Thylacine 1 is expressed segmentally within the paraxial mesoderm of the Xenopus embryo and interacts with the Notch pathway

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Accepted 20 March; published on WWW 6 May 1998

SUMMARY

The presomitic mesoderm of vertebrates undergoes a process of segmentation in which cell-cell interactions mediated by the Notch family of receptors and their associated ligands are involved. The vertebrate homologues of Drosophila Delta are expressed in a dynamic, segmental pattern within the presomitic mesoderm, and alterations in the function of these genes leads to a perturbed pattern of somite segmentation. In this study we have characterised Thylacine 1 which encodes a basic helix-loop-helix class transcription activator. Expression of Thylacine is restricted to the presomitic mesoderm, localising to the anterior half of several somitomeres in register with domains of X-Delta-2 expression. Ectopic expression of Thylacine in embryos causes segmentation defects similar to those seen in embryos in which Notch signalling is altered, and these embryos also show severe disruption in the expression patterns of the marker genes X-Delta-2 and X-ESR5 within the presomitic mesoderm. Finally, the expression of Thylacine is altered in embryos when Notch signalling is perturbed. These observations suggest strongly that Thylacine 1 has a role in the segmentation pathway of the Xenopus embryo, by interacting with the Notch signalling pathway.

Key words: Xenopus laevis, paraxial mesoderm, bHLH protein, Thylacine, segmentation, somites, Notch, Delta, Mesp2.

INTRODUCTION

One important process underlying the development of the vertebrate embryo is the segmentation of the paraxial mesoderm into somites. Somitogenesis entails an orderly sequence of events that pass wave-like down the axis of the paraxial mesoderm in a rostral to caudal direction (Hamilton, 1969; Youn and Malacinski, 1981; Wilson et al., 1989). The early stages of this process occur within the presomitic mesoderm, where a periodic pattern is thought to be generated, and nascent, segmental structures, the somitomeres, are first established (Jacobson, 1988). Studies in the chick embryo suggest that the early segmental organisation within the presomitic mesoderm depends on the formation of a rostral and caudal half segment (Stern and Keynes, 1987; Aoyama and Asamoto, 1988; Goldstein and Kalcheim, 1991, 1992). Although somites themselves differ among vertebrate species in their morphology, the cell types they contain, and the morphogenetic events underlying their formation; the events that occur within the presomitic mesoderm that generate a segmental pattern are likely to be evolutionarily conserved (Jen et al., 1997).

Recent evidence indicates that segmentation of the presomitic mesoderm involves cell-cell interactions mediated by the Notch family of receptors and their associated ligands. For example, components of the Notch signalling pathway have been shown to be expressed in a dynamic, segmental pattern within the presomitic mesoderm of different vertebrate embryos, including several putative Notch ligands encoded by the vertebrate homologues of Drosophila Delta. In the mouse embryo, two homologues of Drosophila Delta, Dll1 and Dll3, are expressed in a broad domain of paraxial mesoderm, becoming restricted to the boundaries of the forming somite, with Dll1 at the posterior boundary and Dll3 at the anterior (Dunwoodie et al., 1997). Similar dynamic patterns of segmental expression have also been described for the Delta homologues, X-Delta-2 and deltaD, in Xenopus and zebrafish respectively (Jen et al., 1997; Dornseifer et al., 1997). Moreover, alterations in function of these Notch ligands, as well as in components of the Notch signalling pathways, has been shown to lead to segmental defects. Such segmental defects are seen in mouse embryos with targeted inactivation of the genes encoding Dll1 (Hrabe de Angelis et al., 1997), Notch1 (Conlon et al., 1995), or a component of the Notch signalling pathway called RBP-Jk or Su(H) (Oka et al., 1995). Similarly, misexpression of the wild-type Delta homologues causes defects in the formation of the somites in Xenopus and zebrafish embryos (Jen et al., 1997; Dornseifer et al., 1997).

Finally, in Xenopus, segmental defects are also produced when the Notch signalling pathway is blocked by ectopic expression
of dominant negative forms of X-Delta-2 or of *Xenopus* Suppressors of Hairless (X-Su(H); Jen et al., 1997). Importantly, alterations of Notch signalling in these different experiments does not appear to affect cytodifferentiation within the paraxial mesoderm, but rather produces phenotypes consistent with a loss of segmental boundaries and/or segmental polarity. However, the exact nature of the defects is not fully understood, and enough variation exists to raise the strong possibility that Notch signalling may serve several functions during the process of segmentation.

