more caudal genes *En1*, *En2*, and *Pax5*, as well as *Gbx2*.

The model shown in Figure 8.1 builds on these observations by suggesting that Gbx2 directly represses *Otx2* in the region posterior to the isthmus, but that OTX2 does not reciprocally repress *Gbx2* in the anterior region. Kowenz-Leutz et al. (1997) identified a consensus sequence (C/GATTAAG/C) in the promoter of *cGMF* to which Gbx2 binds. The first hypothesis, that Gbx2 directly represses *Otx2*, resulted from an examination of the *Otx2* regulatory region (Genbank accession # U96488). It was found to contain two Gbx2 consensus binding sequences at position –403 (reverse orientation) and at –505 (forward orientation) which may serve as transcriptional regulatory sites (Fig. 8.2). Two recent reports describing the ectopic expression of *Gbx2* under the control of the *Wnt1* enhancer region (Millet et al., 1999) and ectopic expression of *Otx2* under the control of the *En1* locus (Broccoli et al., 1999) support

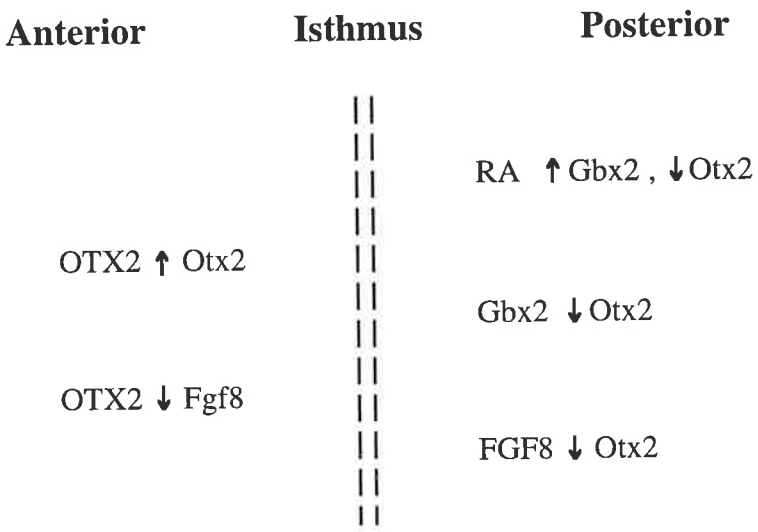


**Figure 8.1** A model of gene regulation in the neurectoderm of the midbrain-hindbrain region during early neurogenesis (7-8 d.p.c.)

```
-749 tgttttaaat gttgaatgcg tttacatttt aggttcacag cagttgaaag tggtaataat
-689 tttaaaaaaa aaaatcagct aagataatta aaacctctgc cagggaaagg caacagtctg
-629 ggaagaggta gatttcttga attgtttctt tgtttctcac caatgggctt tgttatcagc
-569 attatttatt tagccaaaga gttctttgtc tctgcaaatg gccccaatca agttttgttt
-509 gagacaatta gctagtgcca gccaatgagt cctatgcaaa tgttgggtta ggaatgtaga
-449 gcgtgcattt ggaggcgtgg ggttttgttc ttggggaagg cagattgtaa ttgcttttcct
-389 cgggtcggtt ttctttcttt ctttcttttt ttaagtttag ggtgggggtg gggaagacag
-329 gtttatctgg tctcactcca tcccctctag ttttggagct gctggggggt gggggggacg
-269 gcgggggtgg gggacgcatc tgcaactcct ttaaaagcct gtgcccagcg tctcccgggt
-209 tctttttagt tagtgctgga acgtggagga agctgctccc tccgaagcag taaaccagca
-149 tttctgtttg tttgtttgtt ttgcccttag ttccgtcact ccaaatctac ccaccaagga
 -89 ccctgaccct gtccactcca ggcgaatcga gaccgtccgg ctgggtcccc ccaatttggg
 -29 ccgactttgc gcctccaaac aaccttagca tg
```

**Figure 8.2 Gbx2 binding sites in *Otx2* 5' flanking region**

5' flanking region of murine *Otx2* gene, (Genbank accession #U96488). Gbx2 consensus binding sequences ($^{C}/_{G}$ATTAA$^{G}/_{C}$) at - 505 and -404 are shown boxed.

this hypothesis. When *Gbx2* was transiently expressed in the midbrain at 8-9 d.p.c. the *Otx2* border was shifted rostrally. *Wnt1* and *Fgf8* expression was also shifted rostrally, although their positions relative to each other and to the *Otx2* border remained the same. When *Otx2* was ectopically expressed in the midbrain *Wnt1*, *Pax2*, *Fgf8*, and *Gbx2* expression was shifted caudally, suggesting that the juxtaposition of the *Gbx2* and *Otx2* borders establishes the midbrain-hindbrain boundary and subsequent downstream gene expression further defines the isthmic organizer region.

Based on the finding of potential Gbx2 binding sites in the regulatory region of *Otx2*, a search was made of the *Gbx2* genomic sequence for OTX2 consensus binding sequences (CCTAATCCT) (Briata et al., 1999). No OTX2 binding sites were found in the 440 bp region immediately 5' of the *Gbx2* transcriptional start site or in the 1kb intron, leading to the second hypothesis, that OTX2 does not directly reciprocally repress *Gbx2*. While it is apparent from *Otx2* ectopic expression experiments (Broccoli et al., 1999) that *Gbx2* is repressed under the influence of OTX2, this repression may be mediated by another factor such as FGF8, which has been demonstrated to have a rostrally shifted expression pattern in $Otx1^{+/-}/Otx2^{+/-}$ and $Otx1^{-/-}/Otx2^{+/-}$ embryos (Suda et al., 1999; Acampora et al., 1997). OTX2 repression of *Gbx2* cannot be excluded as there may be OTX2 binding sites outside the *Gbx2* sequence examined and *Gbx2* expression in $Otx2^{-/-}$ embryos or embryoid bodies has not been reported.

## 8.3 Future work

While gene targeting studies have contributed much to our understanding of the site and timing of critical gene functions, they often reveal little about the regulatory cascades controlling the formation of the affected structures. Genes which regulate *Gbx2* expression, as well as *Gbx2* target genes, merit further investigation. The isolation of the *Gbx2* genomic locus, including potential regulatory regions, has provided a valuable tool for investigation of genes which regulate *Gbx2*.

In order to resolve whether *Gbx2* and *Otx2* are capable of reciprocal repression, in vitro binding studies would need to be performed. Gbx2 and OTX2 proteins would need to be expressed and purified. These proteins could then be used to induce expression of a reporter gene driven by the relevant regulatory region and in gel shift or DNA footprinting studies to define the specific binding sites. Purified Gbx2 protein could also be used to investigate other potential *Gbx2* target genes such as *Sox1*. Reporter gene assays could be used to investigate candidate *Gbx2* regulatory factors such as retinoic acid or FGF.

In order to study other *Gbx2* target genes, an inducible expression system such as the one described for Myb activation in Chapter 7 could be developed in which Gbx2 activation in was under the control of an inducible promoter. A construct containing *Gbx2* coding region fused to the ligand binding domain of the estrogen receptor would be introduced into a *Gbx2*$^{-/-}$ ES cell line. This cell line would then be differentiated as embryoid bodies, with or without induction at various time points and candidate target gene expression examined. The reproducible, ordered progression of embryoid body differentiation and its similarity to embryonic differentiation events makes this an ideal system for investigating the genes controlling these events. For example, induction could be pinpointed to gastrulation stage embryoid bodies by adding estrogen to the medium at day 4 of culture. In addition, an inducible *Gbx2* construct could be introduced into Early Primitive Ectoderm-like (EPL) cells, which do not express *Gbx2* (Rathjen et al., 1999) and which are severely restricted in their ability to form neurectoderm in embryoid bodies (Lake et al., in press). It is thought that this inability is a result of the lack of visceral endoderm in these bodies, however it would be interesting to determine whether ectopically expressed Gbx2 could provide an appropriate signal to induce the formation of neurectoderm in EPL cell embryoid bodies.

Functional equivalence of homeobox gene family members has been investigated in families consisting of 2 members expressed in slightly different spatial and or temporal patterns by substituting the coding region of one family member in the genomic locus of the of the other family member. This has provided novel information about the functions of both family members. For example, the *En1* mutant phenotype can be completely rescued by the replacement of *En1* coding sequence with *En2* coding sequence, despite only a 55% amino acid identity between the two proteins (Hanks et al., 1995). In the case of *Otx2* mutants, *Otx1* was able to rescue some, but not all of the mutant phenotype (Suda et al., 1999). Functions of *Otx2* in the anterior visceral endoderm, mesendoderm, cephalic neural crest cells, and regionalization of anterior neurectoderm were able to be replaced by *Otx1*, but *Otx2* function in the establishment of anterior neurectoderm is not replaceable by *Otx1*. Little research has been conducted on *Gbx1* beyond chromosomal localization (Frohman et al., 1993) and mapping its expression to the forebrain at 11.5 d.p.c. (Bulfone et al., 1993). It would be interesting to investigate whether *Gbx1* could rescue the *Gbx2* mutant phenotype by the creation and analysis of *Gbx1*$^{-/-}$ and *Gbx2*$^{-/Gbx1}$ mutant mice. It would also be interesting to create *Gbx1*$^{-/-}$,*Gbx2*$^{-/-}$ ES cells to determine whether *Gbx1* compensates for *Gbx2* function in the inner cell mass or in differentiating neurectoderm.

# References

Acampora, D., Mazan, S., Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A., and Brûlet, P. (1995). Forebrain and midbrain regions are deleted in *Otx2*$^{-/-}$ mutants due to defective anterior neurectoderm specification during gastrulation. *Development* **121,** 3279-3290.

Amaya, E., Musci, T. J., and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. *Cell* **66,** 257-270.

