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Expression, purification and DNA-binding activity of tilapia muscle-specific transcription factor, MyoD, produced in *Escherichia coli*☆

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Abstract

MyoD is one of several helix-loop-helix proteins regulating muscle-specific gene expression. Using a reverse transcription-polymerase chain reaction, 5′-rapid cDNA end amplification, and plaque hybridization, MyoD cDNA was cloned from the mRNA of tilapia dorsal skeletal muscle. The 1015 bp MyoD cDNA product contained an 846 bp open reading frame with flanking regions of 115 and 64 bp at the 5′- and 3′-ends, respectively. Results showed that the tilapia MyoD sequence, which includes one polypeptide of 281 amino acids, shared sequence identities of 64.3, 64.1, 62.6 and 62.4% with those of zebrafish, carp, and two rainbow trout, respectively. Results from a molecular phylogenic tree assay showed that the tilapia MyoD was more closely related to those of other fishes than of higher vertebrates. Using *Escherichia coli*, a pET expression system, and an Ni^{2+}-NTA column, we purified ~35 kDa recombinant tilapia MyoD. Results from an electrophoretic mobility shift assay demonstrated that the purified *E. coli*-produced tilapia MyoD was capable of binding to the DNA fragment sequence CA(C/T)(C/A)TG.

Keywords: cDNA; Fish; MyoD; Ni^{2+}-NTA column; pET expression system; Phylogenic tree; Plaque hybridization; Tilapia

1. Introduction

Muscle development provides an ideal environment for studying the many unique biological events involved in myogenesis, including cell migration, changes in morphology, and the activation of muscle-specific regulatory factors (MRFs). Four MRFs play important roles in myogenesis: MyoD (Davis et al., 1987); Myf-5 (Braun et al., 1989); Myogenin (Edmondson and Olson, 1989); and MRF4 (Rhodes and Konieczny, 1989). The MRF family is part of a large group of structurally related proteins that utilize a basic helix-loop-helix (bHLH) motif for oligomerization and DNA binding. These bHLH factors are nuclear proteins that transactivate the expression of muscle-specific genes [e.g. the muscle creatine kinase (Jaynes et al., 1988) and myosin light chain genes (Faerman and Shani, 1993)], which contain one or more E-box motifs and a DNA-binding site containing the general consensus sequence CANNTG.

Davis et al. (1987) reported that the forced expression of mice MyoD allows for the transformation of a C3H10T1/2 fibroblast into a myoblast. MyoD is capable of inducing skeletal muscle terminal differentiation in a variety of non-muscle cell types. Pinney et al. (1995) and Pearson-White (1991) have described the cloning of mice and human *myo*D cDNA, respectively. Ma et al. (1994) characterized the crystalline structure of the bHLH domain of mice MyoD, and reported that (a) the

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basic region interacts with the major DNA groove, and (b) the HLH region is involved in protein–protein interaction. In addition to this bHLH domain function, Bergstrom and Tapscott (2001) found that mice MyoD contains three other functional domains—an N-terminal transcription activation domain, a His/Cys-rich domain, and a C-terminal helix III domain. Each plays an important role in MyoD biological activity: the N-terminal transcription activation domain activates the downstream gene, the C-terminal helix III domain is responsible for cell-type specification, and the His/Cys-rich domain is required for chromatin remodeling; the His/Cys domain may also serve a synergistic function with the C-terminal helix III domain.

Compared to the extensive literature on mammal MyoD, very little is known about the characteristics of fish MyoD proteins. The molecular structure of MyoD cDNA has been cloned in trout (Rescan et al., 1994; Rescan and Gauvry, 1996), zebrafish (Weinberg et al., 1996) and carp (Kobiyama et al., 1998). It is often used as a molecular marker to trace cell lineages (Devoto et al., 1996), to compare evolutionary origins (Neyt et al., 2000) to respond to environmental changes (Xie et al., 2001), and to regulate other muscle-specific genes (Sawada et al., 2000; Xu et al., 2000; Kajihara et al., 2001; Rescan, 2001). In order to expand our knowledge of comparative piscine muscle physiology, we decided to study the MyoD of tilapia (*Oreochromis aurea*), a common fish farm species. After examining the molecular structure of tilapia MyoD, we determined that purified recombinant tilapia MyoD produced by *Escherichia coli* is capable of specific E-box binding.

2. Materials and methods

2.1. RNA isolation

The brain, dorsal skeletal muscle, gill, intestine, spleen and stomach of two sacrificed tilapia adults were excised and immediately frozen in liquid nitrogen. Frozen tissue was homogenized with TRIzol reagent (Gibco BRL). RNA extraction was performed according to methods described in Chen et al. (2000, 2001).

