PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2007, 34(11): 1169~1174
www.pibb.ac.cn

Expression, Purification of Recombinant Flounder MRF4 Protein in *Escherichia coli* and Analysis of Its Polyclonal Antibodies*

XU Peng[1,2], TAN Xun-Gang[1)**], ZHANG Pei-Jun[1)**], ZHANG Yu-Qing[1,2], WANG Bo[1,2)]

([1)]*Key Laboratory of Experimental Marine Biology, Institute of Oceanology, The Chinese Academy of Sciences, Qingdao* 266071, China;
[2)] *Graduate University of Chinese Academy of Sciences, Beijing* 100049, China)

Ab stract *MRF4* is one of muscle regulatory factors and plays critical roles during skeletal muscle development. The muscle development is important for the fish growth which is an important economic factor for the fish culture. To analyze the function of *MRF4* in fish, the founder MRF4 antibody was prepared. The flounder *MRF4* was cloned, ligated into prokaryotic expression vector pET-30b and expressed in strain *E. coli* BL21 (DE3). The recombinant flounder MRF4 fusion protein was soluble and purified with cobalt IMAC resins. To prepare MRF4 polyclonal antibodies, rabbits were immunized with the soluble protein and the increasing level of antibodies was determined by Western blot. Also, the endogenous flounder MRF4 was recognized by the anti-serum. The result further proved the existence of the anti-MRF4 antibody in the anti-serum, which will be useful for studies on the function of flounder MRF4.

Keywords: flounder, expression, purification, MRF4 antibody

MRF4 [1,2] is one of the basic helix-loop-helix muscle regulatory factors (including *MyoD* [3], *Myf5* [4], *myogenin* [5] and *MRF4*) which are master genes for skeletal muscle development. Among these four MRFs, *MyoD* and *Myf5* are required for the determination of skeletal myoblasts [6], whereas myogenin acts as differentiation factor in the later program of myogenensis [7,8]. The function of *MRF4* is more complicated. The recent studies on mouse have demonstrated that *MRF4* can determine skeletal muscle identity in *Myf5/MyoD* double mutant mice and drive myogenesis in embryonic trunk and limbs, which revises the epistatic relationship of the MRFs [9]. This work clearly demonstrated a role for *MRF4* in the early stages of myogenesis. Studies on *Xenopus lavis* show that there are two *MRF4s* which are differentially expressed during development and muscle regeneration, which suggests a functional difference [10]. These new discoveries break the traditional conception that *MRF4* only plays a role in terminal differentiation of myogeninsis [11]. So the exactly function of *MRF4* has not been confirmed. Although it has been cloned from several fish species including zebrafish (*Danio rerio*), fugu rubripes (*Takifugu rubripes*), Atlantic salmon (*Salmo salar*) and pufferfish (*Tetraodon nigroviridis*), the function of *MRF4* in fish has not been demonstrated yet, and no studies have been performed on *MRF4* protein level.

Flounder (Paralichthys olivaceus) is widely cultured and one of the most important economic fish in East Asia. The growth rate is an important factor for the flounder and the muscle growth contribute mostly to the growth. *MRF4* plays role in the muscle development but the exactly role is unclear. To understand the role of *MRF4*, flounder MRF4 recombinant protein was expressed and purified, its polyclonal antibodies was prepared and analyzed.

*Th is work was supported by grants from National Basic Research Program of China (2004CB117402) and Hi-Techn Research and Development Program of China (20060110A4013).
**Corresponding author.
TAN Xun-Guang. Tel: 86-532-82898559, Fax: 86-532-82969735,
E-mail: tanxgj@yahoo.com
ZHANG Pei-Jun. Tel: 86-532-82898551, Fax: 86-532-82969735,
E-mail: pjzhang@ms.qdio.ac.cn
Received: March 12, 2007 Accepted: May 8, 2007

Also, the endogenous MRF4 was detected by the polyclonal antibody. Thus, further *MRF4* function studies on protein level can be performed.

