A gene with homology to myogenin is expressed in developing myotomal musculature of the rainbow trout and in vitro during the conversion of myosatellite cells to myotubes

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Abstract **We report the cloning of a new trout myogenic cDNA which encodes helix-loop-helix protein homologous to the myogenic factor myogenin. Northern analyses indicate that trout myogenin (Tmyogenin) transcripts accumulate in large amounts in the myotomal musculature of embryos and frys. In adults, transcripts concentrate within the thin lateral layer of red (slow oxydative) muscle fibres. They are present only in low amounts in white (fast glycolytic) muscle fibres which constitute the major part of the trunk musculature. Using an in vitro myogenesis system, we observed that the trout myogenin encoding gene is not activated until myosatellite cells fuse to generate multinucleated myotubes, indicating that Tmyogenin lies downstream of muscle determination factors. All these observations show that in a major taxinomic group like teleosts, a gene with homology to myogenin exists. Its activation during myogenesis suggests that it acts as a major developmental regulator of muscle differentiation.**

Key words: Myogenin; Satellite cell; Myogenesis; Teleost

1. Introduction

A family of cell type-specific basic helix-loop-helix (b-HLH) proteins, which includes MyoD [1], myogenin [2,3], Myf-5 [4], and MRF4/herculin/Myf-6 [5–7] has been shown to play an important role in the regulation of myogenesis. These proteins are nuclear phosphoproteins that activate muscle specific transcription through binding to a DNA consensus sequence known as an E-box found in the control regions of numerous muscle genes [8]. Each of these proteins has the ability to induce skeletal muscle phenotype in a wide range of cell types, when it is constitutively synthetized from transfected expression vectors [8]. From genetic knockout experiments, it appears that either MyoD or Myf5 is required for the determination of skeletal myoblast [9], while Myogenin has a function in the transition from a determined myoblast to a fully differentiated myotube [10,11]. Although a myogenin-like genomic fragment including the b-HLH domain was obtained in *Xenopus* and *Torpedo* [12,13], no corresponding transcripts have been detected in the muscle of these animals. Therefore it is not evident whether the myogenin encoding gene is actually transcribed in lower vertebrates. Fish muscle fibres offer an interesting problem in differentiation because of the presence of two major types of fibres (white and red) which are involved in two kinds of swimming activities. However, very little is known concerning the processes that lead to the maintenance of different fibre phenotypes in adult myotomal musculature. In this respect, we do not know whether there is a differential expression of myogenic regulators in white and red muscles. We have previously identified a teleost fish homologue of MyoD [14]. In this study, we report the cloning of a teleost cDNA related to myogenin. We show furthermore, that the gene encoding Tmyogenin is expressed at high levels in developing trout myotomal muscle, and in vitro during the differentiation of satellite cells. In adult, the expression of Tmyogenin appears restricted to the red muscle.

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2. Materials and methods

2.1. Trout embryonic cDNA library construct

A λgt10 cDNA library was constructed from poly(A$^+$) RNA from myotomal muscle of rainbow trout (*Oncorhynchus mykiss*) embryos (eyed stage). The double strand cDNAs synthetized by the method of Gubler et al. [15] were size fractionated by gel filtration on a Sepharose 4B column (Pharmacia). The largest fractions were pooled and inserted into λgt10 vector (Stratagene) and encapsided using an in vitro packaging kit (Amersham).

2.2. Library screening

The embryonic cDNA library was probed at low stringency with the previously described cDNA TMyoD [14]. After hybridization at 42°C for 16 h in 40% formamide, 6 × SSPE, 5 × Denhart's solution, 0.5% SDS and 0.1 mg/ml sheared denatured calf thymus DNA, filters were washed twice in 2 × SSPE, 0.5% SDS for 30 min at 45°C. Positive clones were purified by standard methods. λDNA was analysed by Southern blotting and subcloned into Bluescript plasmid (Stratagene) for sequencing both strands by the dideoxynucleotide termination method (Pharmacia kit) described by Sanger et al. [16]. Besides the universal and reverse primers, oligonucleotides corresponding to the determined sequence were synthetized to obtain the complete sequence.

