Further analysis of *Brachyury (T)*: a gene required during gastrulation

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Declaration

This thesis has been composed by myself

and

all results reported in this thesis have been obtained by myself except where indicated

Penny Rashbass 1994

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Abstract

In order to understand how gastrulation occurs, it is necessary not only to isolate the genes involved, but also to define their function. There are now a large number of cloned genes which are known to be expressed during murine development Gene defects which cause inappropriate differentiation and therefore produce a mutant phenotype are likely to reveal which genes control tissue diversification. However, the subsequent defective tissue interactions and cell death observed in an abnormal phenotype often masks the primary function of the mutated gene.

This thesis is concerned with further analysis of the classical mouse mutant *Brachyury(T)*. Developmental genetics has shown that this gene plays a key role in mesoderm formation during murine gastrulation (Dobrovolskaia-Zavadskaia (1927) *N. Comp. Rend. Seanc. Soc. Biol 97*, 114-119; Chesley P(1935) *J. Exp Zool 70*, 429 - 459; Gruneberg, H. (1958) *J. Embryol. exp. Morph. 6,* 424-443). Although the *Brachyury* gene is only expressed in the primitive streak, nascent mesoderm and notochord of the gastrulating mouse embryo (Wilkinson, D., Bhatt, S. & Herrmann, B.G.(1990) *Nature 343*, 657-659), homozygous null *T/T* mutants have a defective allantois, kinked neural tube and disorganized somites in addition to a bulky primitive streak and disrupted notochord (Chesley P(1935) *J. Exp Zool 70*, 429 - 459; Gruneberg, H. (1958) *J. Embryol. exp. Morph. 6,* 424-443; Spiegelman, M. (1976) in *Embryogenesis on Mammals* (eds. Elliott, K. & O'Connor, M.) 199-226). To study the cell autonomy of the *T* mutation, we have isolated and genetically characterised embryonic stem (ES) cell lines derived from heterozygous *T/+* matings and studied their behaviour in chimaeras. T/+ ‹–› +/+ form normal chimaeras whereas T/T ‹–› +/+ chimaeras mimic the T/T mutant phenotype.

In order to assess how *Brachyury* may interact with other genes which may also be required for mesoderm formation and both rostrocaudal and dorsoventral patterning, I decided to compare the expression patterns of several genes in homozygous and wildtype embryos using a non-radioactive wholemount *in situ* hybridisation technique. The expression pattern of three *Pax* genes (*Pax-1,Pax-3* and *Pax-6*) which exhibit dorsoventral patterning in the neural tube and/or somites are altered in *T/T* embryos, whilst the pattern of *Msx-1* which is expressed in the dorsal part of the neural tube remains unaltered. The *T* gene is also necessary to maintain the normal expression level of *Wnt-5a* and *Wnt-3a* in 8.5dpc embryos and *Evx-1* in 9.5 day embryos. In contrast *BMP-4* expression appears to be unaffected by the absence of the *T* gene product.

The results indicate that the *T* gene acts cell autonomously in the primitive streak and notochord and may be required to maintain, rather than activate, a signalling pathway involved in the specification of other mesodermal tissues and in the patterning the dorsoventral axis of the neural tube.

Chapter 1

General Introduction

The genetic and cellular control of pattern formation in mammals

The cellular and molecular mechanisms underlying pattern formation and tissue diversification of the developing mammalian embryo are poorly understood. However, it is known that the epiblast (Fig 1.1c) is the sole source of all the fetal tissues as well as several components of the extraembryonic structures (the visceral yolk sac mesoderm and mesodermal constituents of the chorioallantoic placenta; see (Beddington 1983b)). At gastrulation, this apparently homogeneous epithelial sheet first forms a definitive anteroposterior axis and then by extensive cell proliferation (Lewis and Rossant 1982; Snow 1977), cell mixing, differentiation and cell or tissue movements (Lawson et al. 1991) transforms into a complex multilayered structure. All the major processes which the embryo undergoes during development, that is cell differentiation, morphogenesis and pattern formation, occur during gastrulation. Therefore by understanding how gastrulation occurs, one may well gain insight into how these processes are executed in other situations.

In order to understand how gastrulation occurs, it is necessary not only to isolate the genes involved, but also to define their function. This thesis is concerned with the analysis of the functional role of one gene, *Brachyury(T)*, which plays an important part in gastrulation and early organogenesis (see below).

In the broadest sense, a gene can be considered as important in development if perturbing that gene's expression causes an abnormal embryonic phenotype. However there are many genes, for example those controlling metabolism, whose products are likely to be vital for most cells

and when disrupted result in cell, and eventually embryonic, death. These should not be considered as developmental regulatory genes. On the other hand, genetic mutations which cause inappropriate differentiation or cell movements (and may or may not be compatible with embryonic viability) are likely to reveal the underlying genetic control of tissue diversification.

The primary effect of a genetic mutation is often masked by subsequent defective tissue interactions and cell death. Therefore, the developmental function of a gene can only be unravelled by combining an intimate knowledge of its molecular and cellular biological properties with a precise understanding of its source and site of action in the embryo and the effects it has on a particular developmental sequence. It should, however, be noted that it is possible to get reproducible defects from quite non-specific teratogenic agents applied at particular stages of development. For example massive cell death at gastrulation caused by the teratogen Mitomycin C interrupts the growth equilibrium producing minor vertebral abnormalities (Gregg and Snow 1983; Snow and Tam 1979).

If one wants to assign causative roles to particular molecules in development, one must first know the sequence of determinative events at the cellular level. In both *Drosophila* and *C.Elegans,* clonal lineage analysis has defined when cells become committed to particular developmental fates (Deppe et al. 1978; Lawrence 1981; Sulston et al. 1983; Technau 1987). The knowledge about the predictable and progressive restrictions that occur in specific groups of cells, has provided an accurate baseline for testing the effects of molecular perturbation and thus identifying which molecules are instrumental in cell commitment and pattern formation. This lineage information combined with the production

of developmental mutations by deliberately saturating the *Drosophila* genome with point mutations (Nusslein-Volhard and Wieschaus 1980), and subsequently with transposable elements (Cooley et al. 1988; Rubin and Spradling 1982; Wilson et al. 1989), has meant that it is possible to perform a molecular dissection of *Drosophila* development.

However, direct extrapolation of developmental mechanisms responsible for laying down the body pattern in lower organisms to the mouse embryo may be misleading. This is because mammalian embryos exhibit several unique features during pattern formation and tissue diversification. First, the body pattern emerges and the organ primordia begin to form only after implantation occurs (see Kaufman 1992), therefore it is possible that there is a direct maternal uterine influence on the developing embryo. Second, there is extensive cell division during gastrulation in the mouse (Snow 1977). Third, the impressive regulatory properties of the preimplantation mouse embryo which extend into postimplantation development (for example Snow and Tam 1979) combined with the extensive cell mixing during early organogenesis (Lawson et al. 1991) means the commitment to a particular spatial domain may be less synchronous and more labile than in lower organisms. Finally, transcription in the early postimplantation mammalian embryo appears to be completely under zygotic control (see Schultz 1986).

The relative inaccessibility and cellular complexity of the mammalian embryo *in utero* also means that we have relatively poor knowledge of the cellular rules required to generate the different tissues in their correct spatial and temporal pattern. However, careful descriptive anatomy of the developing embryo provides a framework from which to start (see Kaufman 1992) and direct manipulation of the embryo *in vitro* (Beddington 1981; Beddington 1982; Copp et al. 1986; Lawson et al. 1991; Tam 1989;

Tam and Beddington 1987) has provided some insights into cell fate and commitment during gastrulation. The next section describes the process of gastrulation. It also includes a description of early organogenesis. This is because failure of correct tissue alignment early in development may cause inappropriate interactions and abnormal subsequent organogenesis.

Gastrulation and early organogenesis in the mouse

Gastrulation

Gastrulation is the process by which the embryo changes from a single epithelial sheet into a complex multilayered structure. Figure 1.1 is a diagram of mouse development showing the sequence of events from just prior to implantation through to the earliest stages of organogenesis. The pre-streak embryo (Fig 1.1c) can be subdivided into two portions: the extraembryonic region which lies mesometrially in the maternal decidual swelling, and the distally situated embryonic region. All the fetal primordia are formed from the inner layer of the embryonic region which lines the proamniotic cavity and is known as the epiblast or primitive ectoderm. At this stage, the embryo is radially symmetrical about its proximodistal axis and currently, it is not possible to predict the future position of the primitive streak.

Although the formation of the primitive streak at the posterior part of the embryo marks the start of gastrulation and defines the anteroposterior axis (Fig 1.1d), very little is known about how it is actually formed or maintained in mammals. The streak is first apparent in a localised region at the junction of the epiblast and extraembryonic ectoderm (Hashimoto and Nakatsuji 1989; Tam and Meier 1982) when epiblast cells lose their epithelial continuity and become more densely packed with an increase in

Figure 1.1

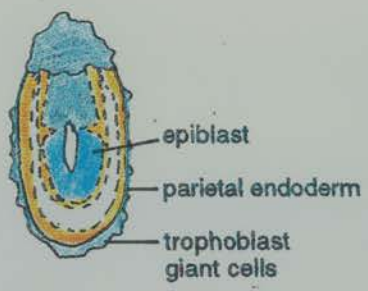
Schematic representation of mouse development between 4.5 and 8.5 days post coitum.

Midline sagital sections of postimplantation embryonic development from pre-gastrulation through to early organogenesis (Not to scale).
Drawing of 6.5 (d) and 7.0dpc (e) embryos include the lateral embryonic wings of mesoderm in a three dimensional effect whilst that of 8.5 dpc (g) embryo includes the position of the paraxial somites.
Embryos older than 6.0dpc (c) are shown with the parietal endoderm removed and embryos older than 7.0dpc (e) are drawn with the ectoplacental cone removed.
Adapted from R. Beddington

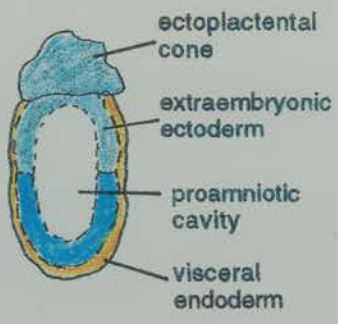
blue -	epiblast/primitive ectoderm
red -	embryonic and extraembryonic mesoderm
orange -	primitive endoderm
brown -	notochord
grey -	extra-embryonic ectoderm

a) 4.5dpc

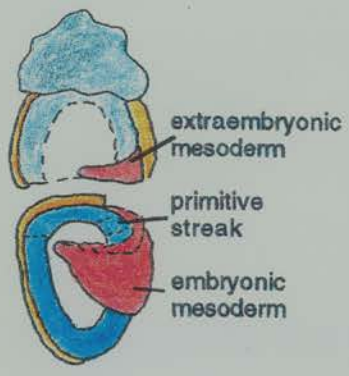
b) 5.5dpc



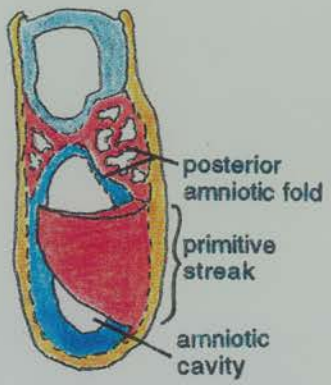
c) 6.0dpc



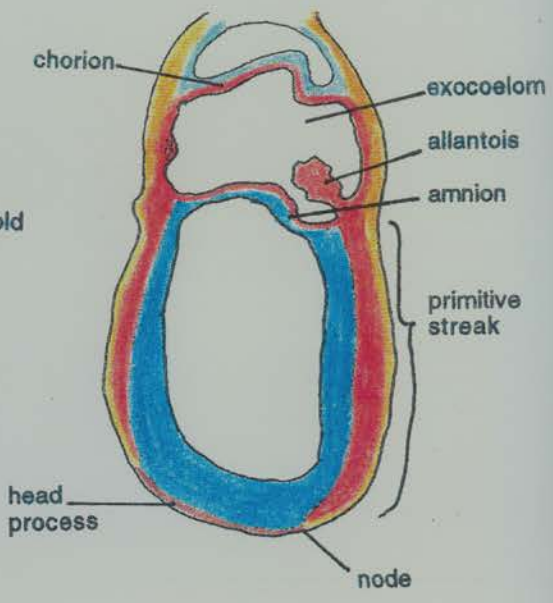
d) 6.5dpc



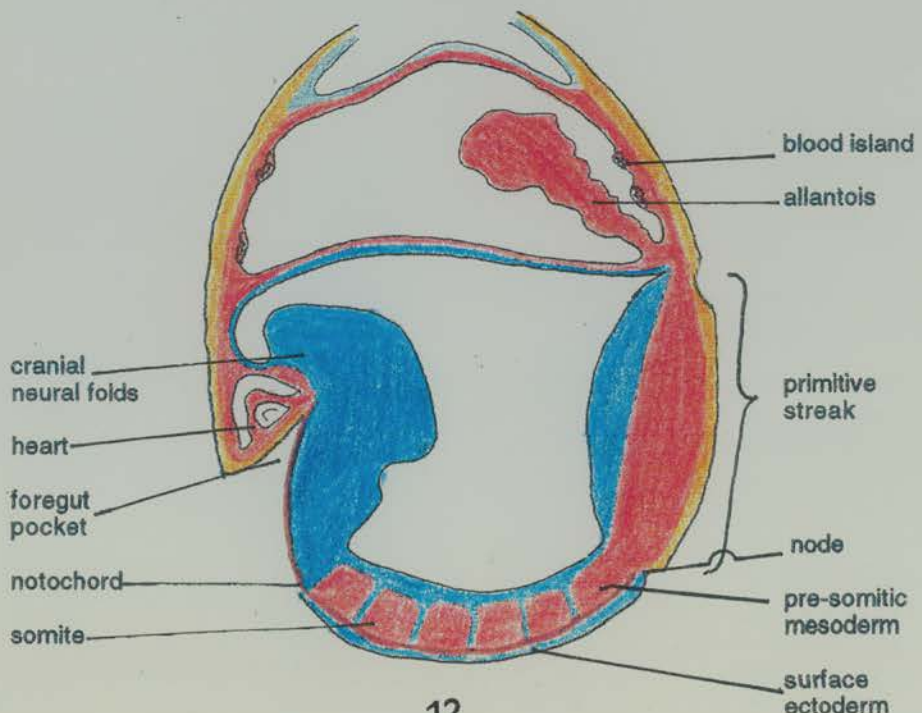
e) 7.0dpc



f) 7.5dpc



g) 8.5dpc



adhesive plaques and gap junctions (Batten and Haar 1979). At the same time there is extensive disruption of the basal lamina (Poelman 1981a) as the epiblast cells invaginate through the streak to produce mesoderm which lies between the visceral endoderm and epiblast layers. It has been suggested that the apparent movement of mesoderm cells away from the streak may be due to displacement of the streak away from the delaminated mesoderm caused by growth of the rapidly proliferating epiblast (Poelman 1981a; Tam and Meier 1982). Although the embryo may employ this mechanism for depositing mesoderm cells, observations made on clonal descendants of individual epiblast cells, labeled with horseradish peroxidase *in situ,* suggest that mesoderm cells also actively migrate away from the streak (Lawson et al. 1991). This agrees with cinematographic studies of gastrulating embryos (Nakatsuji et al. 1986).

Whilst those mesoderm cells which emerge from the lateral sides of the primitive streak spread anteriorly beneath the epiblast as two "wings" of tissue (Fig 1.1d), some of the newly formed mesodermal cells from the posterior part of the streak cross the boundary between the epiblast and the extraembryonic ectoderm (the inner layer of the extraembryonic region) to form the extraembryonic mesoderm (Tam and Meier 1982). Intercellular lacunae form throughout the extraembryonic mesoderm, principally in the posterior regions (Fig 1.1e). These lacunae coalesce and thus increase the volume of the extraembryonic mesoderm. This in turn pushes folds of tissue consisting of the proximal rim of the epiblast and adjacent extraembryonic mesoderm into the proamniotic cavity to form the amniotic folds. By the late primitive streak stage (Fig 1.1f), the expansion of the extraembryonic mesoderm separating the extraembryonic ectoderm from the epiblast and the fusion of the amniotic folds combine to produce a conceptus with three cavities. The amniotic cavity lies distally

(antimesometrially) and is surrounded by the embryonic part of the egg cylinder, but separated from the exocoelom by the amnion, which is itself formed from epiblast and extraembryonic mesoderm cells. The exocoelom is the internal cavity in the extraembryonic mesoderm formed from the coalescing lacunae. The extraembryonic mesoderm and visceral endoderm surrounding the exocoelom will form the yolk sac. The third cavity, the ectoplacental cavity, lies proximally (mesometrially) in the extraembryonic portion and is separated from the exocoelom by the chorion, which consists of extraembryonic mesoderm and extraembryonic ectoderm.

Whilst these three cavities are forming (Fig 1.1d-f), the primitive streak increases in length until the anterior end of the streak reaches the distal tip of the embryonic cylinder at about 7.5 dpc (Fig 1.1f). Soon after the primitive streak has reached its full length, the allantois emerges from the posterior extreme of the primitive streak. The allantois is an extraembryonic mesodermal tissue which grows across the exocoelom to fuse with the chorion. This establishes a connection, between the embryo and the future placenta and later incorporates the umbilical vessels.

During the period of primitive streak elongation, the head process mesoderm originates as a rostral protrusion from the primitive streak through several hiatuses in the primary endoderm (Poelman 1981b). Unlike the stellate-shaped mesoderm cells originating from the lateral edges of the primitive streak, the head process cells are single ciliated, conical shaped cells arrayed in fan-like condensations with their base containing the nucleus facing the ectoderm (Fujimoto and Yanagisawa 1983; Poelman 1981b). The head process originates from the anterior part of the primitive streak, which is a specialised region known as the archenteron or node (Fujimoto and Yanagisawa 1983). The underlying

basal lamina at this point is absent so that the overlying epiblast is continuous with the head process mesoderm. As the primitive endoderm has degenerated from this region, head process mesoderm cells are also exposed to the yolk cavity (Poelman 1981b). Morphological observations (Poelman 1981b), grafting experiments (Beddington 1981) and analysis of the descendants of labeled cells (Lawson et al. 1986; Lawson et al. 1991; Tam and Beddington 1992), all strongly suggest that the head process is the precursor of the prechordal plate and the notochord. Mesoderm delaminating from the primitive streak behind the node region moves laterally and anteriorly to form a continuous intermediate layer underlying what can now be called the definitive ectoderm and overlying the endoderm.

Cell fate studies in chick and mouse show that the definitive endoderm does not arise from the primary hypoblast or primitive endoderm (Gardner and Rossant 1979; Sanders et al. 1978), but arises *de novo* from ectoderm or epiblast at gastrulation (Lawson et al. 1987; Lawson et al. 1991; Poelman 1981b; Rosenquist 1971; Rosenquist 1972; Schoenwolf et al. 1992). Single cell marking studies in the mouse have shown that at the early primitive streak stage, there is a subpopulation of cells in the axial ectoderm overlying the anterior of the streak that contributes to the gut endoderm (Lawson et al. 1987). A second phase of definitive endoderm formation occurs at the mid-streak stage, when the head process appears to contribute to definitive endoderm by displacing primary endoderm cells in the midline (Poelman 1981b). Cell studies in chick and mouse have shown that cells in the early node can contribute to gut endoderm as well as to notochord and the floorplate of the neural tube (Lawson et al. 1991; Schoenwolf et al. 1992; Selleck and Stern 1991). A forkhead domain gene, *hepatocyte nuclear factor 3β (HNF3β)* is expressed in the earliest

stage of gastrulation in a group of cells that include the progenitors of the definitive endoderm (Ang et al. 1993; Monaghan et al. 1993). Slightly later, at the late primitive streak stage, a related gene, *HNF3α* begins to be expressed in the endoderm (Ang et al. 1993; Monaghan et al. 1993) and along with *HNF3β* continues to be expressed similarly thoughout the subsequent differentiation of the gut. (Ang et al. 1993; Monaghan et al. 1993; Sasaki and Hogan 1993). These transcription factors show great homology to the *Drosophila forkhead* gene which is required for normal development of terminal regions, including foregut and hindgut (Weigel et al. 1989), and therefore the mechanisms for specifying the developing gut may be conserved through evolution.

Organogenesis

This section gives a brief description of early organogenesis, focusing in particular on the function of the notochord, which is disrupted in *Brachyury* mutant embryos (see later and chapter 4).

Organogenesis in the anterior part of the embryo begins before the process of gastrulation is completed posteriorly (Fig 1.1e).

Somitogenesis

The paraxial mesoderm starts to segment as neurulation is occurring (see below) at about 8.0 dpc. This segmentation occurs in a craniocaudal sequence to form repeated paired condensations, the somites. Even before overt segmentation occurs, Meier and co-workers have observed in many different vertebrate embryos (using scanning electron microscopy) that a metameric arrangement of cells exist in the presomitic mesoderm (see Jacobson and Meier 1986). These potential somites, known as somitomeres, are formed from concentric circles of mesenchymal cells and

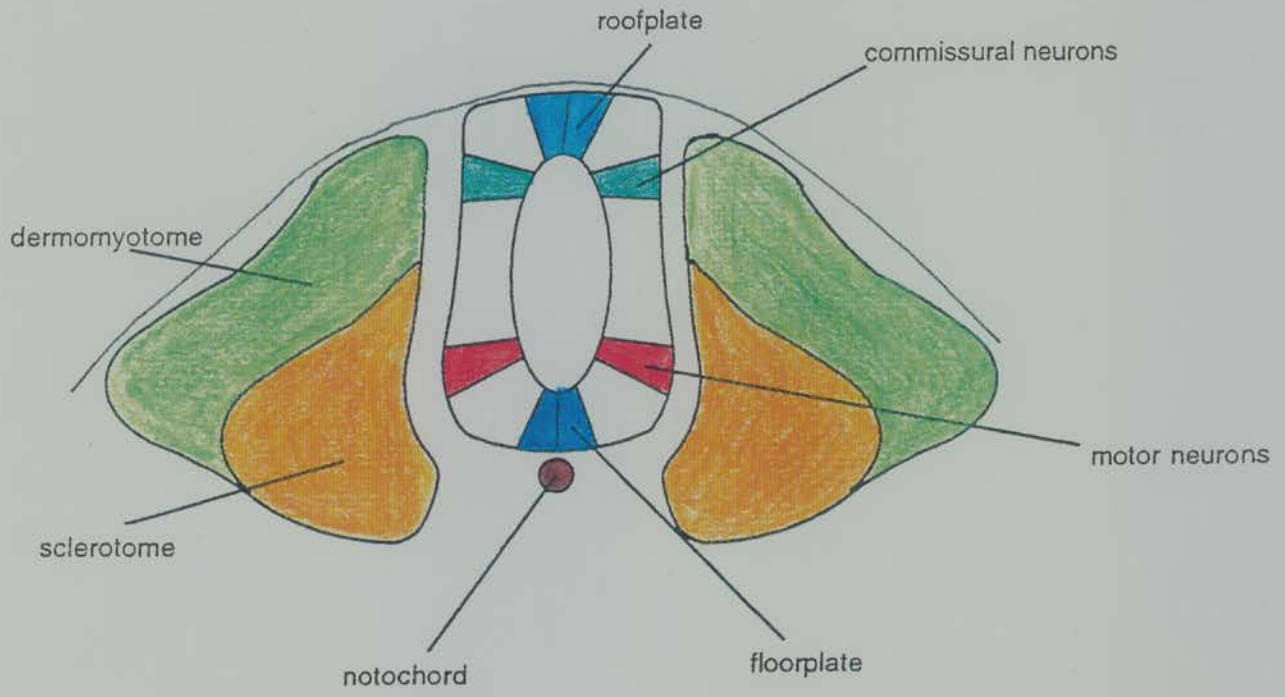
are first observable in the lateral mesoderm wings of the mouse egg cylinder (Tam and Meier 1982). Similar to somites, somitomeres are also formed in a craniocaudal succession. The first seven somitomeres visible in the lateral mesodermal wings have differentiated into cranial mesenchyme by the time the 8th somitomere becomes the first somite (Meier and Tam 1982). It is not known how the somitomere is formed, nor how its configuration is maintained prior to somite segmentation, although primitive streak regression has been implicated (Bellairs and Veini 1984). Nor is it clear whether somitomeres are truly the immediate precursors of somites. It is known, however, that the somites are dependent on the adjacent neural tube and notochord for maintenance of somitic pattern (Bellairs 1979; Packard Jr and Jacobson 1976). Chick somites rapidly lose their structural integrity when separated from the neural tube and notochord (Lipton and Jacobson 1974; Stern and Bellairs 1984) and are unable to differentiate further (Teillet and Le Douarin 1983).

The somites differentiate into a ventromedial sclerotome and dorsolateral dermomyotome (reviewed by Keynes and Stern 1988; Fig 1.2). The sclerotome cells lose their epithelial structure first. These form loosely arranged mesenchyme which disperses and then later recondenses around the notochord and neural tube to form the vertebrae. The dorsolateral dermomyotome cells, on the other hand, retain their epithelial nature longer and subsequently develop into two structures, an outer dermatome cell layer and an inner myotome cell layer. The dermatome cells differentiate into the connective tissue of the dermis in such a way that each somite contributes to a defined area of skin whilst the myotome cells delaminate and differentiate into myoblasts which fuse to form striated muscle fibres. In the chick embryo, initially both the lateral and medial somite halves are capable of generating limb or trunk muscles (Ordahl and

FIGURE 1.2

Diagram of the dorsoventral axis of the neural tube and somites.

In the neural tube, motor neurons (red) differentiate ventrally and are separated at the ventral midline by the floor plate (dark blue).
Commissural neurons (dark green) differentiate dorsally, whilst the roof plate (light blue) forms at the dorsal midline.
In the somite, the ventromedial compartment differentiates into the sclerotome (orange), whilst the dorsolateral part forms the dermomyotome (light green)
The notochord (brown) is positioned ventral to the floor plate.


roofplate
commissural neurons
dermomyotome
motor neurons
sclerotome
notochord
floorplate

Le Douarin 1992). However, two populations of myogenic precursors have been identified in more mature somites: the axial musculature is derived from the medial part of the somite whilst the limb muscles develop from the lateral portion of the dermomyotome adjacent to the limb bud. (Chevallier et al. 1977; Ordahl and Le Douarin 1992).

Experiments manipulating chick somites or notochord *in vivo* and *in vitro* are consistent with cell fate in the early somite being determined by the interaction of somitic cells with adjacent tissues (Aoyama and Asamoto 1988; Ordahl and Le Douarin 1992; Pourquié et al. 1993; Rong et al. 1992). For example, transplantation experiments reversing the last three somites in chick embryos have demonstrated that position in the dorsoventral axis determines the commitment of sclerotome and dermomyotome (Aoyama and Asamoto 1988). The dorsoventral axis appears to be determined about three hours after somite formation because when the somites were rotated about this axis, only the most rostral of these three somites gave rise to abnormally situated dorsal sclerotome (Aoyama and Asamoto 1988). It has also been observed that a signal arising from the neural tube/ notochord complex is required for survival and differentiation of axial myotome, whereas the somitic derived myoblasts of the limb bud survive, migrate and differentiate in the complete absence of these axial organs (Goulding et al. 1994; Rong et al. 1992). In contrast, implanting an extra notochord adjacent to somites causes the loss of dermomyotome from regions adjacent to the graft and the development of ectopic vertebrae (Goulding et al. 1994; Pourquié et al. 1993). The neural tube and skin may also provide signals that are necessary for the development of the myotome, since myogenesis is increased in isolated somites when these tissues are co-cultured (Kenny-Mobbs and Thorogood 1987).

Neurogenesis

At approximately 8.0 dpc, the tissues at the anterior end of the embryo start to fold into the amniotic cavity, to produce the headfolds (Fig 1.1e). Soon afterwards the ectoderm lying along the midline anteroposterior axis thickens to form the neural plate. This subsequently rolls up, to give neural folds which then fuse to form the neural tube, the precursor to the brain and spinal cord. Initially the neural folds fuse above the 4th or 5th somite and then the neural tube closes in both rostral and caudal directions in a zipper-like fashion. Differentiation within the neural tube is a highly ordered process such that specific cells are found at distinct dorsoventral positions (Fig 1.2). Floor plate cells occupy the ventral midline of the neural tube and motor neurons appear in a ventrolateral position. Cells in the dorsal neural tube give rise to sensory relay neurons and neural crest cells whilst those at the dorsal midline, form roof plate cells.

The organisation of cell types along the dorsoventral axis of the neural tube appears to be controlled by signals from the ventral midline. In chick embryos, a localised inductive signal from the notochord is responsible for differentiation of floor plate cells at the ventral midline of the neural tube. Thus, in the chick embryo, if an additional notochord is ectopically grafted such that it is situated beside dorsolateral neurectoderm a second, ectopic floorplate and the expression of ventral markers and additional motor neurons are induced in this region (Goulding et al. 1993; Placzek et al. 1990; van Straaten et al. 1988; Yamada et al. 1991). At the same time such ectopically grafted notochord suppresses the expression of dorsal markers (Goulding et al. 1993; Placzek et al. 1990; van Straaten et al. 1988; Yamada et al. 1991, Ericson, 1992). Conversely, surgical ablation of chick notochord or U.V irradiation of *Xenopus* notochord causes

a neural tube to form which lacks a floor plate (Clarke et al. 1991; Placzek et al. 1990; van Straaten and Hekking 1991; Yamada et al. 1991) and prevents the differentiation of motor neurons and other ventral neural types.

Analysis of *no tail* zebrafish mutants have shown that these embryos lack a properly differentiated notochord, but still form a floor plate (except at the most caudal extremity), suggesting that, at least in this species, although notochord precursor cells may be required for induction of the floor plate, a differentiated notochord is not (Halpern et al. 1993). The chick notochord appears to lose its floor plate-inducing activity at the time that the floor plate cells, themselves, acquire inducing activities. Once induced the chick floor plate is able to effect dorsoventral patterning of the neural tube regardless of whether or not a notochord is still present. However the specification of cell fate and dorsoventral patterning of cell types begins at the neural plate stage and *in vitro* studies suggest that the notochord does provide the initial source of signals responsible for floor plate and motor neuron differentiation. There are at least two distinct signals, one contact mediated and the other diffusible, responsible for this differentiation (Placzek et al. 1993; Yamada et al. 1993). However it seems that both signals are produced by the notochord and floorplate suggesting that there is some redundancy in the system.

Recently four groups have cloned members of a family of extracellular signaling molecules which are homologues of the *Drosophila* segment polarity gene, *hedgehog (hh* : Echalard et al. 1993; Krauss et al. 1993; Riddle et al. 1993; Roelink et al. 1994). The expression pattern, both temporally and spatially, of *sonic hedgehog (shh)* in mouse, rat, chick and zebrafish is consistent with this gene acting as a notochord-derived and then floor-plate derived inducing factor (Echalard et al. 1993; Krauss et al. 1993; Roelink et al. 1994). *shh* is detected initially in the node and

notochord and then extends to the floor plate of the spinal cord and hindbrain. In the midbrain and forebrain, transcripts are also ventrally restricted, but in the former they extend beyond the floorplate region and in the latter there is no morphologically distinguishable floorplate. The early expression pattern of *shh* is similar to that of the mouse *HNF3β* and zebrafish *axial* both members of the forkhead domain transcription factor family (Monaghan et al. 1993; Sasaki and Hogan 1993; Strahle et al. 1993). Sasaki and Hogan (1994), showed that ectopic expression of *HNF3β* in transgenic mice activates several floor plate markers. As *HNF3β* expression precedes that of *shh*, it has been suggested that the former may regulate initiation of the expression of the latter (Smith 1994).

To further assess the role of *shh* as the putative signal for floor plate induction, Krauss et al (1993) examined the expression of *shh* in *no tail* and *cyclops* mutant zebrafish. In *no tail* embryos, *shh* is expressed in notochordal precursor cells and the floorplate. In contrast, in *cyclops* zebrafish mutants, which have a normal notochord but lack a floor plate, *shh* is expressed in the notochord but not in the ventral neural tube. This is consistent with the *cyclops* mutation being caused by a cell autonomous inability of ventral spinal cord cells to respond to a floor-plate inducing signal. Ectopic expression of *shh* in the developing brain and spinal cord in mouse, zebrafish and *Xenopus* embryos resulted in the activation of floorplate specific genes in inappropriate regions of the brain or spinal cord (Echalard et al. 1993; Krauss et al. 1993; Roelink et al. 1994). Also co-culture of neural tube explants with cells transfected with *shh* cDNA resulted in induction of floorplate specific gene expression in the former (Roelink et al. 1994).

Although the experiments described above demonstrate that *shh* can induce the floor-plate, they do not prove that this is their role in normal

development. In the chick, the inducing activity of the floorplate correlates strongly with *shh* expression in this structure. In contrast, however, the floor plate-inducing activity of the notochord is lost at the ten somite stage, whilst *shh* expression continues in the notochord until late development. Thus, the notochord derived signal that induces floorplate induction must include other factors which are temporally regulated and may be contact dependent.

There is also growing evidence that a dorsal signal as well as a ventral one is required to pattern the neural tube. It is known that the neural crest is not a presegregated population and the fate of neural crest and dorsal neural tube cells are intimately interlinked (Bronner-Fraser and Fraser 1988; Bronner-Fraser and Fraser 1989). Ectopically situated notochord grafts do not suppress the formation of neural crest cells or commissural axons (Artinger and Bronner-Fraser 1992), suggesting that such grafts are unable to completely ventralise the neural tube. The isolation of a novel member of the TGF-ß family, *dorsalin-1*, which can act as a potent dorsalising signal in the developing spinal cord of the chick (Bassler et al. 1993) provides further evidence that a dorsal signal is also necessary to correctly pattern the neural tube. The source of this dorsal signal is not known, but anatomical considerations would suggest that such a signal could arise from the overlying surface ectoderm or paraxial mesoderm.

Fate maps of the mouse epiblast

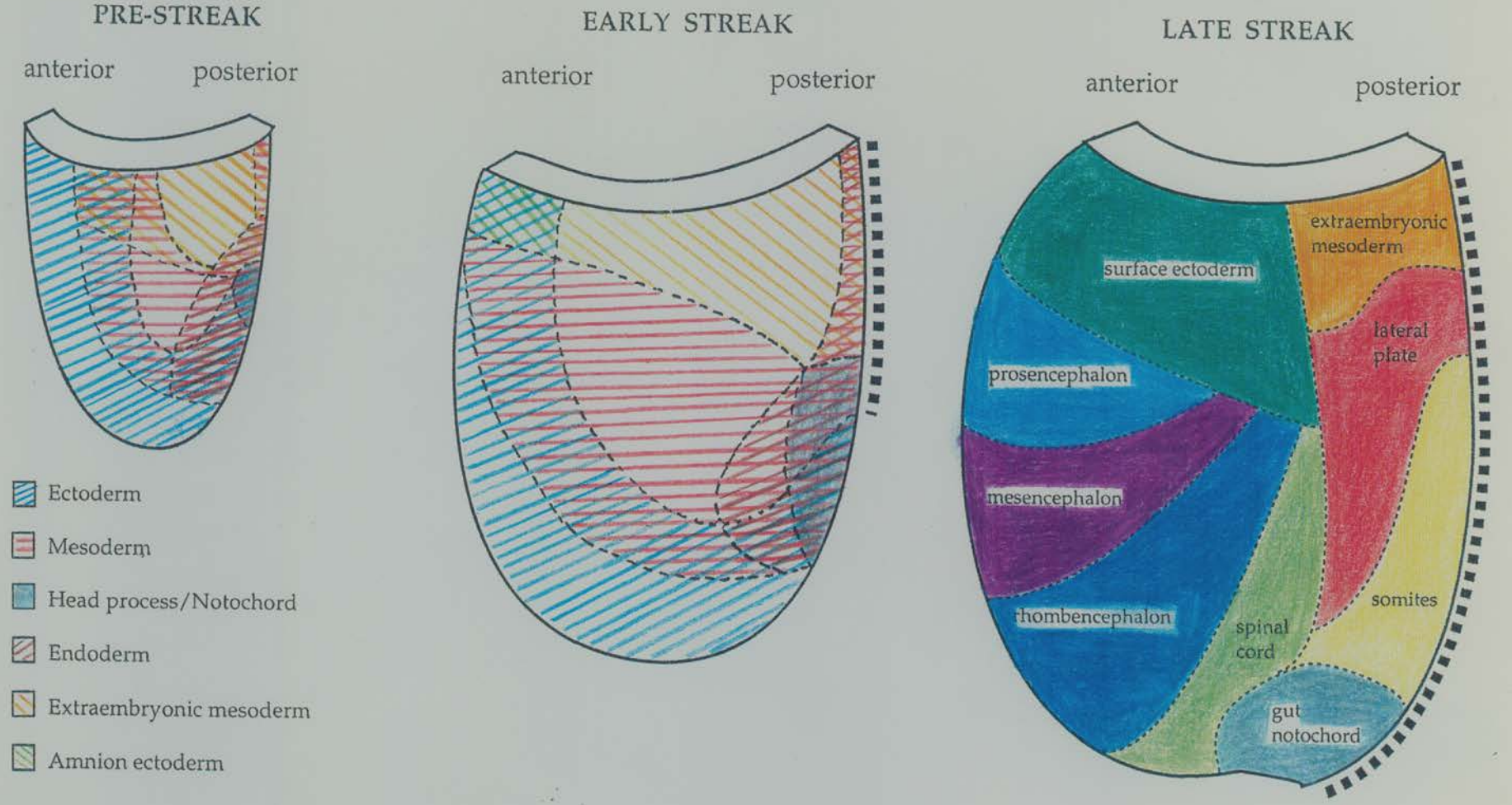
If the epiblast is the sole source of all the fetal tissues, an amazing diversification of cell fate must occur over a comparatively short time. Therefore, when do different cell lineages become committed to different compartments and what determines the fate for any given cell?

Figure 1.3

Fate map of the epiblast of the pre, early and late primitive streak stages.

