**no tail (ntl) is the zebrafish homologue of the mouse T (Brachury) gene**

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**SUMMARY**

The mouse T (Brachury) gene is required for normal mesoderm development and the extension of the body axis. Recently, two mutant alleles of a zebrafish gene, no tail (ntl), have been isolated (Halpern, M. E., Ho., R. K., Walker, C. and Kimmel, C. B. (1993) Cell 75, 99-111). ntl mutant embryos resemble mouse T/T mutant embryos in that they lack a differentiated notochord and the caudal region of their bodies. We report here that this phenotype is caused by mutation of the zebrafish homologue of the T gene. While ntl embryos express mutant mRNA, they show no nuclear protein product. Later, expression of mRNA in mutants, but not in wild types, is greatly reduced along the dorsal midline where the notochord normally forms. This suggests that the protein is required for maintaining transcription of its own gene.

Key words: Brachury, no tail, notochord, zebrafish

**INTRODUCTION**

Among vertebrates, significant progress has been made during recent years in analysing the expression patterns of numerous genes during early development. However, while many genes have been identified by means of their sequence similarity with other previously identified genes, only very few have been shown by mutational analysis to be crucial for early development. Consequently, with the notable exception of a number of mouse mutants, our knowledge about the function of early acting vertebrate genes at the cellular level is still restricted.

A gene whose importance for vertebrate gastrulation was recognized by virtue of its mutant phenotype is the T, or Brachury, gene (Dobrovolskaia-Zavadskaia, 1927). Mouse embryos mutant for T fail to form sufficient mesoderm and lack all but the most anterior seven somites. The notochord does not differentiate, and the embryos die around day 10 post coitum (Chesley, 1935; Gluecksohn-Schoenheimer, 1944; Grüneberg, 1958; for review, see Beddington et al., 1992). The T gene has been cloned (Herrmann et al., 1990), and its embryonic expression pattern was examined in mouse (Wilkinson et al., 1990; Herrmann, 1991). Xenopus (Smith et al., 1991) and zebrafish (Schulte-Merker et al., 1992). In those species, the gene is expressed transiently in cells of the presumptive mesoderm, with a more stable expression in cells of the future notochord. The T gene protein product accumulates in nuclei (Schulte-Merker et al., 1992), acts as a genetic switch (Cunliffe and Smith, 1992) and has been shown to respond to mesoderm inducing factors in both Xenopus and zebrafish (Smith et al., 1991; Schulte-Merker et al., 1992).

Two mutant alleles at the zebrafish no tail (ntl) locus, ntl\textsuperscript{b160} (gamma-ray induced) and ntl\textsuperscript{b195} (spontaneous), have been identified during screens for early embryonic lethal mutations (Walker and Streisinger, 1983; Kimmel, 1989). ntl mutant embryos lack differentiated notochord and the most posterior 11-13 of their normal 30 somites (Halpern et al., 1993). In many respects, the ntl phenotype resembles that of the mouse T gene. This similarity prompted us to ask whether the ntl mutations specifically affect the zebrafish homologue of the T gene, which had been cloned previously and termed Zf-T (Schulte-Merker et al., 1992).

In this paper, we show that ntl is the homologue of the mouse T gene. In both alleles, alterations of the nucleotide sequence lead to truncated protein products, with no detectable accumulation of protein in nuclei of mutant embryos. Our study, together with the paper by Halpern et al. (1993), provides the basis for the analysis of T function in zebrafish development. As mutant ntl embryos die well after hatching and therefore relatively late in development (unlike T/T embryos, which die during midneurulation probably due to the lack of an allantois), it has now become possible to examine the effects of the mutation on later events, thus extending our knowledge about the effect of T (ntl) on early vertebrate development. Anterior somite patterning, especially the formation of muscle pioneer cells, is disturbed in ntl mutant embryos, but the floorplate and other neural structures do not seem to be affected (Halpern et al., 1993). Moreover, the high degree of similarity in phenotype between T mouse mutants and ntl fish mutants provides the strongest evidence so far that homologous genes can serve the same function in members of different vertebrate classes, even if they are evolutionarily as separate as mammals and teleosts. This finding has a strong impact for successfully applying information obtained in one experimental system to another system, as it demonstrates directly that much of the basic biology of vertebrate development is underpinned by common genetic mechanisms.
MATERIAL AND METHODS

