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A novel family of T-box genes in urodele amphibian limb development and regeneration: candidate genes involved in vertebrate forelimb/hindlimb patterning

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SUMMARY

In certain urodeles, a lost appendage, including hand and foot, can be completely replaced through epimorphic regeneration. The regeneration process involves cellular activities similar to those described for embryogenesis. Working on the assumption that the morphological pattern specific for a forelimb or a hindlimb is controlled by different gene activities in the two limbs, we employed a mRNA differential display screen for the detection of candidate limb identity genes. Using this approach, we have isolated a newt gene which in regenerating and developing limbs reveals properties expected of a gene having a role in controlling limb morphology: (1) it is exclusively expressed in the forelimbs, but not hindlimbs, (2) during embryonic development its expression is co-incident with forelimb bud formation, (3) it has an elevated message level throughout the undifferentiated limb bud and the blastema, respectively, and (4) it is expressed only in mesenchymal, but not in epidermal tissues. This novel newt gene shares a conserved DNA-binding domain, the T-box, with putative transcription factors including the *Brachyury* (*T*) gene product. In a following PCR-based screen, we used the evolutionarily conserved T-box motif and amplified a family of related genes in the newt; their different expression patterns in normal and regenerating forelimbs, hindlimbs and tail suggest, in general, an important role of T-domain proteins in vertebrate pattern formation.

Key words: limb development, regeneration, pattern formation, T-box, retinoic acid, amphibian, urodele, *Brachyury*, *Notophthalmus*

INTRODUCTION

Adult urodeles, such as the newt (*Notophthalmus viridescens*), can replace lost appendages through the formation of a regeneration blastema. The blastema cells, which are derived by local dedifferentiation of adult mesenchymal tissue in the stump, proliferate and redifferentiate to replace the lost structure. Experiments in which nerves are deflected from the limb to the flank can induce ectopic limbs; the nature of the induced limb (arm or leg) is dependent upon the location to which the nerve is deviated, indicating the existence of limb-specific territories (Guyenot and Schotte, 1926; Guyenot et al., 1948; Kiortsis, 1953). Moreover, transplantation experiments indicate that blastema cells themselves carry the information that directs them to make the appropriate specific appendage (Stocum and Dearlove, 1972).

Limb regeneration and limb development are distinguished by the respective origins of the cells that make up the undifferentiated bud or blastema. However, regeneration uses mechanisms similar to development for limb outgrowth and pattern formation (Muneoka and Bryant, 1982; Muneoka and Sasson, 1992) resulting in a perfect phenocopy of the structures produced during embryogenesis. Recent studies of the developing limb (see Tabin, 1991 and Duboule, 1994 for reviews) as well as the regenerating limb (Savard et al., 1988; Tabin, 1989; Brown and Brockes, 1991; Simon and Tabin, 1993; Simon et al., 1995; Gardiner et al., 1995; Savard and Tremblay, 1995) identified homeobox-encoding genes as having important roles in limb patterning. While the same *Hoxa* and *Hoxd* genes are expressed in both the forelimbs and hindlimbs, different sets of *Hoxc* genes were found to be expressed in the forelimbs or in the hindlimbs (Savard et al., 1988; Tabin, 1989; Peterson et al., 1992, 1994; Simon and Tabin, 1993; Nelson et al., 1996) suggesting a possible instructive role in specifying the identity of anterior/posterior structures. However, none of these *Hox* genes are expressed ubiquitously throughout the limb bud. Thus, while the unique expression of *Hox* genes in the forelimbs or hindlimbs reinforce the hypothesis that limb

identity is controlled by differential gene expression, candidates for upstream regulators of limb identity remained elusive.

As a novel inroad to identification of such limb identity genes, we employed mRNA differential display (Liang and Pardee, 1992; Liang et al., 1993). We have optimized this technology to facilitate its use as a sensitive and efficient molecular screen for the detection of differentially regulated mRNAs (Simon and Oppenheimer, 1996) and obtained a library of cDNA-tags for genes predominantly expressed in forelimbs or hindlimbs. Making use of the possibility to study both limb development and regeneration in one system, we investigated one of the forelimb-specific isolates which, based on homology studies, contains a sequence motif encoding a novel DNA-binding domain, the T-box. This evolutionarily conserved peptide domain has been recently described in an ancient family of genes including the *Brachyury* (*T*) gene (Bollag et al., 1994). Subsequently, we used this T-box motif to screen for additional related genes and identified a family of novel T-box genes in the newt.

MATERIALS AND METHODS

Unless otherwise noted, all standard cloning techniques were performed according to Sambrook et al. (1989) and all enzymes and molecular biology reagents were obtained from Boehringer Mannheim Biochemicals.

Animals and treatment

Newt (*N. viridescens*) embryos were spawned and reared in the laboratory as described by Khan and Liversage (1995a,b). Adult newts were supplied by M. Tolley, Donelson, TN. Anesthesia, surgical procedures and retinoic acid (RA) treatments were as described (Simon and Tabin, 1993). Regenerating limbs were staged according to Iten and Bryant (1973). All tissue samples were quick frozen in liquid nitrogen and stored at –80°C prior to RNA preparation.

RNA preparation

Total RNA was isolated from frozen tissue by homogenizing the tissue in 4 M guanidinium thiocyanate, followed by ultracentrifugation through a 5.7 M CsCl cushion. Poly $(A)^+$ RNA was purified using the Poly(AT) tract System III (Promega, Madison, WI) following the manufacturer's instructions.

mRNA differential display

Differential displays (DD)s were performed as described (Simon and Oppenheimer, 1996). In short, for reverse transcription (RT) 1 μg of total DNA-free RNA was reverse transcribed using various oligo(dT)-anchored 3′ primers (see below). For PCR, 1/20 volume of the RT reaction was amplified in the presence of [^{35}S]dATP in a two-step PCR: 94°C for 30 seconds, ramp of 36°C to 40°C for 2 minutes and 72°C for 30 seconds for five cycles (low stringency), followed by 94°C for 30 seconds, 42°C for 2 minutes and 72°C for 30 seconds for 25 cycles (high stringency) with an extension at 72°C for 5 minutes. The PCR products were resolved on sequencing gels and exposed to Kodak XAR-5 film for 16 hours. Bands representing potentially differentially expressed mRNAs were excised from the gel and eluted in H_2O. The cDNA PCR products were then reamplified using DD fusion primers to incorporate M13 and reverse M13 (RM13) sequence tags at their 5′ and 3′ ends, respectively. RT and DD 3′ primers: $(T)_{12}$MN where M represents either G, A or C, and N represents either G, A, T, C. DD 5′ primers: 172: 5′-CATTCCTCTC; 177: 5′-CTGGATAGAG; 178: 5′-GCCTACAAGA; 179: 5′-AAGGCATCTG; 183: 5′-ACACCAATGC; 210: 5′-CACTTAGCAG; 211: 5′-TCTTGGTCAG; 222: 5′-ATGCTCTCAC; 223: 5′-TGTGACTGCA; 232: 5′-ATGGTCGTGT. DD fusion primers: Composite primers with M13 sequence tag (5′-TGTAAAACGACGGCCAGT) added to the 5′ end of the DD 5′ primers and RM13 sequence tag (5′-CAGGAAACAGCTATGACC) added to the 5′ end of the anchored oligo(dT) 3′ primers. All oligo primers were synthesized by the biopolymers facility of the Department of Genetics, Harvard Medical School, Boston, MA.

Isolation of cDNA clones and sequence analysis

Longer T-box containing cDNAs were isolated from a plasmid cDNA library derived from newt forelimb mid-bud-stage blastema mRNA (Simon and Tabin, 1993). The 211/169/350 DD fragment was used for the production of random primed probes to screen 10^6 colonies at high stringency by standard procedures. Twelve cDNA clones were isolated and partially sequenced; the two longest cDNAs (#3.1, 1.5 kb and #30.1, 1.7 kb) were fully sequenced on both strands using vector-derived and cDNA-specific primers. Double-stranded sequencing was performed employing Sequenase (US Biochemicals, Cleveland, OH) or using an ABI 373A DNA sequencing system with dye terminator chemistry as described by the manufacturer (Applied Biosystems, subdivision of Perkin Elmer, Foster City, CA). The tagged DD PCR products were directly sequenced on the ABI 373A DNA sequencer with M13 (sense strand) or RM13 (antisense strand) sequencing primers. Sequence data were analyzed using the sequence analysis software package (version 8.1), Genetics Computer Group, Inc. (University Research Park, Madison, WI) and the MacDNASIS sequence analysis software system (Hitachi, San Bruno, CA). Searches for related sequences were done through the BLAST (Altschul et al., 1990) network service provided by the National Center for Biotechnology Information.

Northern blot analysis

A Northern blot with 5 μg of poly $(A)^+$ RNA per tissue sample was prepared, hybridized and washed as described (Simon and Tabin, 1993). The filter was exposed to Kodak XAR-5 film for 6 days at –80°C with intensifying screens after hybridization to the newt *Tbox1* probe (cDNA #3.1), or for 30 minutes at room temperature after hybridization to the newt translation elongation factor 1-α (*EF-1α*) probe (Simon and Tabin, 1993). Before being reprobed, the blot was stripped of probe in boiling water.

