Two novel chick T-box genes related to mouse Brachyury are expressed in different, non-overlapping mesodermal domains during gastrulation

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SUMMARY

The mouse Brachyury (T) gene plays critical roles in the genesis of normal mesoderm during gastrulation and in the maintenance of a functioning notochord. Abrogation of Brachyury (T) expression within the chordamesoderm of homozygous null mutants nevertheless spares anterior axis formation. An intriguing possibility to explain the preservation of anterior axis formation in these mutants would be the existence of other genes compensating for the loss of Brachyury. This compensation and the recent demonstration that Brachyury is the prototype for an evolutionarily conserved family, prompted a search for other T-box genes participating in axis formation. The chick Brachyury orthologue and two related chick T-box genes that are expressed at the onset of gastrulation have been isolated. One of these novel genes (Ch-Tbx6L) becomes restricted to the early paraxial mesoderm lineage and is a potential candidate for complementing or extending Brachyury function in the anterior axis (formation of the head process, prechordal plate). The other gene (Ch-Tbx6L), together with chick T, appears to mark primitive streak progenitors before gastrulation. As cells leave the primitive streak, Ch-Tbx6L becomes restricted to the early paraxial mesoderm lineage and could play a role in regulating somitogenesis.

Key words: gastrulation, notochord, primitive streak, somitogenesis, T-box, Brachyury, chick embryo, activin, FGF, retinoic acid

INTRODUCTION

The embryonic axis is formed through the process of gastrulation, by organized and highly coordinated cell movements giving rise to primary axial and paraxial mesoderm. Formation of the primitive streak has been considered the earliest hallmark of gastrulation in amniotes and includes two separable events: (1) establishment of a localized site at which nascent mesoderm will invaginate and delaminate (Sanders, 1982; Tam and Meier, 1982; Sanders, 1984), and (2) formation of mesoderm proper. While the work of a number of laboratories has led to the identification of a group of candidate regulatory and signalling factors involved in this process, the list is clearly insufficient for the full elucidation of the molecular basis of gastrulation.

The gene Brachyury (T) is one of the first and most extensively studied developmental control genes to be directly implicated in the formation of primitive streak mesoderm (Chesley, 1935 and references therein; Herrmann et al., 1990), and is subsequently required for the maintenance and function of notochord (Conlon et al., 1995a). The T gene encodes a transcription factor very highly conserved throughout chordate evolution (Smith et al., 1991; Schulte-Merker et al., 1992; Kispert and Herrmann, 1993; Yasuo and Satoh, 1993; Schulte-Merker et al., 1994; Yasuo and Satoh, 1994; Kispert et al., 1995a,b; Holland et al., 1995; reviewed by Herrmann and Kispert, 1994). During normal development, the T gene, first identified via semidominant mouse mutants, is expressed transiently in all mesoderm while in transit through the streak, and persisently throughout the axial chordamesoderm (Wilkinson et al., 1990; Herrmann, 1991; Kispert and Herrmann, 1994). The role of the T gene appears to be related more to the maintenance of a functioning primitive streak and notochord than to mesoderm formation per se (Conlon et al., 1995a). Homozygous mutant (T/T) embryos initially form a primitive streak and mesoderm (Chesley, 1935), but subsequently mesodermal cells fail to move away from the streak and accumulate there instead (Hashimoto et al., 1987; Beddington et al., 1992; Wilson et al., 1993, 1995). It is noteworthy that in homozygous null mutants, a notochord also initially forms and the development of the anterior axis up to the level of the forelimb bud is relatively spared (Chesley, 1935), indicating that either the T gene product is not required for anterior axis formation, or that it is functionally redundant in this part of the embryo.

It has been recently shown that Brachyury is a member of a family of diverse genes that all share a conserved region of about 200 amino acids which includes the DNA binding domain (denoted the ‘T-box’) (Kispert and Herrmann, 1993; Bollag et al., 1994; Agulnik et al., 1995; Bulfone et al., 1995). This raises the intriguing possibilities that T function during gastrulation may be complemented by other T-box genes with overlapping expression domains, and perhaps also supplemented by T-box genes with different, but related, roles in mesoderm specification. Because of its size and accessibility, the avian embryo has provided a very attractive model for studying gastrulation in higher vertebrates. To utilize this system for studying T-family gene function, we have isolated the chick orthologue of Brachyury (Ch-T) and two related
chick T-box genes (Ch-TbxT and Ch-Tbx6L). This paper reports the expression domains of these genes prior to the onset of, and during, gastrulation and demonstrates their regulation by axis modulating and mesoderm-inducing signals.