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized using the SuperScript Pre-amplification System (Gibco BRL). Degenerate oligonucleotide primers were designed in reference to known vertebrate MyoD polynucleotide sequences. One forward primer, TMD-116F (5′-ATGGAGTTG(C/T)CGGATATTCC(G/T/C)TTCCC-3′), and one reverse primer, TMD-853R (5′-CTCCAC-GATG(C/T)T(G/T)GA(A/C)AG(A/G)CA(A/G)TCCAAAC-3′) were synthesized according to the conserved amino acid sequences MELPDISF and IREVISSL, respectively. Thirty PCR amplification cycles with Taq DNA polymerase (Viogene) were performed. Each cycle consisted of denaturing for 40 s at 94 °C, 1 min of annealing at 54 °C, and 1 min of extension at 72 °C; final extension lasted for 15 min at 72 °C. Amplified DNA fragments were ligated with pGEM T-Easy vector (Promega) and transformed into *E. coli* DH5α. A bigdye-terminator cycle sequencing reaction kit (Perkin–Elmer Applied Biosystems) equipped with a DNA sequencer (Model 310, Perkin–Elmer) was used for the DNA sequencing of both strands. A RT-PCR product was obtained, confirmed, and named pTMD (116–853). The primers Tβ-F5 (5′-TGCGGTATCCATGAGAC-CAC-3′) and Tβ-R6 (5′-GAAGCA-TTTGCGGTGGACGA-3′), synthesized based on the β-actin cDNA of tilapia (Huang et al., 1999), were used as internal controls.

2.3. cDNA library construction and putative clone isolation

A skeletal muscle cDNA library was prepared from poly (A)-selected RNA using oligo (dT) cellulose. First-strand cDNA was prepared using an oligo (dT) primer and reverse transcriptase (Stratagene); second-strand cDNA was synthesized using RNaseH, DNA polymerase I, and *E. coli* DNA ligase. Following the ligation of *Eco*RI-*Xho*I adaptors, the cDNA was inserted into the *Eco*RI site of a Lambda ZAP II bacteriophage vector (Stratagene). The library was screened with the DIG-labeled DNA probe pTMD (116–853), which contained a fragment corresponding to nucleotides 116–853 of the tilapia *myo*D cDNA; approximately 600 ng of DNA probes were prepared with a PCR DIG Probe Synthesis Kit from Roche. Hybridization was performed using a standard hybridization buffer at 42 °C for 16 h according to the manufacturer's guidelines. Washing conditions were 0.5×SSC at 70 °C for 30 min, followed by 0.1×SSC at 55 °C for 30 min. CDP-

```
  1
TCCCCCCCCCCGAAAGCCCCGAATCCTGGTTCACGTT  36
  37
TCAGTTCTTCCCTAAAAAAAGAGGGAAAAGTTTGGTCATAACTGGATTTTCTTCTTCCTGCTGTGTTCTGGACT
GAACC  115
  116 ATG GAG TTG CCG GAT ATC TCT TTC CCC ATC CCC ACC GCT GAT GAT TTC TAT GAC GAC CCC  175
   1  M  E  L  P  D  I  S  F  P  I  P  T  A  D  D  F  Y  D  D  P   20
  176 TGC TTT AAC ACC AGT GAC ATG CAC TTC TTT GAG GAC CTG GAC CCG CGG CTG GTC CAT GTG  235
   21  C  F  N  T  S  D  M  H  F  F  E  D  L  D  P  R  L  V  H  V   40
  236 GGG CTG TTG AAG CCG GAC GAC TCC TCC TCT TCA TCC TCA TCC TCC CCT TCC TCT TCT TCC  295
   41  G  L  L  K  P  D  D  S  S  S  S  S  S  S  S  P  S  S  S  S   60
  296 TCC TCC CCG TCC TCC CTC CTG CAT CTC CAC CAC CAT GCC GAG GTG GAG GAC GAC GAG CAC
355
   61  S  S  P  S  S  L  L  H  L  H  H  H  A  E  V  E  D  D  E  H   80
  356 GTC CGC GCC CCC AGC GGG CAC CAC CAG GCG GGC CGC TGC CTG CTC TGG GCC TGC AAG GCC
415
   81  V  R  A  P  S  G  H  H  Q  A  G  R  C  L  L  W  A  C  K  A  100
  416 TGC AAG AGG AAG ACG ACC AAC GCG GAC CGG CGG AAG GCG GCC ACG CTG CGG GAG CGC CGG
475
  101  C  K  R  K  T  T  N  A  D  R  R  K  A  A  T  L  R  E  R  R  120
                    |--------------------basic---------------------------
  476 CGG CTC AGC AAG GTC AAC GAC GCC TTC GAG ACC CTG AAG CGC TGC ACG ACG GCC AAC CCC
535
  121  R  L  S  K  V  N  D  A  F  E  T  L  K  R  C  T  T  A  N  P  140
    ---||----------------------helix1-------------------------------||-----loop------
  536 AAC CAG AGG CTG CCC AAG GTG GAG ATC CTG CGC AAC GCC ATC AGC TAC ATC GAG TCC CTG
595
  141  N  Q  R  L  P  K  V  E  I  L  R  N  A  I  S  Y  I  E  S  L  160
     -------------------||-----------------------helix2------------------------------
  596 CAG GCG CTG CTG CGC GGT GGC CAG GAA GAC GGC TTC TAC CCG GTG CTG GAG CAC TAC AGC
655
  161  Q  A  L  L  R  G  G  Q  E  D  G  F  Y  P  V  L  E  H  Y  S  180
     ------------------|
  656 GGG GAC TCG GAC GCA TCC AGC CCC CGC TCC AAC TGC TCC GAC GGC ATG ACG GAT TTT AAC
715
  181  G  D  S  D  A  S  S  P  R  S  N  C  S  D  G  M  T  D  F  N  200
  716 GGC CCC ACC TGT CAG ACA ACC AGA AGA GGA AGC TAT GAC AGC AGC TCT TAT TTC TCC GAG  775
  201  G  P  T  C  Q  T  T  R  R  G  S  Y  D  S  S  S  Y  F  S  E  220
  776 ACT CCA AAC GGC GGT CTG AAG AGC GAA CGC AGT TCA GTG GTC TCC AGT CTG GAC TGC CTG
835
  221  T  P  N  G  G  L  K  S  E  R  S  S  V  V  S  S  L  D  C  L  240
  836 TCC AGC ATC GTG GAG CGG ATC TCC ACC GAT AAC AGC AGC CTG CTG CCA CCT GCT GAC GGC  895
  241  S  S  I  V  E  R  I  S  T  D  N  S  S  L  L  P  P  A  D  G  260
  896 CCA GGA TCC CCG ACG ACG ACA ACA ACT GTG CCG ATG CAG TTT GCT GAT CCT ACA CGG AGA  955
  261  P  G  S  P  T  T  T  T  T  V  P  M  E  F  A  D  P  T  R  R  280
  956 CGC TAA TAAAAGGAGGAGCCTGAATAAAATGATTTTAAAAAGAAAAAAAAAAAAAAAAAA
1015
  281  R  *                                                        281
```

