

## Chimeric analysis of *T* (*Brachyury*) gene function

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### SUMMARY

To investigate *T* (*Brachyury*) gene function, a chimeric analysis of midgestation (9.5-11.5 days post coitum) embryos has been performed. Embryonic stem (ES) cell lines homozygous or heterozygous for the *T* gene have been introduced into wild-type host embryos by blastocyst injection, and the resulting chimeras scored for morphological abnormality and extent of colonization by *T/T* cells. As observed previously in earlier stage chimeras (Rashbass, P., Cooke, L. A., Herrmann, B. G. and Beddington, R. S. P. (1991) *Nature* 353, 348-350), 9.5-11.5 dpc *T/T* ↔ *+/+* chimeras exhibit many morphological features of intact *T/T* mutants. In addition, a dramatic bias of *T/T* cells towards caudal regions (such as tail and allantois) was observed in all chimeras tested. This is likely to result from accumulation of nascent *T/T* mesoderm cells with time near the primitive streak, possibly because of altered migration or adhesion properties. *T/+* cells colonized rostral regions efficiently, but a slight bias towards the distal end of the tail was still evident. No such bias was observed in control chimeras. The presence of *T/T* cells in the allantois resulted in its

failure to form a correct placental connection and thus arrested later development. In contrast, chimeras in which *T/T* cells were present predominantly in the tail developed normally but exhibited severe tail abnormalities such as foreshortening, branching and haemorrhagic cavities. Moreover, in these embryos, much higher levels of chimerism were present in the distal end of the tail than in younger (9.5 dpc) embryos. Later in gestation, such abnormal tails probably degenerated, giving rise to neonates with absent or severely abnormal tails but no evidence of chimerism. In situ analysis of *T* expression in the tail reveals that normally *T* is expressed highly in the tailbud (the growing portion of the tail) during its elongation between 9.5 and 11.5 dpc. Thus, evidence both from chimeras and from *T* expression in the tail suggest that *T* may play a role in the correct deployment of cells emerging from the tailbud.

Key words: *T* (*Brachyury*), mouse embryo, gastrulation, tail, tailbud, allantois, ES cell, chimera

### INTRODUCTION

Genes that act during gastrulation are of considerable interest because major aspects of the embryonic body plan are laid down during this period. *T* (*Brachyury*) is one such gene that is required for normal gastrulation. Morphological features of *T/T* homozygotes, in which the *T* gene is deleted, appear between mid and late gastrulation (Chesley, 1935; Gruneberg, 1958). The allantois fails to grow as a coherent structure to connect with the ectoplacental cone (Gluecksohn-Schonheimer, 1944) and the primitive streak is abnormally thickened. From sectioned *T/T* embryos, it is apparent that frequently the notochord is either absent or incorporated into the adjacent gut or neural tube, and therefore undetectable, along some or all of the length of the embryo. Pyknotic granules (an indication of cell degeneration) are also observed in cells that occupy a notochordal position. As somites form and the neural folds close, additional abnormalities in these tissues become apparent: somites appear disorganized or absent posterior to somite 7, while the closing neural tube begins to kink dramatically. During the ninth and tenth days of gestation, cell death

increases, first in the posterior end of *T/T* embryos, but subsequently becomes more widespread and the embryo dies on the 11th day of gestation. The immediate cause of death is probably nutrient deficiency as a result of failure of the embryo to form a placental connection (Gluecksohn-Schonheimer, 1944).

Cloning of the *T* gene (Herrmann et al., 1991) showed that the *Brachyury* defect is a deletion encompassing the *T* gene, thus confirming its status as a loss-of-function allele. *T* expression in the primitive streak and notochord (Wilkinson et al., 1991) suggested that abnormalities in these structures are fundamental to the *T/T* phenotype, while those in the neural tube, surface ectoderm, somites and allantois are downstream effects. These could arise either from interactions with abnormal cells, or as a consequence of the failure of a progenitor cell to express the *T* product.

Cells emerging from the primitive streak of *T/T* embryos indeed behave abnormally. *T/T* mesoderm in culture exhibits significantly reduced motility on ECM substrates (Hashimoto et al., 1987). Aggregates formed in rotation culture after trypsinization of either anterior or posterior regions of *T/T* embryos are consistently smaller than those

formed by normal embryos (Yanagisawa and Fujimoto, 1977b). In contrast, no differences in mitotic index (Yanagisawa et al., 1981) or [<sup>3</sup>H]thymidine incorporation (Yanagisawa and Fujimoto, 1977a) into *T/T* mesoderm are observed. Necrosis in *T/T* mesoderm is not observed until the 9th day of gestation (Chesley, 1935; Yanagisawa et al., 1981), when cell pyknosis increases in posterior regions of the mesoderm. In vitro studies of *T/T* cells show that their differentiative capacity is not significantly impaired, and the viability of *T/T* cells in whole embryo and organ culture, or in teratomas, extends well past the time of embryonic death (Ephrussi, 1935; Gluecksohn-Schonheimer, 1944; Bennett et al., 1977; Yanagisawa and Fujimoto, 1977a).

Taken together, these results suggest that the defect in *T/T* mesoderm is an organizational failure, perhaps resulting from altered adhesion or migration properties, rather than any inability of *T/T* mesoderm to survive, divide or differentiate. The possibility that *T/T* mesoderm becomes lodged near the primitive streak has been examined by Yanagisawa et al. (1981). Comparison between the mesoderm/ectoderm ratio of sectioned *T/T* and normal embryos reveals a reduced ratio in *T/T* embryos from the early gastrulation stage. Furthermore, although the number of embryos used in the study was small, the distribution of mesoderm cells appeared to be abnormally skewed towards the posterior end of *T/T* embryos at 8 days post coitum (dpc).

Heterozygous *T/+* mice are viable but have short tails (Dobrovolskaia-Zavadskaia, 1927), due to degeneration of the end of the tail after the 12th day of gestation. The heterozygous *T/+* embryo exhibits a phenotype interpreted as intermediate between *T/T* and wild-type (Chesley, 1935; Gruneberg, 1958). The notochord is delayed in its separation from the dorsal gut endoderm and, in the cloacal region of 9.5-10 dpc embryos, may fail to delaminate altogether (Gruneberg, 1958). Additional branching of the notochord or adherence to the neighbouring neural tube or gut frequently occurs in the caudal region (Chesley, 1935; Gruneberg, 1958). In caudal (but not in lumbar or sacral) regions, notochord abnormality correlates well with abnormalities such as fusions between neural tube and gut, and localized duplications in one or both structures (Chesley, 1935). Gruneberg (1958) observed that the ventral ectodermal ridge of the tail tip (Gruneberg, 1956) was reduced to approximately 75% of its normal axial length in 9.75-10.5 dpc *T/+* embryos. At 11.5 dpc, gross abnormality in the tail tip is observed. Reduction in the diameter of the tail tip is accompanied by extensive cell death; no somites are distinguishable in this region, which by 14 dpc has degenerated into a thin tail filament, lacking vertebrae. Subsequently, it is usually lost altogether (Chesley, 1935; Gruneberg, 1958).

In regions of the tail where no notochord is discernible as a separate entity, somitic mesoderm frequently undergoes necrosis (Yanagisawa, 1991). However, necrosis of other tissues such as gut, ventral mesoderm and neural tube is not correlated with absence of the notochord. Indeed, the tail tip necrosis has not been observed to coincide with notochord abnormality (Chesley, 1935). Necrosis in the ventral mesoderm at 12 dpc may be the cause of abnormal

infiltration of blood corpuscles into the tail tip mesoderm (Yanagisawa, 1991). Thus, a complex situation exists in which notochord abnormalities can be correlated with some but not all of the tail defects. In particular, the tail tip death after apparently normal outgrowth remains unexplained by any notochord abnormality.