**MATERIALS AND METHODS**

**Embryos**

White Leghorn chick embryos were incubated at 38°C and staged as described by Eyal-Giladi and Kochav (EG&K: roman numerals) for pregastrulation stages, and by Hamburger and Hamilton (HH: Arabic numerals) for all later embryos (Hamburger and Hamilton, 1951; Eyal-Giladi and Kochav, 1976). Embryos were dissected in phosphate-buffered saline (PBS) and processed for experiments as described below.

**Preparation and analysis of RNA and DNA**

Total RNA was extracted from embryonic chick tissues or primary blastoderm cultures using ULTRASPEC RNA (Biotecx Labs, Inc.). RNAs were separated on 1.2% formaldehyde gels, transferred to nylon membranes and hybridized with 32P-labeled random-primed RNAs were separated on 1.2% formaldehyde gels, transferred to nylon membranes and hybridized with 32P-labeled random-primed DNA probes using standard methods (Sambrook et al., 1989). Hybridizations were carried out in Rapid-Hyb buffer (Amersham) as recommended. A chick beta actin probe was used for control northern hybridizations as previously (Knezevic et al., 1995). Hybridized membranes were exposed to a phosphor screen and signals quantitated with ImageQuant software (Molecular Dynamics). Nucleotide sequence analyses of cDNA clones were performed using the dyeoxy technique (Sanger et al., 1977).

**Cloning of T-box sequences**

Initial reverse transcription and polymerase chain reaction (PCR) were done using a GeneAmp RNA PCR Kit (Perkin Elmer Cetus). 20 μg of total RNA from stage 9-11 chick embryos was reverse transcribed with oligo-dT and amplified through three rounds of PCR using nested primers. Fully degenerate oligonucleotide primers were used in each round of amplification and were derived from highly conserved regions within the T-box and corresponded to the following peptide sequences. 1st round: FKELTNEM for sense, and DAKERND for antisense. 2nd round: NEMIVTK for sense, and NPFAKAF for antisense. 3rd round: DPNAMY (with 5¢ Spel site) for sense, and YQNEEIT (with 5¢ SalI site) for antisense. For each PCR round, standard conditions were used except that, for the first five cycles, the annealing temperature was 45°C and included a two minute ramp to 72°C extension. PCR products were gel purified and cloned into Bluescript SK- using standard methods (Sambrook et al., 1989). These clones (corresponding to Ch-TbxT and Ch-Tbx6L T-box sequences) were used to screen a stage 5-9 chick embryo cDNA library (described by Knezevic et al., 1995) from which clones for Ch-T, -TbxT, and -Tbx6L were isolated. The sequences for Ch-T, -TbxT and -Tbx6L have been deposited in GenBank (accession numbers U67086, U67087, and U67088, respectively).

**Whole-mount in situ hybridization of embryos**

Digoxigenin-UTP riboprobes were generated from either 3' untranscribed sequences (Ch-T and Ch-TbxT) or from coding sequences 3' to the T-box (Ch-Tbx6L). Embryos were prepared for whole mount in situ hybridization, hybridized with antisense riboprobes, washed, and the hybrids visualized with alkaline phosphatase conjugated antidigoxigenin as described by Conlon and Rossant (1992), except that the length of proteinase K digestion varied from 1-5 minutes depending on the size of the embryos. Upon visualization of the reaction product (usually 0.5 to 6 hours), the reaction was stopped and embryos were stored at 4°C in PBS containing 2.5 mM EDTA and 0.1% Tween 20, and photographed without clearing. Stained embryos were embedded in OCT compound (Tissue-Tek) and 10 μm frozen sections were cut and mounted in Immunon (Thomas Scientific). As a control, sense riboprobes for each gene were also used, and none of these gave a detectable hybridization signal at any of the stages analyzed in this report (data not shown).