1 Materials and methods

1.1 Host strains and vectors

The *E.coli* XL1-Blue was used for propagation of the recombinant plasmid and the *E.coli* BL21 (DE3) was used for the expression of the recombinant MRF4 fusion constructs. The pUCm-T vector (Sangon, Shanghai) was used for the cloning of the PCR product. The pBluescript Ⅱ SK vector (Stratagene, USA) was used for cloning of cDNA. The pET-30 vector (Novagen, Germany) was used for expression of the recombinant MRF4 fusion constructs in *E.coli*. IPTG (isopropyl-beta-D-thiogalac-topyranoside) was used to induce production of the recombinant protein in *E.coli*.

1.2 Isolation of flounder *MRF4* cDNA

MRF4 cDNA was isolated by RT-PCR. Total RNA was extracted from flounder embryos using Trizol reagent (Invitrogen, USA) according to manufacturer's instructions. cDNA was synthesized using the first strand cDNA synthesis kit (Promega, USA). PCR was performed with specific primers 5′ CCACTGTCAAAACAAGGGAGAG 3′ and 5′ GTT-GTCGTCGGGTATGAAGGTC 3′, and *pfu* enzyme (Promega, USA) to minimize mutations. The PCR products were gel purified, cloned into pBluescript Ⅱ SK *Sma* Ⅰ site (MRF4/BSSK) and sequenced.

1.3 Construction of expression vectors

The *MRF4* cDNA was released from MRF4/BSSK by *EcoR* Ⅰ and *Not* Ⅰ (Takara, Japan), ligated into pET-30b *EcoR* Ⅰ and *Not* Ⅰ site, and resulted in pET-30b-MRF4. The positive plasmid were determined by restricted enzyme digestion and sequenced to confirm the correct read frame.

1.4 Expression of recombinant MRF4 protein in *E.coli*

The constructed pET-30b-MRF4 plasmids were transformed into competent *E.coli* BL21 (DE3) cells and transformants were selected on LB/kanamycin (50 mg/L) agar plates. Single colony was picked and incubated in 2.5 ml of LB/ kanamycin (50 mg/L) overnight at 37℃with shaking at 200 r/min. An aliquot of 100 μl of overnight culture was used to inoculate in 10 ml of fresh LB medium (containing 50 mg/L kanamycin) in a 50 ml flask and grow under the same condition until the A_{600} reached 0.8. Then IPTG was added to a final concentration of 0.6 mmol/L and the culture was continued to be incubated for another 2 h. Prior to the addition of IPTG, 1 ml of the culture was harvested by centrifugation at 6 000 r/min for 5 min. The pellet were resuspended with 50 μl PBS and 50 μl 2×loading buffer, boiled for 5 min and stored at –20℃, which was considered as the zero time sample T_0. In order to determine the optimal conditions for overproduction of the fusion protein, IPTG concentrations and induction time were optimized. Similarly, the induced culture was harvested at various time intervals (1 h, 3 h and 5 h) and these samples were considered as T_1, T_2 and T_3. The tested concentrations of IPTG were 0.2 mmol/L, 0.4 mmol/L, 0.6 mmol/L, 1.0 mmol/L, 1.5 mmol/L, 2.0 mmol/L and 3.0 mmol/L, and the samples were harvested at the optimal time. All the samples were treated the same way as the zero point time and confirmed by 12% SDS-PAGE which were stained with Coomassie blue R-250.

A large scale induction was performed for getting enough purified MRF4 protein. Cells were incubated in 1 000 ml LB medium based upon the optimal induction time and IPTG concentration determined above. At the end of the induction, the cells were harvested by centrifuged at 10 000 *g* for 10 min, the supernatant was decanted, and the wet weight of cells was determined.

1.5 Solubility analysis

Recombinant protein solubility determination followed B-PER® Bacterial Protein Extraction instruction (Pierce, USA). The samples were analyzed by SDS-PAGE and Western blot using anti-His antibody.

