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The protein product of the zebrafish homologue of the mouse *T* gene is expressed in nuclei of the germ ring and the notochord of the early embryo

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Summary

Embryos mutant for the *T* gene, in mice, make insufficient mesoderm and fail to develop a notochord. We report the cloning and sequencing of the *T* gene in the zebrafish (*Brachydanio rerio*) and show the nuclear localization of the protein product. Both RNA and protein are found in cells of the germ ring, including enveloping layer cells, prior to and during gastrulation of zebrafish embryos. Nuclei of the yolk syncytial layer do not express *Zf-T*. High levels of expression are maintained throughout early development in the notochord, while in paraxial mesoderm cells the gene is turned off during gastrulation. Exposure of animal cap cells to activinA induces *Zf-T* expression, as does transplantation into the germ ring.

Key words: *Brachydanio rerio*, zebrafish, mesoderm, embryogenesis, notochord, *Brachyury*, *T*.

Introduction

Gastrulation is the complex process that leads to the formation of distinct germ layers, endoderm, ectoderm and mesoderm. In vertebrates, special attention has been given to the formation of the mesoderm because it is thought to arise as the result of the earliest induction known in the embryo. Furthermore, dorsal mesoderm itself can act as an organizer (Spemann, 1934) and possesses inducing capabilities (Yamada et al., 1991) for the overlying ectoderm.

Mesoderm induction is best understood in amphibian embryos where experimental manipulations are easy to perform. In amphibia, animal cap cells (prospective ectoderm) can be induced to become mesoderm by juxtaposing them with vegetal blastomeres (prospective endoderm). This shows that an inducing signal is provided by vegetal cells in embryos of the blastula stage (Nieuwkoop, 1969). Its molecular nature is not known, but a whole range of peptide growth factors have been shown to have the capacity to induce mesoderm in vitro (for review see Green and Smith, 1991). The peptide growth factors so far studied belong either to the FGF or TGFβ families (Kimelman and Kirschner, 1987; Rosa et al., 1988; Smith et al., 1990; Thomsen et al., 1990). A number of genes have been identified whose expression can be induced by incubating animal pole cells (animal caps) in medium containing peptide growth factor (Rosa, 1989; Ruiz i Altaba and Melton, 1990; Cho and DeRobertis, 1990; Cho et al., 1992). One of these target genes is the frog homologue of the mouse *T* gene, *Xbra* (Smith et al., 1991).

The correlation between mesoderm induction and expression of *Xbra* is remarkable in respect to the observation that the *T* or *Brachyury* gene is crucial for mesoderm development in the mouse (Chesley, 1935). Embryos homozygous for the mutation *T* fail to produce sufficient mesoderm and lack all posterior structures. Most strikingly, they lack the entire notochord (Chesley, 1935; Gluecksohn-Schoenheimer, 1944; Yanagisawa et al., 1981). Mutant embryos die during midneurulation, probably due to the absence of an allantois.

The murine *T* gene has been cloned (Herrmann et al., 1990) and its expression pattern has been analyzed by in situ hybridization (Wilkinson et al., 1990). The gene is expressed from early gastrulation (day 7 p.c.) throughout mesoderm formation in nascent and migrating mesoderm, and in the notochord at least until day 17.5 of gestation (Wilkinson et al., 1990; Herrmann, 1991). At day 9.5 p.c., expression declines in the primitive streak region, but transcripts can be detected in the notochord through all stages of embryonic development (Wilkinson et al., 1990). The regulation of the *T* gene and the biochemical function of its product are not known. Inductive events that might regulate *T* expression are difficult to study during mammalian embryogenesis since embryonic material is not easily acces-

sible in large amounts. In amphibian embryos, however, it has recently been shown that the transcription of the *T* gene can be induced by both FGF and activin A (Smith et al., 1991).

The zebrafish (*Brachydanio rerio*) combines the advantages of the amphibian and the mammalian system. It already has proven to be a useful experimental system for studying a wide range of developmental processes. As in mice, genetic analysis in zebrafish is possible (Streisinger et al., 1981, 1986; Kimmel, 1989). Screens for mutations affecting early embryonic development have led to the isolation of a number of valuable mutants (Grunwald et al., 1988; Kimmel et al., 1989; Felsenfeld et al., 1991; Hatta et al., 1991). As in amphibia, experimental manipulations in the embryo can be performed readily (Ho and Kane, 1990; Hatta et al., 1991). The transparency of the embryos not only facilitates in vivo observation, but also allows cell lineage analysis by dye tracing (Kimmel and Warga, 1987).

We have analyzed the distribution of both the RNA and the protein product of the zebrafish homologue of the *T* gene (*Zf-T*). Our results demonstrate that the protein encoded by *Zf-T* is a nuclear protein with putative helical domains in its N-terminal half. By using antibodies against the *Zf-T* protein, we have been able to examine the expression pattern of *Zf-T* in much more detail than previously possible. In this report, we correlate the expression pattern with morphogenetic movements during gastrulation. Prior to and during gastrulation, both mRNA and protein are found in cells in the region of the germ ring, where cells involute to yield endoderm and mesoderm. *Zf-T* expression is transient in cells of the germ ring, but stable in cells of the presumptive notochord cells during early embryogenesis. By using the animal cap assay originally developed for amphibia (Nieuwkoop, 1969), we have found that, as in the case of *Xbra* (Smith et al., 1991), *Zf-T* gene expression is induced by activin A. Our results are consistent with the hypothesis that the molecular mechanisms that regulate mesoderm formation during gastrulation in such divergent vertebrates as fish and mammals, are very similar and that, at least in fish and amphibia, the same basic inductive events lead to the formation of mesoderm.

Materials and methods

Maintenance of fish

Fish were kept either in 20 or 40 liter tanks at a density of up to 2 adults per liter. The water used was charcoal-filtered tap water heated to 28.5°C. Embryos were raised in 10% MBS (modified Barth's solution; Winklbauer, 1988) prior to hatching and after hatching in supplemented selmarin (100 mg ocean salt, 400 mg NaCl/l).

Embryos were usually collected from single pair matings of wild-type fish. For collection of large numbers of embryos (more than 500), eggs were collected from 30-80 fish in a 40 liter tank. Embryos were normally collected at 20 minutes intervals, transferred to 10% MBS, and staged according to 'The Zebrafish Book' (Westerfield, 1989).

Isolation of genomic and cDNA clones

Genomic DNA was prepared from a wild-type male following standard procedures (Berger and Kimmel, 1987). The DNA was used to construct a genomic library in EMBL3 phage (Stratagene) following standard procedures (Berger and Kimmel, 1987) and the manufacturers instructions (Stratagene). After packaging (Gigapack, Stratagene), 6×10^5 independent phage clones were screened using a ^{32}P-labelled, random-primed probe derived from a mouse cDNA clone (pme75; Herrmann et al., 1990). Four independent phage clones were isolated, mapped with restriction enzymes and partially sequenced. A fragment containing sequences corresponding to exons 2 and 3 of the mouse *T* gene (B. G. Herrmann, unpublished) was used to screen a cDNA library of 0-48 hour old fish embryos (kindly provided by A. Fjose). 11 clones were isolated and restriction mapped. The longest insert was cloned into the *Eco*RI site of pBluescript II KS to yield plasmid pBSCT-ZFc1. The clone was sequenced via the chain termination method by cloning restriction fragments into vectors M13 mp18/mp19 (Yanisch-Perron et al., 1985) and using either primers specific to the vector or to insert sequences. Both strands were sequenced at least twice. Sequence analysis was carried out using the PCGENE software package (GENOFIT, Switzerland).