The existence of abnormalities in the notochord, and more general disturbances of the primitive streak create difficulties in assigning causal relationships between the various *T/T* abnormalities. The notochord has been implicated in patterning of the neural tube, and possibly in the differentiation of somites (e.g. Clarke et al., 1991; Hemmati-Bravanlou et al., 1990; Kitchin, 1949; Placzek et al., 1990; Smith and Schoenwolf, 1989; van Straaten et al., 1985; Yamada et al., 1991). The mouse short-tailed mutants Danforth's short-tail (*Sd*), Pintail (*Pt*) and truncate (*tc*) also have defects in the notochord (Gruneberg, 1963). In *Sd*, the disappearance of the notochord from posterior regions of the embryo (Paavola et al., 1980) disrupts posterior axon outgrowth (Boloventia and Dodd, 1991), formation of normal vertebrae from sclerotomes, and probably causes vertebral fusions and tail degeneration (Gruneberg, 1958; Theiler, 1959). A precedent thus exists for a notochordal involvement in the *T* phenotype. Chesley (1935), and subsequently Gruneberg (1958), have suggested that abnormal notochord is responsible for the *T/T* phenotype, based on the following observations. Firstly, notochord is invariably abnormal to some extent in the homozygote. It is the only tissue type to be at least partially absent in homozygotes, and is one of the earliest tissues to become recognizably abnormal in both homozygotes and heterozygotes. Indeed, in the heterozygote, Chesley (1935) observed that neural tube abnormalities were always correlated with a notochordal defect, although notochord defects could occur without apparent abnormality of the neural tube.

The dissection of cause and effect in the *T* mutation cannot rely merely on observation of large numbers of histological sections of *T* mutants. For example, even if two abnormalities always occur together, do they derive from a common cause, or does one abnormality cause the other? The study of chimeras between *T/T* or *T/+* cells and normal cells should be a useful tool in determining which abnormalities are cell autonomous, and which arise as a consequence of interaction with *T/T* cells. Previous studies (Rashbass et al., 1991) have shown that chimeras made between *T/T* embryonic stem (ES) cells and normal embryos reproduce aspects of the mutant phenotype at 8.5-9.5 dpc, suggesting that the *T* mutation behaves cell autonomously. In the present work, we have extended this study to investigate the frequency and extent of abnormalities in *T/T* ↔ *+/+* and *T/+* ↔ *+/+* chimeras at later stages and in neonates. We have analysed the relative amount of ES cell contribution to various embryonic tissues and positions along the rostrocaudal axis, to investigate the behaviour and interaction of abnormal cells with normal host cells. Lastly, we have demonstrated that the *T* gene is highly expressed in the tailbud and that reduced expression in this structure in heterozygotes may account for tail abnormalities independent of notochord function.

## MATERIALS AND METHODS

### Mouse strains and matings

The BTBR mouse colony (strain 129/Sv, agouti, *Gpi 1<sup>a</sup>/1<sup>a</sup>*) was bred from mice obtained from ICRF Clare Hall, Oxford. Mice were maintained as *T/+* × *T/+* breeding pairs for all experiments reported here. For blastocyst injection, C57Bl/6 (nonagouti, *Gpi 1<sup>b</sup>/1<sup>b</sup>*) were used. PO (Pathology, Oxford) mice were used as control (*+/+*) mice in studies on *T* expression, and as pseudopregnant recipient females for injected blastocysts. Timed matings were set up in normal light cycle (5 am–7 pm light). Noon on the day of finding a copulation plug was designated 0.5 dpc, and the day of blastocyst transfer as 2.5 dpc.

### ES cell lines

ES cell lines were those derived from *T/+* × *T/+* matings for the experiments reported in Rashbass et al. (1991; BTBR6, *T/T*; BTBR1.3, *T/+*), and three additional lines (BTBR10, *T/T*; BTBR7, *T/+*; BTBR4, *+/+*) derived at the same time were genotyped for these experiments with respect to *T* by Southern analysis (see Rashbass et al., 1991). They were maintained in medium supplemented with foetal and newborn calf serum and DIA/LIF (Smith et al., 1990; Pease et al., 1990).

### Blastocyst injection

Blastocyst injection and transfer of embryos to pseudopregnant recipient females was carried out as described in Robertson (1987).

### Embryo dissection and GPI analysis

Embryos were dissected in PB1 medium (Whittingham and Wales, 1969) containing 10% foetal calf serum. Phenotypic abnormalities were recorded and the embryos were separated into fractions for GPI analysis, as shown in Fig. 1. To separate neural tube from somitic mesoderm, the unseparated trunk sample was incubated in 0.5% trypsin/2.5% pancreatin in PBS (Svajger and Levak-Svajger, 1975) for 20–40 minutes, then replaced in PB1+10% FCS.

After 1 minute, the tissues were gently separated, using forceps and/or a pulled-out Pasteur pipette. All fractions were washed in PBS once and frozen in microwell dishes at  $-20^{\circ}\text{C}$  in about 5 volumes of PBS. The dish was thawed and refrozen twice to disrupt tissues and release GPI enzyme. GPI assay was performed as described in Rashbass et al. (1991) and the ratio of GPI-A:GPI-B was estimated by eye. To assist quantitation, a 50:50 mixture of *Gpi-1<sup>a</sup>/Gpi-1<sup>a</sup>:Gpi-1<sup>b</sup>/Gpi-1<sup>b</sup>* blood was run in parallel with the embryonic tissue fractions. In addition, samples from the same embryo were run together to aid the comparison of relative proportions of GPI-A:GPI-B. Each sample was assessed by two independent observers, and these estimates never varied by more than 10%, but were generally within 5%. We thus assume that this method of quantitation is accurate to within at least 10 percentage points.

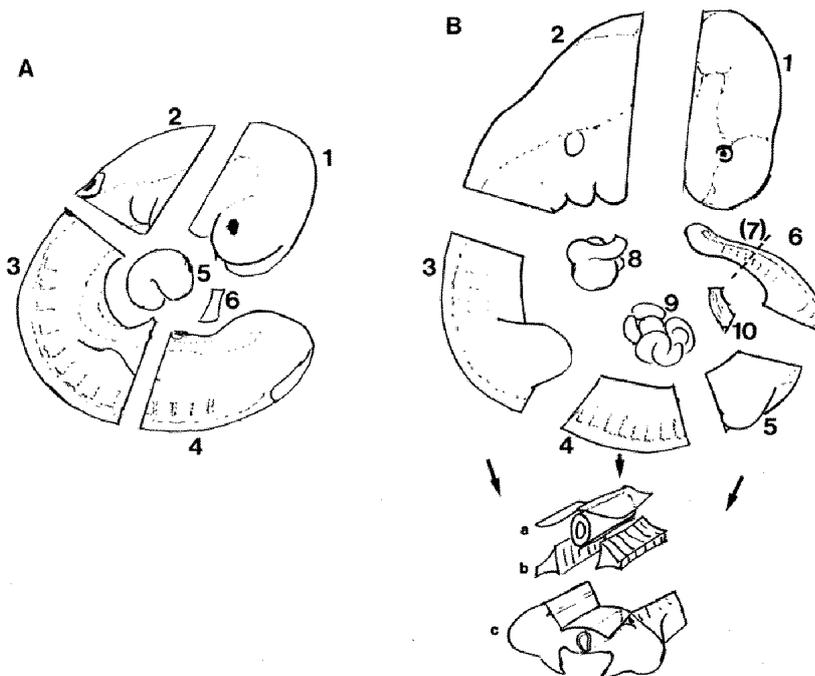
### Whole-mount in situ hybridization

In situ hybridization to *T* RNA in whole-mount tails was carried out according to Rosen and Beddington (1992, unpublished data). The probe used was an antisense digoxigenin-labelled riboprobe transcribed from the full-length *T* cDNA (gift of Dr B. Herrmann).

