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Short Contribution

Differential expression of two skeletal muscle beta-tropomyosin mRNAs during *Xenopus laevis* development

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ABSTRACT A cDNA clone for a *Xenopus laevis* skeletal muscle β-tropomyosin (β-TMad) isoform was isolated from an adult skeletal muscle cDNA library. Sequence analysis revealed that this clone corresponded to a second β-tropomyosin mRNA distinct from the one that was previously characterized (β-TMemb). The two skeletal β-TM mRNAs originate from distinct genes and are differentially expressed during development. β-TMemb mRNA is expressed only in the somites of the early embryo while β-TMad mRNA is expressed in pre-metamorphic tadpoles and adult skeletal muscles. We have isolated the promoter region of the *β-TMemb* gene and shown that a DNA construct containing 2.9 kb of promoter region is properly expressed after injection in the embryo.

KEY WORDS: *tropomyosin, gene expression, skeletal muscle, Xenopus laevis*

Tropomyosins (TMs) constitute a family of ubiquitously conserved structural proteins. In striated muscle cells, TMs mediate Ca^{++} regulated actomyosin contraction while in non-muscle cells they stabilize F actin proteins (Smillie, 1979; Pittenger *et al.*, 1994). TMs isoform diversity is generated by alternative processing of the primary transcript RNA and for some genes by the use of alternate promoters and multiple polyadenylation signals (Lees Miller and Helfman, 1991). There are four TM genes in higher vertebrates namely *α-TM*, *β-TM*, *TM-4* and *TMnm* and we have shown that three of them (*α-TM*, *β-TM* and *TM-4*) are expressed in the amphibian *Xenopus laevis* in a complex temporal stage and tissue specific pattern (Hardy *et al.*, 1991, 1995; Hardy and Thiéband, 1992; Gaillard *et al.*, 1998a, b). Our studies revealed that the amphibian *α-TM* gene was structurally related to its mammalian counterpart while the amphibian *TM-4* gene was more closely related to the homologous chicken gene.

The cDNA clones XTMβ4 and XTMβ5 we have previously isolated, encoded respectively a skeletal and a smooth muscle β-TM isoform, but nucleotide sequence comparison suggested they were originated from distinct genes. (Hardy and Thiéband, 1992) RNAse protection analysis with a XTMβ4 3'UTR probe indicated that the corresponding mRNA was expressed exclusively in the embryo, while Northern blot data with a coding sequence probe revealed a strong signal with adult skeletal muscle RNA (data not shown) (Hardy and Thiéband, 1992). These data suggested the presence in adult skeletal muscle of β-TM transcripts that are related but distinct from XTMβ4. To isolate such sequences, we screened an adult skeletal muscle cDNA library with a XTMβ4 probe. One positive clone termed β-TMad, was further characterized. β-TMad is a 1550bp full length cDNA clone containing an 852bp open reading frame encoding a skeletal muscle β-TM isoform. β-TMad and XTMβ4 showed 94% nucleotide sequence identity over their coding region, giving rise to a 19 amino acid difference. The 3'UTR regions of the cDNAs are divergent, presenting stretches of conserved sequences interrupted by gaps (Fig. 1). These features indicate that the two skeletal muscle β-TM cDNA clones are derived from separate genes. Therefore, there are at least two non-allelic *β-TM* genes in *Xenopus laevis* and both encode a skeletal muscle isoform. This is not uncommon for *Xenopus laevis*, since it is a pseudotetraploid species whose genome has been duplicated during evolution (Kobel and Du Pasquier, 1986). Most of the genes that have been described in *Xenopus laevis* exist as two copies by haploid genome (Graf and Kobel, 1991).

We next analyzed the expression of the two skeletal β-TM transcripts. Because of the sequence homology between the two cDNA clones, it was difficult to generate gene specific probes allowing distinction of the corresponding transcripts by Northern blot or RNase protection analysis. We therefore used RT-PCR analysis

Abbreviations used in this paper: bp, base pair(s); CAT, Chloramphenicol acetyl transferase; cDNA, DNA complementary to RNA; kb, kilobase (s); nt, nucleotide (s); ODC, ornithine decarboxylase; RT-PCR, reverse transcription-polymerase chain reaction; TM, tropomyosin (s); UTR, untranslated region.

