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Original Article

Alterations in gene expression during mesoderm formation and axial patterning in *Brachyury (T)* embryos

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ABSTRACT The mouse *T (Brachyury)* deletion causes defective mesoderm formation and notochord morphogenesis, and abnormalities in the caudal neural tube and somites. To investigate the effect of the wild type *T* gene on concurrently expressed genes, we have compared expression of a panel of such genes in homozygous *T* mutants with that in wild type and heterozygous *T/+* control embryos. Two classes of genes were used in this study: those implicated in primitive streak or mesoderm formation, and those which are differentially expressed in regions of the neural tube and somites. Results of wholemount *in situ* analysis show that the mRNA levels of *Evx-1*, *Wnt-3a* and *Wnt-5a* decrease in *T/T* embryos late in gastrulation, although earlier expression patterns are similar to control embryos. In contrast, *BMP-4* and *Msx-1* expression patterns remain similar throughout the period studied. *Pax-3* and *Pax-6*, which are expressed in specific dorsoventral domains of the neural tube, both have ventrally extended expression domains in caudal *T/T* neural tube. This is consistent with a missing ventral signal provided by the notochord. However, the expression of *Msx-1* in the most dorsal domain of the neural tube is unaltered in *T/T* embryos. *Pax-1* and *Pax-3*, which are expressed in the sclerotome and dermamyotome respectively, are expressed correctly in anterior *T/T* somites, although the *Pax-3* expression domain is widened ventromedially. This extension into ventromedial somite domains is more pronounced caudally, supporting a function for the notochord in ventralizing somites.

KEY WORDS: *Brachyury, primitive streak, gastrulation, Pax*

Introduction

Deletion of the *T* gene in the mouse *Brachyury* mutant results in defective gastrulation culminating in the failure of normal caudal axis formation. While axial pattern and tissue differentiation appear overtly normal, rostral to the forelimb bud region (up to the level of somite 7 or 8), more caudal somites are absent or grossly abnormal: the neural tube is present but often extremely convoluted and no discrete notochord can be identified (Chesley, 1935; Gluecksohn-Schonheimer, 1944; Gruneberg, 1958). Since the *T* gene is normally expressed in the primitive streak from the onset of gastrulation (Wilkinson, *et al.*,1990; Beddington, *et al.*, 1992) it is likely that this aberrant development has an early, if subtle, inception during gastrulation. Furthermore, the notochord has been implicated as an early inducer of both dorsoventral and anteroposterior patterns in vertebrates (van Straaten, *et al.*,1985, 1988, 1989; Smith and Schoenwolf, 1989; Hemmati-Bravanlou *et al.*, 1990; Placzek, *et al.*, 1990; Yamada, *et al.*,1991). Therefore, the *Brachyury* mutant serves as a convenient subject for examining the development of these axes in the absence of a normal notochord.

To examine the hierarchical relationship between *T* expression and that of other known genes during early development, we have analyzed the expression patterns of 4 genes normally expressed coincidently with *T* in the primitve streak from mid-gastrulation to early organogenesis in the mouse. The genes selected for study *(BMP-4, Evx-1, Wnt-3a,* and *Wnt-5a),* like *Brachyury* itself, have homologues in *Xenopus* which influence amphibian mesoderm formation. Transcription of the *Xenopus* homologue of *Brachyury (Xbra)* has been shown to be an immediate response to mesoderm induction by activin A (Smith, *et al.*,1991). Furthermore, injection of *Xbra* mRNA into the animal pole of single-cell *Xenopus* embryos leads to the normal initiation of gastrulation but the marginal zone mesoderm fails to involute and an excess of posteroventral mesoderm is formed (Cunliffe and Smith, 1992). This aberrant gastrulation is associated with the induction of *Xhox-3* expression, a putative transcription factor normally expressed in posterior mesoderm, and the *Xenopus* homologue of *Evx-1* (Ruiz i Altaba and Melton, 1989; Bastian and Gruss, 1990; Dush and Martin,

Abbreviations used in this paper: PCR, polymerase chain reaction; dpc, days *post coitum*.

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1992). A somewhat similar result is obtained when mRNA from the *Xenopus* homologue of *BMP-4, XBMP-4* (Jones *et al.*, 1991), is injected into the animal pole of 1-cell *Xenopus* embryos (Dale *et al.*, 1992; Jones *et al.*, 1992). Ectopic expression of *XBMP-4* leads to disruption of gastrulation movements and the formation of ectopic posteroventral mesoderm, again associated with *Xhox-3* expression. In addition, there is a transient induction of *Xbra* transcription. Injection of *Xhox-3* mRNA into the animal pole of early cleaving *Xenopus* embryos results in the abnormal development of anterior structures following gastrulation, which is consistent with *Xhox-3* having an important role in posterior mesoderm differentiation (Ruiz i Altaba and Melton, 1989). These experiments in *Xenopus* suggest that *XBMP4* is involved in the differentiation of posteroventral mesoderm and the onset of *Xhox3* expression, either via induction of *Xbra* transcription, or in association with it. In contrast, injection of *XWnt-8* mRNA into the vegetal pole of single-cell *Xenopus* embryos, or into ventral blastomeres of early cleaving embryos, can produce a duplicated embryonic axis and an alteration in the fate of ventral cells, such that they differentiate into dorsal rather than ventral mesoderm (Smith and Harland, 1991; Sokol *et al.*, 1991). However, if *XWnt-8* is expressed only after the mid-blastula stage, its effects are reversed and it now acts to ventralize mesoderm (Christian and Moon, 1993). *Wnt-3a* and *Wnt-5a*, which were isolated due to their homology to *Wnt-1* (Gavin *et al.*, 1990) and thus are presumably secreted proteins, are expressed in the primitive streak region of gastrulating mouse embryos. (Takada *et al.*, 1993) and are part of the large gene family to which *XWnt-8* also belongs. Since the effects of late expression of *XWnt-8* in *Xenopus* probably most closely mimic its normal role, homologues of all the genes selected for study in the mouse influence caudal and ventral mesoderm formation in the frog, as does *T* itself.