## RESULTS

### Chimerism and tail abnormality in liveborn *T/T* ↔ *+/+* and *T/+* ↔ *+/+* mice

Liveborn litters from blastocyst injection of *T/T* and *T/+* ES cells exhibited characteristic and distinct phenotypes (see Table 1). Three individuals derived from injection of *T/T* ES cells had very short, curly or absent tails. These animals died within three days of birth, but as some normal littermates also died perinatally, it was not clear whether neonatal mortality was related to embryo manipulation. No evidence of contribution from *T/T* ES cells was found either by coat colour or GPI analysis, except for a trace (5% by



**Fig. 1.** Dissected embryonic fractions for GPI analysis. (A) Fractions for 9.5 dpc and '9.5 dpc' embryos. Fractions 1, 4, 5 and 6 are referred to as head, tail, heart and allantois respectively in Figs 3, 4, 5 and 6. (B) Fractions for 10.5 and 11.5 dpc embryos. Fraction 7 (distal 10 somites in the tail of 11.5 dpc embryos), was only separated from fraction 6 (tail) in embryos 4–12. Fractions 1, 6, 8 and 10 are referred to as head, tail, heart and allantois respectively except in embryos 4–12, where fraction 7 is denoted as tail. Trunk neural tube and surface ectoderm (a) were separated from somitic mesoderm (b) in fractions 3 and 5 or fraction 4. Where fractions 3 and 5 were separated in this manner, lateral tissue consisting mainly of forelimb or hindlimb buds (c) were also assayed.

**Table 1. Phenotype and levels of chimerism of liveborn offspring derived from blastocysts injected with *T/T* or *T/+* ES cells**

Cell line	No. liveborn/ blastocysts transferred	No. abnormal tail (No. chimeric*)	No. normal tail (No. chimeric*)
BTBR6 & BTBR10	23/49	8 (1**)	15 (0)
<i>T/T</i>	46.9%	34.8% (12.5%)	65.2% (0%)
BTBR 1.3 & BTBR 7	32/52	2(2)	30(6)
<i>T/+</i>	61.5%	6.3% (100%)	93.7% (20%)

\*chimerism is judged by coat colour except as indicated  
\*\*trace only by GPI analysis

GPI analysis) in tail skin of one neonate. Another two individuals, also with no evidence of chimerism, had slightly shortened tails and survived to adulthood. No coat colour chimerism was detected in any of the surviving normal littermates of these individuals. In contrast, liveborn young from injection of *T/+* ES cells gave rise to animals that were chimeric by coat colour. A proportion of these (6.3%) had kinked or short tails of approximately 2/3 normal length. No pups with absent tails were born from this series of injections.

It was likely that the severe tail abnormalities observed in neonates were caused by the *T/T* ES cells, since two independent *T/T* ES cell lines (BTBR6 and BTBR10) gave rise to these mice, and no such extreme abnormality had been observed after injection of wild-type (results not shown) or *T/+* ES cells. This raised the possibility that *T/T* cells were selected against during the latter stages of gestation, since extensive chimerism had been detected at 9.5 dpc (Rashbass et al., 1991). We therefore dissected embryos injected with the same ES cell lines between 9.5 and 11.5 dpc, to examine tail outgrowth and levels of chimerism.

#### Abnormalities in 9.5-11.5 dpc embryos injected with *T/T* ES cells

Examination of 9.5-11.5 dpc embryos resulting from injection of *T/T* ES cells revealed that a high proportion exhibited reproducible abnormalities, with no significant differences between embryos derived from injection of BTBR6

and BTBR10 (see Table 2). In more than 86% of abnormal embryos, chimerism could be detected by GPI assay, whereas only one embryo classified as normal was chimeric. The majority of abnormal embryos could be assigned to one of three distinct classes: (1) defective tail or tailbud only, (2) abnormalities associated with allantois only and (3) multiple abnormalities including both defective tail/tailbud and allantois. In the latter class, additional abnormalities were often present in neural tube, somites, or heart.

#### (1) Tail-only abnormalities

At 10.5-11.5 dpc, tail defects ranged from branching, kinking, shortening and the presence of blood-filled sacs, to small lumps of tissue near or at the tail tip. In one extreme case, a 10.5 dpc embryo exhibited a duplication of the tail extending to the cloacal region. Both branches contained somites, but were approximately half normal diameter. A more typical example is shown in Fig. 2A, where the branch is short and does not contain somites. While embryos with only tail abnormalities were common at 10.5-11.5 dpc, no embryos were observed at 9.5 dpc where only the tailbud was abnormal.

#### (2) Allantois-only abnormalities

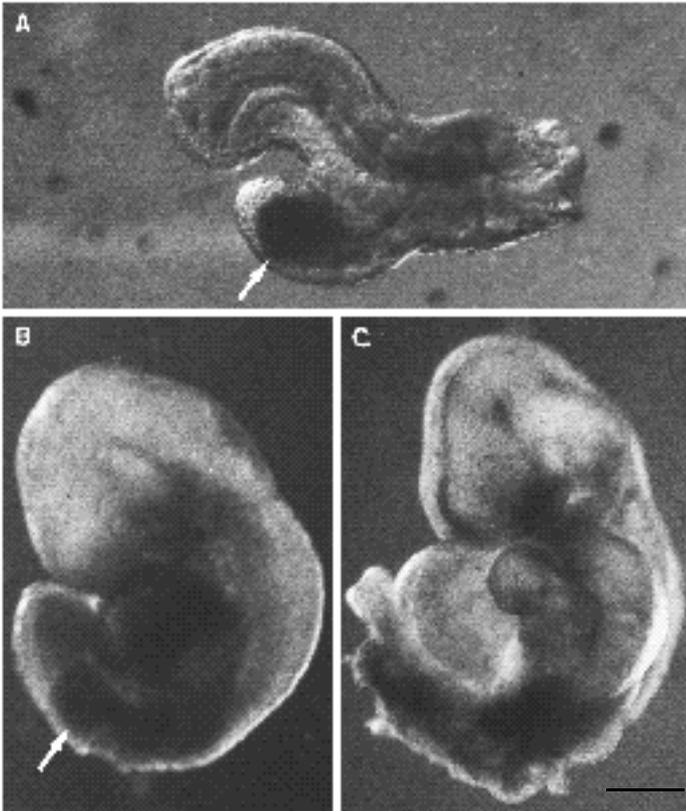
Embryos in which the allantois had failed to fuse with the ectoplacental cone were anaemic and smaller than their 10.5 dpc and 11.5 dpc littermates, resembling 9.5 dpc embryos in size. In addition, using the criteria of somite number, forelimb and hindlimb bud development, tail extension and enlargement of head structures, these embryos were more similar to 9.5 dpc stage than 10.5 or 11.5 dpc stage embryos (see Fig. 2B). Similar retardation has occasionally been observed in PO (+/+×+/-) embryos at 10.5-11.5 dpc, where the allantois had not formed a correct placental connection (results not shown). Embryos were therefore classified as having exclusively allantois abnormalities if the tailbud, neural tube, somites, heart and head resembled a normal 9.5 dpc embryo. Embryos with unfused allantois dissected at 10.5-11.5 dpc are referred to as '9.5 dpc' in the text to differentiate them from true 9.5 dpc embryos. In most of these embryos, the allantois was visible as a small stump-like structure.

#### (3) Multiple abnormalities

This represents the most severely affected class of embryos,

**Table 2. Phenotype and frequency of chimerism in 9.5-11.5 dpc embryos recovered from blastocysts injected with *T/T* or *T/+* ES cells**

Cell line	No. implantation sites containing embryos	No. normal	No. normal chimeric	No. abnormal	No. abnormal chimeric
BTBR 10	76	51	1	25	21
BTBR 6 ( <i>T/T</i> )	23	5	0	18	16
Total	99	56	1 (1.8%)	43	37 (86.1%)
BTBR 7 ( <i>T/+</i> )	34	21	3 (14.3%)	13	10 (76.9%)
BTBR 4 (+/-)	29	22	2 (9.09%)	7	3 (42.8%)



**Fig. 2.** Abnormalities in *T/T*  $+/+$  embryos dissected at 10.5 dpc from a single recipient female. (A) Fraction 6 (tail) from embryo 3, showing branch containing a blood-filled sac (arrow). The tail end is abnormally bent and the tip is malformed. (B) Embryo 16, in which the allantois has not fused with the ectoplacental cone and development is retarded relative to non-chimeric littermates. An arrow indicates the position of the allantois. (C) Embryo 25, in which the tail and allantois are abnormal, and the pericardium is enlarged. Bar, 0.2 mm (A); 0.4 mm (B,C).

in which a variety of abnormalities were observed. Coincident tail and allantois abnormalities were present in 10/12 cases, and in many of these embryos, kinking of the neural tube, irregularity or deficiency of somites and heart abnormalities were also observed. One embryo (embryo 31, Fig. 4) which showed all of these characteristic abnormalities also had open cephalic neural folds. Tailbud abnormalities in this class ranged from failure of posterior neural folds to close, to disorganized posterior tissues where the tailbud was absent or much smaller than normal. In the most severely affected of these, the allantois could not be separated from the tailbud and extraembryonic membranes. A typical example is shown in Fig. 2C.