The pre and early streak fate maps shows the derivation of germ layers up to the mid/late streak and neural plate stages respectively as demonstrated by clonal analysis of the work of Lawson and her colleagues (Lawson et al 1991; Lawson & Pederson 1992). Adapted from Lawson & Pederson (1992)
The late streak stage is based on orthotopic grafting experiments (Beddington 1981, 1982 1983b, Beddington & Tam 1978 and Tam 1989) and clonal analysis (Lawson et al 1991; Lawson & Pederson 1992) Adapted from R. Beddington

FATE MAPS OF EPIBLAST BEFORE AND DURING GASTRULATION



Clonal analysis of individual prestreak and early primitive streak epiblast cells labeled with horseradish peroxidase *in situ* has shown that areas of the mouse epiblast differentiate during gastrulation in a reproducible and coordinated way such that a fate map of the presumptive germ layers and extraembryonic mesoderm can be drawn (Lawson et al. 1991; Lawson and Pederson 1992: Fig 1.3). These movements occur in the presence of extensive, but not indiscriminate, cell mixing in the epiblast such that descendants of a single progenitor may be spread widely and also be present in the different germ layers (Lawson et al. 1991), thus demonstrating that individual epiblast cells are pluripotent. This study also showed that cells that are situated at the anterior part of the streak in early streak stage embryos (Fig 1.1c) have descendants a day later remaining in the anterior part of the streak and the node as well as in more anterior located mesoderm and endoderm (Lawson et al. 1991). This suggests that a subpopulation of cells in the anterior streak maintain their position until late in gastrulation and perhaps comprise a stem cell population whose progeny will populate successively more posterior regions of the embryo. DiI labeling of the node has also indicated that there is a stem cell population in this region (Beddington 1994).

The fate of small groups of epiblast cells from mid to late gastrulation has also been traced by labelling cells with ^{3}H- thymidine or wheat germ agglutinin - gold conjugate and transplanting them orthotopically. (Beddington 1981; Beddington 1982; Copp et al. 1986; Tam and Beddington 1987; Tam 1989; Fig 1.3). Potency at this stage was also tested by transferring groups of cells to heterotopic sites. The grafts were able to contribute to some, but not all tissue types outside their normal fate and did not form structures inappropriate to their new position, thereby demonstrating considerable potency of the epiblast at mid-gastrulation

(Beddington 1981; Beddington 1982). In contrast, full thickness explants containing either both epiblast and visceral endoderm from midstreak stage embryos or epiblast, mesoderm and endoderm from late primitive streak stage embryos, show pronounced regional autonomy *in vitro* (Snow 1981). The complementary results obtained in these two sets of experiments suggest that tissue interactions maintained in the full thickness explants are important in stabilising normal cell fate.

Whilst grafting and transplantation experiments suggest that a gradual regional restriction of potency does occur during gastrulation, prospective fate and time of commitment of individual cells cannot necessarily be inferred from experiments in which groups of cells are manipulated. It is theoretically possible that cell type specification occurs earlier and that gastrulation involves either sorting out or selection of previously specified cells, as has recently been suggested for the chick embryo (Stern and Canning 1990). However, the clonal analysis of early gastrulation epiblast cells done by Lawson and her colleagues demonstrates that single epiblast cells are pluripotent and therefore suggests that this is not the case (Lawson et al. 1991; Lawson and Pederson 1992).

The pluripotent nature of the epiblast

Experiments in which murine epiblast was transferred to an ectopic site, where it forms a teratoma or teratocarcinoma demonstrate the potency of the epiblast to form derivatives of all three germ layers. This capacity is present during gastrulation as well as in pre-primitive streak embryos (Solter et al. 1979; Solter et al. 1980; Solter et al. 1981; Stevens 1970). Before and during the early stages of gastrulation, epiblast transplanted to a well vascularised site in an adult syngeneic mouse (for example beneath

either the testicular or renal capsule) will form transplantable teratocarcinomas containing embryonal carcinoma (EC) stem cells. These tumours, as well as giving rise to neoplastic stem cells, also contain a variety of differentiated tissues from all three germ layers. No regional differences in the histogenic potential of the epiblast have been demonstrated (Beddington 1983a). Embryos transplanted after the early stages of gastrulation produce only benign differentiated teratomas lacking EC cells but still containing a wide range of differentiated tissues representative of all three germ layers. About half a day later in development, when the neural folds have elevated and the foregut indentation is evident, the epiblast has lost its capacity to form endoderm derivatives in ectopic sites, but is still able to form mesoderm. These results indicate that the epiblast as a whole still retains considerable potency until late in gastrulation (Damjanov et al. 1981; Damjanov and Solter 1974).

Genetic control of gastrulation

Recognising developmental genes from mutant phenotypes and determining their function

The problem still remains as to how one finds the molecular and cellular cues involved in pattern formation. Table 1.1 lists different methods available for finding murine genes which have a putative role in development. However, the relative inaccessibility and cellular complexity of the mouse, means that any screening programme for informative developmental mutants is difficult. Despite this, there are now a large number of cloned genes which are known to be expressed during murine development. Although *in situ* hybridisation and antibody studies may show interesting stage- and tissue-specific expression patterns, the

Table 1.1: Methods for identifying genes which control murine development

Strategy	Method	Example	References
1. Homology	i. Genes implicated in other developmental systems e.g. growth factors; transcription factors	*Hox, Pax,* FGF families	see Kessel & Gruss (1990)
	ii. Genes involved in cellular proliferation and tumorigenesis i.e. proto-oncogenes	*Wnt* family	see McMahon (1992); Nusse and Varmus (1992)
2. Random mutation	i. Radiation induced	kr, Mi^{Or}, $T^{c\text{-}2H}$	see Lyon & Searle(1989)
	ii. Chemical induced	T^{kt1},Pt	see Lyon & Searle(1989)
	iii. By replication defective retrovirus infected into ES cells, or into preimplantation or postimplantation embryos	Mov-13 (α1-collagen); 413-d (nodal)	Jaenisch et al (1983);Conlon et al (1991); Zhou et al (1993)
	iv. Enhancer/ gene trap using weak promoter/splice acceptor with reporter gene: screening for differential gene expression	GT4-2(zinc finger protein) fug1 (RNA1)	Gossler et al (1989); Skarnes et al (1992); DeGregori et al (1994)
	v. Any DNA via pronuclear injection	Id^{TgHd};Hβ58	Woychik et al (1985); Radice et al (1991)
3. Generation of novel germ-line mutations in cloned genes	i. Gain of function/ dominant negative transgenes	*Hoxa-7; Hoxd-4*	Balling et al (1989); Kessel et al (1990); Lufkin et al (1992)
	ii. Gene targeting/ loss of function	*Hoxa-3; Wnt-3a*	Chisaka & Capecchi (1991);Takada et al (1993)
	iii. Antisense RNA	inhibition of maternal tPA	Richards et al (1993)
4. Molecular definition of mutant phenotypes	i. association with previously cloned genes	*Pax-1(un); Pax-3(Sp); Pax-6(Sey); c-kit(W)*	see Gruss & Walther (1992); Besmer et al (1993)
	ii. Physical mapping starting from closely linked marker	*T, Sry*	Herrmann et al (1990);Gubbay et al (1990)
5. Identification of novel tissue or stage specific genes	i. Differential or subtractive screening of cDNA libraries	*Glut1; Glut3*	Smith & Gridley (1992)

function of these genes during development remains unknown. As discussed earlier, gene defects which cause inappropriate differentiation and therefore produce a mutant phenotype are likely to reveal which genes control tissue diversification. Null mutants, produced by altering the coding sequence of a gene using homologous recombination in embryonic stem (ES) cells, may demonstrate disturbances in pattern or form, but may not reveal the specific mode or exact site of action. The resultant phenotype may be less severe than expected as redundancy within gene families present in the mammalian genome may serve to decrease the frequency of recognizably abnormal phenotypes. The phenotype of the *Mov-13* mutant illustrates the potential for one gene to take the place of another during development. In these mutants, a retrovirus has interrupted the α1(I) collagen gene (Schnieke et al. 1983; Lohler et al. 1984) causing complete absence of type I collagen synthesis (except in odontoblasts, where the gene may be reactivated (Kratochwil et al 1989)) In normal embryos, type I collagen is first detected just after gastrulation at 8.5dpc and is present in the cranial, cardiac and somitic mesoderm as well as some extraembryonic membranes, including the amnion, chorion and visceral yolk sac whilst later in development it is produced by most mesodermal tissues and is abundant in various epithelial- mesenchymal organs (Leivo et al. 1983). However, the *Mov-13* mutant dies surprising late (between 12 and 14 days gestation),due to haemorrhage caused by weakness in the major blood vessels. Not only is the time of lethality later than expected but the epithelial branching morphogenesis in organs such as the salivary gland is entirely normal despite the absence of type I collagen (Kratochwil et al 1986). The probable explanation for this is that other collagens, particularly type III can substitute for the type I deficiency.

It is likely that evolution may have selected for those genes which are particularly efficient in coordinating specific events within cell populations (for example growth factors) and these molecules may be used repeatedly in embryogenesis for different purposes. If such genes are required for early embryonic survival, some of their later functions may be inaccessible to analysis by mutation unless conditional mutants can be made. For example, *Evx-1* null mutants die shortly after implantation but prior to gastrulation as they fail to undergo further differentiation or morphogenesis (Spyropoulos and Capecchi 1994). Therefore these mutants cannot be used to assess the function (if any) of *Evx-1* expression in the embryonic ectoderm around the primitive streak, the posterior neurectoderm and the limb buds (Bastian and Gruss. 1990; Dush and Martin 1992). Thus, studying mutant embryonic phenotypes is only a start in trying to unravel the primary function of a gene from its secondary consequences and therefore the following problem still remains: how does one ascertain the primary function of a gene from studying its aberrant expression in an abnormal embryo?

Chimaeric/ Mosaic analysis

The mixing of wildtype and mutant cells can reveal information about the nature of a phenotype which is not apparent in intact mutant embryos. Firstly, it should be possible to determine whether a gene product acts cell autonomously or can be rescued by neighbouring wildtype cells, and this in turn may help resolve gene function at a cellular level. Secondly, if the mutant cells are rescued either completely or partially by the presence of wildtype ones, it may be easier to identify which tissue is primarily affected by the mutation since some chimaeric / mosaic embryos should survive beyond the normal stage of lethality. It is also possible that abnormalities

in certain tissues may correlate with particular mutant population patterns. In the mouse, specific selection against mutant cells in certain tissues has been observed. For example, individual androgenetic blastomeres, marked with a transgenic β-globin nuclear marker, aggregated with eight cell embryos only rarely contributed to the embryo proper or to the extraembryonic mesoderm and endoderm in the early postimplantation conceptus (Thomson and Solter 1988a). In contrast, single parthenogenetic and gynogenetic blastomeres did contribute to the embryo and extraembryonic mesoderm, but were only rarely observed in the extraembryonic endoderm. Similar results have been obtained with aggregating whole parthenogenetic eight cell embryos with wild-type ones except that there was no apparent exclusion from the extraembryonic ectoderm (Clarke et al. 1988). This discrepancy may reflect the use of double sized embryos in the latter study. A separate example of selection against mutant cells has been observed in adult mice chimaeric for trisomy 16. In this case, the abnormal cells are excluded from the blood, spleen, thymus and bone marrow (Cox et al. 1984).

In *Drosophila*, mosaic analysis has been highly successful in determining not only whether a gene functions cell autonomously, but also in defining those regional subsets of gene expression that are essential for normal function. A good example of how important it is to combine molecular, genetic and mosaic approaches is illustrated by aspects of the development of the *Drosophila* compound eye (reviewed by Cagan 1993; Rubin 1991).

In a normal ommatidium, the eight photoreceptors differentiate in a fixed sequence (see Fig 1.4a). There is no fixed lineage relationship within an ommatidium (see Tomlinson 1988). Therefore, this pattern of developmental fate is not due to ancestral directives, but instead is

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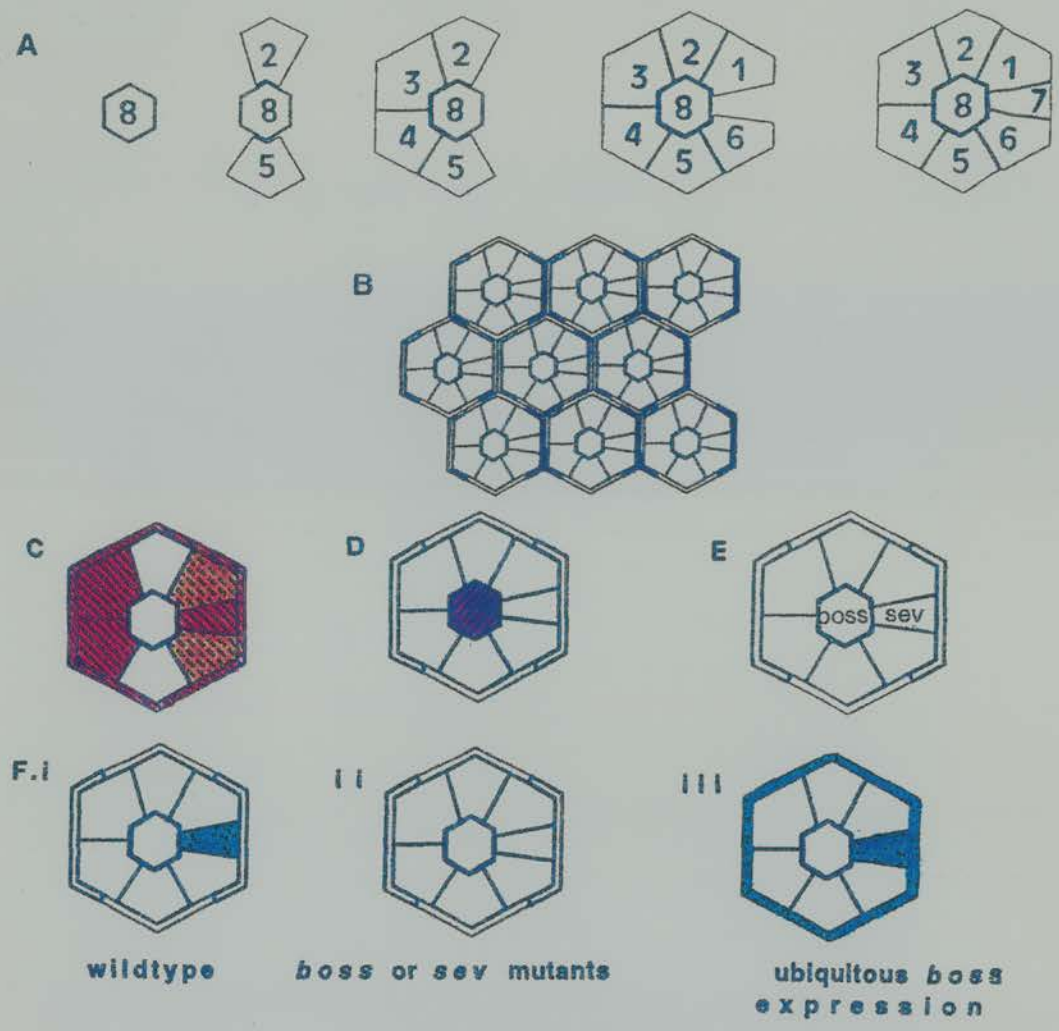
Figure 1.4

Summary of ommatidial development and the expression patterns and genetic data used to infer the role of *sevenless (sev)* and *bride of sevenless (boss)*

A. Sequence of recruitment of the eight photoreceptors in a developing ommatidium. Each group of photoreceptors is surrounded by 4 lens secreting cone cells (not shown)
B. Schematic representation of part of the normal *Drosophila* eye showing several ommatatidia grouped together
C. Expression of *sev* in the ommatidium. High expression levels are observed in R3, R4, R7 and the cone cells; low expression levels are observed in R1 and R6.
D. *boss* transcripts are only observed in R8.
E. The cells in which the function of *sev* and *boss* is required, as determined by mosaic analysis
F. Cells which adopt an R7-like fate in the indicated genotypes are shown by the hatched shading
i. Normal position and fate of R7 (that of a photoreceptor) in the wildtype ommatidium
ii. In *boss* or *sev* mutants R7 becomes a lens secreting cone cell, rather than a photoreceptor
iii. Ubiquitous boss expression, using *hsp70-boss* fusion genes causes the cone cells to differentiate as R7 cells.
G. Rescue of a subset of ommatidia in mutant larvae lacking the wildtype *sevenless* gene by ubiquitously expressing *sev* by linking it to the heat shock promoter, *hsp70*.

Adapted from Rubin 1991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sev
F.i
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i i i
wildtype
boss or sev mutants
ubiquitous boss expression
G
high expression levels of R7
low expression levels of R7
expression of R8
Cells that adopt an R7- cell fate in the indicated genotypes

determined by the environment or sequential inductive interactions. In at least two mutants, *sevenless (sev)* and *bride of sevenless (boss)*, the cell that normally differentiates into the last photoreceptor (R7), fails to do so and instead forms a lens-secreting cell (Reinke and Zipursky 1988; Tomlinson and Ready 1986). However, although they produce the same phenotype, *sev* and *boss* complement each other, and therefore must have different functions. Thus, even an apparently simple abnormal phenotype (change of fate of one cell per ommatidium) does not resolve the function of a gene. Nor does the expression pattern of *sevenless* explain the very specific phenotype of the mutant (Fig 1.4; Tomlinson, 1987). The *sevenless* protein is expressed in all photoreceptors except R2, R5 and R8. It is abundant in R3, R4 and R7 at their junctional contacts with R8, and is more sustained in R7. *sevenless* is also strongly expressed in the surrounding lens-secreting cone cells. It is however possible to rescue a subset of ommatidia in mutant larvae lacking the wildtype *sevenless* gene by ubiquitously expressing *sev* by linking it to the heat shock promoter, *hsp70*. The rescued ommatidia have to be at the appropriate stage of differentiation when the *sevenless* product is induced by heat shock (Basler, 1989; Bowtell, 1989; Fig 1.4). However, this widespread expression of *sevenless* does not result in any other detectable abnormality in the retina, or in any other structure, regardless of when or how long ectopic expression is induced. This not only supports the argument that widespread expression of *sevenless* is redundant (and cannot change the fate of other photoreceptor cells), but also suggests that *sevenless* is only required during a limited sensitive phase of ommatidia development. In contrast, ubiquitous *boss* expression, achieved by fusing *boss* cDNA to an inducible promoter, results in transformation of developing cone cells into R7 neurons (van Vactor et al. 1991),

demonstrating that cone cells are competent to respond to the *boss* inductive cue. Cone cells do not contact R8 early in their development and therefore are not normally in contact with the *boss* inductive ligand.

Mosaic analysis has demonstrated that *sevenless* acts cell autonomously; R7 having an absolute requirement for this gene product (Tomlinson, 1987; Fig 1.4). In contrast, *boss* is non- autonomous within a single ommatidium and inappropriate differentiation of R7 only occurs when the adjacent R8 is lacking the *boss* gene product (Reinke, 1988; Fig1.4). Therefore, mosaic analysis suggests a mechanism whereby *sevenless* codes for a receptor, as its DNA sequence suggests, which must interact with a ligand (the *boss* product) in order for R7 to form a photoreceptor. The fact that the sevenless product is not required elsewhere in the ommatidium, especially in R3 and R4, which are adjacent to R8, suggests that *sevenless* is a redundant component of other aspects of pattern formation.

The power of chimaeric analysis in the mouse has been demonstrated in the analysis of two mutant mouse strains. Both the *Dominant White Spotting (W)* and *Steel (Sl)* loci result in deficiencies in three cell systems: the pigmentary system, the germ cell and the haematopoietic system in the embryo as well as the adult animal (see Russell 1979; Silvers 1979). There are several alleles at each of these loci which have differing degrees of severity in the heterozygous and homozygous state (see Besmer et al. 1993). During normal development, melanoblasts arise from the neural crest and migrate to the periphery where they enter the epidermis, colonise hair follicles and postnatally differentiate to become pigmented melanocytes (Silvers 1979). Primordial germ cells are formed from the cells of the epiblast that move through the posterior primitive streak and first appear in the allantois at 7.5 dpc

(Ginsburg et al. 1990). They then move into the hindgut and laterally to the genital ridges where they arrive by 11.5 dpc. In haematopoesis, *W* and *Sl* mutations affect cells within the stem cell hierarchy responsible for distinct cell populations in the erythroid cell lineage and mast cells both during development and in the adult animal (see Russell 1979). Prior to the molecular characterisation of the mutations, a chimaeric analysis of the two mutations demonstrated that *W* mutations are cell autonomous while *Sl* mutations affect the microenvironment (McCulloch et al. 1964; McCulloch et al. 1965). The *W/Sl* experimental model also demonstrates that useful information about cell autonomy can be gained from mixing genotypes in organ culture, for example normal skin can rescue homozygous *Steel* melanoblasts (Mayer 1973). Molecular characterisation of these mutations has demonstrated that the *W* locus codes for the *c-kit* receptor (Chabot et al. 1988; Geissler et al. 1988; Nocka et al. 1989), whilst *Sl* encodes its ligand (Copeland et al. 1990; Huang et al. 1990; Williams et al. 1990; Zsebo et al. 1990). The defects in *W* and *Sl* mice are consistent with a role of the *c-kit* receptor system in facilitating cell proliferation and survival of precursor cells as well as promoting cell migration and other functions in differentiated cells (see P. Besmer et al. 1993).

Embryonic stem (ES) cells, which are usually derived from the inner cell mass of blastocysts (Evans and Kaufman 1981; Martin 1981), can act as an ideal vehicle to perform a chimaeric analysis for several reasons. First, they have been shown to colonize all tissues of the conceptus (Beddington and Robertson 1989). Second, ES cells can either have specific mutations introduced *in vitro* (for example *c-abl* :Schwartzberg et al. 1989; *Wnt-3a*: Takada et al, 1993) or they can be derived directly from mutant embryos (for example t^{w5}/t^{w5}: Magnuson et al. 1982). Third, the ES cells can be genotyped prior to creating chimaeras. Fourth, it is possible to

mark ES cells with an independent cell marker so that the mutant cells can be followed at a single cell level (for example Beddington 1989).

One drawback of chimaeric analysis in the mouse is that, except for certain cell types e.g. early haemopoietic cells (Weissman et al. 1978) and neural crest cells (Jaenisch 1985), at present it is only possible to create chimaeras during the preimplantation period. This means that mutant cells are present in every fetal tissue, whereas in some cases it might be easier to assess gene function if only certain lineages were populated with mutant cells.

Identifying of other genes involved in the same pathway

A complementary approach to analysing the function of a gene, is to assess alterations in the expression pattern of other genes putatively involved in the same developmental process affected by a particular mutation. This will not only extend the descriptive studies of a phenotype, it may also indicate where the mutant gene lies in the hierarchy of gene expression necessary for pattern formation. Any gene acting downstream of the gene of interest will have altered expression patterns. Conversely, the expression domain of any gene acting upstream or independently of the mutated gene will remain unchanged. For example in *Drosophila* two segment polarity genes *wingless (wg)* and *engrailed (en)* are transcribed in fourteen stripes which correspond to the posterior and anterior margins of each parasegment primordium respectively (Baker 1988; Lawrence et al. 1987). Although *wg* and *en* are expressed in mutually exclusive adjacent domains, the continual expression of each gene requires expression of the other (DiNardo et al. 1988; Martinez-Arias et al. 1988). Thus towards the end of the extended germband stage of embryogenesis *en* expression disappears from the presumptive epidermal cells (though not from the cells

of the nervous system) in *wg* mutant embryos, and at a similar stage *wg* ceases to be expressed in *en* mutant embryos. Further analysis of this system suggests that a pathway of interactions mediated by other segment polarity genes is required to maintain this reciprocal relationship between the two cell populations at each parasegment boundary (see Forbes et al. 1993; van den Heuvel et al. 1993). A contrasting example is demonstrated in zebrafish, where *goosecoid (gsc)* and *no tail (ntl)* show overlapping domains of expression and are co-expressed in cells at the dorsal midline of the early gastrula. However, studies with mutant *no tail* embryos suggest that *gsc* is independent of *ntl* function *in vivo* (Schulte-Merker et al. 1994b). In the end, presumptive gene hierarchies and interactions need to be confirmed genetically, but detailed expression analysis can provide useful pointers as to which genes are likely components of the same developmental pathway.

Analysis of the Brachyury mutant

We chose to extend previous analyses on the classical mouse semidominant mutant *Brachyury (T)*, which acts as a homozygous lethal during early development. Descriptive studies of the mutant embryos along with molecular analysis of the gene and its product suggest that the *T* gene protein acts as a transcription factor which is important in mesoderm formation and caudal axis formation.

Brachyury(T)

Morphological features of *Brachyury*

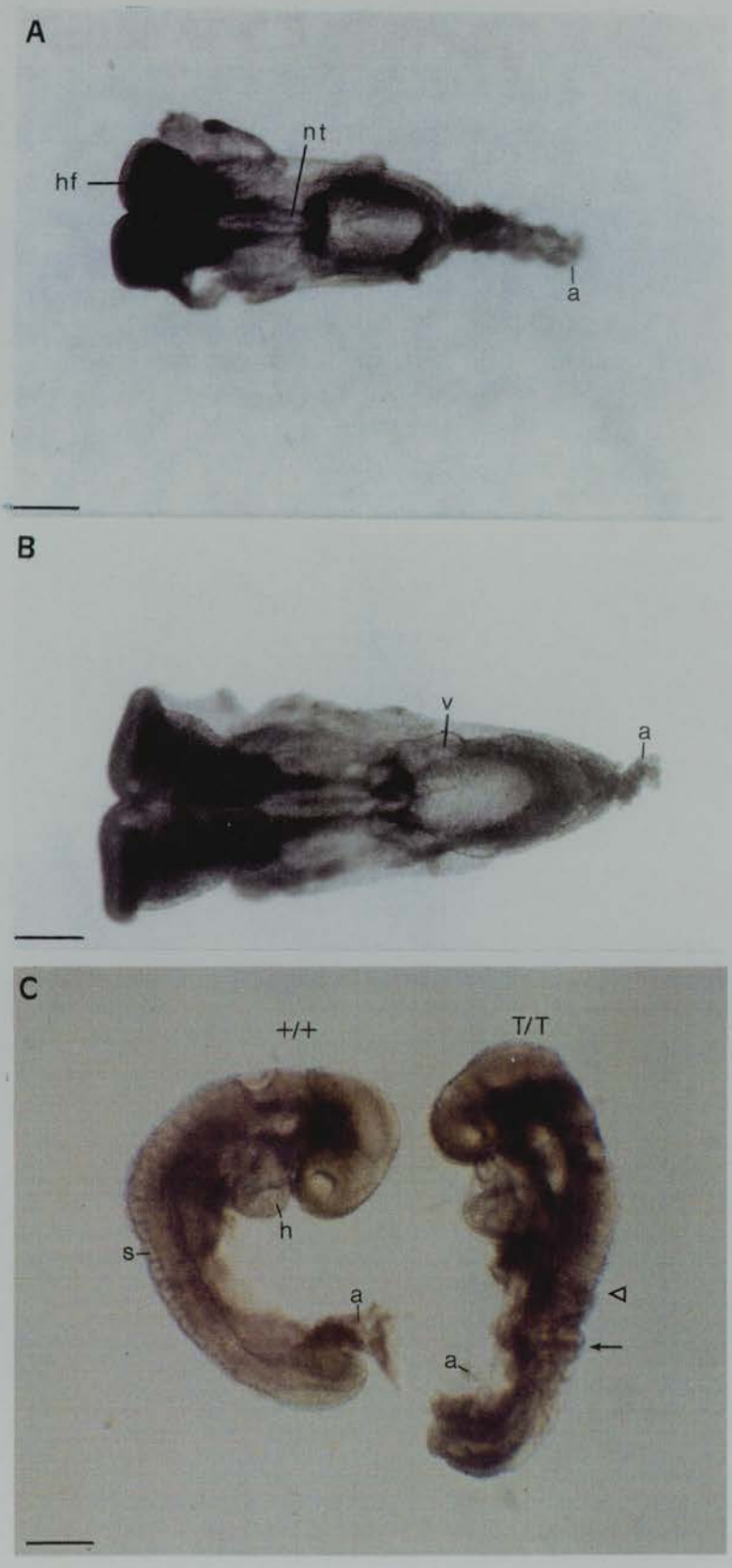
The *Brachyury (T/+)* heterozygote, having as its name suggests a short and often kinked tail, was first described almost 70 years ago by Dobrovolskaia-Zavadskaia (Dobrovolskaia-Zavadskaia 1927). Genetic

Figure 1.5

***Brachyury* phenotype at 8.5 and 9.5 dpc.**

A. 8.5dpc Wildtype embryo. Bar, 400μm
B. 8.5dpc *T/T* embryo with normal rostral end but short allantois and ectodermal vesicles (blebs) and slightly kinked neural tube Bar,300μm
C. 9.5 dpc T/T embryo (right) with wildtype littermate. Homozygous mutant looks normal anterior to somite 7 (open arrow head), but caudally displays a kinked neural tube (arrow), lack of somites and a very rudimentary allantois. Bar,550μm

Abbreviations
hf - headfold
nt - neural tube
a - allantois
v - ectodermal vesicle
s - somite
h - heart


A
nt
hf
a
B
v
a
C
+/+
T/T
h
s
a
a

analysis demonstrated that the homozygote was an embryonic lethal (Chesley 1932; Dobrovolskaia-Zavadskaia et al. 1935). Subsequently it was shown that *T/T* embryos died just prior to 11 days post coitum (dpc) and have distinctive caudal abnormalities posterior to the forelimb bud (Chesley 1935). These abnormalities include a bulky primitive streak, a deranged or absent notochord, a kinked neural tube, surface ectodermal "blebs", irregular and indistinct somites posterior to the seventh somite and an incomplete allantois which fails to cross the exocoelom and fuse with the chorion (Chesley 1935; Gluecksohn-Schonheimer 1944; Gruneberg 1958; Fig 1.5). The failure of the allantois to form a nutritive umbilical connection between the embryonic and maternal circulation is believed to be the physiological cause of death (Gluecksohn-Schonheimer 1944). Although there is no macroscopic abnormality detectable before 8.25 dpc, it has been observed using electronmicroscopic techniques, that putative *T/T* embryos also have an indistinct node at 7.5 dpc (Fujimoto and Yanagisawa 1983).

It has recently been discovered that *no-tail* (*ntl*) is the zebrafish homologue of *Brachyury* (Schulte-Merker et al. 1994a). Unlike *Brachyury* mutants, *ntl* mutants die well after hatching and therefore relatively late in development (Schulte-Merker et al. 1994a). *ntl* mutant embryos resemble *T/T* mutant embryos in that they lack both a differentiated notochord and caudal region to their bodies (Halpern et al. 1993). However, notochordal precursor cells are present in these mutants and CNS differentiation appears largely unaffected in the head and trunk (Halpern et al. 1993).

It has been proposed that the primary defects in *Brachyury* mutants are the thickened primitive streak and abnormal notochord with many of the other embryonic abnormalities being secondary to these. It is unlikely, however, that the deranged notochord directly or indirectly affects allantoic

development. The abnormality in this tissue is more likely due to an inappropriate deployment of cells as they leave the primitive streak or aberrant differentiation of cells in the allantoic bud.

The hypothesis that there are abnormal morphogenetic movements during gastrulation in *T/T* embryos is supported by the observation that the mesoderm:ectoderm ratio is not only increased in the caudal 15% of 8.0 dpc putative homozygous mutant embryos but that the same ratio is decreased immediately anterior to the primitive streak when compared to wildtype embryos (Yanagisawa et al. 1981). However, there is no significant difference in either the mitotic index (Yanagisawa et al. 1981) or in the incorporation of ^{3}H-thymidine labeling in different axial regions (Yanagisawa and Fujimoto 1977b) indicating that general cell viability and proliferation are unaffected. Furthermore, at this stage of embryonic development, these scientists did not note any increase in cell death in the region underlying the primitive streak.

In vitro assays examining the active migration of mesoderm cells on extracellular matrix have also demonstrated that there is a slight but significant reduction in the migration rate of mesoderm from *T/T* embryos compared with that from wildtype embryos (Hashimoto et al. 1987). This decrease in migratory rate may also be compounded by a reduction in the extracellular matrix of *T/T* embryos which has been demonstrated by scanning electron microscopy (Jacobs-Cohen et al. 1983a). It has been suggested that different components of the extracellular matrix selectively influence the activity of galactosyltransferase which is expressed on the cell surface of migratory cells (Shur 1989). Spatial and temporal differences in the activity of cell surface galactosyltransferase in *T/T* embryos compared to wildtype embryos have been described(Shur 1982). Embryos from pooled *T/+* x *T/+* litters at the primitive streak stage show only

65% the galactosyltransferase activity of pooled wildtype litters (corrected per embryo or per microgram DNA; Shur, 1982). In 1982, there was no independent marker to identify *T/T* embryos at the primitive streak stage, thus Shur could only hypothesise that at this stage of development, mutant embryos lacked surface galactosyltransferase activity. Autoradiographs of older *T/T* embryos, when it is possible to distinguish homozygotes phenotypically, demonstrated reduced surface galactosyltransferase activity in the presomitic mesoderm but increased signal intensity in the more mature anterior mesoderm derivatives compared to control embryos (Shur 1982).

The cell adhesive properties of mutant cells have been compared to those of wildtype cells by measuring the diameter of aggregates formed in suspension culture from disaggregated cells from the head, trunk and forelimb regions of 9.5 dpc embryos (Yanagisawa and Fujimoto 1977a). Mutant cells from any one of these regions consistently formed smaller aggregates, implying a difference in cell adhesion properties. However, the actual relevance of this observation is unclear as cells from both affected and unaffected tissues were mixed together.

Histogenic potential of *T/T* embryos

In 1935, Ephrussi explanted mutant *T/T* embryonic tissues *in vitro* and showed that these could survive beyond the time of embryonic death and could differentiate into an array of tissue types from all three germ layers. Similar results were obtained from ectopic transfer of the posterior third of 8.5 - 9.5 dpc homozygous *Brachyury* mutants (Bennett et al. 1977; Fujimoto and Yanagisawa 1979). As with wildtype embryonic tissue transferred at these stages, no embryonal carcinoma (EC) cells were found in *T/T* derived tumours (Bennett et al. 1977; Fujimoto and Yanagisawa

1979). As the epiblast is the progenitor of EC cells (Diwan and Stevens 1976), this result indicates that deranged posterior primitive streak region of mutant *T/T* embryos is not significantly developmentally delayed. When the histogenic potential of anterior and posterior regions of *T/T* embryos was compared, a decrease in frequency, but not complete absence of bone and cartilage were detected in tumours formed from the posterior regions (Fujimoto and Yanagisawa 1979). No such discrepancy was found in other mesodermal derivatives such as striated muscle, adipose and connective tissue. It should also be noted that neither notochord nor allantoic differentiation (two of the tissues which are dramatically affected in *T/T* embryos) can be recognized by histological inspection of experimental teratomas.

The genetics of *Brachyury*

The different alleles known at the T locus are summarised in Table 1.2. Two alleles, T^{2J} and T^{kt1}, at the *T* locus exhibit identical phenotypes to the original *Brachyury (T)* mutation. *T* and T^{2J} are both large deletions, 160-200kb and 81-110kb respectively. T^{kt1} on the other hand was induced by ethylnitrosourea and therefore is likely to be a point mutation or small deletion.

Three further *T* alleles, T^{wis} and T^{c} and $T^{c\text{-}2H}$ have a more severe phenotype than the original *Brachyury* mutant. Heterozygotes for these alleles usually have no tail [Searle, 1966; Herrmann, 1991: Herrmann and Kispert, 1994). Homozygous T^{wis}/T^{wis} embryos have no discernible somites (Herrmann 1991) and T^{c}/T^{c} embryos have more extreme abnormalities in the posterior body with loss of the forelimb buds, an open truncal neural tube and enlarged pericardium (Searle 1966). Therefore, it

Table 1.2: Different *T* alleles

Allele	Homozygous phenotype	Heterozygous phenotype	Molecular lesion	References
T *Brachyury*	comparatively normal anterior to forelimb bud thickened primitive streak ectodermal blebs discontinuous notochord stunted allantois indistinct anterior somites no somites posterior to 7th convoluted neural tube die late 10dpc	tail length 0-99% normal tendency to fewer presacral vertebrae	160-200kb deletion	Chesley 1935; Dobrovolskaia-Zavadskaia 1927; Gluecksohn-Schonheimer 1944; Gruneberg 1958; Herrmann et al.1990
T^{2J} *Jackson*	similar to *T/T*	similar to *T/+* tail length 0-75% normal	80-110kb deletion	Herrmann et al. 1990
T^{c} *Curtailed*	more extreme than *T/T* loss of forelimb bud open neural folds over trunk kinks in spinal cord next to open NT pericardial distention die 10dpc	tail length 0-20% normal; absence of axis odontoid process; absence of nucleus pulposi of intervertebral disks; fewer presacral vertebrae; tendency for rib and vertebral fusions; occasional hindlimb paralysis and atresia ani;	19bp deletion in last exon causing truncated carboxy-terminal	Searle 1966; Stott et al. 1993
$T^{c\text{-}2H}$ *Curtailed-Harwell*	Not reported	Similar to T^{c}	altered last exon causing truncated carboxy-terminal	Cattanach and Rasberry 1987; Herrmann and Kispert 1994
T^{h} *Harwell*	embryonic ectoderm ceases to proliferate, becomes pyknotic and dies die at 7-8dpc	indistinguishable from *T/+*	large deletion includes *qk*	Lyon 1959; Bennett 1975
T^{hp} *hairpin*	die at 7 days gestation	imprinted maternal effect (Tme) T^{hp}maternally derived die late gestation *Thp* paternally derived viable similar to *T/+*	includes deletion of *qk* *Tme* at same locus as *Igf2r*	Bennett 1975; Bennett et al. 1975; Johnson 1974; Johnson 1975;Barlow et al 1991
T^{kt1}/ T^{kt4}	similar to *T/T* but no ectodermal blebs occasional non-closure of anterior neural tube	similar to *T/+* except smaller than normal littermates	probable point mutation	Justice and Bode 1990
T^{Or} *Oak Ridge*	similar to T^{h}	similar to T^{h}	group of six mutations T^{1Or}-T^{6Or} ?deletion not extend as far as qk	Bennett et al. 1975
$T^{t}Orl$	indistinguishable from T^{hp}	indistinguishable from T^{hp}-but no maternal effect	includes deletion of qk:+ inseparable assoc with a t-haplotype	Moutier 1973; Silver et al. 1983
T^{wis} *Wisconsin*	Similar to *T/T* but fewer somites	tail length 0-20% normal	5.5kb insertion in exon 7 causing truncated carboxy-terminal	Herrmann 1991; Herrmann et al. 1990; Shedlovsky et al. 1988

could be considered that the boundary of defects shifts rostrally in both heterozygotes and homozygous T^{wis} and T^{c} animals.