1.6 Purification of His-tagged MRF4 protein under nature conditions

To prepare lysates under nature condition, 5 ml B-PER buffer/g (wet) were added into the tube with pipetting up and down until the cell suspension was homogeneous. Then the solution was stored at room temperature for 30 min. After 30 min centrifugation at 15 000 r/min, the supernatant were poured into a new tube carefully. The pellet was continued to be treated twice with half of B-PER buffer of the first time and the supernatant was collected. SDS-PAGE was applied to determine the quantity of the protein of each fraction.

To purify the recombinant protein under nature

conditions, BD TALON metal affinity resins (BD Biosciences Clontech, US) was used. According to the manufacturer's instructions, 2 ml of resuspended BD TALON resin were added into empty column and ethanol flowed through. At the same time, the lysates were diluted with wash buffer (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 20 mmol/L imidazole, pH 7.5, 1∶1, *v*/*v*). After 4 ml diluted B-PER (1∶1, *v*/*v*) was added and flowed through, the lysates (preparation above) was added and the flow-through was collected and assigned F. The column was washed twice with 4 ml wash buffer and each wash fractions were named W1 and W2. The recombinant protein was eluted eight times with 0.5 ml elution buffer (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 250 mmol/L imidazole, pH 7.5) each time and the eluted fractions were marked as E1 to E8. All of the collected fractions were then analyzed by SDS-PAGE to determine the eluted recombinant protein. The purified fusion protein was confirmed by Western blot analysis. The amount of purified proteins was measured with folin phenol reagent.

1.7 Rabbit immunization and preparation of polyclonal anti-MRF4 antibodies

Polyclonal antibody against recombinant MRF4 was produced by one basal subcuticular injection to rabbits. The first injection contained 0.5 mg purified protein plus an equal volume of Complete Freund's adjuvant. Two fortified subcuticular injections each containing 1 mg purified protein plus an equal volume of Freund's adjuvant (incomplete) were subsequently given at 1 week intervals. The titer for the polyclonal antibody was detected by Western blot assay with the second antibody, goat anti-rabbit IgG-HRP conjugate.

1.8 Analysis of flounder endogenous MRF4 by polyclonal antibody

To further analyze the polyclonal antibodies, about 80 new hatched flounders (5 days after fertilizer) were collected in each tube and treated with 10 μl 2× loading buffer by pipetting up and down until the embryos was homogenous. Western blot was used to detect the expression of endogenous MRF4 with MRF4 polyclonal antibody serum and goat anti-mouse IgG-HRP conjugate (Zhongshan Golden Bridge, Beijing).

1.9 Western blot

Western blot was performed as follows: protein in the gel was transferred to nitrocellulose membrane with constant current 100 mA overnight at 4℃. The membrane was blocked by incubating with 5% lipid-free milk and 1% BSA in PBST at room temperature for 2 h, and incubated with primary antibodies at room temperature for 1 h, followed by the second antibodies (1∶1 000), at room temperature for 1 h. The membrane was reacted with freshly prepared 0.2 g/L DAB in PBS containing 0.1% H_2O_2. The result was recorded by camera or scanner.

2 Results

2.1 Construction of expression vectors

A 720 bp fragment was isolated by RT-PCR and cloned into pBluescript Ⅱ SK. Sequence analysis confirmed that it was flounder *MRF4* gene. The result (Figure 1) showed that restricted enzyme digestion of the clones gave the expected size of the two bands. DNA sequencing confirmed that the target fragment was successfully cloned into pET-30b vector with the correct reading frame.

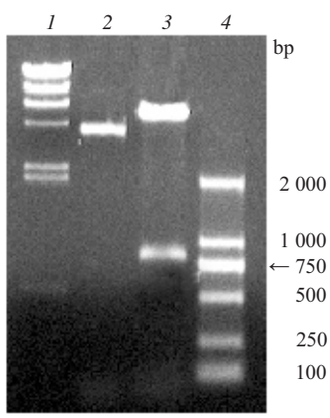

Fig. 1 Agarose gel analysis of restriction enzyme digestion
1: λ/*Hin*d Ⅲ marker; *2*: pET-30b-MRF4 without digestion; *3*:Digested pET-30b-MRF4 plasmid using *EcoR* Ⅰ and *Xba* Ⅰ; *4*: D2000 marker.