Preparation of RNA

Total RNA was prepared by the hot phenol method (Brown and Kafatos, 1988). 300 embryos or one adult were ground up in liquid nitrogen and transferred to 50 ml Falcon tubes. After the liquid nitrogen had boiled off, 8 ml of a boiling mixture (1:1) of unbuffered phenol (pH 4-5) and 2×NETS (0.2 M NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1% SDS) were added. The tube was vortexed immediately for 1 minute. After cooling, phases were separated by centrifugation at 5000 revs/minute for 15 minutes. The organic phase was reextracted with one volume 2×NETS. The combined aqueous phases were extracted once more with phenol, once with phenol:chloroform:isoamylalcohol (25:24:1, v:v:v) and once with chloroform:isoamylalcohol (24:1, v:v), before they were finally precipitated with ethanol.

Northern blot analysis

Northern blot analysis was performed following standard procedures (Sambrook et al., 1989). 6 μg of total RNA were loaded onto 1% agarose (BRL) formaldehyde gels and run under denaturing conditions. Following transfer onto HybondN membranes (Amersham), filters were exposed to UV light for 1 minute and stained with methylene blue (Sambrook et al., 1989) to visualize rRNA. Hybridization to ^{32}P-labelled, random primed probes was carried out at 45°C overnight in the presence of 50% formamide. Washes were performed at 68°C in 0.3×SSPE/0.1%SDS. The gel-purified probes used for hybridization were either the *Pst*I-*Eco*RI fragment (nucleotides 1381-2238) or the complete cDNA (*Eco*RI fragment of pBSCT-ZFc1). Kodak X-ray films were used for detection.

Whole-mount in situ hybridizations

After an initial overnight fixation with PBS-based 4% paraformaldehyde at 4°C and subsequent manual dechorionation, embryos were transferred to vials filled with 100% methanol. The methanol was exchanged after 10 minutes and the vials were stored at −20°C until further treatment. All further steps were performed at room temperature unless stated otherwise.The embryos were incubated for 5 minutes each in 50% MeOH in PBST (PBS/0.1% Tween-20), 30% MeOH in PBST and twice in PBST. A second fixation with 4% paraformaldehyde in PBS (20 minutes) was followed by two more washes with PBST (as above). Embryos were treated with proteinase K (10 μg/ml) for 8-12 minutes. The actual time of digestion was determined empirically; it depends on the batch of enzyme used and on the embryonic stage. After a brief rinse with PBST, the embryos were washed for 5

minutes in PBST and fixed for another 20 minutes with 4% paraformaldehyde in PBS. After two washes with PBST (as above), the PBST was replaced by distilled water. 2.5 ml acetic anhydride and 1.0 ml triethanolamine (pH 7.0) were mixed vigorously and added immediately to the embryos after removal of the distilled water. This treatment helped reduce endogenous phosphatase activity and was therefore only necessary in cases where background was a problem. After a 10 minutes incubation, the embryos were washed twice in PBST (5 minutes each) and transferred to small Eppendorf tubes containing HYB^- (50% formamide, 5×SSC, 0.1% Tween-20).

Embryos were incubated at 55°C for 5 minutes. The HYB^- solution was replaced by HYB^+ (HYB^- with 5 mg/ml torula RNA and 50 mg/ml heparin added) and the embryos were prehybridized in HYB^+ at 55°C for one hour up to three days.

As much of the HYB^+ solution as possible was removed without allowing the embryos come in contact with air. An equal volume of fresh HYB^+ containing 20 to 100 ng of digoxigenin-labelled RNA probe (heated to 68°C for 5 minutes prior to addition) was added to the tubes. Probes were synthesized according to the manufacturers instructions (Boehringer) and subsequently hydrolized to an average length of 150-300 nucleotides in 40 mM $NaHCO_3$/60 mM Na_2CO_3, pH 10.2 at 60°C (Cox et al., 1984). After an overnight incubation at 55°C the probe was removed and the embryos were washed for 20 minutes in 50% formamide/2×SSC at 55°C. Following three washes with 2×SSC at 37°C (10 minutes each) and one wash with PBST (5 minutes at 37°C), unhybridized RNA was removed by digestion with RNAase A (20 mg/ml PBST) and RNAase T1 (100U/ml PBST) for 30 minutes at 37°C.

After further washes with 2×SSC (10 minutes, 37°C), 2×SSCT (SSC/0.1% Tween-20) for 15 minutes at 55°C, 0.2×SSCT (twice at 55°C, 15 minutes each) and PBST (5 minutes at RT), embryos were transfered to microtiter dishes.

Embryos were blocked for 1 hour in NCS-PBST (PBST plus 5% newborn calf serum). Alkaline-phosphatase coupled anti-digoxigenin Fab-fragments (1:8000) were added in fresh NCS-PBST. After 4 hours of incubation with gentle agitation, embryos were washed four times with NCS-PBST (25 minutes each) and three times 5 minutes each with AP-reaction buffer (100 mM Tris pH 9.2, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20, 1 mM fresh levamisol). Detection was performed in AP-reaction buffer with 4.5 µl NBT/ml and 3.5 µl X-phosphate/ml added (as described in the Boehringer instruction manual). The reaction was monitored under a binocular and stopped when the signal was satisfactory. The specimen were dehydrated in methanol and mounted in 2:1 benzyl benzoate: benzyl alcohol (Hemmati-Brivanlou et al., 1990).

Production and purification of antibodies

The pET3a-vector (Studier and Moffat, 1986) was used to express full-length *Zf-T* protein. Using the appropriate primers for oligonucleotide site-directed mutagenesis, an *Nde*I site was introduced at the initiator ATG of the opening reading frame. The *Nde*I-*Bam*HI fragment containing the open reading frame plus 3′ untranslated sequences was cloned into the pET3a-vector yielding plasmid pET-ZfT and transformed into *E.coli* BL 21 DE3 cells. Colonies containing the plasmid were picked and the protein expressed as described by others (Roth et al., 1989). Cells carrying the plasmid without an insert and untransformed BL 21 DE3 cells served as negative control for PAGE and western blot analysis.

After Coomassie-blue staining, a prominent band of the expected relative molecular mass of roughly 45×10^3 was excised from a preparative 10% polyacrylamide gel and homogenized by several passages through a fine mesh. The homogenate was mixed in a 1:1 ratio with complete Freud's adjuvant (Gibco) and injected into a rabbit. Four weeks later, the rabbit was boosted by injection with a 1:2 mixture of homogenate and incomplete Freud's adjuvant (Gibco). One week later, an initial bleed was taken. Thereafter, the rabbit was bled on a weekly schedule. Affinity purification of the antiserum was performed as described by others (Driever and Nüsslein-Volhard, 1988) on CNBr-Sepharose (Pharmacia) with minor modifications.

