行政院國家科學委員會專題研究計畫　成果報告

斑馬魚胚胎早期轉錄因子 Mfy-5 之生物特性:以繼代遺傳轉殖品系作為研究模式(2/2)

計畫類別： 個別型計畫
計畫編號： NSC92-2313-B-002-056-
執行期間： 92 年 08 月 01 日至 93 年 07 月 31 日
執行單位： 國立臺灣大學漁業科學研究所

計畫主持人： 蔡懷楨

報告類型： 完整報告

處理方式： 本計畫可公開查詢

中　華　民　國 93 年 11 月 3 日

A BAC:Myf5:gfp Transgenic Zebrafish Line Revealed the Original Compartment of Cranial Muscle Precursors and the Combinations of Multiple Regulatory Modules within the Upstream 80 kb Region

Yau-Hung Chen[1], Yun-Hsin Wang[1], Cheng-Yung Lin[1], Hung-Chieh Lee[1], Chih-Wei Weng[1], Monte Westerfield[2] and Huai-Jen Tsai[1]*

[1]Institute of Molecular and Cell Biology, National Taiwan University, Taipei, Taiwan

[2]Institute of Neuroscience, University of Oregon, Eugene, OR, USA

1 Roosevelt Road, Sec. 4, Taipei, Taiwan

Tel: 886-2-2364-1705

Fax: 886-2-2363-8483

*Corresponding author

E-mail: hjtsai@ntu.edu.tw

Running title: zebrafish *myf*5 BAC germ line

Keywords: adaxial cell, bacterial artificial chromosome, cranial muscle, gene regulation, muscle regulatory factors, presomitic mesoderm, transgenic fish.

Summary

Myf5 functions as a myogenic factor, which is important in the specification of muscle cells. Because the expression of *myf*5 is somite- and stage-dependent, the regulation is delicately orchestrated. A 156-kb bacterial artificial chromosome (BAC) clone was isolated, in which an upstream 80 kb region of zebrafish *myf*5 was included. We generated a transgenic line carrying -80 kb segment fused GFP reporter gene. The GFP signals were faithfully recapitulated the endogenous expression patterns of *myf*5 during somitogenesis. Interestingly, we found at early larvae stage, GFP signals were restricted at occipital somite, where is the original compartment of cranial muscle precursors. Then, GFP was detected in some cranial muscles, such as adductor hyomandibulae, adductor mandibulae, dilatator operculi, dorsal pharyngeal wall, hyohyoideus, medial rectus, sternohyoideus and superior oblique. This is the first report to demonstrate that zebrafish Myf5 controls some subsets of cranial muscles specification. Furthermore, we also generated and analyzed five transgenic lines derived from embryos injected with -9977/-1, -6212/-1, -2937/-1, -2456/-1 and -290/-1 segments. Compared the GFP-expression patterns of each line, we found (1) -80/-10 kb segment contained a cranial muscle-specific enhancer and a notochord-specific repressive element; (2) The -9977/-6213 segment contained a repressive element; (3) The -6212/-2938 segment contained tissue-specific elements

for fin muscle, jaw muscle, and spinal cord, as well as a lens-specific enhancer; (4) The -2937/-291 segment contained a pupil-specific element and the -2937/-2457 segment is required for notochord- and myocyte-specific expression; and (5) The -290/-1 segment was responsible for basal transcription of somite- and presomitic mesoderm-specific expression. Therefore, we conclude the regulatory *cis*-elements within -80 kb segment include several repressive elements and tissue-specific enhancers, which are involved in regulating the somite- and stage-specific expression of zebrafish *myf*5.