RNase protections

RNase protection was performed as described (Simon and Tabin, 1993). In addition to the *NvTbox1* cDNA, a probe for the newt *EF1-α* (Simon and Tabin, 1993) was employed for normalization. Appropriate fragments of the cDNAs were derived by PCR and cloned into Bluescript KS+. Antisense RNA probes labeled with [α-^{32}P]UTP were hybridized to 5 μg of each total RNA sample. The reaction mixtures were treated with RNase, proteinase K and phenol extracted. The protected fragments were separated on sequencing gels and exposed to Kodak XAR-5 film for 2 days at –80°C with intensifying screens in experiments with the *NvTbox1* probe, or for 15 hours at room temperature in experiments with the *EF-1α* probe, respectively. All RNA samples were initially normalized by measurement of their absorbance at 260 nm. The autoradiographic intensity of the protected fragments was quantitated by use of a Fujix PhosphorImager. The signals were normalized with reference to the signals of the *EF-1α* probe for each sample.

Whole-mount in situ hybridization

To avoid potential cross-hybridization with other T-box mRNAs, a *NvTbox1*-specific subclone of 626 bp was constructed spanning nucleotide 472 to 1098 of the cDNA (Fig. 2). Digoxigenin-labeled RNA probes were synthesized using a Boehringer Mannheim DIG RNA-labeling kit. Whole-mount in situ hybridizations on various stages of newt larvae were carried out essentially as described by Gardiner et al. (1995). To improve visualization of the blue alkaline phosphatase reaction product over the pigment spots, embryos were

treated with 90% MeOH: 10% H_2O_2 at room temperature, prior to being cleared in methyl salicylate and photographed.

RT-PCR

For the amplification of novel T-box sequences with degenerate primers, 5 μg total RNA from forelimb blastemas, hindlimb blastemas and tail blastemas was reverse transcribed into cDNA using a first strand cDNA kit (Pharmacia) with oligo(dT) primers. For PCR, 1/15 volume of the RT reaction was used in a 20 μl reaction containing 1 μM of each degenerate primer, 10 mM Tris pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.1 mg/ml gelatin, 200 μM deoxynucleotides, 1 u *Taq* polymerase. Amplification conditions were 94°C for 2.5 minutes, followed by 40 cycles of 94°C for 1 minute, 54°C for 2 minutes and 60°C for 3 minutes. Amplified fragments were digested with *Cla*I and *Xho*I. Products of expected size (~260 bp) were gel purified and cloned into Bluescript KS+ (Stratagene, La Jolla, CA). Degenerate primers were directed against the peptide motifs as given in Fig. 6A; the sequences were as follows: T-box-FWD: 5′-CACATCGATG-TAC/TATICAC/TCCIGAC/TT/AC/GICC; T-box-REV: 5′-CACCTC-GAGTG/ATC/GG/ATTT/CTGG/ATAIG/CCIGTIAC; (I=inosine).

Quantitative RT-PCR

Quantitative RT-PCR was performed on cDNA generated from various newt tissues using the first strand cDNA kit (Pharmacia) and oligo(dT) primers as described above. Using cloned templates, PCR conditions were determined that allowed only amplification of the respective target gene with each set of T-box-specific primer pairs (data not shown). PCR was performed under constant conditions with a 'hot start': 1/15 volume of the RT reaction was used in a 20 μl reaction containing 1 μM of each forward (FWD) primer and reverse (REV) primer, 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 200 μM deoxynucleotides, 0.1 μM [α-^{32}P]dCTP (3000 Ci/mmol, Amersham, Arlington Heights, IL), 1 u *Taq* polymerase. Cycle conditions were 94°C for 2.5 minutes, followed by 26 to 32 cycles (*Tbox1* to *4*) or 10 to 16 cycles (*EF-1α*) of 94°C for 45 seconds, 72°C for 1 minute. Linear amplification was verified by running a range of different amplification cycles (data not shown). PCR products were separated on native 6% polyacrylamide, 5% glycerol gels and exposed to Kodak XAR-5 film for 7 hours at −80°C with intensifying screens. For quantitation, radioactive signals were scanned as total counts per minute using a Fujix PhosphorImager. The background levels of radioactivity were subtracted from each signal and the relative levels of expression in different tissues were determined by dividing the *T-box* signals by the signal obtained with *EF-1α* in a given reaction. Employed primer pairs specific for the individual genes were: *Tbox1*-FWD: 5′-GGATGCGACAACTTGTATCTTTCC (421), *Tbox1*-REV: 5′-GTCTCAGAAAAGACGTGAGTGCA (430); *Tbox2*-FWD: 5′-AGCAGATTGTGTCCTTCGACAAG (423), *Tbox2*-REV: 5′-GAAAGTCTTGAAGTTCTCCTCGG; *Tbox3*-FWD: 5′-GTCGTCAGTTTCGACAAACTTAAG (425), *Tbox3*-REV: 5′-AACGTCTTCACTCCATCTCCTACC (426); *Tbox4*-FWD: 5′-GCAAGTGATCAGCTTTGACAAACTC (427), *Tbox4*-REV: 5′-ATGGTTTCAGGGAAGGAGAAGGC (428); *EF-1α*-FWD: 5′-CCATGTGTGTGGAGAGCTTCTCA, *EF-1α*-REV: 5′-GGCTCTTGTGATGGACCCTAATG.

Table 1. Differential gene expression patterns observed by mRNA DD in normal and regenerating forelimbs, and normal and regenerating hindlimbs

Expression pattern		Primary isolates	Unique sequences
Expressed in	Not expressed in		
NHL	Elsewhere	27	21
BHL	Elsewhere	11	8
NFL	Elsewhere	13	8
NFL/BFL	NHL/BHL	10	6
BFL	NHL/BHL	18	16
DFL	NHL/BHL	5	2
RFL	NHL/BHL	16	5

DDs were performed with 10 different 5′ primers in combination with 12 poly(A) anchored 3′ primers (see Materials and methods). Primary cDNA PCR products were directly sequenced to eliminate redundant isolates. NHL, normal hindlimb; BHL, blastema hindlimb (proximal); NFL, normal forelimb; BFL, blastema forelimb (proximal); DFL, early digit stage regenerate (proximal); RFL, regenerated forelimb (proximal).

RESULTS

Identification of a forelimb-specific transcript by mRNA differential display

To identify transcriptionally regulated genes potentially involved in the patterning of forelimbs and hindlimbs, we compared mRNA differential display (DD) patterns for normal and regenerating forelimbs with those for normal and regenerating hindlimbs. We performed mRNA DDs with 12 individual 3′ primers in combination with 10 different 5′ primers and identified 100 cDNA PCR products that appeared to be differentially expressed between forelimbs and hindlimbs (Table 1). All DD products were eluted from the acrylamide gels, re-amplified and directly sequenced as described (Simon and Oppenheimer, 1996). This enabled us to eliminate redundant isolates and reduce the amount of DD fragments by approxi-

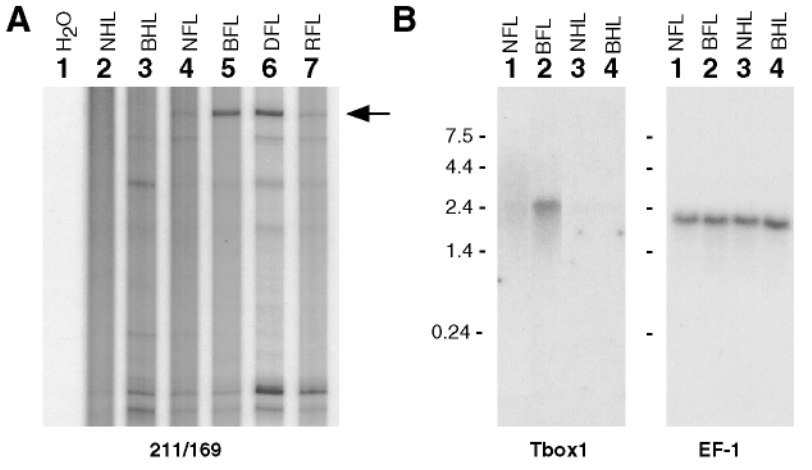

Fig. 1. Differential expression of the 211/169/350 isolate. (A) mRNA differential display using primer pair 211/169 with RNA from normal and regenerating forelimbs, and normal and regenerating hindlimbs. Lanes: (1) H_2O control; (2) normal hindlimb [NHL]; (3) blastema hindlimb [BHL]; (4) normal forelimb [NFL]; (5) blastema forelimb [BFL]; (6) digit-stage forelimb [DFL]; (7) regenerated forelimb [RFL]. The arrow on the right indicates the position of the differentially expressed 350 bp PCR product. (B) Northern blot analysis with cDNA probes specific for the 211/169/350 isolate (*Tbox1*) and *EF-1α*. Lanes: (1) normal forelimb [NFL]; (2) blastema forelimb [BFL]; (3) normal hindlimb [NHL]; (4) blastema hindlimb [BHL]. Size markers (in kb) of the BRL RNA ladder (BRL, Gaithersburg, MD) are on the left.

mately one third. Gene-specific primers were designed for seven isolates with a forelimb/hindlimb difference in expression and used for quantitative RT-PCR (data not shown). Only two isolates confirmed the gene regulation patterns observed in the DD, the remainder revealed expression in both forelimbs and hindlimbs. However, when we analyzed RNA samples representing a time course in regeneration, the majority of the isolates showed the expected differential expression (Simon and Oppenheimer, 1996). For this study, we selected one of the two forelimb-specific candidates, its characteristic expression profile is shown in Fig. 1A. The 350 bp cDNA PCR product, designated as 211/169/350, appeared as a weak band in normal forelimbs. During the course of regeneration, the band intensity showed a maximal level in the blastema and early digit stage and became weaker in later stages. However, the PCR product could not be detected in normal hindlimbs or hindlimb blastemas.