## Maintenance of fish
Wild-type strains (+/+) and +ind/+ind and mutant alleles (ntlb160 and ntlb160, Halpern et al., 1989) were maintained under conditions previously described (Schulte-Merker et al., 1992). Eggs were obtained by natural matings (as described by Culp et al., 1991), or by in vitro fertilization to obtain haploid offspring (as described in Westerfield, 1993).

For the initial segregation analysis (Fig. 1A), a female heterozygous for the mutation no tail (+ b160/b160) was crossed to an unrelated wild-type male (+ ind). Individual fish of the resulting F1 generation (with the genotype ntlb160/+ind) were identified as carrying the ntl mutation and then used for in vitro fertilizations to obtain haploid wild-type (+/ind) or haploid mutant (ntlb160) F2 offspring. For analyzing large numbers of mutant chromosomes (Fig. 1B), two F1 individuals were mated and the diploid mutant offspring were collected.

## Southern analysis
DNA was isolated from whole animals or embryos by standard methods (Sambrook et al., 1989). After digestion with the restriction enzyme BglII or MscI, DNA restriction fragments were separated on 0.8% agarose gels, blotted and fixed onto Hybond N+ (Amersham) according to the manufacturer’s instructions. The EcoRI fragment of pBSCT-ZfC1 (Schulte-Merker et al., 1992) was labelled (Megaprime Kit, Amersham) with [32P]CTP and used for probing the filters. Exposure was overnight, unless otherwise stated.

## Western analysis
Embryos from wild-type or heterozygous parents were collected at the early gastrula stage, i.e. at a point where we were unable to distinguish mutant from wild-type embryos. After freezing on dry ice, 2 ml of 2x Laemmli buffer (Laemmli, 1970) were added per embryo. The samples were heated to 100°C for 5 minutes, put briefly on ice, and centrifuged for 2 minutes. The supernatants (equaling 10 embryos) were loaded onto a 15% gel, electrophoresed (LKB midget unit) and transferred onto nitrocellulose membranes (Schleicher & Schuell). Immunodetection was carried out using the ECL system (Amersham) as previously described (Schulte-Merker et al., 1992).

## Whole-mount in situ hybridisations and immunohistochemistry
Detection of ntl RNA in situ was carried out as previously described (Schulte-Merker et al., 1992) with the following modifications: treatment with RNAse was found to reduce the signal intensity in the case of the antisense ntl RNA and was therefore omitted. Hybridization was followed by five washes at 55°C (50% formamide/2x SSCT, 2x 30 minutes; 2x SSCT, 1x 15 minutes; 0.2x SSCT, 2x 30 minutes). Samples were then transferred to microtiter dishes and incubated for 30 minutes in blocking solution, followed by incubation in blocking solution containing anti-digoxigenin antibody (Boehringer). After terminating the colour reaction, the embryos were fixed in 4% paraformaldehyde/PBS for at least 1 hour. If necessary, mutant embryos were distinguished from sibling wild-type embryos by antibody staining.

Detection of Ntl protein was performed exactly as previously described (Schulte-Merker et al., 1992).

## Molecular analysis
Subgenomic libraries were prepared by digesting genomic DNA with the restriction enzyme HindIII, separating it on 0.8% agarose gels, and cutting out the desired region from the gel. The DNA was isolated from the gel slice (QuiaEx Kit, Quiagen) and ligated into plasmid pBluescript SK (Stratagene). The libraries were screened according to the protocol from Buluwela et al. (1989). RT-PCR was carried out essentially as described previously (Schulte-Merker et al., 1992).

Sequencing was performed on an ALF sequencer (Pharmacia). Either genomic DNA from a subgenomic HindIII library (ntlb160), or genomic DNA from PCR reactions (ntlb160, ntlb195), or PCR products from reverse-transcribed total RNA (ntlb160) were sequenced. In all cases, both strands were sequenced at least twice.