Later patterning events dependent on *T* have been assessed by examining the expression patterns of 5 further genes *(Hox-A.7* (formerly known as *Hox 1.1*), *Msx-1* (formerly known as *Hox 7.1*), *Pax-1*, *Pax-3* and *Pax-6*) which normally show restricted expression domains in one or more of these axes. In particular, the role of the notochord in neural and somitic patterning can be evaluated in *T/T* embryos where the notochord is disorganised or absent. The anterior boundary of *Hox-A7* expression in paraxial mesoderm coincides with the 14th somite, while in the neurectoderm the boundary is further rostral and adjacent to the 8th somite (Mahon *et al.*, 1988; Püschel *et al.*, 1990, 1991). Consequently, this gene provides a convenient marker for anteroposterior patterning in the most rostral region affected in *Brachyury* mutants. *Msx-1* is differentially expressed in the mediolateral axis, being transcribed predominantly in a lateral domain, and it also serves as an early dorsal marker in the neural tube (Robert *et al.*, 1989). The *Pax* genes are also restricted with respect to the dorsoventral axis: *Pax-1* expression becomes confined to the ventromedial part of somites (the sclerotome) (Deutsch *et al.*, 1988; Balling *et al.*, 1992), whereas *Pax-3* expression predominates in the dorsal part of the neural tube (Goulding *et al.*, 1991) with *Pax-6* being expressed in a more ventral sector, although not in the cells of the ventral midline (the floor plate; Walther and Gruss, 1991).

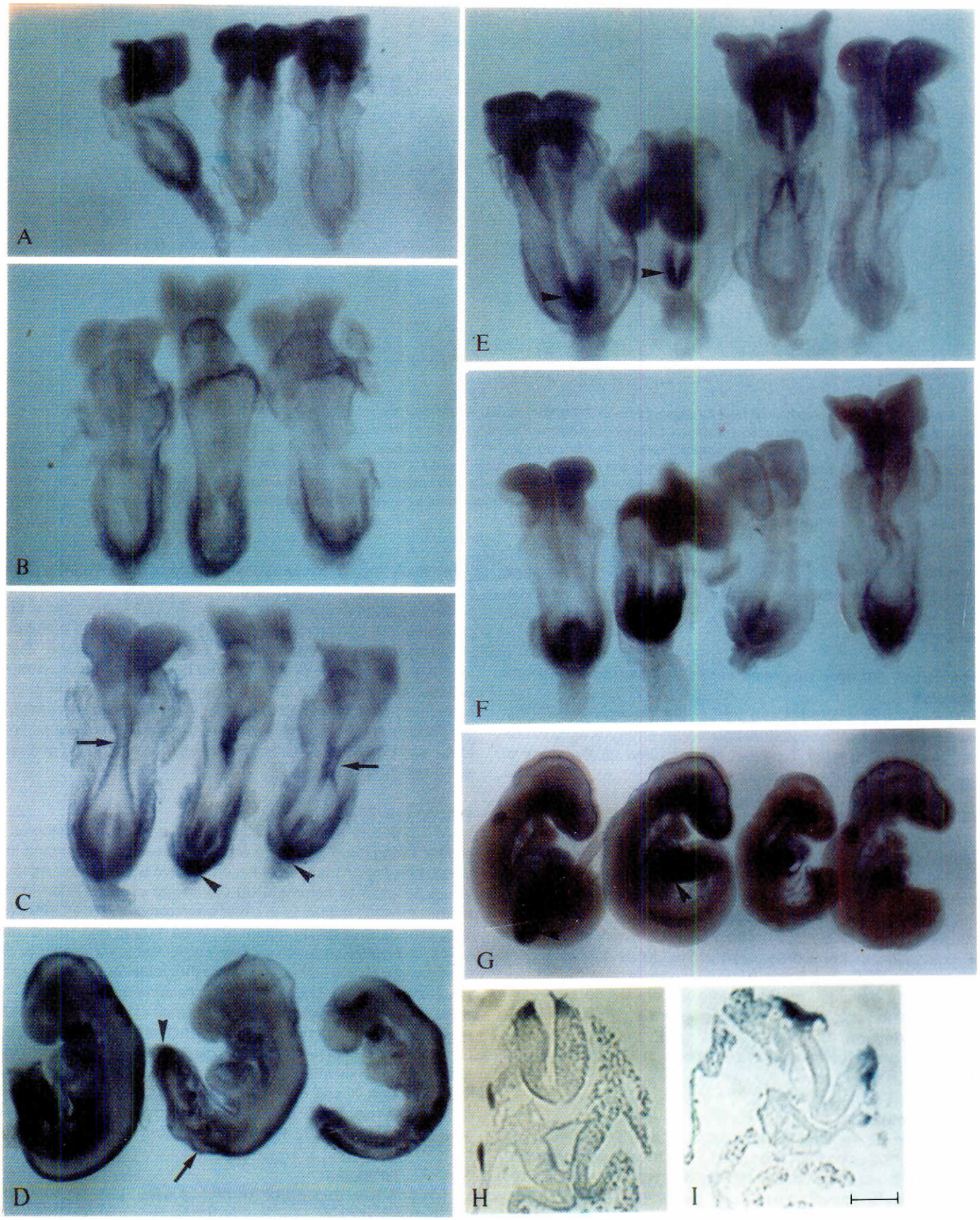
Results

The results described in this paper focus on those embryonic structures and regions which are affected in *T/T* embryos, including the caudal part of the embryo, the neural tube and somites. They do not provide a comprehensive description of gene expression patterns in wild type embryos since these are 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); *Hox-1.1* (Mahon *et al.*, 1988; Püschel *et al.*,1990, 1991); *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). In general, only observed deviations from the normal patterns of gene expression are illustrated.

Polymerase chain reaction (PCR) analysis of 113 extraembryonic portions of 7.5 dpc (days *post coitum*) embryos identified 21 unequivocal *T/T* embryos and 74 control embryos. The *T/T* embryos were divided into 3 groups of 7 and probed with *BMP-4*, *Wnt-3a* and *Wnt-5a*. The control embryos were subdivided into 5 groups (~15 embryos) and probed not only with the same probes as *T/T* embryos, but also with control *T* and α-*actin* probes. 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 obtained from 2 separate hybridizations.

