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Short Sequence-Paper

Identification of a muscle factor related to MyoD in a fish species

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Abstract

We have isolated the cDNA encoding a myogenic factor expressed in embryonic trout muscle by hybridization with a *Xenopus* MyoD cDNA. Nucleotide sequence analysis and amino acid comparison showed that this cDNA called TMyoD encodes a polypeptide of 276 amino acids with 70% identity to the entire *Xenopus* MyoD protein and 92% identity within the basic and myc-like region. Results from Northern blotting showed that the corresponding transcript is expressed both in adult and embryonic skeletal musculature and in an in vitro myogenesis system, but is undetectable in cardiac and smooth muscles and in non muscle tissues.

Key words: MyoD; Myogenesis; Teleost; (Satellite cell)

The MyoD gene identified by subtract cloning for myoblast specific RNA is the prototype of a family of master regulators of skeletal myogenesis which includes in vertebrates three other members namely myogenin, Myf5 and MRF4 identified subsequently [1–5]. All these genes encode proteins that share a highly conserved central region termed the basic/helix-loop-helix (B-HLH) domain related to the c-myc superfamily and contain sequences essential for both dimerisation and DNA binding [6]. In multipotential 10T1/2 cells, transfection experiments have shown that forced expression of these exogenous myogenic factors is sufficient to drive them down the muscle differentiation pathway suggesting their functions in myogenic lineage determination [1–5]. In contrast to vertebrates whose genome encodes multiple members of the MyoD family, invertebrates, including Sea urchin [7], *C. elegans* [8] and *Drosophila* [9], appear to contain only a single myogenic factor encoding gene. However, the myogenic factor for Sea urchin and *C. elegans* activates myogenesis in 10T1/2 cells indicating a highly conserved mechanism for muscle genes activation.

Myogenic factors have been studied in mammals, amphibians and birds [10], but nothing is known to date in fish. To analyse early developmental events leading to muscle formation in fish, we set out to isolate myogenic regulatory factors from Rainbow trout (*Oncorhynchus mykiss*) embryos (398 degree days) using a probe which spanned functional domains of the *Xenopus* MyoD cDNA. For this purpose, a λ gt10 cDNA library was constructed from poly(A)⁺ RNA of the trunk of rainbow trout embryos. The double strands cDNAs synthesized by the method of Gubler et al. [11] were size fractionated by gel filtration on a sepharose 4B column (Pharmacia) and the largest fractions were pooled, inserted into λ gt10 vector (Stratagene) and encapsided using an in vitro packaging kit (Amersham). After amplification of the cDNA library, approximately $5 \cdot 10^5$ plaques were screened at low stringency with a fragment from the *Xenopus* MyoD cDNA encompassing the B-HLH domain [12]. From 9 positive clones, we identified a single cDNA of 1.5 kb which had two *Eco*RI fragments of approximately 1.2 and 0.3 kb. Restriction analysis and sequencing showed that this internal *Eco*RI site was not situated near a *Not*I site which is contained in the linker used for the ligation of the cDNAs in the λ gt10 vector, so we did not think that the two fragments were inadvertently ligated during the construction of the library. The nucleotide sequence of our cDNA, determined by standard

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The sequence data reported in this paper have been submitted to the EMBL/Genbank/DBJ Nucleotide sequence Databases under the accession number X75798.