To confirm the differential regulation of the selected gene and to obtain an estimation of its message size, we performed northern blot hybridizations. Fig. 1B shows the northern analysis with RNA from unamputated (normal) forelimbs, blastema forelimbs, unamputated (normal) hindlimbs and blastema hindlimbs using cDNA probes specific for the 211/169/350 isolate, or as a control for the newt *EF-1α* (Simon and Tabin, 1993). The 211/169/350 specific probe detected a single band of ~2.8 kb in RNA from forelimb blastemas and very weakly in normal forelimbs, but no signal was visible in RNA from normal hindlimbs and hindlimb blastemas.

Newt *Tbox1* is a new member of the family of T-box genes

In order to isolate longer cDNAs, we used the DD fragment 211/169/350 as a probe to screen a forelimb blastema cDNA library. The longest of twelve independent cDNA isolates (#30.1, 1.7 kb) revealed an open reading frame of 411 amino acids and a 3′ untranslated portion of 498 bp (Fig. 2). The predicted protein contained a domain at its amino terminus, which showed a high degree of homology to proteins encoded by the following genes: *Drosophila optomotor blind* (*omb*) gene (Pflugfelder et al., 1992), the *Brachyury* or *T* gene of various species such as mouse (Herrmann et al., 1990), *Xenopus* (Smith et al., 1991) and zebrafish (Schulte-Merker et al., 1992), and the more recently described T-box genes in the mouse (Bollag et al., 1994). In their study, Bollag and colleagues (1994) described a family of embryonically expressed mouse genes that share a highly conserved protein motif, the T-domain, with otherwise unrelated proteins including Brachyury. Biochemical data on Brachyury has demonstrated

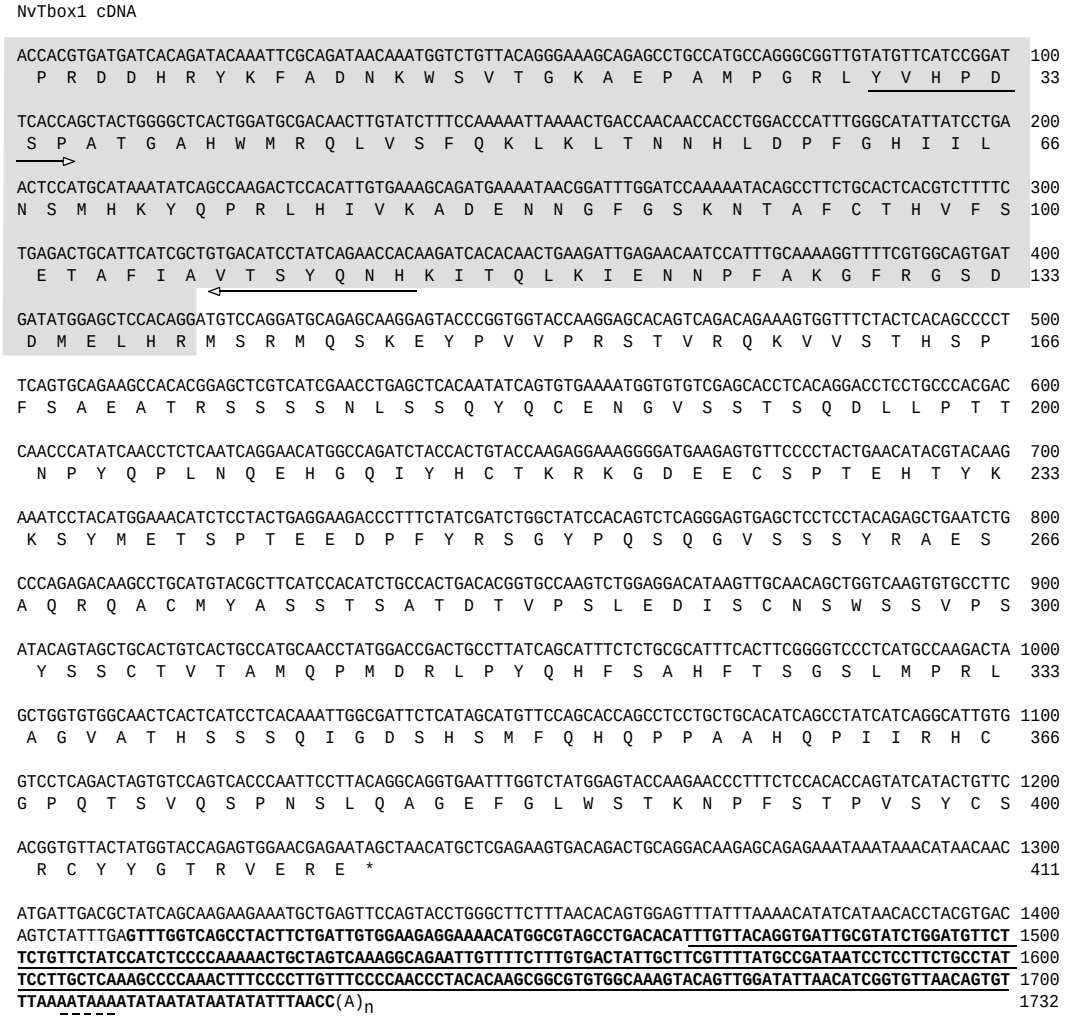

Fig. 2. Structure of the newt *Tbox1* cDNA. Nucleotide and predicted amino acid sequence of the composite newt *Tbox1* cDNA. The aminoterminal 139 amino acids of the conserved T-domain motif contained in the cDNA are shaded. Peptides selected for the design of degenerate oligonucleotides to amplify T-box sequences are indicated by arrows (see also Fig. 6A). The cDNA fragment used as RNase protection probe is underlined. Nucleotides originally PCR amplified in the DD screen are printed in bold. The asterisk indicates the predicted termination codon. The location of a potential polyadenylation signal (AATAAA) is indicated by a dashed underline. The sequence data for the newt T-box genes are available from GenBank; *NvTbox1*, accession number U64433; *NvTbox2*, accession number U65639; *NvTbox3*, accession number U65640; *NvTbox4*, accession number U65641.

DNA-binding activity of its T-domain (Kispert, 1995); however, it is not yet clear whether this property is also conserved in other T-domain proteins. The newt cDNA lacks a potential initiator methionine at its 5′ end and, by comparison to other T-domains, the conserved sequences span only 139 amino acids of the approximately 200 amino acids in other T-domain proteins. Thus, it appears that the cDNA clones obtained do not contain the complete sequence for this gene. This is also in agreement with the mRNA size of ~2.8 kb predicted from the northern blot (Fig. 1B). Beyond the T-box region, we could not detect any homology between the newt and other T-box genes described to date. Since there are no apparent orthologues in other species, we have named it *NvTbox1*.

Tissue distribution and temporal expression pattern of *NvTbox1* during limb regeneration

When we analyzed the expression pattern in normal and regenerating appendages as well as in different organs using the sensitive and quantitative method of RNase protection, we could extend the confirmation of a differential regulation of *NvTbox1* (Fig. 3). We found the gene to be expressed almost exclusively in the forelimb territory. In line with the DD data (Fig. 1A), we detected a faint protected band in RNA of normal forelimbs indicating a weak basal activity of this gene (Fig. 3B). After amputation at a proximal level (mid humerus), we found a strong induction of the message when the regenerates reached mid-bud-stage blastemas. In early digit-stage regenerates, we saw a significantly lower level of expression and, in fully regenerated forelimbs, the expression was further reduced and equivalent to the level detected in normal unamputated forelimbs. Note that the expression was highest in the blastema, the stage when the limb pattern is believed to be respecified. In contrast to the forelimb territory, we did not detect a protected band in normal hindlimbs and hindlimb blastemas or in normal tail and tail blastemas (Fig. 3C). Moreover, in experiments with RNA from a variety of organs and tissues, we could not detect protected fragments of the *NvTbox1* probe in any organ except heart (Fig. 3D). However, the signal was only visible after an extended exposure time of 7 days compared to 2 days with blastema RNA; thus, the expression of this gene in adult tissues is very limited.