Introduction

In vertebrates, the determination and differentiation of trunk skeletal muscle is controlled by the basic-helix-loop-helix (bHLH) family of transcription factors, such as Myf5, MyoD, Myogenin and MRF4 (reviewed by Pownall *et al*., 2002). These myogenic regulatory factors (MRFs) activate muscle-specific transcription via binding to an E-box, which is present in the promoter of numerous muscle-specific genes (reviewed by Rescan *et al*., 2001; Pownall *et al*., 2002). Following the whole-mount *in situ* hybridization of zebrafish embryos, myogenic MRF gene expression in somites has been shown to occur in the order of *myf*5 at 7.5 hours post-fertilization (hpf) (Chen *et al*., 2001; Coutelle *et al*., 2001), *myo*D at 8 hpf (Weinberg *et al*., 1996), *myogenin* at 10.5 hpf (Weinberg *et al*., 1996), and *mrf*4 at 12 hpf (Chen *et al*., unpublished results). The same expression sequential relationships are also found in mice (reviewed by Pownall *et al*., 2002). These observations strongly indicate that *myf*5 is the first MRF expressed during vertebrate's myogenesis.

Promoter mapping of the mouse *myf*5 has been performed using YAC (Hadchouel *et al*., 2000) and BAC DNA (Carvajal *et al*., 2001). Several regulatory *cis*-elements have been proposed: a distal regulatory element at -96/-63 kb, a proximal promoter at -58/-48 kb, a central nervous system-specific enhancer at -0.5/-0.1 kb, a hypaxial myotome enhancer at 0.5/3.5 kb, an epaxial myotome enhancer at -5.6/-4.6

kb, and a branchial enhancer at -1.5/-0.5 and 0.5/3.5 kb (Summerbell *et al*., 2000; Hadchouel *et al*., 2003). In *Xenopus myf*5, an interferon regulatory factor-binding element within the *Xenopus myf*5 promoter is responsible for the elimination of *myf*5 transcription in the mature somitic mesoderm of *Xenopus* embryos (Mei *et al*., 2001). Recently, Lin *et al.* (2003) find that a T-box binding site enables to mediate the dorsal activation of *Xenopus myf*5 during the gastrula stage. These findings highlight the complicated and dispersed nature of the regulatory *cis*-elements of *myf*5. However, compared to the extensive literature on higher vertebrate *myf*5, very little is known about the regulatory mechanism of fish *myf*5, especially the *cis*-regulating elements involved in the somite-, presomitic mesoderm (PSM)- and head muscle-specificity.

Head muscles originate from the so-called paraxial mesoderm/occipital somites, where ventral dermomyotomal cells move into the hypoglossal cord and migrate rostrally to form pharyngeal and tongue muscles (Schilling and Kimmel, 1994; Mackenzie *et al*., 1998, Huang *et al*., 1999). Despite their localizations in head, myogenic precursors from occipital somites essentially follow the trunk programs. In mice, *myf*5 is expressed in the branchial arches where the cells are derived from anterior paraxial mesoderm which will contribute to the formation of facial muscles (Hadchouel *et al*., 2003). Recently, a segment located at -58/-48 kb of mice *myf*5 is identified to direct the transgene to express in the hypoglossal cord (Buchberger *et al*.,

2003; Hadchouel *et al*., 2003). In chicken, *myf*5 transcripts are detected in the epaxial region of the occipital somites at early embryonic stage. Within unsegmented head mesoderm, *myf*5 is expressed in the branchial arch myoblasts, the lateral rectus and eye muscles (Noden *et al*., 1999). In zebrafish, *myo*D transcripts are detected in the precursors of the medial and inferior rectus extraocular muscles, the adductor mandibulae and sternohyals (Schilling and Kimmel, 1997). However, little is known how MRFs regulate cranial myoblast differentiation and how many cranial muscles are controlled by Myf5.

