Int. J. Dev. Biol. 51: 745-752 (2007)
doi: 10.1387/ijdb.072375fn

Developmental Expression Pattern

Differential expression of two *TEF-1 (TEAD)* genes during *Xenopus laevis* development and in response to inducing factors

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ABSTRACT Transcription enhancer factors 1 (TEF-1 or TEAD) make a highly conserved family of eukaryotic DNA binding proteins that activate not only viral regulatory elements but muscle specific genes and are involved in several developmental processes. In this study, we report the identification and the expression pattern of *NTEF-1 (TEAD1)* and *DTEF-1 (TEAD3),* two members of this family in *Xenopus laevis.* Both *X. laevis* NTEF-1 (XNTEF-1 or XTEAD1) and DTEF-1 (XDTEF-1 or XTEAD3) possess a 72 amino acid TEA domain characteristic of TEF-1 proteins. XNTEF-1 is a 426 amino acid protein that has 96% identity with the avian or the mammalian NTEF-1 proteins, while XDTEF-1 is a 433 amino acid protein with 77 to 80% identity with the avian and mammalian DTEF-1 sequences respectively. Temporal expression analysis by RT-PCR indicated that the two genes are expressed maternally and throughout embryonic development. In the adult, the two genes are broadly expressed although they showed differences of expression between tissues. Spatial expression analysis by whole mount *in situ* hybridization showed that the *XNTEF-1* and *XDTEF-1* mRNAs were predominantly detected in eye, embryonic brain, somites and heart. In animal cap assay, the two genes are activated by bFGF but are differently regulated by BMP4 and the muscle regulatory factor Mef2d.

KEY WORDS: *TEF-1/ TEAD, myoD, BMP4, mef2, Xenopus*

TEF-1 (Transcription enhancer factor-1 or TEAD) proteins constitute a family of transcription factors that contain a highly conserved TEA DNA binding domain (Burglin, 1991). TEF-1 protein was first identified and then cloned from HeLa cells by its ability to bind tandemly repeated GT-IIC or Sph motifs from the SV40 enhancer (Davidson *et al*., 1988; Xiao *et al*., 1991). TEF-1 proteins are encoded by a family of genes that contains four members in mammals namely *NTEF-1* (*TEAD1*), *ETEF-1* (*TEAD2*), *DTEF-1* (*TEAD3*) and *RTEF-1* (*TEAD4*) (Yasunami *et al*., 1995; Jacquemin and Davidson, 1997; Kaneko and DePamphilis, 1998; Larkin and Ordahl, 1999). However, not every member seemed to be present in the genome of all species. In mouse, the *TEF-1* genes are differentially expressed during early development showing a complex pattern of expression between early and late developmental stages, which suggests their involvement in several developmental processes (Jacquemin *et al*., 1996; Kaneko *et al*., 1997). For instance, homozygous null mice for *NTEF-1* die from cardiac insufficiency suggesting that TEF-1 is required for late events in cardiogenesis (Chen *et al*., 1994). TEF-1 protein was found to be able to bind the MCAT sequence that has been implicated in the cell-specific regulation of many muscle genes (Farrance *et al*., 1992; Larkin and Ordahl, 1999). For instance, we recently showed that the *Xenopus laevis α-tropomyosin* gene was regulated, in the three muscle lineages, by an MCAT sequence that is essential for the correct temporal and spatial expression of the gene (Pasquet *et al*., 2006). TEF-1 protein also regulates non muscle genes like the human somatomammotropin gene whose expression is controlled by DTEF-1 which is strongly

Abbreviations used in this paper: NTEF, nominal TEF; DTEF, divergent TEF; TEA, transcriptional enhancer activator; TEAD, TEA domain; TEF, transcription enhancer factor.

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[1]Supported by a PhD fellowship awarded by the MENRT. The nucleotide sequence reported in this paper has been submitted to the GenBankTM/EMBL databank unit with accession number EF422418.

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0214-6282/2007/$30.00

Printed in Spain
www.intjdevbiol.com

expressed in placenta (Jacquemin *et al*., 1997; Jiang and Eberhardt, 1997).

TEF-1 orthologs of other eukaryotes have been shown to regulate distinct developmental processes. In *Drosophila melanogaster*, the unique *TEF-1* ortholog gene, namely *scalloped*, is involved in wing development as well as neural development (Campbell *et al*., 1991; Campbell *et al*., 1992). In *Caenorhabditis elegans*, the *TEF-1* ortholog *egl-44*, regulates differentiation of touch-sensitive cells and egg-laying motor neurons (Desai and Horvitz, 1989; Wu *et al*., 2001).