#### Chimerism in *T/T* $+/+$ embryos at 9.5-11.5 dpc

In the majority of cases, abnormal embryos were chimeric (Table 2). Exceptions to this were: one embryo that had a small lump near the tail end and one resorbing 10.5 dpc embryo that had an unfused allantois and abnormal hindbrain. This suggested that the exceptions were those in which chimerism was too low to be detected, or that the

embryo was not abnormal because of the presence of ES progeny. Three further 10.5 dpc embryos were classified as having aberrant heart development only, but were not detectably chimeric. Conversely, the one normal chimeric embryo (embryo 32) had a low contribution (20%) from *T/T* cells. It thus appeared that the great majority of the abnormalities were caused by the presence of *T/T* cells. To gain further information about the tissue-specificities or preferences, if any, of ES cell-derived *T/T* cells, levels of chimerism were assayed by GPI typing of embryo fractions shown in Fig. 1. Figs 3 and 4 show the extent of contribution in head (forebrain and midbrain), tail, heart and allantois for chimeras with abnormal tail or allantois (Fig. 3) and chimeras with multiple abnormalities (Fig. 4). A striking pattern of chimerism was evident. Almost without exception, ES cell contribution was higher in the tail than in the head, with the greatest percentage of ES-derived cells in the distal half of the tail. Results for viscera, hindbrain, trunk and proximal tail are not shown in these diagrams since, in general, equal ES cell contribution to viscera, hindbrain and forebrain was almost always observed. Trunk contribution tended to correspond either to forebrain and hindbrain contribution, or was midway between forebrain and tail contribution. Often a gradient existed where chimerism in head < trunk < proximal tail < distal tail (Beddington et al., 1992).

#### Abnormalities in chimeric embryos

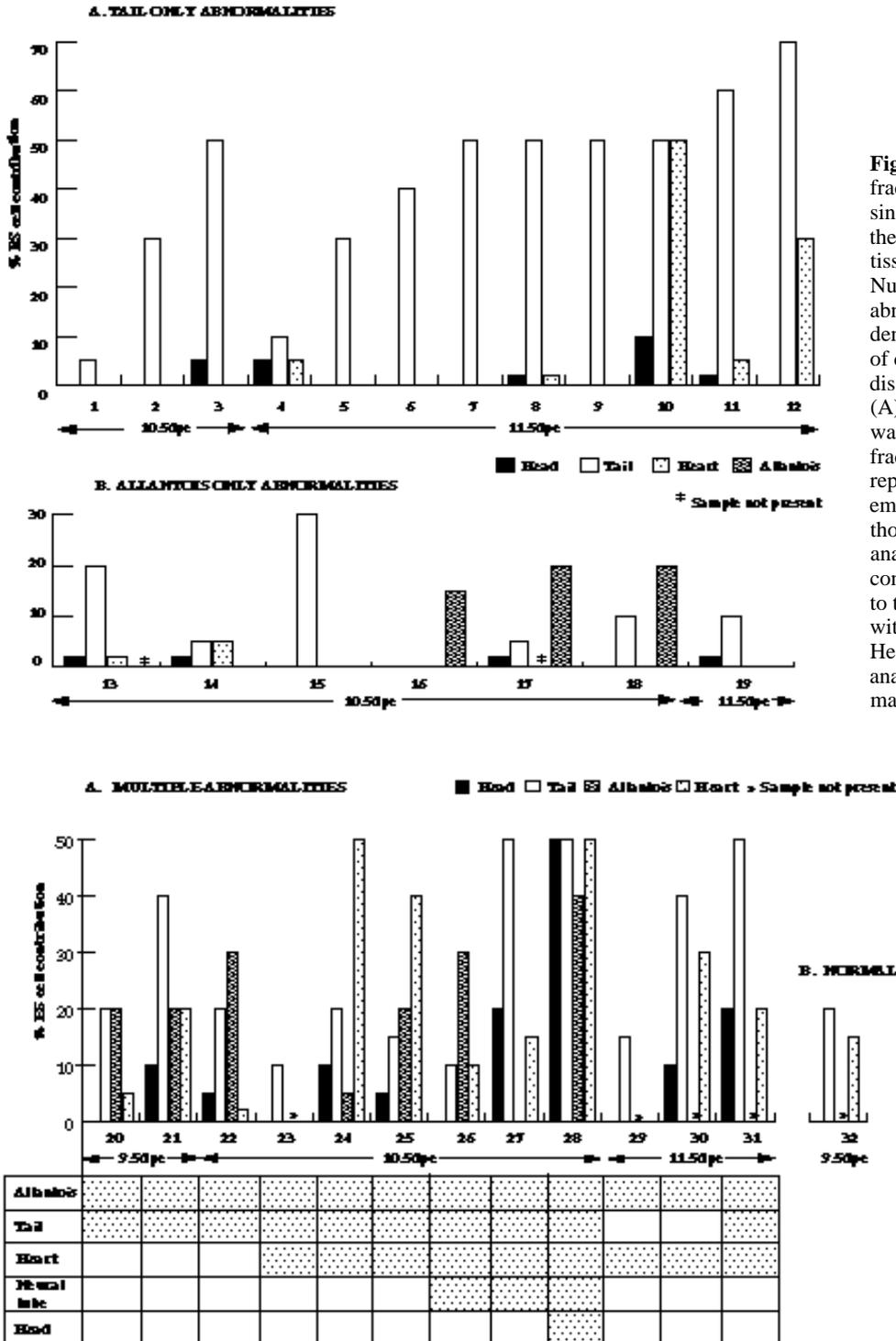
ES cell contribution was, in general, detectable in structures scored as morphologically abnormal (Table 3). In all chimeric embryos except one, ES cell progeny colonized the tail or tail bud. Without exception, an ES cell contribution greater than 40% correlated with a defective tail or tailbud and, in some cases, even a relatively low level of chimerism was sufficient to generate tail abnormalities (chimeras 1, 4, 23 and 26). Chimeric embryos with no apparent tailbud abnormalities, but with a *T/T* cell contribution up to 40% in the caudal region, were in the '9.5 dpc' class (chimeras 13-19, 29 and 30), apart from the single normal 9.5 dpc chimera (no. 32). Therefore, at these earlier stages, overt morphological abnormalities in the tail may not yet be apparent or are difficult to recognize. In four embryos (1, 2, 5 and 7) exhibiting only tail abnormalities, chimerism was only detectable in the tail and, in three of these (2, 5 and 7), the ES cell contribution was relatively high (30-50%).

A high proportion (59.3%) of *T/T*  $+/+$  chimeras had an abnormal allantois. As with tail-only abnormalities, embryos with defects exclusively in the allantois usually had the highest *T/T* cell contribution in the allantois (chimeras 16-18). However, in more than 25% of chimeras that had an abnormal allantois, no *T/T* cells could be detected. This contrasts with the absolute correlation between overt tail defect and the presence of *T/T* cells in the tail.