Bacterial artificial chromosome (BAC) is an *Escherichia coli* F factor-based vector, which is capable of harboring the cloned DNA fragment up to 300 kb stably in bacteria (Shizuya *et al*., 1992). Jessen *et al.* (1998) reported a chi-stimulated homologous recombination method of BAC particularly for transgenic zebrafish containing BAC clones. However, it is too laborious because a chi-based plasmid is needed and the recombination targeting regions for both ends are up to 2 kb. Here, we develop a highly efficient method for zebrafish BAC engineering, which is modified from mice BAC system (Yu *et al*., 2000; Lee *et al*., 2001), and generate transgenic lines containing various lengths of *myf*5 upstream sequences. Results show that some cranial muscles are specifically regulated by Myf5. We also find that -80/-10 kb segment contains a cranial-specific enhancer and a notochord-specific repressor. We

believe that these transgenic lines should provide us excellent materials for studying the PSM-, cranial muscle-specific elements and *trans*-acting factors of *myf*5, and for tracing the muscle progenitors.

Experimental Procedures

BAC library screening

Zebrafish BAC library were brought from RZPD (http://www.rzpd.de) (Germany) and the screening protocols were followed the manufacture's instructions with minor modifications. The primary BAC library pools were screened by PCR using zebrafish *myf*5 intron 1-specific primers, 1261F (5'-TGTTCATTCACTCATTTTCTTTTCA-3') and 2582R (5'-GCAGTCTTCCTACAATGACAA-3'). The positive clones isolated from the primary pools were further confirmed by screening the secondary pools from RZPD. A *myf*5-containing BAC clone was finally isolated.

Pulsed field gel electrophoresis (PFGE)

DNA from a *myf*5-contained BAC clone was extracted, and digested with *Eco*RI, *Hin*dIII and *Sac*I. The resultant DNA was separated by 0.8% agarose gel electrophoresis in the PFGE electrophoresis tank (Biometra). The electrophoresis conditions were: 200 volts at 10℃ for 24 h with electrode angles at 120°, and rotor speed for 2-6 sec. After electrophoresis, the BAC DNA size was determined by Kodak 1D image analysis software.

Bioinformation

For chromosome mapping, we used zebrafish *myf*5-specific primers, 1261F and 2582R to carry out PCR reaction for mapping *myf*5 against the LN54 radiation hybrid

(RH) panel. The RH panel was scored according to Hudson et al. (1995). The mapping results were analyzed by using website: http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi.

For screening *myf*5-containing BAC clone, the junctions of BAC DNA were sequenced by using T7 and SP6 primers. These junctions' sequences and coding region were blasted by using BlastN program on the following website (http://www.ensembl.org/Danio_rerio/). After the blast results between the junction sequences and *myf*5 coding region were compared, and the location of the Myf5-containing BAC clone was characterized.

Generation of a *myf*5-BAC clone containing GFP reporter

Plasmid pZMYP-BAC80E contained an around upstream 80 kb region of zebrafish *myf*5 fused with GFP reporter (Fig. 1a). Basically, we followed the protocols described by Lee *et al*. (2001) with some modifications. The cassette used for targeting the *myf*5 locus was amplified from template pZMYP-82E (Chen *et al*., 2001) with primers ZMFP-82F (5'-CTCTTAGCTCTGTCCTGGCCA-3') and Kan-817R (5'-***ATTTACAAATGAGCAAGCAGTGTGAATAAAGCGTTGGCCTGA***GTCGGTC ATTTCGAACCCCAG-3') by using Deep Vent (NEB) to carry out PCR at following conditions: 94°C, 40s; 58°C, 30s; 72°C, 150s; for 35 cycles. PCR products were digested with *Dpn*I to remove contaminated template.