Spatial expression pattern of *NvTbox1* in normal and regenerating tissues

To obtain further insight into the transcriptional regulation of

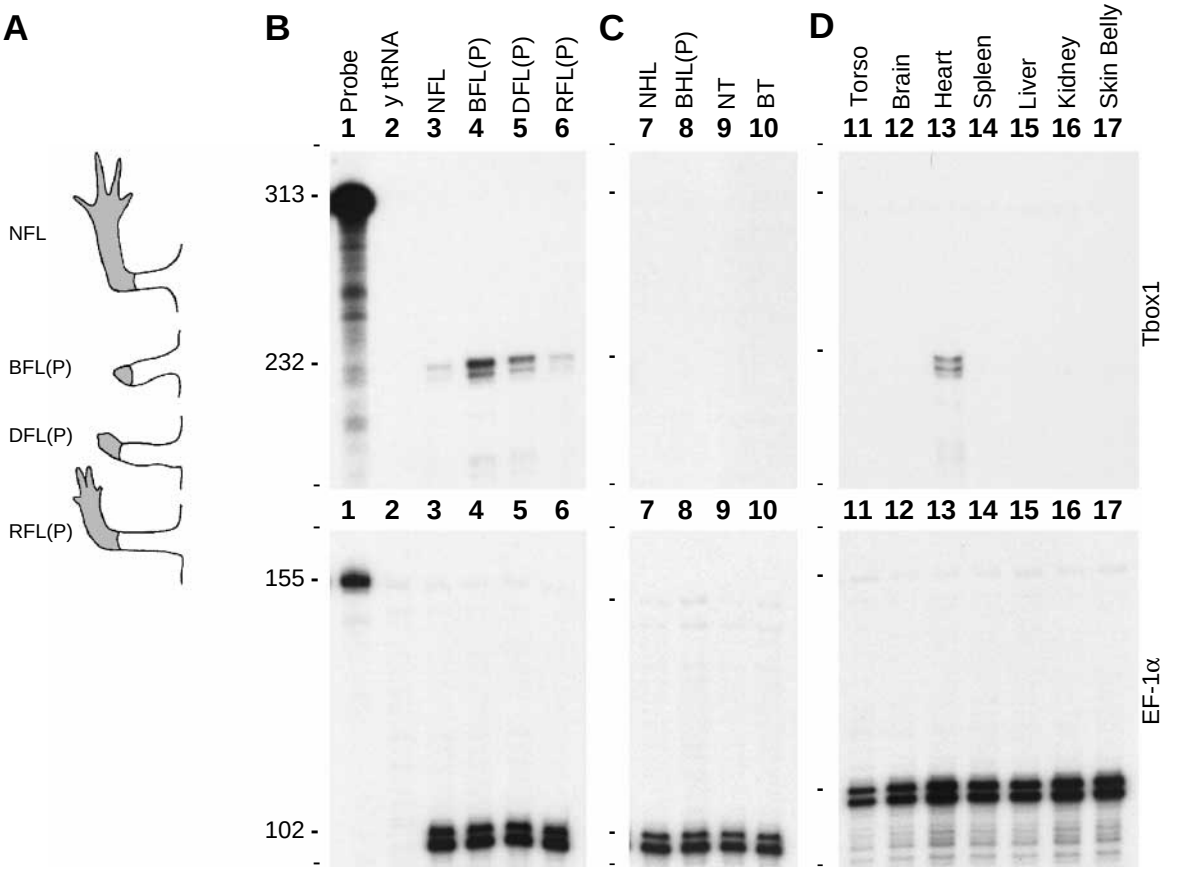

Fig. 3. Tissue distribution and temporal expression of *NvTbox1*. RNase protection analysis with RNA of the respective tissue samples employing the *NvTbox1*-specific probe and the normalizing newt *EF-1α* probe. (A) Schematic diagram representing the tissue (shaded) used to determine the temporal expression pattern. (B) Expression in normal and regenerating forelimbs. Lanes: (1) input probe; (2) yeast tRNA; (3) normal forelimb [NFL]; (4) proximal blastema forelimb [BFL(P)]; (5) proximal digit-stage forelimb [DFL(P)]; (6) proximal regenerated forelimb [RFL(P)]. (C) Expression in normal and regenerating hindlimbs and tail. Lanes: (7) normal hindlimb [NHL]; (8) proximal blastema hindlimb [BHL(P)]; (9) normal tail [NT]; (10) blastema tail [BT]. (D) Tissue distribution. Lanes: (11) whole torso; (12) brain; (13) heart; (14) spleen; (15) liver; (16) kidney; (17) skin belly. The size of the input probes and the respective protected fragments are indicated.

NvTbox1, we obtained a variety of tissue samples as indicated in Fig. 4A. Since northern (Fig. 1B) and RNase protection (Fig. 3) data indicated a restricted expression of *NvTbox1* in the forelimb territory, we tested how far the expression of the gene extends posteriorly along the flank by including surgically dissected flank tissue in our analysis. As shown in Fig. 4B, no protected fragment was detected in anterior or posterior flank tissue. Experiments in Fig. 3 revealed a basal expression of *NvTbox1* in normal forelimbs and an up-regulated expression in mid-bud blastemas. When examining expression profiles at various proximodistal levels of normal forelimbs, we observed a higher level of expression in proximal than distal limb segments. Comparing proximal versus distal blastemas, we also found a 2- to 3-fold higher level of message in proximal blastemas. Transplantation experiments have shown that the information for producing limb pattern resides in the mesodermally derived 'blastema' cells rather than in the wound epidermis that covers them (Stocum and Dearlove, 1972). Therefore, proximal forelimb mid-bud blastemas were dissected into epithelial and mesenchymal fractions. As would be expected for genes that are involved in the determination of pattern during limb regeneration, *NvTbox1* expression was found exclusively in the blastema mesenchyme (Fig. 4C).

The finding that *NvTbox1* appeared to be expressed at a higher level in proximal versus distal blastemas raised the possibility that it might be involved in providing positional information to the blastema cells. The regenerating limb system facilitates experimental testing of this hypothesis by using retinoic acid (RA) to reset the positional value of a distal blastema to a more proximal one (Stocum and Crawford, 1987). Newt forelimbs were amputated distally at the level of mid-radius/ulna, and 5 days later the animals received an intraperitoneal injection of a proximalizing dose of RA or the vehicle DMSO alone. Blastemas were harvested and analyzed for *NvTbox1* expression at two time points: 7 days after RA treatment, at the end of the delay period in regeneration provoked by RA (Maden, 1983) and when they had reached the mid-bud stage: 20 days after RA treatment or 15 days after

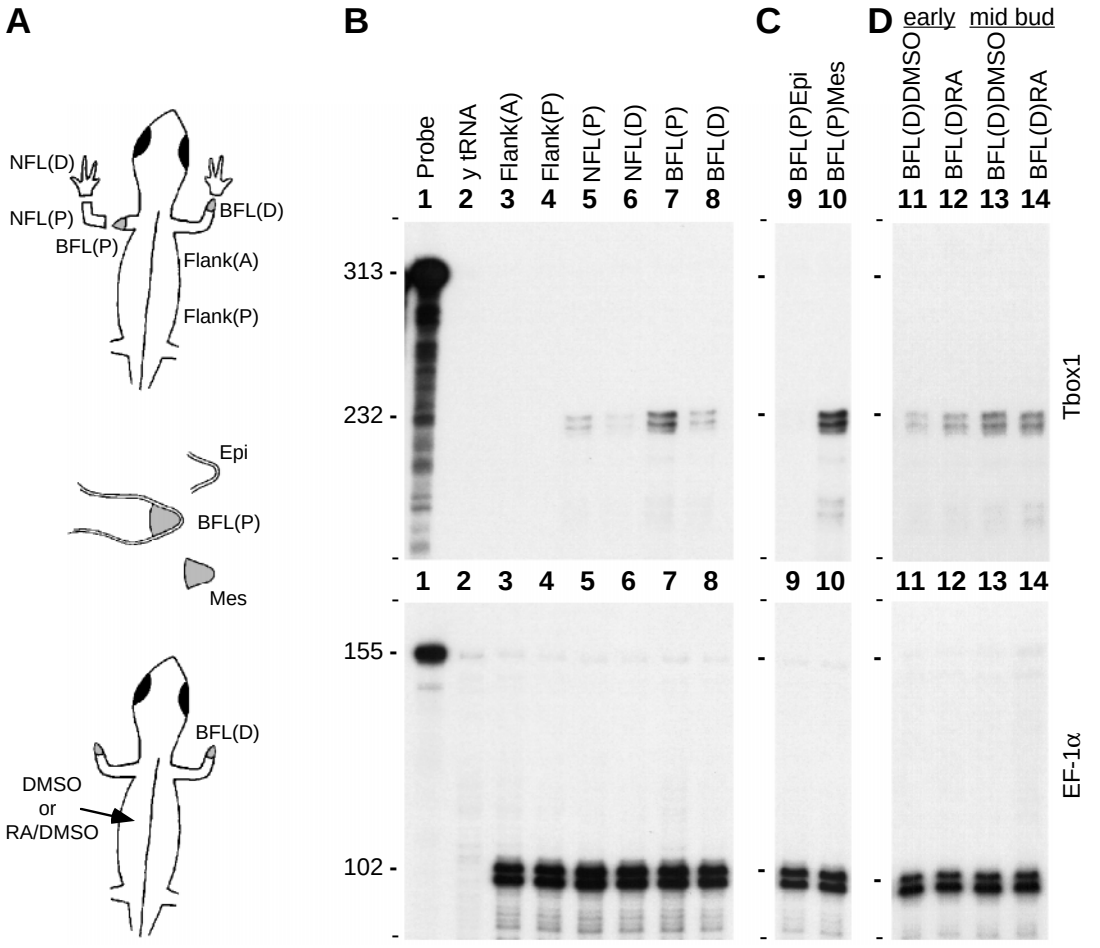

Fig. 4. Spatial expression pattern of *NvTbox1*. RNase protection analysis with the *NvTbox1*-specific probe and the *EF-1α* probe. (A) Schematic diagram of dissection of tissues for RNA preparation. (B) Proximal/distal and anterior/posterior expression patterns in normal and regenerating tissues. Lanes: (1) input probe; (2) yeast tRNA; (3) anterior flank tissue [Flank (A)]; (4) posterior flank tissue [Flank (P)]; (5) proximal normal forelimb [NFL(P)]; (6) distal normal forelimb [NFL(D)]; (7) proximal blastema forelimb [BFL(P)]; (8) distal blastema forelimb [BFL(D)]. (C) Epidermal/mesenchymal expression. Lanes: (9) proximal blastema forelimb - epidermis [BFL(P)Epi]; (10) proximal blastema forelimb - mesenchyme [BFL(P)Mes]. (D) Expression after retinoic acid treatment. Distal forelimb blastemas [BFL(D)]. Lanes: (11) DMSO group, early; (12) RA group, early; (13) DMSO group, mid-bud; (14) RA group, mid-bud. The size of the input probes and the protected fragments are shown.

DMSO treatment (Fig. 4D). The early and mid-bud blastemas differed in their response to RA. In mid-bud-stage blastemas RA seemed to have no effect on the expression level of *NvTbox1*. However, early blastemas showed an increased level of expression, approximately 2-fold higher as compared with the DMSO-treated control animals. Within this time window, RA is able to induce the up-regulation of *NvTbox1*. The results demonstrate that proximalization of distal blastemas by RA correlates with a proximalized expression level of *NvTbox1* transcripts at a time when blastema cells accumulate and start proliferating.