Embryos with multiple abnormalities tended to have generally higher levels of chimerism in head, heart and allantois. In chimera 28, where the head was abnormal, the level of cranial ES contribution equalled that in the tail. However, the extent of chimerism in the heart and trunk neural tube did not always coincide with overt heart and

neural tube abnormalities (see for example chimeras 10 and 12). Abnormalities in the heart and pericardium were common amongst chimeras that displayed multiple abnormalities (28% of all chimeras; 50% of chimeras with an abnormal allantois). These defects included enlargement of the pericardium and failure of the heart tube to rotate correctly. Enlargement of the pericardium similar to that observed in chimeras has been observed in embryos homozygous for the *T<sup>c</sup>* allele (Searle, 1966) and some 9.5-

10.5 dpc intact *T/T* mutant embryos also show defective heart rotation (V.W. and P.R., unpublished observations). Such cardiac defects were not observed in *T/+* or *+/+* chimeras. Since a high *T/T* cell contribution can be tolerated in chimeric hearts that appear normal (chimeras 10, 12, 16-18, 20-22 and 32), it is possible that cardiac defects may be a secondary consequence of impaired circulation or failure of the allantois to fuse with the chorion.



**Fig. 3.** Chimerism in separated fractions from *T/T* *+/+* embryos with single defects. The Y-axis represents the percentage of ES cell progeny in tissue fractions analysed by GPI assay. Numbers along the X-axis represent abnormal embryos, and each column denotes a fraction assayed. The number of days p.c. at which the embryos were dissected is indicated below the X-axis. (A) Chimeras in which an abnormal tail was the only defect observed. Three fractions: head, tail and heart are represented. Allantoic fractions from embryos 1-3 were not chimeric, while those from embryos 4-12 were not analysed. Embryo 6 had 10% ES cell contribution to proximal tail in addition to the fractions shown. (B) Chimeras with abnormality in allantois only. Head, tail, heart and allantois were analysed except in those fractions marked with an asterisk.

**Fig. 4.** (A) Chimerism and abnormalities in *T/T* *+/+* embryos with multiple abnormalities. The upper half of the diagram depicts percentage ES cell contribution to tissue fractions as in Fig. 3B. In the lower half, the presence (shaded area) or absence (blank area) of abnormalities in specific structures are shown for each embryo. (B) Chimerism in the single normal *T/T* *+/+* chimera.

**TABLE 3. Incidence of abnormalities and chimerism in regions of *T/T*  $+/+$  chimeras 1-32**

	Structure			
	Tail	Allantois	Heart	Head
No. with abnormal structure (% of total chimeras)	22 (68.7)	19 (59.3)	9 (28.1)	1 (3.1)
No. assayed by GPI	22	14	9	1
No. chimeric in structure (% of abnormal)	22 (100)	10 (71.4)	7 (77.8)	1 (100)
No. with normal structure (% of total chimeras)	10 (28.1)	12 (37.5)	23 (68.7)	31 (96.9)
No. assayed by GPI	10	2	22	31
No. chimeric in structure (% of normal)	9 (90.0)	0 (0)	10 (45.5)	13 (41.9)

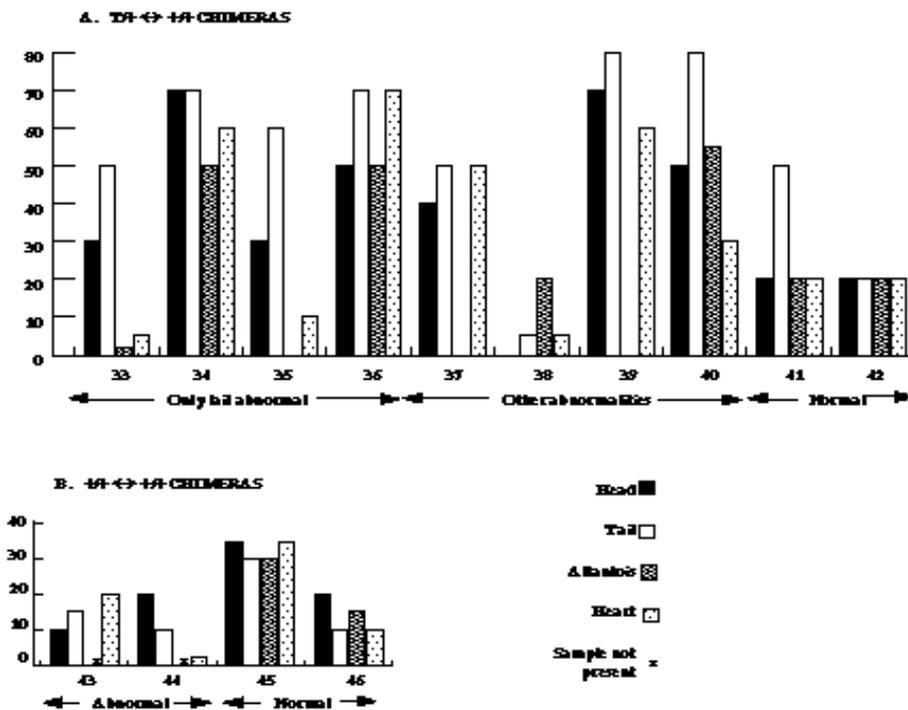
**Chimerism and abnormality in *T/+*  $+/+$  and control embryos**

Injection of a *T/+* ES cell line, BTBR7, gave rise to consistent abnormalities in d11.5 embryos, which were much less severe than those obtained from injection of *T/T* ES cells. These included slight irregularity of the tail tip, or bending near the tail tip. Those with irregular tail tips were, however, chimeric (Fig. 5, embryos 33-36), Three additional abnormal embryos were chimeric. However, the abnormalities observed (resorption in embryo 37, open trunk neural tube in embryos 38 and 39, and slight retardation in embryo 40) did not resemble any of the chimeras

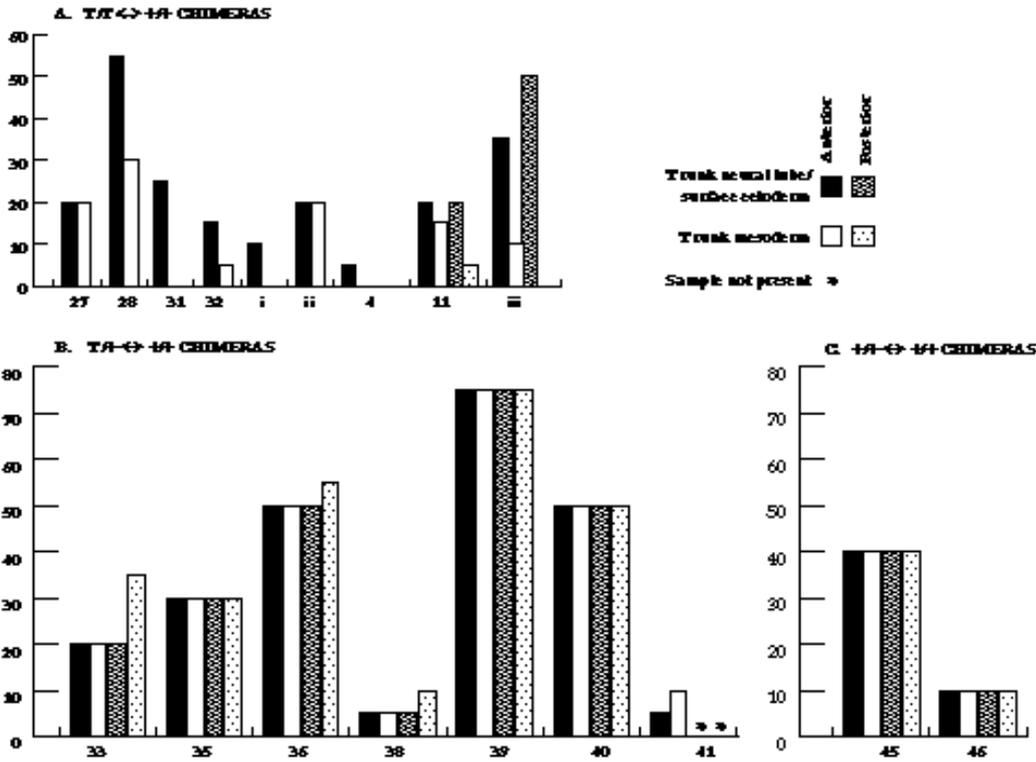
from injection of *T/T* ES cell lines. Additionally, two embryos that appeared normal were chimeric (embryos 41 and 42). All chimeric embryos had significant levels of chimerism in the head, and these corresponded well with extent of chimerism in all fractions except the distal tail, which had a consistently higher level than other fractions. Embryos injected with a control ( $+/+$ ) ES cell line, BTBR4, did not show any tissue or anteroposterior bias, although levels of chimerism varied by around 10 percentage points between fractions. The abnormal chimeras produced with this cell line were either retarded or resorbing and did not exhibit the specific defects associated with the presence of *T/T* ES cell progeny.