For *myf*5-containing BAC clone transformation, 1 μg of *myf*5-containing BAC DNA was used to transform electrocomponent cells *E. coli* DY380. For *myf*5-containing BAC:gfp modification, putative transformants harboring myf5:BAC were isolated and grown on LB medium containing 25 μg/ml of chloramphenicol (CAM) overnight at 32°C with shaking at 150 rpm. After 0.1% inoculum in a 50 ml of LB-CAM, bacteria were continuously cultured to an OD_{600} of 0.8. Then, bacteria were cultured at 42°C for 15 minutes, transferred to ice slurry for 30 minutes, washed 5 times in ice-cold water and electroporated immediately. Around 2 μg of targeting cassette DNA were mixed with the freshly prepared electrocomponent cells of DY380 carrying myf5:BAC. Electroporation was performed at 1.8 kV, 200 Ohms, 25μF for 3 times with a 30 sec interval (Gene Pulser Xcell, BioRad). After electroporation, cells were dispersed on the LB plates with 30 μg/ml of kanamycin and incubated at 32°C overnight. Then, recombinants were picked and checked by PCR using primers of ZMFP-117F (TTTGGGTGGGGATCTAGATGGTG) and GFP-407R (GTTGCCGTCCTCCTTGAAGT), and Kan-F (ATGATTGAACAAGATGGATTGC) and ZMF-1000R (AGCGAGTTAAGTTTAAAGTCTGACCC), to check both integration ends.

Plasmids constructs for promoter analysis

(A) pZMYP-9977E

Following the procedures described by Chen *et al*. (2001), a 3.7-kb *Sac*I-cut fragment from a *myf*5-positive recombinant bacteriophage was ligated with a *Sac*I-digested plasmid pZMYP-6212E, which containing a zebrafish *myf*5 upstream -6212/-1 segment fused with EGFP cDNA. The resultant plasmid, pZMYP-9977E (Fig. 1b), contained a zebrafish *myf*5 upstream -9977/-1 segment fused with EGFP cDNA.

(B) pZMYP-9977E-ITR

A *Sal*I/*Eco*RV fragment from pZMYP-6212E (Chen *et al*., 2001) was ligated with a Klenow-digested (NEB) pGEMT-easy vector (Promega), in which the *Eco*RV site was recovered but the *Sal*I site was not, to produce an intermediate plasmid, p(6212/1984). Then, a 3.8 kb *Sac*I fragment from a *myf*5-positive phage clone (Chen *et al*., 2001) was ligated with *Sac*I-treated p(6212/1984) to produce an intermediate plasmid, p(9977/1984). Finally, a *Sac*I/*EcoR*V fragment cut from p(9977/1984) was ligated with *Sac*I/*EcoR*V-treated pZMYP-6212E-ITR (Fig. 1d). The resultant plasmid, pZMYP-9977E-ITR (Fig. 1c), contained the -9977/-1 segment of the *myf*5 gene and was flanked at both ends with inverted terminal repeats of adeno-associated virus (AAV-ITR).

(C) pZMYP-6212E-ITR and pZMYP-2937E-ITR

A *Sal*I/*Age*I fragment from either pZMYP-6212E or -2937E (Chen *et al*., 2001)

was ligated with a 4.2-kb *Sal*I/*Age*I fragment obtained from pCMV-EGFP-ITR (Chou *et al*., 2001). The resultant plasmids, pZMYP-6212E-ITR and pZMYP-2937E-ITR, contained the zebrafish *myf*5 -6212/-1 and -2937/-1 segments, respectively. Each plasmid was flanked with AAV-ITR at both ends (Figs. 1d and 1e).

(D) pZMYP-290E-ITR

Plasmid pZMYP-290E (Chen *et al*., 2003) was cut with *Hin*dIII, blunted and then cut with *Age*I. The resultant 0.3 kb fragment was ligated with a 4.2-kb fragment, which was produced by cutting pCMV-EGFP-ITR (Chou *et al*., 2001) with *Sal*I, blunting and then cutting with *Age*I. The resultant plasmid, pZMYP-290E-ITR, contained a zebrafish *myf*5 -290/-1 segment and was flanked with AAV-ITR at both ends (Fig. 1g).