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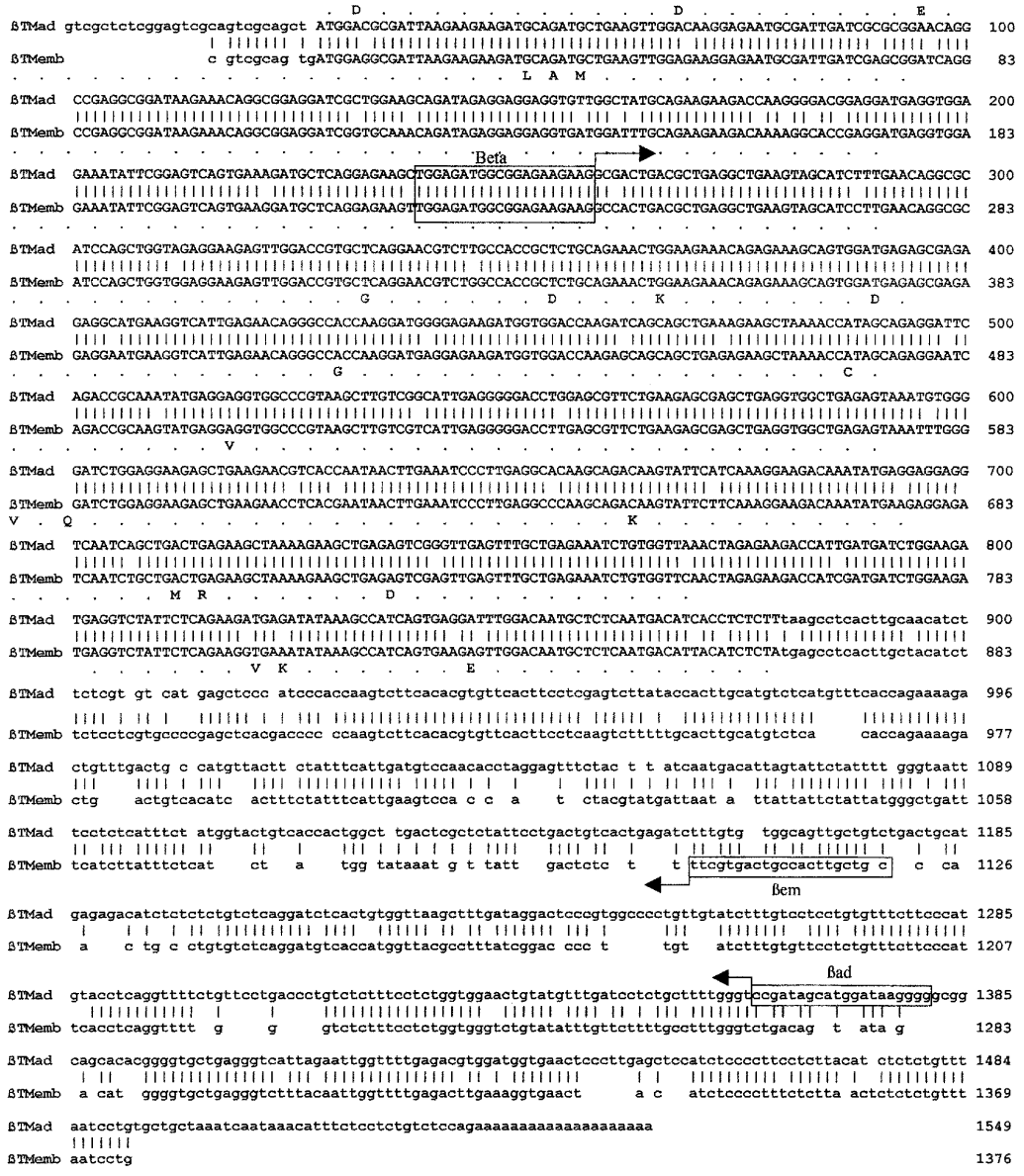