Fig. 1. Nucleotide and deduced amino acid sequences of tilapia *myo*D cDNA. Nucleotides were numbered beginning at the transcription start site (+1). Numbers on the second line of each row indicate the amino acid sequences, and the annotations on the third line indicate the structural motifs. The stop codon is marked with an asterisk, and the polyadenylation signal is shaded in gray. This nucleotide sequence can be found in the GenBank database under accession number AF270790.

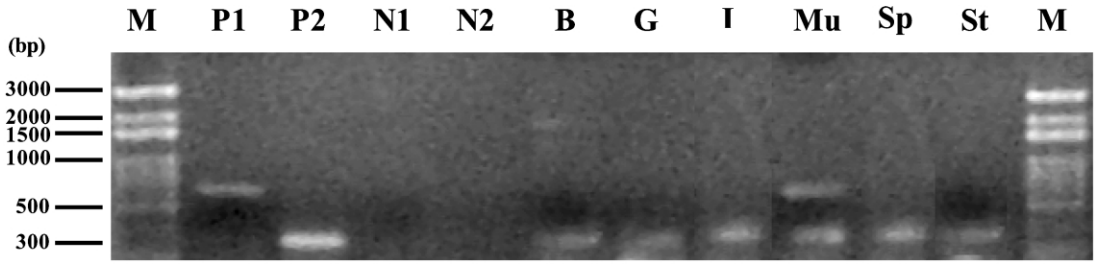

Fig. 2. RT-PCR analysis of the tilapia *myo*D gene transcript tissue distribution, using total RNA extracted from tilapia brain (B), gill (G), intestine (I), skeletal muscle (Mu), spleen (Sp) and stomach (St). Products were analyzed by 1% agarose gel electrophoresis. The primers TMD-116F and TMD-853R were used for detecting *myo*D transcripts: a 735 bp product was expected to be generated (lane P1). The primers Tβ-F5 and Tβ-R6 were also used as internal control for detecting β-actin transcripts and resulting in a 312 bp product (lane P2). Lane M, DNA markers; lanes N1 and N2, negative controls in which either TMD-116F and TMD-853R or Tβ-F5 and Tβ-R6 primers were, respectively, added without reverse transcriptase.

STAR (Tropix) was used as a substrate to visualize the positive bands after 30 min of autoradiography. Positive clones containing phagemids in pBlue-Script (KSII) were selected following in vivo excision according to the supplier's instructions (Stratagene).

2.4. 5′-Rapid amplification of cDNA ends (5′-RACE)

After performing 5′-rapid amplification of cDNA ends (5′-RACE) with first-strand cDNA, terminal transferase TdT (Roche) and dGTP were used to add poly dG residue to the ends of each cDNAs. The tailed cDNAs were then used to generate double-stranded DNA by PCR amplification in the presence of a forward primer, RAAPC(5′-GGCCACGCGTCGACTAGTACT(C)9-3′), and a reverse primer, TMD240R (5′-GTGCTCGTCGTCCTCCACCTCGG-3′). PCR amplification was also performed as described above, with the exception of annealing at 56 °C. Amplified DNA fragments were subcloned and sequenced as described in a previous section.

2.5. Analyses of polypeptide identities and phylogenic dendrograms

The presumptive amino acid sequence was determined with the Wisconsin Sequence Analysis Package v.10.0 (GCG). The Gap program of that package was used for pair comparisons, and the Pileup and Prettybox programs used for multiple comparisons. The Clustalw molecular evolution genetic program was used for our phylogenic tree analysis (http://www.ebi.ac.uk/clustalw/).