(E) pEGFPm(2937/2457) and pEGFPm(2457/2937)

For notochord-specific *cis*-acting element identification, forward (5'-TCTAGAACAGATTCTCATCCAA-3') and reverse (5'-AACTGCACACTGGAGATTCATAAG-3') primers were used to generate cassette -2937/-2457. The cassette was ligated with pGEM T-Easy vector (Promega) and then treated with *Eco*RI to obtain an insert. *Eco*RI-cut pEGFPmTATA, which contained a minimal TATA-box of cytomegalovirus (CMV) promoter to direct the EGFP gene (Chen *et al*., 2003), was ligated with one copy of the *Eco*RI-cut cassette

-2937/-2457 to generate pEGFPm(2937/2457)(Fig. 6). Plasmid pEGFPm(2457/2937), containing one copy of cassette -2457/-2937, was also constructed (Fig. 6).

DNA preparation for microinjection and transient GFP expression

The procedures of microinjection and transient green fluorescence detection were described by Chen *et al.* (2001) except that we observed the GFP-expression of transgenic embryos hourly, especially from 6 to 36 hpf.

Identification of germ-line transmitted zebrafish

All GFP-positive embryos at 24-hpf were raised to adulthood. Transgenic founders (F0) mated with wild-type individually to confirm the parents that transmitted BAC through the germ-line. We tried to acquire at least 3 pairs of germ-line in order to prevent positional effect. At least 200 embryos from each pair were examined their green fluorescences.

After screening, GFP-positive F1 embryos were raised to adulthood and crossed with wild-type zebrafish to generate a heterozygotic F2 generation. GFP-positive F2 individuals were then crossed each other to generate homozygotic F3 fish, which were used to produce 100% GFP-positive F4 offspring.

Cryosectioning and whole-mount *in situ* hybridization

The procedures of cryosectioning and whole-mount *in situ* hybridization were described by Chen and Tsai (2002) except the embryos at 16-120 hpf were used.

Antibody staining

Embryos were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS, pH 7.0) for 4 h at room temperature, or overnight at 4°C. Then, embryos were washed in 0.1 M PBS twice for 15 min each, soaked in 100% acetone at -20°C for at least 10 min, and rehydrated with 0.1% (v/v) Tween 20 in PBS 3 times for 15 min each. Subsequent steps for labeling anti-*myf*5-antibody were described by Du *et al*. (1997).

Forskolin treatment

In order to activate protein kinase A (PKA), forskolin was treated to increase cellular cAMP levels by direct stimulation of adenyl cyclase that will inhibit Hh signaling (Seamon and Daly, 1981). Wild-type embryos at 5.5-hpf were treated with 0.3 mM forskolin (Sigma) dissolved in 4% dimethyl sulfoxide. We changed the freshly prepared forskolin when embryos developed at 24-hpf, then were continuously changed the fresh one every 6 h until 48-hpf. Control groups were treated without containing forskolin.

Results

Screening the BAC library and identification of a Myf5-containing BAC clone

Ten primary pools of zebrafish BAC library were screening by using PCR strategy, one of them (P1) was positive. Forty-eight secondary pools derived from P1 were screened in which five pools were positive. Lastly, we obtained single BAC clone which was *myf*5-positive. After PFGE analysis, the insert size of the myf5-containing BAC clone was around 156 kb. After searching the databank, a contig, ctg9418, contained the whole sequence of the myf5-containing BAC clone was found. When the *myf*5 coding region and the junction sequences of T7 and SP6 were blasted, we found that the sequences located at the ctg9418 were 1380, 1460 and 1304 kb, respectively. Therefore, we conclude that the 5' and 3' regions on this myf5-containing BAC clone were 80 and 70 kb, respectively.

Chromosome mapping and syntenic relationship of zebrafish *myf*5

Radiation hybrid method to map zebrafish *myf*5 revealed that *myf*5 was located at linkage group 4 (LG4), between two EST markers, fb62d08 and fb78c03, and at 5.87 centiRay from EST marker z9667 (data not shown). The syntenic relationship showed that zebrafish LG4 fragment between EST markers fa05f06 and fk68a09 (including *myf*5) was related to human chromosome 12q13-12q21.