A second class of genes which appear to be involved in segmentation are those encoding basic-helix-loop-helix (bHLH) transcription factors. One class of these bHLH genes appear to encode transcriptional repressors with several characteristic structural features, namely a conserved proline in the basic region and a C-terminal WRPW tetrapeptide necessary for interaction with the co-repressor groucho (Paroush et al., 1994). Among the WRPW-bHLH genes expressed in the paraxial mesoderm are *her-1* in zebrafish (Müller et al., 1996), *Hairy2A* in *Xenopus*, (Jen et al., 1997) and *e-hairy 1* in the chick (Palmeirim et al., 1997). Interestingly, all three are expressed segmentally but vary greatly in the exact pattern of expression within the presomitic mesoderm. In zebrafish, *her-1* is expressed in two stripes within the presomitic mesoderm that correspond to alternating prospective somites, beginning with somitomeres 5 and 7, and so forth. In *Xenopus*, *Hairy 2A* is expressed in two stripes that correspond to the posterior halves of somitomeres 2 and 3. In the chick, *e-hairy 1* expression in the tailbud region oscillates with a regular periodicity of 90 minutes (corresponding to the formation time of a single somite), becoming restricted to the posterior half of each somite as it forms. Functional experiments to test the role of these bHLH-WRPW transcription factors have not been reported to date. The second class of bHLH genes expressed in the paraxial mesoderm encode transcriptional activators that are likely to act by forming heterodimers with the ubiquitous bHLH partner, E12. One of these genes, *paraxis*, in the mouse is expressed widely within the paraxial mesoderm, and when inactivated by gene targeting leads to defects in epithelialisation of the somites, but not to segmental defects (Burgess et al., 1996). In contrast, the bHLH protein Mesp2 is expressed in a single stripe in the presomitic mesoderm of mouse embryos, approximately one segment-width from the posterior boundary of the forming somite (Saga et al., 1997). Targeted disruption of the *Mesp2* gene produces defects in somite formation that suggest the Mesp2 protein is required for the formation of the anterior half of each somitic compartment. Thus these observations suggest that the bHLH genes of both activator and inhibitor classes are important issues for understanding how paraxial mesoderm is segmented in different vertebrate species.

Here we describe *Thylacine 1*, a bHLH protein that plays a role in the segmentation of *Xenopus* embryos. *Thylacine* is a member of the Mesp family but differs in both sequence and expression from both Mesp1 and Mesp2. *Thylacine* transcripts are restricted to the presomitic mesoderm, localising to the anterior half of several somitomeres. Ectopic expression of *Thylacine* in embryos does not appear to affect cytodifferentiation but rather leads to segmentation defects, which are already apparent within the presomitic mesoderm, based on the expression of marker genes. One of the genes affected by ectopic *Thylacine* is X-Delta-2, suggesting that *Thylacine* interacts with the Notch pathway. Conversely, the expression of *Thylacine* is altered in embryos when Notch signalling is perturbed. These observations indicate that *Thylacine 1* has a role during segmentation in *Xenopus* embryos, perhaps via its interactions with the Notch signalling pathway. Strikingly, the effects of ectopic *Thylacine* expression are limited to the paraxial mesoderm, and other tissues requiring the Notch signalling pathway for their patterning (such as primary neurons) remain unaffected. Finally, our results indicate that different members of the Mesp family act at distinct points in the process of segmentation, thus mirroring the manner by which families of related bHLH genes regulate myo- and neurogenesis.

**MATERIALS AND METHODS**

**Yeast two-hybrid screen**

A cDNA library was made in yeast vector pGAD10 from poly(A)+ RNA derived from neurula stage *Xenopus laevis* embryos (stage 18) using the Clontech MATCHMAKER kit according to the manufacturer’s instructions. A library of approximately 3×10⁶ independent clones with an average insert size of approximately 1.0 kb was generated, using a combination of oligo(dT) and random priming. A bait for the yeast two-hybrid screen (XE12-pGBT9) was constructed by fusing codons 507-658 encompassing the bHLH and carboxy-terminal domains of *Xenopus* E12 (Rashbass et al., 1992) in frame to the Gal4 DNA binding domain. The library was screened using this bait as previously described (Sparrow et al., 1998). Three independent clones encoding the *Xenopus Thylacine 1* gene were isolated. The longest of these was termed pThyl-14.1 (nucleotides 1-920 of the full length cDNA).

**Isolation of full-length cDNA clones**

A neurula (stage 17) cDNA library (Kintner and Melton, 1987) was screened with an oligo-labelled probe of the entire pThyl-14.1 clone, at a stringency of 0.2× SSC 60°C. Several positively hybridising clones were analysed and found to contain sequences overlapping the original clone. The entire *Thylacine 1* sequence was derived from one of these clones (pThyl-2s7). Several cDNA clones representing a second closely related gene, called *Thylacine 2*, were also isolated from this library and the entire sequence derived from one of these clones (pThyl-4s1). Sequences were analysed using the Lasergene suite of programs (DNASTAR Inc) and BLAST (at the NCBI using the BLAST network server: http://www.ncbi.nlm.nih.gov/). The *Thylacine 1* and *Thylacine 2* nucleotide sequences have been submitted to GenBank under accession numbers Y14446 and Y14447.