T/+ and *Tgm6lacZ1* mice were intercrossed as described in Materials and Methods, to give control and *T/T* embryos whose anterior boundary of *Hox-A7* expression was demarcated by

Fig. 1. Expression of *Wnt-3a, Wnt-5a, Evx-1, BMP-4* and *Msx-1* in control and *T/T* mutant embryos. *Anterior is to the top of the Figure. In all figures of whole-mount preparations two T/T mutant embryos are located to the right of the photomicrograph.* **(A)** *Dorsal view of Wnt-5a expression at 8.5 dpc. In the control embryo 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 T/T embryos by the 5-6 somite stage. The staining seen in the cranial region is non-specific. Bar, 400 μm.* **(B)** *Ventral view of BMP-4 expression at 8.5 dpc. In both mutant and control embryos BMP-4 is expressed in caudal ectoderm and mesoderm and in the amnion (which is responsible for the staining seen at the periphery of the embryo) and overlying the heart. Bar, 300 μm.* **(C)** *Dorsal view of Msx-1 expression at 8.5 dpc. Expression in both control and mutant occurs in the dorsal neural tube and neural plate (arrow), the primitive streak and lateral mesoderm. In mutant embryos there is some indication of elevated expression in the caudal primitive streak (arrowhead). Bar, 300 μm.* **(D)** *Lateral view of Msx-1 expression at 9.5 dpc. In both control and mutant expression is restricted to the dorsal aspect of the neural tube or neural plate, even where this is severely contorted in mutant embryos (arrow). The most caudal tissue of mutant embryos strongly expresses Msx-1 (arowhead). Bar, 750 μm.* **(E)** *Dorsal view of Wnt-3a expression at 8.5 dpc. In controls, specific staining is restricted to the primitive streak (arrowhead), that seen in the head and heart being background staining. In T/T embryos no expression is seen in the primitive streak. Bar, 290 μm.* **(F)** *Dorsal view of Evx-1 expression at 8.5 dpc. Expression is seen in the primitive streak, caudal ectoderm and mesoderm. The level of expression in T/T embryos was consistently reduced by the 7 somite stage. This is somewhat obscured in the embryo on the right because the hindgut invagination is more advanced and causes more non-specific light absorption. Bar, 300 μm.* **(G)** *Lateral view of Evx-1 expression at 9.5 dpc. Despite high background staining in embryonic cavities, it is clear that all caudal Evx-1 expression is extinguished in T/T embryos. Control embryos show high expression in the tailbud. Bar, 800 μm.* **(H)** *Transverse section through the trunk region of 9.5 dpc control showing Msx-1 expression restricted to the dorsal margins of the neural tube. Bar, 200 μm.* **(I)** *Transverse section through the trunk region of a T/T embryo also showing Msx-1 expression restricted to the dorsal aspect of the neural tube. Some staining is observed immediately lateral to the dorsal neural tube and this probably marks neural crest cells displaced by extensive kinking of the neural tube. Bar, 200 μm.*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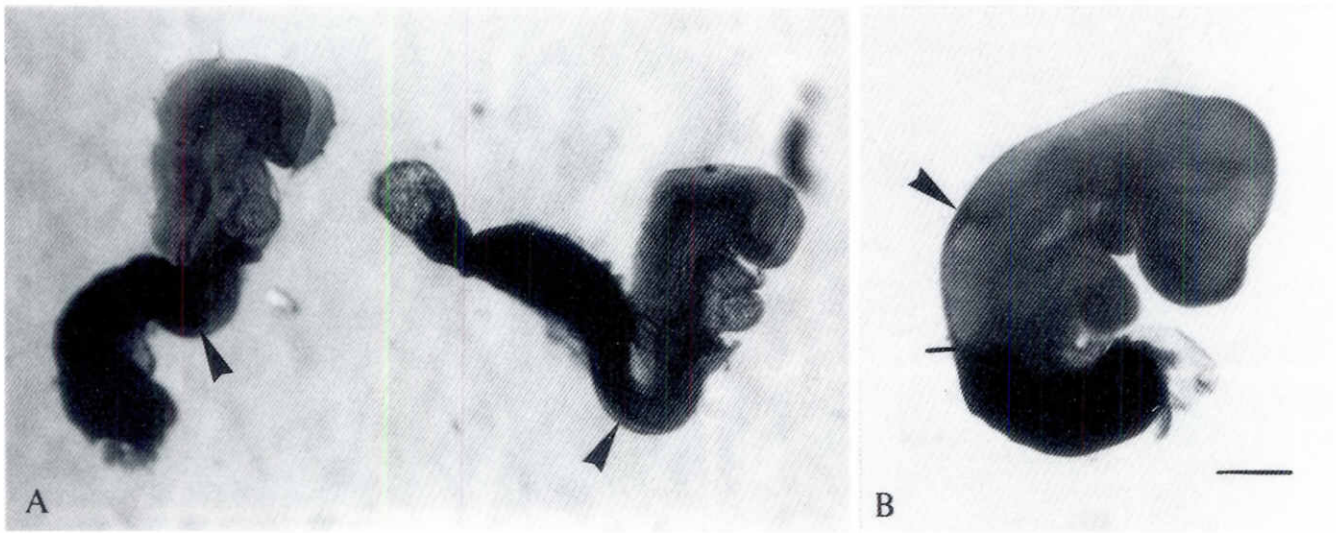

Fig. 2. Normal rostral boundary of *Hox A7* expression in *T/T* embryos. (A) *An 8.5 dpc control (right of picture) and T/T embryo (left of picture) carrying the m6lacZ1 reporter construct showing identical anterior boundaries of staining for E.coli ß-galactosidase activity (arrowheads). Bar, 200 μm.* **(B)** *9.5 dpc Tg.m6lacZ1 transgenic T/T embryo showing the correct anterior boundary of ß-galactosidase staining corresponding to the level of the 7th somite in the neural tube (solid bar) and a more caudal boundary in the paraxial mesoderm (arrow). Even though the paraxial mesoderm is not segmented caudal to somite 7 in T/T embryos, a boundary of lac Z expression still forms in the mesoderm at approximately the same position that it does in control embryos (at the level of the 14th somite). Ectopic staining is seen rostrally in the dorsal neural tube (arrowhead) but on sectioning this proved to be in the lumen of the developing spinal cord rather than intracellular. Bar, 250 μm.*

Escherichia coli ß-galactosidase activity. 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 expressed during mesoderm formation

The expression patterns of 5 genes co-expressed with *T* in the primitive streak region of gastrulating mouse embryos were examined to assess whether T protein was required for the onset or maintenance of their expression.

BMP-4 expression

At 7.5 and 8.5 dpc both control and homozygous *T/T* embryos displayed similar staining intensity and localization of expression of *BMP-4*. 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. 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. It is also expressed in the fore- and hindgut endoderm. After the 4 somite stage, staining is also seen in the most posterior mesoderm and ectoderm (Jones *et al.*, 1991; Fig. 1B).