Spatial expression pattern of *NvTbox1* in developing forelimbs

To appreciate the three-dimensional expression of *NvTbox1*, we performed whole-mount in situ hybridizations on various stages of developing newt forelimbs. We did not detect a hybridization signal in the prospective forelimb field at any stage prior to the emergence of a morphologically defined limb bud (Fig. 5A). With the finer resolution of in situ analysis, we were able to localize gene expression to the forelimb buds just as they became discernable in larval development (Fig. 5B). Interestingly, *NvTbox1* gene activity was also evident in the distal portion of the developing tail at the time of forelimb bud emergence (Fig. 5B) whereupon tail expression gradually reduced with further embryogenesis. A control hybridization with a sense riboprobe did not result in any staining above background (Fig. 5C). When the limbs had elongated to a rod-shape structure, *NvTbox1*-specific signal appeared to be distributed evenly throughout the mesenchyme forming a distinct boundary at the base of the developing limb (Fig. 5D). The tail staining was virtually absent at this developmental stage (data not shown). The limb mesenchyme-specific signal remained as embryogenesis progressed through the palette (Fig. 5E) and digit-forming (Fig. 5F) stages. Additional gene activity in the head, pharynx and lower jaw regions appeared to be associated with areas rich in mesenchyme (Fig. 5D-F). In all cases of forelimb development from limb bud emergence (Fig. 5G)

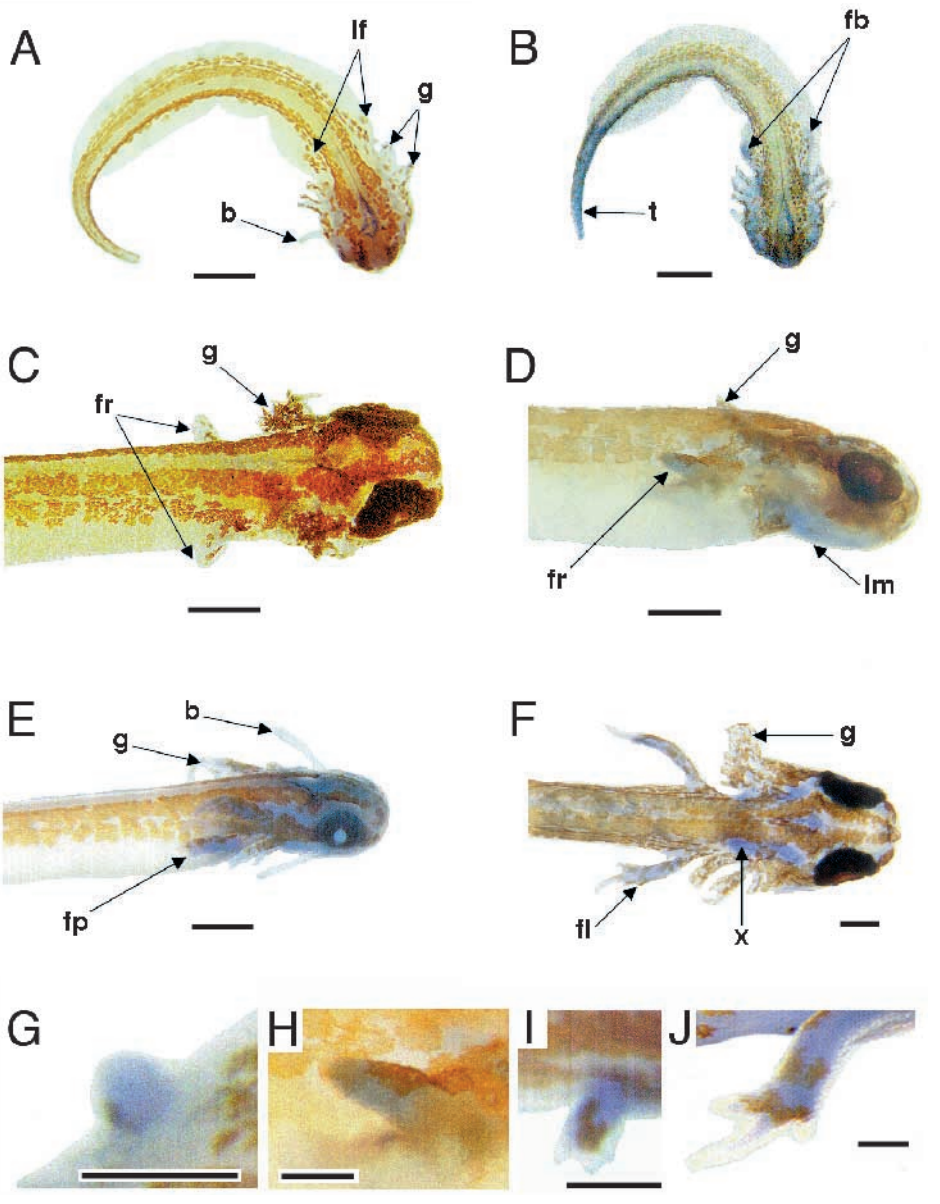

Fig. 5. Expression of *NvTbox1* in developing newt forelimbs as visualized by whole-mount in situ hybridization. *NvTbox1* expression pattern in successive stages of newt larval development. (A) Dorsal view of embryo prior to the emergence of forelimb buds. Forelimb field (lf), balancer (b), developing gills (g). (B) Dorsal view of embryo at the time the forelimb buds form. Forelimb buds (fb), developing tail (t). (C) Control sample hybridized with sense RNA probe. Lateral, slightly dorsal view of an embryo with rod-shape forelimb buds (fr). Gills (g). (D) Lateral view of embryo of comparable stage as in C. Rod-shape forelimbs (fr), gills (g), developing lower mandible (lm). (E) Lateral, slightly dorsal view of embryo with forelimbs developed to a palette stage (fp), balancer (b), gills (g). (F) Dorsal view of embryo with developing limb digits. Forelimb (fl), mesenchymal tissue in head region (x). (G) Higher magnification of an early forelimb bud similar to that depicted in B. (H) Higher magnification of a rod-shape forelimb bud as seen in D. (I) Higher magnification of a palette-shape forelimb similar to E. (J) Higher magnification of a 3-digit forelimb comparable to F. Note that blue staining in the flank is the result of signal in the heart and trapped reaction product in the gut seen through the transparent body wall. Probe-specific staining is given as the blue alkaline phosphatase reaction product. Brownish staining is caused by pigmentation. Anterior is to the right in all images. Scale bars: 0.5 mm (A-F); 0.2 mm (G-J).

through rod (Fig. 5H), palette (Fig. 5I) and digits (Fig 5J), careful inspection revealed the hybridization signal localized in mesenchyme tissue, but not in the almost translucent epithelium covering it.

Identification of a family of novel T-box genes in the newt

Having identified a novel, differentially regulated T-box gene in the newt, we next examined the possibility that more family members might be expressed during limb and tail regeneration. To this end, we designed degenerate oligonucleotides against the most conserved T-domain regions (Fig. 6A) to identify additional T-box-containing genes using a PCR-based strategy. We performed low-stringency PCRs on cDNA of forelimb, hindlimb and tail blastemas, and cloned amplification products of approximately 260 bp, a size predicted from the other T-box motifs. Sequencing revealed four distinct T-box sequences in the PCR clone pool including the previously isolated *NvTbox1*. Fig. 6A shows the homology alignment of the amplified portions of the four newt gene products together with corresponding sequences of the most related genes in other species, mouse *Tbx1*, *Tbx4* and *Tbx5*. Extensive sequence conservation among all of these cDNA PCR products identified the newly cloned genes as members of the T-box family. Interestingly, within the amplified region, the putative NvTbox1 protein shares 98% of its amino acids with Tbx5 and 96% with Tbx4. However, NvTbox2 revealed a 94% identity to mouse Tbx1. NvTbox3 and NvTbox4 are also more closely related to mouse Tbx1 than to any other known T-domain, although they have only 58% and 61%, respectively, of their residues in common with Tbx1. However, the deduced peptides, which share 82% sequence identity, both contain a unique insertion of four amino acids (position 145 to 149), which distinguishes them from all other known T-domains (see also Kispert, 1995).