Dosage studies have shown that *T* alleles which delete the *T* gene can be complemented by the *T* locus duplication t^{wLub2}. (MacMurray and Shin 1988)· However T^{c} is only partially complemented by this duplication which indicates that the T^{c} product antagonises wildtype activity. In addition it has been shown that T^{c}/T embryos has a less severe phenotype than T^{c}/T^{c} homozygotes (Searle 1966). However, the original *T* mutation is a loss of function deletion (Herrmann et al. 1990) and therefore residual *T* activity cannot account for this lessening of severity. Therefore it appears that the T^{C} gene product acts as a dominant negative mutation (MacMurray and Shin 1988). If the *T* gene product is only active with a second gene product, perhaps acting as a dimer, the T^{C} protein may interfere with this association or affect the biological activity of resulting protein complexes (see below). Increasing the dosage of *T* in an otherwise tailless $T^{C}/+$ mutant mouse by introducing transgenes into the genome results in a dose dependent extension of the tail to almost its normal length suggesting that the normal *T* gene product can compete with that of the T^{C} allele (Stott et al. 1993).

Cloning the T gene

The *T* mutation maps to chromosome 17 about 15 centimorgans from the major histocompatibility H-2 complex. Herrmann and his colleagues (1990) cloned the *T* gene via a multistep process. They mapped random DNA markers, which had been isolated from the *t*-complex region by microdissection, onto subregions of the chromosome. Two duplicated markers 119I and 119II, 66E and 66EII proved to be closely linked to the *T* mutation. Both 119I and 119II are deleted in the *T* alleles

T^{tOrl} and T^{Or4} whereas 119II but not 119I was missing in the *T* deletion T^{hp}. This suggested that *T* was located near 119II. In addition the centromeric limit of chromosomal duplication t^{Tu3} was mapped to within the cloned region 119II. Since t^{Tu3} duplicates the *T* gene and complements the *T* mutant phenotype, this demonstrated that the *T* gene must lie on the telomeric side of 119II. A combination of chromosome walking and jumping was used to bridge a region 400kb on the telomeric side of 119II. The end fragment of the second jumping clone identified deletions in the original *T* allele and in T^{2J}. This localised the *T* gene to a genomic region of no more than 100kb. Cosmids were isolated from this region and were used to screen a cDNA library prepared from 8.5 dpc embryos. One cDNA clone, pme75 was used to probe different *T* alleles and revealed the insertion of a transposable element in the T^{wis} allele, which results in the alteration of the *T* gene product and thus pinpoints the locus of the *T* gene.

The *T* gene and it product

Although the sequence of the cloned mouse *T* gene did not immediately reveal the nature of the protein product, it did show that the gene has an open reading frame of 436 amino acids (Herrmann et al. 1990) and shows limited homology to *MyoD1* (Willison 1990). It lacks a signal peptide and a membrane-spanning region (Herrmann et al. 1990) and subsequent antibody studies in both mouse (Kispert and Herrmann 1994) and zebrafish (Schulte-Merker et al. 1992), have demonstrated that the protein has a nuclear location. The amino-terminal portion of the protein-coding sequence is very highly conserved between mice (Herrmann et al. 1990), *Xenopus* (Smith et al. 1991) and zebra fish (Schulte-Merker et al. 1992), suggesting a conserved function for this region. Recently, gel shift data have demonstrated that the N-terminal half

of 229 amino acid residues of all three vertebrate T proteins bind to a novel palindromic DNA target (Kispert and Herrmann 1993). In the same study, Kispert and Herrmann showed that a monomer of T is sufficient for DNA binding as only two sizes of DNA-protein complex were observed despite altering the ratio of the complete protein with respect to the N-terminal DNA binding domain. They also observed that the strength of weak T protein -DNA complexes detected in electrophoretic mobility shift assays was strongly increased when antiserum is added to the reaction. This shows that the DNA binding behaviour of T protein can be modulated by interactions with other proteins which might be important for the action of T protein *in vivo*. Therefore all available data are consistent with the *T* protein being either a transcription factor or an auxiliary protein for transcriptional activation.

As discussed above, heterozygous $T^{wis}/+$, $T^{C}/+$ and $T^{c\text{-}2H}$ animals show a more severe anterior mesoderm and axial abnormalities than those carrying a single wild-type gene. Therefore, the mutant protein from these alleles, appears to reduce the amount of functional T protein available in the embryo. All three of these alleles have an altered open reading frame which results in proteins with a truncated carboxy-terminal end. T^{wis} has a 4.5kb insertion of a retroviral-like element in the seventh exon (Herrmann et al. 1990), whilst T^{C} has a 19bp deletion of the last exon (Stott et al. 1993) and $T^{c\text{-}2H}$ has a point mutation which causes a frame shift in the last exon. However, since the N-terminal DNA binding domain is intact in these mutant proteins, they most likely can bind to DNA, but they may not be able to activate transcription because the abnormal C-terminal competes or interferes with the wildtype protein at its site of action.

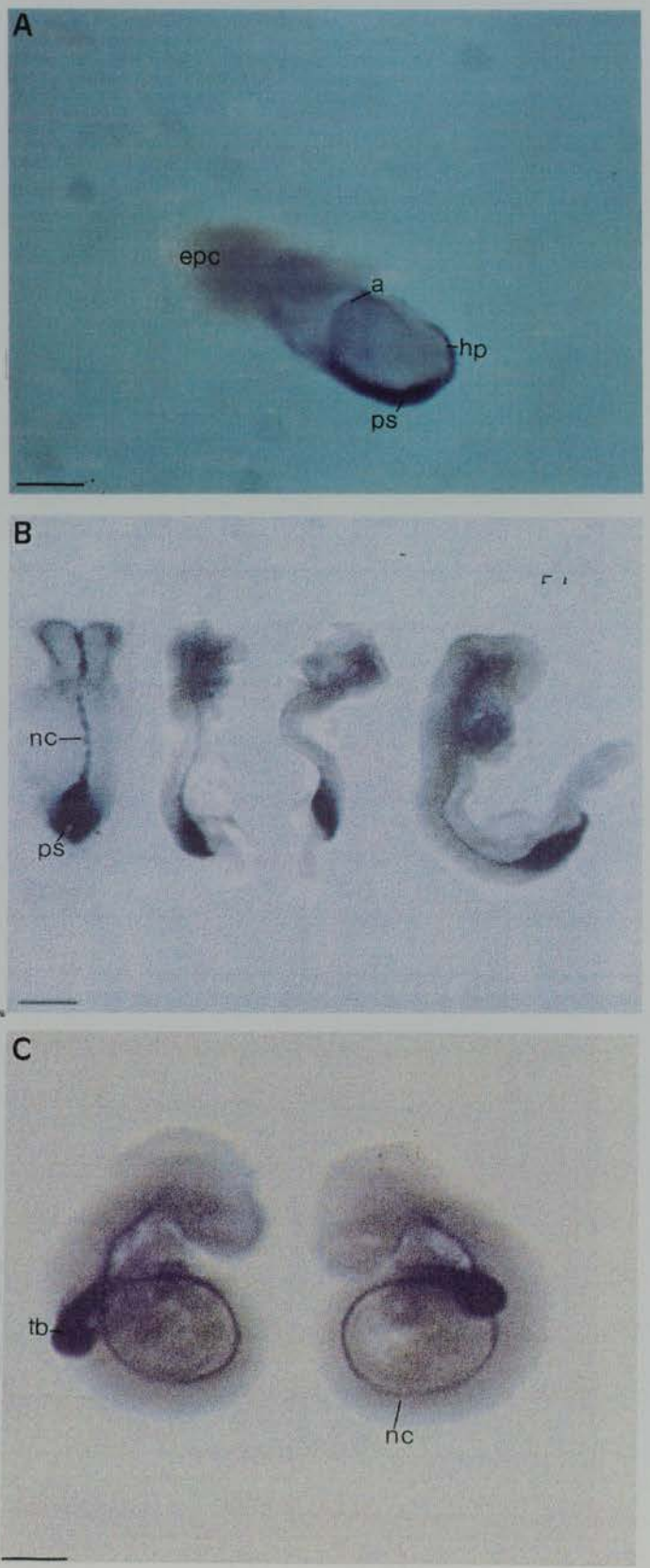
Figure 1.6

Expression pattern of *T* transcripts in wildtype embryos between 7.5 and 9.5 dpc

T mRNA displays a very restricted expression pattern during development.
A. Lateral view of 7.5 dpc embryo with Reichert's membrane removed. *T* transcripts are observed in the primitive streak and head process. Bar 250 μm
B. Dorsal and lateral views of 8.5 and 8.75 dpc embryos which have had the surrounding extra-embryonic membranes removed. *T* expression is observed in the notochord, primitive streak, adjacent ectoderm and nascent mesoderm. Bar 320 μm
C. Lateral view of 9.5 dpc embryos. *T* expression is restricted to the tail bud and notochord. Bar 650 μm

Abbreviations

a	amnion
epc	ectoplacental cone
ps	primitive streak
hp	head process
nc	notochord
tb	tail bud


A
epc
a
hp
ps
B
nc
ps
C
tb
nc

T gene expression pattern

The expression pattern of the *T* gene in mouse (Herrmann 1991; Wilkinson et al. 1990),*Xenopus* (Smith et al. 1991), and zebrafish (Schulte-Merker et al. 1992) embryos is largely consistent with the observed pattern of abnormalities seen in mouse *Brachyury* and zebrafish *no-tail* mutants. In the mouse, it is first expressed in the primitive streak at the onset of gastrulation (Wilkinson et al. 1990). Expression continues in the primitive streak throughout gastrulation and can be detected in ectoderm adjacent to the streak and nascent mesoderm underlying the streak (Fig 1.6). The head process and notochord continue to express *T* upto at least 17.5 dpc where it is seen in the intervertebral discs which are notochordal remnants, (Wilkinson et al. 1990). However, other mesodermal cells and derivatives, with the possible exception of a very early basal component of the allantois, no longer express *T* once they migrate away from the primitive streak. Thus, the expression pattern of the gene supports the notion that its primary sites of action are the primitive streak and notochord, although it does little to explain the defects in allantois development.

T protein expression pattern

The T protein is located in the nucleus (Kispert and Herrmann 1994; Schulte-Merker et al. 1992). In the developing mouse embryo, it is first detected at approximately 6.5 dpc in the primitive streak (Kispert and Herrmann 1994). The extension of the primitive streak and the notochordal plate are marked by T protein. All cells delaminating and ingressing through the primitive streak express *T* transiently (Herrmann 1991; Wilkinson et al. 1990), but the expression domain of T protein appears wider than that of the RNA and includes migrating extraembryonic

mesoderm (Kispert and Herrmann 1994). This probably reflects the longer persistence of T protein as compared to RNA in migrating cells. T protein has also been detected in the early endoderm and in prospective neurectoderm of late primitive streak stage embryos and in the tail bud. The transient presence of T protein in non-mesodermal cells suggests that *T* expression alone is not sufficient to commit epiblast cells to differentiate into mesoderm.

The T protein persists only in the extending notochordal plate and notochord. From about 11.5 dpc onwards, T protein gradually vanishes from the notochord in an anterior to posterior manner. By 14.5 dpc no T protein is observed (Kispert and Herrmann 1994). However, as already noted *T* transcript was found in the intervertebral disks at 17.5 dpc (Wilkinson et al. 1990). Kispert and Herrmann (1994) did not examine the distribution of T protein in embryos older than 14.5 dpc, therefore it remains unclear whether either T gene transcripts are inactive in later stages or whether the T protein is rapidly degraded in older embryos or whether T protein is again made in the intervertebral discs.

T gene and protein distribution in mutant embryos

As discussed, earlier the T^{wis} mutation results from the insertion of a transposable element into the *T* gene (Herrmann et al. 1990). The T^{wis} allele is transcribed and translated and thus allows the analysis of the expression of the *T* gene and protein product in mutant embryos by *in situ* hybridisation and immunohistochemistry (Herrmann 1991; Kispert and Herrmann 1994). In early T^{wis}/T^{wis} embryos, *T* gene and protein expression is normal, but T transcripts cease prematurely during early organogenesis (between 8 and 8.5 dpc) when the disruption of the node becomes apparent and extension of the notochordal precursor comes to a

halt (Herrmann 1991). In these mutants, the expression of *T* decreases over several hours in the primitive streak and head process: the posterior part of the primitive streak is the last structure where transcripts of the *T* gene are detectable. The T protein is still detectable in the primitive streak at 8.5 dpc and in the anterior notochordal plate. At this stage, the notochordal plate cells are not in the correct ventral position with respect to the hindbrain and have a lower level of T protein than normal (Kispert and Herrmann 1994). Posterior to the hindbrain, Kispert and Herrmann only occasionally detected notochordal cells. The densely packed cells in the bulky posterior primitive streak at 8.5 dpc still possess some residual T protein. By 9.5 dpc, no T protein is detectable. The disappearance of transcripts in the head process/notochord precursor correlates with its ability to differentiate into notochord suggesting that a functional *T* gene product is required for this to occur (Herrmann 1991).

The two known *ntl* zebrafish mutants (*ntl*^{b160} and *ntl*^{b195}: Halpern, 1993) also produce altered protein products (S. Schulte-Merker et al. 1994a). *ntl*^{b160} has a change of sequence at the intron-exon boundary of exon 6. This alteration leads to a potential frame shift and a potential premature protein chain termination. *ntl*^{b195}, on the other hand, has a 1.5kb insertion which causes a disruption in the second exon. Ntl^{b195} protein cannot be detected by western blots, unlike Ntl^{b160} protein which can. However, both these alleles are likely to be functional null alleles(Schulte-Merker et al. 1994a). In *ntl* embryos of both alleles, the level of *ntl* mRNA is reduced compared to wild-type embryos at the early gastrula stage. During later development, *ntl* transcripts in mutant embryos are further reduced in the dorsal midline where the notochord normally forms (Schulte-Merker et al. 1994a). In embryos from both *ntl* alleles, Schulte-Merker and colleagues were unable to demonstrate the presence of Ntl

protein irrespective of the stage examined and despite the fact that a truncated protein from ntl^{b160} could be detected on western blots.

T gene and protein expression in the tail bud of heterozygous animals

Wholemount *in situ* hybridisation of the developing tail region of 9.5 to 12.5 dpc wildtype embryos shows that *T* is highly expressed not only in the notochord but also in the tail bud (Wilson et al 1993) and at 10.5 dpc, the T protein is also present in almost all cells of the tailbud of wildtype embryos, except for a surface layer (Kispert and Herrmann 1994). In contrast, 10.5 to 11.5 dpc embryos from *T/+* x *T/+* matings show diminished or undetectable tailbud *T* expression with or without visible defects in the notochord of *T/+* embryos. This suggests that tailbud abnormalities may occur independently of a disrupted notochord (Wilson et al 1993). Thus, an absence or reduction in *T* expression may have a direct effect on tail outgrowth, independent of any secondary influence of the notochord. However, in $T^{wis}/+$ embryos analysed between 10.5 -13.5 dpc, no reduction in T protein was reported in the tail bud itself although expression in the notochord was discontinuous and fragments of T expressing cells were occasionally found integrated in a malformed neural tube (Kispert and Herrmann 1994). By 13.5 dpc the axial structures in the tail of $T^{wis}/+$ embryos have completely degenerated. The apparent discrepancy of the expression pattern of T in *T/+* and $T^{wis}/+$ tailbuds may reflect the longer persistence of T protein as compared to T transcripts in this region.

The role of T in mesoderm formation and caudal axis formation

Although the *T* gene is expressed thoughout the primitive streak, its absence in homozygous *Brachyury* mouse embryos does not completely inhibit mesoderm formation or gastrulation: structures anterior to the

forelimb bud form comparatively normally, suggesting that T is primarily involved in caudal axis formation. The role of T in mesoderm production and caudal axis formation will be discussed in Chapter 4.

Overview of experimental chapters

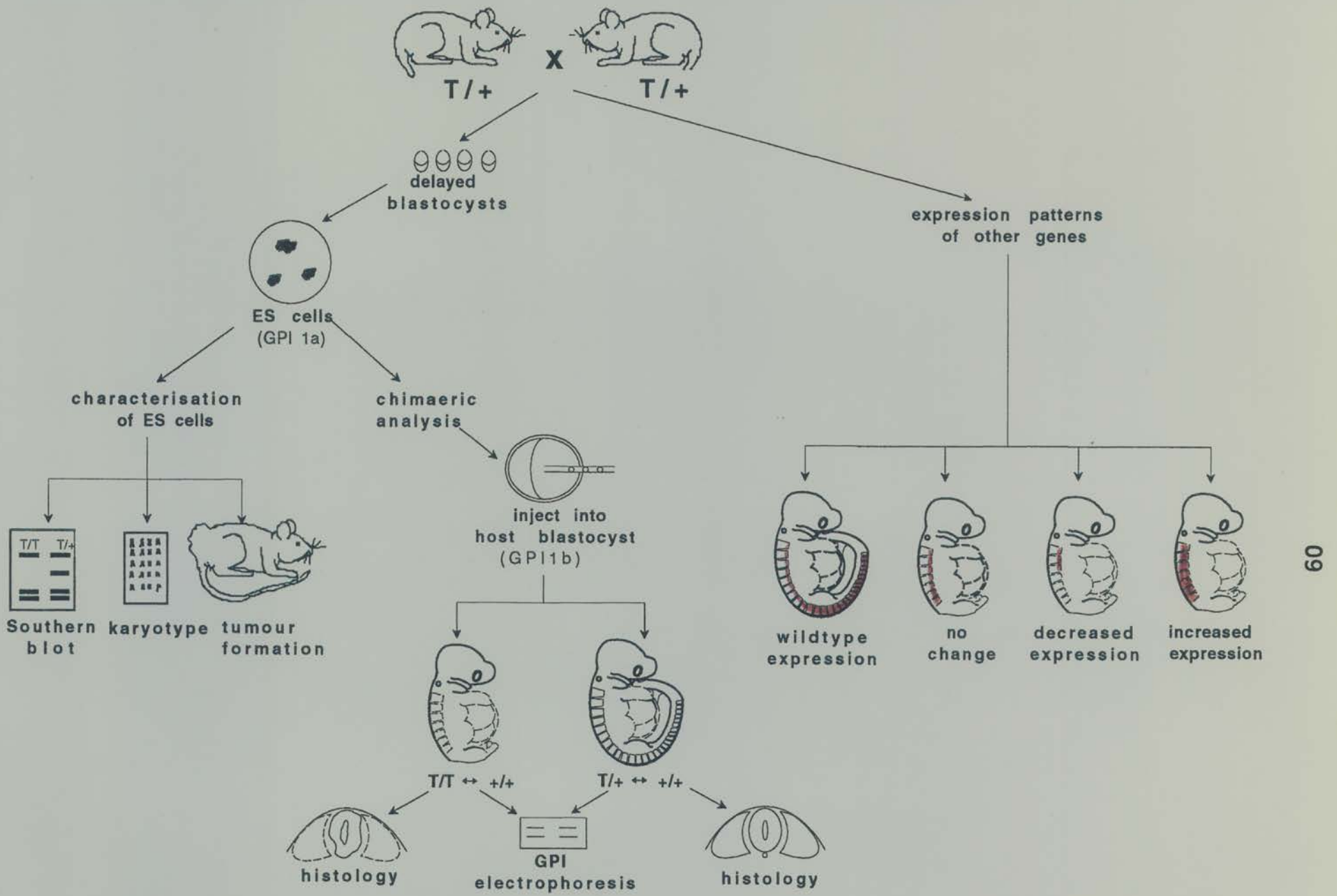
Figure 1.7 summarises the different experiments presented in this thesis.

In order to further analyse the primary function of Brachyury, it was decided to derive ES cells from *T/+* x *T/+* matings. The derivation and characterisation of these lines is described in the Chapter 2.
Chapter 3 describes the chimaeric analysis of *T/T* and *T/+* ES cells at midgestation and in live born animals. The mutant phenotype is mimicked in *T/T* ↔ +/+ chimaeras demonstrating that the *T* gene acts cell autonomously in the primitive streak and notochord.

In order to assess how *Brachyury* may interact with other genes which may also be involved in mesodermal formation and dorsoventral patterning of the neural tube, chapter 4 compares the expression patterns of several genes in homozygous and wildtype embryos using a non-radioactive wholemount *in situ* hybridisation technique. The results indicate that the *T* gene may maintain, rather than activate a signaling pathway required for these processes.

Figure 1.7
Overview of experimental protocol used in this thesis

See text for details


T/+
X
T/+
delayed blastocysts
ES cells (GPI 1a)
characterisation of ES cells
chimaeric analysis
expression patterns of other genes
T/T
T/+
Southern blot
karyotype
tumour formation
inject into host blastocyst (GPI1b)
wildtype expression
no change
decreased expression
increased expression
T/T ↔ +/+
T/+ ↔ +/+
histology
GPI electrophoresis
histology

Chapter 2

Derivation and Characterisation ES cells from *T/+ x T/+* matings

Introduction

The number of developmental mutants which cause an abnormal phenotype in the mouse (Lyon and Searle 1989) make it an attractive experimental model to identify and study those genes required for normal tissue diversification and pattern formation in the embryo. However, this advantage is counteracted by the relative inaccessibility and cellular complexity of the mammalian embryo. Therefore, it would be extremely useful to have a permanent and limitless source of normal and deficient mutant embryonic cells, which could serve as valid populations to study normal and mutant cell behaviour both *in vivo* and *in vitro*. Embryonic stem (ES) cells (Evans and Kaufman 1981; Martin 1981) are such a resource. These cells, which are isolated from blastocysts, can be cultured for long periods *in vitro* and subsequently resume normal development if returned to a carrier embryo (Beddington and Robertson 1989; Bradley et al. 1984). As they often contribute to the germ line as well as to the somatic tissues (Robertson et al. 1986; Robertson 1986), it is possible to use this system as a means of generating transgenic animals either via the addition of new genetic material (Freidrich and Soriano 1991; Gossler et al. 1989) or by alteration of the host gene sequences (Chisaka and Capecchi 1991; Le Mouellic et al. 1992). However, production of new mutations will result in unknown embryonic phenotypes which may take some time to identify and characterise. It is also possible to derive ES cells from blastocysts obtained from matings between animals heterozygous for a particular developmental mutation (Magnuson et al. 1982; Magnuson et al. 1983). However, one can only genotype the resulting ES cells if the mutant gene itself is cloned or very closely linked marker sequences are known.

We decided to derive ES cells from the classical developmental lethal mutation, *Brachyury (T)* (Dobrovolskaia-Zavadskaia 1927). The *T* gene has been cloned (Herrmann et al. 1990) and been shown to be expressed in the primitive streak, nascent mesoderm and notochord (Wilkinson et al. 1990). However, although the gene is known to be important in mesoderm formation during gastrulation (Chesley 1935; Gruneberg 1958; Yanagisawa et al. 1981), neither the gene's sequence nor its expression pattern defines its developmental function (Herrmann et al. 1990; Willison 1990). Isolating and characterising ES cells from heterozygous *Brachyury* matings is appealing for several reasons. Firstly, once established and genotyped, these matings provide heterozygous and wildtype lines on the same genetic background as those null for the *T* gene and which can serve as experimental controls. Secondly, such lines provide a continuous source of mutant cells whose development can be monitored either *in vivo* or *in vitro*. Thirdly, the mixing of wildtype and mutant cells to form chimaeras should reveal information about the nature of the phenotype which is not apparent in intact mutant embryos (see chapter 3). Finally, cells null for the *T* gene present an ideal substrate for genetic manipulation of *T* expression or of genes acting downstream of it.

Materials and Methods

Tissue culture solutions

Prior to use fetal and new born calf serum batches were selected that supported optimum growth of established ES cell lines (tested by Derek Rout and Douglas Colby).

All solutions were made using water which had been purified by passing it through an Elgastat Prima Reverse Osmosis Filter followed by an Elgastat UHP water purifier to ensure good quality.

Culture medium for ES cells (Robertson 1987)

1x Dulbecco-modified Eagles medium (DMEM; GIBCO-BRL) was buffered with 0.37 % $NaHCO_3$ and adjusted to pH 7.2. The medium was supplemented with 10% fetal calf serum (FCS), 5% newborn calf serum (NCS) 10^{-4}M 2-mercaptoethanol (Sigma), 1mM glutamine and 1mM non-essential amino acids (GIBCO-BRL).

Differentiation Inhibiting Activity/Leukaemia Inhibitory Factor (DIA/LIF)

DIA/LIF (either human or murine)was used at a concentration of 1000iu/ml. This had been prepared by transfection of Cos-7 cells with the appropriate human (pC10-6R) or murine (pDR10) plasmid (see Smith, 1991) and harvesting the culture medium.

DIA/LIF was kindly provided by Austin Smith.

Culture medium for STO cells

1x DMEM was buffered with 3.7% H_2CO_3 and supplemented with 10% new born calf serum (NCS) and then brought to pH 7.2 using 1N NaOH.

Ca^{2+},Mg^{2+}-Phosphate buffered saline (PBS)

One PBS - Dulbecco.'A' (Oxoid) tablet was dissolved per 100mls of ultra high pure water to give a PBS solution (pH 7.3) that was calcium and magnesium free.

Trypsin/EDTA (Robertson 1987)

A solution of 0.25% trypsin (Porcine; Difco) in 0.04% EDTA was used for routine subculture of both embryonic stem cell lines and fibroblasts. The other ingredients in this aqueous solution were: 0.7% (w/v) NaCl, 0.03% (w/v) $Na_2HPO_4.12H_20$; 0.024% (w/v) KH_2PO_4; 0.037% (w/v) KCl; 0.1% (w/v) D-glucose; 0.3% (w/v) Tris. A few drops of Phenol red (Difco) were added as a pH indicator and the solution was filter sterilised through a 0.22 μm nitrocellulose filter.

HAT (hypoxanthine, aminopterin, thymidine) medium

A 50x solution of HAT ($5x10^{-3}$M hypoxanthine, $2x10^{-5}$M aminopterin, $8x\ 10^{-4}$M thymidine) was prepared by dissolving the contents of 1 vial of HAT (Sigma) in 10mls of distilled water.

Mitomycin C

2mg of Mitomycin C (Sigma) was dissolved in 1ml of PBS. The volume was made up to 150mls with DMEM plus 10% NCS. This gives a final concentration of 0.013% which is higher than the 0.010 % concentration recommended by Robertson (1987). Once made up the Mitomycin C solution was kept for 3 weeks at 4^oC.

Gelatin

0.1% gelatin (from bovine or porcine skin: Sigma) was dissolved in ultrapure water and autoclaved.

Routine Tissue culture methods

All cells were maintained at 6% CO_2, at 37°C in a humidified incubator. The routine tissue culture methods used were based on those described by Robertson (1987)

Preparation of feeder layers cells

STO cells were maintained in DMEM supplemented with 10 % NCS and passaged when confluent.

To prepare feeder layers, confluent flasks of STO cells were treated for 4 hours with fresh 0.013% Mitomycin C(Sigma). The cells were then trypsinised for 4 minutes at 37°C before resuspending in culture medium. Cells were plated onto gelatinised flasks at a density of 2 $x10^4$ cells/cm^2. Feeder layers were used for up to a week after their preparation.

Routine culture of ES cells

Some ES cell cultures were initially maintained on feeder layers, but subsequently transferred into DIA/LIF supplemented medium (see below). Other cell lines were derived and grown continually in the presence of DIA/LIF. However the routine maintenance for both methods was basically the same, except cells grown in the presence of DIA/LIF were passaged onto gelatinised flasks rather than onto feeder cell monolayers.

Cells were refed with ES cell culture medium every day and subcultured every second to third days when the flasks were confluent.

Subculturing cells

ES cells were refed 1-2 hours prior to passaging. After this time, the medium was aspirated and the cells washed with PBS. The plate was flooded with 0.5ml trypsin/EDTA and incubated for a further 4-5 minutes at

37°C. The flasks were tapped to dislodge the cells from the bottom and 1ml of ES cell medium was added to the flask to stop the trypsin digestion. The suspension was pipetted vigorously with a sterile plugged pasteur pipette. The cell suspension was checked visually under low-power magnification to ensure that it was relatively free of cellular aggregates before replating out at a density of 1:10.

Subculturing fibroblasts used the same procedure but STO culture medium was substituted for the ES culture medium.

Freezing cells

Cells were harvested by trypsin digestion and then centrifuged at 1000rpm for 5 minutes. The pellet from a confluent 25cm^2 flask of cells (approximately 10^7 cells) was resuspended in 0.5mls ES cell culture medium. Whilst the cell suspension was gently shaken, an equal volume of 2X freezing medium (20% dimethyl sulphoxide (DMSO: Sigma),20%FCS,60% DMEM) was added dropwise to the cell suspension. 0.5mls of the final cell suspension was quickly aliquoted into freezing vials (Nunc) which were then placed in a -70°C freezer for 24 hours before being transferred to a liquid nitrogen cell bank.

Thawing ES cells

Frozen vials were retrieved from storage and placed directly into a 37°C water bath. When all the ice crystals had melted, the ampoule was removed from the bath and sterilised with 70% industrial methylated spirits (IMS). The contents were gently transferred using a sterile Pasteur pipette into a 15ml centrifuge tube (Corning) containing 5mls ES cell medium. The cells were pelleted at 1000rpm for 5 minutes and the supernatant aspirated. The cells were resuspended in 10mls ES cell medium and

transferred to either gelatinised 25cm^2 flasks or onto fresh feeder layers as appropriate. The medium was changed after 8 hours and subsequently the cells were maintained as above.

STO cells were frozen and thawed in a similar fashion except that STO culture medium was used instead of ES cell medium.

Removal of ES cells from "live" feeders

Many of the original ES cell lines derived by Lesley Cooke were found to be contaminated with feeders which were not mitotically inactive. It is not certain what caused this problem, but there may either have been a faulty batch of Mitomycin C or the STO cells themselves may have changed character due to long periods of culture without subculture. These "live" feeders gradually overgrew the ES cells in the flasks. They also appeared not to respond to HAT (hypoxanthine/ aminopterin/ thymidine) selection. This may have been caused by metabolic cooperation from the neighbouring ES cell colonies, although it is possible that the treatment actually selected for "live" feeders that were themselves HAT-resistant.

In order to overcome this problem, early passage BTBR ES cell lines were thawed onto feeders made using a 0.013% Mitomycin C solution (Sigma). The ES cell culture medium used contained both DIA/LIF and Ix HAT ($1x10^{-4}$M hypoxanthine, $4x10^{-7}$M aminopterin, $1.6x10^{-5}$M thymidine). The cells were passaged 2-3 days post thawing when the ES cells were judged to be at a high enough density to survive passaging, but before the live feeders had overgrown the flask. They were passaged from a 25cm^2 flask into two or three 75cm^2 flasks which had been previously flooded with 0.1% gelatin (bovine or porcine skin: Sigma). The larger sized flask was used to increase the surface area onto which the cells could attach

and to decrease the likelihood of ES cells saving the feeders from HAT selection. After 2-3 hours when the feeders had settled, but before the ES cells had done so, the supernatant was removed and transferred into new gelatinised 75cm^2 flasks. The original flasks were discarded. Examination of the flasks the next day indicated that this procedure removed most, if not all, the fibroblastic cells. However to ensure that the fibroblasts were eradicated, HAT selection was maintained for another 4-5 days, that is until the cells had been maintained in a selective medium for a total of 7 days. Over the next four days, the HAT concentration was gradually decreased, after which the cells were grown in normal ES cell medium.

At all stages of this procedure the ES cells were maintained in DIA/LIF supplemented culture medium.

Derivation of cell lines

The initial BTBR lines may have undergone a selection procedure when they were removed from feeder layers and subsequently maintained in DIA/LIF. New lines were derived directly into DIA/LIF using the method described by Nichols et al. (1990).

Recovery of embryos

The founder members of the BTBR/Pas colony used were obtained from ICRF Clare Hall Laboratories. The BTBR/Pas heterozygote *T/+* mouse colony was maintained in a stabilised environment on a regime of 14 hours light:10 hour dark. The midpoint of the dark cycle was 12.00 midnight. Females were mated overnight and in order to induce blastocyst delay, pregnant females were ovariectomized 2 days later, between 11.00 am and 3.00 pm and a sub-cutaneous injection of 10μg of Depo-Provera

(UpJohn) given. 4 days after ovariectomy, the female was killed by cervical dislocation and the delayed blastocysts flushed from the uterine horns into glass embryological dishes with PB1 solution (Whittingham and Wales, 1969) supplemented with 10% fetal calf serum.

Culture of embryos and isolation of cell lines

Blastocysts were transferred to 0.1% gelatin-coated 16mm tissue culture wells (Nunc) and cultured in ES cell culture medium supplemented with DIA/LIF. Up to 5 blastocysts were cultured per well as they attached separately and their inner cell masses (ICMs) remained discrete. Cultures were maintained in a humidified atmosphere of 6% CO_2 in air at 37°C. After 5 days of culture, the ICMs were individually disaggregated by physical dislodgement from the trophoblast with a fine glass rod and transferred to separate drops of trypsin. Following incubation for 5 minutes, the trypsin was inactivated by dilution with ES cell medium. Cells were dissociated into small groups by repeated aspiration through a finely drawn-out siliconized pasteur pipette and then transferred into a gelatinised 16mm culture well containing 0.5ml pre-equilibrated ES cell medium containing DIA/LIF. The cells from each blastocyst were placed into separate wells. Thereafter, where appropriate, fresh DIA/LIF was added every 3-4 days.

During the second week of culture, when primary stem cell colonies contained 50-250 cells, they were dissociated by the same procedure and replated into fresh gelatinised 16mm wells. (Fresh ES medium was also put into the wells which had contained the primary colony. Some of these produced second "primary" colonies, either from cells seeding from the first primary colony or forming independently from the inner cell mass.) The cultures were refed as necessary. If small colonies of stem cells were

apparent then the cultures were passaged at a ratio of 1:1 into 25mm wells (Nunc) 4 to 5 days later, using the passage procedure for established cell lines (see above). When the cells were confluent, they were passaged into $25cm^2$ flasks. Frozen aliquots were stored as soon as sufficient cells ($\sim 10^7$) were available.

Karyotype analysis

This part of the analysis was done with the help of Linda Manson.

Preparation of mitotic spreads (Robertson 1987)

Subconfluent cells growing in a $25cm^2$ were trypsinised. The disaggregated cells were spun at 1000rpm in a $15cm^3$ centrifuge tubes (Corning) and the supernatant then aspirated. The pellet was resuspended in 1ml of freshly prepared hypotonic 0.56% KCl. A further 5 mls of hypotonic KCl was added and the tube inverted to ensure thorough mixing. The cells were left at room temperature for 5 minutes. Following recentrifugation at 500rpm for 5 minutes, the hypotonic solution was aspirated and replaced with 5mls ice-cold fixative (3:1 Methanol: Acetic Acid). This was added drop-wise whilst flicking the tube to prevent the cells from clumping together. The tube was left for 5 minutes at room temperature. The cells were repelleted by centrifuging them at 500rpm and the fixative changed 3 times in a similar manner as above except that the final volume of the third mix was 1ml instead of 5ml. Whist the cells were being fixed, glass slides were prepared by washing them with 70% Industrial Metholated Spirits (IMS) and dried with lens tissue. 1-2 pipette drops of cell suspension were dropped onto the slides from a height of at least three feet. Slides were air-dried and then immersed in a 3% (v/v) solution of Gurr's Giemsa stain (BDH) in PBS for 15 minutes. The slides

were rinsed twice in distilled water and allowed to air dry. Chromosomes were counted from 10 or 20 metaphase spreads.

G-band analysis (Robertson 1987)

Unstained slides were baked overnight at 60°C and the following morning incubated in a solution of 2x standard saline citrate (2x SSC; 0.03M NaCl, 0.03M trisodium citrate) for 1 hour at 60°C. The slides were rinsed extensively in several changes of distilled water and stored in a rack under water. Slides were individually immersed in trypsin (0.25% (w/v) trypsin powder (Porcine, Difco) dissolved in Gurr's pH 6.8 phosphate buffer (BDH) at 4°C for 3-4 minutes. Each slide was then rinsed twice in pH 6.8 buffer before staining it for 5-10 minutes in freshly prepared staining solution (5% (v/v) solution of Giemsa Gurr's R-66 stain (BDH) in pH 6.8 buffer). After staining, the slides were washed twice in pH 6.8 buffer followed by two rinses in distilled water. The slides were allowed to air dry before examining and photographing metaphase spreads under oil immersion with a high-power objective photographic microscope (Olympus, Vanox).

Genetic characterisation

Preparation of high molecular weight genomic DNA (Sambrook et al, 1989)

Confluent cells in a 25cm^2 flask (approximately 10^7 cells) were washed once with PBS and then trypsinised for 5 minutes at 37°C. The cells were centrifuged for 5 minutes at 1000rpm and the supernatant removed. The pellet was washed in PBS and again centrifuged. After removal of the supernatant, the pellet was completely resuspended in 0.5mls TEN and 0.5% (w/v) SDS was added, followed by 0.5mg/ml proteinase K (Boehringer). The mixture was incubated over night at 60°C.

The following morning, 10μg/ml RNase A (Boe inger) was added and the mixture was incubated at 37°C for one hour. The DNA was phenol-chloroform extracted to remove any impurities before precipitating it with isopropanol at -20°C for one hour. After centrifugation, the DNA pellet was washed with 70% ethanol and air dried before being resuspended in 0.1ml TE buffer.