The function of *TEF-1* genes in vertebrate development might be difficult to explore due to the presence of four genes and their potential redundancy. The amphibian *Xenopus laevis* can be a suitable model organism to investigate the function of the different *TEF-1* genes during early development. Indeed, this model allows gain-of-function studies by mRNA over-expression and loss-of-function studies due to the availability of antisense morpholinos oligos that permit inhibition function of a specific gene or even all members of a gene family (Heasman *et al*., 2000; Reversade *et al*., 2005). In the present study, we report the identification and the expression pattern of *NTEF-1* and *DTEF-1*, two members of the *TEF-1* family, in *Xenopus laevis.*

Results and Discussion

We performed blast searches in databases to identify *Xenopus laevis* orthologs of mammalian TEF-1 proteins. We identified an expressed sequence tag (EST) (GenBank Accession No. BI444402, IMAGE clone 4434995) which encodes an open reading frame containing a TEA motif. The complete sequence of the retrieved cDNA clone was determined on both strands and registered with the GenBank (Accession No. EF422418). This cDNA encodes a 426 amino acid protein containing a 72 amino acid sequence identical to the TEA DNA binding domain of the avian and mammalian TEF-1 genes. Moreover, this protein shows 96% identity with the chick, human and mouse NTEF-1 proteins but only 67%, 76% and 72% identity with the mammalian ETEF-1, RTEF-1 or DTEF-1 proteins respectively (Fig. 1). We concluded that this clone codes for the *Xenopus laevis* NTEF-1 protein and named it *XNTEF-1*. This *XNTEF-1* clone corresponds to the alternative isoform TEF-1α or TEF-1 zeta described by Jiang *et al*. (2000) and Zuzarte *et al*. (2000) respectively. We also found in database a partial cDNA sequence, previously released with the Accession No. BG354634 and described in an expression cloning strategy, that corresponded to the *XNTEF-1* sequence (Grammer *et al*., 2000). One striking feature of the *Xenopus NTEF-1* sequence is that its initiates translation with a methionine codon while mammalian and chicken orthologs use an isoleucine codon.

In a search for other *Xenopus lae*vis *TEF-1* related sequences we identified an expressed sequence tag (GenBank Accession No. BM180109, IMAGE clone 4959564) whose predicted amino acid sequence contained a 72 amino acid TEA domain. However, this sequence has only 76% identity at the nucleotide level with the *XNTEF-1* sequence and thus indicates that it corresponds to a second *Xenopus TEF-1* gene. We sequenced the retrieved clone and found that it encodes a 433 amino acid protein showing 70-72% identity with the chick, human and mouse DTEF-1 proteins. When allowing for conservative changes, the homology between the amphibian sequence and avian or mammalian DTEF-1 sequences raises up to 82-84% (Fig. 2). When compared to the mammalian ETEF-1 and RTEF-1 proteins, the *Xenopus* protein shows 68% of identity. Moreover, the N-terminal region of the *Xenopus* protein that precedes the TEA domain has 10-13, 9 and 4 amino acids identical with the DTEF-1, RTEF-1 and ETEF-1 vertebrate sequences respectively. In addition, ETEF-1 has an extra 10 amino acids stretch that is absent from other TEF-1 proteins (Fig. 2). Therefore, we concluded that this clone represents the *Xenopus laevis DTEF-1* and we named it *XDTEF-1*. The complete sequence of XDTEF-1 was found identical to the sequence Accession No BC082362, which has been previously released in the databank. In a search for *Xenopus tropicalis* TEF-1 related proteins in databases, we found a protein that has 98% identity at the amino acid level with the *Xenopus laevis* XDTEF-1, thus indicating the presence of a highly conserved *DTEF-1* gene in *Xenopus* (Fig. 2).

The expression of the two *TEF-1* genes during early *Xenopus* embryogenesis was investigated by RT-PCR analysis with RNA isolated from various stages of development (Fig. 3A). *XNTEF-1* and *XDTEF-1* mRNAs were detected in unfertilized egg indicating that the corresponding genes are maternally expressed. *XNTEF-1* mRNA level is constant during development while *XDTEF-1* mRNA level increases between stages 7 and 12 and then remains constant during embryogenesis. This increase in mRNA level corresponds to the zygotic transcription activation that occurs at the mid-*blastula* transition. The maternal expression of *X. laevis NTEF-1* and *DTEF-1* genes differs from the situation observed in mouse were *NTEF-1* and *DTEF-1* genes are not expressed in oocytes at levels detectable by *in situ* hybridization (Kaneko *et al*., 1997). In fact, among the four mammalian *TEF-1* genes, only *ETEF-1* has been found to be expressed during the first seven days of mouse development (Kaneko *et al*., 1997).

The expression of the two *X. laevis TEF-1* genes in adult tissues revealed that both genes were detected most prominently and at a similar level in the ovaries, lungs and skeletal muscle (Fig. 3A). They are also expressed, albeit at a lower level, in the skin but are barely detectable in the liver. In the other tissues analysed the two genes show differences in their expression. *XNTEF-1* gene is more expressed in the stomach, brain, heart and testes while *XDTEF-1* gene is more expressed in the intestine. The expression pattern of *NTEF-1* and *DTEF-1* mRNAs in adult tissues is very similar between *X. laevis* and those reported in mouse and chick with the exception of the *DTEF-1* gene, which in the chick, is highly expressed in the heart but barely detectable in the lung (Azakie *et al*., 1996). Although RT-PCR analysis has shown the presence of *XNTEF-1* and *XDTEF-1* mRNAs in stages earlier than stage 17 (see Fig. 3A), we did not detect any localized expression of both genes before stage 17 embryo by whole mount *in situ* hybridization (data not shown). In order to confirm these data, we have dissected *gastrula* stage 10 embryos into animal cap and mesodermal regions and analysed *XNTEF-1* and *XDTEF-1* mRNAs levels in dissected pieces by RT-PCR (Fig. 3B). There are no major differences in the expression of *XNTEF-1* and *XDTEF-1* mRNAs in the different embryonic regions tested confirming the ubiquitous expression of both genes in early stages (Fig. 3B). In stage 17 embryo, expression of *XNTEF-1* is found at the most antero-lateral edge of the neural plate that overlaps the eye field and the olfactory placodes (Fig. 4A). At stage 23,