Germ-line transmitted zebrafish of *myf*5

We selected and cultured 549 transgenic zebrafish carrying *myf*5 BAC segment. After crossing with wild-type, 4 lines, 80k-5, -18, -21 and -23, possessed GFP-positive F1 embryos: 5 out of 103, 5 out of 96, 3 out of 211, and 101 out of 439 embryos, respectively (Table 1). In addition, we also generated 4 lines from -9977/-1 group (10k-2, -13, -9R, -15R), 4 lines from the -6212/-1 group (6k-9R, -10R, -11R, -16R), 2 lines from the -2937/-1 group (2.9k-18R, -92R), 2 lines from the -2456/-1 group (2.4k-3, -8), and 1 line from the -290/-1 group (0.3k-14R). The F2 inheritance rates for these transgenic lines ranged from 47.5 to 52.9% (Table 1) indicating there was a single transgene insertion site.

The GFP Expression patterns of BAC:Myf5:gfp transgenic lines during early somitogenesis

In embryos derived from BAC:Myf5:gfp transgenic lines 80k-23, the green fluorescent signal was first appeared at 5.5-hpf but extremely weak (data not shown); then reached to detectable level in the segmental plates at 10.5-hpf (Fig. 2a) and expanded to 14 somite pairs in 16-hpf embryos (Fig. 2b). Comparison with the results from whole mount *in situ* hybridization, similar expression domains were observed in the 16-hpf (Fig. 2e), indicating that the BAC transgene was capable of recapitulating the endogenous *myf*5 expression patterns. At 28-hpf, the green signals were

down-regulated in the elder embryonic somites. Unlike F1 germ-line fish, the GFP expression patterns were slightly mosaic in F0 founders (Figs. 2c and 2f). Results from cryosection indicated that GFP signals were detected at both slow- and fast-muscle precursor (Fig. 2d). We also examined the GFP expression patterns of the remaining 3 BAC:Myf5:gfp lines (80k-5, -18 and -21), and found no significant differences among them, indicating that position effects would be excluded.

GFP expression revealed the development of head skeletal muscles

When embryo developed at 30-hpf, green signals were detected in pectoral fin muscle precursors (pm), dorsal anterior myotomes (dam) and hypaxial muscle progenitors (hy)(Figs. 3a and 3b). At 42- and 48-hpf, the cells carrying green signals moved toward anterior parts of the head, and concentrated into occipital somite (os) that is proposed as the original of cranial muscles (Figs. 3c-e). At the same stage, two eye-muscles precursors, superior oblique (so) and adductor mandibulae (am), were also displayed green signals (Figs. 4c and 4h). From 60- to 72-hpf, green signals were only observed in some specific muscles, such as adductor hyomandibulae (ah), adductor mandibulae (am), dilatator operculi (do), dorsal pharyngeal wall (dpw), hyohyoideus (hh), medial rectus (mr), sternohyoideus (sh) and superior oblique (so)(Figs. 3f, 3g-i). The green signals were gradually degraded when embryos were elder than 8-dpf (data not shown). The *myo*D transcripts in 72-hpf zebrafish embryos

were detected by whole-mount *in situ* hybridization, and found that *myo*D were expressed in all cranial muscles (Fig. 3i), indicating that *myf*5 and *myo*D might play distinct roles during head skeletal muscles formation.