2.3. Northern hybridization analysis

Total RNA was extracted by the guanidium thiocyanate/cesium chloride method [17]. RNA(10 μg) were resolved on a formaldehyde gel and transferred to Hybond membrane. Consistency of RNA loading was verified by visualization of ribosomal RNA bands on ethidium bromide stained parallel gels. To avoid cross hybridization with other myogenic factors, a *Pst*I–*Eco*RI fragment that does not encompass the b-HLH domain was labelled with ^{32}P[dATP] by random priming and used for blot hybridization. The conditions of hybridization and washing were the same as described by Sambrook et al. [18]. Filters were exposed to X-ray film for 48–96 h at −80°C using intensifying screen.

2.4. Satellite cells and obtaining cultures

Satellite cells were isolated and cultivated according to the procedure described by Koumanns et al. [19]. Briefly white epaxial muscle from 50 mm long trout was excised under sterile conditions and collected in DMEM (gibco) medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml) and gentamicin (75 μg/ml) The tissue was minced into small pieces, centrifuged and treated 1 h with a 0.2% collagenase solution. After centrifugation, the pellet was

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resuspended twice in a 0.1% trypsine solution during 20 min. Fragments were then mechanically dissociated and the resulting suspension was filtered through a 50 μm filter gauze. Collected cells were seeded on a laminin substrat (Sigma) and cultured in DMEM medium continuously supplemented with 10% calf serum and antibiotics.

3. Results

3.1. Cloning and sequencing of a new trout myogenic factor: Tmyogenin

The low-stringency screening of a trout embryo muscle library with the full-length trout MyoD cDNA probe yielded a 1.4 kb cDNA. Sequence analysis of this cDNA is shown in Fig. 1. Although we were unsuccessful in isolating a full-length

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                15                  30                  45
CGG AAA GCG GCC ACA ATG CGT GAG AAG AGG AGG CTG AAG AAG GTG AAC GAG GCA
Arg Lys Ala Ala Thr Met Arg Glu Lys Arg Arg Leu Lys Lys Val Asn Glu Ala

     60                  75                  90                 105
TTC GAG GCC CTG AAG AGG AGC ACC CTG ATG AAC CCC AAC CAG AGG CTG CCC AAG
Phe Glu Ala Leu Lys Arg Ser Thr Leu Met Asn Pro Asn Gln Arg Leu Pro Lys

            120                 135                 150
GTG GAG ATC CTG AGG AGT GCC ATC CAG TAC ATT GAG AGG CTG CAG GCA CTT GTC
Val Glu Ile Leu Arg Ser Ala Ile Gln Tyr Ile Glu Arg Leu Gln Ala Leu Val

165                 180                 195                 210
TCC TCC CTC AAC CAG CAG GAG AAC GAC CAG GGA ACA CAG GGC TTA CAA TAC CGC
Ser Ser Leu Asn Gln Gln Glu Asn Asp Gln Gly Thr Gln Gly Leu Gln Tyr Arg

        225                 240                 255                 270
ACC GGA CCT GCT CAA CCC AGG GTG TCG TCG TCG AGT GAG CAG GGA TCA GGC AGC
Thr Gly Pro Ala Gln Pro Arg Val Ser Ser Ser Ser Glu Gln Gly Ser Gly Ser

            285                 300                 315
ACC TGC TGT AGC AGC CCA GAG TGG AGC AAC ACC TCA GAC CAC TGT GCC CAG AGC
Thr Cys Cys Ser Ser Pro Glu Trp Ser Asn Thr Ser Asp His Cys Ala Gln Ser

    330                 345                 360                 375
TAC AGC AAC GAG GAC CTC CTG AGT GCA GAC TCT CCA GAG CAG ACT AAC CTG CGC
Tyr Ser Asn Glu Asp Leu Leu Ser Ala Asp Ser Pro Glu Gln Thr Asn Leu Arg