## RESULTS

### ntlb160 and T are closely linked
To investigate whether ntl and T are genetically linked, a recombination analysis was carried out using a restriction fragment length polymorphism (RFLP) within the T gene. In preliminary experiments, genomic DNA of adult fish from ntlb160/+ and a number of unrelated wild-type strains was digested with various restriction enzymes, transferred to filters and probed with the Zf-T cDNA (Schulte-Merker et al., 1992). BglII-digested DNA from ntlb160/+ showed a fragment of...
8.4 kb (Fig. 1A, lane b), while DNA from a particular wild-type strain (+/ind) showed two fragments of 5.7 and 3.3 kb, respectively (Fig. 1A, lane d). This RFLP enabled us to distinguish whether the T gene originated from the mutant or the wild-type background during the following linkage analysis. A cross between fish from both strains (ntl<sup>b160</sup>+/+ × 4<sup>ind</sup>/4<sup>ind</sup>) yielded an F<sub>1</sub> generation that was heterozygous for the RFLP (Fig. 1, lane c). F<sub>1</sub> females heterozygous for the ntl mutation were identified and haploid F<sub>2</sub> offspring obtained from them. F<sub>2</sub> embryos were sorted according to their phenotype and their DNA digested with BglII. Southern analysis revealed that the 8.4 kb fragment indicative of the mutant background always segregated with the mutant phenotype (Fig. 1A, lane a), and that this fragment could not be seen in DNA from wild-type embryos (Fig. 1A, lane e).

In order to determine how closely ntl<sup>b160</sup> and the T gene are linked, it was necessary to analyze a large number of mutant F<sub>2</sub> offspring. 270 homozygous ntl<sup>b160</sup>/ntl<sup>b160</sup> progeny from a ntl<sup>b160</sup>+/4<sup>ind</sup> × ntl<sup>b160</sup>/4<sup>ind</sup> cross (scoring 540 recombination events) were collected, divided into groups of 30 and subjected to the same analysis as in Fig. 1A. As a sensitivity control, the DNA equivalent of another 30 mutant embryos was mixed with the DNA equivalent of half a wild-type embryo. The wild-type fragment of 5.7 kb was detectable on a Southern blot (Fig. 1B, left lane), meaning that we would have been able to detect one recombination event in the DNA from 30 homozygous mutants, had it occurred. However, no recombinants were detected (Fig. 1B). This shows that the cloned T gene and the ntl mutation reside on the same chromosome and are no more than 0.2 cM (1/540) apart (provided unrestricted recombination).

### A frameshift leads to an altered protein product in ntl<sup>b160</sup>

As mutant ntl<sup>b160</sup> embryos express T RNA (see below), we expected the molecular cause of the mutation to reside in the translated part of the gene. We sequenced the whole translated region of the T gene, using as templates either clones from a subgenomic HindIII library or clones obtained by PCR reactions (see Material and Methods). In both cases, genomic DNA obtained either from wild-type or from mutant embryos served as the DNA source. Comparing the sequence of PCR products revealed a change (wild-type: CG→mutant: AGC TGT) at the intron-exon boundary of exon 6. We confirmed this observation by using reverse transcribed RNA from embryos as a PCR substrate, showing that the exchange of two nucleotides in the wild type against six nucleotides in the mutant is also present at the RNA level (Fig. 2A). This change in sequence leads to a potential frame shift and a potential premature protein chain-termination. In agreement with the sequencing data (which predict a truncated protein product of 245 amino acids and of a relative molecular mass of 27x10<sup>3</sup>), a truncated protein product of relative molecular mass of 23x10<sup>3</sup> was detected in embryos from heterozygous, but not from wild-type parents of the same genetic background (Fig. 2B). The wild-type protein is predicted to contain 423 amino acids (Mr 45x10<sup>3</sup>).