Ang, S. L., and Rossant, J. (1994). HNF-3β is essential for node and notochord formation in mouse development. *Cell* **78,** 561-574.

Arcioni, L., Simeone, A., Guazzi, S., Zappavigna, V., Boncinelli, E., and Mavilio, F. (1992). The upstream region of the human homeobox gene HOX3D is a target for regulation by retinoic acid and HOX homeoproteins. *EMBO J.* **11,** 265-277.

Austyn, J. M., and Gordon, S. (1981). F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immun.* **11,** 805-815.

Bain, G., Kitchens, D., Huettner, J.E. and Gottlieb, D.I. (1995). Embryonic stem cells express neuronal properties in vitro. *Dev. Biol.* **168,** 342-357.

Bain, G., Ray, W. J., Yao, M., and Gottlieb, D. L. (1996). Retinoic acid promotes neural and represses mesodermal gene expression in mouse embryonic stem cells in culture. *Biochem. Biophys. Res. Commun.* **223,** 691-694.

Baker, R. K., Haendel, M. A., Swanson, B. J., Shambough, J. C., Micales, B. K., and Lyons, G. E. (1997). In vitro preselection of gene-trapped embryonic stem cell clones for characterizing novel developmentally regulated genes in the mouse. *Dev. Biol.* **185,** 201-214.

Bally-Cuif, L., and Wassef, M. (1995). Determination events in the nervous system of the vertebrate embryo. *Curr. Opin. Genet. Dev.* **5,** 450-458.

Basler, K., Edlund, T., Jessell, T. M., and Yamada, T. (1993). Control of cell pattern in the neural tube: regulation of cell differentiation by dorsalin-1, a novel TGF beta family member. *Cell* **73,** 687-702.

Beddington, R. S. P. (1982). An autoradiographic analysis of tissue potency in different regions of the embryonic egg cylinder. *J. Embryol. Exp. Morph.* **69,** 265-285.

Beddington, R. S. P., and Robertson, E. J. (1989). An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* **105,** 733-737.

Beddington, R. S. P. (1994). Induction of a second neural axis by the mouse node. *Development* **120,** 603-612.

Beddington, R. S. P., and Robertson, E. J. (1998). Anterior patterning in mouse. *Trends Genet.* **14,** 277-284.

Belo, J. A., Bouwmeester, T., Leyns, L., Kertesz, N., Gallo, M., Follettie, M., and De Robertis, E. M. (1997). *Cerberus-like* is a secreted factor with neuralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech. Dev.* **68,** 45-57.

Ben-Shushan, E., Thompson, J. R., Gudas, L. J., and Bergman, Y. (1998). *Rex-1*, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1 binding to an adjacent site. *Mol. Cell. Biol.* **18,** 1866-1878.

Biedenkapp, H., Borgmeyer, U., and Sippel, A. (1988). Viral myb oncogene encodes a sequence-specific DNA-binding activity. *Nature* **335,** 835-837.

Blum, M., Gaunt, S. J., Cho, K. W. Y., Steinbeisser, H., Blumberg, B., Bittner, D., and De Robertis, E. M. (1992). Gastrulation in the mouse: the role of the homeobox gene *goosecoid*. *Cell* **69,** 1097-1106.

Blumberg, B., Bolado, J., Moreno, T. A., Kintner, C., Evans, R. M., and Papalopulu, N. (1997). An essential role for retinoid signaling in anteroposterior neural patterning. *Development* **124,** 373-379.

Boehm, T., Gonzales-Sarmiento, R., Kennedy, M. A., and Rabbitts, T. H. (1991). A simple technique for generating probes for RNA *in situ* hybridisation: An adjunct to genome mapping exemplified by the RAG-1/RAG-2 gene cluster. *Proc. Natl. Acad. Sci. USA* **88,** 3927-3931.

Boncinelli, E., and Mallamaci, A. (1995). Homeobox genes in vertebrate gastrulation. *Curr. Opin. Genet. Dev.* **5,** 627.

Bonifer, C., Faust, N., Geiger, H., and Müller, A. M. (1998). Developmental changes in the differentiation capacity of haematopoietic stem cells. *Immunol. Today* **19,** 236-241.

Bottomley, S. S. (1982). Sideroblastic Anaemia. *In* "Wintrobe's Clinical Hematology" (G. R. Lee, Ed.), pp. 1022-1045. Williams and Willein, Baltimore.

Bottomley, S. S., and Muller-Eberhard, U. (1988). Pathophysiology of heme synthesis. *Seminars in Hematology* **25,** 282-302.

Bouillet, P., Chazaud, C., Oulad-Abdelghani, M., Dollé, P., and Chambon, P. (1995). Sequence and expression pattern of the *Stra7* (*Gbx-2*) homeobox-containing gene induced by retinoic acid in P19 embryonal carcinoma cells. *Developmental Dynamics* **204,** 372-382.

Bradley, I., M., E., Kaufman, M. H., and Robertson, E. J. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature (London)* **309,** 255-256.

Brandon, E. P., Idzera, R. L., and McKnight, G. S. (1995a). Targeting the mouse genome: a compendium of knockouts (part I). *Curr. Biol.* **5,** 625-634.

Brandon, E. P., Idzera, R. L., and McKnight, G. S. (1995b). Targeting the mouse genome: a compendium of knockouts (part II). *Curr. Biol.* **5,** 758-765.

Brandon, E. P., Idzera, R. L., and McKnight, G. S. (1995c). Targeting the mouse genome: a compendium of knockouts (part III). *Curr. Biol.* **5,** 873-881.

Briata, P., Ilengo, C., Bobola, N., and Corte, G. (1999). Binding properties of the human homeodomain protein OTX2 to a DNA target sequence. *FEBS Lett.* **445,** 160-164.

Brighton, V. W., and Krummel, T. M. (1996). The fetal macrophage: a review. *J. Amer. Coll. Surgeons* **182,** 270-276.

Broccoli, V., Boncinelli, E., and Wurst, W. (1999). The caudal limit of *Otx2* expression positions the isthmic organizer. *Nature* **401,** 164-148.

Bronson, S. K., and Smithies, O. (1994). Altering mice by homologous recombination using embryonic stem cells. *J. Biol. Chem.* **269,** 27155-27158.

Bulfone, A., Puelles, L., Porteus, M. H., Frohman, M. A., Martin, G. R., and Rubenstein, J. L. R. (1993). Spatially restricted expression of *Dlx-1*, *Dlx-2* (*Tes-1*), *Gbx-2*, and *Wnt-3* in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. *J. Neurosci.* **13,** 3155-3172.

Burk, O., and Klempnauer, K. H. (1991). Estrogen-dependent alterations in differentiation state of myeloid cells caused by a v-myb/estrogen receptor fusion protein. *EMBO J.* **12,** 3713-3719.

Burkert, U., von Ruden, R. T., and Wagner, E. F. (1991). Early fetal hematopoietic development from *in vitro* differentiated embryonic stem cells. *New Biol.* **3,** 698-708.

Bürglin, T. R. (1994). A comprehensive classification of homeobox genes. *In* "Guidebook to the Homeobox Genes" (D. Duboule, Ed.), pp. 25-71. Oxford University Press, Oxford.

Capecchi, M. R. (1989). The new mouse genetics: altering the genome by gene targeting. *Trends Genet.* **5,** 70-76.

Capecchi, M. R. (1997). Hox genes and mammalian development. *Cold Spring Harbor Symp. Quant. Biol.* **62,** 273-281.

Chan, S.-K., Jaffe, L., Capovilla, M., Botas, J., and Mann, R. (1994). The DNA binding specificity of Ultrabithorax is modulated by cooperative interactions with extradenticle, another homeoprotein. *Cell* **78,** 603-615.

Chang, C.-P., Shen, W.-F., Rozenfeld, S., Lawrence, H. J., Largman, C., and Cleary, M. L. (1995). Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. *Genes Dev.* **9,** 663-674.

Chapman. (1994). Isolation and characterisation of the homeobox gene *Mmox-B*. University of Adelaide.

Chapman, G., and Rathjen, P. D. (1995). Sequence and evolutionary conservation of the murine *Gbx2* homeobox gene. *FEBS Lett.* **364,** 289-292.

Chapman, G., Remiszewski, J. L., Webb, G. C., Schulz, T. C., Bottema, C. D. K., and Rathjen, P. D. (1997). The mouse homeobox gene, *Gbx2*: Genomic organization and expression in pluripotent cells *in vitro* and *in vivo*. *Genomics* **46,** 223-233.

Chen, C., and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7,** 2745-52.

Chen, U. (1992). Differentiation of mouse embryonic stem cells to lympho-hematopoietic lineages in vitro. *Dev. Immunol.* **2,** 29-50.

Chen, W. S., Manova, K., Weinstein, D. C., Duncan, S. A., Plump, A. S., Prezioso, V. R., Bacharova, R. F., and Darnell Jr., J. E. (1994). Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic endoderm and impaired gastrulation of mouse embryos. *Genes Dev.* **8,** 2466-2477.

Chiang, C., Young, K. E., and Beachy, P. A. (1995). Control of *Drosophila* tracheal branching by the novel homeodomain gene *unplugged*, a regulatory target for genes of the bithorax complex. *Development* **121,** 3901-3912.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function. *Nature* **383,** 407-413.

Chisaka, O., Musci, T., and Capecchi, M. (1992). Developmental defects of the ear, cranial nerves and hindbrain resulting from targeted disruption of the mouse homeobox gene *Hox-1.6*. *Nature* **355,** 516-520.

Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Herrmann, B., and Robertson, E. J. (1994). A primary requirement for *nodal* in the formation and maintenance of the primitive streak in the mouse. *Development* **120,** 1919-1928.