Antibody staining of embryos

All steps were carried out at room temperature. Embryos were fixed for 5-6 hours with 4% paraformaldehyde in PBS. Following two washes with PBS and one wash with distilled water (5 minutes each), the chorions were removed manually using watchmaker forceps. For embryos older than the tailbud stage, at this point a 7 minutes acetone treatment at −20°C was included. After a 30 minutes incubation in blocking solution (NCS-PBTD: 10% heat-inactivated new-born calf serum in 0.05% Tween-20, 1% DMSO, PBS), the embryos were incubated 5 hours at room temperature (or longer in the coldroom) in a 10^4-fold dilution of antiserum in NCS-PBTD. Following four washes with NCS-PBTD (25 minutes each), detection of the antigen was carried out using the Avidin/Biotin ABC system (Vector Laboratories) according to standard procedures (Roth et al., 1989). Dehydration was carried out in two changes of MeOH. Dehydrated embryos were mounted in a 2:1 mixture of benzylbenzoat:benzylalcohol. Photographs were taken using Kodak Ektachrome (200 ASA) films.

Immunoprecipitation

Plasmid pBSCT-ZFc1 was linearized by digestion with either *Bam*HI or *Xho*I. Following the protocol by Driever et al. (1990), sense and antisense transcripts were obtained using either T3 polymerase (*Bam*HI linearized template) or T7 polymerase (*Xho*I linearized template), respectively. The mRNAs were translated in vitro in the presence of [^{35}S]methionine using wheat germ extract (Amersham) according to the supplier's protocol.

5 µg of protein A-Sepharose (Pharmacia), equilibrated with washing buffer (300 mM KCl, 0.5% Tween-20, PBS), were incubated overnight at 4°C with 5 µl of either antiserum or preimmune-serum. After three washes with an excess volume of washing buffer, the pellet was resuspended in 200 µl washing buffer and incubated with 10 µl (one third) of the in vitro translation reaction mixture for 3 hours at 4°C. After three more washes as described above, the pellet was resuspended in 20 µl of Laemmli buffer and heated to 100°C for 10 minutes. After cooling, the suspension was centrifuged and 10 µl of the supernatant were loaded onto a gel. After the run, the gel was dried using a vacuum drier and exposed to Kodak film for 24 hours.

RT-PCR

6 µg of total RNA derived from blastula stages (2.5-3.0 hours, 3.0-3.4 hours, 3.4-3.7 hours, 3.7-4.0 hours, 4.2 hours) were mixed with 5 µl 5× M-MLV RT buffer (BRL), 1 µl 0.1M DTT, 1 µl BSA (1 mg/ml), 2.5 mM dNTPs, 36 units RNAase Guard (Pharmacia), 200 ng oligonucleotide primer 1 and 200 units M-MLV reverse transcriptase (BRL). After incubation at 40°C for one hour, 5 µl of this mixture were amplified in a total volume of 100 µl containing 2.5 mM dNTPs, 10 µl 10× PCR buffer (Perkin Elmer Cetus), 10 µl BSA (1 mg/ml), 1 µg of each oligonucleotide primer, and 5 units Taq polymerase (Pharmacia). Amplification was performed for 30 cycles of 1 minute 94°C, 1 minute 62°C and 2.5 minutes 72°C. The following primers were used:
p1: 5′-CCATGTAGTTATTGGTGGTAGTG-3′
p2: 5′-TCCTCGATGCCAAAGAGAGAAGTG-3′.

Western blot analysis

All gels, unless stated otherwise, were 12.5% acrylamide gels run

at constant current (30 mA) using the buffer system described by Laemmli (1970). The gel casting and blotting system used was the LKB midget unit (2050/2051). Blotting was carried out for one hour at constant current (400 mA) using nitrocellulose membranes (Schleicher & Schuell) essentially as described by Towbin et al. (1979).

Membranes were incubated in blocking buffer (BB: 5% dried milk, 0.5% Tween-20, PBS) for at least 30 minutes and thereafter incubated for 2 hours in BB containing a 10^6-fold dilution of antiserum. Following three washes in BB (20 minutes each), signal detection was carried out using peroxidase-coupled goat anti-rabbit secondary antibodies and the ECL system (Amersham) according to the suppliers' instructions.

Animal cap assays

Using an eyelash as a dissecting tool, the animal-pole most quarters (animal caps) of 3.5 hour old embryos were cut off and transferred immediately to agarose-coated dishes kept at 28.5°C, containing either unconditioned medium, or medium conditioned for 2-3 days by XTC-cells (Smith, 1987). The medium used was 85% RPMI (Gibco) supplemented with 10% FCS (Gibco).

The animal caps were incubated for 2 hours, and were then processed for immunostaining essentially as described above except that incubation periods with both serum and secondary antibody were reduced to 90 minutes.

Cell transplantation

Transplantations were performed using methods similar to those described in Ho and Kane (1990). Briefly, donor embryos were labeled at the 2- to 4-cell stage by the intracellular injection into the yolk cell of a mixture of rhodamin-dextran and fixable biotin-dextran (RDA and BFD in a 3:1 ratio, both dyes from Molecular Probes; both dyes 5% solution in 0.2 M KCl). Using a tooled microcapillary glass pipette, cells were taken directly from the animal pole of a labeled donor embryo at 50% epiboly and transplanted into either the animal pole or the marginal zone of unlabeled host embryos at 50% epiboly. Host embryos were allowed to develop further for 1.5 to 2.0 hours and then fixed in 4% paraformaldehyde in PBS for 4 hours at room temperature. Immunohistochemical staining was performed using the PAP method of Sternberger et al. (1970) for the Zf-T antigen. The biotin-dextran in the cytoplasm of the transplanted cells was visualized using standard procedures for a Vectastain goat anti-rabbit IgG alkaline phospatase kit and using the Vectastain substrate kit for 'Vecta Red'.

Results

Structure of the Zf-T *cDNA*

Probing with a mouse *T* cDNA clone, pme75, we isolated two clones from a zebrafish genomic library, and subsequently eleven clones from a 0-48 hour zebrafish cDNA library constructed in λgt10 (Njolstad et al., 1988). The longest clone was sequenced and found to be 2238 nucleotides in length. There is a single long open reading frame of 1269 nucleotides which extends from nucleotide 84 to nucleotide 1353. The start codon at position 84 is preceded by two in frame stop codons at nucleotides 39 and 42. At position 2186 there is a polyadenylation site (Fig. 1).

Comparison of the deduced amino acid sequences of the fish, the frog and the mouse *T* genes reveals extensive conservation between the three proteins (Fig. 2A). The overall degree of amino acid identity is 69.7% (fish/mouse). However, at the N terminus the amino acid identity is 84.5% (fish/mouse; aminoacids 9-222). As in the other two species, the *Zf-T* protein is rich in serine (68/423 aminoacids, 16.1%). The calculated relative mass of the *Zf-T* protein is 45,833 and its isoelectric point is 6.77.

Using the methods of Garnier et al. (1978) and Novotny and Auffray (1984), we performed secondary structure analyses. The predictions suggest a number of conserved helical conformations for the putative protein products of both fish and mouse *T* gene. Helical conformations are, with one exception, restricted to the highly conserved N-terminal half of the *Zf-T* protein (Fig. 2B). Interestingly, the secondary structure predictions for the regions (amino acids 14-24 and 30-59) which are less well conserved (81% and 65%, respectively) than the overall conservation in the N-terminal half (84.5%) are identical for the two proteins. This strict conservation of secondary structure even in regions of moderately conserved primary structure is suggestive of strong structural constraints necessary for the proper function of the respective proteins.

Zf-T *RNA is expressed before gastrulation*

Northern blot analysis reveals a single transcript of about 2.5 kb. On northern blots (Fig. 3), the message is detected at the late blastula/doming stage (4.2 hours). A very weak signal is detectable prior to the beginning of epiboly (4 hours) after prolonged exposure of the blot, but not earlier (data not shown). Maximum levels of RNA are expressed during gastrulation (5.2 to 9.5 hours). The amount of mRNA declines slowly after gastrulation, but mRNA is still detectable in 48 hours embryos. We never observed expression in adult fish (Fig. 3).