To appreciate the evolutionary conservation and relationship of the various T-domain peptides to each other, we performed a phylogenetic tree analysis (Fig. 6B). The similarity plot revealed three major branches. One branch consisted of the Brachyury homologs of chick, mouse, *Xenopus*, zebrafish, *Drosophila* and Ascidia, plus a recently reported brain-specific T-domain in mouse and human (Bulfone et al., 1995). Another branch was made up of three of our novel newt gene products,

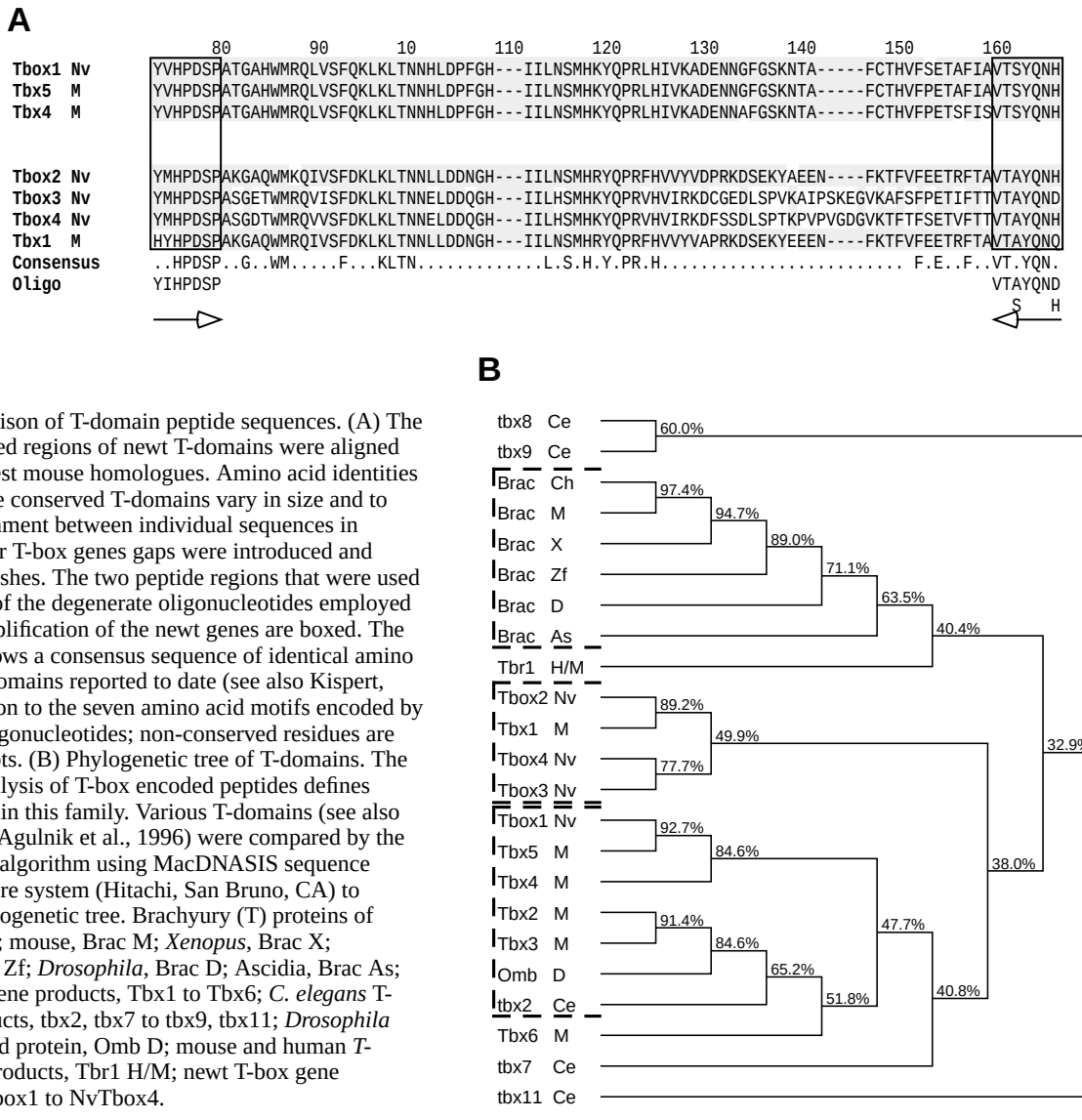

Fig. 6. Comparison of T-domain peptide sequences. (A) The highly conserved regions of newt T-domains were aligned with their closest mouse homologues. Amino acid identities are shaded. The conserved T-domains vary in size and to maximize alignment between individual sequences in relation to other T-box genes gaps were introduced and indicated by dashes. The two peptide regions that were used for the design of the degenerate oligonucleotides employed in the PCR amplification of the newt genes are boxed. The bottom line shows a consensus sequence of identical amino acids in all T-domains reported to date (see also Kispert, 1995) in addition to the seven amino acid motifs encoded by the selected oligonucleotides; non-conserved residues are indicated by dots. (B) Phylogenetic tree of T-domains. The dendogram analysis of T-box encoded peptides defines subgroups within this family. Various T-domains (see also Kispert, 1995; Agulnik et al., 1996) were compared by the Higgins-Sharp algorithm using MacDNASIS sequence analysis software system (Hitachi, San Bruno, CA) to generate a phylogenetic tree. Brachyury (T) proteins of chick, Brac Ch; mouse, Brac M; *Xenopus*, Brac X; zebrafish, Brac Zf; *Drosophila*, Brac D; Ascidia, Brac As; mouse T-box gene products, Tbx1 to Tbx6; *C. elegans* T-box gene products, tbx2, tbx7 to tbx9, tbx11; *Drosophila* optomotor-blind protein, Omb D; mouse and human *T-brain-1* gene products, Tbr1 H/M; newt T-box gene products, NvTbox1 to NvTbox4.

NvTbox2, NvTbox3 and NvTbox4, grouped together with mouse Tbx1. A third branch contained the mouse Tbx2/3/4/5 subfamily (Agulnik et al., 1996) together with the forelimb-specific newt gene product NvTbox1. This newt sequence seemed to be most homologous to mouse Tbx5 and Tbx4 (see also Fig. 6A). However, whether the newt gene product represents a homolog of mouse Tbx5 or Tbx4 must await additional sequence information for the mouse genes beyond the conserved T-box.

Expression patterns of newt T-box family members

In order to examine the expression of the additional isolates during limb and tail regeneration, we carried out quantitative RT-PCR on various RNA samples (Fig. 7). As expected from the RNase protection data, the *NvTbox1*-specific primers produced a weak band in normal unamputated forelimbs and a considerably stronger band in the respective forelimb blastemas. The three new T-box isolates revealed a different expression pattern in comparison with the original isolate *NvTbox1* and to each other. *NvTbox2* was found to have a basal expression in all appendages, but revealed an elevated expression level in normal hindlimb tissue. Interestingly, the two other isolates, *NvTbox3* and *NvTbox4*, demonstrated maximal expression in hindlimb blastemas. Although we found *NvTbox3* to be expressed in both limbs and normal tail, it showed a significant up-regulation in hindlimb blastemas, comparable to the induction of *NvTbox1* in forelimb blastemas. *NvTbox4* seemed to be expressed at a low level in both normal forelimbs and hindlimbs and very weakly in normal tail. However, this gene was up-regulated in mid-bud blastemas of both limbs, approximately 2-fold stronger in hindlimbs as compared to forelimbs. Currently, we are examining the expression of these novel T-box genes in other tissues, but no transcriptional activity was detected in the liver, a physiologically very active organ.

DISCUSSION

The major difference between forelimbs and hindlimbs is expected to be one of pattern, rather than of cell types. Our demonstration of significant differences in gene expression using mRNA DD of forelimbs and hindlimbs strongly suggests that differential gene regulation plays an important role in controlling limb morphology. Our differential expression screen identified a novel member of a recently reported family of genes that share a single structural motif, the T-box (Bollag et al., 1994). Subsequently, we employed this evolutionarily conserved motif to identify a family of related newt genes (this report and unpublished data). The limb-specific expression patterns of individual members of this family suggest they may be candidates for the control of forelimb-specific and hindlimb-specific morphology in vertebrates. Making use of recent achievements in breeding newts in the laboratory (Khan and Liversage, 1995a,b), we performed a study on the original forelimb-specific gene comparing limb development and regeneration.

To date, phylogenetic studies have identified the T-domain in a variety of otherwise unrelated proteins in different species ranging from nematodes to human. The prototypical member of this family, the original *T* gene or *Brachyury* in the mouse (Herrmann et al., 1990), has been shown to encode a transcription factor composed of an amino terminal DNA-binding domain (DBD), termed the T-domain, and a novel type of transactivation domain (Kispert et al., 1995). The newt *NvTbox1* contains the conserved motif for the DBD; however, we did not find evidence for a structurally conserved transactivation domain downstream of the DBD. This lack of sequence conservation in the carboxyterminal portion is characteristic of all other T-domain proteins. Thus, it is possible that the T-box

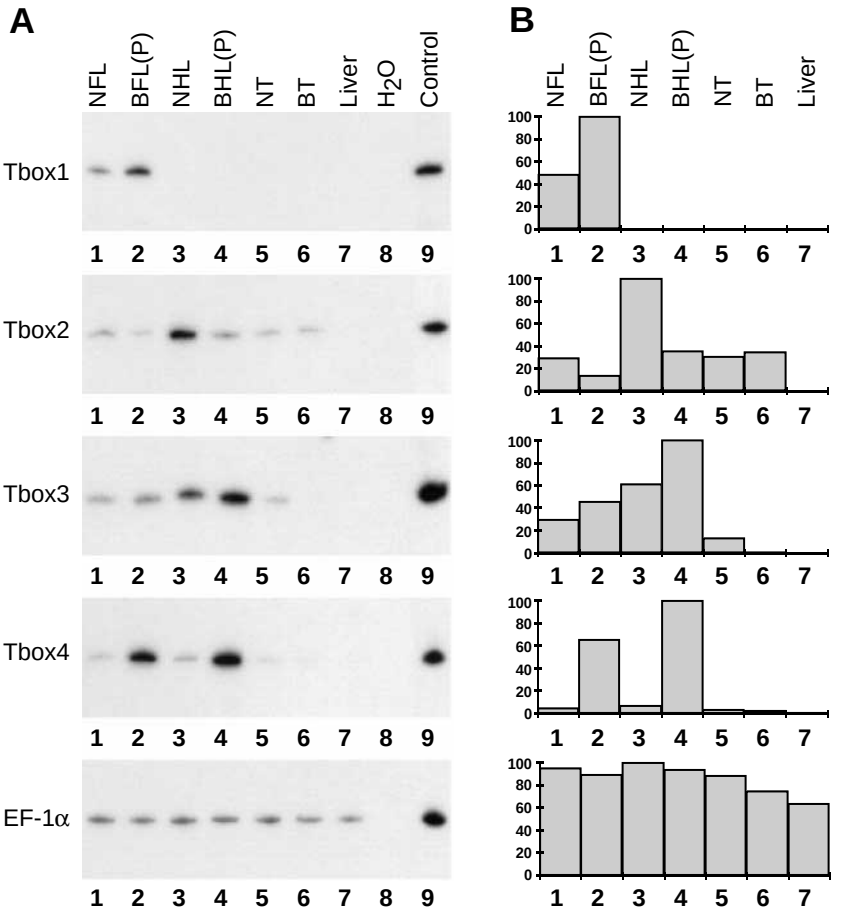

Fig. 7. Spatial expression pattern of newt T-box genes. (A) RT-PCR analysis employing RNA from various tissues with primers specific for *NvTbox1*, *NvTbox2*, *NvTbox3*, *NvTbox4* and the normalizing *EF-1α*. Lanes: (1) normal forelimb [NFL]; (2) proximal blastema forelimb [BFL(P)]; (3) normal hindlimb [NHL]; (4) proximal blastema hindlimb [BHL(P)]; (5) normal tail [NT]; (6) blastema tail [BT]; (7) liver, (8) H_2O; (9) cDNA control. (B) Quantitation of RT-PCR. For each gene, relative levels of expression in different appendages and liver were determined by RT-PCR and PhosphorImager scanning. Each data point in the bar diagram represents the ratio of the PCR products amplified from the genes under analysis to the *EF-1α* normalizing gene and is given as percentage of the respective peak values.