Conover, J. C., Ip, N. Y., Poueymirou, W. T., Bates, B., Goldfarb, M. P., DeChiara, T. M., and Yancopoulos, G. D. (1993). Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. *Development* **110,** 559-565.

Conquet, F., and Brûlet, P. (1990). Developmental expression of myeloid leukemia inhibitory factor gene in preimplantation blastocysts and in extraembryonic tissue of mouse embryos. *Mol. Cell. Biol.* **10,** 3801-3805.

Coucouvanis, E., and Martin, G. R. (1995). Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. *Cell* **83,** 279-287.

Craig, R. W., and Bloch, A. (1984). Early decline in *c-myb* oncogene expression in the differentiation of human myeloblastic leukemia (ML-1) cells induced with 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res.* **44,** 442-446.

Crossley, P. H., and Martin, G. R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121,** 439-451.

Crossley, P. H., Martinez, S., and Martin, G. R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature* **380,** 66-68.

Cumano, A., Dieterlen-Lievre, F., and Godin, I. (1996). Lymphoid potential, proved before circulation in the mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell* **86,** 907-916.

Dattani, M. T., Martinez-Barbera, J. P., Thomas, P. Q., Brickman, J. M., Gupta, R., Martensson, I. L., Toresson, H., Fox, M., Wales, J. K., Hindmarsh, P. C., Krauss, S., Beddington, R. S., and Robinson, I. C. (1998). Mutations in the homeobox gene

HESX1/Hesx1 associated with septo-optic displasia in human and mouse. *Nature Genet.* **19,** 125-33.

Davis, C. A., and Joyner, A. L. (1988). Expression patterns of the homeobox-containing genes *En-1* and *En-2* and the proto-oncogene *int-1* diverge during mouse development. *Genes Dev.* **2,** 1736-1744.

de Jong, R., and Meijlink, F. (1993). The homeobox gene *S8*: Mesoderm-specific expression in presomite embryos and in cells cultured in vitro and modulation in differentiation pluripotent cells. *Dev. Biol.* **157,** 133-146.

de Jong, R., van der Heijden, J., and Meijlink, F. (1993). DNA-binding specificity of the S8 homeodomain. *Nucleic Acids Res.* **21,** 4711-4720.

DeLorenzo, R. J., and Ruddle, F. H. (1969). Genetic control of two electrophoretic variants of glucose phosphate isomerase in the mouse (Mus musculus). *Biochem. Genet.* **3,** 151-162.

Deng, C., and Capecchi, M. (1992). Re-examination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Mol. Cell. Biol.* **12,** 3365-3371.

Dickson, M. C., Martin, J. S., Cousins, F. M., Kulkarni, A. B., Karlsson, S., and Akhurst, R. J. (1995). Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* **121,** 1845-1854.

Dietrich, W., Katz, H., Lincoln, S. E., Shin, H., Friedman, J., Dracololi, N. C., and Lander, E. S. (1992). A genetic map of the mouse suitable for typing intraspecific crosses. *Genetics* **131,** 423-447.

Dini, P. W., Eltman, J. T., and Lipsick, J. S. (1995). Mutations in the DNA binding and transcriptional activation domains of v-Myb cooperate in transformation. *J. Virol.* **69,** 2515-2524.

Doetschman, T. C., Eisetter, H., Katz, M., Schmidt, W., and Kemler, R. J. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands, and myocardium. *J. Embryol. Exp. Morphol.* **87,** 27-45.

Dollé, P., Dierich, A., LeMeur, M., Schimmang, T., Schuhbaur, B., Chambon, P., and Duboule, D. (1993). Disruption of the *Hoxd-13* gene induces localized heterochrony leading to mice with neotenic limbs. *Cell* **75,** 431-441.

Doniach, T. (1993). Planar and vertical induction of anteroposterior pattern during the development of the amphibian central nervous system. *J. Neurobiol.* **24,** 1256-1275.

Doniach, T. (1995). Basic FGF as an inducer of anteroposterior neural pattern. *Cell* **83,** 1067-1070.

Duncan, S. A., Manova, K., Chen, W., Hoodless, P., Wienstein, D. C., Bacharova, R. F., and Darnell Jr., J. E. (1994). Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst. *Proc. Natl. Acad. Sci.* **91,** 7598-7602.

Duncan, S. A., Nagy, A., and Chan, W. (1997). Murine gastrulation requires HNF-4 regulated gene expression in the visceral endoderm: tetraploid rescue of Hnf4(-/-) embryos. *Development* **124,** 279-287.

Durston, A., Timmermans, J. P. M., Hage, W. J., Hendriks, H. F. J., de Vries, N. J., Heideveld, M., and Nieukoop, P. D. (1989). Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* **340,** 140-144.

Dush, M. K., and Martin, G. R. (1992). Analysis of mouse *Evx* genes: *Evx-1* displays graded expression in the primitive streak. *Dev. Biol.* **115,** 273-287.

Dynan, W. S. (1986). Promoters for housekeeping genes. *Trends Genet.* **2,** 196-197.

Dynan, W. S., Sazer, S., Tjian, R., and R.T., S. (1986). Transcription factor Sp1 recognizes a DNA sequence in the mouse dihydrofolate reductase promoter. *Nature* **319,** 246-249.

Dyson, P. J., Poirier, R., and Watson, R. J. (1989). Expression of *c-myb* in embryonal carcinoma cells and embryonal stem cells. *Differentiation* **42,** 24-27.

Dzierak, E., Medvinsky, A., and de Bruijn, M. (1998). Qualitative and quantitative aspects of haematopoietic cell development in the mammalian embryo. *Immunol. Today* **19,** 228-235.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A., and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75.**

Elefanty, A., Robb, L., Birner, R., and Begley, C. G. (1997). Hematopoietic-specific genes are not induced during in vitro differentiation of scl-null embryonic stem cells. *Blood* **90,** 1435-1447.

Ericson, J., Morton, S., Kawakami, A., Roelink, H., and Jessell, T. M. (1996). Two critical periods of Sonic hedgehog signaling required for the specification of motor neuron identity. *Cell* **87,** 661-673.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M., and Briscoe, J. (1997). *Pax6* controls progenitor cell identity and neuronal fate in response to graded Shh signalling. *Cell* **90,** 169-180.

Escary, J.-L., Perreau, D. D., Ezine, S., and Brûlet, P. (1993). Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation. *Nature* **363,** 361-364.

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature (London)* **292,** 154-156.

Fainsod, A., and Gruenbaum, Y. (1989). A chicken homeo box gene with developmentally regulated expression. *FEBS Lett.* **250,** 381-385.

Fibi, M., Zink, B., Kessel, M., Colberg-Poley, A. M., Labeit, S., Lehrach, H., and Gruss, P. (1988). Coding sequence and expression of the homeobox gene *Hox 1.3*. *Development* **102,** 349-359.

Fletcher, C., Neintz, N., and Roeder, R. G. (1987). Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. *Cell* **51,** 773-781.

Fraichard, A., Chassande, O., Bilbaut, G., Dehay, C., Savatier, P., and Samarut, J. (1995). In vitro differentiation of embryonic stem cells into glial cells and functional neurons. *J. Cell Sci.* , 3183-3188.

Friedrich, G., and Soriano, P. (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* **5,** 1513-1523.

Frischer, L. E., Hagen, F. S., and Garber, R. L. (1986). An inversion that disrupts the Antennapedia gene causes abnormal structure and localization of RNAs. *Cell* **47,** 1017-1023.

Frohman, M. A., Dickinson, M. E., Hogan, B. L. M., and Martin, G. R. (1993). Mapping of *GBX1* to mouse Chromosome 5 and *GBX2* to mouse Chromosome 1. *Mouse Genome* **91,** 323-325.

Gajovic, A., Chowdhury, K., and Gruss, P. (1998). Genes expressed after retinoic acid-mediated differentiation of embyroid bodies are likely to be expressed during embryo development. *Exp. Cell Res.* **242,** 138-143.

Gardener, R. L. (1983). Origin and differentiation of extraembryonic tissues of the mouse. *Int. Rev. Exp. Pathol.* **24,** 63-133.

Gardiner-Garden, M., and Frommer, M. (1987). CpG islands in vertebrate genomes. *J. Mol. Biol.* **196,** 261-282.

Gassmann, M., Donoho, G., and Berg, P. (1995). Maintenance of an extrachromosomal plasmid vector in mouse embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **92,** 1292-1296.

Gearing, D. P., Gough, N. M., King, J. A., Hilton, D. J., Nicola, N. A., Simpson, R. J., Nice, E. C., Kelso, A., and Metcalf, D. (1987). Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). *EMBO J.* **6,** 3995-4002.

Gehring, W. J., Affolter, M., and Bürglin, K. (1994). Homeodomain proteins. *Annu. Rev. Biochem.* **63,** 437-526.

Gemel, J., Jacobsen, C., and MacArthur, C. A. (1999). Fibroblast growth factor-8 expression is regulated by intronic Engrailed and Pbx1binding sites. *J. Biol. Chem.* **274,** 6020-6026.

Godsave, S. F., Koster, C. H., Getahun, A., Mathu, M., Hooiveld, M., van der Wees, J., Hendriks, J., and Durston, A. J. (1998). Graded retinoid responses in the developing hindbrain. *Developmental Dynamics* **213,** 39-49.

Gonda, T. J., and Metcalf, D. (1984). Expression of *myb*, *myc*, and *fos* protooncogenes during the differentiation of a murine myeloid leukemia. *Nature* **310,** 249-251.

Graham, A., Papalopulu, N., Lorimer, J., McVey, J. H., Tuddenham, E. G. D., and Krumlauf, R. (1988). Characterization of a murine homeobox gene, *Hox-2.6*, related to the *Drosophila deformed* gene. *Genes Dev.* **2,** 1424-1438.

Green, J. B. A., New, H. V., and J.C., S. (1992). Responses of embryonic *Xenopus* cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. *Cell* **71,** 731-739.