**Blastoderm cell stage**

A suspension of stage XI-XII blastoderm cells was obtained and cultured as described previously (Knezevic et al., 1995). Additions to the cultures were as follows: FGF-4 (gift from Genetics Institute, Inc.) at 300-750 ng/ml with 100 ng/ml of heparin sulfate (Sigma); activin A (BioSource International, Inc.) at 50-100 U/ml with units of activity determined by the manufacturer using the Xenopus animal cap assay; all-trans retinoic acid (Sigma) at 10-6 M; and cycloheximide (Sigma) at 5 μg/ml. Where cycloheximide was used, cells were pre-treated with cycloheximide for 1 hour prior to the addition of growth factors or retinoic acid followed by incubation with factors for an additional 4 hours in the continued presence of cycloheximide. Under these conditions, a 95% inhibition of protein synthesis is achieved (Knezevic et al., 1995). Three independent sets of culture experiments were analyzed for expression levels of Ch-T, Ch-TbxT, and Ch-Tbx6L using northern blot or quantitative RT-PCR analysis.

**RESULTS**

**Cloning of chick T-box genes**

RT-PCR using degenerate oligonucleotide primers corresponding to highly conserved regions of the T-box was employed to identify T-box genes expressed during early development. The percentage (% identity) of alignment. The percentage (%) amino acid identity (ID) for each gene compared to mouse T and to Drosophila Omb are indicated.
embryogenesis. Two different T-box sequences were isolated, that shared 85% (\textit{Ch-TbxT}) and about 51% (\textit{Ch-Tbx6L}) amino acid identity with the mouse \textit{T} gene (Fig. 1). These sequences were each used to screen a stage 5-9 chick cDNA library. cDNA clones including the entire coding regions of \textit{Ch-T} (chick \textit{Brachyury} orthologue) and \textit{Ch-TbxT} were isolated using the \textit{Ch-TbxT} probe. \textit{Ch-Tbx6L} clones isolated were missing part of the C-terminal coding region. \textit{Ch-T} is the orthologue of \textit{Brachyury} and is virtually identical to the chick \textit{T} gene isolated by Kispert et al. (1995b), differing in only four nucleotides (two of them within the coding region result in amino acids changes: D to P at residue 11 and T to A at residue 250). \textit{Ch-TbxT} has been so named because it is more closely related in its T-box to \textit{Brachyury} than to any other known \textit{Tbx} genes and also has tracts of conservation (about 67% identity over 30 amino acid stretches) in regions both N-terminal and C-terminal to the T-box (Fig. 2). \textit{Ch-TbxT} is however clearly nonallele to \textit{Ch-T}, as indicated by a lack of homology in nontranslated regions and distinct genomic restriction digests (data not shown), and by substantial differences in expression pattern (see below). \textit{Ch-TbxT} is also unlikely to be the result of a recent gene duplication event peculiar to birds, since its amino acid sequence is about equally diverged from \textit{Ch-T}, and from \textit{Mm-T} and \textit{Xbra} (the two closest vertebrates for which sequence is known; see Fig. 2).

\textit{Ch-Tbx6L} (short for ‘Tbx6-like’) has been so named because it is similar in its T-box sequence and its expression pattern to a recently identified mouse gene (\textit{Tbx6}; Agulnik et al., 1996; Chapman et al., 1996) and zebrafish gene (\textit{Zftbx6}; B. Hug and D. Grunwald, personal communication). Because of a fairly high degree of sequence divergence outside of the T-box and differences in time of onset of expression, it is presently uncertain whether these vertebrate genes are true orthologues or represent related genes that define a new T-box subfamily. \textit{Ch-Tbx6L} is equally divergent from the two major classes of T-box genes identified to date, sharing about 50% amino acid identity with both \textit{Brachyury} and with \textit{Drosophila Omb} in the T-box region. In contrast, many of the other \textit{Tbx} genes are more closely related to \textit{Omb} than to \textit{Brachyury} in the T-box (eg. 87% identity for \textit{Tbx2}, Bollag et al., 1994; Agulnik et al., 1995).