### A 1.5 kb insertion causes a disruption in the second exon of ntl<sup>b195</sup>

The T gene consists of at least 8 exons, which contain all the translated information (Fig. 3B). Genomic DNA from wild-type fish, digested with the restriction enzyme HindIII, shows two fragments of 1.3 and 3.6 kb, respectively, if probed with the Zf-T cDNA (Fig. 3A, lane c). In the case of a heterozygous ntl<sup>b195</sup> individual, however, there appeared an additional fragment of 2.8 kb (Fig. 3A, lane b). Analysis of DNA from either haploid mutant embryos (Fig. 3A, lane a) or haploid wild-type embryos (Fig. 3A, lane d) revealed that the 2.8 kb fragment always segregated with the mutant phenotype, while the 1.3 kb fragment was always found in DNA from wild-type embryos. Using PCR we found the size difference to be due to the insertion of a 1.5 kb fragment in the second exon of T (Fig. 3B).

Northern analysis confirmed that the insertion is part of the T mRNA: RNA from offspring of ntl<sup>b195</sup> heterozygous parents showed a transcript of 4.0 kb (data not shown), in addition to the 2.5 kb wild-type transcript (Schulte-Merker et al., 1992). The resulting predicted sequence encodes a truncated protein, containing the first 103 N-terminal amino acids of the T protein (normal length 423 amino acids) and an additional 35 amino acids encoded by the insert. We have not been able to detect a truncated protein on western blots in the case of ntl<sup>b195</sup>, in contrast to ntl<sup>b160</sup>. This, most likely, is due to epitopes in the ntl<sup>b160</sup> protein which are not present in the much shorter ntl<sup>b195</sup> protein.

The above data demonstrate the identity of ntl and T. Following recent naming conventions the name for the zebrafish T gene will be ntl.

### The inserted fragment contains features of a transposable element

The 1.5 kb fragment contains a number of intriguing characteristics. First, at the site of insertion there is a duplication of 9 nucleotides of host sequence (nucleotides 82-90 of the cDNA; Fig. 3C,D). Second, there is another pair of direct
repeats in immediate proximity to the 9 bp repeats, separated from them by one nucleotide (at the 5’ end) and 4 nucleotides (at the 3’ end), respectively. Third, there is a striking accumulation of 8 ten-basepair repeats in the distal-most regions of the element, with two repeats at the 5′ end and six at the 3′ end of the inserted fragment. The sequence of the 10mer is GGG GTG GXXX, where the last three nucleotides are CCA (five cases), CCG, TCA, or CAC (one case each). No other 10mer is found in the intervening 1376 bp. Fourth, a recognition site (TGG CCA) for the restriction enzyme MscI was found in the ten-basepair repeat. When genomic DNA from either wild type or ntlb195 was digested with MscI, transferred to filters, and probed with a radioactively labelled subfragment of the 1.5 kb fragment, it became obvious that the fragment is a member of a family of middle repetitive elements (Fig. 3E). Under conditions where a single copy gene was not detectable, a strong signal was observed. Analysis of other members of the family is in progress (A. Fritz and M. E. Halpern, unpublished data).

Ntl protein cannot be detected in nuclei of mutant embryos

We performed immunohistochemistry to address the question whether Ntl protein is present in the nuclei of mutant embryos. In wild-type and heterozygous sibling embryos, Ntl protein can be detected in the nuclei of cells of the germ ring (Fig. 4A) and of the notochord and extending tailtip (Fig. 4C). In mutant embryos of either allele (Fig. 4B,D), we were unable to demonstrate the presence of Ntl protein, irrespective of the stage examined. We could also not see the nucleus of staining in the cytoplasm of cells expected to express the gene. Particularly in the case of ntlb160, where a truncated protein was detectable on western blots, this was somewhat surprising. There are at least two explanations for this: first, the antiserum was raised against a denatured form of the Ntl protein (Schulte-Merker et al., 1992), and therefore there might be more epitopes being recognized after denaturing gel electrophoresis on blots than in the embryo. Second, the nuclear...
localization signal of Ntl might reside in the C-terminal part of the protein. This would lead to a failure of protein accumulation in the nuclei of ntl embryos, which in turn would make it more difficult to detect the protein.