Preparation of probe

The different probes were released from the plasmid DNA by digestion with the appropriate enzyme: p66MRT (Herrmann et al. 1986; Herrmann et al. 1990) was cut with EcoR1 plus Taq1 to give a 1.8kb fragment; p190R10RS (Herrmann et al. 1990) was cut with Taq1 plus SacII to give a 1.6kb fragment and p119AR (Herrmann et al. 1986; Herrmann et al. 1990) was cut with EcoR1 to give a 3.2kb fragment. The digests were run into a 1% low melting point (LMP) agarose Tris Borate/EDTA gel. The amount of DNA in each fragment was determined by comparing it by eye to a known amount of DNA run in parallel. The appropriate fragment was cut out of the gel and weighed. An equivalent volume of water was added to the DNA and the mixture was heated to 70°C to dissolve the LMP agarose. The probes were prepared using a random primed DNA labeling kit (Boe inger) as follows. 25ng DNA fragment was denatured by heating it at 100°C for 10 minutes and subsequently cooling it on ice. The following was added to the DNA and made upto a final volume of 20μl: 2μl of 10x reaction mix buffer (Boe inger) 0.5μM dATP,0.5μM dGTP, 0.5μM dTT 0.1unit/μl Klenow enzyme and 50μCi [α^{32}P] dCTP. After incubating the mixture for 30 minutes at 37°C, the reaction was stopped by adding 20mM EDTA (pH8.0) and heating the mix to 65°C for 10 minutes.

Unincorporated nucleotides were removed by passing the mixture through a Sephadex G-50 (Pharmacia) column.

Southern blot analysis (Sambrook et al, 1989)

The genomic DNA which was to be probed with p190R10RS and p66MRT, was first digested with *Taq1* restriction enzyme whilst that for the p119AR probe was digested with *BamHI* restriction enzyme. The digested DNA samples were run into a 0.7% agarose Tris Borate/EDTA (TBE) gel. The gel was then photographed, rinsed in distilled H_2O and washed for 20 minutes in 0.2M HCl in order to nick the DNA. The DNA was denatured for 45 minutes in a solution containing 0.5M NaOH and 1.5M NaCl. The gel was rinsed twice with distilled H_2O before neutralising it for 1 hour in 1M Tris HCl (pH5.5);1.5M NaCl. The gel was again rinsed in distilled H_2O and the DNA was transferred to a nylon filter (Hybond N, Amersham). This was achieved by laying the gel on a sheet of Whatman No. 3 filter paper soaked in 20 x SSC. The nylon filter, which had been presoaked in 2 x SSC, was laid on top of it. This was covered with three sheets of Whatman No.3 filter paper, the first having been soaked in 2x SSC and the other two being dry. A pile of paper towels and a 1Kg weight were then put on top and the apparatus left overnight for transfer of the DNA to occur.

The following morning the filter was rinsed with 2x SSC, blotted dry and baked at 80^{o}C for an hour. The filter was wetted with 2x SSC, before soaking it in a solution of 0.2% Denhardts, 1x SSC solution for 30 minutes at 65^{o}C. The filter was then washed in 0.2% Denhardts, 1x SSC, 50mg/ml herring sperm DNA, 0.1% SDS for 30 minutes at 65^{o}C. The filter was prehybridised for 30 minutes in 10-15ml in 0.2% Denhardts, 1x SSC, 50mg/ml herring sperm DNA, 0.1% SDS, 9% dextran sulphate and again incubated at 65^{o}C for 30 minutes. The relevant probe was added to the

containers and incubated overnight on rollers at 65°C. The next day the filter was washed at 65°C for 35 minutes in the following solutions: firstly, 2x SSC, 0.1% SDS, then 1x SSC, 0.1% SDS and finally twice with 0.1x SSC, 0.1% SDS. The filter was then blotted dry, wrapped in clingfilm and exposed to autoradiographic Kodak film for different lengths of time varying from overnight to one month at -70°C.

Polymerase Chain Reaction (PCR) to genotype cell lines

In order to distinguish homozygous null cell lines from those containing a wildtype copy of the T gene, the ES cell lines derived using DIA/LIF (BTBR 14 - BTBR 18) were genotyped using a PCR reaction. The presence of the *T* gene was detected using primers which amplified a 310bp region which includes the 3' coding region. The *T* primers used were:

CCA GTT GAC ACC GGT TGT TAC A

TAT CCC AGT CTC TGG TCT GT

As *T/T* cells lack this region, identification relies on absence of an amplified band. Therefore, *Hoxb-5* (previously Hox-*2.1;* Scott, 1992) amplification was used as a control to ensure that there was DNA in the original sample. The *Hoxb-5* primers were:

GCG CCA GTG CAG GGA AGA TTG GAA

GAT ATG ACT GGG CCA GAC GGA AA

These amplify most of the second exon to give a 350bp fragment spanning the homeodomain.

One microgram of DNA was PCR amplified in a final volume of 50µl containing 10µM of each dNTP, 1x PCR buffer (Boehringer), 1 unit *Taq* polymerase (Boehringer) and either 500ng *Hoxb-5* or 500ng *T* primers. The reaction mixtures were overlaid with mineral oil and amplified using a

Techne PHC-3 amplifier as follows: 5 minutes at 95°C then 25 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes and finally 20 minutes at 37°C. 10µl of the reaction was run into a 1.5% agarose gel prepared using Tris Borate/EDTA (TBE) electrophoresis buffer. If both *T* and *Hoxb-5* primers amplified a band, these were classified as *T/+* or *+/+*. Samples which had a *Hoxb-5* but lacked a *T* specific band were classed as *T/T*.

Tumour formation and analysis

4 independent cell lines which were on the same genetic background were used for this study: Two *Brachyury* homozygous lines, BTBR 6 and BTBR 10, one *Brachyury* heterozygous line, BTBR 1.3, and one wildtype line, BTBR 4.

Cells which were subconfluent on a $25cm^2$ tissue culture flask were used. Cells were trypsinised in the usual manner but then resuspended as a single cell suspension in 5mls of ES cell medium and centrifuged at 1000rpm. The supernatant was removed and the cells were resuspended in 0.5ml of culture medium without DIA/LIF.

Once the cells were prepared, isogenic BTBR/Pas males were anaesthetised with Avertin anaesthetic (2.5% (w/v) tribromoethyl alcohol ; 2.5% (v/v) tertiary amyl alcohol). The dose used was 0.02ml Avertin/gm body weight. When the animal was fully anaesthetised, he was placed under a low power dissecting microscope and both testes were exteriorised through an incision in the midline of the lower abdominal wall. A small hole was made into one testes capsule with a 26G syringe needle. Approximately 10^4-10^5 ES cells were injected through this hole in a tract under the testis capsule using a finely pulled pasteur pipette. The inoculation was then repeated with the other testis. Both testes were then

returned into the scrotal sac and the body wall was sutured and the skin wound sealed with a skinclip. The animal was left to recover on a heat pad.

3 or 4 weeks post inoculation the animal was culled and both testes removed. All testes were then fixed in Bouins fluid before dehydrating through an ethanol (Analar Grade: Hayman Ltd) series (70%,80%,87%,95%) with two 30 minute washes in each solution. These were followed by three 30 minute washes in 100% ethanol, one 30 minute wash of equal volume 100% ethanol :Histoclear (National Diagnostics). Before embedding in Histoplast paraffin wax (Shandon) the embryos had two 30 minute washes in Histoclear at room temperature followed by three 30 minute wax changes at 60^{o}C. 8μm serial sections were cut using an Anglia Scientific microtome and dried at 37^{o}C overnight. The following day the sections were dewaxed in xylene and counterstained with Masson's Trichrome stain before mounting under coverslips with DPX mountant (BDH). Every tenth section was examined for the different tissues (table 2.3 shows which tissues were scored). If a particular tissue was not found then every section in that tumour was examined to assess its presence or absence.

Results

Removal of ES cells from "live" feeders

ES cells removed from the feeders by the relative adhesion properties of feeders versus fibroblasts often appeared to divide less rapidly for 1-2 days after transfer onto DIA/LIF. However, all the BTBR lines, except one (BTBR 2) which was severely contaminated with "live" feeders, survived. After the initial period of slower growth, all the surviving ES cell lines as well as the BTBR lines derived directly into DIA/LIF

Figure 2.1

Morphology of BTBR blastocyst culture and primary ES cell colony.

A. 5 days culture of a single blastocyst on a gelatanised tissue culture grown in the presence of DIA/LIF. ICM has formed a mass of cells whilst the trophectoderm cells have spread out along the surface. Bar, 45μm.
B. Morphology of a primary ES cell colony 8 days after disaggregation of the cultured ICM. Bar, 45μm.

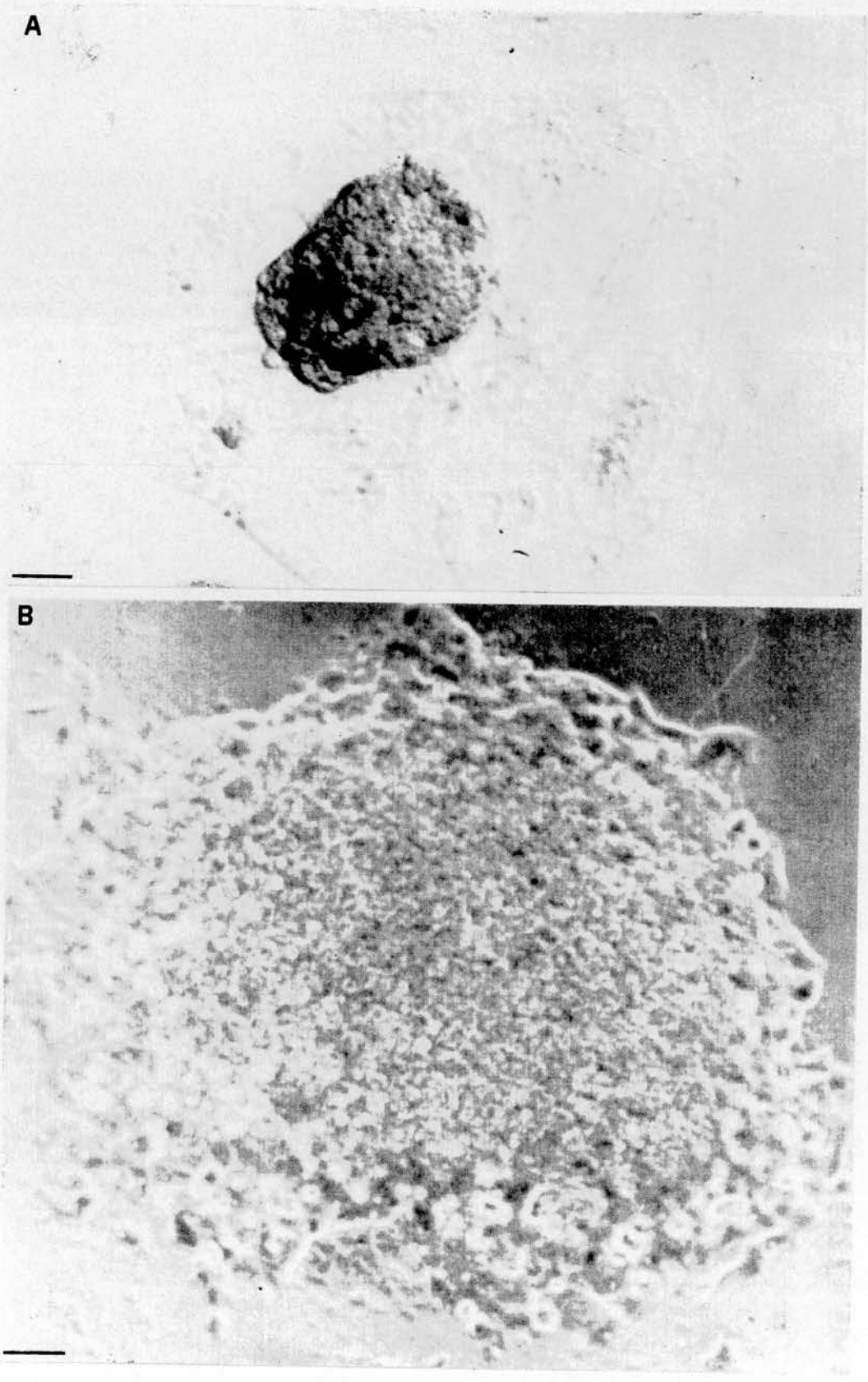

A
B

Table 2.1: Derivation of ES cells from BTBR *T/+* x *T/+* matings

Series	Number of Cultured Blastocysts	Primary Colonies (% of blastocysts)	Established cell lines (% of blastocysts)
On DIA/LIF	187	33 (17.6)	4.[+ 3†] (2.1)
On STO feeders*	433	72 (16.6)	12 [+ 2¥] (2.8)

* Data from Lesley Cooke
[†] Sublones BTBR14.2,BTBR15.2 and BTBR 16.2
[¥]subclones BTBR 1.2 and BTBR 1.3

supplemented media, when plated down with a split ratio of 1 in 10 required passaging every two to three days (i.e. approximately 3.5 generations per passage). These cells had a similar growth rate to a 129/Ola wildtype line (EFC-1), which had been derived and maintained on DIA/LIF (Nichols et al. 1990).

Using DIA/LIF, 4 separate ES cell lines, three of which were comprised of two sister clonal lines, were isolated from BTBR/Pas *T/+* matings. Table 2.1 gives the frequency of primary colonies and established ES cell lines isolated using ES cell culture media containing DIA/LIF and compares it with isolation of isogenic lines derived on feeder layers (data from Lesley Cooke). Blastocysts readily attached to gelatin-coated tissue culture plastic with the ICMs tending to remain central as the trophoblast cells grew out around them (Fig 2.1). After 5 days the ICMs were individually detached and dissociated and plated separately into 16mm wells. Some of the disaggregated ICMs in media supplemented with DIA/LIF produced primary stem cell colonies. These colonies had characteristic ES cell-like morphology, that is tight clusters of small rounded cells with a large nucleus and minimal cytoplasm (Fig 2.1). Once identified, the primary stem cell colonies were again subcultured after 7-11 days. A cell line was considered to be established once there were sufficient cells (~10^7) to allow aliquots to be frozen, that is at passage 4. The difference in numbers of primary cell colonies versus that of established cell lines was due to two factors. Firstly, several of the primary colonies failed to grow on passaging or underwent a significant degree of differentiation even in the presence of DIA/LIF. Secondly, there was a problem with contamination as the cells were grown in the absence of antibiotics. One of the major causal factors for loss of primary colonies appeared to be the difficulty in handling Leydon tubes (Nunc) which were

used as intermediate sized containers when the number of stem cells were being expanded out of 16mm well plates. The level of contamination dropped considerably when the stem cells were passaged into 25mm wells (Nunc) instead. Some blastocysts provided a second primary colony (eg BTBR 16.1 and BTBR 16.2). As these clones were produced from the same blastocyst they had to have the same genotype, but not necessarily the same karyotype.

Genotype analysis of BTBR cell lines.

BTBR 1 - 12 were genotyped with respect to *T* using p190R10RS which is a single copy 1.2kb fragment from the promoter region of the T gene (Herrmann et al 1990). As the *T* mutation is a deletion, this probe only hybridises to *T/+* or wildtype DNA which has been digested with *Taq1* restriction enzyme (Fig 2.2) To confirm that the DNA from the *T/T* lines was intact, the filter was rehybridised with p66MRT (Herrmann et al 1986; Herrmann et al 1990) which recognises flanking sequences on the centromeric(T66E and T66EII) and telomeric (T66D) regions flanking the *T* deletion (Fig 2.2). The p66MRT probe also acts an internal control to demonstrate how much DNA was loaded in each gel.

Not all the feeder layer cells had been removed from the BTBR lines when these Southern blots were performed. T-Gen also recognizes the T gene in the STO feeder cells which is polymorphic between the 129/Sv genetic background of BTBR/Pas and SIM, from which STO cells are derived. Consequently T-Gen identifies two bands in some of the lines.

Subsequently to differentiate *T/+* from wildtype lines, the DNA was genotyped using the flanking clone p119AR, This recognises a restriction length polymorphism (RFLP) closely linked to the *T* gene (Herrmann et al. 1990; Herrmann et al. 1990). Probed with p119AR, *BamH1* digestion of

Figure 2.2

Southern blot to determine the genotype of 5 BTBR ES cell lines (1)

The filter was first probed with p190R10RS which is a single-copy 1.2kb fragment from the promoter region of the T gene (T-Gen; Herrmann et al 1990)
The lower band is specific to the ES cells whereas the upper band is specific to the remaining STO feeder lines. (This probe recognises a polymorphism between SIM, from which STO cells are derived, and the 129/Sv genetic background of BTBR/Pas mice)

The blot was rehybridised with p66MRT which recognises flanking sequences on the centromeric (T66E and T66EII) and the telomeric (66D) sides of the T gene.

BTBR 6 and BTBR 10 are homozygous for the *T* deletion.
BTBR 4, BTBR 7 and BTBR 1.3 contain at least one copy of the wild-type *T* allele

Less DNA was loaded from the BTBR 7 cell line.
See Appendix (Rashbass et al. 1991) for a photograph of superior quality which demonstrates that BTBR 7 does contain at least one copy of T-Gen

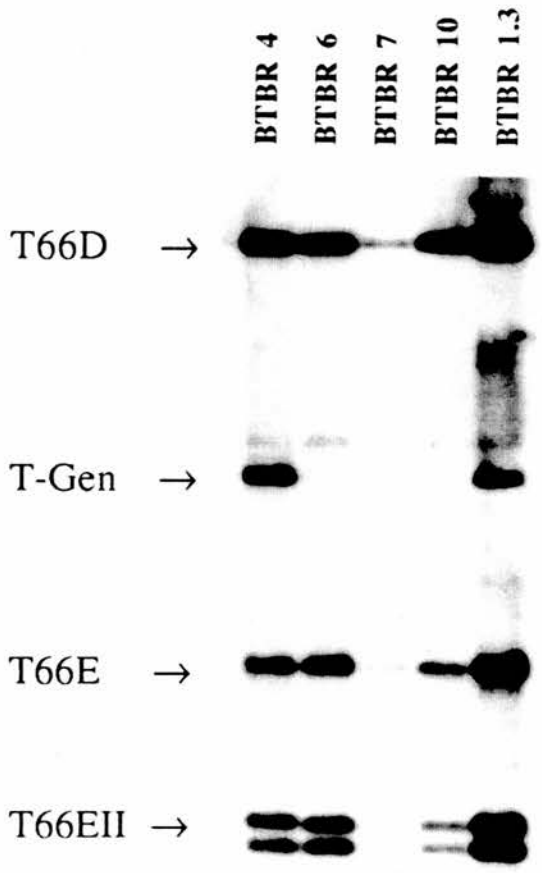

BTBR 4
BTBR 6
BTBR 7
BTBR 10
BTBR 1.3
T66D →
T-Gen →
T66E →
T66EII →

Figure 2.3

Southern blot to determine the genotype of BTBR ES cell lines (2)

The filter was probed with p119AR which is diagnostic for the deletion junction region (Herrmann et al. 1990). *BamH1* digestion of the *T* chromosome gives two diagnostic bands at 7 and 5.5kb,whereas the wildtype chromosome gives a single 9kb band. However, this probe also recognises a further 9kb fragment which is present in all the genomic samples. Thus both *T/T* and *T/+* lines have three bands, but the 9kb band in the *T/+* lines is twice as intense as that in *T/T* lines. Therefore this analysis also distinguishes between *T/T* and *T/+* lines.

A. Filter exposed to autoradiographic film overnight
B. Same filter exposed to autoradiographic film for 2 weeks

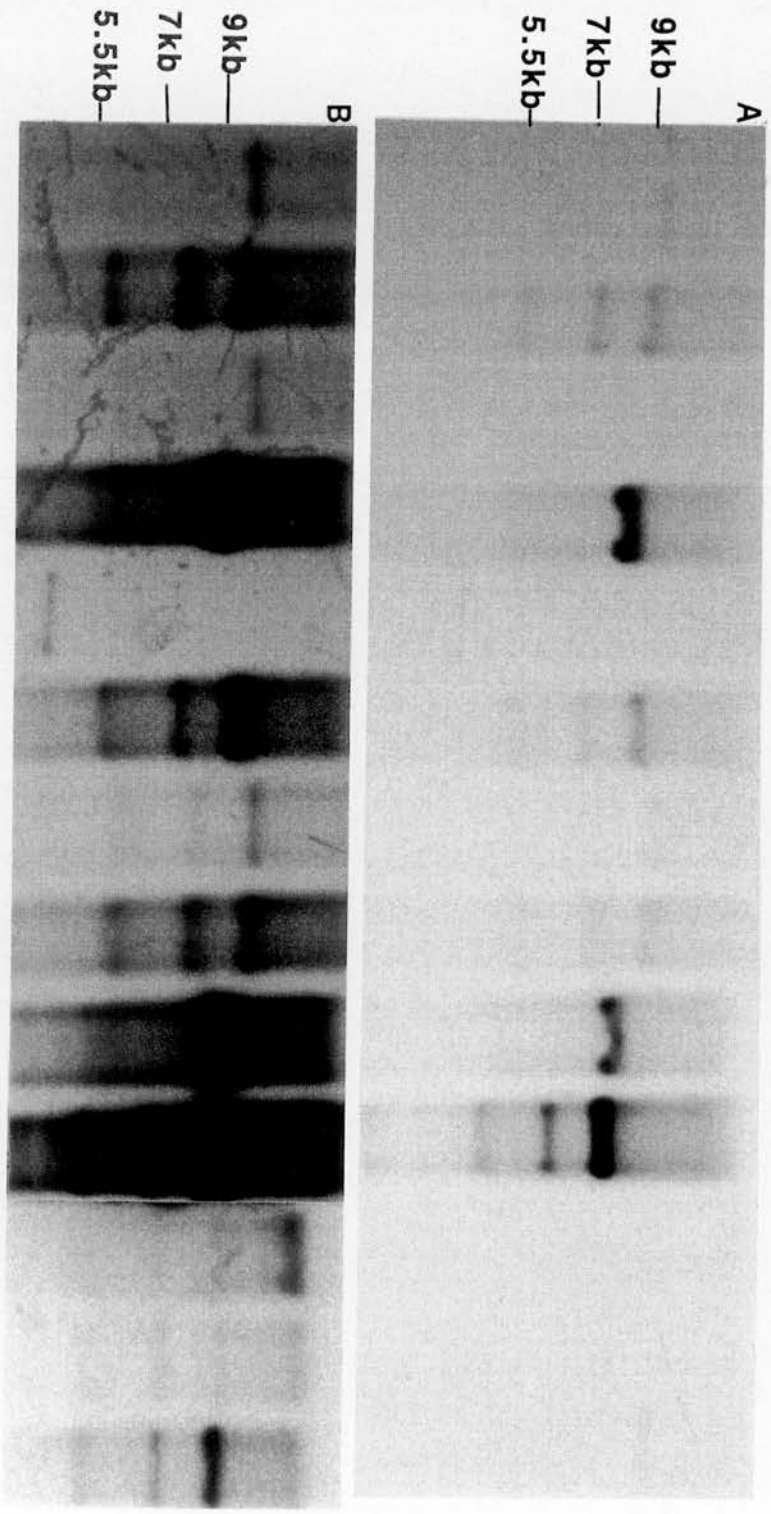
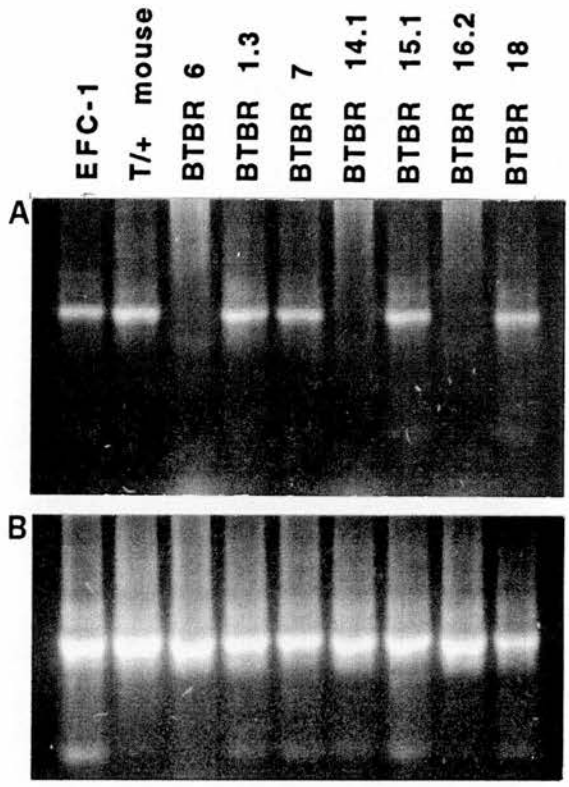

5.5kb
7kb
9kb
B
5.5kb
7kb
9kb
A
EFC 1 (+/+)
BTBR 6 (T/T)
BTBR 4 (+/+)
BTBR 4 (+/+)
λ MARKER
BTBR 8 (T/+)
BTBR 9 (T/+)
BTBR 15 (T/+)
BTBR 3 (+/+)
BTBR 5 (T/+)
BTBR 14.1 (T/T)
BTBR 16.2 (T/T)
BTBR 18 (T/+)

Figure 2.4

PCR determination of genotype of BTBR ES cell lines

A. 310bp PCR products run into a 1.5% gel to determine the presence or absence of a wildtype *T* gene in BTBR ES cell lines
B. 350bp PCR products amplifying a region of *Hoxb-5* acting as a control to ensure the presence of DNA in the original sample.

Presence of a band in both A and B indicates the presence of a wildtype *T* allele, whilst absence of a band in A but not B demonstrates that the cell line is homozygous
DNA from EFC-1 (a wildtype ES cell line) and the tail tip of a heterozygous *T/+* mouse act as positive controls
DNA from BTBR 6 acts a null control as this line had previously been shown to have a *T/T* genotype


EFC-1
T/+ mouse
BTBR 6
BTBR 1.3
BTBR 7
BTBR 14.1
BTBR 15.1
BTBR 16.2
BTBR 18
A
B

the *T* chromosome gives two diagnostic bands at 7 and 5.5kb,whereas the wildtype chromosome gives a single 9kb band. However, this probe also recognises a further 9kb fragment which is present in all the genomic samples (Fig 2.3). Therefore both *T/T* and *T/+* lines have three bands, but the 9kb band in the *T/+* lines is twice as intense as that in *T/T* lines. Therefore analysis with probe p119AR can also distinguish between *T/T* and *T/+* lines. In order to confirm the homozygous nature of BTBR 14.1, BTBR 6 and BTBR 16, samples were assayed using a polymerase chain reaction which detected the presence or absence of the *T* gene(Fig 2.4).

Combining the results from the Southern blots and PCR, 4 cell lines were wildtype (BTBR 3, BTBR 4, BTBR 11 and BTBR 12); 7 lines were heterozygous (BTBR 1.3, BTBR 5, BTBR 7, BTBR 8, BTBR 9, BTBR 15 and BTBR 18) whilst 4 lines were homozygous *T/T* lines (BTBR 6, BTBR 10, BTBR 14 and BTBR 16)

Chromosomal analysis

Chromosomal spreads for the majority of the BTBR lines which were examined for chromosomal abnormalities were prepared between passage 4 to 8. This was after the earlier lines (BTBR 1.3 to BTBR 12) had been removed from live feeders. These data are summarised on Table 2.2. Most of the lines contained significant populations of tetraploid and aneuploid cells. However, 4 of the lines (BTBR 1.3, BTBR 6, BTBR 7 and BTBR 15.1) were euploid in at least 55% of the chromosome spreads counted. BTBR 6 was 85% euploid at passage 6 and subsequent subclones of this line were 100% euploid (V. Wilson & L. Manson- personal communication) G- band analysis of 4 of the lines (BTBR 1.3, BTBR 6, BTBR 7 and BTBR 10) indicated that chromosome duplications in different cells was not uniform throughout the population, that is different

Table 2.2: Chromosomal number for different BTBR ES cell lines

Cell-line (genotype at *T* locus)	% spreads with diploid chromosome number	% of spreads with chromosome number other than 40			
		39	41	42	tetraploid
BTBR 1.3 # *(T/+)*	55	5	-	15	25
BTBR 6 * *(T/T)*	85	-	15	-	
BTBR 7 *(T/+)*	55	-	35		10
BTBR 10† *(T/T)*	20	-	-	65	15
BTBR 14.1‡ *(T/T)*	-	-	-	20	80
BTBR 14.2 *(T/T)*	20	50			25
BTBR 15.1 *(T/+)*	55	-	20		25
BTBR 15.2# *(T/+)*	30	5	-	60	5
BTBR 16.1# *(T/T)*	-	-	-	40	60
BTBR 16.2 *(T/T)*	40	50	-	-	10
BTBR18‡ *(T/+)*	-	-	-	-	100

Chromosome number was determined on ES cells between passage 4 and 8 except where indicated. 20 metaphase spreads were counted for each cell line except where indictaed.

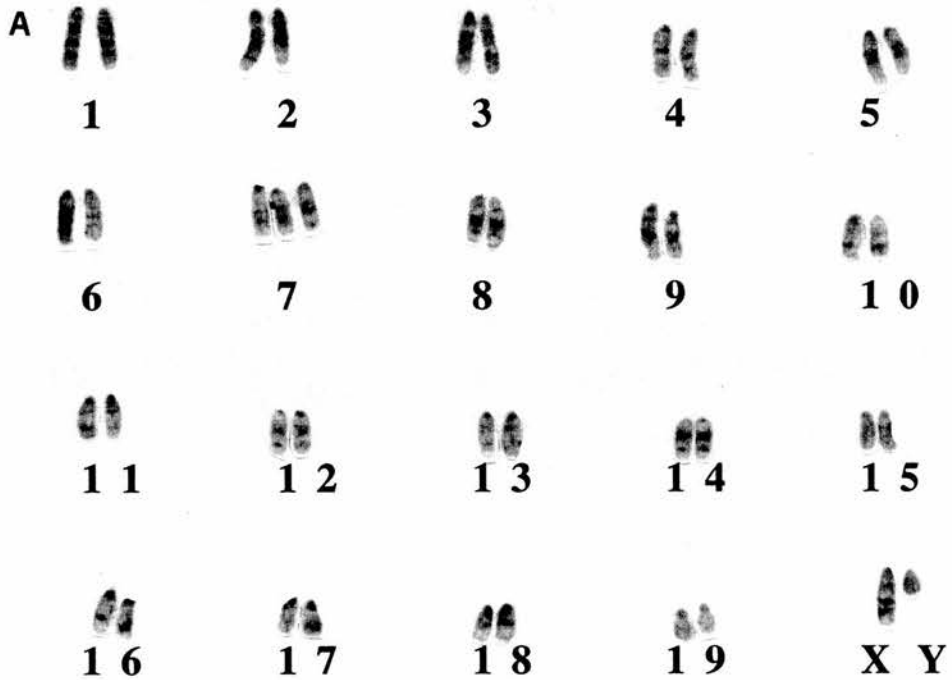
Chromosomes counted after 10 passages
† Chromosomes counted after 15 passages
‡ Only 10 spreads counted
*Subsequent subclones of BTBR6 were 100% euploid (V.Wilson &L.Manson, personnal communication)

Figure 2.5

G band analysis of two metapahase spreads of BTBR 7

BTBR 7 is a male cell line:

A. showing trisomy 7
B. showing trisomy 14


A
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17 18 19 X Y

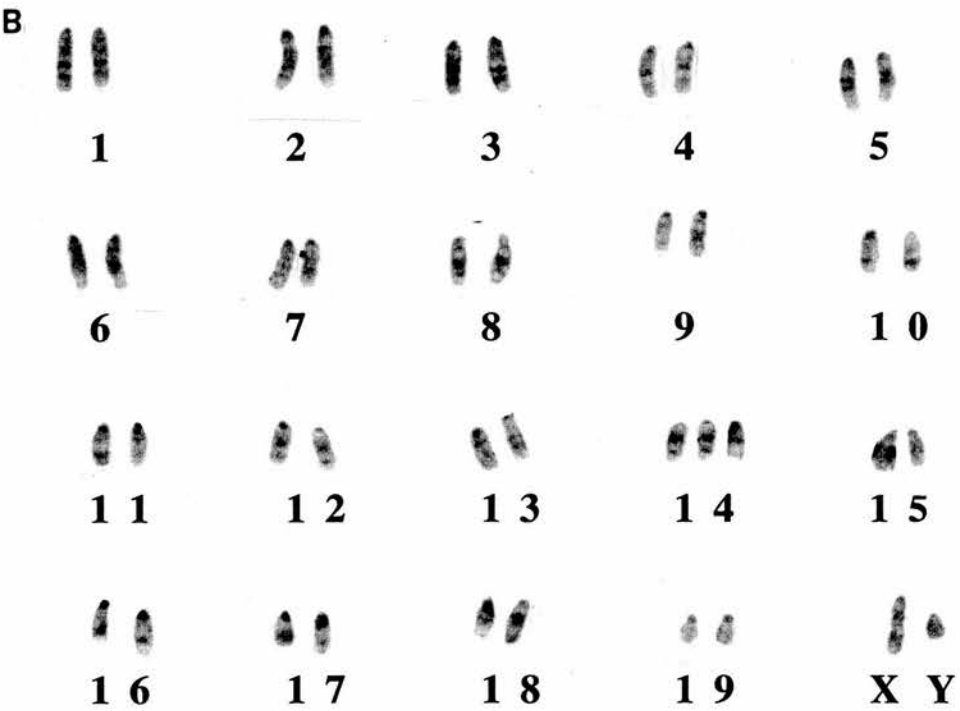
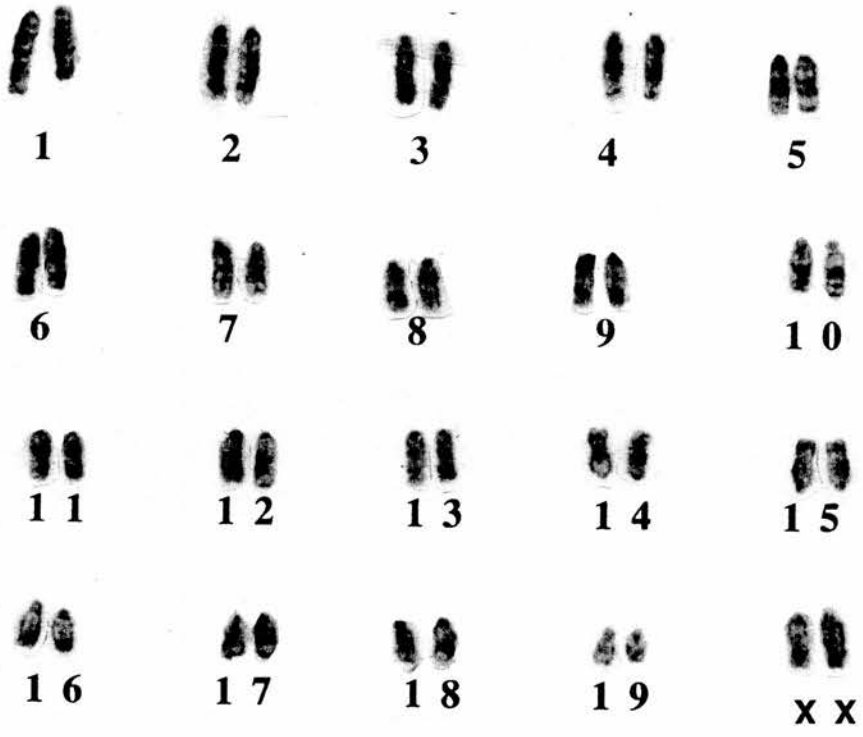

B
1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
16 17 18 19 X Y

Figure 2.6

G band analysis of metaphase spread of BTBR 15.1

BTBR 15 is a female cell line with a normal autosomal component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chromosomes were duplicated in different cells in the same passage. Figure 2.5 illustrates the G-band analysis of two chromosome spreads from BTBR 7, whilst a normal karyotype for BTBR 15.1 is shown in Figure 2.6

Tumour analysis

In order to determine that the BTBR ES cell lines could differentiate into tissues derived from all three germ lines, testes from isogenic males were inoculated with different genotypic BTBR ES cell lines. Following inoculation under the testis capsule, all the four lines tested, regardless of genotype formed teratocarcinomas as demonstrated by the presence of small undifferentiated embryonal carcinoma cells (Fig 2.7). Histological sections of these tumours were examined to determine the cell types present (Table 2.3). Both the heterozygous *T/+* (BTBR 1.3) and the wildtype (BTBR 4) cell lines produced tissues from all germ layers, as did one of the homozygous null lines (BTBR 10). However the other *T/T* line (BTBR 6) failed to produce tumours containing striated muscle and produced fewer tumours containing cartilage and bone.