To confirm the result of the northern analysis with respect to the onset of transcription, we assayed RNA preparations of various stages by cDNA synthesis using M-MLV reverse transcriptase and subsequent PCR reactions using nested primers. The result of two independent experiments showed that *Zf-T* RNA is transcribed as early as 3.4 hours, but not earlier (0 to 3 hours; data not shown).

The Zf-T *transcript becomes confined to the notochord*

A protocol originally devised to perform whole-mount in situ hybridizations in mouse embryos (Herrmann, 1991) was adapted for use in zebrafish embryos. Digoxigenin-labelled sense and antisense RNA probes of an average length of 150-300 nucleotides were hybridized to embryos of various stages. RNA was first detected at the doming stage (4.2 hours), when the yolk has just started to bulge upwards towards the animal pole and the blastomeres show their first epibolic movements towards the vegetal pole (Warga and Kimmel, 1990). During doming and early gastrulation, the cells expressing *Zf-T* RNA (Fig. 4E) are localized in a restricted ring-like area, the marginal zone of the blastoderm, present at the borderline between the blasto-

Fig. 1. Nucleotide sequence of *Zf-T*. The DNA sequence of the coding strand of cDNA pBSCT-ZFc1 is shown. Using the single letter amino acid code, the predicted amino acid sequence of the long open reading frame is shown. A polyadenylation signal at the 3′ end of the transcript is underlined.

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   1
GAATTCCCGCTGTCAAAGCAACAGTATCCAACGGGATTTAGTAGGATCGTCGGACTTATCTCAAGCTTTATTTGATCGGAAAT

  84
ATGTCTGCCTCAAGTCCCGACCAGCGCCTGGATCATCTCCTTAGCGCCGTGGAGAGCGAATTTCAGAAGGGCAGCGAGAAAGGGGACGCG
   1  M  S  A  S  S  P  D  Q  R  L  D  H  L  L  S  A  V  E  S  E  F  Q  K  G  S  E  K  G  D
A

 174
TCCGAGCGGGATATTAAACTTTCGCTTGAAGACGCGGAGTTGTGGACCAAATTTAAAGAGCTCACCAATGAAATGATTGTCACCAAGACT
  31  S  E  R  D  I  K  L  S  L  E  D  A  E  L  W  T  K  F  K  E  L  T  N  E  M  I  V  T  K
T

 264
GGGAGACGAATGTTTCCCGTGCTCAGAGCCAGTGTCACCGGTCTCGACCCTAATGCAATGTACTCGGTCCTGCTGGATTTTGTGGCGGCC
  61  G  R  R  M  F  P  V  L  R  A  S  V  T  G  L  D  P  N  A  M  Y  S  V  L  L  D  F  V  A
A

 354
GATAATAATCGGTGGAAATACGTGAACGGTGAATGGGTGCCCGGTGGGAAACCCGAACCCCAAAGCCCGAGCTGCGTCTACATCCACCCG
  91  D  N  N  R  W  K  Y  V  N  G  E  W  V  P  G  G  K  P  E  P  Q  S  P  S  C  V  Y  I  H
P

 444
GACTCACCCAACTTCGGCGCGCACTGGATGAAAGCACCCGTATCTTTCAGCAAAGTCAAACTCTCCAATAAACTCAACGGAGGAGGACAG
 121  D  S  P  N  F  G  A  H  W  M  K  A  P  V  S  F  S  K  V  K  L  S  N  K  L  N  G  G  G
Q

 534
ATTATGTTAAACTCATTGCACAAATACGAACCCAGGATACACATCGTGAAAGTCGGTGGGATTCAGAAAATGATCAGCAGTCAGTCTTTT
 151  I  M  L  N  S  L  H  K  Y  E  P  R  I  H  I  V  K  V  G  G  I  Q  K  M  I  S  S  Q  S
F

 624
CCTGAGACACAGTTTATTGCAGTCACAGCATATCAGAATGAAGAGATTACCGCTCTGAAAATCAAACACAATCCTTTTGCCAAAGCTTTC
 181  P  E  T  Q  F  I  A  V  T  A  Y  Q  N  E  E  I  T  A  L  K  I  K  H  N  P  F  A  K  A
F

 714
CTCGATGCCAAAGAGAGAAGTGACCACAAGGAAGTCCCAGACCACAGCACTGACAACCAGCAATCTGGATATTCACAACTCGGTGGCTGG
 211  L  D  A  K  E  R  S  D  H  K  E  V  P  D  H  S  T  D  N  Q  Q  S  G  Y  S  Q  L  G  G
W

 804
TTCCTGCCCAGTAACGGCCCCATGGGCCCCAGCAGCAGCCCTCCTCAGTTCAATGGGGCCCCTGTTCACTCCTCGGGTTCGTACTGTGAG
 241  F  L  P  S  N  G  P  M  G  P  S  S  S  P  P  Q  F  N  G  A  P  V  H  S  S  G  S  Y  C
E

 894
AGATACTCCAGCTTGAGGAACCACAGAGCTGCTCCATATCCCAGCCATTACTCCCACCGCAGCACTACCACCAATAACTACATGGACAAC
 271  R  Y  S  S  L  R  N  H  R  A  A  P  Y  P  S  H  Y  S  H  R  S  T  T  T  N  N  Y  M  D
N

 984
TCTTCCGGAAGTCTTGCGTCTCATGACAGCTGGTCAGCCCTGCAGATCCCCAACTCCAGCGGGATGGGAACCCTGGCCCACACCACAAAC
 301  S  S  G  S  L  A  S  H  D  S  W  S  A  L  Q  I  P  N  S  S  G  M  G  T  L  A  H  T  T
N
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A

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  1  MSSPGTESAG KSLQYRVDHL LSAVESELQA GSEKGDPTER ELRVGLEESE LWLRFKELTN  60
  1    MSATESCA KNVQYRVDHL LSAVENELQA GSEKGDPTEK ELKVSLEERD LWTRFKELTN  58
  1        MS-A SSPDQRLDHL LSAVESEFQK GSEKGDASER DIKLSLEDAE LWTKFKELTN  53

 61  EMIVTKNGRR MFPVLKVNVS GLDPNAMYSF LLDFVTADNH RWKYVNGEWV PGGKPEPQAP 120
 59  EMIVTKNGRR MFPVLKVSMS GLDPNAMYTV LLDFVAADNH RWKYVNGEWV PGGKPEPQAP 118
 54  EMIVTKTGRR MFPVLRASVT GLDPNAMYSV LLDFVAADNN RWKYVNGEWV PGGKPEPQSP 113

121  SCVYIHPDSP NFGAHWMKAP VSFSKVKLTN KLNGGGQIML NSLHKYEPRI HIVRVGGPQR 180
119  SCVYIHPDSP NFGAHWMKDP VSFSKVKLTN KMNGGGQIML NSLHKYEPRI HIVRVGGTQR 178
114  SCVYIHPDSP NFGAHWMKAP VSFSKVKLSN KLNGGGQIML NSLHKYEPRI HIVKVGGIQK 173