Fig. 1. Sequence comparison between β-TMad and β-TMemb (formerly XTMβ4) clones. *Nucleotide and amino acid identity are indicated by dashes and dots respectively. Gaps have been introduced to maximize the alignment in the 3'UTR. Primers used in RT-PCR analysis have been boxed.*

and chose downstream primers in the divergent 3'UTR regions of the two clones and a common forward primer in their 5' coding sequence (see Fig. 1 for the location of the primers). As shown in Figure 2A, the two skeletal β-TM mRNAs have a distinct pattern of expression. XTMβ4 transcripts are not detected in the egg and appeared between stage 22 and 25 of embryogenesis. The steady state level of the transcripts peaks around stage 33 before decaying and being barely detectable by stage 48. XTMβ4 transcripts are not expressed in skeletal muscle tissues nor are they found in larvae or in the adult. In contrast, β-TMad transcripts are not detected in the embryo before stage 48 but are present in the body and tail muscles of the metamorphic larvae and in adult skeletal muscle (Fig. 2A). A control RT-PCR with ODC specific primers showed no variations in the RNA level between the samples (data not shown). These data indicate that the two skeletal β-TM mRNAs have a distinct temporal pattern of expression that appears to be mutually exclusive. In several reports it has been shown that the two *Xenopus laevis* non-allelic genes are differentially expressed (e.g., *MyoD* gene or preproinsulin gene) (Scales *et al.*, 1990; Perfetti *et al.*, 1994). However, to our knowledge, there is no example where the duplicated genes show complementary patterns of expression in a manner comparable with our findings. The presence of the two transcripts in the late embryo (stage 48) could correspond to the situation where the *XTMβ4* gene is turned off and the corresponding transcripts are decaying while the β-TMad transcripts start to accumulate. Because of its pattern of expression we have changed the name *XTMβ4* for *β-TMemb*, thus indicating its restricted expression to the embryo.

To analyze the spatial expression of the β-TMemb transcripts in the embryo, we performed RNA whole-mount *in situ* hybridization. To avoid cross hybridization with transcripts from the *α-TM* gene that is highly expressed in the embryo (Hardy *et al.*, 1991; Gaillard *et al.*, 1998a), we used a 3'UTR probe derived from the β-TMemb cDNA clone. As shown in Figure 2B, β-TMemb transcripts are solely detected in the somites following an anteroposterior gradient as the embryo developed. This was confirmed by *in situ* hybridization with ^{35}S antisense probe on embryo sections (Fig. 2C).

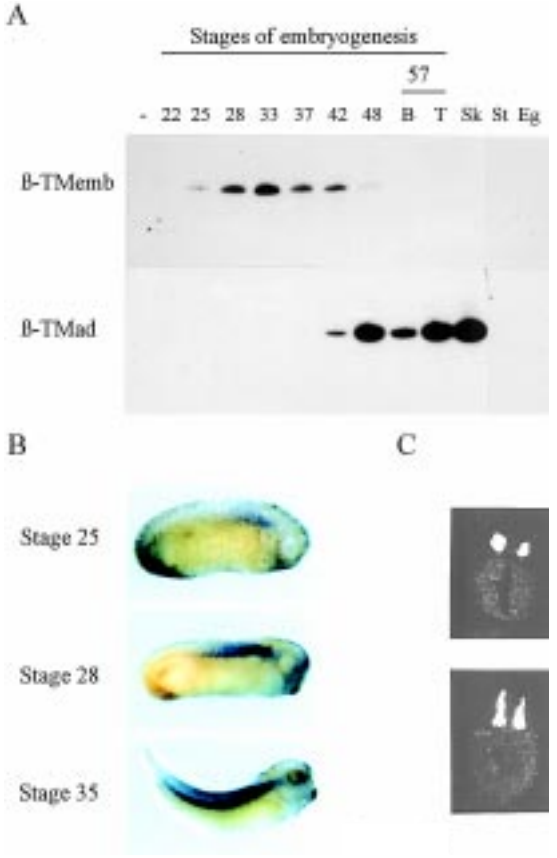

Fig. 2. Expression of β-TMemb and β-TMad transcripts in embryos and adult tissues. (A) *The distribution of β-TMemb and β-TMad transcripts was analyzed by RT-PCR. RNA was from eggs (Eg), different staged embryos (22-48), body (B) and tail (T) muscles of pre-metamorphic stage 57 tadpoles, adult skeletal muscle (Sk) and stomach (St). tRNA (-) was used as control.* **(B)** *Whole-mount in situ analysis of β-TMemb transcripts. Embryos were stained at stage 25, 28 and 35.* **(C)** *In situ hybridization on transverse sections of a stage 25 (upper) and stage 35 (lower) embryo.*