family of genes encodes a heterogeneous set of transcription factors that each play distinct roles in developmental signalling. Indeed, while both mouse and zebrafish Brachyury proteins have been demonstrated to play an important role in differentiation of the notochord and formation of posterior mesoderm (Herrmann et al., 1990; Halpern et al., 1993; Schulte-Merker and Smith, 1995), the expression domains of the mouse *Tbx1* to *Tbx6* genes correlate with regions of other active tissue interactions during embryogenesis, implicating them in a variety of inductive processes (Chapman et al., 1996).

The four novel newt T-box genes described in this study all demonstrated different expression profiles in normal and regenerating appendages, indicative of different functional roles in the regeneration process. In particular, *NvTbox1* shows expression patterns consistent with it having a role in controlling limb identity. In developing newts, the onset of expression correlates with the forelimb bud being formed from flank mesoderm. Importantly, during limb bud formation and subsequent growth, the mRNA appears to be uniformly distributed throughout the limb mesenchyme. Thus, probably all mesenchymal limb cells express this transcription factor, which in turn might control gene activities directly or indirectly involved in the specification of forelimb morphology. Similar, but slightly different gene activities might be directed by a hindlimb identity gene giving rise to hindlimb-specific patterns. On the basis of its activity profile, *NvTbox3* might be a candidate for such a gene; however, more work is needed to clarify this possiblity. In a parallel study on T-box gene expression during mouse development, Chapman et al. (1996) and Gibson-Brown et al. (1996) described similar limb-specific expression profiles for *Tbx4* and *Tbx5*. The authors demonstrated that *Tbx5* is expressed specifically in the forelimbs whereas *Tbx4* is expressed predominantly in the hindlimbs and only weakly in the forelimbs. However, there are also significant differences in the gene activities. The mouse in situ hybridization data revealed an early expression in the flank mesoderm at a time when the prospective limb fields are being established, and a later expression throughout the mesoderm of the developing limb bud. In newt larval development, we did not find evidence for *NvTbox1* gene activity in the lateral plate mesoderm of the forelimb field. The reason for this discrepancy is currently unclear, but it is possible that the transcriptional activity of the newt gene was too low or the *NvTbox1*-positive cells too dispersed to be detected in the presumptive forelimb field. Alternatively, lower and higher vertebrates might have developed a different regulation for these genes. Similar to the newt *NvTbox1*, the two mouse genes showed transcriptional activity in the head, in mandible mesenchyme and in mesenchyme in the pharyngeal region and the heart, suggesting a common functional role in these tissues. In addition to the aforementioned developmental data, our work on regeneration supports the notion that *NvTbox1* plays a crucial role in the specification of the forelimb territory: it has forelimb-specific expression and moreover, it is expressed in the correct cell type during the appropriate time window that would be expected of a gene involved in the specification of a limb territory.

In limb regeneration, RA causes a distal blastema to regenerate as if it had been transformed to a proximal one, leading to the formation of duplicated pattern along the proximodistal axis (Maden, 1983; Stocum and Crawford, 1987). When we tested whether the proximalization of blastema cells had an effect on the expression of *NvTbox1*, we also demonstrated that its message level became proximalized. The fact that the RA treatment results in concurrent shifts in *NvTbox1* expression and regenerative pattern supports the view that this T-box gene plays a role in providing axial positional information. Our data provide evidence for a very early and transient effect of RA on *NvTbox1* gene expression at a time when progenitor cells are being recruited from stump tissue by dedifferentiation and stimulated to enter the cell cycle yielding a blastema. These findings are reminiscent of previous reports of RA effects on *Hox* gene expression during limb regeneration (Simon and Tabin, 1993; Gardiner et al., 1995) and might suggest that certain positional cues in the limb modulate the activity of several gene systems involved in different aspects of limb patterning. We note that an obvious difference of *NvTbox1* message in proximal versus distal portions of developing limbs could not be detected. However, a 2-fold higher expression level in proximal limb segments as indicated by RNase protections with normal adult limbs might not be detectable by in situ analysis. Previously, similar limb-specific expression profiles have been reported only for members of the *Hoxc* gene cluster, characterizing them as candidates for specifying forelimb versus hindlimb morphologies (Savard et al., 1988; Tabin, 1989; Peterson et al., 1992; Simon and Tabin, 1993; Peterson et al., 1994; Nelson et al., 1996). Similar to these homeobox transcription factors, *NvTbox1* remained expressed at a low level in adult newt tissue, which might suggest a general requirement for limb-identity genes to maintain positional information in the event of regeneration. Preliminary expression data for the other newt T-box genes described here also indicate that persistence of expression in adult limbs and differential regulation between forelimbs and hindlimbs are common themes.

It is not clear whether any of the newt T-box genes represent homologs of the recently identified mouse *Tbx* genes, although this is suggested in at least one case by the expression data. Our homology studies indicate a close structural relationship of *NvTbox1* with the mouse genes *Tbx4* and *Tbx5*. However, due to limited sequence data for the mouse genes, we cannot ascertain whether *NvTbox1* is the newt ortholog of mouse *Tbx5* or whether it is an independent forelimb-specific T-box gene co-expressed in this territory. The two hindlimb-specific T-box genes, newt *NvTbox3* and mouse *Tbx4*, are only distantly related, suggesting that, for this territory, a minimum of two limb-specific T-box genes are expressed in the developing and/or regenerating vertebrate limb. The regeneration-specific *NvTbox4* represents a novel T-box gene, its T-domain being considerably diverged from mouse *Tbx4* and the other members of the *Tbx2/3/4/5* subfamily (Agulnik et al., 1996); together with the other two newt T-box genes and mouse *Tbx1*, it forms a separate subfamily. Interestingly enough these genes form a new branch based on structural relationship, nevertheless they seem to have different functions since mouse *Tbx1* is not expressed in the limbs (Chapman et al., 1996).

To date, our understanding of how limb-specific morphologies are controlled on a molecular level is limited. However, based on the data available it is unlikely that a single forelimb- or hindlimb-specific gene controls the different limb morphologies; rather a number of differentially expressed genes including members of the T-box family and *Hox* family, and

still unknown genes are likely part of a complex pathway controlling limb identity. The novel T-box transcription factors with a distinct forelimb/hindlimb expression profile could be key regulators initiating and guiding downstream molecular events which orchestrate the growth and shaping of muscle, connective tissue, and bone into the slightly different patterns which represent the morphological differences between forelimbs and hindlimbs. Among the players that direct growth and pattern are the *Hox* genes, which already have been shown to have a role in the specification of morphology. In particular, some of the *Hoxc* genes are also differentially regulated in forelimbs and hindlimbs; given their prominent anterior/proximal expression in developing limbs, it has been speculated that *Hoxc* gene expression might affect the patterning of the pectoral and/or pelvic girdle, respectively (Nelson et al., 1996). With regard to the question of what leads to activation of T-box transcription factors, it is interesting that, in the chick embryo, FGF-soaked beads can induce the formation of ectopic wings or legs from anterior or posterior flank tissue, respectively (Cohn et al. 1995). To date, in limb development/regeneration, no transcription factors downstream of FGF signalling have been identified. However, Schulte-Merker and Smith (1995) have recently shown that *Xenopus* Brachyury regulates, and in turn is regulated by, eFGF during mesoderm induction. In this regard, it is intriguing that *NvTbox1* upregulation in the regenerating forelimb is contemporaneous with elevated aFGF activity and expression of FGF receptor 1 and FGF receptor 2 (Boilly et al., 1991; Poulin et al., 1993). In addition, the *Drosophila* T-box gene *omb*, which is a close relative of the mouse *Tbx2* and *Tbx3* genes (Chapman et al., 1996; Gibson-Brown et al., 1996), is expressed in the wing imaginal disk. Ectopic misexpression of *omb* results in the production of a second pair of wings, indicating that *omb* is an essential factor promoting wing outgrowth and perhaps wing identity (Grimm and Pflugfelder, 1996). Moreover, its gene activity is controlled by two secreted signalling molecules, wingless (wg) and decapentaplegic (dpp). In vertebrates, it has been clearly demonstrated that homologs of these fly proteins are involved in limb formation. It will be interesting to find out whether the striking parallels previously reported for molecules involved in vertebrate limb development and their homologs in wing and limb imaginal disks in the fly embryo (Ingham, 1994; Yang and Niswander, 1995) might also lead to new insights into the regulation of this new family of T-domain transcription factors.