181  MITSHCFPET QFIAVTAYQN EEITALKIKY NPFAKAFLDA KERNDHKDVM EEPGDCQQPG 240
179  MITSHSFPET QFIAVTAYQN EEITALKIKH NPFAKAFLDA KERNDYKDIL DEGIDSQHSN 238
174  MISSQSFPET QFIAVTAYQN EEITALKIKH NPFAKAFLDA KERSDHKEVP DHSTDNQQSG 233

241  YSQWG-WLVP GAGTLCPPAS SHPQF-GGSL SLPSTHGCER YPALRNHRSS PYPSPYAHRN 298
239  FSQLGTWLIP NGGSLCSPN- PHTQF-GAPL SLSSPHGCER YSSLRNHRSA PYPSPYTHRN 296
234  YSQLGGWFLP SNGP-MGPSS SPPQFNGAPV H-SSGSYCER YSSLRNHRAA PYPSHYSHRS 291

299  SSPT-YADNS SACLSMLQSH DNWSSLGVPG HTSMLPVSHN ASPPTGSSQY PSLWSVSNGT 357
297  NSPNNLADNS SACLSMLQSH DNWSTLQMPA HTGMLPMSHS TGTPPPSSQY PSLWSVSNSA 356
292  TTTNNYMDNS SGSLA---SH DSWSALQIPN SSGMGTLAHT TNTTSNTSQY PSLWSVAGTT 348
358  ITP-GSQTAG VSNGLGAQFF RGSPAHYTPL THTVSAATSS SSGSPMYE-G AATVTDISDS 415
357  ITP-VSQSGG ITNGISSQYL LGSTPHYSSL SHAVP---SP STGSPLYEHG AQTE--IAEN 410
349  LTPSGSASGS ITGGLTSQFL RGSSMSYSGL TSSLPVSSPS SMYDPGLSEV GVGDAQFESS 408

416  QYD-TAQSLL IASWTPVSPP SM                                         436
411  QYDVTAHSRL SSTWTPVAPP SV                                         432
409  IARLTA---- --SWAPVAQS Y                                          423
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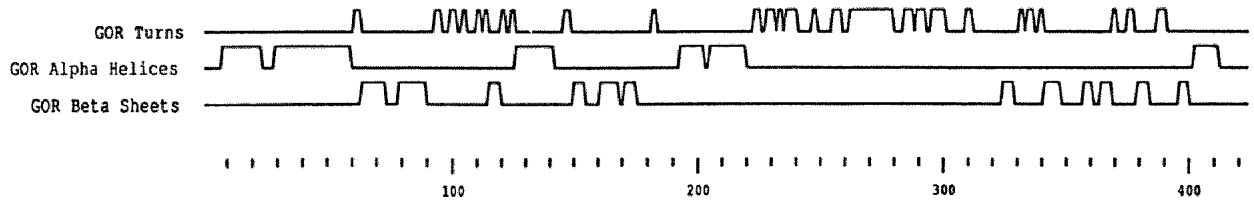
B



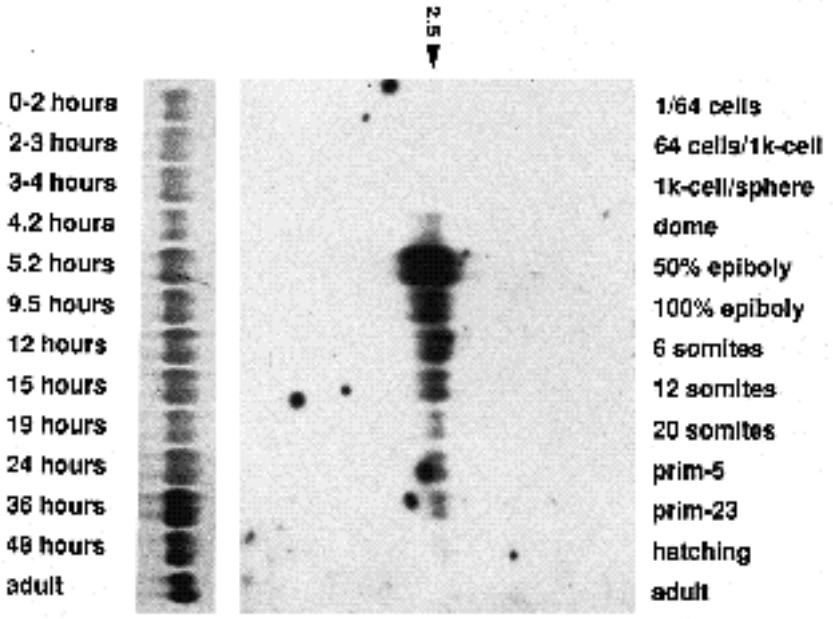


Fig. 3. Temporal expression pattern of *Zf-T*. 10 μg of total RNA of the indicated stages were blotted. Staging was done according to Westerfield (1989). A single transcript of about 2.5 kb was detected upon probing with the entire insert of pBSCT-ZFc1. The left panel shows methylene blue staining of ribosomal RNA of the same blot.

derm and the yolk cell. Already at this stage only cells in the position of the future germ ring (see below) express the *Zf-T* gene, whereas more central blastomeres do not. During gastrula stages (5.2-9.0 hours), both future endodermal and future mesodermal cells change their pathway of migration and move back towards the animal pole, possibly using the membrane of the yolk cell as a substratum (Fleig, 1990). This process leads to a thickening of the embryo in the marginal region, which is called the germ ring (see Fig. 4B). The *Zf-T* RNA is localized in cells closest to the vegetal pole, i.e. the area where cells are involuting. At the embryonic shield stage (Fig. 4B), just after the onset of involution, a band of cells expresses high levels of the *Zf-T* RNA all around the equator of the embryo (Fig. 4E). This region contains presumptive endodermal and mesodermal cells that involute during gastrulation (Kimmel et al., 1990), as well as cells of the enveloping layer. Sections through embryos of this stage reveal that both epiblast (cells that have not involuted) and hypoblast cells (cells that have involuted) in the germ ring express high levels of *Zf-T* RNA (data not shown). The resolution is not high enough to determine whether also enveloping layer cells express *Zf-T* RNA. After having involuted, most hypoblast cells gradually lose expression of the *Zf-T* gene. However, a subset of hypoblast cells, namely presumptive notochord cells, retain high levels of *Zf-T* RNA. This becomes obvious in the midgastrula at about 75% epiboly, and is even more striking at later stages (Fig. 4F,G).

At the tailbud stage (10 hours, Fig. 4D), when the blastoderm has completely covered the yolk cell, *Zf-T* RNA is present in cells of the presumptive notochord and in cells of the tailbud itself (Fig. 4F). By this stage, the majority of the presumptive posterior mesoderm and ectoderm is contained in the tailbud. This pattern of expression (i.e. *Zf-T* expression in the presumptive notochord and in the most posterior cells) continues throughout somitogenesis. Fig. 4G shows a spread of a 5-somite-stage embryo. Staining is much stronger at the posterior end of the prospective notochord. As confirmed by DAPI staining, higher intensity of staining is not due to a higher cell density in this region, but to higher amounts of message present in the most posterior axial cells (data not shown).

The expression of *Zf-T* RNA is detectable up to 36 hours of development, when expression is confined solely to notochord cells.