2.6. Tilapia MyoD expression vector construction

A 517 bp *Pst*I fragment digested from pTMD (116–853) and a 3547 bp *Pst*I fragment digested from pTMD (321–1015) were ligated to establish pTMD (1–1015) containing the full-length tilapia *myo*D cDNA. A forward primer, TMD1F-NdeI (5′-CATATGGAGTTGCCGGATAT-3′), and a reverse primer, TMD281R-XhoI (5′-CTCGAGT-TAGCGTCTCCGTGT-3′), were used for the PCR amplification of the pTMD (1–1015) template, thus allowing for the introduction of two additional *Nde*I and *Xho*I sites in the coding region. Amplification procedures were identical to those described earlier, except that the PCR product was ligated to the pET15b expression vectors (Novogen) after they were digested with *Nde*I and *Xho*I. The resulting plasmid, pTMD (1–281), was used for the expression of recombinant tilapia MyoD.

2.7. Induction, expression, and purification of 6×His-TMD fusion proteins

The pET15b and pTMD (1–281) plasmids were transformed into *E. coli BL21(DE3)pLysS*. Cells were cultured in 50 ml of 2×YT medium (ampicillin 200 mg/ml, chloramphenicol 34 mg/ml, tryptone 20 g/l, yeast extract 10 g/l, and NaCl 10 g/l) at 37 °C to 0.4 OD_{600}. Induction was performed by adding isopropyl-thio-D-galactoside (IPTG) to a final concentration of 1.6 mM. Samples were collected once per hour for protein

```
Tilapia
MELPDISFPIPTADDFYDDPCFNTSDMHFFEDLDPRLVHVGLLKPDDSSSSSSSSPSSSS 60
Common Carp       ...S..P.....S.............N.....................E---------- 47
Zebrafish         ...S..P.....S.............N.....................E---------- 47
Rainbow trout 1    ......P...TSP.....................................---------- 47
Rainbow trout 2   ...S....VTS.....................................---------- 47

Tilapia
SSPSSLLHLHHHAEVEDDEHVRAPSGHHQAGRCLLWACKACKRKTTNADRRKAATLRERR 120
Common Carp       ---------...L.---..... M.... 95
Zebrafish        ---------...I.---.....M.... 95
Rainbow trout 1   ---------...K.---...I.....M.... 95
Rainbow trout 2   ---------..YN.---...I.....S.....M.... 95

Tilapia          RLSKVNDAFETLKRCTTANPNQRLPKVEILRNAISYIESLQALLRG-GQE-
DGFYPVLEH 178
Common Carp       .................SN.....--...-ENY..... 152
Zebrafish        .................ST.....--S..-.NY...M.. 152
Rainbow trout 1   .................ST.....D.....G....A...-GNY...MD. 154
Rainbow trout 2   ..G.....SN.....S...--..DGENY..... 153

Tilapia
YSGDSDASSPRSNCSDGMTDFNGPTCQTTRRGSYDSSSYFSETPNGGLKSERSSVVSSLD 238
Common Carp       ..................M..M.....SR..N....-...ND...ADARNTK..... 211
Zebrafish        ..................M..M.....R..N....-...NDA..ADARNNKN..... 211
Rainbow trout 1   ..................M....QS.PPR..NK...-T..N.A..-DSRHKKN..I.... 212
Rainbow trout 2   .....Q.....M.Y.A...TSA..SN...-...A....ADSR.NKNAA..... 212

Tilapia          CLSSIVERISTDNSS----------LLPPADGPGSPTTTTTVPMEFADP 277
Common Carp       ...........ETPACPVLSVPEGHEGS-PCSPQEGSVLSET.APA.SP..C-.QQQ.RD 269
Zebrafish        ...........ETPACPVLSVPEAHEGS-PCSPHEGSVLSDT.TTA.SP.SC-.QQQ.QE 269
Rainbow trout 1   ...N.....T..T.ACPAVQ-DGSEGSSPCSPGDGSIASENG.API.SPINC..ALHDPN 270
Rainbow trout 2   ...N.....T.ACTVLSGQEGSEGS-PCSPQEGSILSRN.GTV.SP.NC-.QP-SHD 269

Tilapia          TRRR--                                                  281
Common Carp       PIYQVL                                                 275
Zebrafish        TIYQVL                                                  275
Rainbow trout 1   TIYQVL                                                 276
Rainbow trout 2   PIYQVL                                                 275
```

Fig. 3. Comparison of the deduced amino acid sequence of tilapia MyoD with those of other known piscine species. Data were obtained from GenBank nucleotide sequence database with the following accession numbers: common carp (AB012882); zebrafish (Z36945); rainbow trout 1 (X75798); and rainbow trout 2 (Z46924) myoD. Amino acid residues identical to that of tilapia MyoD are represented by dots. Dashes represent gaps created to maximize the degree of identity among all compared sequences.

analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Recombinant tilapia MyoD was purified with a Ni^{2+}-NTA spin column according to the manufacturer's instructions (Qiagen).