**Construction of deletions and transcription activation domain mapping in yeast**

Deletions of the full length *Thylacine 1* gene were made either by exonuclease digestion (Henikoff, 1984) or by PCR and fused to the Gal 4 DNA binding domain. These deletions were transformed into yeast strain Y190 (MATa gal 4 gal 80 his 3 trp1-901 ade2-101 ura3-52 leu2-3,-112 + URA3::GAL→LacZ, LYS2::GAL(UAS)→HIS3 cyh r) by the lithium acetate method (Schiestl and Gietz, 1989). Transformants were plated on SD -Trp, and allowed to grow for 3 days at 30°C. Levels of *lacZ* gene expression were determined by liquid culture assay as described (Johnson et al., 1986). Expression levels were normalised for protein content and expressed as a percentage of transcriptional activity of the full length protein.
RNA preparation and assay
RNA was prepared from embryos and adult tissue as described previously (Logan and Mohun, 1993). *Thylacin 1* transcripts were detected with a probe consisting of sequences spanning nucleotides 740-920 (pThyl-14.1 HinDIII). This was synthesised using XhoI linearised template and T7 RNA polymerase. RNase protection assays were performed as described previously (Chambers et al., 1994). A control probe for XMax2 (Tomissen and Krieg, 1994) was prepared as described.

Whole-mount RNA in situ hybridisation
Albino embryos were used for whole-mount in situ hybridisation as described by (Harland, 1991) using digoxigenin-labelled probes. Two different antisense probes for *Thylacin 1* were used: the first identical to the RNase protection probe (nucleotides 740-920) and the second derived from XhoI linearised pThyl-14.1, synthesised using T3 RNA polymerase (nucleotides 1-920). In each case sense control probes were synthesised from the same plasmids using the complementary RNA polymerase. A *Thylacin 2*-specific probe consisting entirely of 3′ UTR sequence was synthesised from PvuII linearised pThyl-4s1 XbaΔ (nucleotides 1068-1274) using T7 RNA polymerase. The X-Delta 2, X-ESR5 and N-tubulin probes were synthesised as previously described (Jen et al., 1997; Chitnis et al., 1995). Chromogenic reactions were performed using NBT/BCIP tablets (Boehringer).

Expression of synthetic RNA in embryos
For generating synthetic RNA in vitro, the coding region of *Thylacin 1* was inserted into pT7TS (Cleaver et al., 1996) producing a construct in which both the 5′ and 3′ untranslated regions of *Thylacin 1* were replaced by those of *Xenopus β-globin*. RNA was transcribed in vitro using T7 RNA polymerase from templates linearised with XhoI as described previously (Chambers et al., 1994). The templates for synthesis of nuclear lacZ (Turner and Weintraub, 1994), XNGNR1 (Ma et al., 1996), X-Su(H)DBM (referred to as X-Su(H)1DBM in Wettstein et al., 1997) and X-Su(H)Ank (referred to as X-Su(H)1/Ank in Wettstein et al., 1997) were as described previously. Injections were performed as described by Jen et al. (1997). For each set of experiments lacZ RNA alone was injected as a negative control.

RESULTS
Isolation of two related bHLH genes
We screened a *Xenopus* late neurula (stage 18) cDNA library using the yeast two-hybrid system to look for novel members of the basic helix-loop-helix (bHLH) family of transcription factors. Approximately 5x10⁶ yeast transformants were screened using the optimised procedure previously described (Sparrow et al., 1998) and three independent clones of the same gene were isolated. The longest of these (pThyl-14.1) was 920 nucleotides in length and contained a single continuous open reading frame, suggesting that it did not represent the full cDNA. The remainder of the *Xenopus* transcript was isolated by screening a neurula (stage 17) cDNA library (Kintner and Melton, 1987). Several clones representing the entire cDNA were isolated and sequenced (Fig. 1A). The cDNA contained a single open reading frame encoding a 308 residue protein. Sequence analysis of this protein revealed a bHLH domain, as predicted from the bait used in the yeast two hybrid screen. We named this gene *Thylacin 1*. A closely related gene, *Thylacin 2*, was also isolated from the neurula cDNA library. It shares 87% amino acid identity with *Thylacin 1* (Fig. 1B), but diverges significantly in the 3′ UTR (63% nucleotide identity).

The sequences surrounding the first in-frame methionine have a good match to the Kozak consensus (Kozak, 1987) and this is most likely to be the translation initiation site. In vitro translation of the full-length cDNAs confirmed that this methionine is able to function as an initiation codon (data not shown). Comparison of the deduced amino acid sequence with the database revealed several closely related bHLH family members (Fig. 1C). The bHLH regions of *Thylacin 1* and *Thylacin 2* were most similar to those of the mouse Mesp1 and Mesp2 proteins (Saga et al., 1996, 1997; 68% identity). Less closely related proteins included several involved in neurogenesis: *Xenopus* NGNR1 (Ma et al., 1996; 45% identity), mouse NSCL/HEN 1 and 2 (Begley et al., 1992; Brown et al., 1992; 40% identity); as well as two mouse proteins expressed in early mesoderm: bHLH-EC2/mesod-1/paraxis (Quertermous et al., 1994; Blanar et al., 1995; Burgess et al., 1995; 38% identity) and scleraxis (Cserjesi et al., 1995; 40% identity). In contrast, regions of *Thylacin 1* and 2 located outside of the bHLH domain had no significant homologies with any of these proteins, nor any other proteins in the database.