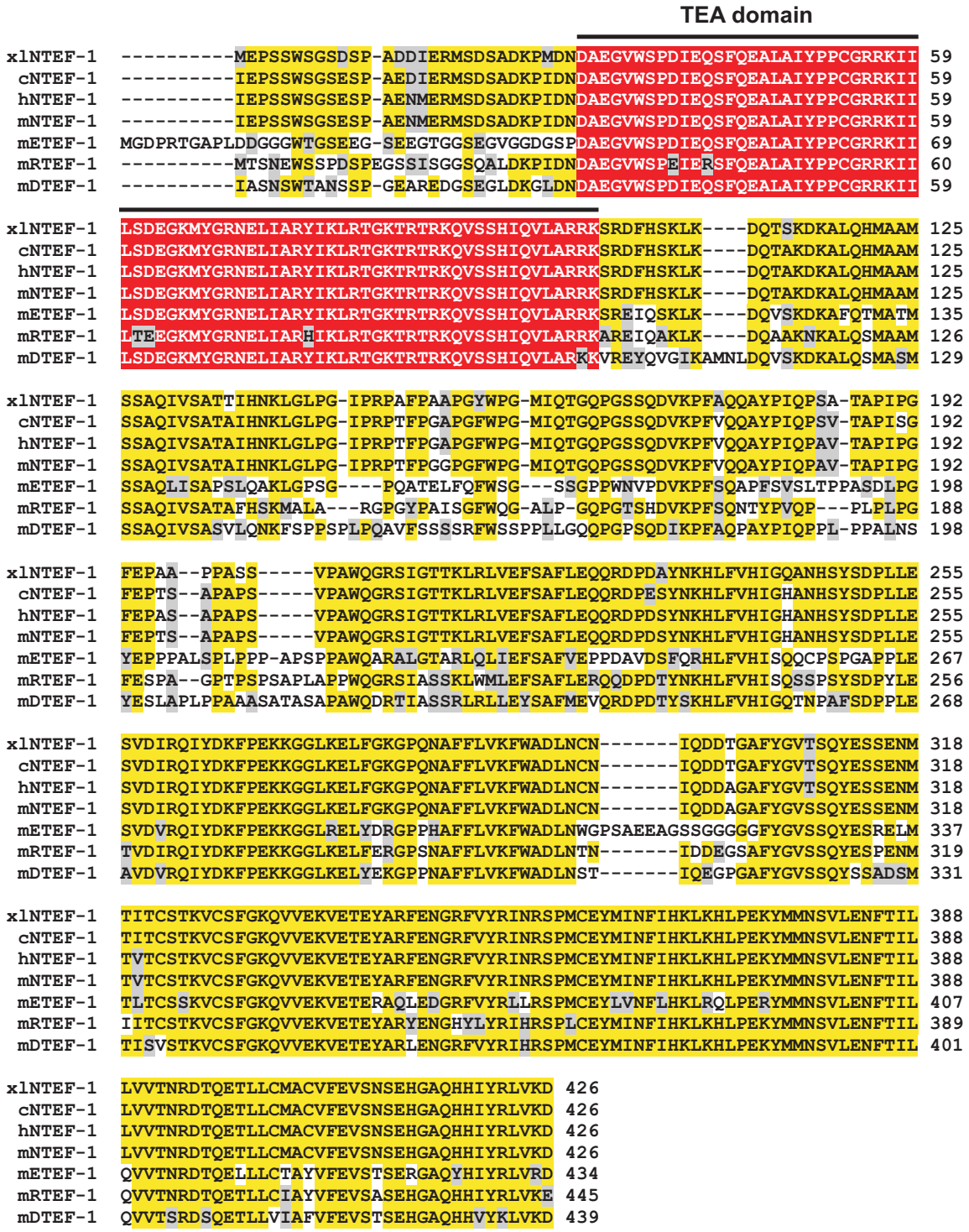

Fig. 1. *Xenopus* NTEF-1 amino acid sequence and comparison with avian and mammalian TEF-1 sequences. Xenopus laevis *NTEF-1 sequence (xlNTEF-1, GenBank Accession No. EF422418) is aligned with chick NTEF-1 (cNTEF-1, GenBank Accession No. XM_420962), human NTEF-1 (hNTEF-1, GenBank Accession No. P28347), mouse NTEF-1 (mNTEF-1, GenBank Accession No. AAA40410), mouse ETEF-1 (mETEF-1, GenBank Accession No. BAA12018), mouse RTEF-1 (mRTEF-1, GenBank Accession No. X94441) and mouse DTEF-1 (mDTEF-1, GenBank Accession No. AAH21364) sequences. The highly conserved TEA domain is overlined and red boxes indicate conserved amino acid residues. Yellow boxes indicate the amino acid residues that are identical in at least four of the seven sequences. Grey boxes indicate the amino acid residues that are conserved. Numbers refer to the amino acid sequences and gaps have been introduced to maximize similarity.*