Grigoriadis, A. E., Wang, Z.-Q., Cecchini, M. G., Hofstetter, W., Felix, R., Fleisch, H. A., and Wagner, E. F. (1994). c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science* **266,** 443-448.

Guthrie, S., Muchamore, I., Kuroiwa, A., Marshall, H., and Krumlauf, R. (1992). Neuroectodermal autonomy of *Hox-2.9* expression revealed by rhombomere transpositions. *Nature* **356,** 157-159.

Han, K., Levine, M. S., and Manley, J. L. (1989). Synergistic activation and repression of transcription by Drosophila homeobox proteins. *Cell* **56,** 573-583.

Hanks, M., Wurst, W., Anson-Cartwrite, L., Auerbach, A. B., and Joyner, A. L. (1995). Rescue of the *En1* mutant phenotype by replacement of *En1* with *En2*. *Science* **269,** 679-682.

Harland, R. M. (1997). Neural induction in *Xenopus*. *In* "Molecular and Cellular Approaches to Neural Development" (W. M. Cowan, T. M. Jessel, and S. L. Zipursky, Eds.), pp. 1-25. Oxford University Press, Oxford.

Haub, O., and Goldfarb, M. (1991). Expression of the fibroblast growth factor-5 gene in the mouse embryo. *Development* **112,** 397-406.

Heath, J. K., and Smith, A. G. (1988). Regulatory factors of embryonic stem cells. *J. Cell Sci. (Suppl)* **10,** 257-266.

Hemmati-Brivanlou, A., Kelly, O. G., and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77,** 283-295.

Herrmann, G. B. (1991). Expression pattern of the *Brachyury* gene in whole mount $T^{Wis}/T^{Wis}$ mutant embryos. *Development* **113,** 913-917.

Hébert, J. M., Boyle, M., and Martin, G. R. (1991). mRNA localization studies suggest that murine FGF-5 plays a role in gastrulation. *Development* **112,** 407-415.

Hébert, J. M., Rosenquist, T., Gotz, J., and Martin, G. R. (1994). FGF-5 is a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. *Cell* **78,** 1017-1025.

Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1986). "Manipulating the mouse embryo, a laboratory manual." Cold Spring Harbor Laboratory,

Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994). "Manipulating the mouse embryo, a laboratory manual." Cold Spring Harbor Laboratory,

Hogg, A., Schirm, S., Nakagoshi, H., Bartley, P., Shunsuke, I., Bishop, J. M., and Gonda, T. J. (1997). Inactivation of a c-Myb/estrogen receptor fusion protein in transformed primary cells leads to granulocyte/macrophage differentiation and down regulation of *c-kit* but not *c-myc* or *cdc2*. *Oncogene* **15,** 2885-2898.

Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987). HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature (London)* **326,** 292-295.

Hunt, P., Gulisano, M., Cook, M., Sham, M.-H., Faiella, A., Wilkinson, D., Boncinelli, E., and Krumlauf, R. (1991). A distinct *Hox* code for the branchial region of the vertebrate head. *Nature* **353,** 861-864.

Inamdar, M., Koch, T., Rapoport, R., Dixon, J. T., Probolus, J. A., Cram, E., and Bautch, V. L. (1997). Yolk sac-derived murine macrophage cell line has a counterpart during ES cell differentiation. *Developmental Dynamics* **210,** 487-497.

Ince, T. A., and Scotto, K. W. (1995). A conserved downstream element defines a new class of RNA polymerase II promoters. *J. Biol. Chem* **270,** 30249-30252.

Isaacs, H., Tannahill, D., and Slack, J. (1992). Expression of a novel FGF in the Xenopus embryo: a new candidate inducing factor for mesderm formation in antero-posterior patterning. *Development* **71,** 711-720.

Izpisúa-Belmonte, J.-C., Tickle, C., Dollé, P., Wolpert, L., and Duboule, D. (1991). Expression of the homeobox Hox-4 genes and the specification of position in chick wing development. *Nature* **350,** 585-589.

Jackson, I. J. (1989). The mouse. *In* "Genes and Embryos" (D. M. Glover and B. D. Hames, Eds.), pp. 165-221. IRL Press, Oxford.

Jaynes, J. B., and O'Farrell, P. H. (1988). Activation and repression of transcription by homeodomain-containing proteins that bind a common site. *Nature* **336,** 744-749.

Johansson, B. M., and Wiles, M. V. (1995). Evidence for involvement of activin A and bone morphogenetic protein-4 in mammalian mesoderm and haematopoietic development. *Mol. Cell. Biol.* **15,** 141-151.

Johnson, M. H., and Maro, B. (1988). Time and space in the mouse early embryo: a cell biological approach to cell diversification. *In* "Experimental approaches to mammalian development", pp. 35-65. Cambridge University Press, Cambridge.

Jones-Villeneuve, E. M. V., McBurney, M. W., Rogers, K. A., and Kallins, V. I. (1982). Retinoid acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell. Biol.* **94,** 253-262.

Kalionis, B., and O'Farrell, P. H. (1993). A universal sequence is bound in vitro by diverse homeodomains. *Mech. Dev.* **43,** 57-70.

Kappen, C., Schughart, K., and Ruddle, F. H. (1993). Early evolutionary origin of major homeodomain sequence classes. *Genomics* **18,** 54-70.

Karn, K., Matthes, H. W. D., Gait, M. J., and Brenner, S. (1984). A new phage cloning vector, λ2001, with sites for XbaI, BamHI, HindIII, EcoRI, SstI and XhoI. *Gene* **32,** 217-224.

Kastner, P., Mark, M., and Chambon, P. (1995). Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life? *Cell* **83,** 859-869.

Kaufman, M. H. (1992). "The atlas of mouse development." Academic Press, Harcourt Brace Jovanovich, London.

Keller, G., Kennedy, M., Papayannopoulou, T., and Wiles, M. V. (1993). Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol. Cell. Biol.* **13,** 473-486.

Kemler, I., and Schaffner, W. (1990). Octamer transcription factors and the cell-type specificity of immunoglobulin gene expression. *FASEB J.* **4,** 1444-1449.

Kessel, M., and Gruss, P. (1990). Murine developmental control genes. *Science* **249,** 374-379.

King, M., and Moore, M. (1994). Novel HOX, POU and FKH genes expressed during bFGF mesodermal differentiation in *Xenopus*. *Nucl. Acids Res.* **33,** 3990-3996.

Kissinger, C. R., Liu, B., Martin-Blanco, E., Kornberg, T. B., and Pabo, C. O. (1990). Crystal structure of an engrailed homeodomain-DNA complex at 2.8A resolution: a framework for understanding homeodomain-DNA interactions. *Cell* **63,** 579-590.

Kolm, P. J., Apekin, V., and Sive, H. (1997). *Xenopus* hindbrain patterning requires retinoid signaling. *Dev. Biol.* **192,** 1-16.

Kowenz-Leutz, E., Herr, P., Knut, N., and Leutz, A. (1997). The homeobox gene *GBX2*, a target of the *myb* oncogene, mediates autocrine growth and monocyte differentiation. *Cell* **91,** 185-195.

Krasnow, M. A., Saffman, E. E., Kornfeld, K., and Hogness, D. S. (1989). Transcriptional activation and repression by *Ultrabithorax* proteins in cultured *Drosophila* cells. *Cell* **57,** 1031-1043.

Krieg, P. A., and Melton, D. A. (1987). *In vitro* RNA synthesis with SP6 RNA polymerase. *Meth. Enz.* **155,** 397-415.

Krumlauf, R. (1993). Hox genes and pattern formation in the branchial region of the vertebrate head. *Trends Genet.* **9,** 106-12.

Lake, J. (1996). Differentiation of pluripotential murine embryonic stem cells. University of Adelaide, Adelaide.

Lake, J., Rathjen, J., and Rathjen, P. D. (in press). Reversible programming of pluripotent cell differentiation: Implications for cell specification during early embryogenesis. *J. Cell Sci.* .

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D., and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* **262,** 713-718.

Lamb, T. M., and Harland, R. M. (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern. *Development* **121.**

Laughon, A. (1991). DNA binding specificity of homeodomains. *Biochemistry* **30,** 11357-

11367.

Lawson, K. A., Meneses, J. J., and Pedersen, R. A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development* **113,** 891-911.

Le Dourain, N. M. (1982). "The Neural Crest." Cambridge University Press, Cambridge.

Le Mouellic, H., Lallemand, Y., and Brulet, P. (1992). Homeosis in the mouse induced by a null mutation in the *Hox-3.1* gene. *Cell* **69,** 251-264.

Leutz, A., Beug, H., and Graf, T. (1984). Purification and characterization of cMGF, a novel chicken myelomonocytic growth factor. *EMBO J.* **3,** 3191-3197.

Leutz, A., Damm, K., Sterneck, E., Kowenz, E., Nexx, S., Frank, R., Gausepohl, H., Pan, Y. C., Smart, J., Hayman, M., and al., e. (1989). Molecular cloning of the chicken myelomonocytic growth factor (cMGF) reveals relationship to interleukin 6 and granulocyte colony stimulating factor. *EMBO J.* **8,** 175-181.

Li, P., He, X., Gerrero, M. R., Mok, M., Aggarwal, A., and Rosenfeld, M. G. (1993). Spacing and orientation of bipartite DNA-binding motifs as potential functional determinants for POU domain factors. *Genes Dev.* **7,** 2483-2496.

Li, M., Sendtner, M., and Smith, A. (1995). Essential function of LIF receptor in motor neurons. *Nature* **378,** 724-727.

Liem, K., Tremml, G., Roelink, H., and Jessel, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82,** 969-979.