**Temporal and spatial distribution of T-box gene mRNAs during chick embryonic development**

Transcripts for all three genes were detected in early stage 9 chick embryos by northern blot analysis (Fig. 3A). Each was expressed as a single major transcript, of 3.2 kb for both \textit{Ch-T} and \textit{Ch-TbxT}, and of 3.8 kb for \textit{Ch-Tbx6L}. The temporal distribution of \textit{Ch-T} and \textit{Ch-Tbx6L} expression during embryonic development was further evaluated by northern blot analysis of different pre- and postgastrulation stages extending to midembryogenesis. \textit{Ch-TbxT} transcripts were not further evaluated using northern blots because of very low levels of expression that were only weakly detectable even with large amounts of RNA.
Ch-T expression
Ch-T transcripts first appeared just prior to primitive streak formation, in a narrow crescent along the posterior border of stage XIII blastoderm (Fig. 4A). Expression was seen exclusively in epiblastic cells within, and just anterior to, the posterior marginal zone (Fig. 4G and not shown). During subsequent stages of primitive streak formation (stages 2-4), hybridization became restricted to the region of the elongating primitive streak (Fig. 4B-D). At this time, expression in mesodermal cells through the streak has begun (Waddington, 1952). Ch-T transcripts were detected in the ectodermal layer of the streak, in mesodermal cells within and immediately adjacent to the streak, and in Hensen’s node (Fig. 4IJ). A small region of both ectoderm and endoderm just anterior to Hensen’s node was also stained (Fig. 4H), an exception to the general restriction of Brachyury expression to cells with mesodermal fates. This observation has been previously noted in mouse (Kispert and Herrmann, 1994) and chick (Kispert et al., 1995b). The axial mesoderm arising from Hensen’s node (head process and notochord) also strongly expressed Ch-T (Fig. 4E,F,K,L). Other than the domain noted in neural plate ectoderm immediately anterior to Hensen’s node, no Ch-T expression was detected in the developing nervous system, including floorplate (Fig. 4K,L and not shown). Ch-T transcripts persisted in the notochord and were later also detected in the developing tail bud and tail, up to stage 26-28 (Fig. 3 and not shown).

Ch-Tbx6L expression
As suggested by northern blot analysis, Ch-Tbx6L transcripts were readily detected in stage X-XI blastoderms (Fig. 6A). Staining was initially distributed uniformly throughout the posterior marginal zone epiblast and the epiblast of the area pellucida just anterior to it (Fig. 6GH). During subsequent prestreak stages, the lateral extent of this rim of expression gradually receded as the central posterior part became more intense and extended more anteriorly, prefiguring formation of the primitive streak (Fig. 6B-D). At the fully elongated streak neuropore (not shown). Elsewhere in the neural tube, notably in the floor plate, no expression was detected. Ch-Tbx6T expression rapidly declined during further development and transcripts were undetectable in embryos by whole-mount in situ hybridization after stage 10-12.
stage (stage 4), stained cells were present in the ectoderm at
and immediately adjacent to the streak (Fig. 6I), but not in the
mesoderm and endoderm. Concomitant with the onset of node
and streak regression (stage 5), expression of Ch-Tbx6L in the
ectoderm adjacent to the streak was lost, and commenced in
mesodermal cells in and around the primitive streak (Fig. 6J).
Ch-Tbx6L expression extended more laterally in the nascent
mesoderm arising from the primitive streak than did Ch-T.
During streak regression, nascent mesoderm arising from
the node and streak becomes organized into two major subpopu-
lations; axial (prechordal plate, head process, notochord) and
paraxial (segmental and lateral plates). Ch-Tbx6L transcripts
were exclusively detected within the mesoderm of the
segmental plate and were rapidly downregulated upon seg-
mentation (Fig. 6F,K,L). Only weak staining of the most
recently formed somite was occasionally seen. The paraxial
mesoderm of the head, clearly distinguishable at this point
(Hamburger and Hamilton, 1951) and normally incapable of
somite formation (Meier, 1979), showed no detectable Ch-
Tbx6L expression. Staining of the segmental plate continued
beyond trunk formation and during the phases of tail bud
formation and tail elongation, and disappeared by stage 26-28
(not shown), when the chick tail undergoes degeneration
(Schoenwolf, 1981).