**Expression of ntl RNA in mutant embryos**

In ntl mutant embryos of both alleles, the levels of ntl mRNA were found to be reduced compared to wild-type embryos (Fig. 5A; two mutant ntl<sup>b160</sup> embryos are on the left). At the germ ring stage the reduction is at least twofold. During later stages of development, there is a dramatic difference in expression levels of the ntl transcript in the cells we take to be the presumptive notochord cells (Fig. 5B, C). While wild-type sibling embryos exhibit high levels of ntl RNA along the dorsal midline (Fig. 5B), both mutant ntl<sup>b160</sup> (Fig. 5C) and ntl<sup>b195</sup> (Fig. 5D) embryos show very low levels of ntl expression along the dorsal midline. For embryos shown in A, the staining reaction was stopped early while still in the linear range; for embryos shown in B-D, the staining reaction was allowed to reach saturation. Therefore, the difference in staining intensity in the non-axial presumptive mesoderm cannot be seen in B-D.

Interestingly, a population of cells at the dorsal margin of the zebrafish gastrula expresses high levels of ntl mRNA (Fig. 5C). These cells are also present in wild-type embryos, but they are somewhat disguised by the strong ntl expression in the cells underlying them. The fate of these cells in wild-type embryos has not been established yet, however, they might be homologous to the terminal node cells in trout (Ballard, 1973).

**DISCUSSION**

We have investigated the molecular cause of mutations in two alleles of the zebrafish gene *no tail*, and have shown that *ntl* is the homologue of the mouse *T* gene. This work establishes that the equivalent genes in fish and a mammal produce remarkably similar phenotypic changes in the embryo when they are mutated. Moreover, our analysis of ntl mutant embryos demonstrates that Ntl protein is required in the axial mesoderm to maintain *ntl* transcription in this tissue.

**Both ntl alleles are likely to be null-alleles**

After confirming that ntl<sup>b160</sup> and *T* are linked, we have revealed the molecular cause of the mutations in both ntl<sup>b160</sup> and ntl<sup>b195</sup>. Even though both ntl<sup>b160</sup> and ntl<sup>b195</sup> embryos produce RNA, and even though we have been able to detect a truncated protein form in the case of ntl<sup>b160</sup>, both alleles are likely to be functional null alleles. There are two lines of argument supporting this notion. First, the mutant phenotype is equally strong in both ntl<sup>b160</sup> and ntl<sup>b195</sup> (Halpern et al., 1993). It is unlikely that two different truncated protein products would lead to the same partial phenotype. Second, it has been shown that the vertebrate T proteins can bind to DNA, and that this property resides in the N-terminal domain of the mouse protein (Kispert and Herrmann, 1993). A truncated form of the mouse protein, containing only the N-terminal 129 amino acids, completely loses its ability to bind its target DNA (Kispert and Herrmann, 1993). Furthermore, a truncated form of *Xbra* RNA is no longer able to lead to transcriptional activation of *Xsna*, muscle actin and *Xhox3* (Cunliffe and Smith, 1992). This truncated form of the Xbra protein is 11 amino acids shorter.
than the truncated Ntl protein predicted for ntl<sup>b160</sup>, but considerably longer than the predicted Ntl protein for ntl<sup>b195</sup>. Therefore, it is reasonable to assume that no functional protein is present in mutant embryos.

The 1.5 kb fragment is a multi-copy fragment

It is possible that the mutation in ntl<sup>b195</sup> is the result of insertional mutagenesis by a transposable element. The mutation occurred spontaneously, and at the site of insertion there is a duplication of host sequence, as is often found as the consequence of a transposition event (Schubiger et al., 1985; Berg and Howe, 1989). Moreover, the fragment is a member of a family of middle repetitive elements with the size of about 1.5 kb. However, the significance of other features of the fragment is not clear to us, and awaits further investigation (A. Fritz and M. E. Halpern, unpublished data).