Together these data revealed that the two skeletal muscle β-TM transcripts have a distinct pattern of expression with respect to the embryonic and adult skeletal muscle lineages. This implies that in these lineages, both activators and repressors of transcription are operating to give the biphasic pattern of *β-TM* gene expression. As a first step towards gaining insights into the molecular mechanisms that control the developmental dependent muscle expression of the *β-TM* genes, we screened a genomic library and isolated a 13 kb genomic clone whose sequence indicated it was corresponding to the *β-TMemb* gene. This genomic clone was covering the first five exons and extended 3.8 kb upstream of the transcription start site that was mapped by RNase protection analysis (Fig. 3A and B). Computer analysis of the genomic sequence between exons 2 and 3 did not reveal the presence of an additional exon (exon 1') corresponding to the internal promoter described in the chicken gene (Libri *et al.*, 1990). Together with our gene expression studies and cDNA screening data, this suggested that the amphibian *β-TM* genes do not generate a low molecular weight non-muscle isoform from an internal promoter as in the case for the avian gene. This makes the amphibian *β-TM* gene structurally closely related to its mammalian counterparts. However, the genomic region covering the first five exons of the *Xenopus* gene is 8.5 kb long compared to 4.5 kb for the rat gene. This difference corresponds to a longer size of the intron I in the *Xenopus* gene.

To determine whether the β-TMemb genomic clone we isolated contained regulatory sequences sufficient for expression in the embryo, the region between -2.9 kb to + 7bp of the promoter region was inserted upstream of the CAT reporter gene. The resulting gene fusion was injected into *oocyte* and fertilized eggs. As shown in Figure 3C, CAT activity is undetectable in the oocyte. There is a non detectable CAT expression in stage 12 and stage 18 embryos but a high activity in stage 24 and 28 (Fig. 3C, lanes 12-24). This is not due to the amplification of the injected DNA as controlled by DNA dot blot (data not shown). When comparing the CAT activity in the dorsal part versus the ventral part of stage 28 microdissected embryos, the activity is detected only in the dorsal part of the embryo that consists mainly of somitic tissues (Fig. 3C, lanes V and D). A control promoterless plasmid gave no CAT activity in the oocyte or in the embryo. This suggested that the 2.9 kb promoter region of the β-TMemb gene contained regulatory sequences for correct expression in the embryo.

The present study showed that two skeletal muscle β-TM mRNAs originating from distinct genes are differentially expressed during development. It is hardly conceivable that the two proteins perform different functions. Nishikawa and Hayashi (1994) have shown there was a nine fold increase of β-TM protein level during *Xenopus* metamorphosis while α-TM level seemed unchanged. According to our data, this β-TM protein increase corresponds to the activation of the *β-TMad* gene as we have showed there was no expression of the *β-TMemb* gene during the progression from larva to adult. The shift in the expression of the *β-TM* genes is occurring during metamorphosis, suggesting a possible control by thyroid hormone. However, we were unable to get an effect of the hormone on either the extinction of the *β-TMemb* gene or the precocious activation of the *β-TMad* gene (data not shown). This is consistent with the study of Nishikawa and Hayashi (1994), showing no differences of the TM protein level in hormonal treated embryos.

In conclusion, the *Xenopus laevis β-TM* genes provide a good model for the study of the embryonic/adult dependant activation of skeletal muscle genes.

Experimental procedures

Isolation and characterization of cDNA and genomic clones

An adult skeletal muscle cDNA library made in pGAD10 plasmid vector was screened at high stringency with the coding region of XTMβ4. Positive clones were screened in duplicate with a XTMβ4 probe and the skeletal α-TM 3'UTR probe XTMβ26 (Hardy *et al.*, 1991) in order to remove α-TM sequences that are highly expressed in adult skeletal muscle. The positive hybridizing clone encoding β-TMad was sequenced by the dideoxy chain-termination method (Sanger *et al.,* 1977) using a sequencing kit (Pharmacia). A *Xenopus laevis* genomic library (courtesy of Igor Dawid) was screened at high stringency with the 5' region of β-TMemb cDNA clone. The positive clones were analyzed by southern blot and DNA sequencing using standard procedures (Sambrook et al., 1989).