Discussion

Derivation and Karyotype of lines

Combining the data obtained from deriving ES cell lines on STO feeder layers (from Lesley Cooke) and using DIA/LIF, the number of established lines from heterozygous *T/+* matings produced 4 *T/T*, 7 *T/+ and* 4 *+/+:* lines (excluding clonal cell lines) which is similar to the predicted frequency of 1 *T/T* : 2 *T/+* : 1 *+/+*. Therefore, there does not appear to be any selective pressure against *T/T* blastocysts themselves to form ES cells. However the relative frequency of primary colonies (17%) and established cell lines (2-3%) (excluding clonal cell lines) derived from the BTBR/Pas

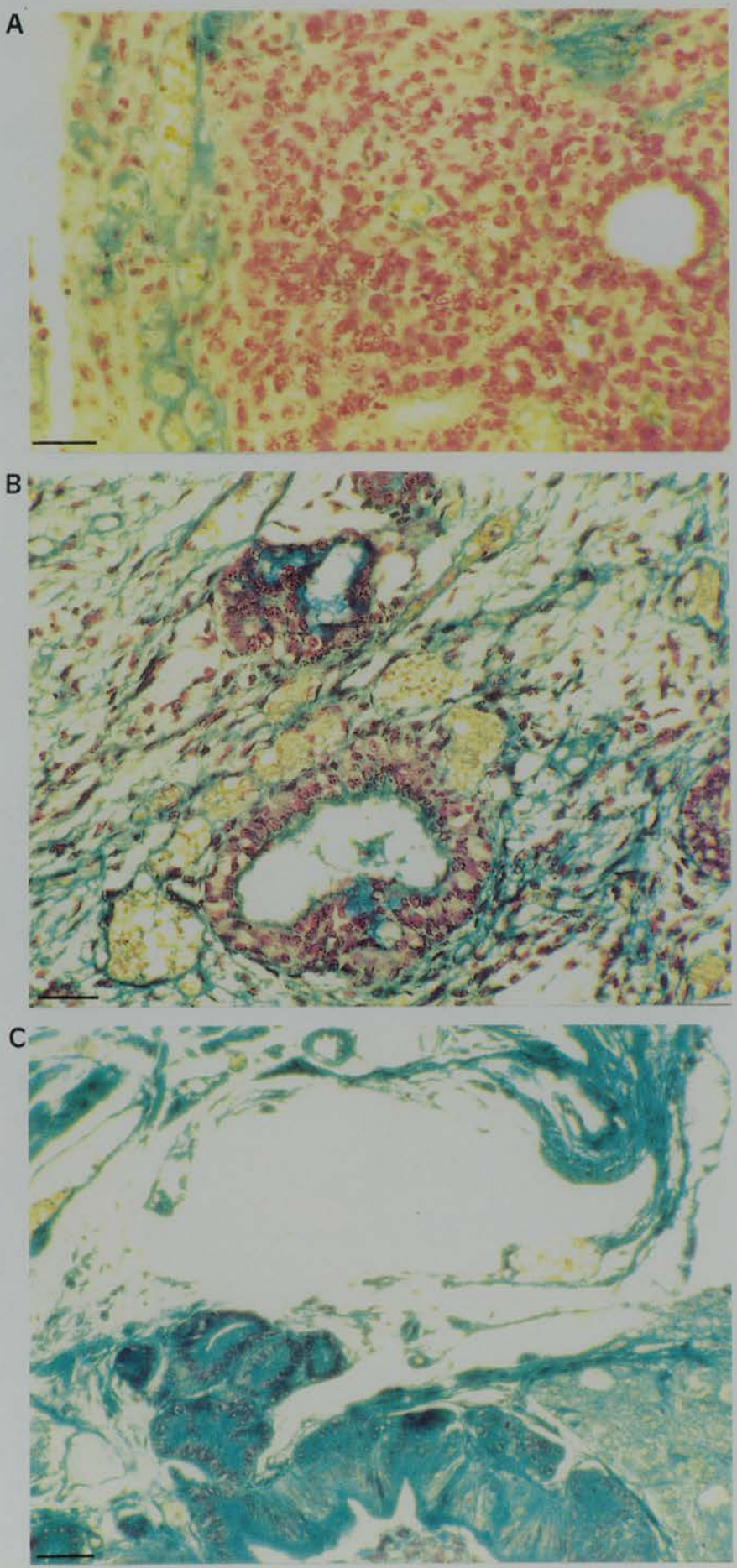
Table 2.3: Histogenic Potential of T/T, T/+ and +/+ ES cells in teratocarcinomas

Cell Line (genotype)	No. Testes analysed	No with tumours (% testes)	EC cells (% tumours)	Epithelial cysts (% tumours)	Neural tissue (% tumours)	Cartilage (% tumours)	Bone (% tumours)	Striated muscle (% tumours)	Keratinous epithelia or fibrous cysts (% tumours)
BTBR6 *(T/T)*	7	7 (100)	7 (100)	7 (100)	6 (86)	2 (29)	1 (14)	0 (0)	4 (57)
BTBR10 *(T/T)*	6	6 (100)	5 (83)	6 (100)	6 (100)	5 (83)	5 (83)	6 (100)	5 (83)
BTBR1.3 *(T/+)*	6	6 (100)	6 (100)	5 (83)	5 (83)	5 (83)	4 (66)	4 (66)	5 (83)
BTBR4 *(+/+)*	4	3 (75)	2 (67)	2 (67)	3 (100)	2 (67)	1 (33)	2 (67)	2 (67)

Figure 2.7

Sections of tumours formed by injecting BTBR ES cell lines into isogenic male testes

A. Embryonic carcinoma (EC) cells from a teratocarcinoma formed from BTBR 6 (*T/T*) ES cells. Bar. 100μm
B. Secretory epithelium and connective tissue from a tumour formed from BTBR 6 (*T/T*) ES cells. Bar. 200μm
C. Muscle and bronchial epithelia from a tumour formed from BTBR4 (T/+) ES cells. Bar. 200μm


A
B
C

blastocysts is lower than the 20-30% reported for isolating ES cell lines from blastocysts from the 129 strain of mice (Robertson 1987; Robertson et al. 1983; Nichols 1990). This may be somewhat surprising because, although ES cell lines produced from other inbred and outbred strains and from F1 embryos are usually obtained with a much lower frequency (Martin 1981; Robertson et al. 1983), the BTBR/Pas colony is on a 129/Sv genetic background. There may be two possible alternative explanations for this. First, the *Brachyury* mutation was first identified over 65 years ago in the Pasteur Institute in Paris. This constitutes at least 260 mouse generations. Thus, if the colony has been kept as a predominantly inbred one, it is highly likely that some of the genetic traits have changed over this period of time. On the other hand, if the BTBR/Pas colony has not been totally inbred, there is the likelihood that other strains have been introduced into the colony over the intervening years. This second alternative may be more probable as one of the sublines of our inbred agouti colony started to produce different coloured offspring in the third generation (data not shown). With further inbreeding of this subline and crossing onto mice with different coat colours, it became apparent that a pink-eyed dilution chinchilla (p , c^{ch})mouse had been introduced into the BTBR stock at some stage prior to our receiving the founder mice.

The growth rates of the *T/T* cell lines (BTBR 6 and BTBR 10) were also comparable to isogenic wildtype (BTBR 4) and another wildtype cell line (EFC-1) grown in parallel. This is not surprising since firstly, the abnormal phenotype is not apparent in *T/T* embryos until midgastrula tion (Chesley 1935; Gruneberg 1958) and secondly, no difference in the mitotic index of the intact mutant embryos has been observed (Yanagisawa and Fujimoto 1977b; Yanagisawa et al. 1981).

36% (4/11) of the lines analysed in this study were modally euploid with one cell line, BTBR 6, having a normal karyotype in 85% of the metaphase spreads (Table 2.2). A similar result (6/15 (40%)) was obtained by Nichols et al (1990) deriving lines using DIA/LIF with only one of their lines having an entirely normal karyotype, although subsequent lines derived using this method have been highly efficient at passing through the germ line (one in 6-8 blastocysts injected with CGR8 are germ-line chimaeras - J. Nichols and W. Skarnes - personal communication). Published data from Robertson and her colleagues (Robertson et al 1983; Robertson & Bradley, 1986) indicates that the majority (27 out of 35) of cell lines derived by this group directly onto feeder layers are initially euploid. Other workers have reported that not all ES cells differentiate normally and that relatively few exhibit high levels of germ line transmission (Frohman and Martin 1989). The poor frequency of the latter is ascribed to the presence of chromosomal aberrations although comprehensive karyotype data is not readily available. The subsequent experiments described in this thesis used cells at a low passage number and none of the cell lines used for these experiments (teratocarcinoma formation and chimaeric analysis) had the same chromosomal abnormalities as assessed by G-banding. Two independent null ES cell lines (BTBR 6 and BTBR 10), displayed similar properties in *T/T* ↔ *+/+* chimaeras (see chapter 3; Wilson, 1993) and mimicked intact *Brachyury* mutants, whereas *T/+* (BTBR 1.3) and *+/+* (BTBR 4) cell lines did not. Therefore, at least in these experiments the abnormalities observed are probably due to the absence of the *T* gene product in the null ES cells rather than any other chromosomal defect.

Tumour Formation

Teratomata and teratocarcinomas are tumours with cells types derived from all three germlayers. The former contain only differentiated cell types, while the latter contain a mixture of differentiated and undifferentiated cell types including multipotential transplantable embryonic carcinoma (EC) cells. These tumours can be generated by grafting 1 - 7.5 dpc embryos into an extrauterine site in a histocompatible host (Solter et al. 1979; Solter et al. 1980; Solter et al. 1981; Stevens 1970). They can also be produced by similar ectopic injections of ES cells (Robertson et al 1983). Thus, this technique permits one to assess the different cell types that a mutant ES cell line can contribute to. This provides useful information if, either the mutation of interest is an early embryonic lethal which causes death before all tissue types are differentiated or if mutant cells have a selective disadvantage in some tissues when mixed with wildtype cells to form a chimaera. Examples of this occur in chimaeras formed with parthenogenetic, androgenetic or trisomy 16 cells (Clarke et al. 1988; Cox et al. 1984; Thomson and Solter 1988a). However, neither notochord, nor allantoic differentiation, both of which are severely deranged in *T/T* mutants can be recognized by histological inspection of experimental teratomas. It should also be noted that certain tissues can be derived from more than one germ layer, for example bone, striated muscle and cartilage are derived from both mesoderm and ectoderm via neural crest cells. Therefore experiments of this type provide limited but potentially useful information.

The teratocarcinomas formed from one of the homozygous *T/T* lines (BTBR 6) were poorly differentiated, with a high contribution of EC cells and an overall low incidence of cartilage and bone formation They also failed to give rise to striated muscle. However, inoculations of a second

independent null *T* gene line (BTBR 10) produced well differentiated tumours which contained striated muscle, cartilage and bone, and were similar to tumours formed from the *T/+* (BTBR 1.3) and wildtype (BTBR 4) lines. Interestingly, other groups have reported similarly conflicting results from ectopic transfer of *T/T* embryonic fractions: one group have found decreased cartilage and bone formation (Fujimoto and Yanagisawa 1979) whilst another has not (Bennett et al. 1977). Therefore taken overall our results demonstrate that in general ES cells lacking the *T* product have the same histogenic potential as mutant homozygous *Brachyury* embryos (Bennett et al. 1977; Ephrussi 1935; Fujimoto and Yanagisawa 1979).

Chapter 3

Chimaeric analysis of a *T/T* (BTBR 6) and *T/+* (BTBR 1.3) ES cell line

Introduction

An intact mutant embryo identifies those genes necessary for normal pattern formation and tissue diversification. However, in the mouse, the observed abnormal phenotype of these mutants often masks the primary gene function by subsequent defective tissue interactions and cell death. Thus, although the Brachyury *(T)* gene is only expressed in the primitive streak, nascent mesoderm and notochord of the gastrulating mouse embryo (Wilkinson et al. 1990), homozygous null *T/T* mutants have a defective allantois, kinked neural tube and disorganised somites in addition to a bulky primitive streak and disrupted notochord. In *Drosophila*, one method that has been highly successful in determining not only whether a gene functions cell autonomously, but also in defining those regional subsets of gene expression that are essential for normal function has been mixing wildtype and mutant cells in a mosaic analysis (for example in ommatidium development; Rubin, 1991). Similarly, in the mouse, embryonic and haemopoietic chimaeric analyses of two developmental mutants with a similar phenotype, *Dominant White Spotting (W)* and *Steel (Sl)*, have determined the cell autonomous gene function of the former and the non-cell autonomous function of the latter (see Besmer, et al 1993). In this chapter, a chimaeric analysis mixing wildtype and *T/T* homozygous cells together was performed to assess whether the *T* gene product functions cell autonomously in the regions where it is expressed. However, in the mouse, chimaerism in all tissues can only be achieved by mixing cells in the preimplantation period either by morula aggregation or injection of inner cell mass (ICM) cells into a host blastocyst. At these stages, however, it is impossible to morphologically distinguish between *T/T*, *T/+* and *+/+* embryos. Therefore, in order to avoid laborious polymerase chain reaction assays on biopsies of individual embryos

(Handyside et al. 1990) or genotyping the chimaeras retrospectively, *T/T*, *T/+* and *+/+* embryonic stem (ES) cells which had already been genotyped (see chapter 2) were utilised instead of ICM cells.

The results described in this chapter are my own work. Corroborating evidence of these results, using independent *T/T* and *T/+* ES cell lines was obtained by V. Wilson. Subsequent experiments were performed in collaboration with V. Wilson and R. Beddington (see appendix: Wilson et al 1993). The bulk of this work was not done by myself and, therefore, not presented in the results section. However, the results are discussed as they further elucidate the role of *T* in mesoderm formation.

Materials and Methods

Formation of chimaeras

Preparation of ES cells for blastocyst injection

The ES cells lines were derived from blastocysts derived from mating heterozygous *T/+* BTBR/Pas mice together (see chapter 2). This mouse strain is on a 129/Sv agouti *GPI-1^a/GPI-1^a* genetic background. Two different lines: BTBR 6 (homozygous *T/T*) and BTBR 1.3 (heterozygous *T/+*) were used. These had been derived on Mitomycin-treated STO fibroblast feeders, but subsequently maintained using Differentiation Inhibitor Activity/Leukaemia Inhibitory Factor (DIA/LIF) in Dulbeccos Modified Eagle's Medium (DMEM-GIBCO) supplemented with 10% fetal and 5% newborn calf serum, non-essential amino acids and 2-mercaptoethanol (see chapter 2).

Cells which were subconfluent on a 25cm^2 tissue culture flask were used for blastocyst injection. 1 hour prior to injection the cells were refed with serum-supplemented DMEM without DIA/LIF. Immediately before injection the cells were washed once with phosphate buffered saline (PBS)

at room temperature and single cell suspension was prepared by adding 0.5mls trypsin (0.25% trypsin, 0.04M ethylenediaminetetra-acetic acid (EDTA)) to the flask and incubating for 4 minutes at 37°C. This digestion was stopped by adding 1ml culture medium without DIA/LIF. Following centrifugation for 5 minutes at 1000rpm, the supernatant was removed and the cells were resuspended in 0.3-0.5ml of culture medium, again without DIA/LIF. Small aliquots of ES cells were transferred immediately to the microinjection chamber for blastocyst injection.

Blastocyst injection and embryo transfer

Embryos for microinjection were obtained from matings between inbred non-agouti C57Bl/6 mice. This strain is homozygous for the *GPI-1^b* allele. Expanded blastocysts were recovered 3.5 days *post coitum* (dpc: morning of plug = 0.5 dpc) and cultured in drops of DMEM plus 10% fetal calf serum in a 37°C, 6% CO_2 humidified incubator. Groups of 3-5 blastocysts were transferred to hanging drops in a microinjection chamber containing PB1 (Whittingham, 1969) supplemented with 10% fetal calf serum. In order to reduce any stickiness, the chambers containing the blastocysts were then put at 4°C for 10-15 minutes whilst the ES cells were being prepared. A small number of ES cells were added either to the same drops as the blastocysts or else to neighbouring hanging drops. 10-20 ES cells were transferred into the blastocoel cavity using the method described by Bradley (1987) except that a flame-polished injection needle was used rather than a sharp point.

Following injection, the embryos were returned to serum-supplemented DMEM and incubated at 37°C, 6% CO_2 for 2-3 hours, after which time they were inspected using a dissecting microscope. Any embryos in which the ES cells had been excluded from the blastocoel

cavity (i.e. clearly visible between the trophectoderm and zona pellucida) were discarded. The remainder were transferred in groups of 6 to 8 into the uterine horns of pseudopregnant Swiss outbred (Pathology Oxford:PO) recipient females.

Analysis of chimaeras

Embryo dissection

Embryos were recovered 6 or 7 days after transfer to pseudopregnant recipients. After these periods of time, the transferred embryos were developmentally equivalent to untransferred 8.5 or 9.5 dpc embryos. The embryos were dissected free from the decidua and Reichert's membrane was removed. The visceral yolk sac was reflected and dissected away from the embryo to give a clear view of the morphology of the embryo and the allantois. After inspection for abnormalities' the allantois and embryonic regions of 8.5 dpc embryos were analysed separately for relative donor and host contributions by GPI analysis. The embryonic portion of the seven abnormal BTBR 6 ↔+/+ 9.25 and 9.5 dpc chimaeras was fixed in Bouin's fluid and processed for routine histology. The allantois and/or amnion from these embryos were removed for GPI analysis (see below).

Liveborn mice

Some injected blastocysts were allowed to go to term. Any pups which died perinatally were dissected into various tissues. These were the body skin, testis, kidney, liver, adrenals, spleen, intestine, leg muscle, heart, lungs, brain and tail skin. The samples were put into eppendorf

tubes and covered with PBS. Samples were then prepared for GPI assay (see below).

Animals which survived to weaning and beyond were assessed for ES cell contribution by the presence or absence of coat colour chimaerism and were examined externally for any abnormalities. After being photographed, the tail tip of two mice with kinked and slightly short tails from a *T/T* ↔ *+/+* set of injections was removed and assayed for *T/T* cell contribution using GPI electrophoresis.

GPI electrophoresis

As the ES cells used in this study do not contain a visible single cell marker, the relative contributions of donor ES cell versus host blastocyst in the embryo and allantois were determined using the different electrophoretic potentials of two isoenzymes, IA and IB, of the enzyme glucose phosphate isomerase (GPI).

Samples were placed separately in microdrops in 96 well plates (Nunc) under paraffin oil (Boots plc). They were frozen and thawed two or three times before loading up to 9 experimental samples in a parallel line onto Titan III cellulose acetate plates (Helena laboratories). Prior to use, these plates had been soaked for 20 - 60 minutes in buffer (25mM Tris, 200mM glycine). A control standard consisting of freeze/thawed blood which was a mixture of equal volumes of blood taken from GPI -1^a/GPI -1^a and GPI -1^b/GPI -1^b mice was also loaded. The plate was run at 200V for 1 hour at 4^{o}C with wicks trailing into reservoirs of buffer (25mM Tris, 200mM glycine). The plates were then removed and placed in freshly prepared stain (see Table 3.1) at room temperature in the dark for 5-15 minutes. When staining was complete the plates were fixed in 5% (v/v) acetic acid

Table 3.1: Recipe for GPI stain

Ingrdedient	Final concentration
Tris -HCl pH 8.0	80mM
Mg-acetate	10mM
Fructose 6- phosphate (F6P)	4mg/ml
NADP (Na_2 salt)	400mg/ml
phenazine methosulphate	50mg/ml
Methylthiazolium tetrazolium (MTT)	400mg/ml
Glucose-6- phosphate dehydrogenase (G6PD)*	0.28U/ml

*added just prior to use

(BDH) for 30 minutes before allowing them to air dry in the dark. Plates were subsequently stored in the dark as the stain is light sensitive.

Histology

After fixing overnight the embryos were dehydrated through an ethanol (Analar grade: Hayman Ltd) series (70%,80%,87%,95%) with two fifteen minute washes in each solution. This was followed by three 20 minute washes in 100% ethanol, one 20 minute wash of equal volume 100% ethanol :Histoclear (National Diagnostics). Before embedding in Histoplast paraffin wax (Shandon) the embryos had three 40 minute washes in Histoclear at room temperature followed by three 20 minute wax changes at 60°C The embryos were oriented in the wax using a dissecting microscope. Wax blocks were stored at 4°C until they were sectioned at 7-8 μm with an Anglia Scientific microtome. After drying overnight the sections were dewaxed in xylene (BDH) and counterstained with 0.5% (w/v) Eosin and 1.5% (w/v) Haematoxylin before being mounted under coverslips using DPX mountant (BDH).

Results

Midgestation embryos from *T/T* ↔ *+/+* and *T/+* ↔ *+/+* injections

Table 3.2 show the development and frequency of chimaerism of midgestation embryos derived from blastocysts injected with BTBR 6 or BTBR 1.3. The embryos dissected seven days after blastocyst injection were divided into two groups, 9.25 and 9.5 dpc, depending on the developmental stage of the normal littermates.

Table 3.2: Development and frequency of chimearism in c57Bl/6 blastocysts injected with BTBR 6 or BRBR 1.3 ES cells

Cell Line	Day recovered	Number transfered	Number implanted (% transfered)	Number resorbed (% implanted)	Number grossly retarded (% implanted)	Number correct embryonic stage (% implanted)	Number Normal embryos (% correct stage)	Number posteriorly abnormal embryos (% correct stage)	Number normal chimaeric (% normal)	Number abnormal chimaeric (% abnormal)
BTBR6 (T/T)	8.5,9.25 and 9.5	128	98 (76)	32 (32.7)	5* (5.1)	61 (62.4)	47 (77.0)	14 (23.0%)	1† (2.1%)	14 (100%)
BTBR1.3 (T/+)	9.25 and 9.5	42	27 (64)	12 (44.4)	0 (0)	15 (55.6)	15 (100)	0 (0)	7 (46.7)	0 (0)

* one grossly retarded embryo was chimaeric
† 5-10% chimaerism in the embryo only

Table 3.3: Extent of chimaerism and developmental characteristics of midgestation chimaeras

a: BTBR 6 *(T/T)* ‹-› +/+ Chimaeras

Chimaera No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Day of recovery	8.5	8.5	8.5	8.5	8.5	8.5	9.25	9.5	9.5	9.5	9.5	9.5	9.25	9.5
Somite number	7	7	6	2	7	6	7+	7	6	7+	7+	7+	15	7+
Abnormal Posterior	+	+	+	++	+	+	+++	+++	++	++	++	+++	-	++
Allantois morphology	+	++	+++	+++	++	+++	+++	+++	+	++	++	+++	-	++
%GPI 1a-embryo	10	25	60	60	30	60	50	ND	ND	ND	ND	ND	10	ND
%GPI 1a-allantois	10	50	60	ND	40	60	90	ND	10	ND	30	80	0	70
%GPI 1a-amnion	ND	ND	ND	ND	ND	ND	ND	100	ND	90	ND	80	ND	70

b: BTBR 1.3 *(T/+)* ‹-› +/+ Chimaeras

Chimaera No.	1	2	3	4	5	6	7
Day of recovery	9.25	9.25	9.25	9.25	9.25	9.5	9.5
Somite number	9	12	15	15	9	18	18
Abnormal Posterior	-	-	-	-	-	-	-
Allantois morphology	-	-	-	-	-	-	-
%GPI 1a-embryo	50	0	10	50	100	50	60
%GPI 1a-allantois	90	15	10	30	100	40	70
%GPI 1a-amnion	ND	ND	ND	ND	ND	ND	ND

Posterior abnormalities classified as follows:

+ thickened or asymmetrical primitive streak
++ kinked neural tube, disrupted somites, thickened primitive streak
+++ neural tubes and somites abnormal posterior to forelimb bud regions
- no abnormalitiy detected

Allantoic morphology classified as follows:

+ blebs on surface of allantois
++ blebs and branching morphology
+++ grossly reduced allantois with a tendency to spread over amnion
- no abnormality detected

ND Not Done

T/T↔+/+ blastocyst injections

All 14 abnormal embryos from BTBR 6 (*T/T*) injections had an anterior morphology that was indistinguishable from their littermates. However, they all had varying degrees of abnormalities in their caudal region and allantois. GPI analysis revealed that all these embryos were chimaeric (Tables 3.2 and 3.3). At all stages examined, the extent of *T/T* ES cell contribution in the allantois appeared to correlate with the severity of the morphological abnormalities in this tissue (Table 3.3). One apparently normal 9.25 dpc embryo proved to be slightly chimaeric with an ES cell contribution in the embryo of less than 10% (Chimaera No. 13 Table 3.2a). However, the allantois of this normal chimaera contained only cells derived from the host blastocyst. All the other normal-looking embryos were not chimaeric, that is they had no ES cell contribution as assessed by GPI analysis

The extent of development at 8.5 dpc meant that in most cases only the primitive streak and allantoic morphology could be accurately scored. Thus, at 8.5 dpc, the most distinctive feature that the chimaeric embryos possessed was an abnormal allantois (Fig 3.1). In the most severe cases, the allantois was severely stunted with a tendency to spread across the amnion (Fig 3.1D). In other abnormal embryos the atypical allantoic morphology consisted of either "branches" and/or surface blebs (Fig 3.1B,C). The embryos themselves had a thickened or asymmetrical primitive streak and in some more severe and slightly developmentally-advanced cases the neural tube was kinked as well.

At 9.25 and 9.5 dpc, again the chimaeric embryos looked normal anterior to the forelimb bud region (Fig 3.2). However, they had fewer somites than would be expected for the stage of cranial development.

Figure 3.1

Allantoic abnormalities in 8.5dpc *T/T* ↔+/+ chimaeras

A. Non-chimaeric 8.5dpc embryo. Bar,350μm
B. BTBR 6 ↔ +/+ Chimaera 1 (table 3.3a) Embryo appears normal but there is a distinctive "bobble" (arrow) on the end of the allantois. Bar,300μm
C. BTBR 6↔ +/+ Chimaera 2 (table 3.3a) with "branched" (arrow) allantois. Bar,350μm
D. BTBR 6 ↔ +/+ Chimaera 3 (table 3.3a) with stunted allantois spreading over the amnion (arrow).Bar,350μm

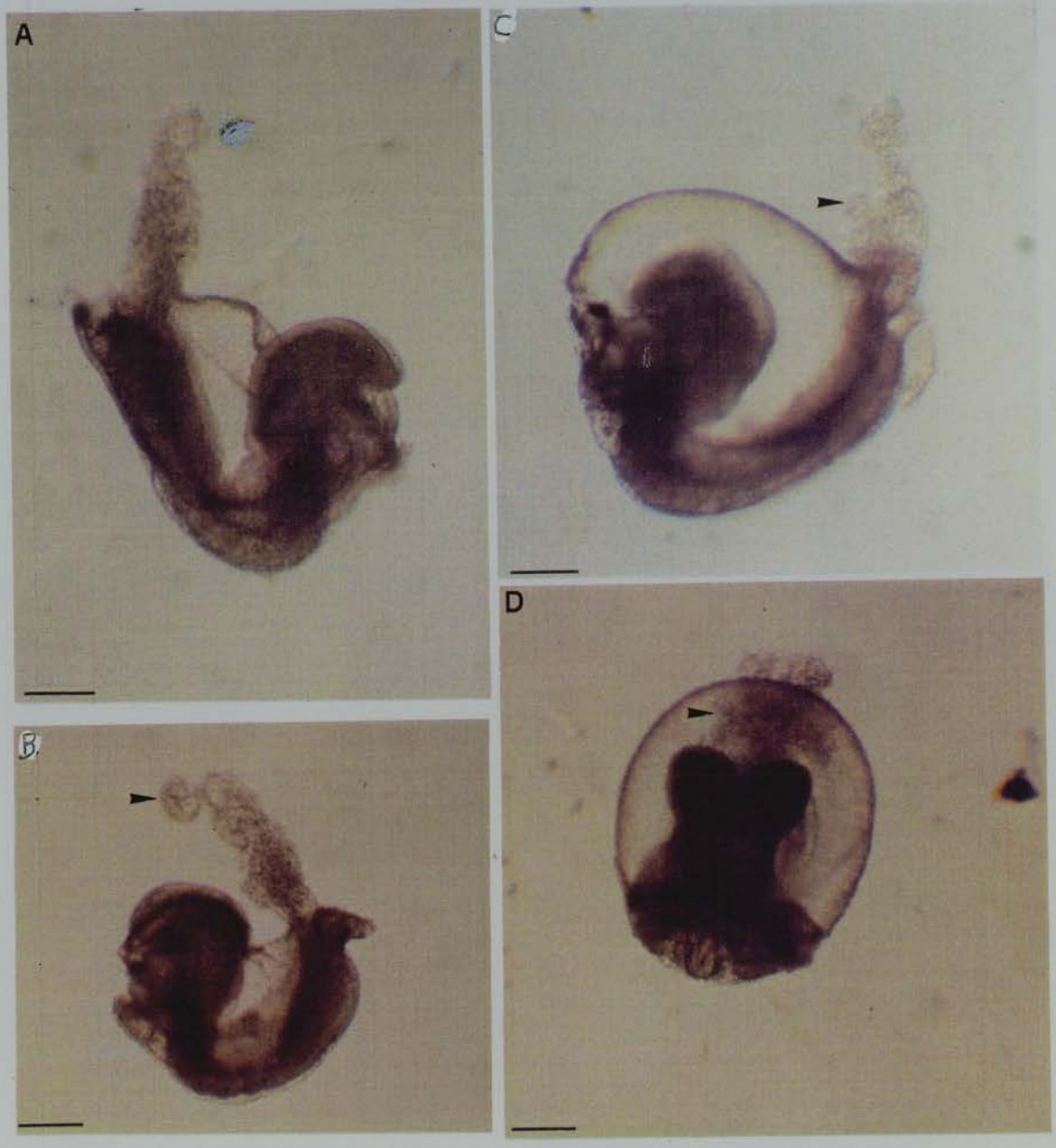

A
C
B
D

Figure 3.2

Development of 9.25 and 9.5 dpc *T/T* and *T/+* chimaeras.

A. 9.5 dpc *T/T* (left) and normal littermate. Homozygous mutant looks normal anterior to somite 7 (open arrow head), but caudally displays a kinked neural tube, lack of somites and a very rudimentary allantois. Bar, 550μm

B. 9.5dpc *T/+* ↔+/+ Chimaera No 6 (Table 3.3b). The embryo appears entirely normal and the allantois has fused with the chorion. Bar, 500μm

C. 9.25dpc *T/+* ↔+/+ Chimaera No 5 (Table 3.3a). The rostral end is normal but posterior to somite 7 the trunk and caudal region are abnormal. The primitive region is enlarged and there is only a minimal allantois. Bar, 500μm

D. 9.5dpc *T/+* ↔+/+ Chimaera No 8 (Table 3.3a) looking very similar to a *T/T* mutant, with an abnormal caudal region characterised by very convoluted neural tube, indistinct somites and rudimentary allantois. Bar, 350μm

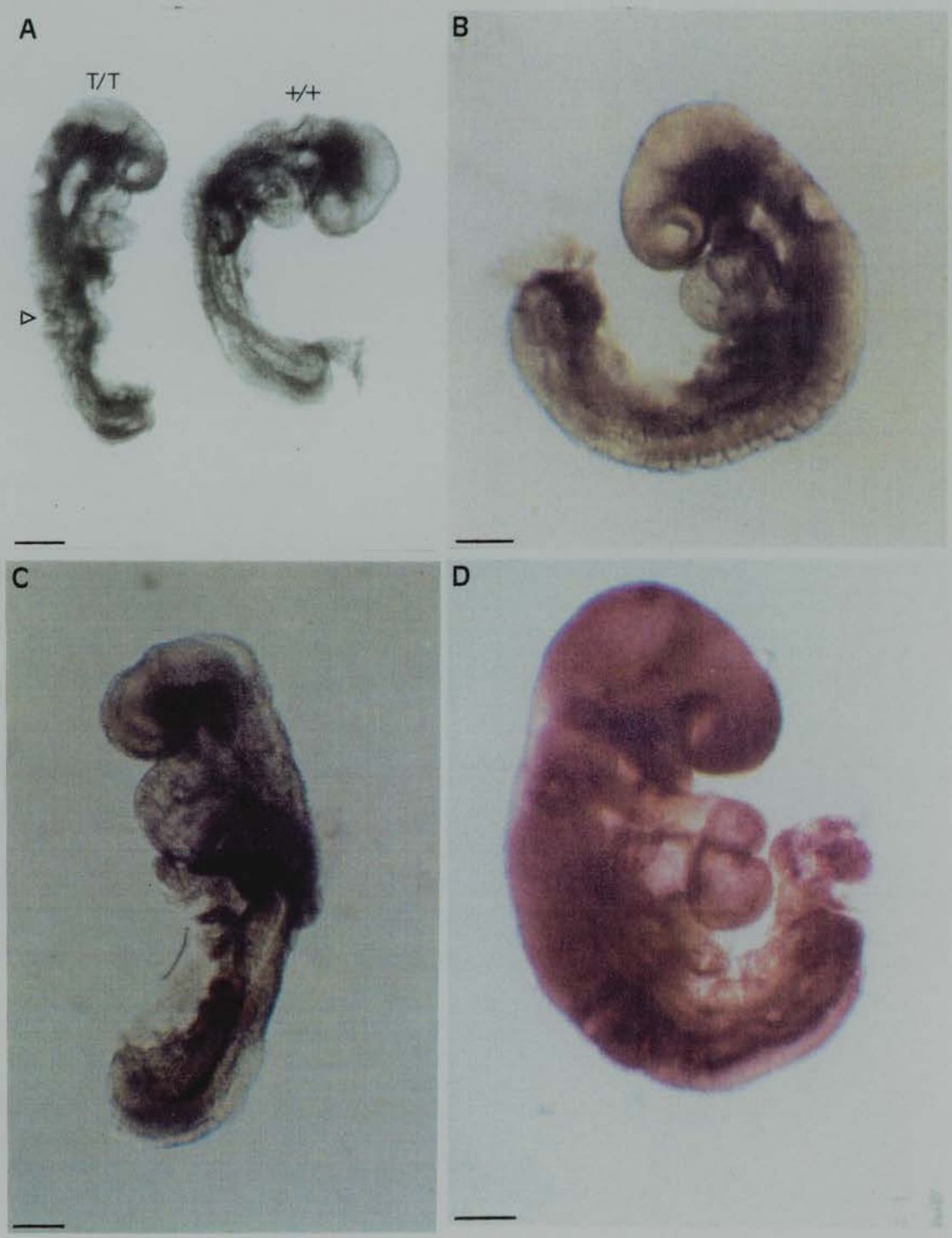

A
T/T
+/+
B
C
D

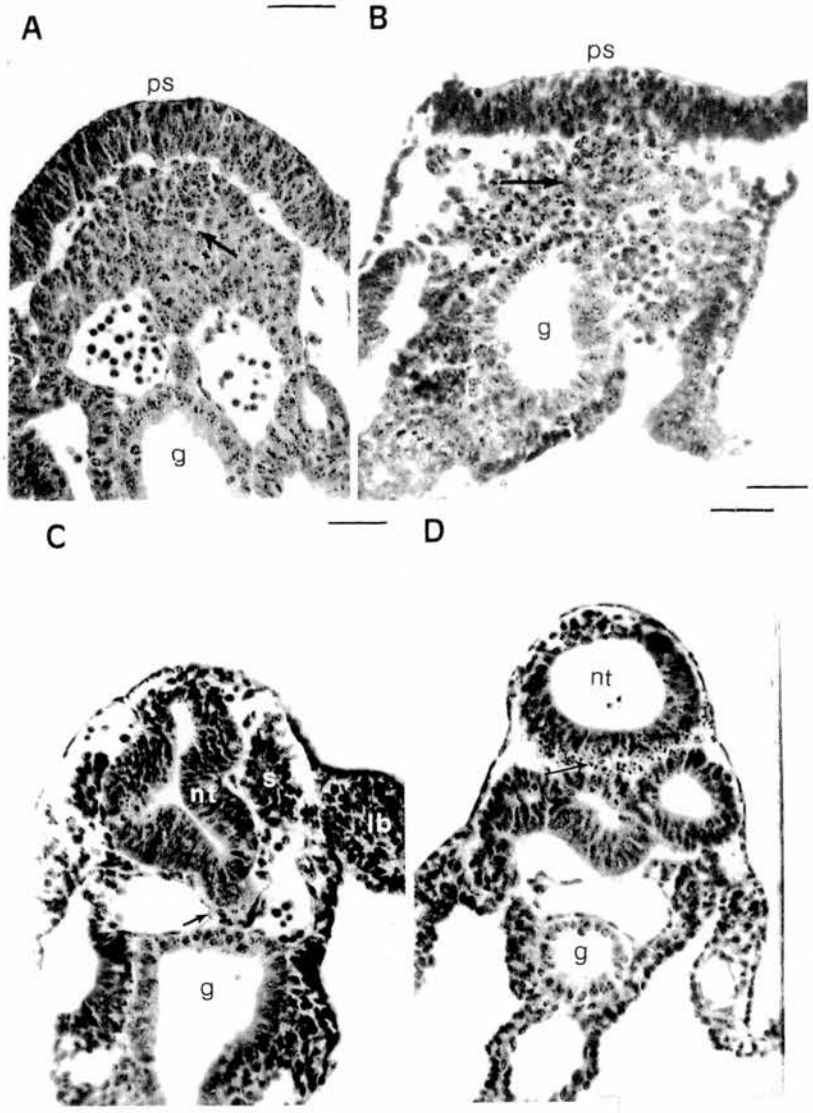
Figure 3.3

Transverse sections through the caudal region of BTBR 6 (*T/T*) ↔+/+ chimaeras

A. 8.5 dpc chimaera showing area of abnormal mass of cells (arrow) situated underneath primitive streak. Bar 40μm
B. 8.5 dpc chimaera. Pyknotic cells are evident in a discrete region (arrow) beneath the primitive streak. Bar 40μm
C. Transverse section at the level of the limb bud in a 9.5dpc chimaera. The neural tube is convoluted and the somites are not properly formed. There are pyknotic cells in the putative notochord (arrow) which is fused with the neural tube. Bar 70μm
D. 9.5dpc chimaera. Pyknotic cells are evident underneath the neural tube (arrow). There is no discernible notochord. Bar 70μm

Abbreviations

g	gut
ps	primitive streak
nt	neural tube
lb	limb bud
s	somite


A
B
ps
ps
g
g
C
D
nt
s
lb
nt
g
g

Also the somites caudal to the seventh appeared irregular in both shape and distribution. The posterior third of the embryo, including the primitive streak region, was severely disrupted and the neural tube caudal to the forelimb bud region was severely kinked. All the abnormal chimaeric embryos at these stages except one had only a minimal allantois. The one exception had only a 10% *T/T* ES cell contribution in the allantois and morphologically had "blebs" on this structure (Chimera No. 9 Table 3.3).

Histological sections of the caudal regions of six 9.25 - 9.5 dpc abnormal embryos showed that the notochord was either discontinuous or contained pyknotic cells (Fig 3.3). Dead cells were also present in the nascent mesoderm emerging from the primitive streak. However, dead cells were not prominent in sections of the abnormal allantoides from chimaeras 4, 8 and 10.

Typically, the phenotype of the *T/T* ↔ *+/+* chimaeras resembles that of the intact *T/T* mutant. Chimaeric embryos with greater than 70% contribution of *T/T* ES cells as judged by GPI isoenzyme activity appear to be almost indistinguishable from intact mutants. However, the disruption to the neural tube and the somites is more variable in chimaeric embryos with less than 70% *T/T* cell contribution, but these embryos do have an abnormal allantois and thickened primitive streak.