**Chimerism in separated trunk ectoderm and mesoderm**

To test whether there was a tissue bias between ectoderm and mesoderm derivatives, trunk fractions were separated into neural tube and surface ectoderm versus somitic mesoderm. An aberrant pattern of chimerism in *T/T*  $+/+$  embryos was also observed in these fractions (Fig. 6). Whereas the chimerism levels were always approximately equal in fractions from *T/+*  $+/+$  and  $+/+$   $+/+$  chimeras, there was consistently less *T/T* ES cell contribution to somitic mesoderm than neural tube and surface ectoderm. The value for the neural tube and surface ectoderm chimerism usually corresponded to that for head fractions, which have a relatively high ectoderm component (Table 4). No *T/T* cells were detected in the four limb bud fractions assayed, (embryos 4 and 11, Fig. 6 legend), while levels of chimerism in the limb buds of all *T/+* and  $+/+$  chimeras corresponded to within 15 percentage points of those in somitic mesoderm.



**Fig. 5.** Chimerism and abnormalities in (A) *T/+*  $+/+$  chimeras and (B)  $+/+$   $+/+$  chimeras at 11.5 dpc. Chimeras 33 and 36 had an uneven pair of somites near the tail tip. The tail tip in chimeras 34 and 35 was slightly malformed.



**Fig. 6.** Chimerism in separated trunk ectoderm and mesoderm fractions. Numbers along the X-axis refer to chimeras represented in Figs 3-5; chimeras i-iii are not represented elsewhere. In embryos 27, 28, 31, 32, i and ii, only fraction 4 (mid trunk) was separated into ectoderm and mesoderm components, and these are represented by black and white columns respectively. In all other embryos, fractions 3a and b (anterior trunk ectoderm and mesoderm) and 5a and b (posterior trunk ectoderm and mesoderm) were separated and are represented by black, white, dark grey and light grey columns, respectively. Limb buds (fractions 3c and 5c) were assayed in embryos

4, 11, and all *T/+* and *+/+* chimeras. No ES cell contribution to these fractions in embryos 4 and 11 was detected. In all other chimeras except fraction 3 from embryo 41, ES cell contribution to fractions 3c and 4c correlated to within 15 percentage points of ES cell contribution to both neural tube and surface ectoderm.

**In situ hybridization to *+/+* and *T/+* tails**

The accumulation of *T/T* cells in the tail and the consequent tail abnormalities seen are difficult to explain solely on the basis of a defective notochord. To investigate this further, we have begun a whole-mount in situ analysis of *T* expression in the tails of wild-type and *T/+* embryos over the period of tail elongation. As illustrated in Fig. 7, *T* is highly expressed not only in the notochord but also in the tailbud of all wild-type embryos at 9.5-12.5 dpc. In contrast, embryos from *T/+* × *T/+* matings with visible defects in the notochord at 10.5-11.5 dpc showed diminished or undetectable tailbud *T* expression (data not shown). Interest-

ingly, reduced or absent *T* expression was also observed in tails with an apparently normal notochord, suggesting that tailbud abnormalities may occur independently of a disrupted notochord.

**DISCUSSION**

Two independent *T/T* ES cell lines have given rise to chimeras that show a characteristic set of abnormalities reminiscent of those found in *T/T* embryos. In contrast, a *T/+* ES cell line gave rise to chimeras that were normal or displayed slight tail abnormalities. None of these defects were observed in chimeras made with a wild-type ES cell line. Thus, it seems likely that the abnormalities observed between 9.5 and 11.5 dpc were due to the absence of the *T* gene in ES cell progeny. These chimeric studies have examined the behaviour of *T/T* cells in embryos at stages beyond the normal time of death of intact *T/T* mutants. This, and particularly the analysis of low level chimeras, has made it possible to detect a skew in the colonisation pattern of *T/T* cells, first evident at 9.5 dpc, with respect both to the rostrocaudal axis and different germ layer derivatives.

**Table 4. Correlation between levels of chimerism in trunk neural tube and head**

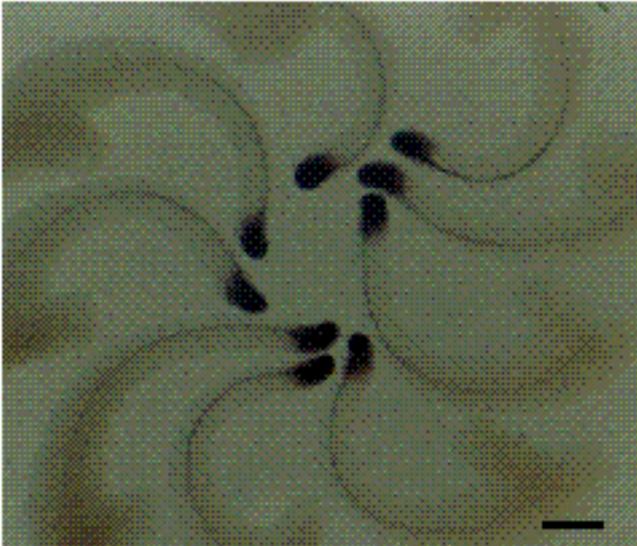
		Number of chimeric embryos		
		TrunkNT* = head	TrunkNT** > head	Trunk NT** < head
<i>T/T</i>	<i>+/+</i>	7	2	0
<i>T/+</i>	<i>+/+</i>	7	0	0
<i>+/+</i>	<i>+/+</i>	2	0	0
Total		16 (88.9%)	2(11.1%)	0

\*Chimeras in which percentage ES cell contribution to trunk neural tube (NT) correlates to within 10 percentage points of ES cell contribution to the head.

\*\*Chimeras in which the ES cell contribution varies by 15 or more percentage points between trunk neural tube and head.

**Skewed colonization patterns in *T/T* and *T/+* chimeras**

The dramatic elevation in *T/T* cell contribution to caudal regions of chimeras could be explained in two different



**Fig. 7.** In situ hybridization to *T* mRNA in wild-type 11.5 dpc tails. Bar, 0.8 mm.

ways: either *T/T* cells present in anterior regions at earlier stages are selected against later in development, or *T/T* cells do not populate anterior regions. It is unlikely that *T/T* cells selectively die in rostral regions because, in intact *T/T* mutants, extensive cell death in the head prior to 9.5 dpc has not been observed (Chesley, 1935; Yanagisawa and Fujimoto, 1977a). If *T/T* cells are failing to reach anterior regions, this could be for a variety of reasons. During gastrulation, they may never populate regions of the epiblast destined to give rise to anterior structures or, alternatively, they may selectively colonize prospective posterior regions. For this to occur, according to the fate maps of Lawson et al. (1992), *T/T* cells would have to occupy a position in the pregastrula epiblast immediately lateral to the prospective anterior end of the axis, so that by the end of gastrulation movements they would reside at the caudal end of the embryo. Since an abnormal rostrocaudal distribution is seen in all chimeras, this would require efficient and reproducible cell sorting within each chimeric epiblast. While we cannot rule this out, such a distribution seems unlikely because such sorting implies that the *T* gene is required in all epiblast for normal intermixing to occur within the epiblast (Beddington et al., 1989). *T* expression is, however, restricted to epiblast cells immediately adjacent to the primitive streak during gastrulation (Wilkinson et al., 1991).