2.8. Western blotting

Following 12% SDS-PAGE analysis, protein samples were transferred to PVDF membranes (Amersham–Pharmacia) via semi-dry transfer

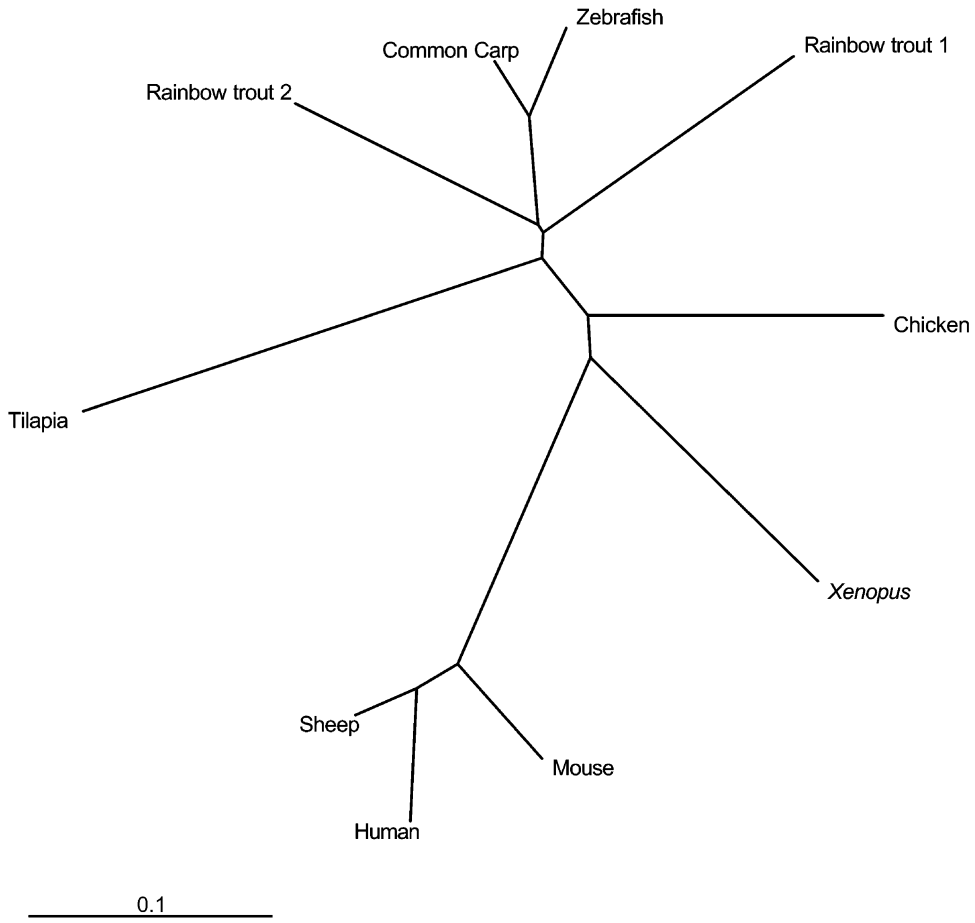

Fig. 4. A molecular phylogenic tree of MyoD polypeptides. Data were obtained from GenBank nucleotide sequence database with the following accession numbers: common carp (AB012882); zebrafish (Z36945); rainbow trout 1 (X75798); rainbow trout 2 (Z46924); Xenopus (X16106); chicken (L34006); mouse (NM010866); sheep (X62102); and human (X56677) MyoD.

cells (BioRad) and allowed to stand for 1 h. Membranes were blocked with PBS (phosphate-buffered saline) containing 5% skim milk at 37 °C for 1 h. Next, 1:1000 dilutions of rabbit anti-mouse MyoD polyclonal antibodies (Santa Cruz) or mouse anti-6×His monoclonal antibodies (Mdbio) were added and allowed to react for 4 h at 4 °C. Mouse anti-rabbit IgG or goat anti-mouse IgG secondary antibodies conjugated with alkaline phosphatase were added, and the resulting mixture incubated at 37 °C for another 1 h. After two washings with PBS, CDP-STAR (Promega) was added to make the positive bands visible.

2.9. Thrombin hydrolysis

Approximately 200 μg of the purified 6×His-TMD fusion proteins were added to thrombin cleavage buffer (50 mM Tris–HCl at pH 7.5, 150 mM NaCl, 2.5 mM $CaCl_2$), mixed with 1 U thrombin, and reacted at 22 °C for 1 h to remove the histidine fusing tag. The purified recombinant proteins were analyzed by SDS-PAGE and Western blot analysis, and partially sequenced with a polypeptide sequencer.

2.10. Electrophoretic mobility shift assay (EMSA)

Three double-stranded oligonucleotides were used as probes for the binding activity of the purified recombinant tilapia MyoD:

1. The E-box (mE-box, formed by 5′-TTTCCCCAACACCTGCTGCCT-3′ and 5′-AGGCAGCAGGTGTTGGGGAAA-3′) of the mouse muscle creatine kinase enhancer (Jaynes et al., 1988).

2. Zebrafish troponin T enhancers 1 (Z1, formed by 5′-TCCTGTGCATCTGTTTTGAG-3′ and 5′-CTCAAAACAGATGCACAGGA-3′) and 2 (Z2, formed by 5′-TTAACGTCACATGAG-GAGGG-3′ and 5′-CCCTCCTCATGTGACGT-TAA-3′) (Huang et al., unpublished data).
3. Non-specific oligonucleotides (Non-30fr, formed by 5′-CACGTCACGAGCTATCGGT-GATCATCTCTG-3′ and 5′-GTGCA-GTGCTCGATAGCCACTAGTAGAGAC-3′).