Fig. 5. Spatial distribution of Ch-TbxT mRNA during gastrulation.
The embryos in A-F are oriented with their anterior border topmost.
Levels of transverse sections (labeled G-L) are indicated on the
whole-mount embryos and are shown in G-L. A hybridization signal
was transiently detected at the posterior end of stage 2-3 primitive
streak (A) and had disappeared by stage 4- (not shown). By stage 4,
transcripts were detected in Hensen's node (hn, B-F,K), early
prechordal plate (D,H), notochord (E-I,J,L) and in the ectoderm in
an arc extending posterolateral to the node (C,G, arrows in E,G). A
small region of neural plate ectoderm, just anterior to the node, also
expressed Ch-TbxT transcripts (J). (A) stage 3; (B) stage 4–;
(C) stage 4; (D,G,H) stage 4+; (E,I-K) stage 5; (F,L) stage 9; ps,
primitive streak; hn,Hensen's node; pp, prechordal plate; no,
notochord; ap, neural plate.

Fig. 6. Spatial distribution of Ch-Tbx6L mRNA prior to and during
gastrulation. The embryos in A-F are oriented with their anterior
border topmost. Levels of sections (labeled G-L) are indicated on the
whole-mount embryos and are shown in G-K (transverse sections)
and L (parasagittal section). Transcripts were readily detected in the
posterior epiblast of stage XI blastoderm including the posterior
marginal zone (A,G,H) and became gradually localized in and
around the primitive streak (B-E). Hensen's node (hn) and notochord
were never stained (E,F,K). Initially only the ectoderm around the
streak was positive for Ch-Tbx6L transcripts (I), but after stage 5,
transcripts were detectable in the mesodermal cells migrating away
from the streak and disappeared from the ectoderm adjacent to the
streak (J). During the regression phase of gastrulation, the mesoderm
of the segmental plate was positive (F,K,L). Expression was lost as
segmentation proceeded, with a weak hybridization signal
occasionally remaining in the most recently formed somite (arrows,
FL). (A,G,H) stage XI, (B) stage XIII, (C) stage XIV, (D) stage 2,
(E,I) stage 4, (F,J,K,L) stage 9; a,anterior; p, posterior; ps, primitive
streak; hn,Hensen's node; sp, segmental plate; no, notochord; epi,
epiblast; hyp, hypoblast; pmz, posterior marginal zone; gw, germ
wall; so, somite.
Mesoderm-inducing factors activate Ch-T and Ch-Tbx6L expression

The T gene is expressed in primary mesoderm and plays roles in the normal functioning of primary mesoderm and in embryonic axis formation/elongation, particularly in the posterior embryo (trunk and tail). Two major classes of signalling factors capable of mediating mesoderm induction have been identified and extensively studied in Xenopus: FGF family members induce ventral/posterior mesoderm (Kimelman and Kirschner, 1987; Slack, 1987), and activin, a member of the TGFβ family, induces dorsal/anterior mesoderm and can also induce a secondary axis (Smith et al., 1990; Thomsen et al., 1990). It has been shown in several organisms that the T gene responds to both of these classes of mesoderm-inducing factors (Smith et al., 1991; Schulte-Merker et al., 1992; Isaacs et al., 1994; Kispert et al., 1995b), and in Xenopus, this appears to be an immediate early response not requiring new protein synthesis (Smith et al., 1991). Another intensively studied type of signalling factor, retinoic acid, has no effect on mesoderm formation per se, but modulates axis polarity, promoting caudalization/posteriorization, which can include duplication of caudal structures (Durston et al., 1989; Kessel and Gruss, 1991; Mohanty-Hejmadi et al., 1992; Maden, 1993; Rutledge et al., 1994). Retinoic acid alone (also see Discussion) does not appear to affect expression of Xbra (Tadano et al., 1993), but its effect on T expression has not been evaluated in other organisms. To determine whether chick T-box genes respond to these classes of signalling factors, primary cultures of dispersed blastodermal cells (stage XI-XII) were treated with these agents, which facilitated controlled, quantitative assays for expression.

The mesoderm-inducing factors FGF-4 and activin both induced expression of Ch-T and of Ch-Tbx6L (Fig. 7). Quantitation of results from three independent culture experiments reproducibly indicated that Ch-T expression was induced about 15-fold by FGF (range of 8- to 25-fold) and 10-fold by activin (range of 8- to 14-fold) while Ch-Tbx6L was induced about 2-fold by FGF and 5-fold by activin (range of 3- to 7-fold) under our culture conditions. Ch-Tbx6L was also induced by retinoic acid (about 2-fold), but this was only evident after short incubation times in culture (compare Fig. 7, 24 hours with Fig. 8, 4 hours). When factors were applied in combinations, only additive and no obvious synergistic effects on gene expression were seen (Fig. 7).