The lack of an in-frame stop codon upstream of the first methionine codon suggested neither cDNA contains the full 5′ end of the RNA. Northern analysis and primer extension were used to investigate whether a significant amount of the transcript was missing from our cDNA clones. From these results we estimated that our *Thylacin 1* clone lacks 167 bp of 5′ UTR (data not shown). We attempted to clone this region by rescreening the early neurula library and by 5′ RACE, but were unable to generate clones encoding these sequences.

Expression of the *Xenopus Thylacin 1* gene during embryonic development
We examined the distribution of *Thylacin 1* transcripts using RNase protection. To minimise possible cross hybridisation with closely related transcripts (such as those from *Thylacin 2*) we used a probe comprising nucleotides 740-920 of the *Thylacin 1* cDNA. This protects 180 nucleotides of *Thylacin 1*, but a maximum of 80 nucleotides from *Thylacin 2*. No maternal *Thylacin 1* transcripts were detected in the unfertilised egg but zygotic expression was readily detected from the late gastrula stage onwards (Nieuwkoop stage 12.5; Fig. 2A, lane 6). Using long exposures, *Thylacin 1* transcripts were first detected in the blastula embryo (stage 8). The level of transcripts increased through subsequent development reaching a peak at neurula stages (stages 14-17; lanes 7 and 8). Transcript levels declined later in development and by the swimming tadpole stage (stage 34; lane 11) were only detectable after prolonged exposure of autoradiograms (data not shown). The same probe was used to assay RNA derived from a number of adult tissues (Fig. 2B), however none of these tissues tested had detectable levels of transcripts, even after prolonged exposure of autoradiograms.
Fig. 1. Sequence of the *Xenopus* Thylacine 1 and Thylacine 2 genes. (A) *Xenopus Thylacine 1* cDNA sequence and deduced amino acid sequence. The amino acids in bold represent the basic helix-loop-helix (bHLH) domain. (B) Comparison of the amino acid sequences of Thylacine 1 and Thylacine 2. Dots indicate amino acid identity, dashes represent gaps introduced to maximise the alignment and black shading indicates the bHLH domain. (C) Comparison of Thylacine 1 and Thylacine 2 bHLH domains with other members of the bHLH family. Black shading indicates amino acid identity with Thylacine 1. Sequences were aligned using the MegAlign program (DNASTAR Inc.). GenBank accession numbers for the Thylacine 1 and Thylacine 2 nucleotide sequences are Y14446 and Y14447 respectively.
Localisation of *Thylacine* transcripts within the embryo

*Thylacine 1* expression was examined in the embryo by RNA whole-mount in situ hybridisation. We were unable to localise transcripts in blastula and gastrula stage embryos, probably as a result of the relatively low levels present. *Thylacine 1* RNA was first detected at late neurula (stage 17) in stripes of cells located on either side of the neural tube in the middle of the embryo (Fig. 3A, B). As the embryo developed, the position of the stripes shifted progressively towards the posterior of the embryo (Fig. 3C-H) and by tadpole stages were localised in the tailbud (Fig. 3I). The number of stripes varied between one and three, but two were most often observed. We also investigated the distribution of *Thylacine 2* transcripts using a specific probe derived entirely from 3’ UTR. This showed an identical expression pattern to that of *Thylacine 1* (data not shown).

To identify the precise location of transcripts, embryos stained in whole-mount were sectioned along the longitudinal axis (Fig. 4B). Strikingly, *Thylacine 1* expression was not detected in the somites, but in the unsegmented presomitic mesoderm in a similar pattern to that observed for X-Delta-2 (Jen et al., 1997), and X-ESR5, a gene of the hairy class (W.-C. Jen and C. Kintner, unpublished observations). Expression seemed to mark a segmental pattern that corresponds to the future somites. In *Xenopus* embryos, a somite consists entirely of mononucleated myotomal cells that span each segmental unit. These form by a morphological process in which cells segregate from the paraxial mesoderm and rotate through 90 degrees (Hamilton, 1969; Youn and Malacinski, 1981). Prior to rotation, each prospective segment consists of approximately 10 cells. This unique property of the *Xenopus* embryo makes the precise positioning of transcripts within somitomeres possible. In all cases, sections from 10-20 embryos were examined to confirm the location of the transcripts. In Fig. 4 brackets are used to indicate the relative positions of somitomeres (numbered) and mature somites (labelled in lower case letters). The forming somite (undergoing rotation) is designated somitomere 1. The expression of *Thylacine 1* was initially broad, filling nearly the whole of a somitomere (Fig. 4B, somitomere 4), but was restricted to the anterior half of the adjacent somitomere (Fig. 4B, somitomere 3). Rarely, a third stripe of expression was visible on the anterior edge of somitomere 2 (immediately posterior to the rotating somite).

In some cases a different number of stripes was visible on either side of the midline (Fig. 3A, indicated by the white arrow). This curious observation may be explained by the fact that in *Xenopus* somites are formed in a staggered manner alternately on the left and right sides of the embryo and this asymmetry of expression may reflect this asynchrony of development.