            390                 405                 420
TCT CTG ACG TCC ATC GTG GAC AGC ATC ACA GCA GCA GAG GGG GCT CCT CTG GCC
Ser Leu Thr Ser Ile Val Asp Ser Ile Thr Ala Ala Glu Gly Ala Pro Leu Ala

435                 450                 465                 480
TAC CCT GTA CCT GTA GGA ACA TTC CCA AAT AAA CCC AGA GCA GCA GAC AGA CAT
Tyr Pro Val Pro Val Gly Thr Phe Pro Asn Lys Pro Arg Ala Ala Asp Arg His

        495         500         510         520         530         540
GTG TGC TGT T AACA  GGTCCAATAC  ATCACAAACC  ATCCTTTTTT  ACTCCCTTCC
Val Cys Cys  *

       550         560         570         580         590         600
ATGAGAAGGT  AGTCACTGAT  GGGATATAAC  TGGATAGGTG  AAAGCTATGC  AGCGAAACCT

       610         620         630         640         650         660
ACTGTACATT  CCACCATCCA  AGCATGTTAG  ATCAGTGACT  ACTTCAATGA  AGGGAGGAAA

       670         680         690         700         710         720
GAAGGCCAGG  CCCTGTGTGT  CCCAGCTAGT  TGCAAGCTAG  CACTTGGACA  CCACACAGCA

       730         740         750         760         770         780
CCATACACAC  CCACACACCA  TACAACACCA  TACACACCAA  CATAGGCCAG  GAGGTTTTTC

       790         800         810         820         830         840
CAGGACTAAC  TCCTGGCCCT  AACCTAAAGC  CCTCACACCT  CTTCCAGGCC  TGAGGCCTTA

       850         860         870         880         890         900
TTCCCTACCT  ACCAAACCCT  GTGGTCCACG  TCTCCCTACC  AGTAGGTTAC  GGGTCAGATC

       910         920         930         940         950         960
CTAACCTGAG  AACCCACTGA  CATCGAATCT  ATGCATGTTT  TAGGACAAAA  ATCCAGTTCC

       970         980         990        1000        1010        1020
CTGTGTGGAT  CTCAAGTTAG  AACTGAGGCC  CTAAGTGCCA  CTGGAAAGGA  GCAACGAGAA

      1030        1040        1050        1060        1070        1080
TTATGGTTTT  CTGTCCTGAC  TTATGCAGAA  AAATATATGT  TTCCTCCTTT  TCTAAATCTT

      1090        1100        1110        1120        1130        1140
TCCCTGATCT  ATCTTGCATT  CTGTTTGCGT  GTGTTATGCT  TAAAAACATT  AGTTTATTAT

      1150        1160        1170        1180        1190        1200
TTGTGGGGCC  AATATGTACC  ATGGCAGTTT  TGACCAATGC  ATCTGGCTAA  TTTAAACTGG

      1210        1220        1230        1240        1250        1260
TAATGAATGC  TAGTGATGTA  AATACGTTCC  GTTTTCTACA  GAGTGGTTTT  GCTATTTTAT

      1270        1280        1290        1300        1310        1320
TTTCTATTTT  TTTTGCTAAC  TTATTTTTGA  GTTTTATAAT  AAAGATTGTT  GTGTATTTGT

      1330        1340        1350        1360        1370        1380
AGATGTCCCA  AGAAGTGATG  TTTCTGTCTA  AACTTGCATT  AAAGACCATT  TTCAATAAAA

1384
AAAA
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Fig. 1. Nucleotide and deduced amino acid sequence of Tmyogenin. The asterisk indicates the termination codon. The polyadenylation signal is underlined.

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                    *           *   *           *
Tmyogenin  RKAATMREKRRLKKVNEAFEALKRSTLMNPNQRLPKVEILRSAIQYIERLQALVSSLNQQ 60
qmf2       .R...L.....................L.............S...T....S.L.....
TmyoD      ........R...S...D...T...C.ST.........D...N..S...S..G.LRGAG-.