Evx-1 expression

Only one experiment, including both 8.5 and 9.5 dpc embryos, was performed using this probe. At 8.5 dpc the expression pattern of *Evx-1* is the same in both mutant and control embryos (Fig. 1F), although by about the 7 somite stage (judged by head morphology rather than somite count since *T/T* embryos do not form more than 7 somites), expression in mutant embryos is consistently lower than in controls. At early somite stages *Evx-1* is expressed in the posterior part of the embryo caudal to the last somite and is also present in the primitive streak, medial mesoderm and ectoderm, including the neurectoderm. 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 incipient tailbud 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 *T/T* embryos at 9.5 dpc (Fig. 1G).

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. 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 normal embryos expression is restricted mainly to the primitive streak but also extends into the posterior mesoderm and ectoderm (Fig. 1E). There is no expression in the allantois. In *T/T* embryos, before clearing specimens, weak expression of *Wnt-3a* was evident corresponding to the normal pattern. However, after clearing, no hybridization signal was seen in the caudal region although dorsal expression in the neural tube was maintained (Fig. 1E). Although based on a total of only ten 8.5 dpc embryos at different developmental stages, it appears that expression of *Wnt-3a* declines rapidly at about the 4-5 somite stage.

Wnt-5a expression

The pattern 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

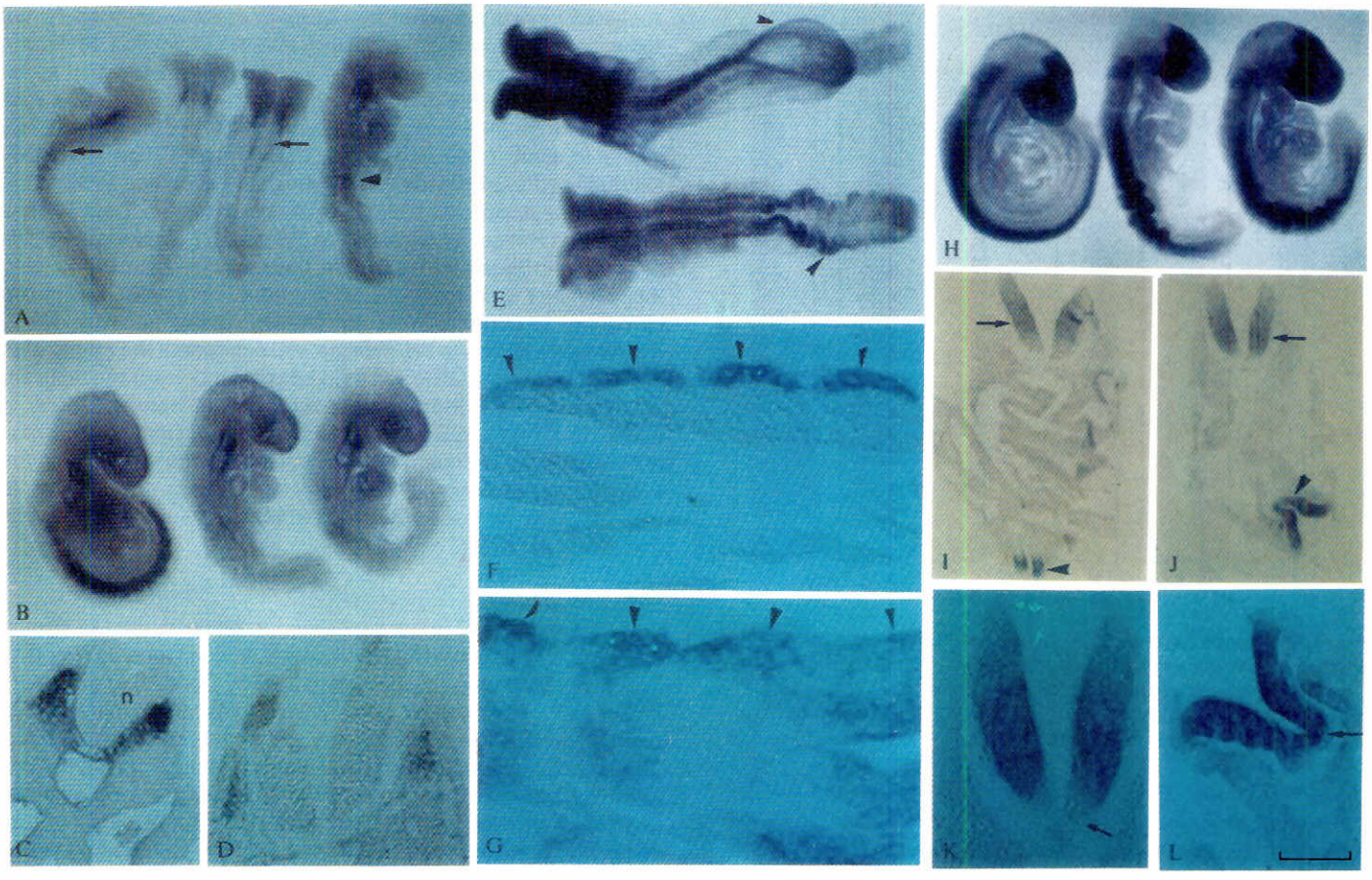

Fig. 3. Expression of *Pax* genes in wholemount preparations. *Two mutant embryos are present on the left of each photomicrograph, except in E where the mutant embryo is located at the bottom of the picture.* **(A)** *Pax-1 expression in 8.75 and 9.0 dpc embryos. Expression in the paraxial mesoderm of both mutant and control embryos commences at the level of the 4th somite but rapidly becomes abnormal in mutant embryos (arrowhead). Expression is also seen in the pharynx. Bar, 400 μm.* **(B)** *Pax-1 expression in 9.5 dpc embryos. Control embryos express Pax-1 in all somites caudal to and including somite 4. Some remnants of Pax-1 expression are evident in somites 4-7 of T/T embryos but at greatly reduced levels. Bar, 750 μm.* **(C)** *Transverse section through the trunk region of a control embryo showing strong Pax-1 expression in the sclerotomal compartment of the somites. N, neural tube. Bar, 150 μm.* **(D)** *Transverse section through the cervical region of T/T embryo showing weak expression of Pax-1 in a reduced ventral compartment of the somites. Bar, 100 μm.* **(E)** *Dorsal view of Pax-3 expression at 8.5 dpc. Expression is evident in the somites and dorsal neural tube of both mutant and control embryos. The staining in the head of the control is non-specific. The dorsal domain of expression in the unfused neural folds of the T/Tembryo is enlarged (compare staining domains indicated by arrowheads). Bar, 250 μm.* **(F)** *Longitudinal section of somites in the trunk region of a control embryo showing Pax-3 expression. Staining is restricted to the dermamyotome compartment of the somite (arrowheads). Bar, 100 μm.* **(G)** *Longitudinal section of somites in the cervical region of a T/T embryo showing Pax-3 expression. The staining is much more diffuse in the somites (arrowheads) and no clear demarcation of the dermayotome is evident. Instead the domain of expression in each somite is enlarged and encroaches on the sclerotome region. n, neural tube. Bar, 100 μm.