To determine whether protein synthesis was required for induction of expression, the effect of these factors was also examined in the presence of cycloheximide. Gnotl expression, which is tremendously increased or stabilized in the presence of cycloheximide (Knezevic et al., 1995), served as a positive control to exclude global toxicity as a result of the cycloheximide treatment. Surprisingly, the induction of both Ch-T by FGF or activin, and of Ch-Tbx6L by FGF, activin, and retinoic acid were all sensitive to protein synthesis inhibition (Fig. 8). However, expression of both genes was clearly stimulated by mesoderm-inducing factors when cycloheximide was not added, even within the short interval of incubation (4 hours) used in this experiment (and in which baseline expression without added factors has not yet fully declined). Ch-T and Ch-Tbx6L can consequently be considered as ‘early’ response genes to these factors.

Ch-TbxT message was undetectable on northern blots of RNA extracted from cultured blastoderm cells under all conditions tested (with or without factor addition; data not shown). Ch-TbxT is not normally detectable in the blastoderm of stage X-XII embryos even by in situ hybridization and hence may not be inducible at this stage. Alternatively, such assays may not be sensitive enough to detect activation of this gene, which, even in later embryos, is expressed as a rare transcript (see Fig. 3A). Using a more sensitive quantitative RT-PCR assay, Ch-TbxT expression was detectable, but only a 2-fold increase in expression after activin treatment and a 2-fold decrease in expression after treatment with retinoic acid in conjunction with activin were observed, and FGF had no effect (data not shown). Given the high sensitivity but lower precision of this assay, the significance of such modest changes in low level expression is difficult to assess, although a mild inhibition by retinoic acid in combination with activin has also been reported for Xbra expression (Tadano et al., 1993).

DISCUSSION

Two new chick T-box genes have been isolated which are expressed in complementary mesodermal subdomains. Each of
these subdomains include part of the zone of $T$ expression during gastrulation. These T-box genes may either compensate, supplement, or modify $T$ function in mesoderm and axis formation. The expression of $Ch-T$ during gastrulation and its response to activin have been previously described (Kispert et al., 1995b). The present report confirms and extends this prior study. $Ch-T$ is already expressed in the posterior blastoderm prior to the onset of gastrulation, and its expression can be rapidly induced by members of both major classes of mesoderm-inducing factors, activin and FGF. Although this induction occurs rapidly, it is nevertheless sensitive to protein synthesis inhibition and is consequently not an immediate early response. This result is somewhat at odds with previously published work indicating that the *Xenopus T* gene, *Xbra*, is induced by these factors even in the absence of protein synthesis (Smith et al., 1991). The discrepancy may be explained as a possible species or cell type difference in regulation, or as a consequence of the use of more stringent conditions of protein synthesis inhibition in the present experiments, in which the reversible protein synthesis inhibitor, cycloheximide, was present throughout the entire incubation period. In fact, Tadano et al. (1993) have also reported sensitivity of *Xbra* induction by activin to cycloheximide using somewhat different conditions. $Ch-TbxT$ is expressed predominantly in the dorsal axial mesodermal subdomain of $T$ expression, with early expression extending anterior to the $T$ domain, to include the prechordal plate. $Ch-TbxT$ expression was only modestly induced by activin under the conditions used in this report. Our results could indicate that $Ch-TbxT$ is not very highly responsive to mesoderm-inducing factors, or more likely, that some other factor critical for the regulated expression of this gene is lacking in the early blastoderm cells in culture, since $Ch-TbxT$ expression is also undetectable in embryos at the same stage by in situ hybridization. This latter explanation seems plausible considering that even $Ch-T$ stimulation is not an immediate response to mesoderm-inducing signals in this system. $Ch-Tbx6L$ expression occurs in the nascent mesodermal subdomain of $T$ expression within the primitive streak. However, $Ch-Tbx6L$ expression during streak regression extends more laterally and includes the segmental plate mesoderm. Like $Ch-T$, $Ch-Tbx6L$ expression is rapidly induced in blastodermal cells exposed to either activin or FGF, consistent with an early role in mesoderm formation, but this stimulation is sensitive to the inhibition of protein synthesis. $Ch-Tbx6L$ is also induced modestly by retinoic acid, a caudalizing factor for mesoderm, suggesting that $Ch-Tbx6L$ expression may be a significant feature for posterior mesoderm and axis formation.