Fig. 2. *Xenopus* DTEF-1 amino acid sequences and comparison with avian and mammalian TEF-1 sequences. Xenopus laevis *DTEF-1 sequence (xlDTEF-1, GenBank Accession No. BC082362) is compared with* Xenopus tropicalis *DTEF-1 (xtDTEF-1, Protein ID: 177240, DOE Joint genome institute), chick DTEF-1 (cDTEF-1, GenBank Accession No. T09284), human DTEF-1 (hDTEF-1, GenBank Accession No. X94439), mouse DTEF-1 (mDTEF-1, GenBank Accession No. AAH21364), mouse ETEF-1 (mETEF-1, GenBank Accession No. BAA12018), mouse RTEF-1 (mRTEF-1, GenBank Accession No. X94441) and mouse NTEF-1 (mNTEF-1, GenBank Accession No. AAA40410) sequences. The highly conserved TEA domain is overlined and red boxes indicate conserved amino acid residues. Yellow boxes indicate the amino acid residues that are identical in at least four of the eight sequences. Grey boxes indicate the amino acid residues that are conserved. Numbers refer to the amino acid sequences and gaps have been introduced to maximize similarity.*

XNTEF-1 expression marks the trunk somites in addition to the eye and the brain, (Fig. 4B). At stage 27, *XNTEF-1* mRNAs are detected in the eye, the brain, the anterior part of the neural tube and in the somites (Fig. 4C). The expression in the brain marks predominantly the forebrain but the midbrain-hindbrain boundary and the hindbrain are also marked (Fig.4C). In transverse section at the level of the brain and eye, expression is observed in the ventricular zone of the midbrain and in the eye (Fig. 4E). At stage 37, *XNTEF-1* is widely expressed in the brain and the head mesenchyme and in the branchial arches (Fig. 4D). The expression in the somites has decreased and there is a widespread expression in the posterior region of the embryo (Fig. 4D). In an anterior section the expression of *XNTEF-1* is detected in the eye and there is a faint labelling of the embryonic heart (Fig. 4F). In a more posterior transverse section, the expression is observed in the otic vesicle and the hindbrain (Fig. 4G).

The expression of *XDTEF-1* is detected at stage 17 in the midbrain region of the neural plate (Fig. 4H). *XDTEF-1* is also weakly expressed in the paraxial mesoderm. At stage 27, expression is observed in the presomitic mesoderm and the posterior somites (Fig. 4I). The eye and the midbrain and hindbrain regions are also stained but there is no detectable expression in the anterior region of the brain (Fig. 4I). At stage 33, *XDTEF-1* expression persists in the midbrain and hindbrain, the eye and the posterior somites, and appears in the branchial arches, the otic vesicle and the anterior region of the brain (Fig. 4J). In transverse section, the expression is detected in the hindbrain, the otic vesicle and the branchial arches (Fig. 4L). At stage 37, *XDTEF-1* is expressed in head mesenchyme, branchial arches, in brain and in otic vesicle. Its expression also uniformly marks the trunk and posterior regions of the embryo (Fig. 4K). A faint labelling is observed in the heart region and this is more noticeable in a transverse section (Fig. 4M). A transverse section in the posterior trunk region shows expression in the most lateral region of the somites (Fig. 4N).

In summary, the two *X. laevis TEF-1* genes show an identical expression in the eye of the early embryo but have distinct expression in the somites with *XNTEF-1* being expressed in the trunk somites and *XDTEF-1* expression marking the presomitic mesoderm and more posterior somites. The two genes also show distinct expression pattern in the brain. *XNTEF-1* is widely expressed in the brain while *XDTEF-1* is dynamically expressed with a posterior expression at early stages and then an overall expression at later stages. The expression of *NTEF-1* and *DTEF-1* in mouse embryo begins between day 7 and day 9. *DTEF-1* is specifically expressed in skeletal muscle precursors whereas *NTEF-1* is expressed not only in developing skeletal muscle but also in the myocardium (Yasunami *et al*., 1995; Jacquemin *et al*., 1996). In chick, *DTEF-1* gene is expressed in the embryonic heart, lung and gizzard (Azakie *et al*., 1996; Azakie *et al*., 2005). We have found a weak expression of *XNTEF-1* and *XDTEF-1* genes in the early stages of embryonic heart (Fig. 4F and 4M). To assess this finding more definitely, we have dissected heart regions of stage 36 and 42 embryo and performed RT-PCR analysis. As shown in Fig. 3C, *XNTEF-1* and *XDTEF-1* mRNAs are detected in embryonic cardiac cells like the cardiac specific *XMLC2* mRNAs. *XNTEF-1* and *XDTEF-1* are also expressed in embryonic brain and gut.