Tmyogenin  ENDQGTQGLQYRTGPAQ--PRVSSSSEQGSGSTCCSSPEWSNTSDHCAQSYSNEDLLSAD 118
qmf2       .R.ERE--.R..PTAP.--.AAP.--.C....SS..-....TQLEFGTNPTDH--...D.
TmyoD      .GNYYPVMDH.SGDSDASS..SNC.DGMMDFNGQSCP.RRR.KY.STYFNEAPN.SRHKK

Tmyogenin  SPEQTNLRSLTSIVDSITAAEGAPLAYPVPVGT...(continues)
qmf2       AA.DR..H..S...E..AVEDV.VTFPEER.QN
TmyoD      NSVISS.DC.SN..ER..TDTS.CP.VQDGSEG...(continues)
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Fig. 2. Amino acid comparison of Tmyogenin, qmf2 and TmyoD. The box surrounds the basic/helix-loop-helix domain. The asterisks indicate residues that are conserved only among the myogenin type homologues (Qmf2, myogenin, myf4 and Xmg) and not among the other myogenic HLH determinants.

Tmyogenin clone, our cDNA includes an open reading frame of 180 amino acids encompassing the basic/helix-loop-helix (b-HLH) domain that has been shown to be involved in DNA binding and heterooligomerization [20]. Within the b-HLH domain, Tmyogenin shows greater identity (87%) with the quail myogenin qmf2 [21] than with the trout myogenic factor TMyoD (75%) and there are conserved amino acids (Fig. 2*) present only among the myogenin type homologues (qmf2 [21], myogenin [2], myf 4 [22] and Xmg [12]). In addition, between qmf2 and Tmyogenin, there are runs of identities outside the b-HLH domain. Using Tmyogenin as the amino acid position reference (Fig. 2), the identities are clustered in regions 56–63, 85–99 and 124–135. These comparisons strongly suggest that our clone encodes a teleost homologue of the myogenic factor myogenin previously described in higher vertebrates.

3.2. The gene encoding Tmyogenin is expressed in trout developing myotomal musculature

Northern analysis of total RNA shows a single transcript of about 1.8 kb. A strong signal is seen in myotomal musculature of embryos at the eyed stage (Fig. 3A, lane 2). Only a faint signal is detected in adult white muscle which constitutes the major part of the trunk musculature (Fig. 3A, lane 1). To determine whether Tmyogenin is expressed preferentially during muscle development, we examined Tmyogenin mRNA levels in muscle from 3 and 5 cm long frys. In both cases, we observed high levels of Tmyogenin transcript, though to a lesser extent than in embryos (Fig. 3B, lanes 2 and 3).

3.3. Tmyogenin transcript accumulates selectively in red muscle fibres from adult trout

To determine whether Tmyogenin is expressed in a tissue-specific manner, RNAs from several adult trout tissues were subjected to northern analysis (Fig. 3C). No detectable Tmyogenin mRNA is observed in cardiac (heart) and smooth (intestine) muscle tissues. Non muscle tissue including liver, kidney and gills were also devoid of detectable transcript. Whereas small quantities of Tmyogenin mRNA were detected in white (fast) muscle fibres (lane 1), red (slow) muscle fibres that surround the white fibres contained large amounts of Tmyogenin transcript (lane 2).

3.4. Tmyogenin is expressed in vitro during the conversion of satellite cells to multinucleated myotubes

To analyse the expression of Tmyogenin on the earliest steps of muscle formation, we isolated myosatellite cells from juvenile trout and cultivated them on laminin substrates. Two days after plating cells lined up and first fusion occurred (Fig. 4A).