T/+ ↔ *+/+* blastocyst injections

The embryos that were injected with BTBR 1.3 *(T/+)* ES cells were recovered only at seven days post transfer. All fifteen were morphologically normal, although 7 were chimaeric (Table 3.2). One of the chimaeric embryos showed no trace of the host blastocyst GPI isoenzyme in the embryo or allantois (Chimaera 5 Table 3.3b)

Table 3.4: Summary of BTBR 6 *(T/T)* ↔ +/+ and BTBR 1.3 *(T/+)* ↔ +/+ liveborn chimaeras

Cell Line	Number transfered	Number born (% transfered)	Number anterior normal (% born)	Number short or kinked tails (% born)	Number abnormal chimaeric (% abnormal)	Number normal chimaeric (% normal)
BTBR6 *(T/T)*	42	19 (45.2)	19 (100)	4# (21.1)	1† (25)	0 (0)
BTBR1.3 *(T/+)*	52	27 (51.9)	26‡ (96.3)	2 (3.7)	2* (100)	6* (24)

two short tailed pups died shortly after birth

‡ one BTBR 1.3 (T/+) ↔ +/+ female with a short tail also had an abnormal head

†-only 5% by GPI analysis in tail skin of one short tailed pup which died shortly after birth

*-detected by coat colour chimaerism

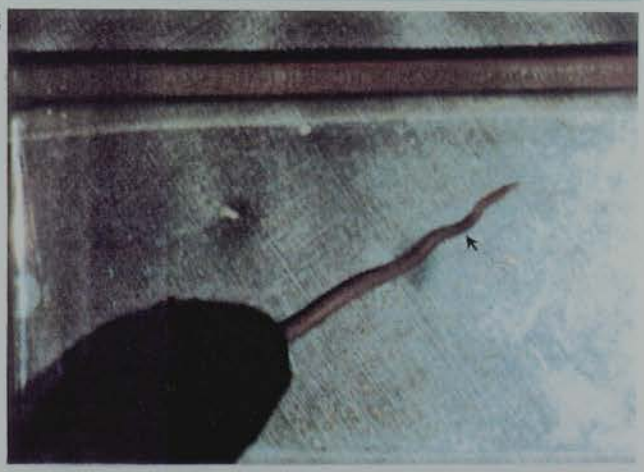
Figure 3.4

Liveborn *T/T* and *T/+* chimaeras

A. The caudal end of three newborn pups derived from injection of BTBR 6(*T/T*)ES cells into C57Bl/6 blastocysts. These died within two days of birth. Two pups have very short curly tail (arrows) but only one had a trace of T/T ES cell contribution in the tail skin
B. Slightly short and kinked tail (arrow) in an adult mouse derived from injection of BTBR 6*T/T* ES cells into a wildtype blastocyst which had no evidence of coat color chimaerism.
C. Female chimaera from injection of BTBR 1.3 (*T/+)* ES cells into a C57Bl/6 blastocyst showing an abnormal asymmetrical head and micropthalmia on the left. She also lacked her upper teeth and had a short, kinked tail.

A

B



C

Liveborn mice from *T/T* ↔ *+/+* and *T/+* ↔ *+/+* injections

Liveborn pups from blastocyst injection of BTBR 6*T/T* and BTBR 1.3 *T/+* ES cells exhibited a characteristic and distinctive phenotype (Table 3.4; Fig 3.4)

T/T ↔ *+/+* injections

Five individuals derived from injection of BTBR 6*T/T* ES cells died within three days of birth, of these two had a very short curly tail (Fig 3.4A). GPI analysis of a variety of organs, including tail, skin, testis, kidney, liver and muscle indicated that there was no *T/T* ES cell contribution except for a trace (5% by GPI analysis) in the tail skin of one of the short-tailed neonates. No coat colour chimaerism was detected in any of the animals derived from blastocyst injection of the *T/T* ES cells which survived to adulthood. However, two of these had slightly shortened kinked tails compared to their littermates (Fig 3.4B). There was no detectable *T/T* ES cell contribution in the tail tip of these animals as assessed by GPI analysis.

T/+ ↔ *+/+* injections

In contrast to the homozygous ES cell injections, liveborn young from injection of BTBR 1.3 *T/+* gave rise to animals which were chimaeric by coat colour. Two of these had kinked and short tails of approximately 2/3 normal length and had white flashes on their bellies. One female chimaera with a short tail also had an abnormal asymmetrical head which was smaller on the left (Fig 3.4C). She also had micropthalmia on this side and lacked her upper teeth. However, she survived for almost two years on a diet of mash with occasional trimming of her lower teeth to stop overgrowth. No pups with absent tails were born from this series of

injections. None of the chimaeras demonstrated germline transmission of the ES cell genotype

Discussion

Midgestation and liveborn *T/+* ↔*+/+* chimaeras

9.5 dpc *T/+* ↔*+/+* chimaeric embryos are indistinguishable from non-chimaeric embryos even when there is no evidence of host cell contribution. At a similar stage of development, *T/+* embryos also have a normal phenotype. In contrast, at term, two chimaeric animals had short tails similar to heterozygous *Brachyury* mutants. Viable *T/+* chimaeras with a similar short tail phenotype have been produced by morula aggregation in experiments designed to rescue the maternally lethal T^{hp} deletion (Bennett 1978). However one of the chimaeras derived from *T/+* ES cells also had an abnormal head and both had white flashes on their chest. Furthermore liveborn coat colour chimaeras produced from blastocyst injection of another independent *T/+* ES cell line had normal length tails (Wilson et al. 1993). Thus, although it is likely that the short tail was caused by lack of one *T* allele, it is not possible to categorically state that this is the cause, especially as the BTBR 1.3 cell line was not 100% euploid (Table 2.2)

Midgestation and liveborn *T/T* ↔*+/+* chimaeras

Similar results to those described in this chapter have also been obtained with another independent *T/T* line (Wilson et al. 1993) and none of the described defects have been observed with wildtype ES cell lines (Beddington and Robertson 1989; Wilson et al. 1993) or *T/+* cell lines. Thus it seems likely that the abnormalities observed in the BTBR 6 ↔ +/+

chimaeras are due to the absence of the *T* gene product in the BTBR 6 ES cell line.

The abnormalities observed in the *T/T* ↔*+/+* midgestation chimaeras mimics those seen in the intact null *T* mutant. Therefore, the results in this chapter demonstrate that the effects of the *Brachyury* mutation cannot be rescued by wildtype cells in *T/T* ↔*+/+* chimaeras. The discontinuous notochord, bulky primitive streak and presence of pyknotic cells in the notochord and primitive streak of 9.25 and 9.5 dpc BTBR 6 *T/T* ↔*+/+* chimaeras indicates that the T gene product acts cell autonomously in these tissues and may also be required for the cell survival and maintenance of the correct morphology of these structures. If the abnormalities in the mutant animals were solely due to an abnormality in the extracellular matrix of *T/T* embryos (Jacobs-Cohen et al. 1983a), then the phenotype should have been at least partially rescued in *T/T* ↔ chimaeras.

Recently the cell autonomy of *ntl*, the zebrafish homologue of *Brachyury*, has also been demonstrated (Halpern et al. 1993). In these experiments donor cells were marked with lineage tracer dyes. Halpern and colleagues showed that the *ntl* mutant environment supports the correct induction and maintenance of Ntl protein expression in wildtype cells. Also wildtype cells transplanted to the dorsal side of the blastula margin were later able to differentiate into notochordal cells in mutant hosts. In contrast *ntl* mutant cells were never found to contribute to notochord in wildtype hosts. However transplanted cells derived from *ntl* donors were noted outside the notochord at positions where the wildtype host displayed a kinked notochord or bent axis. These results strongly suggest that expression of the Ntl protein is an obligatory step in

notochordal differentiation because *ntl* cells fail to be recruited into wild-type notochord.

The studies described in this chapter have now been extended to examine chimaeras with a lower *T/T* cell contribution later in gestation (Wilson et al. 1993; see appendix). Between 9.5 and 11.5 dpc over 85% of the abnormal embryos were chimaeric. There was a range of defects apparent in these embryos varying from localised defects in the allantois or distal tail region which included truncation, branching or abnormal blood filled sacs through to a very severe phenotype similar to intact dying homozygous mutants. Dissections of these embryos into different axial regions and separation of the neurectoderm and surface ectoderm from the adjacent paraxial mesoderm and forelimb buds allowed assessment of the relative GPI contribution to each region. The germ layer separation differentiates between those cells which have invaginated through and migrated away from the primitive streak (the somites and limb buds) and those that have not (the neurectoderm). The distribution of *T/T* ES cell descendants was markedly skewed along the anteroposterior axis and between the ectoderm and mesoderm derivatives: such that the *T/T* cell contribution was increased in the tail region compared to that of the head, and also increased in the neural tube and surface ectoderm as compared to the mesoderm from the same anteroposterior axial level. Therefore, it appears that *T/T* cells accumulate at the caudal end of the embryo because they fail to migrate efficiently away from the streak after invagination during gastrulation. This suggests that nascent mesoderm cells require T product in order to ingress normally and move efficiently from their site of origin. This migratory disability is possibly related to the changes in galactosyltransferase (which is expressed on the cell surface of migratory cells activity; Shur 1989) as spatial and temporal differences in

the activity of cell surface galactosyltransferase in *T/T* embryos compared to wildtype embryos have been described (Shur 1982). The migratory disability of *T* deficient mesoderm cells may also be partly caused by changes in integrin activity as the *Brachyury* phenotype is similar to that observed in embryos lacking α_5 integrin (Yang et al. 1993).

In $T/T \leftrightarrow {}^{+/+}_{\wedge}$chimaeras, the actual migratory defect of the mutant cells may only be mild because there is still a substantial contribution of *T/T* cells to the paraxial mesoderm. However, with time, the gradual accumulation of *T/T* cells at the caudal end means they make a higher contribution to the tail bud region when it begins to form during the early forelimb bud stage.

The abnormalities described in 9.5-11.5 dpc indicate that chimaeras with a large *T/T* contribution die at a similar stage as intact *T/T* mutants and only chimaeras with a low *T/T* contribution, which was usually only at the caudal end, survive to term (Wilson et al. 1993). This may explain the surprising results observed in the liveborn described in this chapter. We found that 4 of the animals left to go to term had a short or deformed tail, but only one of these had any evidence of mutant *T/T* cell contribution as demonstrated by a trace of GPI-1A activity in the tail remnant. This apparent anomaly may be caused by degeneration of the chimaeric distal tail region between 11.5 dpc and term rather than these animals having less than 5% *T/T* cells contribution which would not be detected by the GPI assay.

Although the *T* gene is expressed throughout the primitive streak, its absence in intact homozygous *Brachyury* mutants does not completely inhibit mesoderm formation or gastrulation: structures anterior to the forelimb bud are essentially normal. Injection of the *Xenopus Brachyury* homologue, *Xbra* mRNA into *Xenopus* eggs leads to inhibition of head formation, the frequent development of a second tail, differentiation of

ectopic mesoderm and the induction of *Xhox-3*, a gene normally expressed highly only in the posterior end of the embryo (Cunliffe and Smith 1992). Thus it appears that *T* is required for the formation of caudal tissue. In terms of embryogenesis, therefore, the mutant *T/T* phenotype might in part be explained as a subtle defect in the movement of mesoderm cells, that leads to a gradual buildup of tissue beneath the primitive streak which then causes a mechanical block to gastrulation. In chimaeras with a low contribution of mutant cells or in *T/+* animals with an overall decrease of T product, such failure of migration prevents correct formation and function of a tail bud.

T/T ↔*+/+* chimaeras, with a high contribution of mutant cells, as described in this chapter, also mimic the phenotype of the intact *Brachyury* mutant in the malformations seen in tissues which do not express *T*, that is the allantois, neural tube and somites. If *T* expression is required in the precursor cells as they invaginate through the streak, then it is possible that the abnormalities seen in the somites and allantois are also caused by a cell autonomous effect of the T protein. On the other hand, the somite abnormalities may be caused by aberrant signals from the notochord. The kinked neural tube, which is a characteristic feature in both intact *T/T* mutants and *T/T* ↔*+/+* chimaeras may also be a secondary defect as neither this tissue nor its precursors pass through the primitive streak. There are several explanations as to how this secondary defect could occur, none of which are exclusive. Firstly, the absence of notochord and the reduced amount of mesoderm and somitic tissue flanking the neural tube may reduce the structural support of the neural tube. Alternatively, the failure of the embryonic axis to elongate normally could lead to a concertina effect on the neural tube if the latter tissue continues to elongate in *T/T* mutants and *T/T* ↔*+/+* chimaeras similar to that reported for *curly tail*

mutant embryos (Brook et al. 1991; Copp et al. 1988). Finally, the neurectoderm may be deprived of important signals normally emanating from cells which do express the *T* gene, for example the notochord. If this third reason is correct, then critical threshold effects must operate because wildtype cells cannot rescue this signal to the neurectoderm in *T/T* ↔*+/+* chimaeras.

The allantois is consistently disrupted in *T/T* ↔*+/+* chimaeras, such that the severity of allantoic malformation correlates with the relative contribution of *T/T* cells compared to wildtype cells. However, the malformations observed cannot be explained solely by cell death as there is no evidence of increased cell pyknosis in the chimaeric allantoides, nor is there any apparent selective pressure against recruitment of *T/T* cells into the allantois (Table 3.3). The preference of the chimaeric allantois to spread over the amnion rather than transverse the exocoelom as a coherent structure, suggests that the absence of the *T* product somehow alters the behaviour of this tissue. This observation agrees with other evidence that *T/T* cells have altered adhesion and/or migratory properties (Hashimoto et al. 1987; Yanagisawa and Fujimoto 1977a). *T/T* mesoderm in culture exhibits significantly reduced motility on ECM substrates (Hashimoto et al. 1987) and aggregates formed in rotation culture from either the anterior or posterior regions of *T/T* embryos are consistently smaller than those formed by normal embryos (Yanagisawa and Fujimoto 1977a). As the *T* gene product is probably a transcription factor (Herrmann et al. 1990; Schulte-Merker et al, 1992; Kispert and Herrmann 1993,), then the behavioural alterations of the allantois in *T/T* ↔*+/+* chimaeras suggests that the *T* gene product is an intracellular prerequisite for normal allantoic differentiation perhaps playing a critical role upstream of a signalling pathway required to specify the character of allantoic cells.

Chapter 4

Alterations in gene expression in *Brachyury* embryos

Introduction

The *T* gene is expressed in the primitive streak, the notochord and the ectoderm and mesoderm cells immediately surrounding the primitive streak (Wilkinson et al. 1990). Embryos lacking the *T* gene product start gastrulation apparently normally but develop a distinctive phenotype at about 8.5 dpc. By 9.5 dpc, although *T/T* embryos are grossly normal rostral to the forelimb bud region (somite 7 or 8), they have a foreshortened rostrocaudal axis, a bulky primitive streak, disrupted notochord, indistinct somites and a severely kinked neural tube (Fujimoto and Yanagisawa 1983; Gluecksohn-Schonheimer 1944; Gruneberg 1958; Spiegelman 1976). All these abnormalities are more severe in the posterior part of the embryo. The expression pattern of *T* and the null homozygous phenotype, suggest that the *T* gene product may play a role in both mesoderm formation and axial patterning. Similarly, the severe lethal *T/T* phenotype suggests that no other gene can act in its place. Therefore, the aim of this chapter was to use the *Brachyury* mutant to assess the role of the *T* gene product in mesoderm formation and axial patterning. Wholemount *in situ* hybridisations were used to compare the expression patterns in *T/T* and control embryos of other genes putatively involved in the same processes.

Mesoderm formation

The question can be asked where does *T* lie in the hierarchy of gene expression necessary for mesoderm formation? Any gene acting downstream of *T* will have an altered expression pattern in null *T* mutants. Conversely, the expression domain of any gene acting upstream or independently of *T* will remain unchanged. In order to start addressing this problem, one must find other genes involved in the same morphogenetic

pathway. Thus the genes, chosen for this comparison need to fulfill two criteria: not only do they have to be co-expressed with *T* in the posterior part of the embryo during gastrulation in the mouse, they also have to potentially play a role in mesoderm formation. At present there is no assay available in the mouse to test the latter criterion. However, it is possible to use *Xenopus laevis* embryos to search for genes which when ectopically expressed induce mesoderm. This mesoderm induction assay involves changes in the fate of cells taken from the animal cap of *Xenopus* blastula embryos. These cells do not normally contribute significantly to mesoderm derivatives, but usually form epidermal and neural structures (Dale and Slack 1987). It is possible however, to induce mesoderm formation in animal caps by either incubating isolated animal caps with certain growth factors (reviewed by Smith, 1989) or by injecting specific blastomeres of earlier intact embryos with mRNA of genes putatively involved in mesoderm specification. 4 genes (*BMP-4, Evx-1, Wnt-3a* and *Wnt-5a*) which fulfill those criteria were chosen for this study.

Injection of the *Xenopus Brachyury* homologue (*Xbra*) mRNA into the animal poles of single-cell *Xenopus* embryos leads to the normal initiation of gastrulation, but the marginal zone mesoderm fails to involute and an excess of posterior mesoderm is formed (Cunliffe and Smith 1992). This aberrant gastrulation is associated with the induction of the *Xenopus* homologue of *Evx-1 (Xhox-3). Xhox-3* is a putative transcription factor normally expressed in posterior mesoderm (Bastian and Gruss 1990; Dush and Martin 1992; Ruiz i Altaba and Melton 1989). Injection of *Xhox-3* into the animal pole of early *Xenopus* embryos results in severely deformed anterior structures, but does not induce ectopic mesoderm formation (Dush and Martin 1992; Ruiz i Altaba and Melton 1989). On the other hand, ectopic expression of *XBMP-4*, the *Xenopus* homologue of *BMP-4* (Jones

et al. 1991) disrupts gastrulation movements and forms posteroventral mesoderm (Dale et al. 1992; Jones et al. 1992). This is associated with *Xhox-3* expression and transient induction of *Xbra* transcription. *Wnt-3a* and *Wnt-5a*, together with *Xwnt-8*, belong to a large family of presumed secreted proteins which show homolgy to *Wnt-1* (Christian et al. 1991; Gavin et al. 1990). Injection of *Xwnt-8* mRNA into the vegetal pole of single cell *Xenopus* embryos or into the ventral blastomeres of 16 to 32 cell embryos produces duplication of the embryonic axis and an alteration of the fate of ventral cells such that they differentiate into dorsal rather than ventral mesoderm (Christian et al. 1991; Smith and Harland 1991; Sokol et al. 1991). However, if *Xwnt-8* is expressed after the midblastula stage, its effects are reversed and instead it ventralises mesoderm (Christian and Moon 1993). From the endogenous expression pattern of *Xwnt-8*, it is probable that the late expression of *Xenopus* mimics its normal role.

Axial patterning

It is also possible to use the same experimental approach to study the role of the *T* gene product in patterning the rostrocaudal, mediolateral and dorsoventral axes. However alterations in axial pattern in *T* mutants may stem not so much from a direct effect of the absence of the *T* gene product impairing a signal pathway, but rather from its importance in the production or maintenance of signalling tissues. In particular the role of the notochord in neural and somitic patterning can be evaluated in *T/T* embryos where the notochord is severely disorganised or absent.

The expression domains of the different genes were studied at both 8.5 dpc when the morphological null *T* phenotype first becomes macroscopically apparent and at 9.5 dpc when the *T/T* phenotype is more severe.

Rostrocaudal axis.

The *Hox* genes may play a role in identifying unique positions along the rostrocaudal axis (Holland and Hogan 1988; McGinnis and Krumlauf 1992). The anterior boundary of *Hoxa-7* (formerly known as *Hox-1.1*) expression in paraxial mesoderm coincides with the 14th somite, whilst in the neurectoderm the boundary is further rostral and adjacent to the 8th somite. Consequently the rostral expression of this gene coincides with the level along the anteroposterior axis that the first severe phenotypic changes in *T/T* embryos are observed (Mahon et al. 1988; Püschel et al. 1990; Püschel et al. 1991). However, the *Hoxa-7* riboprobe failed to work in my hands, therefore, a different strategy was employed. This utilised Tgm6lacZ1 mice (Püschel et al. 1990) which contain a *lacZ* reporter gene under the control of 5' *Hoxa-7* sequences. Embryos display ß-galactosidase activity with the same spatiotemporal expression as endogenous *Hoxa-7* with respect to the establishment of correct anterior boundaries in neuroectoderm and paraxial mesoderm (Püschel et al. 1990). A breeding programme was set up to obtain both *T/T* and control embryos which contained the transgene and would, therefore, following staining for ß-galactosidase activity, show the anterior boundary of *Hoxa-7* expression.

Mediolateral patterning

In wildtype embryos, there are obvious differences in the medial tissues compared to lateral ones: for example neurectoderm forms only in the midline ectoderm, notochord in midline mesoderm and somites in the paraxial mesoderm. The molecular and cellular interactions that specify this divergence of tissue fate are poorly understood, although the node and notochord are both implicated (for example Beddington 1994; Goulding et

al. 1993; Hornbruch and Wolpert 1986; Placzek et al. 1990; Yamada et al. 1991). As the primitive streak and notochord are both affected in the *Brachyury* homozygous embryo, it is possible that the patterning of the mediolateral axis is also perturbed. *Msx-1* (formerly known as *Hox-7.1*) was chosen as a marker of this axis, because at 8.5 dpc it is expressed in the lateral ectoderm and mesoderm, but not in somites or notochord (Robert et al. 1989).

Dorsoventral patterning of neural tube and somites

The neural tube and somites are two tissues that are severely affected in the *Brachyury* mutant (Chesley 1935; Gruneberg 1958). The severity of malformations increases in an anteroposterior gradient. The notochord has been implicated in patterning the dorsoventral axis of these structures in both chick and *Xenopus* embryos (Aoyama and Asamoto 1988; Bassler et al. 1993; Clarke et al. 1991; Ordahl and Le Douarin 1992; Rong et al. 1992; Teillet and. Le Douarin 1983; van Straaten et al. 1988; Yamada et al. 1993) and therefore one might expect that this axis is also affected in *T/T* embryos. *Pax-1* (Deutsch et al. 1988), *Pax-3* (Goulding et al. 1991), *Pax-6* (Walther and Gruss 1991) and *Msx-1* (Robert et al. 1989) have been shown to have specific expression domains along the dorsoventral axis of the neural tube and/or somites and were therefore used in this study.

Materials and Methods

Embryo collection

Heterozygous (*T/+*) BTBR/Pas mice were mated together over night. The midpoint of the dark cycle (midnight) was assumed to be the time of

mating. Embryos were recovered at 7.5,8.5 and 9.5 days *post coitum* (dpc: morning of plug = 0.5 dpc).

8.5 and 9.5 embryo collection

For 8.5 and 9.5d embryos most of the extraembryonic membranes and amnion were removed. The homozygous (*T/T*) embryos were identified by their morphological phenotype and segregated into a separate group. The control group of embryos consisted of the rest of the litter and, therefore, contained both heterozygous (*T/+*) and wildtype (*+/+*) embryos as it is not possible to distinguish these phenotypically at these stages of development. The 9.5 dpc embryos had small holes punctured in the heart, fore- and hind-brain using a finely pulled pasteur pipette to prevent trapping of the probe. All the embryos were fixed overnight in freshly prepared 4% paraformaldehyde (PFA, Fisons) in phosphate buffered saline (PBS) at 4°C. In order to ensure that control and homozygous *T/T* embryos were treated with exactly the same conditions they were grouped together in the same tube during the wholemount *in-situ* hybridisation procedure.

7.5 dpc embryo collection and genotyping embryos by polymerase chain reaction *(PCR)*

At 7.5d it is not possible to macroscopically distinguish homozygous *T/T* embryos. Therefore the polymerase chain reaction (PCR) was used to identify homozygous and control embryos. 7.5d embryos were dissected free from the decidua and Reichert's membrane and the ectoplacental cone were removed. Using watchmakers' forceps, the embryo was divided into embryonic and extraembryonic portions and the amnion, which was included in the embryonic portion was pierced with a fine glass

needle. The embryonic and extraembryonic portions were placed into matched wells in two separate 96 well plates (Nunc). The embryonic portion was fixed in 4% PFA overnight at 4°C whilst the extraembryonic portion was placed in 20µl PBS and the polymerase chain reaction was used on this tissue to genotype the embryos.

The same PCR reaction was used as described in chapter 2, that is each sample was tested for the presence of *T* whilst using a parallel reaction which amplified the homeobox region of *Hoxb-5*. as a positive control to ensure that there was DNA in the original sample. However, due to the small amount of tissue, the preparation of the samples for PCR was slightly different from that described previously and was as follows. 1µg/µl proteinase K (Boehringer) was added to each well. The wells were covered with mineral oil (Sigma) and the 96 well plate was incubated at 55°C for 3-4 hours until the tissue had partially disintegrated. The plates were then placed at 95°C for 20 minutes to degrade the proteinase K and denature the DNA. Two 5µl aliquots of this solution were PCR amplified in a final volume of 50µl containing 10mM of each dNTP, 1x PCR buffer (Boehringer), 2 units Taq polymerase (Boehringer) and either 500ng *Hoxb-5* or 500ng *T* primers. The reactions were overlaid with mineral oil and amplified using a Techne PHC-3 amplifier as follows: 5 minutes at 95°C then 30 cycles of 94°C for 1 minute, 60°C for 1 minute, 70°C for 2 minutes and finally 20 minutes at 37°C. 10µl of the reaction was run into a 1.5% agarose gel prepared using Tris Borate/EDTA (TBE) electrophoresis buffer. If both *T* and *Hoxb-5* primers amplified a band, these were classified as *T/+* or *+/+*. Samples which had a *Hoxb-5* but lacked a *T* specific band were classed as *T/T*. Samples which produced any other combination of bands were discarded. Corresponding embryos were grouped into either *T/T* or control (*T/+* or *+/+*) pools and taken through

Table 4.1: Details of probes used

PROBE	PLASMID NAME	FRAGMENT SIZE -(approx bp)	FRAGMENT DETAILS	PARENT VECTOR	ENZYME USED TO LINEARISE PLASMID	RNA POLYMERASE USED	RECEIVED FROM
BMP-4	pSP72/ BMP-4 5'	1000	5'coding region of BMP-4 cDNA	pSP72	Eco RI	Sp6	Mike Jones & Brigid Hogan
Evx-1	pAB11	700	non-coding 3' region	Bluescript	Hind III	T7	Mike Dush
Hox-7.1	Pλ2.2	1200	3'end coding region extendint into 3' untranslated region	pTZ19	Bss HII	T7	Robert Hill
Pax-1	pmprd Hinc11-Sac-1	310	genomic DNA incl. mouse "paired" box		Hind III	T7	Peter Gruss
Pax-3	pc pax3 PstI/ Hind III	520	3'end cDNA incl part of homeonox	Bluescript KS	Hind III	T7	Peter Gruss
Pax-6	pSM-1	1600	3'end of paired box running into 3' untranslated region	Bluescript SK	Xba I	T7	Robert Hill
T	pme75	800	3' untranslated region	Bluesscript SK	EcoR1	T7	Bernhard Herrmann
Wnt-3a	Wnt-3a	750	non-coding 3' region	pgem 32f	Eco RI	T7	Andy McMahon & Jill McMahon
Wnt-5a	373 wnt-5a	360	PCR fragment from 9.5d cDNA	pgem 32f	Eco RI	Sp6	Andy McMahon & Jill McMahon

the wholemount *in situ* procedure simultaneously but in separate containers.

Wholemount *in situ* hybridisation

Plasmids and Probes

The different probes used at the various embryonic stages are shown in Table 4.1. Each experiment also included embryos probed with α-actin and *T* as control for the efficacy of the in *situ* hybridisation. Table 4.2 gives the details of the different probes used. The plasmids were isolated from transformed bacteria using a Qiagen plasmid maxi kit, and linearised with the appropriate restriction enzyme (see Table 4.1). The linearised plasmid was phenol-chloroform extracted to remove impurities.

The antisense riboprobes were transcribed using the appropriate RNA polymerase (see Table 4.1). The probes were synthesised by incubating 1-3μg linearised template DNA with 1x ribonucleotide (NTP) mix (1mM ATP, 1mM CTP, 1mM GTP, 650μM UTP, 350μM Digoxigenin-11-UTP: Boehringer); 1x Transcription buffer (10x Transcription buffer = 400mM Tris(pH 8.0), 60mM $MgCl_2$, 100mM DTT, 20mM Spermidine (Sigma), 100mM NaCl); 50 units RNase inhibitor (Boehringer); 50 units RNA polymerase(Boehringer). The final solution was made up to 50μl with DEPC-treated autoclaved dH_20 and incubated at 37°C for two hours. Subsequently the plasmid DNA was digested by adding 20 units of DNase (RNase-free: Boehringer) for 15 minutes at 37°C. This reaction was stopped by adding 4μl of 250mM EDTA. The riboprobe was precipitated by adding 5.5μl of 4M LiCl and 165μl 100% ethanol at either -70°C for 30 minutes or -20°C overnight. The centrifuged pellets were washed with 70% ethanol, air dried and dissolved in 100μl DEPC-treated water. Probe

length was verified by gel electrophoresis. The probes are stable for at least 6 months at -20°C.

Wholemount *in situ* technique for 7.5 and 8.5d embryos (Rosen and Beddington 1993)

For this procedure all solutions were autoclaved prior to use and the diluent (distilled water or PBS) had 0.1% DEPC (Sigma) added and was heat treated at 37°C for two hours to remove any RNase. All washes were done at room temperature unless otherwise stated.

Embryos which had been fixed overnight in 4% PFA were washed twice on ice with PBS-0.1% Tween-20(PBT; Sigma). They were then dehydrated on ice through 25%, 50%, 75%, and 2x 100% Methanol (Analar grade,BDH): PBS, with 5 - 10 minutes in each solution. Embryos were then stored at -20°C for up to 2 months. When enough embryos had been collected in this way they were pooled and rehydrated on ice through a methanol series and washed 3x with PBT. The embryos were permeabilised 3x for 30 minutes with RIPA (150 mM NaCl, 1% Nonidet P-40 (Sigma), 0.5% Sodium Deoxycholate, 0.1% SDS, 1mM EDTA, 50 mM Tris (pH 8.0)). After permeabilisation the embryos were fixed for 20 minutes in freshly prepared 4% paraformaldehyde/0.2% glutaraldehyde E.M. grade ('Sigma', Grade 1) in PBT. After fixing the embryos were washed for 5 minutes with PBT.

At this point the 8.5 dpc embryos were divided into groups of 6 - 10 *T/T* homozygous embryos plus the same number of controls for each probe and put into 15ml centrifuge tubes (Corning). The embryos were then washed with 1:1 PBT : hybridization buffer (50% Ultrapure Formamide (BDH), 5X SSC(pH 4.5), 50µg/ml heparin, (Sigma), 0.1% Tween-20;100 µg/µl sheared, denatured herring sperm DNA (Boehringer)) for 10 minutes

and then washed 1x with hybridization buffer for 10 minutes. The embryos were prehybridised at 70°C, for 2 hours in 1 ml of hybridisation buffer containing 100 µg/µl tRNA (Boehringer). The prehybridisation solution was then removed and the embryos hybridised with 1:100-1:200 dilution digoxigenin labeled riboprobe in hybridisation buffer overnight at 70°C. To decrease the amount of evaporation a foam rubber insert containing a 0.5 ml eppendorf tube filled with hybridization buffer was placed in the neck of the tube above the embryos to act as a humidifier. The rim of the tubes was coated with vacuum grease and firmly sealed.

The next morning the probe was removed and the embryos washed twice for 10 minutes at 70°C with hybridisation buffer. They were then washed twice at 65°C for 5 minutes with 50% formamide, 2x SSC, 0.1% Tween-20 and then another three times for 30 minutes with the same solution. The embryos were allowed to cool to room temperature before washing twice with 1x TBST (0.8% NaCl, 0.02% KCl, 25mmol Tris-HCl (pH 7.5), 0.1% Tween-20). The embryos were "blocked" to prevent non-specific binding of antibody by incubating them for 1 hour in 10% heat-inactivated sheep serum (Sigma) /TBST at room temperature. The sheep serum was removed and the embryos were then incubated overnight at 4°C with 0.5 - 1ml of 1:2000 dilution of anti-digoxigenin alkaline phosphatase Fab conjugate (Boehringer) in 1% heat inactivated sheep serum/ TBST. This Fab fragment was preadsorbed and diluted immediately before use as follows: several milligrams of embryo acetone powder were heat inactivated in TBST for 30 minutes at 70°C This was then centrifuged and the supernatant removed. The powder was cooled on ice and then dispersed in an appropriate volume of 1:500 dilution of the Fab conjugate in 1% heat inactivated sheep serum/ TBST. This mixture was incubated at 4°C with gentle shaking for one hour. It was then

centrifuged for 10 minutes and the supernatant was transferred to a fresh tube. Finally, the antibody was diluted fourfold with 1% sheep serum/TBST to give a final dilution of 1:2000.

The following morning the antibody was removed and the embryos were washed 3x with TBST. This was followed by five 1 hour washes with TBST. The embryos were washed 3x 10 minutes with freshly made alkaline phosphatase buffer (100mM NaCl, 50mM $MgCl_2$, 0.1% Tween-20, 100 mM Tris (pH 9.5)) and during the final wash they were transferred to a glass embryological dish. The final wash was replaced with 1 - 2 ml freshly prepared staining solution (4.5µl/ml NBT (75 mg/ml nitroblue tetrazolium in 70%(v/v) dimethylformamide: GIBCO-BRL) and 3.5µl/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt, 50 mg/ml in dimethylformamide; GIBCO-BRL)/ml alkaline phosphatase buffer: GIBCO-BRL) and the dishes placed in the dark. The staining was stopped by washing embryos in PBT twice and then putting the embryos in PBT pH 5.5. The reaction was stopped at the same time for both mutant and control embryos

The specimens were refixed in 4% paraformaldehyde/0.2% glutaraldehyde for 20 minutes and then washed twice for 5 minutes in PBT. The embryos were cleared in 50% glycerol in PBT for 20 minutes and then transferred to 80% glycerol before being photographed.

Wholemount *in situ* technique 9.5d embryos (Wilkinson 1992)

The protocol used for 9.5d embryos was largely the same as that for earlier embryos. However, there were some differences which enhanced results in these older embryos

On the first day after rehydrating embryos through methanol, they were bleached in 6% hydrogen peroxide for 1 hour. They were then

washed three times for 5 minutes in PBT. The embryos were permeabilised with 10μg/ml proteinase K for 15 minutes (instead of using the non-ionic detergent RIPA). They were then washed with 2mg/ml glycine in PBT for 5 minutes before being rinsed twice with PBT. The embryos were refixed for twenty minutes with 4% paraformaldehyde/0.2% glutaraldehyde in PBT and then washed twice with PBT for 5 minutes. The wash was then replaced with prehybridisation buffer (50% ultrapure formamide, 5x SSC, 20mM Tris-HCl (pH8), 5mM EDTA (pH8), 0.1% Tween-20). After mixing, the solution was removed and the embryos prehybridised for 1 hour at 70°C with fresh buffer. The prehybridisation mix was replaced with 0.5 ml hybridisation solution (prehybridisation buffer containing 0.2% polyvinylpyrrolidone, 0.2% Ficoll type 400, 2μg/ml tRNA, 2μg/ml heparin)containing a 1:100-1:200 dilution of digoxigenin labeled RNA probe. After mixing, the solution was replaced with 0.5ml fresh hybridisation solution. The embryos were hybridised overnight at 70°C using the same precautions against evaporation as the protocol for younger embryos. The following day the embryos were washed twice with prewarmed solution A (50% formamide, 4x SSC(pH 4.5), 1% SDS)for 30 minutes at 70°C. They were then rinsed for 10 minutes at 70°C with a 1:1 mix of solution A: solution B before washing the embryos three times at room temperature with solution B (500mM NaCl; 10mM Tris HCl(pH 7.5); 0.1% Tween-20) The embryos were treated twice at 37°C with 100μg/ml RNase A (Boehringer) in solution B for 30 minutes. They were then rinsed at 37°C for 5 minutes with a 1:1 mix of solution B: solution C before being washed twice with solution C (50% formamide, 2x SSC(pH 4.5)) at 65°C for 30 minutes.

The rest of the procedure for treating embryos with anti-digoxigenin alkaline phosphatase Fab conjugate and staining was the same as for 7.5 dpc and 8.5 dpc embryos.

Production of BTBR-lac-Z mice

Heterozygous *(T/+)* BTBR/Pas were mated with homozygous Tgm6lacZ1 females. Offspring that had short tails and were therefore *T/+* were tail-tipped. These tails were assayed for the presence of *lac-Z* protein by staining for ß-Galactosidase activity (see below). Positive results demonstrated that the mice were both heterozygous for *T* and hemizygous for Tgm6lacZ1. These mice were then backcrossed onto homozygous Tgm6lacZ1 mice, in order to increase the number of offspring containing the *Hoxa-7lacZ* transgene. The assay for *lacZ* protein was repeated on the short-tailed pups. When old enough these mice were intercrossed and embryos recovered at 8.5 and 9.5 dpc (0.5 dpc = morning of plug). All the embryos were assayed for the presence of *lacZ* protein and the embryos which were phenotypically homozygous *T/T* were identified.

lacZ staining (Beddington et al. 1989)

The samples were rinsed in phosphate buffered saline (PBS) before fixing for 30 minutes at 4^{o}C in 0.2% glutaraldehyde (Sigma) in 0.1M phosphate buffer (0.1M disodium hydrogen orthophosphate : 0.1M sodium dihydrogen orthophosphate in a ratio of 21:4) containing 2mM $MgCl_2$ and 5mM EGTA

The samples were washed 3x for 20 minutes at room temperature in Wash solution (0.1M phosphate buffer containing 2mM $MgCl_2$; 0.01% (w/v) sodium deoxycholate; 0.02% (v/v) Nonidet P-40; (Sigma); 0.05% (w/v) BSA

(Sigma). Specimens were then stained for two hours at 37°C in the dark, positive samples staining blue within this time, before refixing the embryos in 0.2% glutaraldehyde. The stain consisted of mixing two completely dissolved solutions together. Solution A consisted of 25mg 5-Bromo,4-Chloro,3-Indolyl-ß Galactopyranoside (X-gal: Sigma) dissolved in 0.5ml dimethyl formamide and Solution B of 25ml Wash solution containing 1mM spermidine hydrochloride, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 0.001%NaCl. The two solutions were mixed, filtered and stored for up to two weeks at 37°C in the dark.