2.2 Expression of recombinant MRF4 protein

The recombinant plasmid pET-30b-MRF4 was transformed into an expression host *E.coli* BL21 (DE3). The PolyHis-MRF4 fusion protein would have a calculated value about 29 ku. After induction with IPTG, the expected distinct band around 31 ku was observed in T_1, T_2, T_3 and not in negative control on SDS-PAGE.

Through the analysis of SDS-PAGE, the recombinant protein concentration increased from T_1 to T_2, while it was not changed too much from T_2 to T_3 (Figure 2). So the optimum incubation time was determined as T_2, 3 h after IPTG induction. The concentration of recombinant protein did not changed much when the IPTG concentration was altered from

0.2 mmol/L to 3.0 mmol/L. So 0.2 mmol/L was chosen as the optimum IPTG concentration (Figure 2). The solubility analysis with B-PER showed that most of the recombinant protein was in supernatant sample, while a little protein was in the pellet sample (Figure 3a). Western blot further confirmed that the expected fusion protein was soluble (Figure 3b). These optimum conditions were applied for large-scale expression, and the recombinant protein was purified under nature condition.

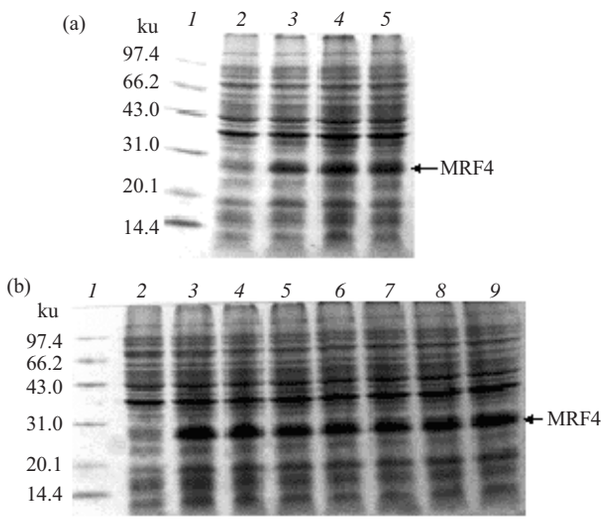

Fig. 2 SDS-PAGE analysis of the optimal conditions for expression of recombinant MRF4 protein

(a) Optimal time for MRF4 expression. The induction time of expression was from T_0 to T_3 (0 h, 1 h, 3 h, 5 h). lane *1* shows the protein marker, and lane *2* shows a sample taken prior to induction (T_0), whereas lane *3*～*5* show samples after 1 h, 3 h and 5 h of expression, respectively. (b) Optimal IPTG concentration for the induction. Lane *1* shows protein marker, and lane *2*～*9* show the samples with different concentrations: 0.2 mmol/L, 0.4 mmol/L, 0.6 mmol/L, 1.0 mmol/L, 1.5 mmol/L, 2.0 mmol/L and 3.0 mmol/L, respectively.

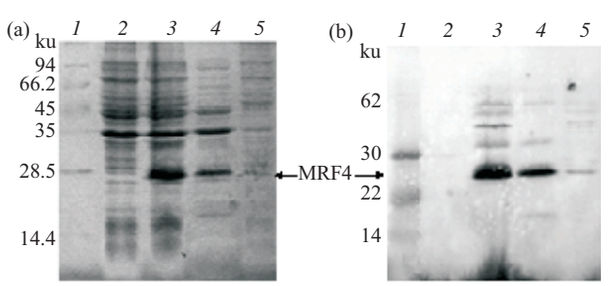

Fig. 3 Analysis of the solubility of MRF4 protein under B-PER buffer treatment

(a) SDS-PAGE analysis. *1*: Protein ladder; *2*: Control sample without induction; *3*: Induction sample without lysis; *4*: Supernatant sample after B-PER treatment; *5*: Pellet sample from the cell lysates. (b) Western blot analysis. Anti-his antibody was used for detection on blot. Lane *1* was prestainned protein ladder, the order of lane *2*～*5* was same to that of SDS-PAGE analysis.