All probes were labeled with γ-[^{32}P]ATP 3000 μCi/ml, using T4 polynucleotide kinase (NEB) according to the supplier's protocols.

Approximately 600 μl of the purified 6×His-TMD fusion proteins were put into a dialysis bag, sealed, and placed in a flask containing 500 ml dialysis buffer (10 mM Hepes at pH 7.9, 100 mM KCl, 0.2 mM EDTA, 5 mM PMSF, 0.5 mM DTT, 10% glycerol). After stirring for 4 h at 4 °C, the dialytic proteins were dispensed and stored at −70 °C until used.

Between 10 ng and 1 μg of the 6×His-TMD fusion proteins and 1 μg of the poly(dIdC) were added to reaction buffer (10 mM Tris at pH 7.5, 50 mM NaCl, 0.5 mM EDTA pH 8.0, 0.5 mM DTT, 5% glycerol) and allowed to stand on ice for 10 min. After adding 1 μl of probe with a specific radioactivity of 10^7 cpm/μg, each mixture was incubated at 30 °C for 30 min and analyzed by 6% acrylamide gel electrophoresis (79:1 acrylamide/bisacrylamide). The gel was dried and exposed to X-ray film for 48 h.

3. Results and discussion

3.1. cDNA and deduced amino acid sequences

A 735 bp fragment amplified by the primers TMD-116F and TMD-853R was labeled with DIG as a probe. The nucleotide sequences of approximately 60 positive clones excised from 2×10^5 plaques were confirmed. All clones were determined to be identical 5′-truncated forms of tilapia *myo*D cDNA; since their sequences corresponded to nucleotides 321–1015, they were labeled pTMD (321–1015). The primers RAAPC and TMD240R were used to perform the 5′-RACE, which produced a 355 bp fragment. The 1015 bp tilapia myoD cDNA encoded an 846 bp open reading frame with 115 and 64 bp flanking regions at the 5′- and 3′-ends, respectively (Fig. 1). The deduced tilapia MyoD amino acid sequence revealed a 281 amino acid polypeptide containing a bHLH domain located between amino acid positions 108 and 165. Although previous reports described two non-allelic MyoD encoding genes for rainbow trout [designated rainbow trout 1 (Rescan et al., 1994) and rainbow trout 2 (Rescan and Gauvry, 1996)], only one tilapia *myo*D cDNA was cloned for the present study.

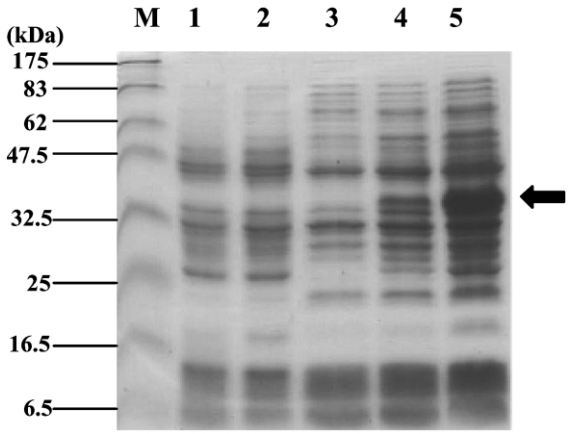

Fig. 5. Expression of recombinant tilapia MyoD by *E. coli BL21*(*DE3*)*pLysS*. Samples were collected and analyzed by 12% SDS-PAGE following coomassie brilliant blue staining. Lane M, protein markers; lanes 1 and 2, lysates from *E. coli* harboring no plasmid and pET15b, respectively, as negative controls; lanes 3, 4 and 5, lysates from *E. coli* harboring plasmid pET15bTMD (1–281) 0, 2 and 4 h following induction, respectively. Arrow indicates an induced recombinant protein.

3.2. Tissue distribution of tilapia MyoD transcripts

RT-PCR experiments were performed to determine the expression of the tilapia *myo*D transcripts in muscle or other tissues. Using the primers TMD-116F and TMD-853R, first-strand DNA was individually synthesized from tissue taken from tilapia brain, dorsal skeletal muscle, intestine, gill, spleen and stomach. A 735 bp RT-PCR product was observed in skeletal muscle tissue only (lane Mu of Fig. 2). We therefore suggest that the *myo*D mRNA clones established during our experiments were muscle-specific.