Patterning the early *Xenopus* embryo relies on limited signalling pathways that influence cell fate and ultimately regulate the expression of transcription factors in a cell type dependent manner (Heasman, 2006). Of major importance are the FGF, activin and BMP signalling pathways. We have tested in the animal cap assay whether these pathways could modulate the expression of the *TEF-1* genes. As shown in Fig. 5A, the expression of *XNTEF-1* and *XDTEF-1* genes is increased at high dose of bFGF (100ng/ml). While *XNTEF-1* gene expression increases between 50 and 100ng/ml of bFGF, the expression of *XDTEF-1* starts to increase at 12.5ng/ml of bFGF and follows the increased expression of *Xmsr* gene that serves as a control of the induction by bFGF. The expression of the two genes are not significantly modified in activin treated explants, even when a high dose of the factor is used (Fig. 5B, lanes 2-4). BMP4 has no effect on *XDTEF-1* gene expression but it reduces, at both concentration tested, the

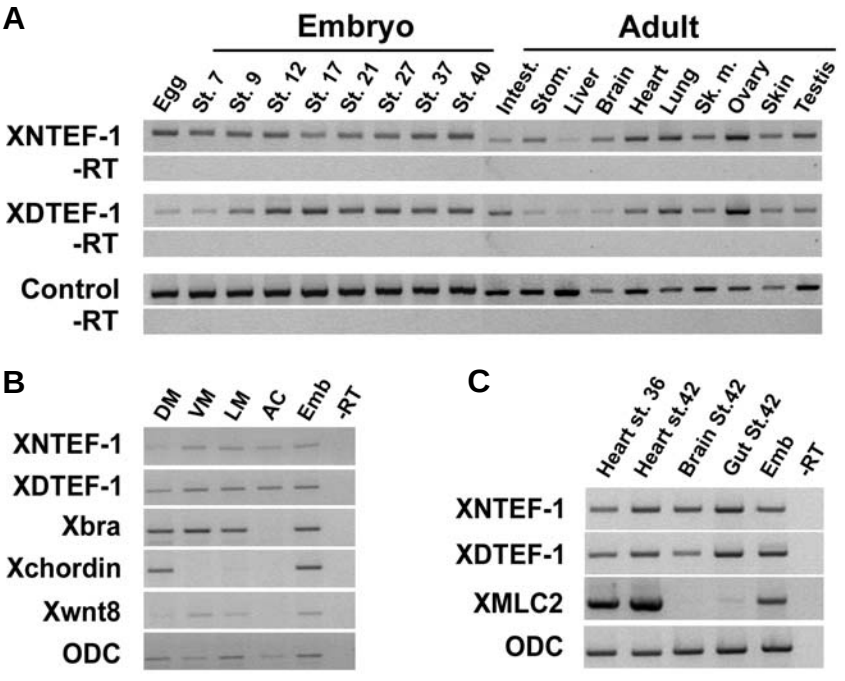

Fig. 3. Expression of *XNTEF-1* and *XDTEF-1* mRNAs during development and in adult tissues. (A) *RT-PCR analysis of developmental stages (Embryo) and adult tissues (Adult). Total RNA was extracted from staged embryos and adult tissues and subjected to RT-PCR using primers specific to* XNTEF-1 *and* XDTEF-1 *sequences. Nieuwkoop and Faber stages are indicated. Intestine (Intest.), stomach (Stom.), skeletal muscle (Sk.m.).* **(B)** *RT-PCR analysis of total RNA from explants dissected from* gastrula *stage embryos. Explants are from dorsal-mesoderm (DM), ventral-mesoderm (VM), lateral mesoderm (LM) and animal cap (AC). Total RNA from stage 10 embryo is analysed in control (Emb).* Xbra *is used as control of pan-mesodermal marker,* Xchordin *as dorsal mesoderm marker and* Xwnt8 *as ventro lateral mesoderm.* **(C)** *RT-PCR analysis of total RNA from dissected embryonic heart, brain and gut. The stage of the dissected embryos is indicated. Embryo (Emb) RNA is used in control in B and C.* ODC *in A (Embryo), in B and in C or* Rpl8 *in A (Adult) were used as loading controls and genomic DNA contamination was assayed by reaction without RT (-RT).*