2.3 Purification of MRF4 protein under nature condition

To produce optimum concentration of the fusion protein, the recombinant protein was purified with BD TALON IMAC system. The targeted recombinant protein was eluted out in fractions E1 to E8 with different concentrations (Figure 4). There was high concentration of recombinant protein in E2, while there was low concentration in fraction E3 and E4. And there was much lower concentration in E5 to E8 (which was not shown in the figure). There was also lower concentration of targeted protein in flow through fractions because too much protein was added to the column and not bound to resins completely. There were no protein in washing samples and E1, which suggested that the column was washed and the unbounded proteins were removed completely. There was lower concentration and low MW protein in the purified samples. This could be the degradation of the fusion protein and confirmed by Western blot with anti-His antibody (Figure 5).

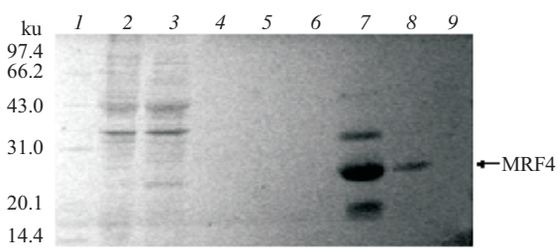

Fig. 4 SDS-PAGE analysis of the purified His-tagged MRF4 fusion protein

1: Protein ladder; *2*: Whole cells (non-induced control); *3*: Whole cells (induced control); *4*: Eluted fraction (E2); *5*: Flow through fraction (F); *6*: Wash fraction (W1); *7*～*9*: Eluted fraction (E1, E3 and E4 respectively).

2.4 Preparation of polyclonal anti-MRF4 antibodies

The rabbit immunized with purified protein produced MRF4-specific antibodies after the second immunization. After the third injection, a stronger increase of antibodies was observed using Western blot. The serum was diluted with 1∶300, 1∶500 and 1∶1000 (*v*/*v*), and all of dilutions could detect the purified protein.

The recognized band by anti-MRF4 serum was the band recognized by anti-his antibody (Figure 5b and c). But some fainter bands were appeared in non-induced control and induced control that because the components of serum were very complicated (Figure 5b).

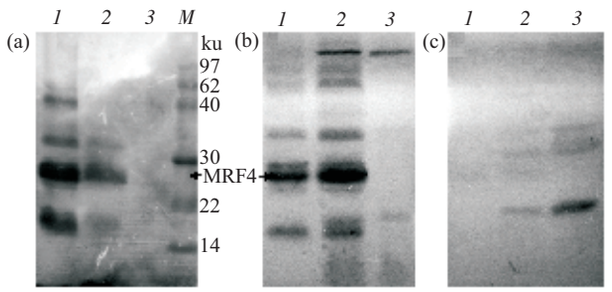

Fig. 5 Western blot analysis of the anti-MRF4 antibody

(a) Anti-his antibody was used for detection on blot. (b) Anti-serum was used on blot. (c) Negative serum was used on blot. *1*: The purified sample; *2*: The induced control; *3*: The non-induced control; *M*: Prestainned protein ladder.

2.5 Analysis of the ability of anti-MRF4 serum through the detection of endogenous MRF4 protein

The flounder MRF4 protein has a calculated value about 25 ku which is about 6 ku smaller than the PolyHis-MRF4 fusion protein. The Western blot analysis showed that the polyclonal antibodies could recognize a protein more than 22 ku in new-hatched flounder sample (Figure 6c). These results indicated that the serum contained anti-MRF4 antibody which could recognize the endogenous expression of MRF4 protein.