Photography and Histology

Embryos were photographed using a SZH Olympus dissecting microscope and camera. Computer images were obtained using a Hamamatsu CCD Vision Camera Module attached to the SZH Olympus dissecting microscope or an Olympus IMT-2 inverted research microscope. The images were processed using The ColourVision programme version 1.2.2 (Image Processing and Vision Company Ltd) run on an Apple Macintosh Quadra 900 Computer.

After photography embryos were washed 3 times for 20 minutes in 40% glycerol:35% ethanol (Analar grade, Hayman Ltd):10% PBT:15% dH_2O and then dehydrated through an ethanol series (70%,80%,87%,95%) with one 30 minute wash in each solution. This was followed by three 20 minute washes in 100% ethanol, one 20 minute wash of equal volume 100% ethanol: Histoclear (National Diagnostics). Before embedding in Histoplast paraffin wax (Shandon) the embryos had three 40 minute washes in Histoclear at room temperature followed by three 20 minute wax changes at 60°C The embryos were oriented in the wax using a dissecting microscope. Wax blocks were stored at 4°C until they were

sectioned at 7-8 μm with an Anglia Scientific microtome. After drying overnight the sections were dewaxed in Xylene before being mounted under coverslips using DPX mountant.

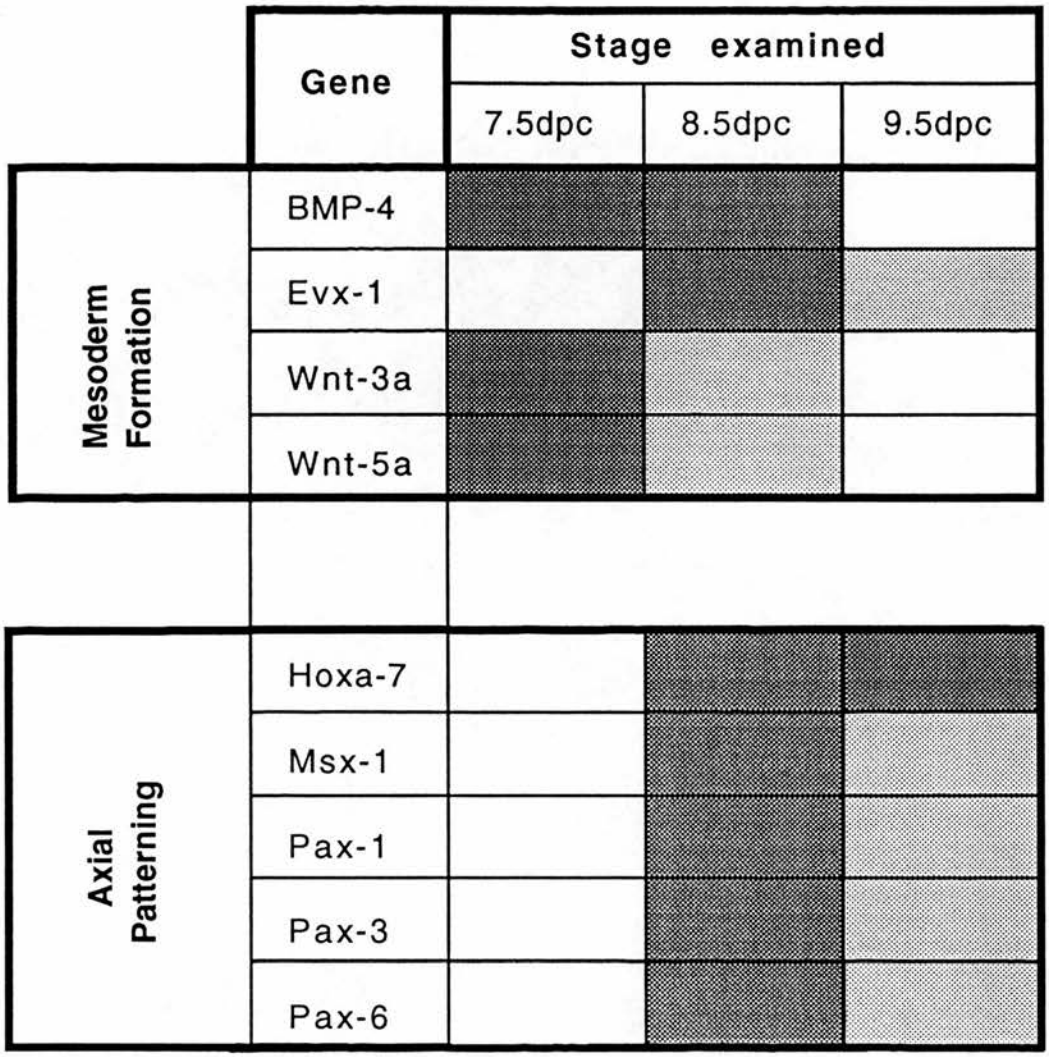
Results

Preliminary experiments showed that the wholemount *in-situ* hybridisation technique using RIPA as the permeabilisation agent (Rosen and Beddington, 1993) gave a higher background signal at 9.5 dpc compared with the method using proteinase K and RNase treatment (Wilkinson, 1992). However, proteinase K treatment digested away the allantoides of control embryos and the ectodermal blebs present on *T/T* 8.5d embryos. This meant that using this protocol the only criteria to distinguish the *T/T* embryos from the controls at this age was a slightly kinked neural tube. It was therefore decided to use the milder protocol (Rosen and Beddington, 1993) which causes less damage to delicate tissues on the younger embryos.

A summary of the data obtained is shown in Figure 4.1. The results focus on those structures and regions which are affected in *T/T* embryos including the caudal end of the embryo, the neural tube and somites. They do not provide a comprehensive description of the wildtype expression patterns of the different genes used as this is available elsewhere: *BMP-4*: (Jones et al. 1991); *Evx-1* (Dush and Martin 1992) ;*Wnt-3a* (Takada et al. 1993); *Wnt-5a* (Gavin et al. 1990); *Hoxa-7* (Mahon et al. 1988; Püschel et al. 1990); *Msx-1* (Hill et al. 1989; Robert et al. 1989); *Pax-1* (Deutsch et al. 1988); *Pax-3* (Goulding et al. 1991); *Pax-6* (Walther and Gruss 1991).

Figure 4.1

Summary of stages that alterations in expression domain of the different genes examined in *T/T* mutant embryos were observed



Altered expression pattern

No change in expression pattern

Stage not examined

Embryo collection

PCR analysis of 113 extraembryonic portions of 7.5 dpc embryos identified 21 unequivocal *T/T* embryos and 74 control embryos. The *T/T* embryos were divided into 3 groups and probed with *BMP-4*, *Wnt-3a* and *Wnt-5a*. The control embryos were subdivided into 5 groups and probed not only with the same probes as the *T/T* embryos, but also with *T* and *α-actin* as controls to ensure that the procedure was working correctly. Figure 1.6 show the expression pattern of T in 7.5, 8.5 and 9.5 dpc control embryos.

For both 8.5 and 9.5 dpc stages, each probe was assayed on 6-10 mutant and 6-10 control embryos. Unless, otherwise stated, the results for each probe were repeated twice.

T/+ and Tgm6lacZ1 mice were intercrossed as described in the methods, to give control and *T/T* embryos whose anterior boundary of *Hoxa-7* expression was demarcated by *lacZ* protein expression. At 8.5 dpc, 22 embryos stained positive for *lacZ* protein and of these 5 were *T/T* embryos. At 9.5 dpc, 27 embryos, including 9 *T/T* mutants, expressed the *lac-Z* protein.

Expression patterns of genes important for mesoderm formation

The expression pattern of 5 genes co-expressed with *T* in the primitive streak region of the gastrulating embryo were examined to assess whether T protein was required for the induction or maintenance of their expression. Although gastrulation starts at 6.5 dpc, the earliest embryos analysed in the experiments described in this chapter were a day older. This is because the small size of 6.5 dpc mouse embryos made it technically difficult to carry out both genotyping and wholemount *in situ* hybridisations on individual embryos.

BMP-4 expression

At 7.5 and 8.5 dpc both control and homozygous *T/T* embryos had similar staining intensity and pattern of expression of *BMP-4* (Fig 4.3 A-C) At 7.5 days *BMP-4* is expressed in both mutant and wildtype embryos in a broad region covering a third of the embryonic portion of the proximal egg cylinder adjacent to the embryonic-extraembryonic junction (Fig 4.3A,B). It is not possible to comment on the extent of expression in the extraembryonic region as this had been removed for PCR. At 8.5 dpc *BMP-4* is expressed in the amnion and base of the allantois and at the junction between the embryonic and extraembryonic tissues (Fig 4.3C). It is also expressed in the fore- and hind-gut endoderm. After the 4 somite stage staining is also seen in the most posterior mesoderm and ectoderm.

Wnt-3a expression

At 7.5 dpc *Wnt-3a* expression in *T/T* and control embryos is similar, being localised to the primitive streak and extending into the posterolateral margins of the embryonic egg cylinder (Fig 4.2A,B). Again, it is not possible to comment on the extent of expression in the extraembryonic region as this had been removed for PCR. At 8.5 dpc, *Wnt-3a* expression differs between mutant and control embryos. In the controls, expression is restricted mainly to the primitive streak, but also extends into the posterior mesoderm and ectoderm (Fig 4.2C). There is no expression in the allantois. In *T/T* embryos, prior to clearing weak expression of *Wnt-3a* was evident and corresponded to the normal pattern. However, after extensive clearing in glycerol, no hybridisation signal was observed in the caudal region although dorsal expression in the neural tube was maintained (Fig 4.3C). Although based on a total of only ten 8.5 dpc embryos at different

Figure 4.2

Expression of *Wnt-3a* and *Wnt-5a* in control and mutant embryos

A-C *Wnt-3a* expression
A. The embryonic portion of 7.5 dpc wildtype embryos. *Wnt-3a* is expressed in a broad band extending over a third of the embryonic portion of the proximal egg cylinder adjacent to the embryonic-extraembryonic junction. Bar 200μm
B. *Wnt-3a* expression in the embryonic portion of 7.5 dpc *T/T* embryos - no difference is observed from the controls shown in A. Bar 200μm
C. Dorsal view of *Wnt-3a* expression at 8.5 dpc after clearing in glycerol. In controls (2 embryos on left) specific staining is restricted to the primitive streak (arrow head). In *T/T* embryos (2 embryos on right) no expression is seen in the streak. Bar, 400μm
D-F *Wnt-5a* expression
D. The embryonic portion of 7.5 dpc control embryos. *Wnt-5a* correlates with the position of the lateral wings of mesodermal tissue. Bar 200μm
E. *Wnt-5a* expression in the embryonic portion of 7.5 dpc *T/T* embryos - no difference is observed from the controls shown in D. Bar 200μm
F. Dorsal view of Wnt-5a expression at 8.5 dpc after clearing in glycerol. In the control embryo (1 embryo on left) *Wnt-5a* expression is restricted to tissue caudal to the last formed somite and can also be detected in the allantois. No caudal expression is seen in the *T/T* embryos (two embryos on right). The staining in the cranial region is non-specific.
Bar 550μm

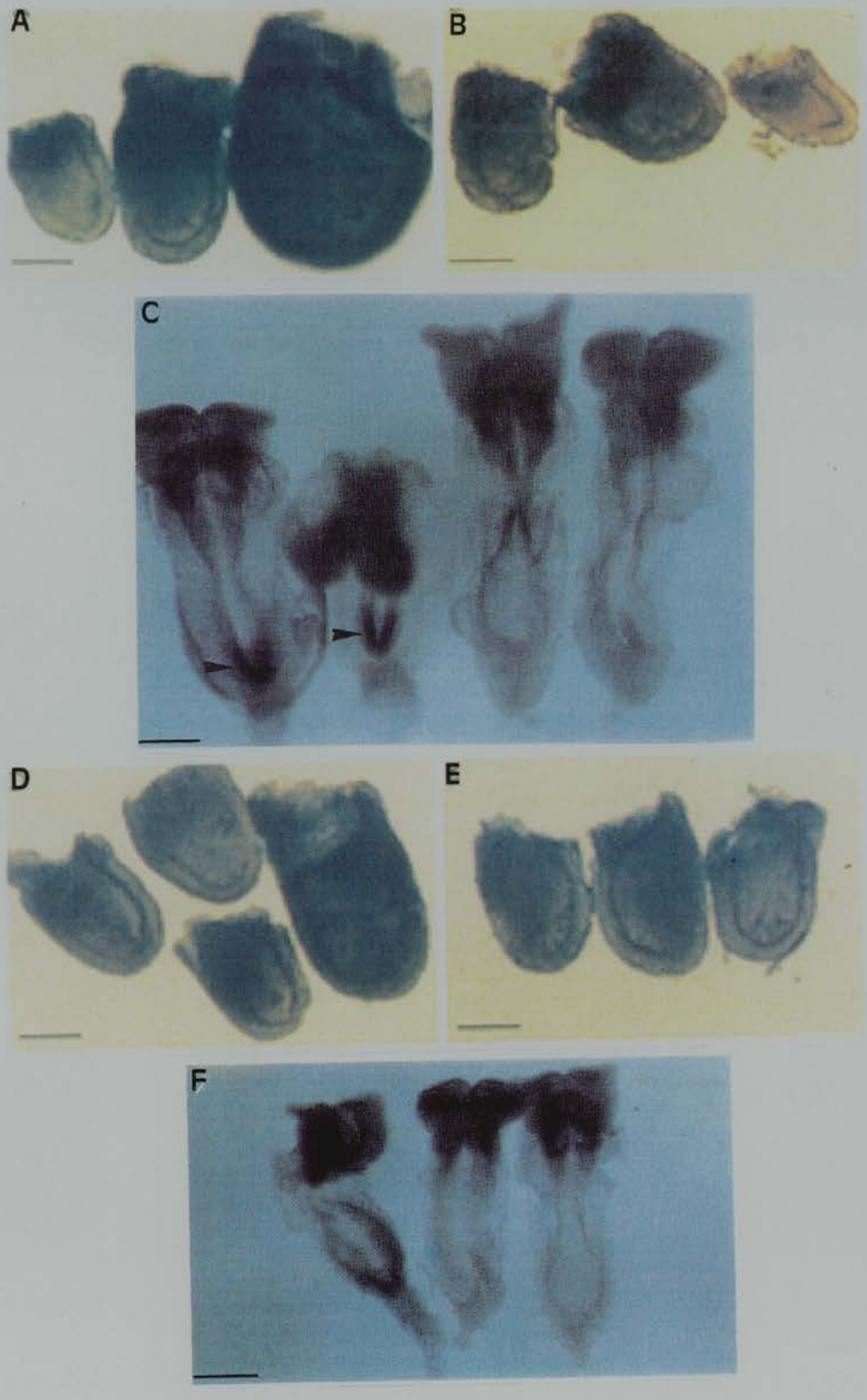

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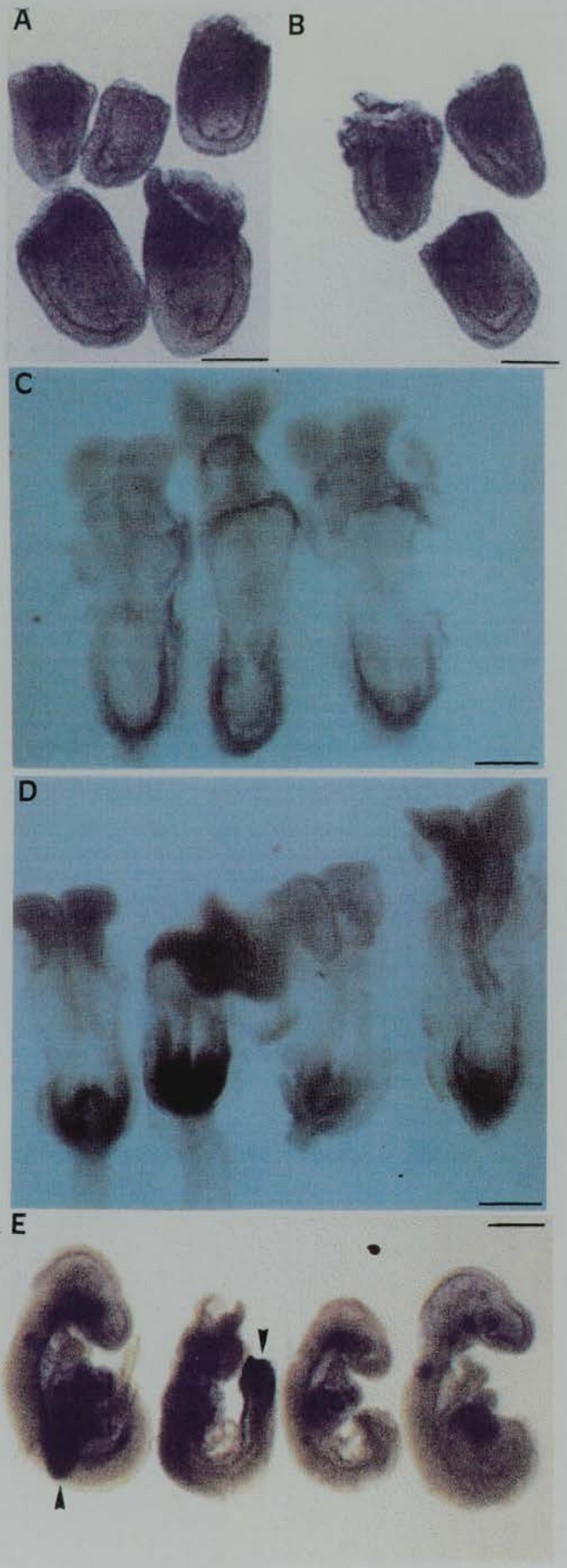
Figure 4.3

Expression of *BMP-4* and *Evx-1* in control and mutant embryos

A-C *BMP-4* expression
A. *BMP-4* expression domain in the embryonic portion of 7.5 dpc wildtype embryos is a broad domain covering a third of the embryonic portion of the proximal egg cylinder adjacent to the embryonic-extraembryonic junction. Bar 200μm
B. *BMP-4* expression in the embryonic portion of 7.5 dpc *T/T* embryos - no difference is observed from the controls shown in A. Bar 200μm
C Ventral view of *BMP-4* expression at 8.5 dpc. In both controls (embryo on left) and *T/T* embryos (2 embryos on right), BMP-4 is expressed in caudal ectoderm and mesoderm. Bar 450μm

D-E *Evx-1* expression
D. Dorsal view of *Evx-1* expression at 8.5 dpc. Expression is seen in the primitive streak, caudal ectoderm and mesoderm. At this stage no difference was apparent between controls (2 embryos on left) and homozygous mutants (2 embryos on right). Bar 450μm
E. Lateral view of *Evx-1* expression at 9.5 dpc. Despite high background staining in embryonic cavities, it is clear that there are no Evx-1 transcripts detectable in T/T embryos (2 embryos on right) unlike the controls (2 embryos on left) which have high expression levels in the tailbud and presomitic mesoderm (arrowhead). Bar 700μm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developmental stages, it appears that expression of *Wnt-3a* declines rapidly at about the 4-5 somite stage.

Wnt-5a expression

The pattern and intensity of expression for *Wnt-5a* is the same in control and *T/T* embryos at 7.5 dpc and is seen in a position that correlates with the lateral wings of mesodermal tissue (Fig 4.2D,E). However as these embryos were not sectioned it is not possible to categorically state which germ layer was expressing *Wnt-5a*. In control embryos at 8.5dpc, Wnt-5a is expressed in all posterior tissues up to and including the most caudal one or two somites. It is also expressed in the allantois, especially at its base and in the amnion. Although the pattern of expression was the same in mutant embryos the intensity of *Wnt-5a* expression in 8.5 dpc *T/T* embryos was reduced in the posterior region compared to that observed in controls. This was observed in two separate experiments. After prolonged clearing, no hybridisation signal was detectable in *T/T* embryos older than the 5-6 somite stage (Fig 4.2F).

Evx-1 expression

Only one experiment was performed at both 8.5 and 9.5 dpc using this probe. At 8.5 dpc the expression pattern of *Evx-1* is the same in both mutant and control embryos. However there are differences between the two groups at 9.5 dpc. In 8.5 day control embryos *Evx-1* is expressed in the posterior part of the embryo caudal to the last somite. It is present in the primitive streak, medial mesoderm and ectoderm including the neurectoderm (Fig 4.3D) In *T/T* embryos at this stage *Evx-1* expression shows the correct anterior boundary of expression. At 9.5 dpc, *Evx-1* is expressed at the caudal end of control embryos, in the remnant of the

primitive streak and adjacent tissue. It is also expressed in the caudal neurectoderm extending to the level of the most recently formed somite. No *Evx-1* transcripts were detected in the caudal region of *T/T* embryos at 9.5 dpc (Fig 4.3E).

Axial patterning

Hoxa-7 was used as a marker to assess whether there was any changes in the rostrocaudal caudal axis. Potential alterations in the mediolateral axis was examined using *Msx-1*, whilst dorsoventral pattern in the neural tube and somites in embryos lacking a normal embryo was evaluated using *Pax-1, Pax-3, Pax-6* and *Msx-1.*

Hoxa-7 expression

At 8.5 and 9.5 dpc, the anterior boundary of *Hoxa-7* expression (as assessed by the anterior boundary of positive ß-galactosidase activity), is the same in both mutant and control embryos. At 8.5 dpc both *T/T* and controls have *lac-Z* staining in all tissues posterior to the 4th or 5th somite. Similarly, at 9.5 dpc both *T/T* and control embryos have the same anterior boundaries for *Hoxa-7* expression in both the neural tube and somites: the anterior boundary in the neural tube is the level of somite 6-7, whereas the most rostral somite to show X-gal staining is the 14th (Fig 4.4A,B). In four out of nine 9.5 dpc mutant embryos, ectopic staining is also seen anterior to the normal boundary of *Hoxa-7* expression (Fig 4.4A). This ectopic staining is never seen in control embryos. On sectioning, the *Brachyury* homozygous embryos, this ectopic ß-galactosidase activity was restricted to embryonic cavities including the neural tube lumen and the lumen of the dorsal aorta (Fig 4.4C). Thus, the ectopic staining appears to result from aberrant diffusion of protein, presumably due to disrupted tissue structure in *T/T* mutant embryos

Figure 4.4

Hoxa-7 expression in 9.5 dpc *T/T and* control embryos

A. Control embryo carrying the m6lacZ1 reporter construct showing correct anterior boundary for *Hoxa-7* expression (arrow).as demonstrated by staining for ß- galactosidase activity. Bar, 350μm
B. Tgm6lacZ1 transgenic T/T embryo showing same anterior boundary of *Hoxa-7* expression as control. Ectopic staining is seen rostrally in neural tube (arrow head) and dorsal aorta (open arrow head). Bar, 350μm
C. Transverse section of embryo in B showing that ectopic staining is in the lumen of both the neural tube and dorsal aorta Bar, 60μm

Abbreviations
nt neural tube
da dorsal aorta

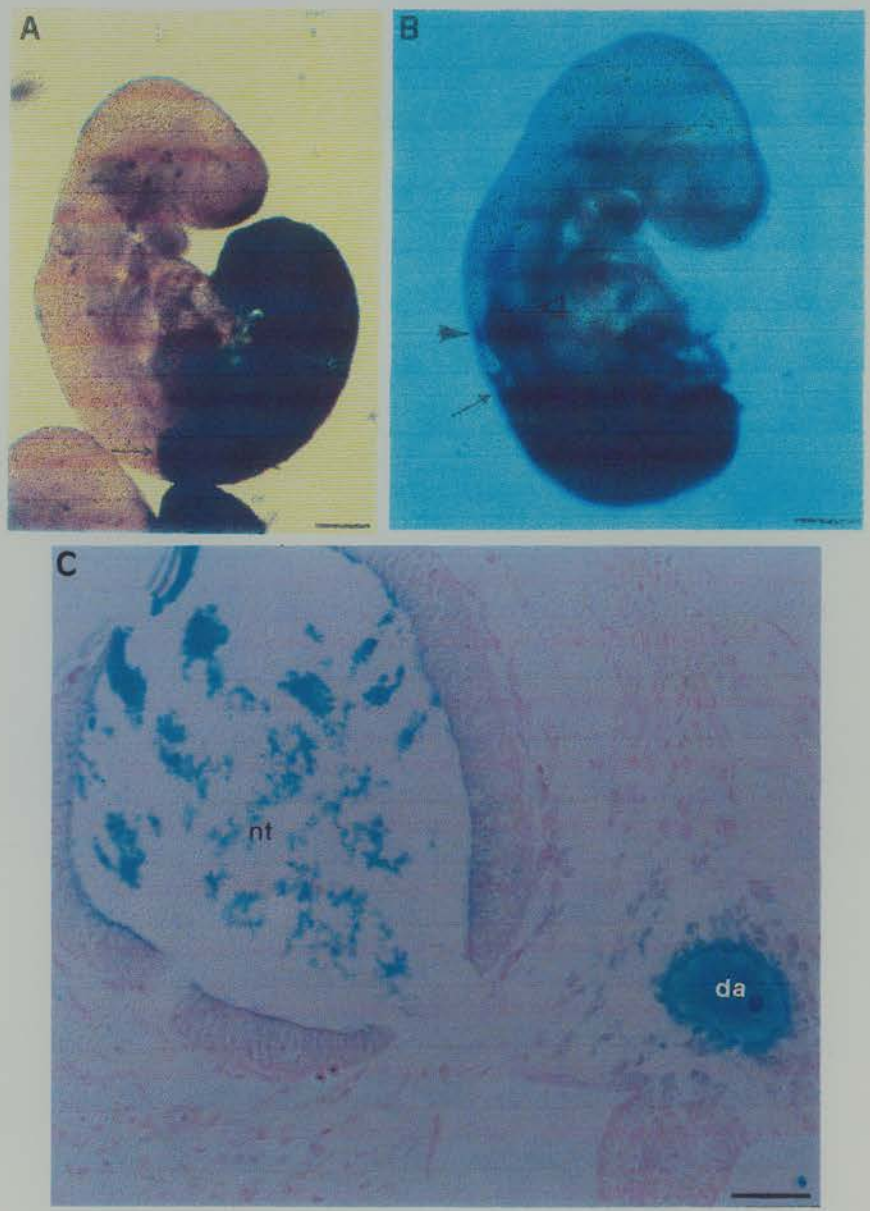
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Figure 4.5

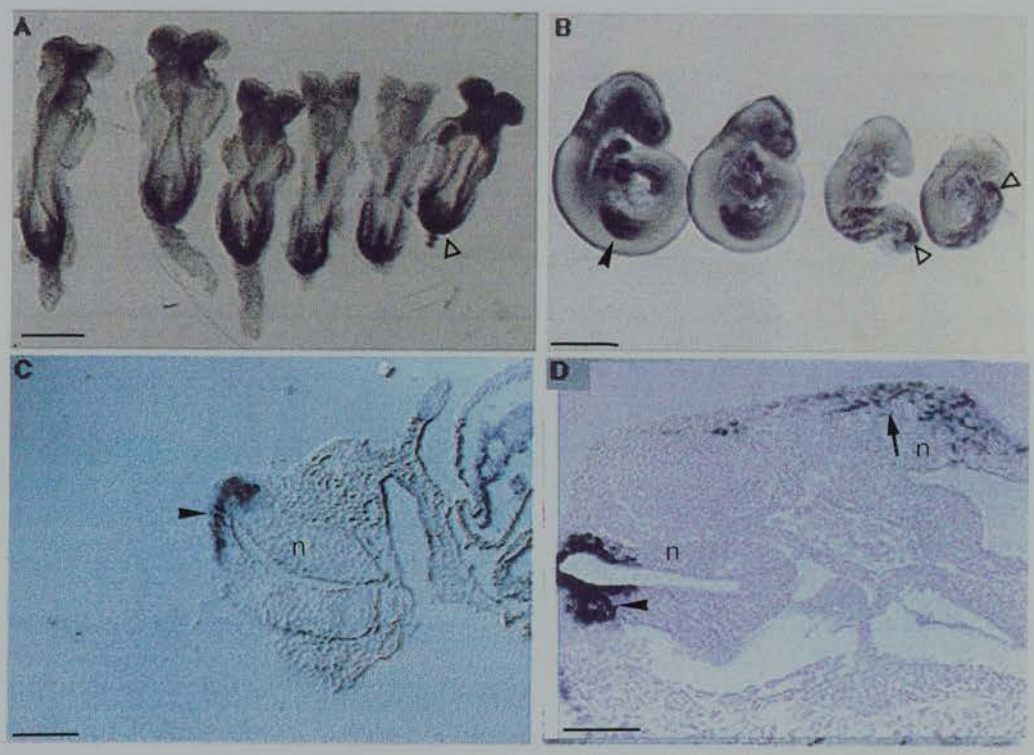
Expression of *Msx-1* in control and mutant embryos

A. Dorsal view of *Msx-1* expression at 8.5 dpc. In control (3 embryos on left) and mutant (3 embryos on right) embryos, expression is observed in the primitive streak, lateral mesoderm and dorsal neural tube and neural plate. In mutant embryos there is elevated expression in the caudal primitive streak (open arrow head). Bar 750μm

B. Lateral view of 9.5 dpc embryos. In both control (2 embryos on right) and *T/T* (2 embryos on left) embryos, *Msx-1* expression is restricted to the dorsal neural tube even when, in the mutants, this structure is highly convoluted. The most caudal tissue of the mutant embryos strongly express *Msx-1* (open arrow head). In control embryos high transcript levels are also seen in the limb bud (closed arrow head). Bar, 1mm

C. Transverse section through trunk region of 9.5 dpc control embryo showing *Msx-1* expression restricted to the dorsal margins of the neural tube (arrow head). Bar 150μm

D. Transverse section through trunk region of 9.5 dpc *T/T* embryo showing *Msx-1* expression is also restricted to the dorsal margins of the neural tube (arrow head).Some staining is observed laterally (arrow) and this probably marks neural crest cells displaced by extensive kinking of the neural tube (n). Bar 150μm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Msx-1 expression

The *Msx-1* expression pattern was identical in both control and *T/T* embryos at 8.5 dpc. *Msx-1* is expressed in the dorsal aspect of the neural tube and neural folds, the lateral mesenchymal tissue, the lateral and caudal ectoderm and in the amnion (Fig 4.5A). In mutant embryos beyond the 5 somite stage, the expression of *Msx-1* was slightly elevated at the caudal extreme of the primitive streak. At 9.5 dpc control embryos show intense staining in the forelimb-buds and in the neural tube (Fig 4.5B), although expression in the neural tube does not extend to the caudal extreme of the neural tube (in contrast to the previous description of *Msx-1* expression; Robert et al. 1989). In 9.5 dpc *T/T* embryos, *Msx-1* is also expressed in the forelimb-buds, and neural tube (Fig 4.5B). However the staining intensity in the forelimb buds in the mutant embryos is reduced compared to controls. Unlike controls, *Msx-1* expression domain extends along the whole length of the trunk neural tube, upto and including its caudal extremity (Fig 4.5B), although as in wildtype embryos, transcripts are always restricted to the dorsal aspect of the neurectoderm (Fig 4.5C, Fig 4.5D). In *T/T* embryos, the hybridisation signal in the tailbud region is considerably stronger than that seen in controls (Fig 4.5B).

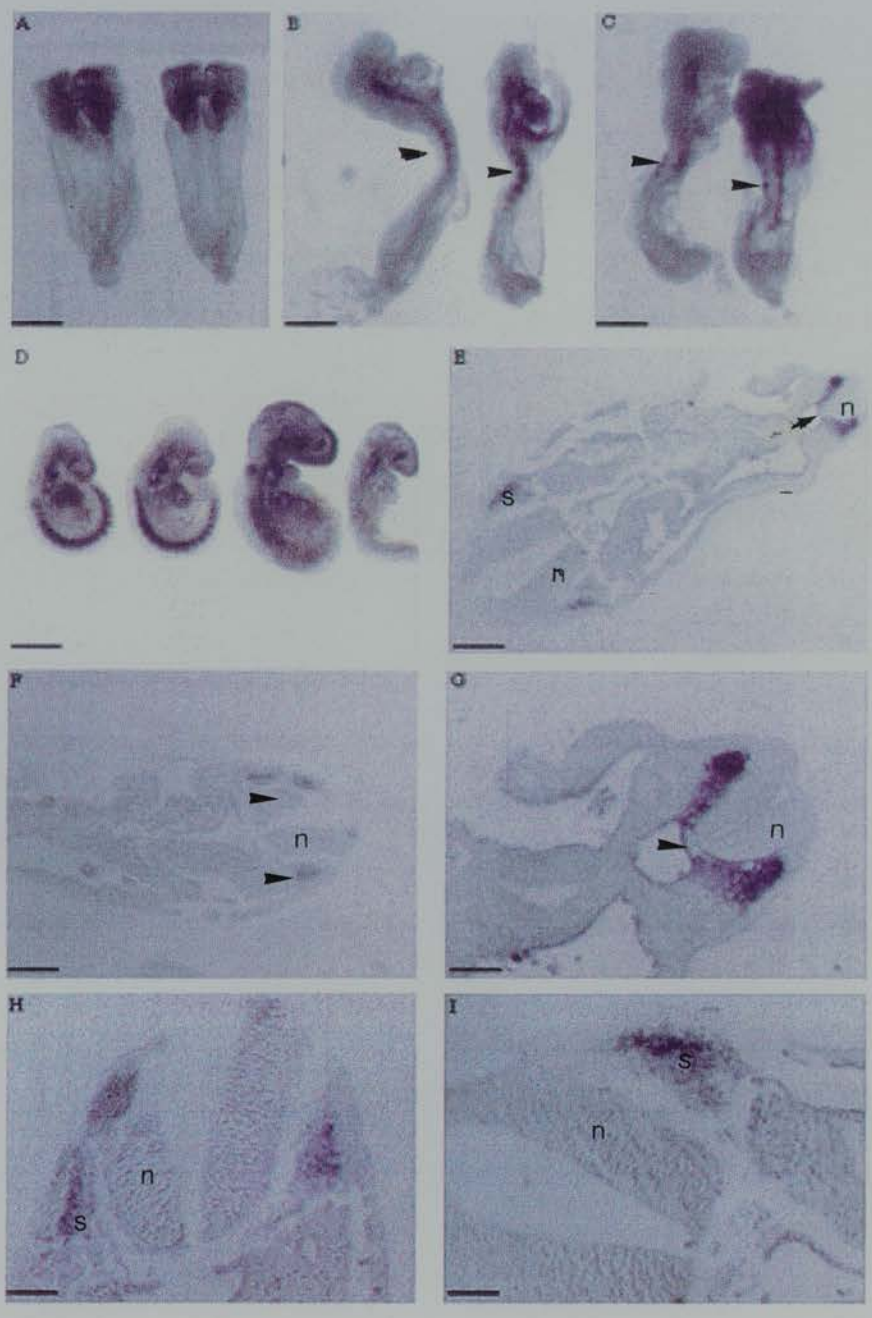
Pax-1 expression

At 8.5 dpc *Pax-1* is not expressed in either mutant or control embryos (Fig 4.6A). *Pax-1* expression is first seen in 8.75 dpc embryos. This is earlier than previously described by Deutsch et al (1988). At this stage, both control and *T/T* embryos show expression in the 4th - 7th somite (Fig 4.6B). There is no apparent difference in staining intensity or distribution between the two groups. However, by 9.5 dpc, mutant and

Figure 4.6

Expression of *Pax-1* in control and mutant embryos

A-C Control embryo on right, T/T embryo on left
A. Dorsal view of 8.5 dpc control and *T/T* embryos- no *Pax-1* expression is detectable in the somites. Bar 300μm
B and C. Lateral view of 8.75 and 9.0 dpc embryos respectively - both control and mutant embryos show *Pax-1* transcripts in paraxial mesoderm (arrow head) commencing at the 4th somite. Expression is also seen in the pharynx. Bar 300μm
D. Lateral view of 9.5 dpc embryos . Control embryos (2 on left) show somitic staining in all somites caudal to and including the 4th whilst *T/T* (2 on right) embryos only have detectable *Pax-1* transcripts in somites 4 -7 but at greatly reduced levels. Bar 750μm
E. Transverse section through control embryo with rostral end to the left. Staining is observed in the ventral sclerotome compartment. *Pax-1* expression in the caudal sclerotome extends towards the midline at the caudal showing staining in sclerotome compartment. Arrow head points to notochord. Bar 150μm
F. Transverse section through cervical region of *T/T* embryo showing weak staining in ventral compartment (arrow head). Bar 150μm
G. Higher magnification of E. Caudal somite of control embryo showing very intense staining in sclerotome compartment extending medially toward notochord (Arrow head). Bar 50μm
H. Higher magnification of F. Weak Staining in the ventral compartment of an anterior *T/T* somite showing correct localisation of transcripts. Bar 50μm
I. Higher magnification of E. Anterior somite of control embryo showing strong staining in the sclerotome compartment .Bar 50μm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control embryos have different expression patterns. At 9.5 dpc, although the control embryos express *Pax-1* in all somites, the mutant embryos express *Pax-1* only in somites 4-7 (Fig 4.6D). In these somites, the staining intensity appears to be more variable between *T/T* embryos than the staining intensity between control embryos(Fig 4.6D-I). Also, the staining intensity of the anterior somites appears, visually, to be weaker in *Brachyury* homozygous embryos compared to corresponding somites in control embryos (Fig 4.6D - I). However, on sectioning the *T/T* and control embryos, the pattern of *Pax-1* expression is the same in both groups. Thus *Pax-1* is only expressed in the sclerotome compartment of somites 4-7 (Fig 4.6E,F)

In 9.5 dpc control embryos, *Pax-1* expression is stronger in the more posterior somites compared to the anterior ones (Fig 4.6D,G). At the caudal end of control embryos, serial sections demonstrate that there is a band of *Pax-1* expression continuous with the sclerotome that crosses the midline ventral to the notochord (Fig 4.6G). No such staining is seen in paraxial mesoderm caudal to the 7th somite in *T/T* embryos.