**Thylacine 1** gene expression overlaps other genes involved in segmentation

The expression pattern observed for *Thylacine 1* and 2 was remarkably similar to that reported for the *Xenopus* Delta-2, *Hairy 2A* (Jen et al., 1997) and X-ESR5 genes (W.-C. Jen and C. Kintner, unpublished observations), as well as the zebrafish *her1* gene (Müller et al., 1996). A comparison of the expression pattern of *Thylacine 1* with that of X-Delta 2 and X-ESR5 by whole-mount in situ hybridisation at tadpole stages is shown in Fig. 4A. As discussed above, *Thylacine 1* was expressed in two strong stripes (Fig. 4A, labelled i and ii), with the more anterior stripe (stripe i) being restricted to the anterior half of somitomere 3 and the more posterior stripe (stripe ii) filling most of somitomere 4 (Fig. 4B). The expression pattern of X-Delta-2 (Fig. 4C) showed four stripes and a broad posterior domain in the tailbud region (labelled i-iv and P respectively). Longitudinal sections of embryos at this stage (Fig. 4D) revealed that stripe iv of X-Delta-2 expression appeared to fill all of somitomere 4. Expression was then downregulated in a posterior to anterior progression within each prospective segment, resulting in a narrow stripe of X-Delta-2-expressing cells at the anterior edge of a somitomere. Stripes ii and iii are in somitomeres 2 and 3 respectively. Finally, stripe i of X-Delta-2 expression was restricted to the anteriolateral portion
of the somite undergoing rotation (somitomere 1). The exact location of the broad posterior domain was difficult to determine because the curvature of the embryo made true longitudinal sections impossible to generate. However, analysis of sections from 10-20 embryos gave the impression that it is separated from stripe iv by a distance equivalent to a little more than a single somitomere. X-Delta-2 was also expressed in a subset of cells in the neural tube, a site where no expression of Thyacine can be detected. X-ESR5 was expressed in two stripes and a broad posterior domain in the tailbud region (Fig. 4E, labelled i, ii and P respectively). Sections in the longitudinal plane showed that the two stripes of X-ESR5 expression were present in somitomeres 3 and 4 (Fig. 4F). Again the exact location of the posterior domain stripe was difficult to determine, but it seemed to extend more anteriorly than that of X-Delta-2, with less than a single somitomere between it and stripe ii.

Thus the two stripes of Thyacine expression exactly overlap the two darkest stripes of both X-Delta-2 and X-ESR5. By contrast, no Thyacine expression was seen that overlaps with the broad posterior domains of X-Delta-2 or X-ESR5. Further support for this conclusion was provided by double labelled whole-mount in situ analysis of Thyacine 1 and X-Delta-2 expression (data not shown). The coincident expression of Thyacine and X-Delta-2 expression contrasts with that of the Hairy 2A gene which is expressed in a complementary pattern in the posterior portion of somitomeres 2 and 3 (Jen et al., 1997).

**Thylacine 1 is a potent activator of transcription**

On the basis of expression pattern, it might seem that Thyacine is another member of the Enhancer-of-split related (ESR) family of bHLH transcriptional repressors since it has similarities to Hairy-2A, X-ESR5 and her-1. However it lacks two of the structural characteristics of this family, namely the conserved proline in the basic region and the C-terminal WRPW tetrapeptide necessary for interaction with the corepressor groucho (Paroush et al., 1994). Indeed, as described above, Thyacine 1 is structurally more closely related to Xenopus NGNR-1 a proneural transcriptional activator, than to the ESR family. To test whether Thyacine could act as an activator of transcription we used a yeast assay system. A series of amino- and carboxy-terminal deletions of Thyacine 1 fused to the Gal 4 DNA binding domain were transformed into a yeast strain (Y190) containing a lacZ reporter gene driven by multiple copies of the Gal 4 operator. β-galactosidase expression levels were assayed by liquid culture assay (Fig. 5). The full length protein was found to be able to activate transcription strongly, as predicted. Progressive removal of the carboxy-terminal 15 amino acids (to Thyacine 1-293) had little effect on transcriptional activity. However deletion of a further 16 amino acids (Thylacine 1-277) reduced transcription levels to background. This suggests a sharp carboxy-terminal boundary for the transcriptional activation domain, lying between amino acids 277 and 293. Removal of the amino-terminal 191 residues resulted in a two-fold increase in activity. This may indicate the presence of an inhibitory domain at the amino terminus, but it may also result from differences in stability that may exist between the different constructs. Further deletion to residue 234 returned activity to full length levels and removal of a further 31 residues (Thylacine 265-308) dropped activity to background levels. Together, these results indicate that full transcriptional activation requires amino acids 234 to 293 of Thyacine 1. Database searches using this domain did not reveal any significant homologies with any other proteins.