Distribution of Zf-T *protein in early embryos*

Bacterial cells transformed with vector pET-ZfT express the full-length protein product of the *Zf-T* gene with an apparent relative molecular mass of 45×10^3 (Fig. 5A). Polyclonal antibodies were raised against this prominent band in rabbits. Affinity-purified serum recognizes two proteins on western blots of extracts from induced bacteria (Fig. 5C), but not of extracts from uninduced bacteria (Fig. 5D). The strongly labelled protein band has an M_r of 45×10^3, whereas the weaker labelled protein has an M_r of 31×10^3. The protein of M_r 31×10^3 may represent a degradation product of the full-length *Zf-T* protein. Immunoprecipitation of in vitro synthesized *Zf-T* protein showed that immune, but not preimmune, serum is capable of precipitating a protein of M_r 45×10^3 (Fig. 5E,F).

In those cells that express *Zf-T* RNA, the protein was always localized to nuclei. Cytoplasmic staining was very weak and was equally intense in controls performed with preimmune serum.

At 4.0 hours (sphere stage; Fig. 4A), we detected the first immunopositive cells (Fig. 6A). About 30 cells in one localized region of the blastoderm margin neighbouring the yolk cell express low levels of the nuclear antigen. From this stage on, RNA and protein expression pattern are identical. In slightly later embryos (4.3 hours), cells in the margin

Fig. 2. (A) Sequence comparison between the predicted amino acid sequences of mouse *T* (top), Xenopus *Xbra* (middle), and *Zf-T* (bottom). (B) Secondary structure prediction of *Zf-T* protein. Numbers at the bottom indicate amino acids.

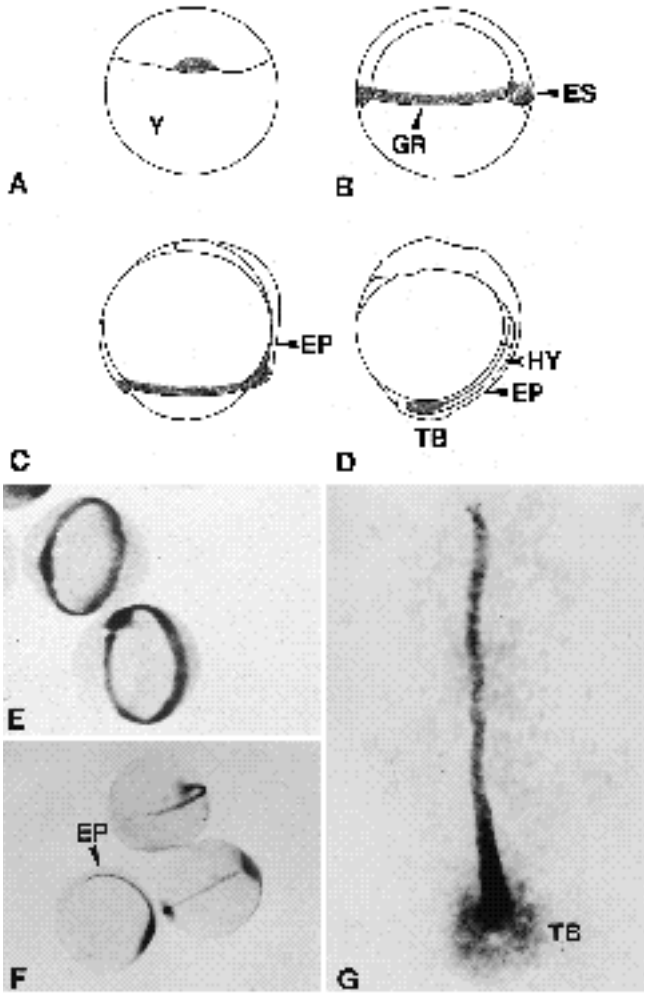


Fig. 4. Distribution of *Zf-T* mRNA in early embryos. (A-D) Schematic drawings of embryos, indicating (shaded) the distribution of Zf-T mRNA as revealed by whole-mount in situ hybridizations. Embryos are oriented with their animal pole up, and with their dorsal side to the right (in A, the dorsal-ventral polarity is not discernable yet). (A) Sphere stage, 4.0 hours. The blastomeres sit on top of the yolk (Y), epiboly is just about to start. (B) Embryonic shield stage, 6.0 hours. Epiboly has led to a spreading of cells over the yolk mass, which has undergone a change in shape. Involution causes a thickening of the embryo around its equator. Dorsal convergence leads to a thickening of the germ ring (GR) at the dorsal side, the embryonic shield (ES). (C) 80% epiboly, 8.0 hours. The embryo is two-layered: cells that have involuted constitute the hypoblast (HY; future endo- and mesoderm), cells that have not involuted constitute the epiblast (EP). (D) 2 somite-stage, 11.0 hours. Epiboly has finished, but gastrulation continues in the tailbud (TB) which is going to extend over the next couple of hours to make up the posterior half of the body. (E-G) Whole-mount in situ hybridizations using antisense RNA probes to reveal spatial expression of *Zf-T*. (E) Embryonic shield stage. Cells in the region of the germ ring express high levels of *Zf-T* RNA. Note the thickening at the dorsal side, the embryonic shield (ES). (F) Tailbud stage. *Zf-T* RNA is expressed in the presumptive notochord and in the tailbud (TB), but not in cells of the paraxial mesoderm. The staining is confined to the hypoblast and cannot be detected in any cells of the epiblast (EP). (G) 5-somite stage. Expression is not uniform along the anterior-posterior axis, but highest in cells of the posterior presumptive notochord. Anterior at the top.

express the nuclear *Zf-T* antigen (data not shown). At 50% epiboly, just prior to the onset of involution, the blastoderm is of uniform thickness and the marginal cells have reached the equator of the embryo due to epibolic movements. Again, as shown above by in situs for mRNA, the cells of the future germ ring show expression of the *Zf-T* protein (Fig. 6B, right half, and 6G) whereas the cells of the animal pole (future ectoderm) are not stained with the antiserum (Fig. 6B, left half).

During gastrulation, both endodermal and mesodermal cells involute (Warga and Kimmel, 1990). We stained early gastrula stages and sectioned embryos sagittally in order to identify which nuclei in the region of the germ ring contain *Zf-T* protein. As a counterstain, we used the flourescent dye DAPI (4,6-diamino-2-phenylindole). DAPI is quenched by the reaction product (3,3′-diaminobenzidine) of horseradish peroxidase which was used to detect *Zf-T* protein. Therefore, DAPI flourescence reveals only the nuclei that do not contain *Zf-T* protein (Fig. 6F; embryonic shield stage). Hypoblast cells (those that already have involuted) as well as epiblast cells (those that have not involuted) express *Zf-T* protein in their nuclei. At this stage, many marginal epiblast cells have yet to involute into the hypoblast.

There are two other types of nuclei at the marginal region besides those of the hypoblast and epiblast: nuclei of the yolk syncytial layer and nuclei of the enveloping layer. Neither in whole embryos nor in sections did we ever observe a nucleus of the yolk syncytial layer that showed any signs of *Zf-T* protein (Fig. 6E). In contrast, enveloping layer cells clearly express *Zf-T* (Fig. 6D,G). Therefore, in the region of the germ ring, epiblast and hypoblast as well as enveloping layer cells express the *Zf-T* gene product, but not the yolk syncytial layer.