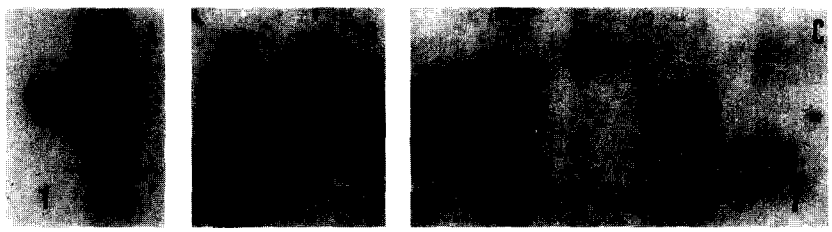

Fig. 3. Tmyogenin expression in vivo. (A) Northern blot of RNA from the myotomal muscle of embryos at the eyed stage (lane 2) and adult white muscle (lane 1). The exposure time was prolonged (96 h) to evidence Tmyogenin transcript in adult. (B) Northern blot of RNA from the epiaxial musculature of 3 and 5 cm long frys (lanes 2 and 3) and for comparison from myotomal muscle of embryos at the eyed stage (lane 1). (C) Northern blot of RNA from white muscle, red muscle, heart, liver, intestine, gills and kidney (lane 1 to 7).

After 5 days of culture, multinucleated and differentiated myotubes were present (Fig. 4B). Taking advantage of this in vitro myogenesis system, we showed that Tmyogenin mRNA was not detectable in isolated myosatellite cells 4 h after seeding (Fig. 4, lane 1), but it was clearly evidenced in 48 h cultures when first fusions occurred (lane 2). Tmyogenin mRNA levels increased in differentiated myotubes (7 and 11 days after seeding, lanes 3 and 4).

4. Discussion

The present study reports the cloning of a fish homologue of myogenin. This novel myogenic factor represents in teleosts the second member of a family of muscle regulatory factors that include the previously described TmyoD [14]. Northern analyses indicate that this novel myogenic factor is expressed in developing myotomal musculature, in red adult muscle layer and in vitro during the conversion of satellite cells to myotubes. These results contrast with previous studies that failed to demonstrate the expression of myogenin in lower vertebrates, although myogenin-like genomic fragments including the b-HLH domain were obtained by polymerase chain reactions [12,13].

We have examined the distribution of the mRNA encoding trout myogenin to determine if its distribution in adult fish muscle makes it a potential regulator of specific muscle fibre types. Indeed, the myotomal musculature of teleosts consists mainly of two clearly distinguishable layers of fibres. Red (slow oxydative) fibres which are superficially located, and white (fast glycolytic) fibres which occupy the deep portion of the fish trunk musculature [23]. Red (slow) muscle fibres are specialized for sustained use, while white (fast) muscle fibres are specialized in short forceful of activity [23]. The functional diversity of the red and white fibres is reflected in their histochemical [23,24] and immunohistochemical properties [25]. Furthermore, the expression of different myosin isoform in these two fibre types has been also established [25,26]. In this study, we show that the red and white fibres display different levels of myogenin mRNA accumulation: Tmyogenin mRNA did not accumulate in the fast glycolytic white muscle fibres, but concentrated in the slow oxydative red fibres. Interestingly, this selective accumulation contrasts with the distribution of TMyoD which is found to be present in equal amounts in both fibre types of fish myotomal musculature [14]. This observation suggests that Tmyogenin takes part in the control of fibre type-specific gene expression. Hughes et al. [27] have also reported an accumulation of myogenin in rat slow fibres, while Myod was found to concentrate in fast fibres. These authors, in addition, demonstrated the correlation between muscle phenotype and MyoD/Myogenin mRNA levels by manipulation of thyroid hormone level or innervation. In both experiments, the alteration of MyoD and Myogenin levels paralleled changes in fibre types. To explain how HLH transcription factors control the fibre