**Ectopic expression of Thyacine 1 produces segmentation defects**

By analogy to Xenopus Delta-2, the expression pattern of Thyacine 1 suggested that it might be involved in the process of segmentation of paraxial mesoderm. To test this hypothesis, we investigated the effects of overexpression of the protein in Xenopus embryos. Since Thyacine 1 is expressed in a dynamic and transitory fashion, it is likely that the transcript is unstable. To overcome this potential problem, we substituted the natural 5’ and 3’ UTRs with those derived from Xenopus β-globin. Synthetic Thyacine 1 RNA was microinjected into one cell at the two cell stage, leading to ectopic expression in one half of the embryo. The uninjected half of the embryo acted as a control. The injected side was marked by co-injection with nlacZ transcripts (which encodes a nuclear-localised form of β-galactosidase). Injected embryos were examined by sectioning along the longitudinal axis after staining in whole-mount with X-gal and the 12/101 monoclonal antibody, which recognises an epitope present on the surface of differentiated...
Thylacine 1 function in Xenopus segmentation

muscle cells (Kintner and Brockes, 1985). In embryos ectopically expressing Thylacine 1, 12/101 antibody staining appeared to be the same on both the injected and control sides, suggesting that overexpression of Thylacine 1 did not affect myotomal differentiation in the paraxial mesoderm (Fig. 6B,C). However, Thylacine 1 had a profound effect on the pattern of segmentation. In nlacZ RNA-injected embryos, the myotomal nuclei line up in each somitic unit (Fig. 6A). By contrast, in embryos injected with both Thylacine 1 and nlacZ the position of the somitic nuclei appeared randomised on the injected side suggesting that the segmentation process had not occurred properly (Fig. 6B,C). In three independent experiments, 35/37 embryos showed segmentation defects, whereas no such defects were observed in 24 embryos injected with nlacZ alone. This phenotype was very similar to the effects seen when the Notch signalling pathway was disrupted in Xenopus embryos by ectopic expression of dominant negative forms of both X-Delta-2 and X-Su(H) (Jen et al., 1997). The degree of the defects varied, perhaps reflecting the amounts of Thylacine 1 RNA expressed. Strikingly, the penetrance of the segmentation defect varied along the anterior-posterior axis. Some embryos had defective segmentation in the anterior somites but not in the posterior, more recently formed, somites (Fig. 6B), probably due to failure of the injected RNA to persist beyond early tailbud stages.

As a test of the specificity of the defects produced by the ectopic expression of Thylacine 1 RNA on the paraxial mesoderm, we also examined the effects seen when the Notch signalling pathway was disrupted in Xenopus embryos by ectopic expression of dominant negative forms of both X-Delta-2 and X-Su(H) (Jen et al., 1997). The degree of the defects varied, perhaps reflecting the amounts of Thylacine 1 RNA expressed. Strikingly, the penetrance of the segmentation defect varied along the anterior-posterior axis. Some embryos had defective segmentation in the anterior somites but not in the posterior, more recently formed, somites (Fig. 6B), probably due to failure of the injected RNA to persist beyond early tailbud stages.

As a test of the specificity of the defects produced by the ectopic expression of Thylacine 1 RNA on the paraxial mesoderm, we also examined the effects of ectopic Thylacine 1 expression on the ectoderm (Fig. 7). Previous studies have shown that ectopic expression of the neural bHLH proteins in the ectoderm results in the formation of ectopic primary neurons. For example, when XNGNR1, a Xenopus gene belonging to the Neurogenin family, was ectopically expressed in Xenopus embryos, it induced ectopic primary neurons as marked by the expression of a neural tubulin gene called N-tubulin (Ma et al., 1996; Fig. 7C, 20/20 embryos). In contrast, when Thylacine 1 was ectopically expressed in the ectoderm, little or no effect on the formation of primary neurons was detected, as scored by N-tubulin expression (Fig. 7B, 14/16 embryos). Thus, this result indicates that the effects of ectopic Thylacine 1 expression on the paraxial mesoderm are relatively specific to this tissue, making it less likely that it acts by soaking up, for example, general factors required for the activity of these bHLH proteins, such as the ubiquitous heterodimer partner Xenopus E12.

Ectopic expression of Thylacine alters segmental expression of X-Delta-2 and X-ESR5

The results described above show that ectopic expression of Thylacine 1 causes segmentation defects in somitic tissue. This suggests that Thylacine 1 acts within the somitomeric region of the paraxial mesoderm to establish a segmental pattern, since this is where Thylacine 1 is normally expressed. As a further test of this idea, we also asked whether ectopic expression of Thylacine 1 disrupted the segmental pattern within the somitomeric region, by examining the expression of segmental markers such as X-Delta-2 and X-ESR5 in embryos injected with Thylacine 1 RNA (Fig. 8). In embryos injected with a mixture of nlacZ and Thylacine 1 RNAs, the segmental pattern of expression of X-Delta-2 was lost on the injected side, and this was also accompanied by a general decrease in the