Altered levels and expression pattern of ntl RNA in mutant embryos

Mutant ntl embryos exhibit a reduction of ntl mRNA levels from early gastrula stages onwards. More strikingly, only very few cells along the dorsal midline show detectable amounts of ntl mRNA. Morphological data and lineage analysis (Halpern et al., 1993), and particularly the remaining expression of ntl in this region, suggest strongly that notochord precursor cells are indeed present along the dorsal midline, but that they subsequently fail to differentiate. This is also supported by the presence of a floor plate (Hatta et al., 1991) in mutant embryos, which we suppose is induced by notochordal mesoderm, as in other vertebrates (Placzek et al., 1990; Yamada et al., 1991; van Straaten and Hekking, 1991). The dramatic reduction of ntl message in cells that we take to be presumptive notochord cells could be due to the instability of ntl message specifically in these cells of the dorsal midline, while the message is more stable in non-axial cells. An alternative explanation would be that functional Ntl protein is required, either directly or indirectly, to ensure normal levels of its own transcript in the presumptive notochord, and to a lesser extent in nonaxial cells. We favour the latter idea of an autoregulatory function of Ntl, as there exists evidence that Ntl may operate as a transcription factor: the nuclear localisation of the Ntl protein (Schulte-Merker et al., 1992), cell autonomous action of mouse T (Rashbass et al., 1991; Wilson et al., 1993) and zebrafish ntl (Halpern et al., 1993), activity as a genetic switch (Cunliffe and Smith, 1992), and DNA-binding of the optomotor-blind gene (Pflugfelder et al., 1992), a close relative of the T gene in Drosophila, and of the mouse T protein (Kispert and Herrmann, 1993), are all consistent with a role for Ntl protein in transcription. The data presented here argue that ntl gene regulatory elements might be a candidate downstream target of its own protein. This finding should allow us to investigate if Ntl protein interacts with putative notochord-specific elements in the ntl promoter.

The ntl phenotype

The phenotype of ntl embryos has been studied in detail in the paper by Halpern and colleagues (1993). Together, our data provide further insight into the biology of the T gene, at both cellular and biochemical levels. As zebrafish ntl mutants survive to later embryonic stages than mouse T homozygotes, it has been possible to establish the presence of a floor plate along almost the entire length of the mutant body axis, and the requirement for a notochord for the differentiation of muscle pioneer cells. Fish embryos may also be especially useful for learning about the consequences of the mutation for tail development and the expression of genes that might be involved in this process (Joly et al., 1993). It is possible that the dorsal marginal cells, which we have observed to express high levels of ntl mRNA in mutant embryos (Fig. 5C), play a role in the extension of the tail bud and consequently of the body axis. It will be interesting to see whether the behaviour of these cells is altered in mutant versus wild-type embryos.

In conclusion, this work establishes that the equivalent genes in a fish and a mammal produce remarkably similar phenotypic changes in the embryo when they are mutated. Previous studies have revealed a surprising evolutionary conservation in the molecules that appear to be guiding development of diverse vertebrates, despite often marked dissimilarities in the overall appearances of the embryos and the arrangements of their cells. The demonstration that T is equivalent to ntl shows that, at least

**Fig. 5.** Expression of ntl RNA in ntl mutant embryos. (A-D) Whole-mount in situ hybridizations detecting ntl mRNA. (A) Mutant ntl<sup>b160</sup> embryos (two embryos on the left) at the embryonic shield stage exhibit reduced levels of ntl transcript if compared to sibling embryos (two embryos on the right). Mutant embryos were identified by their lack of Ntl protein after antibody stainings. (B) Wild-type sibling of embryo shown in (C) at 100% epiboly. There is strong expression of ntl RNA in the axial mesoderm and the germ ring. (C) Mutant ntl<sup>b195</sup> embryo at 95% epiboly. Transcript levels are high in the germ ring, but hardly detectable in the presumptive notochord (arrowhead). Note the staining of dorsal marginal cells (see text for details). (D) Mutant ntl<sup>b195</sup> embryo at tailbud stage (10h), demonstrating that mutant embryos of both alleles behave indistinguishably with respect to ntl mRNA expression in cells of the presumptive notochord (arrowhead).
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