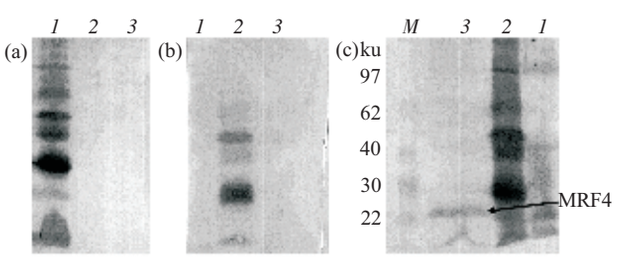

Fig. 6 Western blot analysis of the endogenous flounder MRF4

(a) Negative serum. (b) Anti-His monoclonal antibody and (c) Anti-serum were used as primary antibodies, respectively. *1*: Non-induced control; *2*: Induced control; *3*: New-hatched flounder; *M*: Prestainned protein ladder.

3 Discussion

Many studies have been performed at the protein level of the four MRFs. In 1992, Hopwood *et al*.[12] first prepared anti-MyoD monoclonal antibody to describe the pattern of expression of MyoD in early frog development. Then monoclonal anti-MyoD and anti-myogenin antibodies which were well characterized markers for the early and late phases of the cell cycles were confirmed usefully for diagnosing RMS (rhabdomyosarcoma)[13]. The MRF4 functions in adult and regenerating muscle were found through using anti-MRF4 polyclonal antibodies on protein level. In rat, the rat MRF4 polyclonal antibodies recognized the expression of MRF4 during rat muscle regeneration, and showed that MRF4 shared overlapping functions with the other MyoD family factors after fusion had occurred [14]. In Xenopus, Becker *et al*. [15] developed an affinity-purified polyclonal antibody against Xenopus MRF4 and used it to describe the MRF4 expression pattern in the adult and in regenerating muscles. Also, the Xenopus MRF4 polyclonal antibody could recognize two different MRF4s[10]. All of these studies use antibody to describe the function or discover new function of MRFs that different from the RNA level. So, it is one important step to gain an antibody against interest gene.

In present study, antibody against flounder MRF4 was prepared and proved to specially recognize the endogenous flounder MRF4. An expression host *E. coli* BL21 (DE3) was used because it is a specific bacterial strain designed for expression of genes regulated by the T7 promoter[16]. The expression vector pET-30b was chosen based on the presence of sequences encoding six histidine residues at 5′ of the multiple cloning sites (MCS) and high expression efficiency. The fusion protein was purified with BD TALON resins which were durable, cobalt-based IMAC resins designed to purify rebombinant polyhistidine-tagged proteins[17]. Compared with nickel IMAC resins, BD TALON resins exhibited a significantly reduced affinity for host proteins. Thus the background proteins were reduced, and the tagged protein could be eluted under less stringent conditions. To obtain the soluble protein in nature condition, B-PER reagent was used for the lysis of the bacterial cells. It utilized a proprietary, mild, nonionic detergent in 20 mmol/L Tris•HCl (pH 7.5). This novel reagent provides versatility for different applications and eliminated exogenous contamination of the recombinant protein. The expression and purification system could provide enough high quality protein efficiently.

The endogenous MRF4 protein was detected by the Western blot using the polyclonal antibody. In the endogenous sample, only one band was detected, which suggested that there should be only one form of

MRF4 in the flounder. From this study, the MRF4 polyclonal antibody was found to be specific to the MRF4 protein. So, this polyclonal antibody can be used to study the function of flounder MRF4.

References

1 Rhodes S J, Konieczny S F. Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev, 1989, **3** (12B): 2050～2061

2 Braun T, Bober E, Winter B, *et al*. Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. EMBO J, 1990, **9** (3): 821～831

3 Davis R L, Weintraub H, Lassar A B. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell, 1987, **51** (6): 987～1000

4 Braun T, Buschhausen-Denker G, Bober E, *et al*. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J, 1989, **8** (3): 701～709

5 Wright W E, Sassoon D A, Lin V K. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell, 1989, **56** (4): 607～617

6 Rudnicki M A, Schnegelsberg P N, Stead R H, *et al*. MyoD or Myf5 is required for the formation of skeletal muscle. Cell, 1993, **75** (7): 1351～1359

7 Hasty P, Bradley A, Morris J H, *et al*. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature, 1993, **364** (6437): 501～506