A more likely explanation is that *T/T* mesoderm cells are defective in moving away from the streak. This would mean that they would tend to accumulate in the midline beneath the streak and, therefore, make a greater contribution to derivatives of this region (such as the tail bud and allantois) than to lateral or paraxial mesoderm. This interpretation is consistent with descriptions of anomalous *T/T* mesoderm movements in vitro (Hashimoto et al., 1987) and the observed thickening of the primitive streak in intact mutants (Yanagisawa, 1981) and *T/T*  $+/+$  chimeras (Rashbass et al., 1991). In addition, it is supported by our observations that *T/T* cells contribute less to trunk somitic mesoderm and limb buds than to trunk neurectoderm. The *T/T* contribu-

tion to somitic mesoderm should reflect events in a population of invaginated cells that have to move away from the streak. Conversely, the contribution to neural tube should correlate with the initial level of chimerism in the epiblast, since no invagination movements are required for neurectoderm or surface ectoderm formation. There is a positive correlation between chimerism in trunk neural tube and the head, which is a predominantly ectodermal structure (Table 4), as would be expected if levels of chimerism in the ectoderm were relatively homogeneous. It is important to appreciate that, although somitic chimerism is reduced, *T/T* cells can still make a substantial contribution to paraxial mesoderm and, therefore, the defect in mesoderm movement is probably slight and exposed only by competition with wild-type cells. For example, in intact mutants or high level chimeras, the movement of nascent *T/T* mesoderm is adequate to maintain normal tissue interactions and juxtapositions during the early stages of gastrulation (Yanagisawa, 1981). The increasing accumulation of mesoderm beneath the primitive streak would only later disrupt both the continuation of gastrulation and normal trunk development.

This discrepancy between somitic and neural tube chimerism, which is not evident in *T/+* chimeras, also indicates that the defect in *T/T* cells is cell autonomous, as opposed to an extracellular effect, since the wild-type constituent in chimeras populates paraxial mesoderm normally. Although *T/+* cells have a normal distribution within germ layer derivatives they also show a tendency, albeit reduced, for an elevated contribution to caudal tissues. Therefore, they too are probably compromised in mesodermal cell movements and accumulate posteriorly. This indicates that the level of *T* expression is critical for ensuring adequate production of molecules necessary for normal mesoderm cell adhesion or cell movement. This explanation does not invoke an increased requirement for *T* expression along the rostrocaudal axis (MacMurray and Shin, 1988) but rather points to a single, mild defect in morphogenetic movements being compounded with time.

#### Requirement for *T* in allantois development

Usually allantoic defects correlated with colonization of this tissue by *T/T* cells, although the *T/T* cell contribution was never higher than 20% in embryos showing only an abnormal allantois (Fig. 3B). Furthermore, in some chimeras with a defective allantois, no *T/T* cells could be detected in allantoic fractions. In chimeras analysed at an earlier stage (8.5-9.5 dpc; Rashbass et al., 1991), abnormality in the allantois correlated well with the extent of allantois chimerism and an abnormal allantois was present in all abnormal chimeras at these stages. These chimeras frequently showed a very high *T/T* cell contribution to the allantois (50-80% in 4/8 embryos assayed). In contrast, there was never more than 40% *T/T* cell contribution to the allantois in the embryos analysed here. Therefore, it is possible that *T/T* cells selectively die in the allantois. It is also possible that either extremely low levels of chimerism in the allantois (<5%) are sufficient to cause aberrant development, or that allantoic abnormalities are due to events occurring before this tissue starts to emerge into the exocoelom. Therefore, there may be a phase of *T* expression during the transition from epiblast to extraembryonic mesoderm that is obliga-

tory for normal allantoic differentiation and survival. The main body of the allantois does not express *T*, but since the boundary between allantoic tissue and caudal embryonic mesoderm is unknown, it is possible that basal allantoic cells do. Alternatively, the allantois may fail to develop normally because there is a surfeit of mutant cells accumulating at its base, preventing appropriate cell recruitment into the allantoic bud. However, the abnormalities seen in chimeric allantoides, where cells tend to stick to the amnion or bud off from the periphery of the allantois (see Rashbass et al., 1991), indicate that the defect is not simply one of cell number but rather of inappropriate differentiation. If cell adhesion deficiencies are the cause of defective mesoderm movement, they could also explain the failure of coherent allantoic growth.

### Requirement for *T* in normal tail development

These experiments examine for the first time the effects of the complete absence of *T* gene expression on tail development. The anomalies (foreshortening, branching and haemorrhagic bulli) observed may all be perceived as more severe manifestations of those abnormalities observed in *T/+* and *T/t* tail development. Degeneration of blood vessels near the tail tip has been reported in *T/+* mutants at 11.5-12.5 dpc (Yanagisawa, 1990) and forked tails have occasionally been seen in *T/t* mutants (Gruneberg, 1958). Thus, the tail defects seen in 10.5-11.5 dpc *T/T* *+/+* chimeras, some of which have distal tails composed of 70% viable *T/T* cells, are almost certainly directly attributable to the absence of *T* gene expression during tail development rather than an artefact of chimerism.

In normal development, the tail grows from its distal tip by virtue of a population of mesoderm cells located in the tail bud, the slightly thickened caudal extreme of the tail. This precursor population arises from the last cells to invaginate through the primitive streak (Rugh, 1968; Tam et al., 1982; Gajovic et al., 1989) and has been shown to be responsible for generating tail somites (Tam and Tan, 1992). It also has the potential to generate tail notochord and neural tube (Tam and Tan, 1992). Traditionally, the tail defects seen in *T/+* and *T/t* mutants have been ascribed to a defective notochord (Chesley, 1935; Gruneberg, 1958; Theiler, 1961), although it has always been puzzling why extreme variations in notochord development (from apparently normal to absent) should always result in the sudden degeneration of the tail tip characteristic of *T/+* mutants. In addition, the notochord is not always abnormal at the tail tip prior to its degeneration. It is, therefore, possible that an absence or reduction in *T* expression has a direct effect on tail outgrowth, independent of any secondary influence of the notochord. Certainly, the high level of expression of *T* in the tail bud (Fig. 7) of normal embryos, and the apparent reduction in *T* expression in *T/+* mutants as early as 10.5 dpc, would suggest an involvement of *T* product in tail bud as well as tail notochord function. Comparison of the rostrocaudal gradient of *T* chimerism between 9.5 dpc chimeras and later chimeras shows a dramatic increase in the caudal accumulation of *T/T* cells once tail outgrowth has begun. The tail fractions of embryos 4-12 included only the most caudal 10 somites and, with only a single exception, contained at least 20% more *T/T* cells than did prox-

imal tail fractions (data not shown). The same trend was evident in *T/+* *+/+* chimeras (data not shown). Therefore, it is likely that normal *T* expression is required for the correct deployment of new cells emerging from the tail bud precursor population. Here too, cell autonomous defects in adhesion or cell movement could disrupt this supply, causing foreshortening of the tail or branching due to *T/T* cells forming a physical wedge between growing wild-type tail-bud cells. However, since some *T/t* mutants show similar branching defects to those observed in chimeras, branching may not be due to a failure of two different cell types to intermix, but rather an intrinsic failure of *T/T* cells to produce coherent tail elongation. Thus, the abnormal behaviour of *T/T* and *T/t* cells themselves would produce distal tail branching.

The abnormalities observed between 9.5 and 11.5 dpc show that only chimeras with a low *T/T* cell contribution would survive into the latter stages of gestation. Many of these were chimeric only in the tail, and this coincided with tail defects. Therefore, the liveborn young, which were not overtly chimeric, but which had abnormal tails, are likely to arise from this class of chimeras. Thus, the failure to detect more than a trace of *T/T* cells in the tails of these animals indicates that the chimeric distal tail region must degenerate between 11.5 dpc and birth.