3.3. Deduced amino acid sequence comparison—tilapia/other piscine species

The tilapia MyoD polypeptide shares sequence identities of 64.3, 64.1, 62.6 and 62.4% with the reported MyoD of zebrafish (Weinberg et al.,

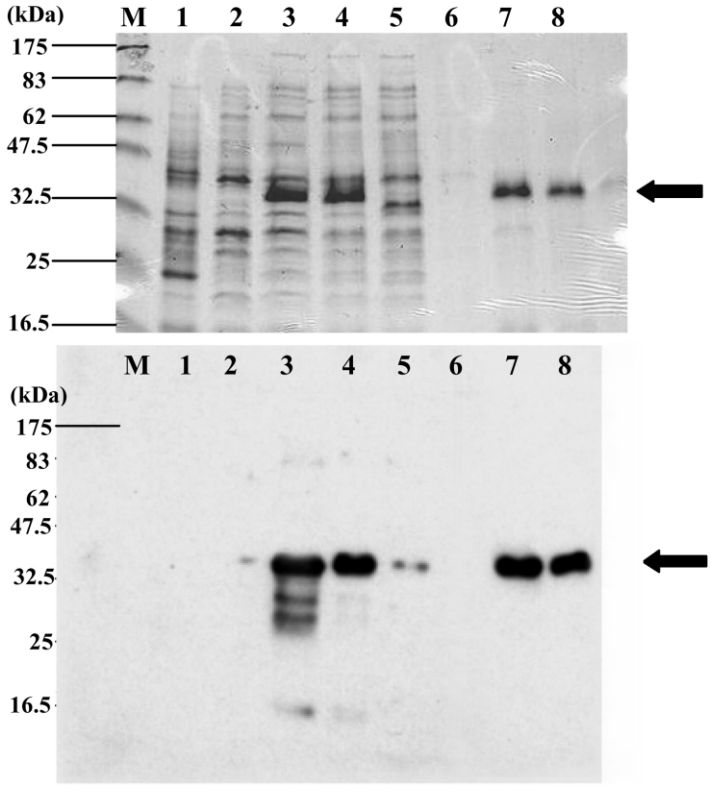

Fig. 6. Protein and Western blot analyses during the recombinant tilapia MyoD purification process. Samples were collected, purified, and analyzed by 12% SDS-PAGE following coomassie brilliant blue staining (upper panel), and Western blot analysis (bottom panel). Lysates obtained from *E. coli* harboring plasmid pTMD (1-281) after 4 h (lane 3) induction were dissolved in buffer B (lane 4), injected into a Ni^{2+}-NTA spin column (lane 5), washed with buffer C (lane 6), and eluted (lanes 7 and 8) (see Section 2 for buffer description). Arrows indicate purified recombinant tilapia MyoD. Lane M, protein markers; lane 1, lysate from *E. coli* harboring plasmid pET15b (negative control); lane 2, lysate from *E. coli* harboring pTMD (1-281) prior to induction.

1996), carp (Kobiyama et al., 1998), and the above-mentioned trout 1 (Rescan et al., 1994) and trout 2 (Rescan and Gauvry, 1996), respectively (Fig. 3). The tilapia MyoD also served the three functional domains (the N-terminal transcription activation domain, the His/Cys-rich domain, and the C-terminal helix III domain) described in mice MyoD by Bergstrom and Tapscott (2001). However, the tilapia MyoD also contained a unique poly-serine region between amino acid positions 48 and 65. A comparison of the MyoD from tilapia and *Trichinella spiralis* (Connolly et al., 1996) revealed a unique glutamine-rich motif in the latter. To our knowledge, no other species shares this amino acid-rich region in MyoD polypeptides with tilapia and *T. spiralis.* Further study is required to identify the biological characteristics of this serine-rich region.

We used the Clustalw program to determine the phylogenic similarities between tilapia MyoD and the common carp, zebrafish, rainbow trout 1 and 2, *Xenopus* (Hopwood et al., 1989), chickens (Dechesne et al., 1994), mice (Pinney et al., 1995), sheep (Huynen et al., 1992) and humans (Pearson-White, 1991). The phylogenic tree generated by the program showed that tilapia MyoD was more closely related to MyoD from other fish species than those from higher vertebrates (Fig. 4). From a phylogenic perspective, tilapia experienced a faster MyoD evolution compared to other fishes.

3.4. Induction, expression and purification of 6×His-TMD fusion protein

Following IPTG induction, polypeptide samples were collected and analyzed by SDS-PAGE and