Lin, X., Swaroop, A., Vaccarino, F. M., Murtha, A. T., Haas, M., Xiao-dong, J., Ruddle, F. H., and Leckman, J. F. (1996). Characterisation and sequence analysis of the human homeobox-containing gene *GBX2*. *Genomics* **31,** 335-342.

Ling, V., and Neben, S. (1997). In vitro differentiation of embryonic stem cells: Immunophenotypic analysis of cultured embryoid bodies. *J. Cell. Physiol.* **171,** 104-115.

Liu, A., Losos, K., and Joyner, A. L. (1999). FGF8 can activate *Gbx2* and transform regions of the rostral mouse brain into a hindbrain fate. *Development* **126,** 4827-4838.

Logan, C., Hanks, M. C., Noble-Topham, S., Nallainathan, D., Provart, N. J., and Joyner, A. L. (1992). Cloning and sequence comparison of the mouse, human, and chicken *engrailed* genes reveal potential functional domains and regulatory regions. *Dev. Genet.* **13,** 345-358.

Lovell-Badge, R., and Hacker, A. (1995). The molecular genetics of *Sry* in mammalian sex determination. *Phil. Trans. R. Soc. Lond. B.* **350,** 205-214.

Lu, J., Lee, W., Jiang, C., and Keller, E. B. (1994). Start site selection by Sp1 in the TATA-less human Ha-ras promoter. *J. Biol. Chem.* **269,** 5391-5402.

Lu, M. F., Cheng, H. T., Kern, M. J., Potter, S. S., Tran, G., Diekwisch, T. G., and Martin, J. F. (1999). *prx-1* functions cooperatively with another paired-related homeobox gene, *prx-2*, to maintain cell fates within the craniofacial mesenchyme. *Development* **126,** 495-

504.

Lufkin, T., Dierich, A., LeMeur, M., Mark, M., and Chambon, P. (1991). Disruption of the *Hox-1.6* homeobox gene results in defects in a region corresponding to its rostral domain of expression. *Cell* **66,** 1105-1119.

Lumsden, A., and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337,** 424-433.

Lumsden, A., Sprawson, N., and Graham, A. (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **121,** 825-837.

Lumsden, A., and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274,** 1109-1115.

Lyon, M. F., and Serle, A. G. (1989). "Genetic Variants and Strains of the Laboratory Mouse." Oxford University Press, Oxford.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L., and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene *Nkx-2.5*. *Genes Dev.* **9,** 1654-1666.

Maden, M., Gale, E., Kostetskii, I., and Zile, M. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Curr. Biol.* **6,** 417-426.

Mann, R. S., and Chan, S.-K. (1996). Extra specificity from *extradenticle*: the partnership between HOX and PBX/EXD homeodomain proteins. *Trends Genet.* **12,** 258-262.

Mansour, S. L., Thomas, K. R., and Capecchi, M. R. (1988). Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature (London)* **336,** 348-352.

Marshall, H., Nonchev, S., Sham, M. H., Muchamore, I., Lumsden, A., and Krumlauf, R. (1992). Retinoic acid alters the hindbrain *Hox* code and induces the transformation of rhombomeres 2/3 into a rhombomere 4/5 identity. *Nature* **360,** 737-741.

Marti, E., Bumcrot, D. A., Takada, R., and McMahon, A. P. (1995). Requirement of 19k form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375,** 322-325.

Martin, G. R. (1981). Isolation of a pluripotential cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78,** 7634-7638.

Martinez, S., Crossley, P. H., Cobos, I., Rubenstein, J. L. R., and Martin, G. R. (1999). FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on *Otx2* expression. *Development* **126,** 1189-1200.

Matsui, T., Hirati, M., Hirano, M., and Kurosawa, Y. (1993). The *HOX* complex neighbored by the *EVX* gene, as well as two other homeobox-containing genes, the *GBX*-class and the *EN*-class, are located on the same chromosomes 2 and 7 in humans. *FEBS Lett.* **336,** 107-110.

May, B. K., Dogra, S. C., Sadlon, T. J., Bhasker, C. R., Cox, T. C., and Bottomley, S. S. (1995). Molecular regulation of heme biosynthesis in higher vertebrates. *Prog. Nucleic Acid Res. Mol. Biol.* , 1-51.

McCormack, M. P., and Gonda, T. J. (1997). Expression of activated mutants of the human interleukin3/interleukin-5/granulocyte-macrophage colony-stimulating factor receptor common beta subunit in primary hematopoietic cells induces factor-independent proliferation and differentiation. *Blood* **90,** 1471-1481.

McGinnis, W., and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68,** 283-302.

McMahon, A. P., and Bradley, A. (1990). The *wnt-1* (*int-1*) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62,** 1073-1085.

McMahon, A. P., Joyner, A. L., Bradley, A., and McMahon, J. A. (1992). The midbrain-hindbrain phenotype of *wnt-1-/wnt-1-* mice results from stepwise deletion of *engrailed*-expressing cells by 9.5 days postcoitum. *Cell* **69,** 581-595.

Means, A. L., and Gudas, L. J. (1995). The roles of retinoids in vertebrate development. *Annu. Rev. Biochem.* **64,** 201-233.

Melzler, M., Chen, N., Helgason, C. D., R.K., G., Nichol, K., McCutcheon, K., Nasir, J., Humphries, R. K., Raymond, L. A., and Hayden, M. R. (1999). Life without huntingtin: normal differentiation into functional neurons. *J. Neurochem.* **72,** 1009-1018.

Mermod, N., O'Neill, E. A., Kelly, T. J., and Tjian, R. (1989). The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. *Cell* **58,** 741-753.

Metz, T., Graf, T., and Leutz, A. (1991). Activation of cMGF expression is a critical step in avian myeloid leukemogenesis. *EMBO J.*. **10,** 837-844.

Meyers, E. N., Lewandoski, M., and Martin, G. R. (1998). An *Fgf8* mutant allelic series generated by cre- and flp-mediated recombination. *Nature Genet.* **18,** 136-141.

Millet, A., Campbell, K., Epstein, D. J., Losos, K., Harris, E., and Joyner, A. J. (1999). A role for *Gbx2* in repression of *Otx2* and positioning the mid/hindbrain organizer. *Nature* **401,** 161-164.

Moore, M. A., and Metcalf, D. (1970). Ontogeny of the haemopoietic system: yolk sac origin of *in vivo* and *in vitro* colony forming cells in the developing mouse embryo. *Brit. J. Haem.* **18,** 279-296.

Morris, L., Graham, C. F., and Gordon, S. (1991). Macrophages in haemopoietic and other tissues of the developing mouse detected by the monoclonal antibody F4/80. *Development* **112,** 517-526.

Morrisey, E. E., Tang, Z., Sigrist, K., Lu, M. M., Jiang, F., Ip, H. S., and Parmacek, M. S. (1998). GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes Dev.* **12,** 3579-3590.

Morriss-Kay, G. M., and Sokolova, N. (1996). Embryonic development and pattern

formation. *FASEB J.* **10,** 961-968.

Mortensen, R. M., Conner, D. A., Chao, S., Geisterfer-Lowrance, A. A. T., and Seidman, J. G. (1992). Production of homozygous mutant ES cells with a single targeting construct. *Mol. Cell. Biol.* **12,** 2391-2395.

Mount, S. M. (1982). A catalogue of splice junction sequences. *Nucleic Acids Res.* **10,** 459-472.

Mountford, P., Zevnik, B., Duwel, A., Nichols, J., Li, M., Dani, C., Robertson, M., and A., S. (1994). Distronic targeting constructs: Reporters and modifiers of mammalian gene expression. *Proc. Natl. Acad. Sci. USA* **91,** 4303-4307.

Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J. J., and Potter, S. S. (1991). A functional *c-myb* gene is required for normal murine fetal hepatic hematopoiesis. *Cell* **65,** 677-689.

Murphy, P., Davidson, D. R., and Hill, R. E. (1989). Segment-specific expression of a homeobox containing gene in the mouse hindbrain. *Nature* **341,** 156-159.

Murtha, M. T., Leckman, J. F., and Ruddle, F. H. (1991). Detection of homeobox genes in development and evolution. *Proc. Natl. Acad. Sci. USA* **88,** 10711-10715.

Nagy, A., and Rossant, J. (1996). Targeted mutagenesis: Analysis of phenotype without germline transmission. *J. Clin. Invest.* **97,** 1360-1365.

Naito, M., Umeda, S., Yamamoto, T., Moriyama, H., Umezu, H., Hasegawa, G., Usuda, H., Schulz, L. D., and Takahashi, K. (1996). Development, differentiation, and phenotypic heterogeneity of murine tissue macrophages. *J. Leuk. Biol.* **59,** 133-138.

Nakai, S., Kawano, H., Yudate, T., Nishi, M., Kuno, J., Nagata, A., Jishage, K., Hamada, H., Fudii, H., and Kawamura, K. (1995). The POU domain transcription factor *Brn-2* is required for the determination of specific neuronal lineages in the hypothalamus of the mouse. *Genes Dev.* **9,** 3109-3121.

Nichols, J., Davidson, D., Taga, T., Yoshida, K., Chambers, I., and Smith, A. G. (1996). Complementary tissue-specific expression of LIF and LIF-receptor mRNAs in early mouse embryogenesis. *Mech. Dev.* **57,** 123-131.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schöler, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor *Oct4*. *Cell* **95,** 379-391.

Nieuwkoop, P. D., Boterenbrood, E. C., Kremer, A., Bloemsma, F. F. S. N., Hoessels, E. L. M. J., Meyer, G., and Verheyen, F. J. (1952). Activation and organization of the central nervous system in amphibians. *J. Exp. Zool.* **120,** 1-108.

Nishiguchi, S., Wood, H., Kondoh, H., Lovell-Badge, R., and Episkopou, V. (1998). *Sox1* directly regulates the γ-crystallin genes and is essential for lens development in mice. *Genes Dev.* **12,** 776-781.