* **(H)** *Lateral view of Pax-6 expression at 9.5 dpc. Expression in the head is identical in control and mutant embryos but the dorsoventral expression domain in the neural tube is enlarged in T/T embryos. Bar, 750 μm.* **(I)** *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 (arrowhead). Bar, 200 μm.* **(J)** *Transverse section through a 9.5 dpc T/T embryo showing an enlarged and ventralised band of Pax-6 expression in the rostral neural folds (arrow) and greatly increased ventral expression in the trunk neural folds (arrowhead). Bar, 200 μm.* **(K)** *Enlarged view of adjacent section to* **J** *showing non-staining 'floor plate region' in the rostral neural folds and the presence of a notochord (arrow). Bar, 100 μm.* **(L)** *Enlarged view of* **J** *showing staining crossing the 'floor plate region' in the trunk neural folds (arrow). Bar, 100 μm.*

the lateral wings of mesodermal tissue. At this stage the staining intensity in *T/T* embryos and controls is the same. At the 1-2 somite stage, the pattern of expression is also the same in both groups. *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. However, in two separate experiments after 2-3 h staining, the intensity of *Wnt-5a* expression in 8.5 dpc embryos was greatly reduced in the posterior region of *T/T* embryos compared to controls, although less so at the base of the allantois. After clearing, no hybridization signal was detectable (Fig. 1A). By the 5-6 somite stage no expression of *Wnt-5a* is detected in the caudal region (Fig. 1A).

Axial patterning

Patterning of the anteroposterior axis in *T/T* embryos was assessed using *Hox-A7* as a marker. Dorsoventral pattern in the neural tube and somites of embryos lacking a normal notochord was evaluated using *Pax-1*, *Pax-3*, *Pax-6* and *Msx-1* as markers.

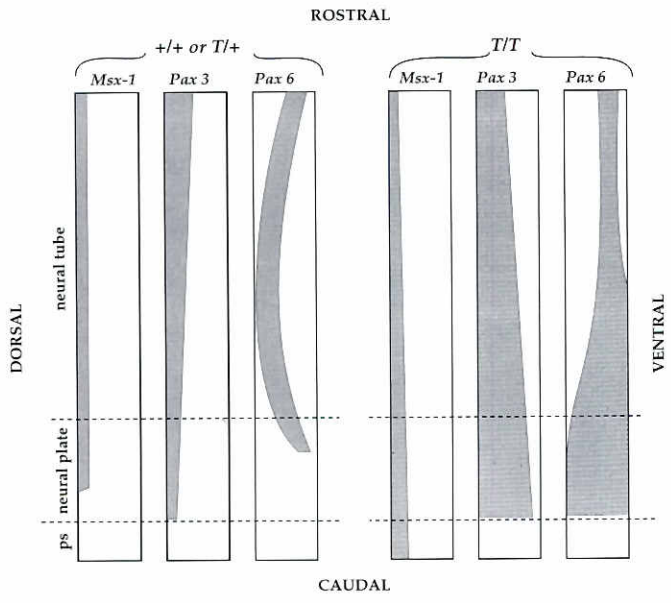

Fig. 4. Schematic representation of differential expression patterns in the dorsoventral axis along the neural tube of *T/T* and control embryos. *The neural tube is represented in each case by a rectangle, and the expression pattern of Msx-1, Pax-3 and Pax-6 in either control (left) or T/T (right) embryos is shown by a shaded area.*

Hox-A7 expression

In the transgenic line *Tgm6lacZ1* , which contains a *lacZ* reporter gene inserted between 3.6 kb of *Hox-A7* promoter sequence and 1.7 kb of 3' *Hox-A7* sequence, *E.coli* ß-galactosidase activity reflects the normal spatiotemporal expression pattern of endogenous *Hox-A7* mRNA with respect to the establishment of correct anterior boundaries in neurectoderm and paraxial mesoderm (Püschel *et al.*, 1990). At 8.5 and 9.5 dpc, the anterior boundary of *Hox-A7* expression (as assessed by the anterior boundary of *E.coli* ß-galactosidase activity), is the same in both mutant and control embryos (Fig. 2A and B). At 8.5 dpc both *T/T* and controls are stained in all tissues posterior to the 4th or 5th somite. Similarly, at 9.5 dpc both *T/T* and control embryos exhibit the same anterior boundaries for *Hox-A7* expression in both the neural tube and paraxial mesoderm: the anterior boundary in the neural tube is at the level of somite 6-7, whereas the most rostral somite to show X-Gal staining is the 14th. In almost half (4/9) of 9.5 dpc mutant embryos, ectopic staining is seen anterior to the normal boundary of *Hox-A7* expression (Fig. 2B). This ectopic staining is never seen in control embryos. On sectioning the *Tgm6lacZ1 Brachyury* homozygous embryos, it was evident that this ectopic ß-galactosidase activity was restricted to extracellular spaces, including the lumen of the neural tube and the lumen of the dorsal aorta. Thus, the ectopic staining appears to result from aberrant diffusion of protein, presumably due to disrupted tissue structure in *T/T* mutant embryos, and no change in *Hox-A7* expression boundaries could be detected.