### Blastodermal expression of $Ch-T$ and $Ch-Tbx6L$ in relation to the primitive streak forming region of the avian embryo

The position of the axis in the chick embryo is already determined prior to its actual formation (reviewed by Khaner, 1993). Cells that will form the early primitive streak are initially recruited from a relatively narrow region of the posterior epiblast (Stern, 1990; Eyal-Giladi et al., 1992). Their recruitment appears to be regulated by the epiblast of the posterior marginal zone and the forming hypoblast (Stern, 1990; reviewed by Khaner, 1993).

$Ch-T$ and $Ch-Tbx6L$ are both continuously expressed in the epiblast from pregastrulation through gastrulational stages ($Ch-T$ beginning at stage XIII and $Ch-Tbx6L$ already present at stage X). The early pregastrulation expression pattern of both of these genes is of interest with respect to commitment of cells to a primitive streak fate. The dynamic expression of these genes correlates well with proposed cell movements during early primitive streak formation (Stern, 1990; Eyal-Giladi et al., 1992; Hatada and Stern, 1994). Expression of both genes is initially restricted to a narrow circumferential arc in the posterior half of the area pellucida epiblast (where presumptive primitive streak progenitors are located) and is present in the marginal zone epiblast as well, a region also reported to contribute to the primitive streak (Stern, 1990; Eyal-Giladi et al., 1992). Since epiblast cells from the entire blastoderm appear to be competent to form primitive streak (Spratt and Haas, 1960; Eyal-Giladi and Spratt, 1965), the restricted expression of $Ch-T$ and $Ch-Tbx6L$ within the posterior blastoderm suggests that both of these genes may mark more committed primitive streak progenitors. The lack of expression of these genes in anterior epiblast cells reported to contain primitive streak progenitors that will join the streak later (Hatada and Stern, 1994, and references therein), may indicate either that these anteriorly located ($Ch-T$ and $Ch-Tbx6L$ negative) epiblast cells in fact do not participate in formation of the primitive streak (as suggested by Kispert et al., 1995b), or that they only begin to express these genes once they arrive at the streak.

### Could a TbxT gene complement or extend T function in the anterior axis?

Mouse homozygous $T$ embryos initiate gastrulation on time and appear to develop normally to the 7-somite stage (Chesley, 1935). Subsequently, anterior structures cephalic to the forelimb bud appear to develop normally. These observations indicate that a functional $T$ gene is not required for initial formation and function of the head process and anterior notochord, or that in early development another, related gene product can partly compensate for the loss of $T$ function anteriorly. At the amino acid level, $Ch-TbxT$ is 85% identical with $Ch-T$ within the T-box region, and about 62% identical overall. It is initially (and transiently) expressed in the early primitive streak (stage 2-3) and also transiently in early prechordal plate (stage 5-6), while later expression is mainly restricted to Hensen’s node and notochord. The observations that $Ch-TbxT$ transcripts become localized to the notochord and are rapidly downregulated after the 7-10 somite stage (stage 9-10) make this gene a potential candidate for complementing or extending $T$ function anteriorly, although primitive streak defects beyond stage 4 (in the chick) would not be expected to be rescued by $Ch-TbxT$. Alternatively, the early-restricted, more transient expression of $Ch-TbxT$ in axial mesoderm may reflect a function for this gene in the initial formation of chordamesoderm, as opposed to the subsequent differentiation and maintenance of the notochord, which is dependent on $T$ function.