Niss, K., and Leutz, A. (1998). Expression of the homeobox gene *GBX2* during chicken development. *Mech. Dev.* **76,** 151-155.

Niswander, L., and Martin, G. R. (1992). *Fgf-4* expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* **114,** 775-768.

Noden, D. M. (1983). The role of neural crest in patterning of avian cranial, connective, and muscle tissues. *Dev. Biol.* **96,** 144-165.

Novina, C. D., and Roy, A. L. (1996). Core promoters and transcriptional control. *Trends Genet.* **12,** 351-355.

Otting, G., Qian, Y. Q., Billeter, M., Müller, M., Affolter, M., Gehring, W. J., and Wüthrich, K. (1990). Protein-DNA contacts in the structure of a homeodomain-DNA complex determined by nuclear magnetic resonance spectroscopy in solution. *EMBO J.*. **9,** 3085-3092.

Palmieri, S. L., Peter, W., Hess, H., and Schöler, H. R. (1994). *Oct4* transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Dev. Biol.* **166,** 259-267.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G., and Boncinelli, E. (1995). The *Xenopus* homolog of *Otx2* is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* **121,** 707-720.

Papalopulu, N., Lovell-Badge, R., and Krumlauf, R. (1991). The expression of murine *Hox-2* genes is dependent on the differentiation pathway and displays collinear sensitivity to retinoic acid in F9 cells and *Xenopus* embryos. *Nucl. Acids Res.* **19,** 5497-5506.

Parameswaran, M., and Tam, P. P. L. (1995). Regionalisation of cell fate and morphogenetic movement of the mesoderm during mouse gastrulation. *Dev. Genet.* **17,** 16-28.

Patterson, K. D., Cleaver, O., Gerber, W. V., Grow, M. W., Newman, C. S., and Krieg, P. A. (1998). Homeobox genes in cardiovascular development. *Curr. Top. Dev. Biol.* **40,** 1-44.

Pellegrini, M., Manouri, A., Simeone, A., Boncinelli, E., and Gruss, P. (1996). Dentate gyrus formation requires *Emx2*. *Development* **122,** 3893-3898.

Pendleton, J. W., Nagai, B. K., Murtha, M. T., and Ruddle, F. H. (1993). Expansion of the *Hox* gene family and the evolution of chordates. *Proc. Natl. Acad. Sci. USA* **90,** 6300-6304.

Perkins, A. C., and Cory, S. (1993). Conditional immortalization of mouse myelomonocytic, megakaryocytic and mast cell progenitors by the Hox-2.4 homeobox gene. *EMBO J.* **12,** 3835-3846.

Pevney, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S. F., D'Agati, V., Orkin, S. H., and Costantini, F. (1991). Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* **349,** 257-260.

Pevny, L. H., Sockanathan, S., Placzek, M., and Lovell-Badge, R. (1998). A role for SOX1 in neural determination. *Development* , 1967-1978.

Phelan, M. L., Rambaldi, I., and Featherstone, M. S. (1995). Cooperative interactions

between HOX and PBX proteins mediated by a conserved peptide motif. *Mol. Cell. Biol.* **15,** 3989-3997.

Piquet-Pellorce, C., Grey, L., Mereau, A., and Heath, J. K. (1994). Are LIF and related cytokines functionally equivalent? *Exp. Cell Res.* **213,** 340-347.

Pöpperl, H., Bienz, M., Studer, M., Chan, S., Aparicio, S., Brenner, S., Mann, R. S., and Krumlauf, R. (1995). Segmental expression of *Hoxb-1* is controlled by a highly conserved autoregulatory loop dependent on *exd/pbx*. *Cell* **81,** 1031-1042.

Puelles, L., Amat, J. A., and Martinez-de-la-Torre, M. (1987). Segment-related, mosaic neurogenetic pattern in the forebrain and mesencephalon of early chick embryos: I. Topography of AChE-positive neuroblasts up to stage HH18. *J. Comp. Neurol.* **266,** 247-268.

Puelles, L., and Rubenstein, J. L. R. (1993). Expression patterns of homeobox and their putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization. *Trends Neurosci.* **16,** 472-479.

Qian, Y. Q., Furukubo-Tokunaga, K., Resendez-Perez, D., Muller, M., Gehring, W. J., and Wuthrich, K. (1994). Nuclear magnetic resonance solution structure of the *fushi tarazu* homeodomain from *Drosophila* and comparison with the *Antennapedia* homeodomain. *J. Mol. Biol.* **238,** 333-345.

Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995). MatInd and MatInspector-New fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucl. Acids Res.* **23,** 4878-4884.

Rathjen, P. D., Nichols, J., Toth, S., Edwards, D. R., Heath, J. K., and Smith, A. G. (1990). Developmentally programmed induction of differentiation inhibiting activity and the control of stem cell populations. *Genes Dev.* **4,** 2308-2318.

Rathjen, J., Lake, J., Bettess, M. D., Washington, J. M., Chapman, G., and Rathjen, P. D. (1999). Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. *J. Cell Sci.* **112,** 601-612.

Renoncourt, Y., Carroll, P., Filippi, P., Arce, V., and Alonso, S. (1998). Neurons derived in vitro from ES cells express homeoproteins characteristic of motoneurons and interneurons. *Mech. Dev.* **79,** 185-197.

Rhinn, M., Dierich, A., Shawlot, W., Behringer, R. R., LeMeur, M., and Ang, S.-L. (1998). Sequential roles for *Otx2* in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. *Development* **125,** 845-856.

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75,** 1401-1416.

Rivera-Pérez, J. A., Mallo, M., Gendron-Maguire, M., T., G., and Behringer, R. R. (1995). *goosecoid* is not an essential component of the mouse gastrula organizer but is required for craniofacial and rib development. *Development* **121,** 3005-3012.

Robb, L., Elwood, N. J., Elefanty, A. G., Kontgen, F., Li, R., Barnett, L. D., and Begley, G. (1996). The *scl* gene product is required for the generation of all haematopoietic

lineages in the adult mouse. *EMBO J.* **15,** 4123-4129.

Robb, L. (1997). Hematopoiesis: Origin pinned down at last? *Curr. Biol.* **7,** R10-R12.

Roberts, C. W., Shutter, J. R., and Korsmeyer, S. J. (1994). *Hox11* controls the genesis of the spleen. *Nature* **368,** 747-749.

Robertson, E. J. (1986). Pluripotential stem cells as a route into the mouse germline. *Trends Genet.* **2,** 9.

Robertson, E., Bradley, A., Kuehn, M., and Evans, M. (1986). Germline transmission of genes introduced into cultured pluripotential cells by retroviral vector. *Nature* **323,** 445-448.

Robertson, E. J. (1987). Embryo-derived stem cell lines. *In* "Teratocarcinomas and embryonic stem cells: a practical approach" (E. J. Robertson, Ed.), pp. 71-112. IRL press, Oxford, Washington DC.

Robertson, M., Chambers, I., Rathjen, P., Nichols, J., and Smith, A. (1993). Expression of alternative forms of differentiation inhibiting activity (DIA/LIF) during murine embryogenesis and in neonatal and adult tissues. *Dev. Genet.* **14,** 165-173.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T., and Dodd, J. (1994). Floor plate and motor neuron induction by *vhh-1*, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76,** 761-775.

Rogers, M. B., Hosler, B. A., and Gudas, L. J. (1991). Specific expression of a retinoid acid-regulated, zinc-finger gene, Rex-1, in preimplantation embryos, trophoblast and spermatocytes. *Development* **113,** 815-824.

Rohwedel, J., Maltsev, V., Bober, E., H.H., A., Hescheler, J., and Wobus, A. M. (1994). Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. *Dev. Biol.* **164,** 87-101.

Rohwedel, J., Guan, K., Zuschratter, W., Jin, S., Ahnert-Hilger, G., Furst, D., Fassler, R., and Wobus, A. M. (1998). Loss of beta1 integrin function results in a retardation of myogenic, but an acceleration of neuronal, differentiation of embryonic stem cells in vitro. *Dev. Biol.* **201,** 167-184.

Rosner, M. H., Vigano, A., Ozato, K., Timmons, P. M., Poirier, F., and Rigby, P. (1990). A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345,** 686-692.

Rowitch, D. H., and McMahon, A. P. (1995). *Pax-2* expression in the murine neural plate precedes and encompasses the expression domains of *Wnt-1* and *En-1*. *Mech. Dev.* **52,** 3-8.

Rubenstein, J. L. R., and Puelles, L. (1994). Homeobox gene expression during development of the vertebrate brain. *Curr. Top. Dev. Biol.* **29,** 1-63.

Rubenstein, J. L. R., and Shimamura, K. (1997). Regulation of patterning and differentiation in the embryonic vertebrate forebrain. *In* "Molecular and Cellular

Approaches to Neural Development" (W. M. Cowen, T. M. Jessel, and S. L. Zipursky, Eds.), pp. 356-390. Oxford University Press, Oxford.

Ruiz i Altaba, A., and Jessel, T. (1991). Retinoic acid modifies mesodermal patterning in early *Xenopus* embryos. *Genes Dev.* **5,** 175-187.

Ruiz i Altaba, A. (1992). Planar and vertical signals in the induction and patterning of the Xenopus nervous system. *Development* **116,** 67-80.

Sasai, Y., Lu, B., Steinbeisser, H., and De Robertis, E. M. (1995). Regulation of neural induction by the *Chd* and *Bmp-4* antagonistic patterning signals in Xenopus. *Nature* **376,** 333-336.

Sasaki, H., Yokoyama, E., and Kuroiwa, A. (1990). Specific DNA binding of the two chicken Deformed family homeodomain proteins, Chox-1.4 and Chox-a. *Nucleic Acids Res* **18,** 1739-1747.