During later stages of gastrulation (Fig. 6C,H; 90% epiboly), the nuclear staining remains most pronounced in cells close to the margin of the blastoderm (which moves in a vegetal direction by epiboly). Hypoblast cells lose expression of the *Zf-T* gene as they move away from the margin. Presumptive notochord cells, however, maintain high levels of the *Zf-T* protein. Fig. 6C and H show whole-mount preparations of 90% epiboly embryos. Note the strongly stained nuclei of cells closest to the area of involution. A specimen was flattened (Fig. 6H; anterior at the top) to show more clearly that cells of the paraxial mesoderm no longer express the *Zf-T* protein, whereas cells of the prospective notochord still do. Sections through the anterior third of an embryo at late gastrula (90% epiboly) reveal staining only in the most dorsal hypoblast cells (not shown), whereas a section through the posterior third shows staining in many cells of the hypoblast.

At 9.0 hours of development, the zebrafish embryo has finished epiboly. One hour later a prominent swelling at the posterior pole, the tail bud, starts to form (Fig. 4D). The tail bud will give rise to all the posterior structures of the embryo. At the same time somitogenesis is commencing.

At the tailbud stage and during somitogenesis, prospective and differentiating notochord, and the cells of the posterior-most mesoderm express *Zf-T* protein. Fig. 6I shows the tail tip of a 15-somite-stage embryo. Note the absence of staining in cells of the enveloping layer and of the ecto-

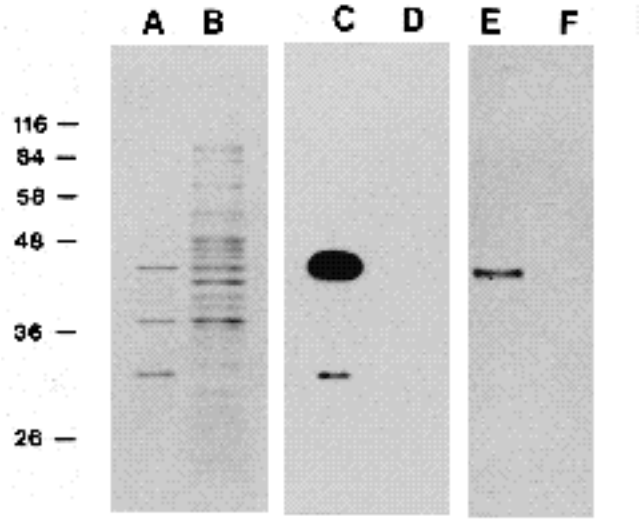


Fig. 5. Specificity of the anti-Zf-T antiserum. Coomassie-blue stained SDS-PAGE gels (lanes A and B) and Western blot (lanes C and D) showing total protein from induced bacteria carrying expression vector pET-ZfT (lanes A and C) and from uninduced bacteria also containing the expression vector (lanes B and D). The band with apparent relative molecular mass of 45×10^3 was used to raise the antiserum. Immunprecipitations using immune (lane E) and preimmune serum (lane F) to precipitate in vitro made *Zf-T* protein further demonstrates specificity of the antiserum.

derm, in contrast to the strong staining in nuclei of the posterior-most mesoderm. Fig. 6K shows the middle region of a 28-somite-stage embryo. *Zf-T* is still expressed even in notochord cells that have already undergone differentiation.

Induction of Zf-T *expression*

It has been shown that a number of genes expressed in the mesoderm of vertebrates respond to induction by activin A and other peptide growth factors (Cho et al., 1992; Cho and DeRobertis, 1990; Smith et al., 1991). We wanted to examine whether also *Zf-T* could be induced by activin A. Animal caps of blastula stage embryos were incubated in control serum or medium conditioned by XTC cells (Smith, 1987). XTC cells have been shown to produce activin A (Smith et al., 1990). The activin A-treated (Fig. 7A), but not the control explants (Fig. 7B), showed strong expression of *Zf-T*. Without exception, activin A-treated explants contained immunopositive nuclei at least in some cells; frequently *Zf-T* was expressed in all cells (Fig. 7A). In control embryos, no significant expression was observed.

We do not know at present whether activin is present in the zebrafish embryo. To learn whether endogenous signals are present in appropriate regions of the early gastrula, we questioned whether the marginal area contains signals capable of inducing *Zf-T* expression. Animal pole cells, which normally never express *Zf-T*, were transplanted into the marginal zone of a host embryo. As a control, animal pole cells were also transplanted to a region that normally does not contain any *Zf-T* expressing cells, i.e. into the animal pole region of another embryo. Donor embryos were labelled with lineage tracer at the one-cell stage to enable us to distinguish between host and donor cells. Cells transplanted into the marginal area of an early gastrula showed strongly induced nuclear staining indistinguishable from staining in neighbouring host cells (Fig. 7C). In contrast to the results from marginal transplants, the cells transplanted to the animal pole never showed label (Fig. 7D).

Discussion

In this study, we have examined the expression pattern of the zebrafish *T* gene. The use of an anti-*T* antiserum and the optical clarity of the zebrafish embryo have allowed us to investigate the spatial expression pattern of the *T* gene in much greater detail than previously possible (Herrmann, 1991; Smith et al., 1991). We have found that the *T* protein is expressed in a transient fashion in marginal cells of blastula and gastrula stages, but stably in notochord cells throughout embryogenesis. Furthermore, we show that the *Zf-T* protein accumulates in the nucleus and that it is expressed not only in mesodermal precursors, but also in other cell types.

The finding in zebrafish of the nuclear localization of *Zf-T* protein gives a first indication of its molecular function. Its dynamic and highly restricted expression pattern, as well as the finding of its cell autonomous requirement in the notochord of mouse embryos (Rashbass et al., 1991) suggest a possible role as a regulatory factor and most likely as a transcription factor.

Previously it has been shown that the *T* gene is expressed in mouse and frog embryos in mesodermal cells (Wilkinson et al., 1990; Herrmann, 1991; Smith et al., 1991). Our findings suggest that the *Zf-T* protein in fish embryos is not only expressed in cells of the future mesoderm, but also in cells of the future endoderm and in cells of the enveloping layer. Even though there are no morphological criteria or markers available which could directly show that the *T* protein is present in the presumptive endoderm, lineage experiments performed by Warga and Kimmel (1990) clearly indicate that the endoderm arises from cells of the germ ring. Since we show here that all cells of the germ ring express the *T* gene, it is clear that *T* expression cannot be restricted to mesoderm precursor cells alone.

The finding of *Zf-T* protein in cells of the gastrula enveloping layer is surprising since these cells are clearly neither endodermal nor mesodermal. Enveloping layer cells also differ from other cells in their behaviour during gastrulation since they do not undergo involution (Kimmel et al., 1990) or dorsal convergence (Keller and Trinkaus, 1987; studies performed in *Fundulus*). The lineage of cells of the enveloping layer is restricted, by the time *T* expression is present, to a superficial skin layer, the periderm (Kimmel et al., 1990). Possibly, *Zf-T* protein is expressed in the enveloping layer cells of the margin because all blastoderm cells (hypoblast, epiblast and enveloping layer cells) in the marginal region become exposed to the same inductive signal. One of the consequences of exposure to such a signal might be the transcription of early genes like *Zf-T*. However, cells of the enveloping layer are the first blastomeres to become restricted to a specific lineage (Warga and Kimmel, 1990).

It is not known if, by the time they express the *T* gene, cells of the enveloping layer are already determined. This question could be addressed by cell transplantations similar to the ones presented in this paper.