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REFERENCES

Agulnik, S. I., Garvey, N., Hancock, S., Ruvinsky, I., Chapman, D. L., Agulnik, I., Bollag, R., Papaioannou, V. and Silver, L. M. (1996). Evolution of mouse *T-box* genes by tandem duplication and cluster dispersion. *Genetics* **144**, 249-254.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410.

Boilly, B., Cavanaugh, K. P., Thomas, D., Hondermarck, H. Bryant, S. V. and Bradshaw, R. A. (1991). Acidic fibroblast growth factor is present in regenerating limb blastemas of axolotls and binds specifically to blastema tissues. *Dev. Biol.* **145**, 302-310.

Bollag, R. J., Siegfried, Z., Cebra-Thomas, J. A., Garvey, N., Davison, E. M. and Silver, L. M. (1994). An ancient family of embryonically expressed mouse genes sharing a conserved protein motif with the T locus. *Nat. Genet.* **7**, 383-389.

Brown, R. and Brockes, J. P. (1991). Identification and expression of a regeneration-specific homeobox gene in the newt limb blastema. *Development* **111**, 489-496.

Bulfone, A., Smiga, S. M., Shimamura, K., Peterson, A., Puelles, L. and Rubenstein, J. L. (1995). *T-brain-1*: a homolog of *Brachyury* whose expression defines molecularly distinct domains within the cerebral cortex. *Neuron* **15**, 63-78.

Chapman, D. L., Garvey, N., Hancock, S., Alexiou, M., Agulnik, S., Brown, J. J. G., Cebra-Thomas, J., Bollag, R. J., Silver, L. M. and Papaioannou, V. E. (1996). Expression of the T-box family genes, *Tbx1-Tbx5*, during early mouse development. *Dev. Dyn.* **206**, 379-390.

Cohn, M. J., Izpisua-Belmonte, J. C., Abud, H., Heath, J. K. and Tickle, C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* **80**, 739-746.

Duboule, D. (1994). How to make a limb? *Science* **266**, 575-576.

Gardiner, D. M., Blumberg, B., Komine, Y. and Bryant, S. V. (1995). Regulation of *HoxA* expression in developing and regenerating axolotl limbs. *Development* **121**, 1731-1741.

Gibson-Brown, J. J. G., Agulnik, S., Chapman, D. L., Alexiou, M., Garvey, N., Silver, L. M. and Papaioannou, V. E. (1996). Evidence of a role for T-box genes in the evolution of limb morphogenesis and the specification of forelimb/hindlimb identity. *Mech. Dev.* **56**, 93-101.

Grimm, S. and Pflugfelder, G. O. (1996). Control of the gene *optomoter-blind* in *Drosophila* wing development by decapentaplegic and wingless. *Science* **271**, 1601-1604.

Guyenot, E. and Schotte, O. E. (1926). Demonstration de l'existance de territoires specifiques de regeneration par la methode de la deviation des troncs nerveux. *C.R. Soc. Biol.* **94**, 1050-1052.

Guyenot, E., Dinichert-Favarger, J. and Galland, M. (1948). L'exploration du territoire de la patte anterieure du triton. *Rev. Suisse Zool.* **55**, Suppl. 2, 1-120.

Halpern, M. E., Ho, R. K., Walker, C. and Kimmel, C. B. (1993). Induction of muscle pioneers and floor plate is distinguished by the zebrafish *no tail* mutation. *Cell* **75**, 99-111.

Herrmann, B. G., Labeit, S., Poustka, A., King, T. R. and Lehrach, H. (1990). Cloning of the *T gene* required in mesoderm formation in the mouse. *Nature* **343**, 617-622.

Inghan, P. (1994). Signal transduciton. Dorsal developments. *Nature* **372**, 500-501.

Iten, L. E. and Bryant, S. V. (1973). Forelimb regeneration from different levels of amputation in the newt *Notophthalmus viridescens*. Length, rate and stages. *Wilhelm Roux' Arch. Dev. Biol.* **173**, 263-282.

Khan, P. A. and Liversage, R. A. (1995a). Spawning of *Notophthalmus viridescens* and rearing of embryos under laboratory conditions. *Herpetol. Review* **26**, 95-96.

Khan, P. A. and Liversage, R. A. (1995b). Development of *Notophthalmus viridescens* embryos. *Develop. Growth Differ.* **37**, 529-537.

Kiortsis, V. (1953). Potentialites du territoire patte chez le triton (adultes, larves, et embryons). *Rev. Suisse Zool.* **60**, 301-410.

Kispert, A. (1995). The Brachyury protein: a T-domain transcription factor. *Dev. Biol.* **6**, 395-403.

Liang, P. and Pardee, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971.

Liang, P., Averboukh, L. and Pardee, A. B. (1993). Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucl. Acids Res.* **21**, 3269-3275.

Maden, M. (1983). The effect of vitamin A on the regenerating axolotl limb. *J. Embryol. Exp. Morph.* **77**, 273-295.

Muneoka, K. and Bryant, S. V. (1982). Evidence that patterning mechanisms in developing and regenerating limbs are the same. *Nature* **298**, 369-371.

Muneoka, K. and Sassoon, D. (1992). Molecular aspects of regeneration in developing vertebrate limbs. *Dev. Biol.* **152**, 37-49.

Nelson, C. E., Morgan, B. A., Burke, A. C., Laufer, E., DiMambro, E., Murtaugh, L. C., Gonzales, E., Tessarollo, L., Parada, L. F. and Tabin, C. (1996). Analysis of *Hox* expression in the chick limb bud. *Development* **122**, 1449-1466.

Peterson, R. L., Jacobs, D. F. and Awgulewitsch, A. (1992). *Hox-3.6*:

isolation and characterization of a new murine homeobox gene located in the 5′ region of the *Hox-3* cluster. *Mech. Dev.* **37**, 151-166.

Peterson, R. L., Papenbrock, T., Davda, M. M. and Awgulewitsch, A. (1994). The murine *Hoxc* cluster contains five neighboring *AbdB*-related *Hox* genes that show unique spatially coordinated expression in posterior embryonic subregions. *Mech. Dev.* **47**, 253-260.

Pflugfelder, G. O., Roth, J., Poeck, B., Kerscher, S., Schwarz, H. Jonschker, B., and Heisenberg, M. (1992). The lethal (1) optomotor-blind gene of *Drosophila melanogaster* is a major organizer of optic lobe development: Isolation and characterization of the gene. *Proc. Natl. Acad. Sci.* **89**, 1199-1203.

Poulin, M. L., Patrie, K. M., Botelho, M. J., Tassava, R. A. and Chiu, I.-M. (1993). Heterogeneity in the expression of fibroblast growth factor receptors during limb regeneration in newts (*Notophthalmus viridescens*). *Development* **119**, 353-361.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Savard, P., Gates, P. B. and Brockes, J. P. (1988). Position dependent expression of a homeobox gene transcript in relation to amphibian limb regeneration. *EMBO J.* **7,** 4275-4282.

Savard, P. and Tremblay, M. (1995). Differential regulation of *Hox C6* in the appendages of adult urodeles and anurans. *J. Mol. Biol*. **249**, 879-889.

Schulte-Merker, S., Ho, R. K., Herrmann, B. G. and Nusslein-Volhard, C. (1992). 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. *Development* **116**, 1021-1032.

Schulte-Merker, S. and Smith, J. C. (1995). Mesoderm formation in response to brachyury requires FGF signalling. *Curr. Biol.* **5**, 62-67.

Simon, H.-G. and Tabin, C. J. (1993). Analysis of *Hox-4.5* and *Hox-3.6* expression during newt limb regeneration: differential regulation of paralogous *Hox* genes suggests different roles for members of different *Hox* clusters. *Development* **117**, 1397-1407.

Simon, H.-G., Nelson, C., Goff, D., Laufer, E., Morgan, B. A. and Tabin, C. (1995). Differential expression of myogenic regulatory genes and *Msx-1* during dedifferentiation of regenerating amphibian limbs. *Dev. Dyn.* **202**, 1-12.

Simon, H.-G. and Oppenheimer, S. (1996). Advanced mRNA differential display: isolation of a new differentially regulated myosin heavy chain-encoding gene in amphibian limb regeneration. *Gene* **172**, 175-181.

Smith, J. C., Price, B. M., Green, J. B., Weigel, D. and Herrmann, B. G. (1991). Expression of a *Xenopus* homolog of *Brachyury* (*T*) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

Stocum, D. L. and Dearlove, G. E. (1972). Epidermal-mesodermal interaction during morphogenesis of the limb regeneration blastema in larval salamanders. *J. Exp. Zool.* **181**, 49-61.

Stocum, D. L. and Crawford, K. (1987). Use of retinoids to analyse the cellular basis of positional memory in regenerating limbs. *Biochem. Cell Biol.* **65**, 750-761.

Tabin, C. J. (1989). Isolation of potential vertebrate limb-identity genes. *Development* **105**, 813 820.

Tabin, C. J. (1991). Retinoids, homeoboxes, and growth factors: Toward molecular models for limb development. *Cell* **66**, 199-217.

Yang, Y. and Niswander, L. (1995). Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell* **80**, 939-947.

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Note added in proof

While the paper was under review two research groups [Quan et al. (1997) *Nature Genetics* **15**, 21-29; Craig et al. (1997) *Nature Genetics* **15**, 30-35] reported the human *TBX5* cDNA which by comparison is the homolog of the newt *Tbox1* gene. The authors showed *TBX5* to be the gene mutated in Holt-Oram syndrome, a dominant mutation causing upper limb malformations and cardiac septation defects.