Fig. 4. Comparison of Thylacine, X-Delta-2 and X-ESR5 expression patterns. Tailbud embryos (stage 27) were stained by whole-mount in situ hybridisation for Thylacine 1 (A,B), X-Delta-2 (C,D) or X-ESR5 (E,F) expression. Lateral views are shown in A,C and E, and sections of the same embryo along the longitudinal axis are shown in B,D and F respectively, viewed from a dorsal aspect. Expression is shown in dark blue and nuclei are counter-stained in red with Fuelgen. Brackets are used to indicate the relative positions of somitomeres (numbered) and mature somites (labelled in lower case letters). The forming somite (undergoing rotation) is designated somitomere 1. In all cases, sections from 10-20 embryos were examined to confirm the precise positioning of the transcripts relative to the somitomeres.
levels of X-Delta-2 expression on this side (Fig. 8D, 31/43 lacking segmental expression, 20/43 decreased expression). While the ectopic expression of Thylacine 1 disrupted the somitomeric expression of X-Delta-2, the tailbud expression domain of X-Delta-2 was not noticeably changed. Similarly, in embryos injected with a mixture of nlacZ and Thylacine 1 RNAs, the segmental expression of X-ESR5 in somitomeres 3 and 4 was severely disrupted while the tailbud expression of X-ESR5 was not noticeably changed (Fig. 8G, 35/47 embryos). In control embryos injected with nlacZ alone, both left and right sides of the neurula embryo expressed equivalent levels of X-Delta-2 (Fig. 8A,B, 38/38 embryos) or X-ESR5 (Fig. 8E,F, 46/46 embryos). Thus these results indicate that altered expression of Thylacine 1 in the paraxial mesoderm causes profound changes in the segmental expression of both X-Delta-2 and X-ESR5 within the somitomeric region, suggesting strongly that Thylacine 1 acts within this region to produce a segmental pattern. Moreover, since both X-Delta-2 and X-ESR5 may be components of the Notch pathway, these results indicate that Thylacine 1 may function during segmentation by regulating Notch signalling.

Alteration of the Notch signalling pathway affects Thylacine 1 expression

The results described indicate that Thylacine 1 is required for proper segmentation by interacting with the Notch signalling pathway within the somitomeric region of the paraxial mesoderm. Conversely, it is possible that the Notch signalling pathway is one component regulating the expression of Thylacine 1 since reciprocal interactions between bHLH genes and the neurogenic genes have been described during neural development. To study the effects of peturbation of the Notch pathway on Thylacine 1 gene expression, embryos were injected with RNAs encoding nlacZ and either activated (X-Su(H)/Ank) or dominant negative (X-Su(H)DBM) versions of X-Su(H) (Fig. 9). The expression of Thylacine 1, as well as X-Delta-2, in the injected embryos was determined at neurula stages by whole-mount in situ hybridisation. As previously described, inhibition of the Notch signalling pathway with the dominant negative X-Su(H) resulted in a loss of the segmental pattern of X-Delta-2 expression (Jen et al., 1997; Fig. 9B, 28/33 embryos) without affecting levels of expression of X-Delta-2 in the tailbud. By contrast, injection of the activated form of X-Su(H) eliminated X-Delta-2 expression in the tailbud (21/29 embryos) and also disrupted and severely reduced the segmental expression pattern (Fig. 7C, 29/29 embryos). Similar injection experiments analysed for Thylacine 1 showed that expression of the dominant negative X-Su(H) reduced overall levels of Thylacine 1 transcripts and also eliminated it’s striped pattern (Fig. 7E, 22/34 embryos with reduced staining, 19/34 embryos lacked striped pattern), whilst injection of the constitutively active form of X-Su(H) caused an almost complete lack of Thylacine 1 expression (Fig. 9F, 46/53 embryos). No changes in X-Delta-2 or Thylacine 1 expression were seen in 222 embryos injected with nlacZ alone (Fig. 9A,D). The loss of the segmental pattern is probably a secondary effect of disruption of the Notch pathway interfering with the formation of a prepattern within the paraxial mesoderm (Jen et al., 1997). The change in levels of expression produced by the X-Su(H) mutants suggests that, like X-Delta-2 (Wettstein et al., 1997) and X-Delta-2 (Jen et al., 1997), Thylacine 1 expression is determined in part by the Notch-signalling pathway.
DISCUSSION

Recent studies in chick, mouse, zebrafish and *Xenopus* suggest that the Notch family of receptors and their associated ligands are required for proper segmentation of the paraxial mesoderm. The exact requirement for Notch signalling during segmentation is not known, but it presumably needs to be restricted in a periodic pattern in order to be effective. Thus, to understand the role of Notch signalling during segmentation, it is essential to identify other components that are targets of, or regulate, Notch signalling within the presomitic mesoderm. Our results indicate that Thylacine is likely to be one such component that is required for segmentation because of its genetic interactions with the Notch signalling pathway within the somitomeric region of the paraxial mesoderm.

**Thylacine expression marks anterior half segments within the somitomeric region**

The striped expression pattern of Thylacine closely parallels that of *X-ESR5* and *X-Delta-2* within the presomitic mesoderm, by localising to the anterior half of somitomeres 3 and 4, thus marking an early periodic pattern corresponding to segmentation. The expression pattern of these three genes, which complements the expression of *Hairy2A* within the posterior half of somitomeres 2 and 3, further supports the idea that somitomeres are established as posterior and anterior half segments. Previous studies indicated that this half segmental pattern of gene expression within the somitomeric region depends on Notch signalling, since when Notch signalling is disrupted the expression of *X-Delta-2* and *Hairy2A* is then altered (Jen et al., 1997). The results reported here show a similar effect on the expression of *Thylacine 1*. Activating the Notch pathway suppresses the expression of both *Thylacine* and *X-Delta-2*. Both genes, however, are expressed when Notch signalling is inhibited in the paraxial mesoderm but their expression no longer shows a segmental periodicity. Thus, the expression of *Thylacine 1* depends on the formation of a segmental pattern within the somitomeric region via the Notch signalling pathway.