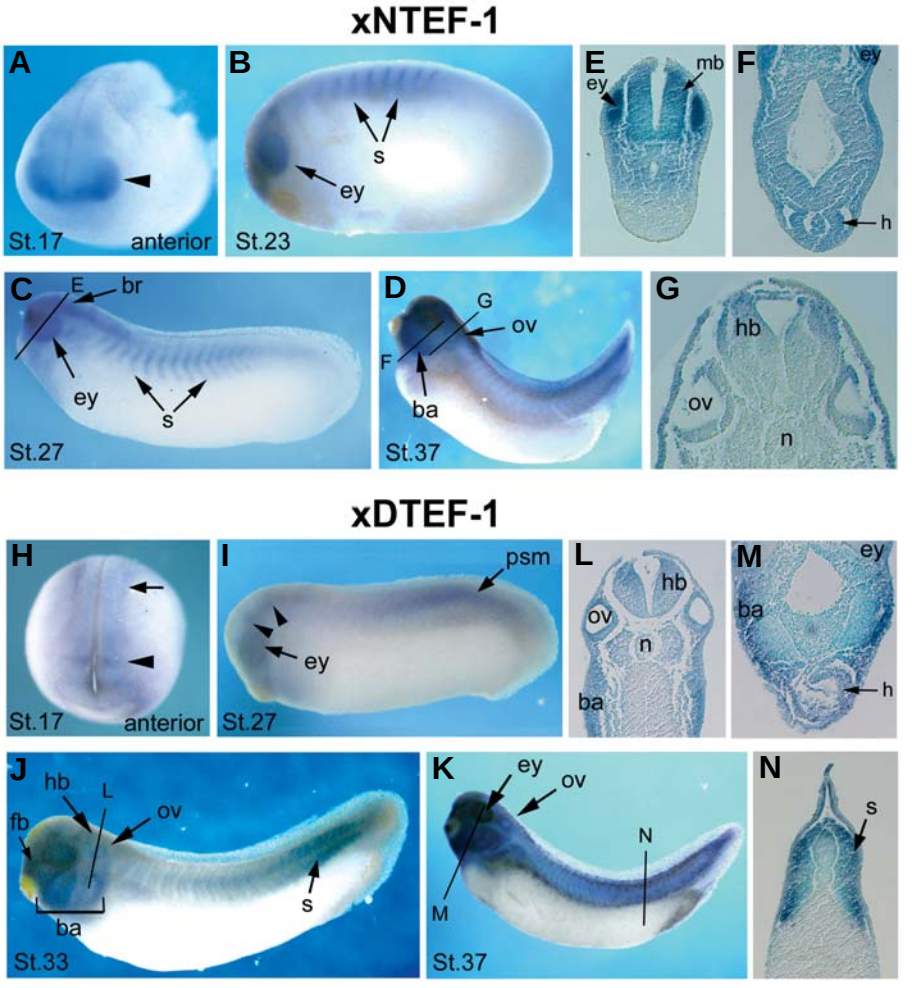

Fig. 4. Spatial expression pattern of *XNTEF-1* and *XDTEF-1* genes in developing embryos. *Whole mount* in situ *hybridization was performed on embryos at various developmental stages to determine the expression pattern of* XNTEF-1 **(A-G)** *and* XDTEF-1 **(H-N)** *genes.* **(A)** *In stage 17 embryo,* XNTEF-1 *mRNA is localized in the anterior region of the neural plate covering the eye field (arrowhead).* **(B)** *In stage 23 embryo, the expression is found in the eye (ey) and the trunk somites (s).* **(C)** *In stage 27 embryo,* XNTEF-1 *is strongly expressed in the brain (br), the eye (ey) and the trunk somites (s). Black line indicates the position of the section shown in (E).* **(D)** *In stage 37 embryo,* XNTEF-1 *is expressed in the brain, the head mesenchyme, the branchial arches (ba) and the otic vesicle (ov). There is also a widespread expression in the trunk and the posterior region. Black lines indicate the position of the sections shown in (F,G).* **(E)** *Transverse section of stage 27 embryo showing* XNTEF1 *expression in the ventricular zone of the midbrain (mb) and in the eye (ey).* **(F)** *Transverse section in the anterior region of stage 37 embryo showed expression in the eye (ey) and in the embryonic heart (h).* **(G)** *In transverse section of stage 37 embryo, the staining is present in the hindbrain (hb) and the otic vesicle (ov) but absent from notochord (n).* **(H)** *In stage 17 embryo,* XDTEF-1 *is expressed in midbrain (arrowhead) and paraxial domain (arrow).* **(I)** *In stage 27 embryo, the expression is detected in the midbrain and hindbrain regions (arrowheads), the eye (ey), the posterior somites and the presomitic mesoderm (psm).* **(J)** *In stage 33 embryo, the staining marks predominantly the forebrain (fb), the eye, the branchial arches (ba), the otic vesicle (ov) and the posterior somites (s). Black line indicates the position of the section shown in (L).* **(K)** *In stage 37 embryo,* XDTEF-1 *expression is found in the brain, head mesenchyme, otic vesicle (ov), trunk and posterior regions. Black lines indicate the position of the sections shown in (M,N).* **(L)** *Transverse section in the head region of stage 33 embryo showed expression in the hindbrain (hb), the otic vesicle (ov) and the branchial arches (ba).* **(M)** *Transverse section in the anterior region of stage 37 embryo showed expression in eye (ey), branchial arches (ba) and embryonic heart (h).* **(N)** *Transverse section in posterior region of stage 37 embryo showed expression in the somites (s).*

expression of *XNTEF-1* gene (Fig. 5C, lanes 2 and 3). In those same explants BMP4 induction is controlled by *Xαglobin* gene expression (Fig. 5C, lanes 2 and 3).