Msx-1 expression

The *Msx-1* expression pattern was identical in control and *T/T* embryos at 8.5 dpc (Fig. 1C). *Msx-1* is expressed in the dorsal aspect of the neural tube and neural folds, the lateral mesenchymal tissue, lateral and caudal ectoderm and in the amnion. In mutant embryos the expression of *Msx-1* was somewhat elevated at the caudal extreme of the primitive streak, first noticeable at about the 5 somite stage. At 9.5 dpc control embryos show intense staining in the forelimb buds and in the dorsal neural tube (Fig. 1H), although expression in the neural tube does not extend as far as the posterior neuropore (in contrast to previous descriptions of *Msx-1* expression Robert, *et al.*, 1989). In 9.5 dpc *T/T* embryos, *Msx-1* is expressed in the the forelimb buds, and neural tube but the staining intensity in the forelimb buds in mutant embryos is reduced compared to controls (data not shown). Unlike controls, *Msx-1* expression extends to the most caudal extreme of the neural tube in the *T/T* embryos, although as in normal embryos transcripts are always restricted to the dorsal aspect of the neurectoderm (Figs. 1I and 4). In *T/T* embryos the hybridization signal in the tailbud region is considerably stronger than that seen in controls. This, and the elevated signal beneath the streak at 8.5 dpc, may be due to accumulation of nascent mesoderm cells in mutant embryos (Wilson *et al.*,1993).

Pax-1 expression

At 8.5 dpc *Pax-1* is not expressed in either mutant or control embryos. Expression is first evident in 8.75 dpc embryos (Fig. 3A). This is earlier than previously described by Deutsch *et al.* (1988). At 8.75dpc, both control and *T/T* embryos show the onset of expression in paraxial mesoderm at the level of the 4th somite. There is no apparent difference in staining intensity or distribution of mRNA between the two groups and both express *Pax-1* in the pharynx. However, by 9.5 dpc mutant and control embryos do display different expression patterns (Fig. 3A and B). At 9.5 dpc, although control embryos express *Pax-1* in all somites (Fig. 3B), *T/T* embryos express it only in somites 4-7 , and more caudal paraxial mesoderm, which is present but diminished in mutants, does not express *Pax-1*. In 9.5 dpc control embryos, *Pax-1* expression is highest in the more caudal somites (Fig. 3B) and in this region a band of *Pax-1* expression, continuous with the sclerotome, crosses the midline ventral to the notochord (Fig. 3C). In the 4 rostral somites which express *Pax-1* in *T/T* embryos, the staining intensity is reduced (Fig. 3D) compared to expression levels in controls. The domain of *Pax-1* expression appears smaller, although this may be a function of the reduced staining intensity. Alternatively, the sclerotome compartment may be diminished in size.

Pax-3 expression

At 8.5 dpc both the control and homozygous mutant groups have a similar *Pax-3* expression pattern (Fig. 3E). At this stage, *Pax-3* is expressed in all somites and along the dorsal aspect of the neural tube and lateral margins of the neural plate. At 9.5 dpc *T/T* and control embryos differ in the distribution of *Pax-3* mRNA. 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 dermamyotome component of the somites (Fig. 3F). However, the extent of *Pax-3* expression in the neural tube varies according to position along the rostrocaudal axis. In the rostral neural tube, *Pax-3* expression extends ventrally to approximately the midpoint of the neural tube dorsoventral axis. Further caudally, this expression is gradually restricted to a smaller dorsal domain in the neural tube (Fig. 4). In mutant embryos, the *Pax-3* expression domain in

anterior regions, where a notochord is still present, includes more ventral neural tube cells than in equivalent sections of control embryos. This ventral extension of expression is accentuated in more caudal regions which lack a notochord. This enlargement of the normal domain of expression in the neural tube is already evident at 8.5 dpc caudal to somite 7 as can be seen in the unfused neural folds of the *T/T* embryo depicted in Fig. 3E. Although the caudal extremity of the neural tube in *T/T* embryos is severely kinked and irregular, *Pax-3* expression is consistently excluded from the ventral midline (normally the floor plate region) in all sections examined.

Unlike control embryos (Fig. 3F), *Pax-3* expression in *T/T* somites at 9.5 dpc is not restricted to the dorsolateral margins, but extends into the ventromedial "sclerotomal" compartment (Fig. 3G). Again, this abnormal expression domain is most marked in the most caudal somites present (somites 4-7). Careful inspection of these *T/T* embryos shows weak Pax-3 expression in 3 additional "somites" (corresponding to somites 8-10) which cannot be distinguished by morphology alone (data not shown).

Pax-6 expression

In 8.5 and 9.5 dpc control embryos *Pax-6* is expressed along the neural tube but not in somites. At 8.5 dpc *Pax-6* expression appears identical in *T/T* and control embryos, being restricted predominantly to the ventrolateral regions of the neuroectoderm (data not shown). However, at 9.5 dpc differences are apparent between control and mutant embryos (Fig. 3H). Serial sections of 9.5 dpc control embryos show that the domain of expression varies along the neural tube such that rostrally there is a band of *Pax-6* expression in the ventral region of the neural tube (Fig. 3I). Further caudally, this band of expression shifts dorsally (Fig. 3I). At the level of the 9th somite expression is seen in the roof plate of the neural tube and in adjacent dorsal regions but there is little *Pax-6* expression in more ventral cells. However, further caudally still, in the more immature part of the embryo, *Pax-6* expression is once more ventral and no dorsal expression is seen (Fig. 4).

Sections of 9.5 dpc *T/T* embryos also show variation in *Pax-6* expression in the neural tube according to rostrocaudal level. Expression in the mutants is similar to that in control embryos in the anterior trunk region rostral to somite 7, where a notochord can be distinguished in some sections. However, in more caudal regions of the neural tube, *Pax-6* expression extends much further ventrally than in controls. In particular, expression extends into the most ventral aspect of the neural tube (Fig. 3J,K and L), the region where the floor plate is normally located, and where *Pax-6* expression is clearly excluded in control embryos (Fig. 3I).

Discussion

By examining the expression patterns of a number of genes, implicated either in mesoderm formation or in axial patterning, in homozygous *Brachyury* mutants a more precise description of the mutant phenotype is obtained. Most alterations in the patterns of gene expression described in tissues which do not themselves normally express the *T* gene can be explained by the lack of a functional notochord and will be discussed only briefly. However, two aspects of the expression patterns in mutant embryos merit detailed consideration: i) the persistent dorsal expression of *Msx-1* in the neural tube; and ii) the reduction in mRNA levels of *Evx-1*, *Wnt-3a* and *Wnt-5a* but not *BMP-4* or *Msx-1* at early somite stages.