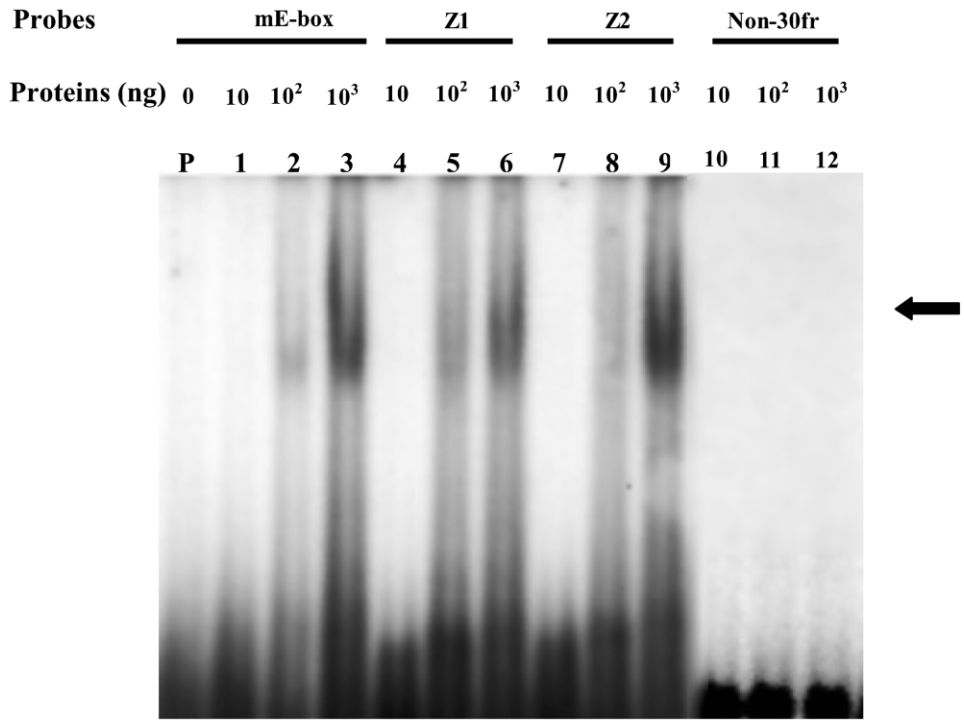

Fig. 7. EMSA experiment for examining the DNA-binding activities of purified recombinant tilapia MyoD protein. Approximately 10 ng (lanes 1, 4, 7 and 10), 100 ng (lanes 2, 5, 8 and 11) or 1 μg (lanes 3, 6, 9 and 12) of purified recombinant tilapia MyoD produced by *E. coli* were reacted with radio-labeled probes (mE-box, Z1, Z2 and Non-30fr) oligonucleotides as indicated. Lane P: radio-labeled probe mE-box only. Arrow indicates a shifting band formed by double-stranded oligonucleotides and recombinant tilapia MyoD.

Western blotting. A ~35 kDa polypeptide was induced 2 and 4 h following IPTG treatment (Fig. 5, lanes 4 and 5). It was recognized by both rabbit anti-mouse MyoD polyclonal antibody and mouse anti-6×His antibody (data not shown). After purifying the 6×His-TMD fusion protein through a Ni^{2+}-NTA column, a single ~35 kDa band was observed on the gel (Fig. 6, lanes 7 and 8), also recognized by both rabbit anti-mouse MyoD polyclonal antibody and mouse anti-6×His antibody. After removing the His tag from the 6×His-TMD fusion protein by thrombin hydrolysis, the N-terminus of the polypeptide was sequenced and identified as GSHMELPDISFPIPTADD—identical to the amino acid residues of the tilapia MyoD polypeptide from 1 to 15.

Maleki and Hurlburt (1997) used High Prep Q and S to increase the purity of their recombinant mice MyoD protein to 90%—a time-consuming and labor-intensive purification process. For the present study, we used a pET expression system and Ni^{2+}-NTA column purification methods with our recombinant tilapia MyoD protein and found that a 95% purity level and 95% recovery rate were both possible, with the purified recombinant MyoD capable of DNA-binding activity. We therefore suggest the combination of the pET expression and Ni^{2+}-NTA column purification systems as a relatively simple but efficient way to produce recombinant fish MyoD.

3.5. EMSA experiments

EMSA experiments were conducted to determine whether the purified recombinant tilapia MyoD was capable of binding with a specific DNA fragment. A complex formed by the recombinant tilapia MyoD protein and mE-box oligonucleotide resulted in a shifted gel band (Fig. 7, lane 2). This positive signal band grew in strength as the amount of protein increased (Fig. 7, lane 3). Shifted bands were also observed when the double-stranded oligonucleotides of Z1 (Fig. 7, lanes 5 and 6) and Z2 (Fig. 7, lanes 8 and 9) probes were

used in the EMSA experiments. When exposure was prolonged to 48 h, very faint signals appeared in lanes 1, 4 and 7 (data not shown). As expected, shifted bands did not form between MyoD and the non-specific oligonucleotide Non-30fr (Fig. 7, lanes 10–12). According to these results, (a) the recombinant tilapia MyoD produced via an *E. coli* expression system is capable of binding to a specific sequence within a DNA fragment, and (b) the 6×His tag does not affect the E-box-binding activity of the recombinant tilapia MyoD protein.

EMSA signals were very faint for recombinant protein amounts below 10 ng (data not shown). Lorenzo-Puri and Sartorelli (2000) demonstrated that the protein kinase C is capable of phosphorylating threonine residue in the basic region of mice MyoD (T115), resulting in the promotion of DNA-binding activity. We therefore believe that the non-phosphorylated form of tilapia MyoD produced by *E. coli* causes weak binding sensitivity between oligonucleotides and MyoD at low concentrations.

Three probes containing different core E-box sequences were used in the EMSA experiments: mE-box (CACCTG) oligonucleotide was designed based on the mouse muscle creatine kinase gene; and Z1 (CATCTG) and Z2 (CACATG) oligonucleotides were designed based on the zebrafish troponin T gene. A comparison of the DNA-binding strength of recombinant tilapia MyoD among these probes showed that the signal formed by the MyoD polypeptide and Z2 oligonucleotide was the strongest, and that the signal formed by the MyoD polypeptide and Z1 oligonucleotide was the weakest. This indicates that tilapia MyoD has different binding affinities with various core E-box sequences. The data support results from Czernik et al. (1996), who found that MRF family members can form homodimers or heterodimers to bind E-boxes in muscle-specific genes with different binding affinities.

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