XNTEF-1 and *XDTEF-1* genes are both expressed in somites, therefore their expression might be controlled by myogenic factors. Moreover a recent report has suggested that *TEF-1* genes could be targets of the myogenic regulatory factor MyoD and the transcription factor Myocyte Enhancer Factor 2 (Mef2) during the differentiation of murine myoblasts (Blais *et al*., 2005). We have tested this hypothesis by analysing the expression of *XNTEF-1* and *XDTEF-1* genes upon overexpression of *MyoD* and *Mef2* mRNAs in animal cap explants. This approach has been widely used for testing the effect of transcription factors on potential genes targets. For example, we have shown that MyoD could activate the α-tropomyosin and the *MLC1f/3f* skeletal muscle genes and that this activation was increased in the presence of Mef2 (Thézé *et al*., 1995; Gaillard *et al*., 1998). We have tested the effect of MyoD and Mef2a and Mef2d, the two known *Xenopus* Mef2 proteins. MyoD has no effect on either *XNTEF-1* or *XDTEF-1* genes expression in such assay (Fig. 5D, lane 2). On the contrary, Mef2d but not Mef2a induces a significant decrease in the expression of *XDTEF-1* gene but not of *XNTEF-1* gene (Fig. 5D, lane 3). Mef2a induces a slight but specific increase in the expression of *XDTEF-1* gene but has no effect on *XNTEF-1* gene expression (Fig. 5D, lane 4). The co-injection of MyoD with Mef2a or Mef2d does not modify the expression of both *TEF-1* genes while it increases, as expected, the expression of *XMLC1f* gene (Fig.5D, lanes 5, 6). Together these data show that the two *X. laevis TEF-1* genes have distinct spatial expression but are also differently regulated by signalling pathways in the early embryo.

Experimental Procedures

Isolation of Xenopus laevis DTEF-1*and* NTEF-1 *cDNAs*

The IMAGE clones #4434995 and # 4959564 were obtained from the MRC Geneservice and the IMAGE Consortium and were fully sequenced on both strands. For sequence comparisons, the following sequences were used: human, Genbank Accession No. P28347 and X94439; mouse, Genbank Accession No. AAA40410, AAH21364, BAA12018 and