8 Nabeshima Y, Hanaoka K, Hayasaka M, *et al*. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature, 1993, **364** (6437): 532～535

9 Kassar-Duchossoy L, Gayraud-Morel B, Gomes D, *et al*. Mrf4 determines skeletal muscle identitiy in Myf5: MyoD double-mutant mice. Nature, 2004, **431** (7007): 466～471

10 Della Gaspera B, Sequeira I, Charbonnier F, *et al*. Spatio-temporal expression of MRF4 transcripts and protein during Xenopus laevis embryogenesis. Dev Dyn, 2006, **235** (2): 524～529

11 Zhu Z, Miller J B. MRF4 can substitute for myogenin during early stages of myogenesis. Dev Dyn, 1997, **209** (2): 233～241

12 Hopwood N D, Pluck A, Gurdon J B, *et al*. Expression of XMyoD protein in early Xenopus laevis embryos. Development, 1992, **114** (1): 31～38

13 Cui S, Hano H, Harada T, *et al*. Evaluation of new monoclonal anti-MyoD1 and anti-myogenin antibodies for the diagnosis of rhabdomyosarcoma. Pathol Int, 1999, **49** (1): 62～68

14 Zhou Z, Bornemann A. MRF4 protein expression in regenerating rat muscle. J Muscle Res Cell Motil, 2001, **22** (4): 311～316

15 Becker C, Della Gaspera B, Guyot M, *et al*. Expression of MRF4 protein in adult and in regenerating muscles in Xenopus. Dev Dyn, 2003, **227** (3): 445～449

16 Fang C M, Zainuddin Z F, Musa M, *et al*. Cloning, expression, and purification of recombinant protein from a single synthetic multivalent construct of Mycobacterium tuberculosis. Protein Expr Purif, 2006, **47** (2): 341～347

17 Bush G L, Tassin A M, Friden H, *et al*. Secretion in yeast: purification and *in vitro* translocation of chemical amounts of prepro-alpha-factor. J Biol Chem, 1991, **266** (21): 13811～13814

重组牙鲆MRF4在大肠杆菌中的表达、纯化及多克隆抗体分析*

徐 芃[1,2] 谭训刚[1)**] 张培军[1)**] 张玉青[1,2] 王 波[1,2]

([1)]中国科学院海洋研究所，实验海洋生物学重点实验室，青岛 266071；[2)]中国科学院研究生院，北京 100049)

摘要 *MRF4* 是肌肉发生调节因子家族的一员，其对肌肉的发育有重要的调节作用．对于养殖鱼类，生长速度是很重要的经济指标，而肌肉的发育对鱼类的生长非常重要．制备了 MRF4 的多克隆抗体以分析在鱼类中 *MRF4* 的功能．通过采用大肠杆菌表达系统及 pET-30 表达载体对牙鲆肌肉发生调节因子 MRF4 进行了体外重组表达．所得到的 MRF4 重组蛋白为可溶性的．在自然条件下，经金属离子螯合柱纯化后，得到了电泳纯的融合蛋白，通过免疫新西兰大白兔制备 MRF4 的多克隆抗体．蛋白质印迹分析显示：重组蛋白能被抗组氨酸标签的抗体及所制备的多克隆抗体识别，而且这一多克隆抗体能检测到内源性的 MRF4 蛋白，从而进一步证明了抗血清中含有 MRF4 的抗体．MRF4 融合蛋白的成功表达及其多克隆抗体的制备为进一步深入研究其在牙鲆肌肉发育过程中的分子机制奠定了基础．

关键词 牙鲆，表达，纯化，MRF4 抗体

学科分类号 Q78

* 国家重点基础研究发展计划(973)(2004CB117402)和国家高技术研究发展计划(863)(20060110A4013)资助项目.

** 通讯作者.

谭训刚, Tel: 0532-82898559, Fax: 0532-82969735, E-mail: tanxgj@yahoo.com

张培军, Tel: 0532-82898551, Fax: 0532-82969735, E-mail: pjzhang@ms.qdio.ac.cn

收稿日期：2007-03-12 接受日期： 2007-05-08