Sauvageau, G., Thorsteinsdottir, U., Eaves, C. J., Lawrence, H. J., Largman, C., Lansdorp, P. M., and Humphries, R. K. (1995). Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. *Genes Dev.* **9,** 1753-1765.

Sauvageau, G., Thorsteinsdottir, U., Hough, M. R., Hugo, P., Lawrence, H. J., Largman, C., and Humphries, R. K. (1997). Overexpression of HOXB3 in hematopoietic cells causes defective lymphoid development and progressive myeloproliferation. *Immunity* **6,** 13-22.

Scheidereit, C., Heguy, A., and Roeder, R. G. (1987). Identification and purification of a human lymphoid-specific Octamer-binding Protein (OTF-2) that activates transcription of an immunoglobulin promotor in vitro. *Cell* **51,** 783-793.

Schillam, M. W., Oosterwegel, M. A., Moerer, P., Ya, J., De Boer, P. A. J., Van de Wetering, M., Verbeek, S., Lamers, W. H., Kruisbeek, A. M., Cumano, A., and Clevers, H. (1996). Defects in cardiac outflow tract formation and pro-B-lymphocyte expansion in mice lacking Sox-4. *Nature* **346,** 240-244.

Schmitt, R., Bruyns, E., and Snodgrass, H. (1991). Hematopoietic development of embryonic stem cells in vitro: cytokine and receptor gene expression. *Genes Dev.* **5,** 728-740.

Schneider-Maunoury, S., Topilko, P., Seitanidou, T., Levi, G., Tannoudji, M., Pournin, A., Babinet, C., and Charnay, P. (1993). Disruption of *Krox-20* results in alteration of rhombomeres 3 and 5 in the developing hindbrain. *Cell* **75,** 1199-1214.

Schöler, H. R., Hatzopoulos, A. K., Balling, R., Suzuki, N., and Gruss, P. (1989). A family of Octamer-specific proteins present during mouse embryogenesis: evidence for germline-specific expression of an Oct factor. *EMBO J.* **8,** 2543-2550.

Schöler, H. R., Dressler, G. R., Balling, R., Rohdewohld, H., and Gruss, P. (1990). *Oct-4*: a germline-specific transcription factor mapping to the mouse t-complex. *EMBO J.* **9,** 2185-2195.

Schöler, H. R. (1991). Octamania: the POU factors in murine development. *Trends Genet.* **7,** 323-329.

Scott, M. P., Tamkun, J. W., and Hartzell, G. W. (1989). The structure and function of the homeodomain. *Biochim. Biophys. Acta* **989,** 25-48.

Scott, E. W., Simon, M. C., Anastasi, J., and Singh, H. (1994). Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* **265,** 1573-1577.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., and Schuh, A. C. (1995). Failure of blood-island formation and vasculogenesis in *Flk-1* deficient mice. *Nature* **376,** 62-66.

Shalaby, F., Ho, J., Stanford, W. L., Fischer, K. D., Schuh, A. C., Scwartz, L., Bernstein, A., and Rossant, J. (1997). A requirement for *Flk1* in primitive and definitive hematopoiesis and vasculogenesis. *Cell* **89,** 981-90.

Shamim, H., and Mason, I. (1998). Expression of *Gbx-2* during early development of the chick embryo. *Mech. Dev.* **76,** 157-159.

Shamim, H., Mahmood, R., C., L., Doherty, P., Lumsden, A., and Mason, I. (1999). Sequential roles for *Fgf4*, *En1*, and *Fgf8* in specification and regionalisation of the midbrain. *Development* **126,** 945-959.

Shawlot, W., and Behringer, R. R. (1995). Requirement for *Lim1* in head-organizer function. *Nature* **174,** 425-430.

Shearwin-Whyatt, L., Remiszewski, J., and Rathjen, P. D. (in press). Gene targeting. *In* "The Encyclopedia of Molecular Biology" (T. E. Creighton, Ed.). John Wiley and Sons, New York.

Shen, M. M., and Leder, P. (1992). Leukemia inhibitory factor is expressed by the preimplantation uterus and selectively blocks primitive ectoderm formation *in vitro*. *Proc. Natl. Acad. Sci. USA* **89,** 8240-8244.

Shier, A. F., and Gehring, W. J. (1992). Direct homeodomain-DNA interaction in the autorregulation of the fushi tarazu gene. *Nature* **356,** 804-807.

Simeone, A., Acampora, D., Arcioni, L., Andrews, P. W., Boncinelli, E., and Mavilio, F. (1990). Sequential activation of HOX2 homeobox genes by retinoic acid in human embryonal carcinoma cells. *Nature* **346,** 763-766.

Simeone, A., Acampora, D., Nigro, V., Faiella, A., D'Esposito, M., Stornaiuolo, A., Mavilio, F., and Boncinelli, E. (1991). Differential regulation by retinoic acid of the homeobox genes of the four *HOX* loci in human embryonal carcinoma cells. *Mech. Dev.* **33,** 215-227.

Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A., D'Apice, M. R., Nigro, V., and Boncinelli, E. (1993). A vertebrate gene related to *orthodenticle* contains a homeodomain of the *bicoid* class and demarcates anterior neurectoderm in the gastrulating mouse embryo. *EMBO J.*. **12,** 2735-2747.

Simeone, A. (1998). *Otx1* and *Otx2* in the development and evolution of the mammalian brain. *EMBO J.* **17,** 6790-6798.

Simon, H., and Lumsden, A. (1993). Rhombomere-specific origin of the contralateral vestibulo-acoustic efferent neurons and their migration across the embryonic midline. *Neuron* **11,** 209-220.

Sivaraja, M., Botfield, M. C., Mueller, M., Jancso, A., and Weiss, M. A. (1994). Solution structure of a POU-specific homeodomain: 3D-NMR studies of human B-cell transcription factor Oct-2. *Biochemistry* **33,** 9845-9855.

Slamon, D. J., Boone, T. C., Murdock, D. C., Keith, D. E., Press, M. F., Lavson, R. A., and Souza, L. M. (1986). Studies of the human *c-myb* gene and its product in human acute leukemias. *Science* **233,** 347-354.

Small, K. S., and Potter, S. (1993). Homeotic transformations and limb defects in *Hoxa-11* mutant mice. *Genes Dev.* **7,** 2318-2328.

Smith, A. G., and Hooper, M. L. (1987). Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. *Dev. Biol.* **121,** 1-9.

Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M., and Rogers, D. (1988). Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature (London)* **336,** 688-690.

Smith, J. L., and Schoenwolf, G. C. (1989). Notochordal induction of cell wedging in the chick neural plate and its role in neural tube formation. *J. Exp. Zool.* **250,** 49-62.

Smith, A. G. (1991). Culture and differentiation of embryonic stem cells. *J. Tiss. Cult. Meth.* **13,** 89-94.

Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A., and Kucherlapati, R. S. (1985). Insertion of DNA sequences into the human chromosomal b-globin locus by homologous recombination. *Nature (London)* **317,** 230-234.

Spyropoulos, D. D., and Capecchi, M. R. (1994). Targeted disruption of the *even-skipped* gene, *evx1*, causes early postimplantation lethality of the mouse conceptus. *Genes Dev.* **8,** 1949-1961.

St-Jacques, B., and McMahon, A. P. (1996). Early mouse development: lessons from gene targeting. *Curr. Opin. Genet. Dev.* **6,** 439-444.

Stanford, W. L., Caruana, G., Vallis, K. A., Inamdar, M., Hidaka, M., Bautch, V. L., and Bernstein, A. (1998). Expression trapping: identification of novel genes expressed in hematopoietic and endothelial lineages by gene trapping in ES cells. *Blood* **92,** 4622-4631.

Stewart, C. L., Kasper, P., Brunet, L. J., Bhatt, H., Gadi, I., Köntgen, F., and Abbondanzo, S. J. (1992). Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature* **359,** 76-79.

Struhl, G., and White, R. A. H. (1985). Regulation of the *Ultrabithorax* gene of *Drosophila* by other *Bithorax* complex genes. *Cell* **43,** 507-519.

Strübing, C., Ahnert-Hilger, G., Shan, J., Wiedenmann, B., Hescheler, J., and Wobus, A. M. (1995). Differentiation of pluripotent embryonic stem cells into the neuronal lineage in

vitro gives rise to mature inhibitory and excitatory neurons. *Mech. Dev.* , 275-287.

Suda, Y., Nakabayashi, J., Matsuo, I., and Sinichi, A. (1999). Functional equivalency between *Otx2* and *Otx1* in development of the rostral head. *Development* **126,** 743-757.

Tam, P. P. L., and Beddington, R. S. P. (1987). The formation of mesodermal tissues during gastrulation. *Development* **101,** 55-67.

Tam, P. P. L. (1989). Regionalisation of the mouse embryonic ectoderm: allocation of prospective ectodermal tissues during gastrulation. *Development* **101,** 55-67.

Tam, P. P. L., and Beddington, R. S. P. (1992). Establishment and organization of germ layers in the gastrulating mouse embryo. *Ciba Found. Symp.* **165,** 27-49.

Tam, P. P. L., and Zhou, S. X. (1996). The allocation of epiblast cells to ectodermal and germline lineages is influenced by the position of the cells in the gastrulating mouse embryo. *Dev. Biol.* **178,** 124-132.

Tam, P. P. L., Steiner, K. A., Zhou, S. X., and Quinlan, G. A. (1997). Lineage and functional analyses of the mouse organizer. *Cold Spring Harbor Symp. Quant. Biol.* **62,** 135-144.

Tam, P. P. L., and Behringer, R. R. (1997). Mouse gastrulation: the formation of a mammalian body plan. *Mech. Dev.* **68,** 3-25.

Tanabe, Y., and Jessel, T. M. (1996). Diversity and pattern in the developing spinal cord. *Science* **274,** 1115-1123.