Xen. MyoD	MELLPPPLADMEVTEGSLCAFPITDDFYDDPCFNTSDMSFFEDDLRLVHVHTLLKPEEPH	60
Tr. MyoD	...FDI.FPI-----TS-----H-----G...DDH.	
Xen. MyoD	HNEHDVRAPSGGHAGRCLLWACKAKRKRTTNADNRKAATMRERRRLSKVNEAFETLKR	120
Tr. MyoD	K...I...-.....D.....	
Xen. MyoD	YTSNPNQRLPKVEILRNATRYIESLQALH--DQEAFFYPVLEHYSGSDASSPSRNCSS	178
Tr. MyoD	C.....D.....S...G...RGAG.EGNY...MD.....	
Xen. MyoD	DGMMDYNSPPCGSRNRSSSYFSDSPNDRSLGKSSVSSLDCLSSIVERISTQSPSCP	238
Tr. MyoD	...F.GQS.FP...K...TYFNEA...HK.N...N...T.DTSA...	
Xen. MyoD	VPTAVDSGSGGS-PCSPLOGTETSERVITPSPNFTQSLQSDPSSTIHYVL	289
Tr. MyoD	...OD...S...GD.SIA..NGAP...I.CVPA..H..N...Q...	

The open reading frame encodes a polypeptide of 276 amino acids with 70% of identity with the *Xenopus* MyoD protein (Fig. 2). Within the B-HLH domain, the deduced polypeptide has higher identity with XMyoD (92%) than other *Xenopus* myogenic determinants as Myf 5 (82%), Myogenin (72%) and MRF4 (70%) [14,15]. These comparisons suggest that our clone encodes a teleost homologue of the myogenic factor MyoD. Moreover, outside the conserved basic and myc-like domain which is found in all myogenic factors of vertebrates and invertebrates, we identified four other blocks of conserved sequences which association is typical of the MyoD protein of all vertebrates studied so far [16]. Using XMyoD as the amino acid position reference (Fig 2), these conserved sequences include an acidic amino-terminal region (24–56), a histidine-cysteine rich domain (60–87) and two regions (163–183, 215–230) clustered in the carboxy-terminal half of the protein. On the whole, these data show that a teleost homologue of the myogenic factor MyoD does exist, its presence in fish indicates furthermore that this protein appeared early in vertebrate evolution as a distinct myogenic factor.

To determine whether TMyoD represents a muscle specific gene product, we examined, by Northern blot, the steady state level of its mRNA in various tissues. As shown in Fig. 3B, TMyoD is present both in red and white fibers which compose the trunk musculature

dideoxymethods [13] is shown in Fig. 1: the total cDNA contains 1469 bases with an open reading frame of 828 nucleotides. In the 3' untranslated region, the internal *Eco*RI site is found at position 1119 and a putative polyadenylation site is apparent at position 1421.

Fig. 3. TMyoD expression. Total RNA (10 μ g) were resolved on a formaldehyde gel and transferred to hybrid membranes. The blots were hybridized with a 1.1 kb *Eco*RI TMyoD insert radioactively labeled by random priming and washed at high stringency. (A) Northern blot of RNA from hatching embryos (lane 1) and adult (lane 2) trout muscles. (B) Northern blot of RNA from red muscle, white muscle, kidney, testis, gills, intestine, liver and heart (lanes 1 to 8). (C) Northern blot analysis of RNA from trout primary cultures of satellite. Total RNA was isolated from growing cells 4 h (lane 1) and 48 h (lane 2) after seeding, and in differentiated myotubes, 7 days (lane 3) and 11 days (lane 4) after seeding.

of teleosts. No detectable TMyoD mRNA is observed in cardiac (heart) and smooth (intestine) muscle tissues. TMyoD mRNA is undetectable in non muscle tissue types including liver, kidney, gills and testis. Thus, our results show that TMyoD expression is restricted to a skeletal muscle lineage in adult animals.

We have also analysed the time course of expression of TMyoD during in vitro differentiation of myosatellite cells isolated from juvenile trouts and cultured at 18°C in DMEM medium supplemented with 10% calf serum. Fig. 3C shows that TMyoD mRNA is slightly detectable in 4-h cultures, its level is dramatically increased in growing myosatellite cell cultures (48 h after seeding) and remains constantly elevated in differentiated myotubes (7 and 11 days after seeding). These observations indicate that TMyoD acts as a major developmental regulator of myogenesis in fish.

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