Alterations in tissues which do not express T

The changes in domains of *Pax* gene expression in the neural tube of *T/T* embryos (Fig. 4) are wholly consistent with the absence of a functional notochord, and consequently lack of a ventralizing signal, in the caudal region of *T/T* embryos (see Jessell and Melton, 1992; van Straaten *et al.*, 1988, 1991; Placzek *et al.*, 1990; Clarke *et al.*, 1991; Yamada *et al.*, 1991; Goulding *et al.*, 1993). Thus, both *Pax-6* and *Pax-3* expression domains are expanded ventrally. However, the neural tube in caudal regions does not seem to be entirely dorsalized because *Pax-3* expression never extends into the ventral midline.

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 on chick embryos have shown that the notochord/neural tube complex is important for normal vertebral morphogenesis and, in particular, for the survival and differentiation of sclerotome and myotome cells (e.g. Kosher and Lash, 1975; Hall, 1977; Rong *et al.*, 1992), as well as definition of the dorsoventral axis (Aoyama and Asamoto, 1988). Although previous investigators have maintained that the normal architecture of sclerotome and dermamyotome is completely lost in *Brachyury* homozygotes (Jacobs-Cohen *et al.*, 1983), our results demonstrate that the anterior somites maintain the correct spatial relationship between *Pax-1* and *Pax-3* expression even if the sclerotome compartment is reduced.

Our results also show that embryos lacking the *T* gene product can still specify the correct anterior boundary for *Hox-A7* even though the mutant phenotype has a greatly foreshortened rostrocaudal axis and severe posterior abnormalities. It has been argued that the notochord imparts anteroposterior information to overlying tissues. For example, anterior *Xenopus* notochord induces *engrailed* expression in animal cap tissue but posterior notochord does not (Hemmati-Bravanlou *et al.*, 1990). The formation of a normal *Hox-A7* boundary in a region of the anteroposterior 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).

Maintenance of normal Msx-1 expression in neurectoderm

Intriguingly, the *Msx-1* expression domain does not extend ventrally in *T/T* neurectoderm, but remains restricted to the roofplate region, as it does in controls (Fig. 1H and I). This means that unlike *Pax-6* and *Pax-3*, *Msx-1* expression is not ventralized in the *T/T* neural tube, even at the caudal aspect of the embryo. This suggests that the correct patterning of the neural tube requires at least two signals: a ventral signal emanating from the notochord and a dorsal signal of unknown origin. This hypothesis is supported by the recent demonstration that a member of the TGF-ß family, *dorsalin-1*, can act as a potent dorsalizing signal in the developing spinal cord of the chick (Basler *et al.*, 1993). However, while *dorsalin-1* is first detected after neural tube closure, *Msx-1* expression is already tightly restricted to the lateral margins of the posterior neural plate before tube closure. Therefore, lateral signals may exist in the embryo which pattern nascent neurectoderm and define the future dorsal margins of the neural tube. The origin of such a signal, or signals, which may also define the lateral limits of neurectoderm differentiation and the prospective neural crest population, are not known but the anatomy of the embryo would suggest that lateral mesoderm or surface ectoderm are potential sources.

Reduction in the expression of genes expressed in the primitive streak

In mouse embryos lacking the *T* gene no anomalies in the expression of *BMP-4*, *Wnt-3a* or *Wnt-5a* could be discerned in the 7.5 dpc egg cylinder, and the normal expression pattern of *Evx-1* at headfold stages argues that its expression was also unaffected in the egg cylinder. This implies that the T protein is not required to establish transcription of any of these genes. In contrast, *Wnt-3a*, *Wnt-5a* and *Evx-1* expression are clearly reduced in *T/T* embryos at 8.5 dpc, the decline in *Wnt-3a* and *5a* mRNA levels slightly preceding that of *Evx-1*. It could be argued that the loss of expression of these genes is due to loss of tissue rather than to an effect on transcription. However, no changes in cell cycle length, nor retardation in growth have been observed before the 27-somite stage in *T/T* embryos (Yanagisawa and Fujimoto, 1977; Yanagisawa *et al.*, 1981) and it is not until the 7-somite stage that the first sign of elevated pyknosis occurs in the streak region (Chesley, 1935). Furthermore, the normal expression of *BMP-4* and *Msx-1* (Fig. 1B and C) is not compatible with extensive cell loss.

Therefore, T may be required either directly or indirectly to maintain normal levels of transcription of *Evx-1*, *Wnt-3a* and *Wnt-5a*. Since their expression domains overlap that of T, this maintenance function could be due to T acting as a direct transcriptional activator or being an essential component of a regulatory pathway. From studies of T^{wis}/T^{wis} mutant embryos, where the level of mutant transcipts rapidly declines at the early somite stage, it has been suggested that wild type T protein is normally required to maintain *T* gene expression (Herrmann, 1991). Interestingly, the time that T^{wis} mRNA can no longer be detected in T^{wis}/T^{wis} embryos coincides with the time that mRNA levels of both *Wnt* genes decrease, shortly followed by reduced levels of *Evx-1* transcripts. This suggests that the early somite stages could mark a general change in the mode of regulation of gene expression in the primitive streak region. Early expression may be initiated and maintained by inducing molecules whereas at 8.5 dpc crossregulatory mechanisms assume the responsibility for ensuring maintencance of expression. Lineage studies (Lawson *et al.*,1986, 1987) indicate that at the onset of gastrulation the primitve streak is overlain by a population of endoderm which continues to be closely associated with the elongating streak but during headfold and early somite stages is displaced by the developing hindgut to a more caudal location in the posteior and posterolateral visceral yolk sac. Thus, as the embryo develops from a simple cylinder to the more complex anatomy of the early somite embryo, so the primitve streak loses a close association with this early overlying population of endoderm cells. If this endoderm population were responsible for inducing and maintaining expression of genes in the primitive streak then an alternative mode of regulation would have to be instigated once hindgut formation commences. Our results would be consistent with such a model: gene expression in the primitive streak being independent of cross regulatory pathways in the egg cylinder but dependent on them thereafter. Consequently, the absence of any component of such regulatory pathways would affect predominantly post-cervical regions. This is the phenotype of embryos lacking normal products of either the *T* (Chesley, 1935), *Wnt-3a* (Takada *et al.*, 1993) or *integrin a5* (Yang *et al.*, 1993) genes.