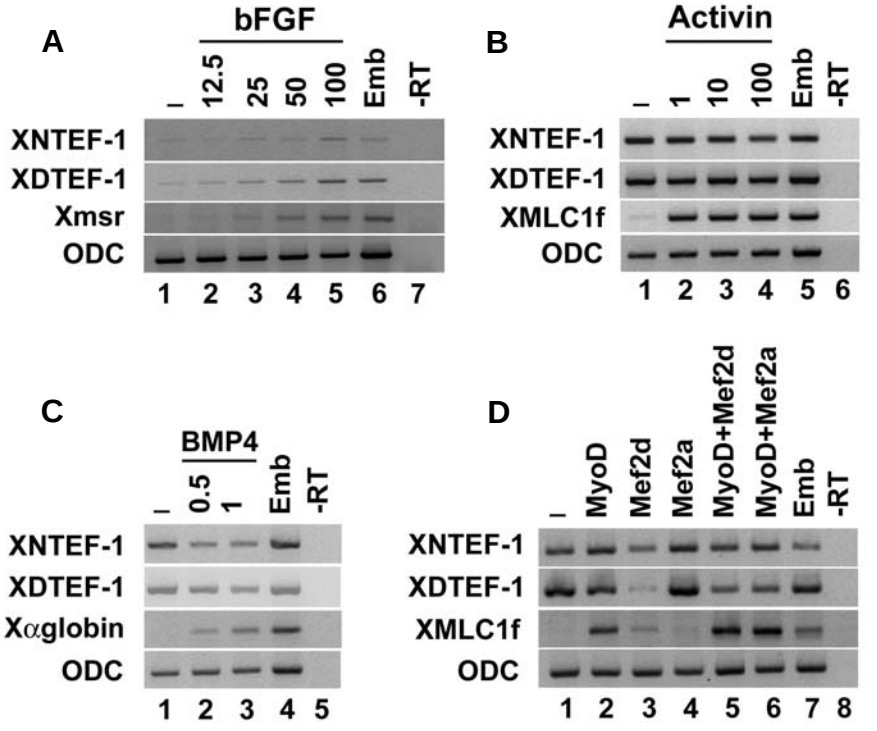

Fig. 5. *XNTEF-1* and *XDTEF-1* expression in animal cap explants. *Animal caps explants, treated with growth factors* **(A,B)** *or derived from injected embryos with mRNAs* **(C,D)**, *were analysed with control embryo (Emb) for* XNTEF-1 *and* XDTEF-1 *mRNAs content by RT-PCR analysis.* **(A)** *Animal cap explants were treated with increasing amounts of bFGF (12.5-100 ng/ml).* Xmsr *mRNA detection was used as a control of induction.* **(B)** *Animal caps were treated with increasing amounts of activin (1-100ng/ml).* XMLC1f *mRNA detection was used as a control of induction.* **(C)** *2-cell stage embryos were injected with 0.5ng or 1ng of* BMP4 *mRNA.* Xαglobin *mRNA detection was used as control.* **(D)** *2-cell stage embryos were injected with the indicated mRNAs: lane 2 (0.4 ng of* MyoD *and 0.4 ng of* E12*), lane 3 (1ng of* Mef2d*), lane 4 (1ng of* Mef2a*), lane 5 (0.4ng of* MyoD, E12 *and* Mef2d*), lane 6 (0.4ng of* MyoD, E12 *and* Mef2a*).* XMLC1f *mRNA detection was used as control. ODC was used as control of loading and a reaction was performed in absence of reverse transcriptase to check for genomic DNA contamination (-RT).*

X94441; chicken, Genbank Accession No XM_420962 and T09284.

Xenopus *embryo collection and whole mount* in situ *hybridization*

Eggs were obtained from *Xenopus laevis* females primed with human chorionic gonadotrophin, fertilized artificially and cultured in 0.1XMBS (Sive *et al*., 2000). Embryos were fixed in MEMFA at the desired stage according to Nieuwkoop and Faber (1994). Whole-mount *in situ* hybridization was carried out using current protocol (Sive *et al*. 2000) with full length anti-sense probes corresponding to *XNTEF-1* and *XDTEF1* sequences. Embryos were embedded in paraplast, sectioned at 25 nm, dewaxed, dehydrated and embedded in permount.

Microinjection of embryos and animal cap assay

For induction assay, animal cap explants from stage 8-9 embryos were treated with increasing amounts of bFGF (kindly provided by Dr. A. Bikfalvi) or with activin (R&D Systems) and cultured until control embryos reached stage 26 before RT-PCR analysis. For microinjection experiments, the capped mRNAs were synthesized *in vitro* by using Ambion mMessage mMachine SP6 and T7 kits (Austin, TX) with plasmids generously provided by H. Brivanlou (BMP4) and T. Mohun (MyoD, E12, Mef2a and Mef2d). Embryos were injected in the animal cap with a known amount of the mRNA solution at the two-cell stage into both blastomeres using a Nanoject system (Drummond Scientific). Animal caps were then dissected from stage 8-9 injected embryos and cultured to stage 26 (for BMP4) or stage 12 (for MyoD, Mef2a and Mef2d) before RNA extraction.

RT-PCR

Total RNA from embryos and animal cap explants was isolated with a proteinase K protocol (Sive *et al*., 2000). RNA from adult tissues was extracted with trizol solution (Invitrogen) according to manufacturer instructions. 1 μg of total RNA was used in first strand synthesis using Promega MMLV reverse transcriptase following manufacturer instructions. PCR reaction was performed as previously described and a detailed protocol is available upon request (Pasquet *et al*., 2006). Reverse transcribed samples without reverse transcriptase were also prepared to check for contamination of genomic DNA. The quantity of input cDNA was verified by similarity of *ornithine decarboxylase* (*ODC*) or *L8 ribosomal protein* gene (*Rpl8*) signals. Linearity of amplification was confirmed by PCR with consecutive doubling dilutions of input cDNA. The following primer pairs were used:

XNTEF-1	5'-ATTCCAGCCTGCAAACTTCC-3 and 5'- GTGACTAGACACCTGTTTCC-3';
XDTEF-1	5'-TTCTGCGATCGCACTAAGAAGC-3' and 5'-GCCATACATTTTGCCTTC-3';
XMLC1f	5'-TTTGACAAGGAAGGCAATGG-3' and 5'- CATTCTGCTGACAGTTCTTG -3';
XMLC2	5'- GGATGAGATGCTCAAGAGG -3' and 5'-TCTCATCTCCATGCGTGATG-3';
Xwnt8	5'-TGGCAAGAACTTGTCCCAGT-3' and 5'-TTCTGGAATGCCGTCATCTC-3';
Xbra	5'-TTAAGTGCTGTAATCTCTTCA-3' and 5'-CTGGAAGTATGTGAATGGAG-3';
Xchordin	5'-CTCCAATCCAAGACTCCAGC-3' and 5'-GGAGGAGGAGGAGCTTTGGGA-3';
Rpl8	5'-GACGACCAGTACGACGAG-3' and 5'-CACCATGCCTGAAGGCAC-3';
ODC	5'-GTCAATGATGGAGTGTATGGATC-3' and 5'-TCCATTCCGCTCTCCTGACCAC-3';
Xmsr	5'-ACATCATTGTCAGCCTGCAC-3' and 5'-AGTCCCTGTTCTGTAATCAG-3';
Xαglobin	5'-GCTGTCTCACACCATCCAGG-3' and 5'- TGTACTTGGAGGTGAGGACG-3'.

Acknowledgements

This work was supported by the CNRS and the University of Bordeaux 2. We thank the DoE Joint Genome Institute for sharing data.

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Published Online: 6th September 2007