The ascidian T-box gene (*As-T*) related to mouse *T* (Yasuo and Satoh, 1993, 1994) is restricted very early in development to blastomeres with an exclusively notochordal fate. The exclusive notochordal localization of $Ch-TbxT$ transcripts raises the possibility that $Ch-TbxT$ is a more archetypal ‘$T$ gene’ than *Brachyury*. However, sequence comparison indicates that $Ch-TbxT$ is neither more nor less closely related to *As-T* (or to lower vertebrate *T* genes) than is the *Ch-T* gene.
It is intriguing that the overall late expression pattern of \textit{Ch-TbxT} is remarkably similar to that of another type of development-
mental regulator expressed primarily in the chordomesoderm. At stage 4 and later, the chick Not-family homeobox genes (\textit{Gnot1} and \textit{Gnot2}), like \textit{Ch-TbxT}, are expressed in Hensen’s node, in adjacent posteriorlateral ectodermal arcs, in notochord, and transiently in the anterior neural folds near the neuropore (Knezevic et al., 1995; Ranson et al., 1995; Stein and Kessel, 1995; Ljubic and Mackem, unpublished observations). Indeed, the zebrafish homologue of \textit{Gnot2}, \textit{floating head}, is essential for specification of the notochord (Talbot et al., 1995). Perhaps this group of genes, that all share this distinctive expression pattern, play some related role in notochord specification and/or function and potentially interact with each other.

\textbf{\textit{Ch-Tbx6L} as a potential marker of somitic precursors}

Expression of \textit{Ch-Tbx6L} correlates with the fate map of presumptive somitic cells. Hatada et al. (1994) showed that these cells are initially located in the posteriorlateral aspect of the pre-
gastrulational epiblast of the area pellucida, which during sub-
sequent stages become concentrated in and around the forming primitive streak. \textit{Ch-Tbx6L} is also expressed in the epiblast of the marginal zone, suggesting that this region also contributes to the somitic lineage. Until stage 5, somitic precursors are located within the streak and in the ectoderm surrounding the anterior end of the streak (Wolff, 1936; Nicolet, 1970; Gallera, 1975). Subsequently these precursors enter the streak proper with no further ectodermal contribution (Nicolet, 1967). These observations correlate strikingly with the complete loss of \textit{Ch-Tbx6L} expression in the ectoderm surrounding the streak and the onset of nascent mesodermal expression that occurs at stage 5-6. However, \textit{Ch-Tbx6L}-expressing cells are also present in the posterior half of the primitive streak region which normally doesn’t contribute cells to the somites (Psychoyos and Stern, 1996), suggesting that \textit{Ch-Tbx6L} expression domains encompass certain other progenitors as well. \textit{Ch-Tbx6L} trans-
scripts are rapidly downregulated upon somite formation in the segmental plate mesoderm, and only weak expression can occasionally be observed in the last, most recently formed somite. \textit{Ch-Tbx6L} expression was never detected in the paraxial mesoderm of the head, which does not become segmented (Meier, 1979). Relatively few genes have been thus far identified that are specifically and selectively expressed
according to presomitic fate in this fashion; among these are the \textit{Notch 1} receptor and its ligand \textit{Delta} (Conlon et al., 1995b; Henrique et al., 1995), as well as one of the Eph family tyrosine kinase receptors and its ligand (Nieto et al., 1992; Bergemann et al., 1995). \textit{Notch 1} has in fact been shown to play a role in somitogenesis (Conlon et al., 1995b).

\textbf{Conclusions}

Two novel chick T-box genes that, like \textit{Ch-T}, are expressed during gastrulation have been isolated. \textit{Ch-T} and \textit{Ch-Tbx6L} are both activated by mesoderm-inducing factors and are expressed in the posterior epiblast and marginal zone in the presumptive primitive streak forming region before the onset of gastrula-
tion. \textit{Ch-Tbx6L} is already expressed in stage X blastoderm, prior to \textit{Ch-T}, and may play some early role in primitive streak formation. \textit{Ch-TbxT} is transiently expressed in early primitive streak and prechordal plate and then becomes restricted solely
to the axial mesodermal lineage. Both the expression domain and the timing of expression of this gene (which is downregu-
lated after the 10-somite stage) raise the possibility that a \textit{Ch-TbxT} equivalent in the mouse could partly complement \textit{T} function in the anterior axis, or play a role in the initiation of notochord formation. The progressive restriction of \textit{Ch-Tbx6L} to the segmental plate mesoderm and the extinction of its expression coincident with segmentation also suggest a role for this gene in somitogenesis.

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