Materials and Methods

Embryo recovery

Heterozygous *(T/+)* BTBR/Pas mice were mated overnight. 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 dpc (dpc: morning of plug= 0.5 dpc). In order to generate *T/T* embryos containing the transgenic reporter construct reflecting *Hox-A7* expression (*m6lacZ1*; Püschel *et al.*, 1990), heterozygous *(T/+)* BTBR/Pas males were mated with homozygous *Tgm6lacZ1* females. Offspring that had short tails *(T/+)* were tail-tipped and the presence of *lac-Z* protein determined by staining for *E. coli* ß-galactosidase activity (Beddington *et al.*, 1989). Mice with short tails and positively staining tail tips were backcrossed onto homozygous *Tgm6lacZ1* mice, and the resulting *lac Z* expressing short-tailed offspring intercrossed. Transgenic embryos recovered at 8.5 and 9.5 dpc were stained with X-Gal (Beddington *et al.*, 1989), and *T/T* mutant embryos identified morphologically.

Extraembryonic membranes and the amnion were removed from 8.5 and 9.5 dpc embryos. The homozygous *(T/T)* embryos were identified by morphology. The control group contained both heterozygous *(T/+)* and wild type *(+/+)* embryos because these cannot be distinguished phenotypically at these stages of development. In order to ensure that control and homozygous *T/T* embryos were treated identically during the wholemount *in-situ* hybridization procedure, they were processed in the same tube. The heart, fore- and hind-brain were punctured using a finely pulled pasteur pipette to prevent non-specific trapping of probe in embryonic cavities. All embryos were fixed overnight in freshly prepared 4% paraformaldehyde (PFA, Fisons) in phosphate buffered saline (PBS) at 4°C.

At 7.5 dpc it is not possible to distinguish homozygous *T/T* embryos morphologically. Therefore, PCR was used to identify homozygous and control embryos. 7.5 dpc embryos were dissected from maternal tissue and Reichert's membrane and the ectoplacental cone was removed. Using watchmakers' forceps, embryos were 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 separate 96 well plates (Nunc). The embryonic portion was fixed in 4% PFA overnight at 4°C, while the extraembryonic portion was placed in 20 ml PBS and used for PCR amplification.

In order to genotype the embryos, each sample was tested for the presence of a 310bp region which includes the 3' coding region of the *T* gene. *T/T* embryos lack this region, and consequently identification of homozygous mutants relies on the absence of an amplified band. Amplification of most of the second exon of *Hox 2.1* (a 350bp fragment spanning the homeodomain) was used as a positive control to demonstrate that DNA was present in the sample.

The *T* primers used were:

CCA GTT GAC ACC GGT TGT TAC A

TAT CCC AGT CTC TGG TCT GT

The *Hox-2.1* primers were:

GCG CCA GTG CAG GGA AGA TTG GAA

GAT ATG ACT GGG CCA GAC GGA AA

20 mg proteinase K (1 ml of a 20 mg/ml stock; Boehringer) was added to each well containing an extraembryonic sample. Wells were covered with mineral oil (Sigma) to prevent evaporation and incubated at 55°C for 3-4 h until the tissue had partially disintegrated. DNA was denatured by incubation at 95°C for 20 min and two aliquots (2x5 ml) of each sample were PCR amplified in a final volume of 50 ml containing 100 mM of each dNTP, 1xPCR buffer (Boehringer), 2 units Taq polymerase (Boehringer) and either 500 ng *Hox-2.1* or 500 ng *T* primers, using a Techne PHC-3 amplifier. The cycle consisted of 5 min at 95°C followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, 70°C for 2 min and finally 20 min at 37°C. 10 ml of the reaction was run into a 1.5% agarose gel. The presence of a 310 and a 350bp band was diagnostic of *T/+* or *+/+* embryos Samples which had a *Hox-2.1* but lacked a *T* specific band were classed as *T/T*. Samples which produced any other combination of bands were discarded. The corre-

sponding embryonic regions were grouped into either *T/T* or control *(T/+* or *+/+)* populations and processed for wholemount *in situ* hybridization simultaneously but in separate containers

Plasmids and probes

Plasmids for *in situ* hybridization were kindly provided by the following: *BMP-4*, M. Jones and B. Hogan; *Evx-1*, M. Dush and G. Martin; *Msx-1* and *Pax 6*, R. Hill; *Pax-1* and *Pax-3*, P. Gruss and *Wnt-3 and Wnt-5a*, A. McMahon. Each experiment also included embryos probed with α-*actin* and *T* to control for the efficacy of the in *situ* hybridization. Plasmids were prepared using a Qiagen plasmid maxi kit (Hybaid). Phenol-extracted, linearized plasmid DNA was transcribed using T7 or SP6 RNA polymerase using standard procedures (Boehringer riboprobe kit). Probe length was verified by gel electrophoresis.

Wholemount in situ T hybridization

Two slightly different protocols (Wilkinson, 1992; Rosen and Beddington, 1993) were used according to the developmental stage of the embryos. Preliminary experiments showed that proteinase K treatment tended to destroy the allantoides of control embryos and the ectodermal blebs present on *T/T* 8.5 dpc embryos. This meant that the only criterion left to distinguish the *T/T* embryos from controls at 8.5 dpc was a slightly kinked neural tube. Therefore, younger embryos (7.5-8.5 dpc) were processed using a milder protocol (Rosen and Beddington, 1993), which causes less damage to delicate tissues, while 9.5 dpc embryos were processed according to Wilkinson (1992). After staining and post-fixation in 4% paraformaldehyde/0.2% glutaraldehyde for 20 min followed by a single wash in PBT, embryos were cleared in 50% glycerol in PBT for 20 min and then transferred to 80% glycerol before being photographed.

Photography

Embryos were photographed using a SZH Olympus dissecting microscope and camera. Digitalized images of intact embryos or sections were obtained using a Hamamatsu CCD Vision Camera Module attached either to a SZH Olympus dissecting microscope or an Olympus IMT-2 inverted research microscope. The images were processed using ColourVision program version 1.2.2 (Image Processing and Vision Company Ltd, Coventry, UK).

Histology

Embryos were washed 3 times for 20 min in 40% glycerol:35% ethanol (Analar grade, Hayman Ltd):10% PBT:15% dH_2O before being dehydrated through an ethanol series (70%, 80%, 87%, 95%, 100%; 30 min in each solution except 3x20 min in 100% ethanol). Embryos were cleared by a 5 min exposure to equal volumes of 100% ethanol:Histoclear (National Diagnostics) and 3x40 min in Histoclear at room temperature. Infiltration with wax was effected by 3x20 min incubations in wax (Histoplast, m.p. 56°C) at 60°C. Embryos were orientated in the wax when embedding using a dissecting microscope. Serial sections were cut 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.

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