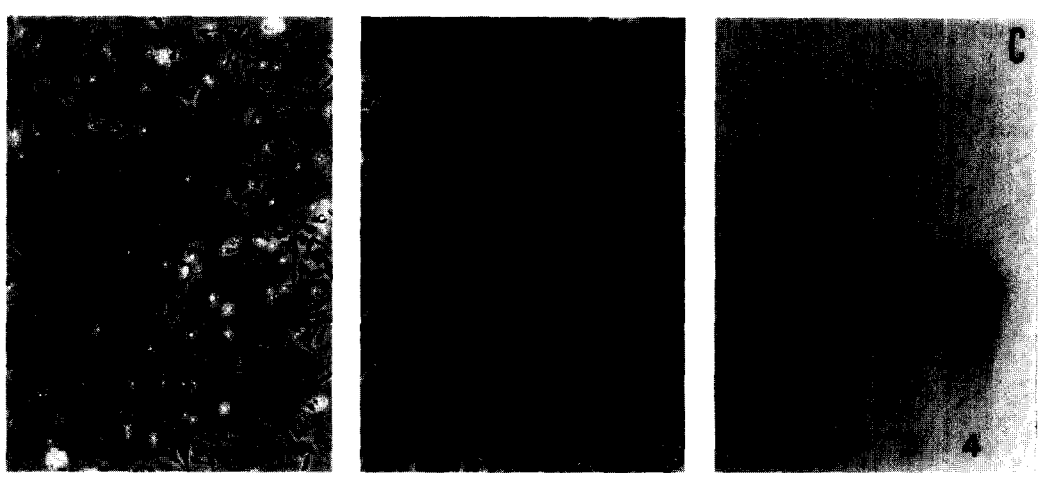

Fig. 4. (A) Morphology of cultivated myosatellite cells 48 h after plating × 200. (B) Morphology of satellite cells derived myotubes (M) formed 7 days following plating × 200. (C) Northern blot analysis of RNA from trout primary cultures of satellite cells. Total RNA was isolated from satellite cells 4 h (lane 1) and 48 h (lane 2) after seeding, and in myotubes 7 days (lane 3) and 11 days (lane 4) after seeding.

type-specific gene expression, it can be assumed that individual myogenic regulatory factors exhibit different capabilities in transcriptional activation of muscle-specific genes by acting on their regulatory elements in distinctive ways. Indeed, in transient transfection assays, it has been shown that MyoD is more effective than myogenin, Myf 5 or MRF4 in activating the fast fibre-specific myosin light chain 1/3 enhancer [28,29].

At the present time, we do not know whether the low amounts of Tmyogenin mRNA within the white muscle is due to a local expression inside small fibres, the smallest being newly formed. Indeed, in contrast with other vertebrates in which normal post natal muscle growth occurs by increase in the size of fibres, teleost muscle may grow by the addition of new fibers generated by the continuous proliferation and differentiation of myosatellite cells [30]. In situ hybridization would provide insights on the local expression of Tmyogenin within the white muscle.

To further analyze the early expression of Tmyogenin during muscle formation, we used an in vitro myogenesis system. In this system, trout dissociated myosatellite cells proliferate on laminin substrate and fuse to form large multinucleated myotubes whose phenotype was confirmed by immunostaining of desmin, a muscle-specific intermediate filament and the sarcomeric myosin heavy chain. (Rescan and Paboeuf, unpublished results). By exploiting this myogenesis model, we demonstrate that Tmyogenin is expressed in differentiated myotubes, but is not detected in satellite cells prior to the initiation of differentiation. This in vitro observation indicates that teleost myosatellite cells retain their potential to differentiate without expressing the myogenin gene. Therefore, the early detection of TMyoD transcripts in proliferating undifferentiated myosatellite cells [14] is consistent with the notion that in teleosteans, Tmyogenin functions downstream of TMyoD in a regulatory pathway. This sequential activation of myogenic regulatory genes is close to mammalian models, but contrasts with avian system in which the myogenin gene has been shown to be activated in both proliferative primary myoblasts and differentiated myofibre cultures [21]. The difference in timing expression of the myogenic regulatory genes, may reflect different control systems regulating their expression in different organisms. In this respect, further studies are needed to elucidate in fishes the potential role of MEF2 related factors [31] in the expression of myogenic helix-loop-helix regulators.

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