**Ectopic expression of Thylacine perturbs segmentation**

Ectopic expression of *Thylacine 1* by RNA injection produces defects that are consistent with a role in segmentation. Upon ectopic expression of *Thylacine 1*, the paraxial mesoderm is unable to segment properly, although myotomal differentiation appears to occur normally. These phenotypes are indistinguishable from those observed in embryos where Notch signalling has been perturbed. Not only did *Thylacine 1* expression not perturb myotomal differentiation of the mesoderm, which presumably involves the myogenic bHLH proteins such as MyoD, it also did not appear to alter the formation of primary neurons, which appears to involve neural bHLH genes such as *Neurogenin*. Thus, as detectable by ectopic expression *Thylacine 1* seems to have relatively specific effects on patterning of the paraxial mesoderm, rather than on cell differentiation as seen with related bHLH proteins.

The effects of ectopic *Thylacine 1* expression on segmentation can be observed within the somitomeric region where the formation of segments is marked by the expression of *X-ESR5* and *X-Delta-2*. Both genes have an expression domain in the tailbud which is not affected by ectopic
expression of *Thylacine 1*, while their segmental expression within the somitomeric region is severely affected. The results from this analysis are complicated and do not lead to a simple picture of how Thylacine 1 might regulate the expression of X-Delta-2 or X-ESR5 within the somitomeres. One likely source for this complexity is that while Thylacine expression affects that of X-Delta-2 and X-ESR5, the expression of these genes is likely to affect the expression of each other as components of the Notch signalling pathway. Thus a number of regulatory feedback loops are likely to occur which greatly complicates the interpretation of ectopic expression experiments. The nature of these regulatory loops and how they might produce a half segment pattern of expression within the somitomeric regions are important questions for the future.

**Is Thylacine 1 the Xenopus homologue of Mesp2?**

Although somites are morphologically distinct in different vertebrate species, it seems likely that the mechanisms that pattern the presomitic mesoderm into segments are evolutionarily conserved. Thus, it may be significant that the predicted amino acid sequence of Thylacine 1 and 2, are most closely related to those of mouse bHLH factors, Mesp 1 and 2. **Mesp1** transcripts are initially expressed in all nascent mesoderm at early streak stage, but at 7.5 dpc expression is downregulated and localised to the base of the allantois and the region of mesoderm that is fated to be lateral. From 3.0-9.5 dpc expression is only present in two faint bands on either side of the node (Saga et al., 1996, 1997). **Mesp2** transcripts are first detected at 8.0 dpc in a pattern overlapping that of Mesp1 and is present in later stages (until 12.5 dpc) in a single stripe in the presomitic mesoderm. This domain appears to overlap partially with the anterior portion of the Dll1 expression domain in the anterior of the somitomere adjacent to the somite undergoing formation (Saga et al., 1997), but the precise location within the somitomere has not yet been described. As mentioned above, this contrasts somewhat with the expression pattern of Thylacine which is expressed in the anterior portion of more than one somitomere. Targeted disruption of the Mesp2 gene produces defects in somite formation, which result in a fused vertebral column and dorsal root ganglia. Analysis of several markers of scleratome polarity (Mox-1, Pax-1 and Dll1, which are all normally expressed in the posterior half of the sclerotalomral component of the mature somite) suggest that the establishment of the segment polarity of the somite is impaired and that the anterior compartment takes on a posterior character. Interestingly, the myotome and dermamte develop with some segmentation suggesting that Mesp2 is not required for the generation of periodicity within the paraxial mesoderm, but is required for the subsequent forming of segmental boundaries. In addition, the expression of Notch 2 is absent and levels of Notch 1 are substantially downregulated in Mesp2 mutant mice suggesting interactions with the Notch signalling pathway.

Given the high degree of similarity between the amino acid sequences of the bHLH regions of Mesp2 and Thylacine 1, the fact that both genes are implicated in the process of vertebrate Notch-dependent segmentation and the observation that they are probably both involved in the specification of the anterior portion of the somitomere, it is likely that Thylacine 1 performs an analogous function to Mesp2. However the lack of amino acid sequence conservation outside the bHLH domain, the differences between the Notch ligands regulated (Dll-1 and X-Delta-2) and the differences in expression patterns between Thylacine and Mesp2 all suggest Thylacine 1 is a distinct member of the Mesp family. Thus this new family of bHLH transcription factors may consist of many members that act at different levels of a conserved mechanism for generating a segmental pattern in vertebrate embryos, in a manner similar to the well established roles of various transcription factors at different levels of the processes of myogenesis and neurogenesis.

This work was supported by the Medical Research Council and the British Heart Foundation (D. B. S., S. K., N. T. and T. J. M.) and the NIH (C. K.). W.-C. J. is the holder of a Markey fellowship. We wish to thank Sally Dunwoodie for critical comments on the text.

**REFERENCES**


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