Pax-3 expression

At 8.5 dpc both the control and homozygous mutant group have a similar *Pax-3* expression pattern, although the dorsal expression domain in the unfused neural folds of T/T embryos is enlarged (Fig 4.7A). At this stage, *Pax-3* is expressed in all the somites and along the dorsal neural tube extending caudally into the lateral neural plate (Fig 4.7A)

At 9.5 dpc, the differences observed in the expression patterns of *T/T* and control embryos are more apparent (Fig 4.7 and 4.8). Serial histological sections of 9.5 dpc control embryos show that *Pax-3* is expressed in the dorsal part of the neural tube and in the dorsolateral

Figure 4.7

Expression of *Pax-3* in control and mutant embryos

A. Dorsal view of *Pax-3* expression in 8.5 dpc embryos, with mutant embryo at bottom of picture. Expression is evident in the somites and neural tube of both mutant and control embryos. The head staining is non specific. The arrow heads indicate that the dorsal domain of expression in the unfused neural folds of the *T/T* embryos is enlarged compared to the control. Bar 400μm

B Lateral view of *Pax-3* expression in 9.5 dpc embryos.. Control embryo (on left) shows intense staining in the somites and dorsal neural tube. The neural tube expression domain in *T/T* embryos (caudal to right .) is enlarged making it difficult to assess somite expression in this picture. Bar 700μm

C. Transverse section through a 9.5 dpc control embryo - *Pax- 3* is expressed in the dorsal neural tube and dorsolateral dermomyotome compartment of the somite. Bar 80μm

D. Transverse section through a 9.5 dpc *T/T* embryo. *Pax-3* expression is still restricted to the neural tube but extends further ventrally (to the left) compared with control in C. Staining is more diffuse in the somite and no clear dermomyotome boundary is evident. Bar 80μm

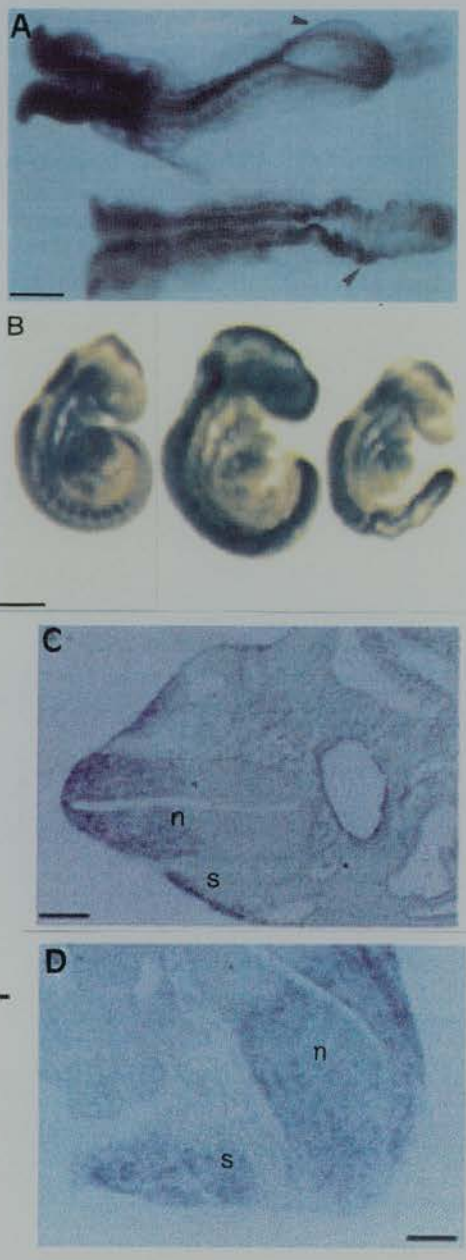
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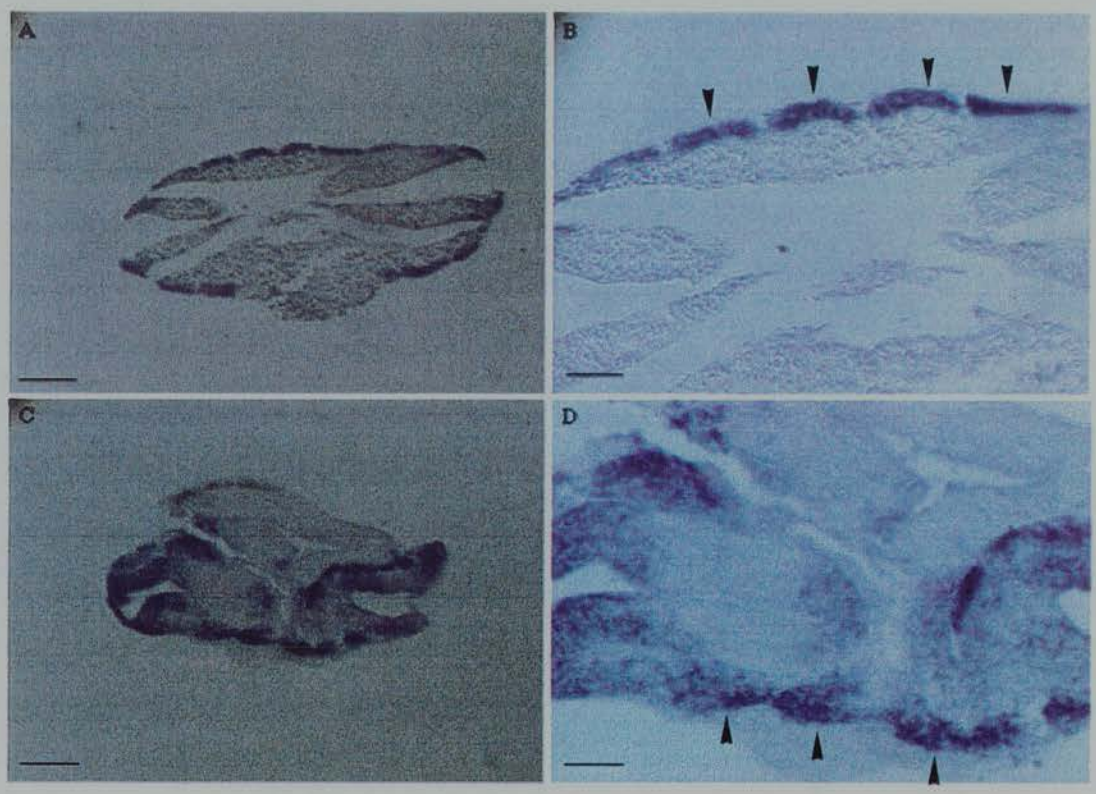
Figure 4.8

Somite Expression of *Pax-3* in control and mutant embryos

A-D. Longitudinal sections through the trunk region (forelimb bud)
A. Control embryos. *Pax-3* expression is restricted to the dermomyotome compartment of the somites. Bar 100μm
B . Higher magnification of A - Arrowheads mark the dermomyotome region. Bar 40μm
C. *T/T* embryo - *Pax-3* expression domain is much more diffuse in the somites and no clear demarcation of the somites is evident. Bar 100μm
D. Higher magnification of C. Arrowheads mark the more extensive diffuse staining in the somites. Bar 40μm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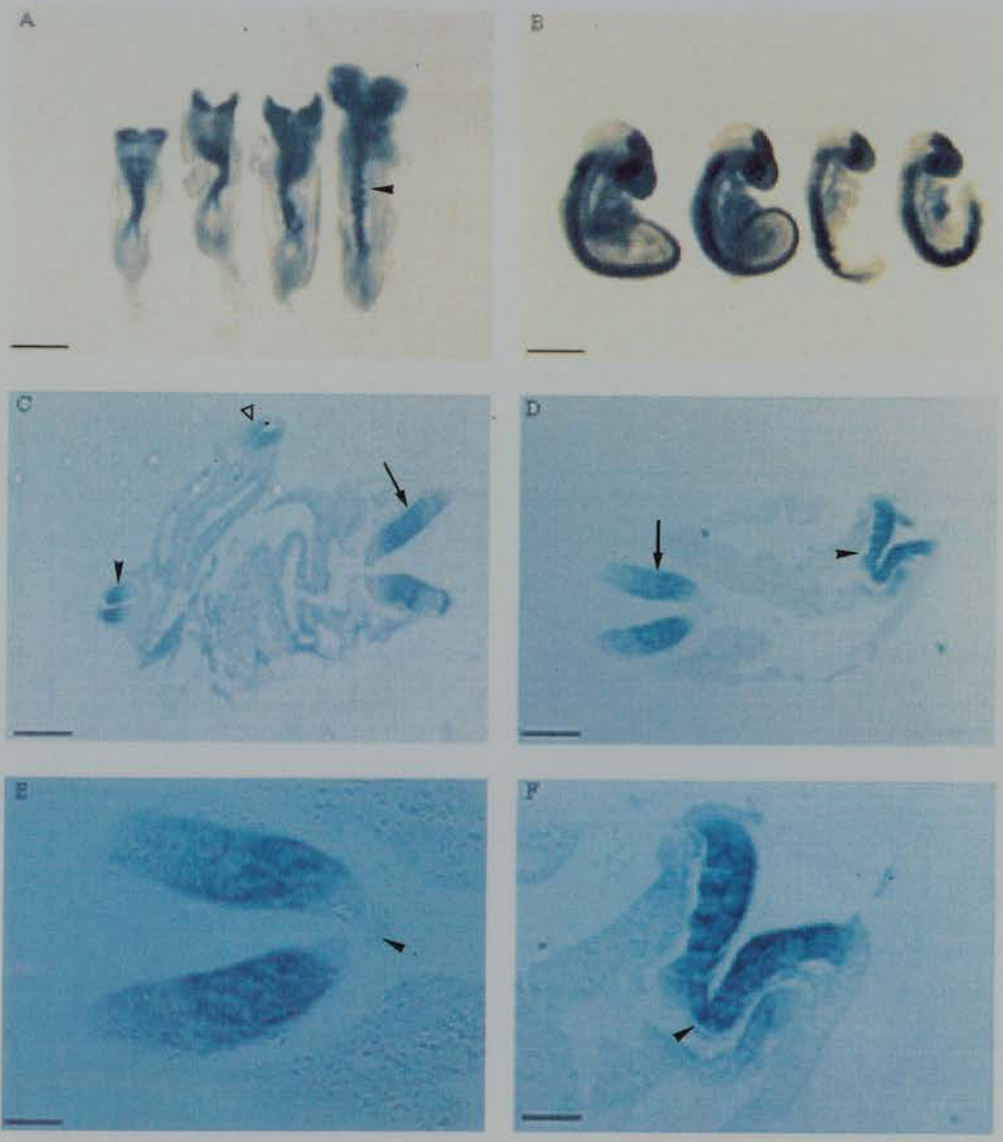
dermomyotome component of the somites. In control embryos, the extent of *Pax-3* expression in the neural tube changes along the rostrocaudal axis. In the more mature, anterior neural tube, *Pax-3* expression includes not only the most dorsal cells of the neural tube but also extends down the lateral margins of the neurectoderm to approximately the midpoint of the dorsoventral axis (Fig 4.7C). However, as one looks further caudally along the axis, this expression gradually becomes restricted to the most dorsal cells in the neural tube. In *T/T* embryos, *Pax-3* is also expressed in the dorsal part of the neural tube. However, in contrast to control embryos, as one looks further caudally along the rostrocaudal axis, the *Pax-3* expression domain gradually expands and includes more ventral regions of the neural tube (Fig 4.7D). Thus, even in anterior sections which contain a notochord the *Pax-3* expression domain in mutant embryos includes more ventral neural tube cells than in equivalent sections of control embryos. Although the caudal end of the neural tube of *T/T* embryos is very kinked and irregular, it appears that *Pax-3* expression is excluded from the ventral midline in all sections examined.

Unlike control embryos, *Pax-3* expression in *T/T* somites is not restricted to the lateral margins, but extends into the medial "sclerotomal" compartment (Fig 4.7 and 4.8). Again, the abnormal expression domains are most marked in the posterior part of the 9.5 dpc embryo: the most posterior somites express *Pax-3* only in the medial part of the somite and not along their lateral margin as in controls (Fig 4.8). It is apparent from the expression of *Pax-3* in *T/T* embryos that there are segmental blocks adjacent to the forelimb-bud. This suggests that *Brachyury* homozygous embryos have 9-10 pairs of somites rather than just 7 as previously reported. It should also be noted that these posterior somites do not express *Pax-1.*

Figure 4.9

Expression of *Pax-6* in control and mutant embryos

A and B. 2 embryos on the left are controls whilst the 2 on the right are homozygous mutants.
A. Dorsal view of 8.5 dpc control and *T/T* embryos. *Pax-6* expression appears the same in mutant and control embryos in both the head and along the neural tube. *Pax-6* transcripts reveal the highly convoluted nature of the neural tube in T/T embryos (arrow head). Bar 300μm
B. Lateral view of 9.5 dpc embryos. The expression domain is identical in the control and *T/T* embryos but the dorsoventral expression of the neural tube is enlarged in the mutants. Bar 750μm
C. Transverse section through a control 9.5 dpc embryo showing a medial band of *Pax-6* expression in the rostral neural folds (arrow '). and a more dorsal band in the mid trunk region (closed arrow head) - towards the caudal end of the embryo *Pax-6* expression again appears to be more ventromedial (arrow). Bar 100μm
D. Transverse section through a mutant 9.5 dpc embryo showing a medial band displaying an enlarged and ventralised *Pax-6* domain in the rostral head folds (arrow ') and greatly increased ventral expression in the trunk neural folds (closed arrowhead). Bar 100μm
E. Enlarged view of section D showing enlarged and ventralised expression of *Pax-6* in rostral neural folds of *T/T* embryo showing non staining in the "floor plate region" and the presence of a notochord (arrowhead). Bar 50μm
F. Enlarged view of section D showing ventralised expression of *Pax-6* in caudal neural folds of T/T embryo extending across ventral midline, the "floorplate region", (arrowhead) in the trunk neural folds. Bar 50μm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Pax-6 expression

In 8.5 and 9.5 dpc control embryos *Pax-6* is expressed along the neural tube but not in somites (Fig 4.9). At 8.5 dpc the wholemount *in situ* hybridisations with *Pax-6* appears to be the same for both *T/T* and control embryos, with expression being detected along the dorsal regions of the neurectoderm (Fig 4.9A). However, at 9.5 dpc there are differences between control and mutant embryos (Fig 4.9B-F). Serial sections of 9.5 dpc control embryos shows that the domain of expression varies along the neural tube (Fig 4.9C). Anteriorly, *Pax-6* is expressed in the ventrolateral regions of the neural tube. Further caudally, both the dorsal and ventral boundaries of the expression domain move dorsally. At the level of the 9th somite, expression is seen in the roof plate of the neural tube and in other dorsal regions whilst there is no *Pax-6* expression in the more ventro-lateral cells. However towards the more posterior, immature part of the embryo, *Pax-6* expression is again more ventral and no dorsal expression is seen (Fig 4.9C)

Sections of 9.5 dpc *T/T* embryos also show that *Pax-6* expression varies along the anteroposterior axis of the neural tube (Fig 4.9D). *Pax-6* expression in the mutants is similar to that in control embryos in the anterior trunk region rostral to somite 7, where a notochord-like structure is seen in some sections (Fig 4.9E). Posteriorly, however, *Pax-6* expression extends much further ventrally than in controls: expression is detected in the most ventral aspect of the neural tube, corresponding to the region where floorplate is located in control embryos (Fig 4.9F). In control 9.5 dpc embryos, *Pax-6* expression is never seen extending into the floorplate region.

Discussion

By examining the expression patterns of a number of genes implicated either in mesodermal formation or axial patterning in homozygous *T/T* mutants a more precise description of the mutant phenotype is obtained.

The *T* gene in Mesoderm formation

No abnormalities were noted in *BMP-4, Wnt-3a* and *Wnt-5a* expression in 7.5 dpc *T/T* egg cylinders. At 8.5dpc, BMP-4 transcription remained unaltered in mutant embryos. However, by 8.5 dpc the level of expression of *Wnt-5a* transcription had decreased in *T/T* embryos compared to that the same stage control embryos. Furthermore, after prolonged clearing it was evident that Wnt-3a expression intensity was also reduced. Similarly, by 9.5 dpc, *Evx-1* expression was no longer apparent in mutant embryos, despite having a normal distribution in headfold-stage embryos. This implies that although T protein is not required to activate transcription of these genes, it is necessary to maintain expression of *Evx-1*,*Wnt-5a* and probably *Wnt-3a* It is unlikely that this is due to tissue loss, as no change in cell cycle, reduction in growth rate and increase in pyknosis has been noted in *T/T* embryos before 9.5 dpc (Yanagisawa and Fujimoto 1977b; Yanagisawa et al. 1981). Furthermore the normal expression of *BMP-4* is not compatible with extensive cell loss.

Interestingly, studies of T^{wis}/T^{wis} mutant embryos have demonstrated that the level of mutant T transcripts rapidly declines at the early somite stage. Therefore wildtype T protein may be required in embryos beyond the early somite stage to maintain *Wnt-3a*,*Wnt-5a, Evx-1* and *T* gene transcription. A similar requirement for T protein to maintain *eve-1*, the zebrafish homologue of *Evx-1*, occurs in the early somite stages of that

organism (Joly et al. 1993). Recently, Cunliffe and Smith (1994) have shown that *Xbra* injected into Xenopus eggs triggered formation of ectopic ventral-type mesoderm and some muscle but not notochord in animal caps. When *noggin* was co-expressed with *Xbra*, dorsal mesoderm (muscle and notochord) and neural tissue were formed. In contrast, when *Xwnt-8* was co-expressed with *Xbra,* the animal caps formed mainly muscle masses. These experiments demonstrate that T protein can switch cells from an ectoderm to ventral mesoderm fate. This ventral mesoderm will in turn differentiate into dorsal mesoderm derivatives in response to dorsalising signals. *Xhox-3*, the *Xenopus* homologue of *Evx-1*, is one of the mesodermal markers transcriptionally activated by *Xbra*. The alteration of gene expression in *T/T* embryos, and the changes in cell fate induced by ectopic T protein combined with the mutant phenotype suggest that posterior mesoderm requires wildtype T, whilst the formation of anterior mesoderm is independent of wildtype T function.

From the data presented in this chapter, it would appear that at least *BMP-4* is expressed independently or upstream of *Brachyury*. Ectopic expression of *BMP-4* in 1-cell stage *Xenopus* embryos causes a transient increase in *Xbra* transcripts once zygotic transcription begins (Dale et al. 1992; Jones et al. 1992), suggesting that *BMP-4* or a related protein may normally activate *T* expression. Similarly, the recent demonstration that mouse embryos lacking a *Wnt-3a* product display a morphological phenotype remarkably similar to homozygous *Brachyury* mutants (Takada et al. 1993) argues that *Wnt-3a* and *T* are both involved in the same pathway. However, in *Wnt-3a* null mutants, scattered *Brachyury* expressing cells are visible even though a distinct notochord is not apparent. This result combined with those presented in this chapter suggest that *T* and *Wnt-3a* act independently of each other early in

gastrulation, although at later stages *Wnt-3a* may require *T* at later stages either directly or indirectly.

Axial Patterning

Most alterations in the axial patterns of gene expression described in tissues which do not themselves express the *T* gene can be explained by the lack of a functional notochord. However, the unaltered anterior expression boundary of *Hoxa-7* and the persistent dorsal expression of *Msx-1* in the neural tube are not consistent with this explanation.

Anteroposterior and mediolateral axial patterning

The results in this chapter show that 8.5 and 9.5 dpc embryos lacking the *T* gene product can still specify the correct anterior boundary for *Hoxa-7* even though the mutant phenotype has a greatly foreshortened rostrocaudal axis and severe posterior abnormalities. Therefore, the presence of an active *T* gene product is not required to specify the anterior boundary of *Hoxa-7* expression domain. It has been argued that the notochord imparts rostrocaudal information to overlying tissues. For example, anterior *Xenopus* notochord induces *engrailed* expression in an animal cap whilst posterior notochord does not (Hemmati-Brivanlou et al. 1990). The formation of a normal *Hoxa-7* boundary in a region of the posterior axis of *T/T* embryos lacking an intact notochord does not support this argument. Instead the results agree with experiments in the chick which show that *engrailed-2* expression is unaffected in the absence of cranial notochord (Darnell et al. 1992). However, if the half life of the *lacZ* protein is relatively long compared to that of *Hoxa-7* mRNA, then my experiments cannot assess whether the *T* gene is required to maintain the correct spatial integrity of endogenous *Hoxa-7* expression.

It is possible that the *T* gene product is only required for those *Hox* genes that are expressed later in development at the very posterior extreme of the embryo. Thus, *Hoxa-7* may not be affected because of it's early induction and anterior expression pattern. Therefore, comparison of other *Hox* genes which are first expressed after 8.5 dpc (for example *Hoxd-9 to Hoxd-12* (formerly known as *Hox 4.4* to *-4.7* : Izpisua-Belmonte et al. 1991) in *Brachyury* and wildtype embryos may give insight as to when, or if, the *T* gene product controls, either directly or indirectly, any *Hox* gene expression.

In contrast to the rostrocaudal axis, the mediolateral axis is altered in *T/T* embryos. Thus, there is an elevated *Msx-1* hybridisation signal both beneath the primitive streak of 8.5dpc embryos and in the tail bud region of 9.5dpc embryos as well as a decrease in signal intensity in the forelimb bud of *T/T* embryos. The changes observed in the mediolateral patterning of *Msx-1* transcripts probably reflects the accumulation of nascent mesoderm cells in the caudal region of mutant embryos (Wilson et al. 1993) with a subsequent decrease in cell number in the developing limb bud.

Dorsoventral patterning in the neural tube and somites

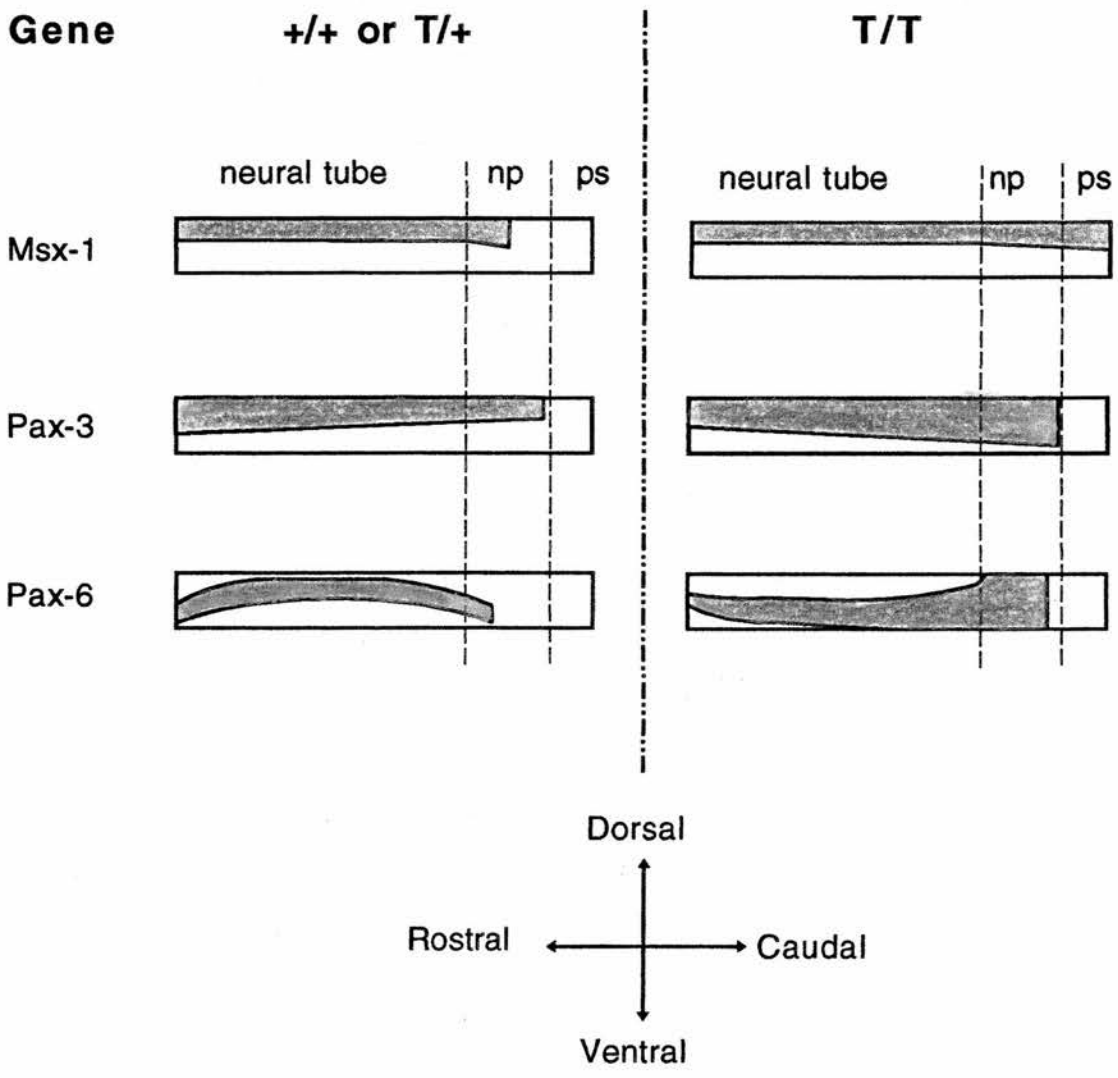
The changes in domain of *Msx-1* and *Pax* gene expression in the neural tube is summarised in Figure 4.10. The alteration in *Pax-1*, *Pax-3* and *Pax-6* expression in somites and neurectoderm in *T/T* mutant embryos is consistent with the loss of a ventralising signal in these embryos. In posterior somites there are no *Pax-1* transcripts detected, whilst the expression domain of *Pax-3* extends ventrally. In the neural tube both *Pax-3* and *Pax-6* expression extends further ventrally than control embryos especially at the caudal extremity. This loss of ventralising signal is most

Figure 4.10

Schematic representation of the differential expression domain along the dorsoventral axis in the neural tube of *T/T* and control embryos

The neural tube is represented in each case by a rectangle, whilst the expression pattern of *Msx-1,Pax-3* and *Pax-6* in either control (+/+ or *T/+*) or *T/T* embryos is shown by a shaded area

np-neural plate
ps- primitive streak


Gene
+/+ or T/+
T/T
neural tube
np
ps
neural tube
np
ps
Msx-1
Pax-3
Pax-6
Dorsal
Rostral
Caudal
Ventral

probably due to the absence of a functional notochord in the caudal end of the mutant embryo (see Clarke et al. 1991; Goulding et al. 1993; Jessell and Melton 1992; Ordahl and Le Douarin 1992; Placzek et al. 1990; Pourquie et al. 1993; Rong et al. 1992; van Straaten and Hekking 1991; van Straaten et al. 1988; Yamada et al. 1991). The extension of *Pax-6* expression domain across the ventral midline at the caudal end of *T/T* mutants, strongly suggests that no floor plate is present here. These results agree with the observation that no floor plate is induced in the caudal most extremity of zebrafish *no tail* mutants (Halpern et al. 1993). Lineage studies in *ntl* mutants suggest that these embryos possess small residual notochord precursor cells which fail to differentiate into a notochord and presumably at the caudal extremity of the embryo too few remain to induce a *bona fide* floorplate (Halpern et al. 1993). Similarly, in *Brachyury* homozygotes, the anterior notochord does form but instead of remaining as a distinct rod-like structure, it becomes sporadic and notochord-like cells are seen forming abnormal connections with the neurectoderm and gut endoderm. Although a definitive notochord does not form in the posterior part of the mutant embryo, it is possible that notochord-like cells are present but are not organised into a distinct midline structure. While floor plate induction may be compromised caudally, allowing *Pax-6* expression to extend across the neural tube midline, sufficient notochordal-like cells may still be able to partially effect neural tube pattern as *Pax-3* expression never extends into the ventral midline of this tissue suggesting that, even at the caudal aspect, the neural tube is not entirely dorsalised. Alternatively *Pax-3* expression may also depends on independent dorsalising signals which are insufficient to allow ubiquitous expression of *Pax-3* in the neural tube.

The disruption of somite patterning may also be attributed to the lack of a functional notochord, although this effect may be indirect and mediated via the neural tube. A number of experiments carried out in chick have demonstrated that the axial structures, that is the notochord and neural tube, are important for normal somite survival and differentiation as well as definition of the dorsoventral and mediolateral axes. Previous investigators have suggested that the normal architecture of sclerotome and dermomyotome is lost in *Brachyury* homozygotes (Jacobs-Cohen et al. 1983b). However, our results demonstrate, that the anterior mutant somites maintain the correct spatial relationship between *Pax-1* and *Pax-3* expression even if the sclerotome compartment is reduced. This suggests that, at least in the 4th - 7th somite, sclerotome differentiation may be normal. As *Pax-1* expression appears to be necessary for normal vertebral chondrogenesis (Balling et al. 1992; Wallin et al. 1994), this finding is consistent with the observation that *T/T* somites are capable of differentiating into cartilage *in vitro* (Jacobs-Cohen et al. 1983b).

The abnormal expression patterns of *Pax-1* and *Pax-3* seen in *Brachyury* homozygous somites could be also be caused by defective migration of mesodermal cells away from the primitive streak which may result in insufficient paraxial mesoderm for appropriate differentiation. If, as in the chick, medial somite mesoderm arises from the node and lateral somite mesoderm from the anterior streak (Selleck and Stern 1991), then abnormalities in the node may selectively reduce the ventromedial cells and a decrease in *Pax-1* expression may occur. However, such a dual origin of somite mesoderm has been challenged by Schoenwolf and co-workers (Garcia-Martinez and Schoenwolf 1992; Schoenwolf et al. 1992). Also other mouse mutants with notochordal defects show similar alterations of *Pax-1* and *Pax-3* expression patterns (Dietrich et al. 1993), suggesting

that the abnormalities observed in *T/T* embryos are due to aberrant signals from the notochord rather the mesodermal migration defects.

More intriguingly, the *Msx-1* expression domain does not extend ventrally in *T/T* neurectoderm, but remains restricted to the roof plate region as it does in control embryos (Fig 4.5D). This means that, unlike *Pax-3* and *Pax-6, Msx-1* expression is not ventralised in the *T/T* neural tube, even at the caudal aspect of the embryo. There are two possible mechanisms which could explain this, neither of which can be excluded. (i) the defective notochord in *T/T* mutants is still capable of restricting *Msx-1* expression or (ii) correct patterning of the neural tube requires at least two independent signals: a ventral signal emanating from the notochord/floorplate and a dorsal signal of unknown origin. The first hypothesis, is possible if a separate or much weaker signal from the wildtype notochord/floorplate restricts *Msx-1* expression and this is sufficiently unaltered to maintain normal *Msx-1* expression, but not *Pax-3* expression, in *T/T* embryos. The second hypothesis is supported by the recent demonstration that *dorsalin-1*, a member of the TGF-ß family, can act as a potent but quite late dorsalising signal of the developing chick spinal cord. *Msx-1* transcripts are also present in neural crest cells whose fate is intimately linked with the cells of the dorsal neural tube (Bronner-Fraser and Fraser 1988; Bronner-Fraser and Fraser 1989). Thus, the observation that the *Msx-1* expression domain in the neural tube of *T/T* embryos is unaltered is consistent with the result that ectopically situated notochord grafts do not suppress the formation of neural crest cells or commissural axons (Artinger and Bronner-Fraser 1992). *Msx-1* expression is restricted to the lateral margins of the posterior wildtype neural plate before tube closure, suggesting that a lateral signal may exist in the embryo which defines the prospective neural crest and future dorsal

neurectoderm. The origin of this signal is not known, but from the anatomy of the embryo the surface ectoderm or lateral mesoderm are potential sources.

Chapter 5

Conclusions

The work presented in this thesis has used two experimental approaches to further analyse of the functional role of one gene, *Brachyury(T)*. Firstly, ES cells, derived from heterozygous *T/+* matings, were used to perform a chimaeric analysis to demonstrate the cell autonomous nature of the *T* gene in the notochord and primitive streak. Secondly, a more detailed description of the *Brachyury* phenotype was performed to assess whether *T* interacts, either directly or indirectly, with other genes which may also be involved in mesodermal formation and dorsoventral patterning of the neural tube.

Introduction of a single cell marker

Brachyury ES cell lines provide a continuous source of mutant cells whose development can be monitored either *in vivo* or *in vitro*. They are a good system to use to make chimaeras because they can be genotyped prior to chimaera formation. This avoids performing polymerase chain reaction assays on biopsies of individual embryos (Handyside et al. 1990) or genotyping the chimaeras retrospectively. In order to extend the experiments described in chapter 3 and further analyse the fate of *T/T* cells in chimaeras, it would be necessary to introduce a visible genetic marker which can be used to distinguish, either immunologically or histochemically, the fate of all progeny of the ES cells on a single cell level. Two kinds of marker have been used in other chimaeric studies: those that rely on gene expression and those that do not.

The *Escherichia Coli lacZ* gene has frequently been used as a cell autonomous reporter transgene when attached to different promoters (for example Püschel et al 1990) as it has little or no effect on development (Beddington 1989). Some ubiquitous promoters, such as herpes simplex thymidine kinase, appear to be too weak to overcome position effects

(Allen et al. 1988). Other "strong" promoters produce unexpected tissue-specific and stage-specific gene expression in transgenic animals: for example the rat *ß-actin* promoter, whilst generating ubiquitous *lacZ* expression in the midgastrulation embryo, does not maintain expression in the latter half of gestation or postnatally (Beddington et al, 1989). Recently, a transgenic strain of mice has been produced by pronuclear injection which caries at least 14 copies of *lacZ* attached to the HMG promoter (Tam and Tan 1992). The *HMG-lacZ* transgene appears to have integrated into a single locus on the X-chromosome and ubiquitous and uniform staining of *lacZ* is observed in all cells of hemizygous males and homozygous females from the morula stage (2.5 dpc) upto at least 13.5 dpc. Random insertion of gene trap constructs, consisting of a reporter gene without a promoter (Gossler et al 1989) may also produce ES cell lines which express the *lacZ* reporter constitutively. Using this method, several investigators (Freidrich and Sorriano, 1991; Skarnes et al, 1992) have produced clones, which on injection or aggregation with preimplantation embryos, appear to display ubiquitous staining of *lacZ* at the embryonic stages examined. It remains to be seen whether any of these ES cell lines not only produce constitutive *lacZ* expressing progeny at all stages of development but also have no deleterious effect to the embryo.

A non-expressing nuclear marker has the advantage that it is not subject to poorly understood transcriptional and translational influences. Such markers have been used to distinguish experimental cells from wildtype in chimaeras. For example, one transgenic strain, produced by pronuclear injection, contains a head-to-tail concatamer of approximately 1000 copies of ß-globin (Thomson and Solter. 1988b). This transgene has been used to assess the tissue-specific selection against androgenetic,

parthenogenetic or gynogenetic cells in chimaeras (Clarke et al. 1988: Thomson et al. 1988a). There are however several disadvantages from using a nuclear marker. Firstly, it may be more difficult to identify cell phenotype and position with a marker that does not define the cellular outline. Secondly, at present, detection by DNA *in situ* hybridisation is restricted to sectioned material. Therefore, in order to regain three dimensional information regarding cell position it is necessary to perform laborious reconstructions. Also different optimal section widths are required to obtain maximal detection from different sized nuclei.

Role of *T* in the primitive streak and notochord

The chimaeric analysis in chapter 3 demonstrates that *T* acts cell autonomously. This result agrees with the studies on zebrafish *ntl* embryos (Halpern et al, 1993) and is supported by the observation that the T protein has a nuclear location (Schulte-Merker et al., 1992; Kispert and Herrmann, 1994). However, the function of T is probably different in the primitive streak and notochord. T is required for proper differentiation of the notochord (and perhaps the allantois). In contrast, in the streak, it appears to necessary for morphogenetic cell movement. Therefore it would be interesting to attempt to rescue the function of one without the other, that is, to produce embryos which have *T* expression in the primitive streak, but not in the notochord or *vice versa*. In order to do this, it will be necessary to identify the separate promoters or enhancers required for the different sites of *T* transcription. Once identified, these could be attached to the *T* genomic coding sequence. The effect of such transgenes could be assessed by producing transgenic animals by pronuclear injection. These would then have to be mated with heterozygous *T/+* animals and bred to remove the endogenous gene. Alternatively, the transgene could

be introduced into null ES cells, which also allows one to easily assess the fate of the manipulated cells in chimaeras.

Analysis of gene expression

Examination of gene transcription patterns indicates that *T* is part of a complex repertoire of gene expression during early gastrulation. So far all genes examined (*Wnt-3a, Wnt-5a BMP-4, Evx-1* and *T* (see chapter4; Herrmann 1991)) seem to be expressed independently early on in gastrulation. However, later development appears to require that genes regulate each other in order for correct differentiation to occur. This would be consistent with the hypothesis that gene expression in the primitive streak of the egg cylinder is under global control whilst later gene expression in the streak is dependent on cross regulatory pathways. Consequently, the absence of any component in this system would predominantly affect the post-cervical regions. Interestingly this phenotype is observed in embryos lacking normal products of either *T* (Chesley, 1935), *Wnt-3a* (Takada et al. 1993) or α_5 integrin (Yang et al. 1993) genes.

ES cells null for the *T* gene present an ideal substrate for genetic manipulation of genes acting downstream of *T*. Cultured cells could be used to assay for the presence or absence of genes by performing RNA *in situ* hybridisations on cell monolayers or on embryoid bodies. However, if the role of T protein is to modify and maintain, rather than to initiate, gene expression, this assay alone may not find many genes acting downstream of *T*. More information about the role of *T* in modulating the function of other genes may be obtained by altering the culture conditions, either by the addition of substances, for example growth factors or mutagens, or by co-culturing *T/T* and wildtype ES cells in the presence of other tissues or

cells. Eventually extensive mutation analysis is required to resolve the gene hierarchies.

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