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### REFERENCES

- Beddington, R. S. P., Morgenstern, J., Land, H. and Hogan, A. (1989). An in situ transgenic enzyme marker for the midgestation mouse embryo and the visualization of inner cell mass clones during early organogenesis. *Development* **106**, 37-46.
- Beddington, R. S. P., Rashbass, P. and Wilson, V. (1992). *Brachyury* - a gene affecting mouse gastrulation and early organogenesis. *Development* **1992 Supplement**, in press.
- Bennett, D., Artzt, K., Magnuson, T. and Spiegelman, M. (1977). Developmental interactions studied with experimental teratomas derived from mutants at the *T/t* locus in the mouse. In *Cell Interactions in Differentiation*. (ed. M. Kaarinen-Jaaskelainen, L. Saxen and L. Weiss). pp. 389-398. New York: Academic Press.
- Bolvent, P. and Dodd, J. (1991). Perturbation of neuronal differentiation and axon guidance in the spinal cord of mouse embryos lacking a floor plate: analysis of Danforth's short-tail mutation. *Development* **113**, 625-639.
- Chesley, P. (1935). Development of the short-tailed mutant in the house mouse. *J. Exp. Zool.* **70**, 429-459.
- Clarke, J. D. W., Holder, N., Soffe, S. R. and Storm-Mathison, J. (1991). Neuroanatomical and functional analysis of neural tube formation in notochordless *Xenopus* embryos; laterality of the ventral spinal cord is lost. *Development* **112**, 499-516.
- Dobrovol'skaia-Zavad'skaia, N. (1927). Sur la mortification spontanée de la queue chez la souris nouveau-née et sur l'existence d'un caractère héréditaire 'non-viable'. *C. R. Soc. Biol.* **97**, 114-116.
- Ephrussi, B. (1935). The behaviour *in vitro* of tissues from lethal embryos. *J. Exp. Zool.* **70**, 197-204.
- Fujimoto, H., and Yanagisawa K. O. (1979). Effects of the *T* mutation on histogenesis of the mouse embryo under the testis capsule. *J. Embryol. Exp. Morph.* **50**, 21-30.
- Gajovic, S., Kostovic-Knezevic, L. and Svajger, A. (1989) Origin of the notochord in the rat embryonic tail. *Anat. Embryol.* **179**, 305-310.
- Gluecksohn-Schoenheimer, S. (1944). The development of normal and homozygous *brachy* (*T/T*) mouse embryos in the extraembryonic coelom of the chick. *Proc. Natl. Acad. Sci. USA* **30**, 134-140.
- Gruneberg, H. (1958). Genetical studies on the skeleton of the mouse.

- XXIII. The development of *Brachyury* and *Anury*. *J. Embryol. Exp. Morph.* **6**, 424-443.
- Gruneberg, H.** (1963). *The Pathology of Development*. Oxford: Blackwell Scientific Publications.
- Hashimoto, K., Fujimoto, H. and Nakatsuji N.** (1987). An ECM substratum allows mouse mesodermal cells isolated from the primitive streak to exhibit motility similar to that inside the embryo and reveals a deficiency in the *T/T* mutant cells. *Development* **100**, 587-598.
- Hemmati-Bravanlou, A., Stewart, R. M. and Harland, R. M.** (1990). Region-specific neural induction of an engrailed protein by anterior notochord in *Xenopus*. *Science* **250**, 800-802.
- Herrmann, B. G., Labeit, S., Poustka, A., King, T. R. and Lehrach, H.** (1990). Cloning of the *T* gene required in mesoderm formation in the mouse. *Nature* **343**, 617-622.
- Kitchin, I. C.** (1949). The effects of notochordectomy in *Amblystoma mexicanum*. *J. Exp. Zool.* **112**, 393-415.
- Lawson, K. A., Meneses, J. J. and Pedersen, R. A.** (1991). Clonal analysis of epiblast during germ layer formation in the mouse embryo. *Development* **113**, 891-911.
- MacMurray, A., and Shin, H.-S.** (1988). The antimorphic nature of the *T<sup>c</sup>* allele at the mouse *T* locus. *Genetics* **120**, 545-550.
- Paavola, L. G., Wilson, D. B. and Center, E. M.** (1980). Histochemistry of the developing notochord, perichordal sheath and vertebrae in Danforth's short-tail (*Sd*) and normal C57BL/6 mice. *J. Embryol. Exp. Morph.* **55**, 227-245.
- Placzek, M., Tessier-Lavigne, M., Yamada, T., Jessell, T. and Dodd, J.** (1990). Mesodermal control of neural cell identity: floor plate induction by the notochord. *Science* **250**, 985-988.
- Rashbass, P., Cooke, L. A., Herrmann, B. G. and Beddington, R. S. P.** (1991). A cell autonomous function of *Brachyury* in *T/T* embryonic stem cell chimeras. *Nature* **353**, 348-350.
- Robertson, E. J.** (1987). *Teratocarcinomas and Embryonic Stem Cells: a Practical Approach*. Oxford: IRL press.
- Rugh, R.** (1968). *The Mouse*. Burgess: Minneapolis.
- Searle, A. G.** (1966). *Curtailed*, a new dominant *T*-allele in the house mouse. *Genet. Res.* **7**, 86-95.
- Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M. and Rogers, D.** (1988). Inhibition of pluripotential embryonic stem cells by purified polypeptides. *Nature* **336**, 688-692.
- Smith, J. L., and Schoenwolf, G. C.** (1989). Notochordal induction of cell wedging in the chick neural plate and its role in neural tube formation. *J. Exp. Zool.* **250**, 49-62.
- Svajger, A. and Levak-Svajger, B.** (1975). Technique of separation of germ layers in rate embryonic shields. *Wilhelm Roux Arch. Dev. Biol.* **178**, 303-308.
- Tam, P. P. L. and Tan, S.-S.** (1992). The somitogenetic potential of cells in the primitive streak and the tail bud of the organogenesis-stage mouse embryo. *Development* **115**, 703-715.
- Tam, P. P. L., Meier, S. and Jacobson, A. G.** (1982). Differentiation of the metamer pattern of the embryonic pattern of the mouse. II. Somitomeric organization of the presomitic mesoderm. *Differentiation* **21**, 109-122.
- Theiler, K.** (1961). Genetisch bedingte Chordaschädigungen bei der Maus. *Arch. Julius-Klaus-Stift* **36**, 118-125.
- van Straaten, H. W. M., Thors, F., Wiertz-Hoessels, E. J. L., Hekking, J. W. M. and Drukker, J.** (1985). Effect of a notochord implant on the early morphogenesis of the neural tube and neuroblasts: histometrical and histological results. *Dev. Biol.* **110**, 247-254.
- Wilkinson, D., Bhatt, S. and Herrmann, B. G.** (1990). Expression pattern of the mouse *T* gene and its role in mesoderm formation. *Nature* **343**, 657-659.
- Yamada, T., Placzek, M., Tanaka, H., Dodd, J. and Jessell, T. M.** (1991). Control of cell pattern in the developing nervous system: Polarizing activity of the floor plate and notochord. *Cell* **64**, 635-647.
- Williams, R. L., Hilton, D. H., Pease, S., Wilson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., and Gough, N. M.** (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336**, 684-687.
- Whittingham, D. G., and Wales, R. G.** (1969). Storage of two-cell mouse embryos in vitro. *Austr. J. Biol. Sci.* **2**, 1065-1068.
- Yanagisawa, K. O.** (1990). Does the *T* gene determine the anteroposterior axis of a mouse embryo? *Jpn. J. Genet.* **65**, 287-297.
- Yanagisawa, K. O., and Fujimoto, H.** (1977). Differences in rotation mediated aggregation between wild-type and homozygous *Brachyury* (*T*) cells. *J. Embryol. Exp. Morph.* **40**, 277-283.
- Yanagisawa, K. O., and Fujimoto, H.** (1977). Viability and metabolic activity of homozygous *Brachyury* (*T*) embryos. *J. Embryol. Exp. Morph.* **40**, 271-276.
- Yanagisawa, K. O., Fujimoto, H. and Urushihara, H.** (1981). Effects of the *Brachyury* (*T*) mutation on morphogenetic movement in the mouse embryo. *Dev. Biol.* **87**, 242-248.