Tarkowski, A. K. (1959). Experiments on the development of isolated blastomeres of mouse eggs. *Nature (London)* **184,** 1286-1287.

te Riele, H., Maandag, E. R., Clarke, A., Hooper, M., and Berns, A. (1990). Consecutive inactivation of both alleles of the *pim-1* proto-oncogene by homologous recombination in embryonic stem cells. *Nature* **348,** 649-651.

te Riele, H., Maandag, E. R., and Berns, A. (1992). Highly efficient gene targeting in embryonic stem cells through homologous recombination with isogenic DNA constructs. *Proc. Natl. Acad. Sci. USA* **89,** 5128-5132.

Thiele, C. J., McKeon, C., Triche, T. J., Ross, R. A., Reynolds, C. P., and Israel, M. A. (1987). Differential protooncogene expression characterizes histopathologically indistinguishable tumors of the peripheral nervous system. *J. Clin. Invest.* **80,** 804-811.

Thiele, C. J., Cohen, P. S., and Israel, M. A. (1988). Regulation of *c-myb* expression in human neuroblastoma cells during retinoic acid-induced differentiation. *Mol. Cell. Biol.* **8,** 1677-1783.

Thomas, K. R., and Capecchi, M. R. (1987). Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51,** 503-512.

Thomas, P. Q. (1994). Homeobox gene expression in murine embryonic stem cells. University of Adelaide.

Thomas, P. Q., Johnson, B. V., Rathjen, J., and Rathjen, P. D. (1995). Sequence, genomic organization, and expression of the novel homeobox gene *Hesx1*. *J. Biol. Chem.* **270,** 3869-3875.

Thomas, P., and Beddington, R. S. P. (1996). Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. *Curr. Biol.* **6,** 1487-1496.

Thomas, P., Brickman, J. M., Popperl, H., Krumlauf, R., and Beddington, R. S. P. (1997). Axis duplication and anterior identity in the mouse embryo. *In* "Cold Spring Harbor Symp. Quant. Biol.", Vol. 62, pp. 115-126. Cold Spring Harbor Laboratory Press.

Thomas, P. Q., Brown, A., and Beddington, R. S. (1998). *Hex*: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. *Development* **125,** 85-94.

Thorsteinsdottir, U., Sauvageau, G., Hough, M. R., Dragowska, W., Lansdorp, P. M., Lawrence, H. J., Largman, C., and Humphries, R. K. (1997a). Overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia. *Mol. Cell. Biol.* **17,** 495-505.

Thorsteinsdottir, U., Sauvageau, G., and Humphries, R. K. (1997b). Hox homeobox genes as regulators of normal and leukemic hematopoiesis. *Hematol. Oncol. Clin. North Am.* **11,** 1221-1233.

Treisman, J., Harris, E., and Desplan, C. (1991). The paired box encodes a second DNA-binding domain in the paired homeodomain protein. *Genes Dev.* **5,** 594-604.

van Deursen, J., and Wieringa, B. (1992). Targeting of the creatinine kinase M gene in embryonic stem cells using isogenic and nonisogenic DNA. *Nucl. Acids Res.* **20,** 3815-3820.

Varlet, I., Collingnon, J., and Robertson, E. J. (1997). *nodal* expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. *Development* **124,** 1033-1044.

Vollmer, J.-Y., and Clerc, R. G. (1998). Homeobox genes in the developing mouse brain. *J. Neurochem.* **71,** 1-19.

von Bubnoff, A., Schmidt, J. E., and Kimelman, D. (1995). The *Xenopus laevis* homeobox gene, *Xgbx-2* is an early marker of anteroposterior patterning in the ectoderm. *Mech. Dev.* **54,** 149-160.

Ware, C. B., Horowitz, M. C., Renshaw, B. R., Hunt, J. S., Liggitt, D., Koblar, S. A., Gliniak, B. C., McKenna, H. J., Papayannopolou, T., Thoma, B., Cheng, L., Donovan, P. J., Peschon, J., Bartlett, P. F., Willis, C. R., Wright, B. D., Carpenter, M. K., Davidson, B. L., and Gearing, D. P. (1995). Targeted disruption of the low-affinity leukaemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. *Development* **121,** 1283-1299.

Wassarman, K. M., Lewandoski, M., Campbell, K., Joyner, A. L., Rubenstein, J. L. R., Martinez, S., and Martin, G. R. (1997). Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on *Gbx2* gene function.

*Development* **124,** 2923-2934.

Wassef, M., and Joyner, A. L. (1997). Early mesencephalon/metencephalon patterning and development of the cerebellum. *Perspect. Dev. Neurobiol.* **5,** 3-16.

Watson, A. J., and Kidder, G. M. (1988). Immunofluorescence assessment of the timing of appearance and cellular distribution of Na/K-ATPase during mouse embryogenesis. *Dev. Biol.* **126,** 80-90.

Watson, A. J., Damsky, C. H., and Kidder, G. M. (1990). Differentiation of an epithelium: Factors affecting the polarised distribution of Na+, K+-ATPase in mouse trophectoderm. *Dev. Biol.* **141,** 104-114.

Weiss, M. J., Keller, G., and Orkin, S. H. (1994). Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells. *Genes Dev.* **8,** 1184-1197.

Weiss, M. J., and Orkin, S. H. (1996). In vitro differentiation of murine embryonic stem cells. *J. Clin. Invest.* **97,** 591-595.

Westin, E. H., Gallo, R. C., Arya, S. K., Eva, A., Souza, L. M., Valuda, M. A., Aaronson, S. A., and Wong-Staal, F. (1982). Differential expression of the *amv* gene in human hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **79,** 2194-2198.

Whyatt, L. (1996). Systems for the analysis of developmental control genes of the mouse. University of Adelaide.

Whyatt, L. M., and Rathjen, P. D. (1997). Introduction of precise alterations into the mouse genome with high efficiency by stable tag-exchange gene targeting: implications for gene targeting in ES cells. *Nucl. Acids Res.* **25,** 2381-2388.

Wilder, P. J., Kelly, D., Brigman, K., Peterson, C. L., Nowling, T., Gao, Q. S., McComb, R. D., Capecchi, M. R., and Rizzano, A. (1997). Inactivation of the FGF-4 gene in embryonic stem cells alters the growth and/or survival of their early differentiated progeny. *Dev. Biol.* **192,** 614-629.

Wiles, M. V., and Keller, G. (1991). Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. *Development* **111,** 259-267.

Wilkinson, D., Bailes, J. A., Champion, J. F., and McMahon, A. P. (1987). Expression of the proto-oncogene *int-1* is restricted to specific neural cells in the developing mouse brain. *Cell* **50,** 79-88.

Wilkinson, D. G., Bhatt, S., Chavrier, P., Bravo, R., and Charnay, P. (1989). Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. *Nature* **337,** 461-464.

Williams, R. L., Hilton., D. J., Pease, S., Williams, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., and Gough, N. M. (1988). Myeloid leukemia inhibitory factor maintain the developmental potential of embryonic stem cells. *Nature* **336,** 684-687.

Williams, T., and Tjian, R. (1991). Analysis of the DNA-binding and activation properties

of the human transcription factor AP-2. *Genes Dev.* **5,** 670-682.

Williamson, M. P. (1994). The structure and function of proline-rich regions in proteins. *Biochem. J.* **297,** 249-260.

Wilson, V., Manson, L., Skarnes, W. C., and Beddington, R. S. P. (1995). The *T* gene is necessary for normal mesodermal morphogenetic cell movements during gastrulation. *Development* **121,** 877-886.

Wilson, P. A., and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376,** 331-333.

Winnier, G., Blessing, M., P.A., L., and B.L.M., H. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.* **9,** 2105-2116.

Wobus, A. M., Grosse, R., and Schöneich, J. (1988). Specific effects of nerve growth factor on the differentiation pattern of mouse embryonic stem cells in vitro. *Biomed.Biochim. Acta* **47,** 965-973.

Wolberger, C., Vershon, A. K., Liu, B., Johnson, A. D., and Pabo, C. O. (1991). Crystal structure of a MAT alpha 2 homeodomain-operator complex suggests a general model for homeodomain-DNA interactions. *Cell* **67,** 517-528.

Wurst, W., Auerbach, A. B., and Joyner, A. L. (1994). Multiple developmental defects in *Engrailed-1* mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. .

Yamada, G., Mansouri, A., Torres, M., Stuart, E. T., Blum, M., Schultz, M., De Robertis, E. M., and Gruss, P. (1995). Targeted mutation of the murine *goosecoid* gene results in craniofacial defects and neonatal death. *Development* **121,** 2917-2922.

Yanagisawa, K. O., Fujimoto, H., and Urushibara, H. (1981). Effects of the Brachyury (*T*) mutation on morphogenetic movement in the mouse embryo. *Dev. Biol.* **87,** 242-248.

Yeom, Y. I., Ha, H. S., Balling, R., Schöler, H. R., and Artzt, K. (1991). Structure, expression and chromosomal location of the *Oct-4* gene. *Mech. Dev.* **35,** 171-179.

Yoshida, K., Taga, T., Saito, M., Suematsu, S., Kumanogoh, A., Tanaka, T., Fujiwara, H., Hirata, M., Yamagami, T., Nakahata, T., Hirabayashi, T., Yoneda, Y., Tanaka, K., Wang, W.-Z., Mori, C., Shiota, K., Yoshida, N., and Kishimoto, T. (1996). Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders. *Proc. Natl. Acad. Sci. USA* **93,** 407-411.

Zhang, M., Kim, H. J., Marshall, H., Gendron-Maguire, M., Lucas, D. A., Baron, A., Gudas, L. J., Gridley, T., Krumlauf, R., and Grippo, J. (1994). Ectopic Hoxa-1 induces rhombomere transformation in mouse hindbrain. *Development* **120**.