Interestingly, we observed an initial asymmetry in *Zf-T* expression along the dorsal-ventral axis of zebrafish blastulae (Fig. 6A). Since in all later stages expression of *T* protein is always highest in cells of the notochord, the most likely possibility is that this initial asymmetry marks the dorsal side of the zebrafish embryo, right at the time when *T* protein can first be detected. At this stage, as in other vertebrates, notochord precursors are localized to the dorsal marginal region (Kimmel et al., 1990). This would mean that some dorsal blastomeres express *Zf-T* earlier and/or at higher levels than their ventral siblings, suggesting an early role for *Zf-T* in dorsal versus ventral and lateral prospective mesoderm determination. Since some cells of the dorsal mesoderm ultimately differentiate to form the notochord, it is noteworthy that only cells of the dorsal mesoderm maintain high levels of *Zf-T* RNA and protein after involution. *Zf-T* protein is expressed prior to *and* after differentiation only in cells of the notochord. In contrast, e.g. in cells of the paraxial mesoderm that will form somites, expression of the *T* gene is regulated down long before they have formed somites and before they start to differentiate.

Our observations support the notion that the function of the *T* protein may well be different in cells of the notochord versus other *T*-expressing cells. Rashbass et al. (1991) have recently shown that *T* function, at least in the dorsal mesoderm, is cell autonomous. Our finding that *T* is a nuclear protein is consistent with a cell autonomous function. Furthermore, the phenotype of mutant mouse embryos suggests that the presence of a functional *T* protein is required for differentiation of the notochord, but not necessarily for all types of mesoderm (Chesley, 1935; and see below).

With the exception of enveloping layer cells, *T* protein can be found in those cells that become endoderm and mesoderm. All posterior cells of the margin that are destined to involute express the *T* gene at high levels prior to involution, but turn down expression after involution. It is only the presumptive notochord cells that are different in this respect. Gastrulation and involution probably continue in the tail bud after yolk plug closure. There, a large number of cells extends the tail during somitogenesis. The cells actively undergoing morphogenesis in the tail region continue to express very high levels of *Zf-T* protein. Despite the striking correlation between *Zf-T* expression and the process of involution, at least in the mouse, the *T* gene is clearly not the sole gene necessary for gastrulation and specification of mesoderm. Homozygous mutant embryos make at least head mesoderm and some somitic mesoderm (Gluecksohn-Schoenheimer, 1944; Chesley, 1935). It has been suggested that posterior mesoderm requires higher levels of *T* gene product than anterior mesoderm to develop normally (Yanagisawa, 1981), and that mesoderm formation in mice is biphasic. A first phase of anterior mesoderm formation does not require the product of the *T* gene, while a second phase during which posterior mesoderm forms, depends critically on normal levels of *T* protein (Herrmann, 1991). Here we present evidence that in zebrafish there are higher levels of *Zf-T* RNA in the posterior part of embryonic mesoderm early during somitogenesis (Fig. 4G). We also show that there are high levels of *Zf-T* protein present until the very end of gastrulation in those cells that are being recruited to become mesoderm in the most posterior region of the embryo, the growing tail tip.

We observed that animal pole cells, which normally never express *Zf-T*, do so if transplanted into the marginal area of the early gastrula (5 hours). We can at present not exclude that, in the marginal area, blastomeres that express *Zf-T* protein induce their neighbours or, in this instance, ectopically placed cells to express *Zf-T* protein. It seems more likely though, that the transplanted cells respond to the same inductive signals as the other marginal blastomeres.

To address what the nature of the putative inductive signal might be, we examined the effects of activin A on animal caps of zebrafish blastulae. Over the last few years, a number of peptide growth factors have been shown to possess mesoderm-inducing properties (Thomsen et al., 1990; Smith, 1987; Sokol et al., 1990), but their endogenous role remains unclear. We used activin A, because it is the most potent of the many peptide growth factors capable of inducing mesoderm (for review, see Green and Smith, 1991) and has been shown to behave as a morphogen (Thomsen et al., 1990). The *Zf-T* gene is ectopically induced by activin A. Animal caps, containing only cells that in their normal environment would become ectoderm, express *Zf-T* protein after exposure to activin A. The varying number of cells induced per explant probably depends on the number of cells exposed to activin A. This in turn depends on how fast the caps close up after having been cut.

Our data are in agreement with the data obtained by Smith et al. (1991). They have recently shown that the homologue of the *T* gene in *Xenopus*, termed *Xbra*, can be induced by both activin A and bFGF even in the presence of cycloheximide. This suggests that the expression of the *T* gene in vertebrates is an immediate early response to mesoderm induction, not requiring protein synthesis.

Support for differential expression of the *Zf-T* gene comes from the observation that the *Zf-T* protein can be detected first in a restricted population of blastula cells, while shortly thereafter all marginal cells of the blastula express the gene (see above). This initial asymmetry might indicate different concentrations of an inductive signal. In gastrula embryos, we have observed that the yolk syntycial layer on the dorsal side (Fig. 6E) is larger than ventrally (Fig. 6D). Future studies will show whether such a difference can also be observed in embryos prior to gastrulation, and whether there is a correlation with initial *Zf-T* expression.

The antiserum against the *Zf-T* protein will be a useful marker for future studies concerning induction and formation of endoderm and mesoderm in vertebrates. The possibility of following cells of the marginal area that have been transplanted into ectopic positions, and examining whether non-marginal cells have been induced to express *T* following various treatments, makes a number of experiments on the single cell level feasible. Molecular markers such as the anti-*Zf-T* antiserum or other molecular probes (Molven et

al., 1990; Patel et al., 1989; Hatta et al., 1990; Krauss et al., 1991) will also help in exploiting the advantages of zebrafish as a genetic system. Recently, a zebrafish mutation has been discovered that closely mimics the *T* phenotype (Halpern and Kimmel, unpublished observation). Homozygous mutant embryos lack the notochord and all posterior structures. We are currently investigating the relationship between this mutation and the *Zf-T* gene.

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Fig. 6. Whole-mount antibody stainings using anti-Zf-T antiserum. (A) Sphere stage. The nuclear antigen is restricted to a few cells at the margin. (B) 50% epiboly, animal view. Plane of focus is on the animal pole (left half) and on the margin (right half). Cells in the future ectoderm are devoid of nuclear staining. (C) 90% epiboly. Ventral view. (D and E) Germ ring stage. An enveloping layer cell and all blastomeres of the marginal area are stained (D), but not the nuclei of the yolk syncytial layer (E). Note the size difference between the yolk syncytial layer at dorsal (E) and ventral (D) positions of the same embryo. (F) Sagittal section through an embryo of the germ ring stage. DAPI counterstaining reveals that all nuclei of blastomeres in the margin contain *Zf-T* protein. (G) 50% epiboly, side view. Note staining of the enveloping layer cells. (H) Dorsal view of a 90% epiboly embryo. The specimen has been flattened. Anterior is to the top. (I) Tailtip of a 15-somite-stage embryo. (K) Trunk region of a 28-somite-stage embryo (side view). The focus is on the level of the notochord. Animal pole to the top in A, C, D, E and G. Abbreviations: EL, enveloping layer; YSL, yolk syncytial layer; NT, neural tube.

Fig. 7. Induction of Zf-T expression as detected by anti-Zf-T immunostaining. (A) Animal caps respond to incubation in XTC-MIF (*Xenopus* activin A). (B) Animal caps do not express *Zf-T* protein if incubated in control serum. (C) Alkaline phosphatase-labelled cells (arrowheads) from the animal pole of a donor embryo express *Zf-T* protein after transplantation into the margin of another embryo. (D) Upon transplantation into the animal pole region of a host embryo, donor cells do not express *Zf-T*.