RNA preparation and analysis

RNA was prepared from embryos and adult tissues as described previously (Gaillard *et al.*, 1998a). RT-PCR was performed as described in Wilson and Melton (1994) and the cycling parameters were as follows: the initial cycling consisted of a 94°C denaturation for 5 min, 1 min at 55°C annealing temperature and 1 min at 72°C extension temperature. The remaining 25

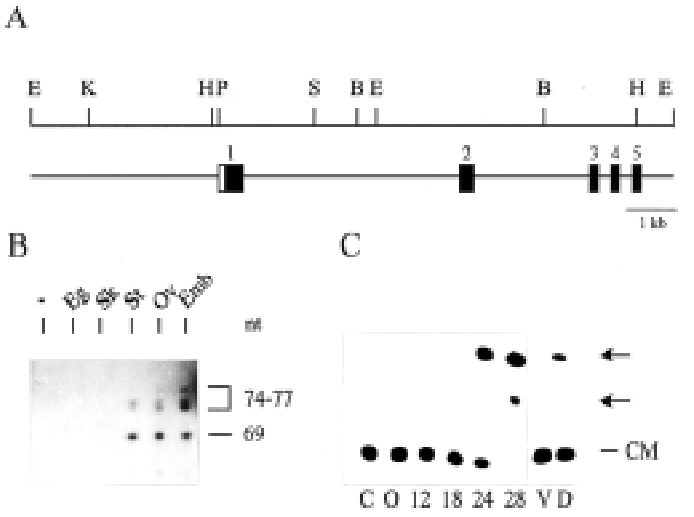

Fig. 3. *Xenopus laevis* β*-TMemb* gene 5' structure and transcriptional activation of the promoter region in the embryo. (A) *Schematic representation of the 13 kb genomic clone. Exons are figured by black boxes and 5'UTR by an open box. Major restriction enzymes sites are BamHI (B), EcoRI (E), HindIII (H) and KpnI (K).* **(B)** *RNase mapping of the transcription start site. Total RNA from eggs (Eg), skeletal muscle (Sk), stomach (St), oviduct (Ov), embryo (Emb) or tRNA (-) were analyzed as described in Experimental Procedures. Similar size major products of 69 and 74-77 nt are detected in embryo and smooth muscle tissues.* **(C)** *CAT activity detection in Xenopus oocytes and embryos injected with the 2.9 kb β-TM/CAT plasmid construction. Embryos are from stage 12 (12), 18 (18), 24 (24) 28 (28), and ventral (V) or dorsal (D) part of a stage 28 embryo. The promoterless plasmid PBCOCAT was used as control (C). The migration of the acetylated forms of chloramphenicol (CM) is indicated by arrows.*

cycles were for 30 sec at 94°C, 1 min at 55°C and 1 min at 72°C. Primers for PCR were as follows:
Beta common forward primer: 5'TGGAGATGGCGGAGAAGAAG3'; βem primer 5'GCAGCAAGTGGCAGTCACGA3'; βad primer: 5'CCCCTTATCCATGCTATCGG3'.

RNase protection analysis was performed as described in Gaillard *et al.* (1998a) using a radiolabeled antisense probe that covers a 163 nt genomic region upstream of the third amino acid of β-TMemb.

In situ hybridization

Whole-mount RNA *in situ* hybridization was performed as described by Harland (1991), using a digoxygenin-labeled probe. The antisense β-TMemb probe was derived from the subclone XTMβ44 that covers 380 nt of 3'UTR (Hardy and Thiéband, 1992). *In situ* hybridization of sectioned embryos with a radiolabeled RNA probe was carried out as described by Thézé *et al.*, (1995). ^{35}S-labeled probe was synthesized from the plasmid XTMβ44.

DNA construction and injections

A 2.9 kb KpnI/PstI genomic fragment including the transcription start site of the *β-TMemb* gene was subcloned into pBCOCAT vector (Clark *et al.*, 1989) to give the β-TM/CAT plasmid construction. Oocytes were injected with 20 nl of a solution containing 2.5 ng of circular β-TM/CAT plasmid or pBCOCAT promoterless plasmid. For embryo injections, 5 nl of a solution containing 250 pg of circular plasmid DNA were injected at the two-cell stage. CAT analysis was performed as described by Gorman *et al.* (1982) and analyzed by thin layer chromatography.

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