Dynamic GFP-Expression patterns in transgenic lines carrying the -9977/-1, -6212/-1 , -2937/-1, -2456/-1 and -290/-1 segments

In order to further study the biological functions of -80 kb fragment, we dissected the -80 kb segment to generate zebrafish that transmitted the -9977/-1, -6212/-1, -2937/-1, or -290/-1 segments through the germ-line. All the expression patterns and expression domains of different transgenic lines were summarized in Table 2. In embryos derived from lines -9977/-1 (10k), the green fluorescent signals were detected at notochord, somite and PSM. The deletion of the -9977/-6213 segment from -9977/-1 led to a broad expansion of expression domains in the transgenic lines (-6212/-1; 6k). GFP signals were first detected 3-hpf, with the intensity increasing in all parts of the embryos, especially the head, somites and notochord, between 16- and 20-hpf. GFP signals were gradually down-regulated after 30-hpf, and green signals were only found in the urogential opening at 32- and 44-hpf. GFP signals were also broadly expressed in the jaw, fin muscles (levator pinnae pectoralis, levator pinnae abdominalis internus and carinatus ventralis), gills, bones, jaw cartilages, lens, and other domains. These data implicates that -9977/-6213

segment is a repressive *cis*-element. In the following parts, we will discuss the PSM-, notochord-, bone, eyes- and olfactory-pits-restricted expression in different transgenic lines.

GFP expression patterns in the presomitic mesoderm of different transgenic lines

The unique characteristic of *myf*5 expression is PSM-specific (Chen *et a*l., 2001). However, elements required for *myf*5 PSM-specific control element is unknown. At 10.5-hpf embryos of line BAC:Myf5:gfp (80k-23), the GFP signals were strongly expressed in adaxial cells and presomitic mesoderm (Fig. 4a) and expanded to the somites when embryos developed at 16-hpf (Fig. 4b). These observations were similar to the endogenous Myf5 protein expression (Fig. 4c). We also found that transgenic lines carrying the -9977/-1, -6212/-1 and –2937/-1 segments (lines 10k-13, 7k-14R and 2.9k-18R) displayed strong GFP signals in the PSM (Figs. 4d-f). However, in the transgenic line carrying the –290/-1 segment (line 0.3k-14R), the GFP signal in the PSM was weak (Fig. 4g, arrow). Thus, we propose the minimal PSM-specific element of *myf*5 is located within the -290/-1 segment.

Cassette -2937/-2457 is able to direct notochord-specificity

Transgenic lines carrying the -9977/-1 (Figs. 5a and 5b), -6212/-1 (Figs. 5c and 5d) and -2937/-1 (Figs. 5e-h) segments displayed notochord-restricted GFP expression patterns. However, no green fluorescent signals were detected in the

notochord of zebrafish bearing a germ-line transmitted -2456/-1 segment (Table 2). Therefore, we think that a notochord-specific element is located within the -2937/-2457 segment. To test this hypothesis, we constructed and injected three different expression plasmids that used GFP as a reporter gene. Plasmid pEGFPmTATA contained a cytomegalovirus (CMV) mini-promoter (TATA box only) fused with GFP. This plasmid was used as a backbone plasmid for constructing pEGFPm(2937/2457) and pEGFPm(2457/2937). Plasmid pEGFPm(2937/2457) contained one copy of two directions of cassette 2937/2457 and plasmid pEGFPm(2457/2937) contained one copy of cassette 2457/2937. Only 5.1% (6 of 104) of pEGFPmTATA-injected-embryos were GFP-positive, and none were notochord- or myocyte-specific (Fig. 6). However, the notochord- and myocyte-specific expression rates in pEGFPm(2937/2457)- and pEGFPm(2457/2937)-injected embryos were 48.4% and 44.5%, respectively (Fig. 6). Thus, the -2937/-2457 segment is a typical, orientation-independent enhancer for notochord- and myocyte-specificity.

GFP expression patterns in the spinal cord, bones, eyes and olfactory pits of different transgenic lines

In transgenic line carrying -6212/-1 segment (6k), GFP signals reappeared in the axial and dorsal-medial lips of embryonic somites at 2-dpf (Fig. 7a), and migrated from the rostral to caudal region along the axial lips at 3-dpf (Fig. 7b). Cryosection of

the anterior of the dorsal-mediate lips revealed that GFP was expressed in the cells of the ectoderm and spinal cord (Fig. 7c). However, the GFP signal in the spinal cord was restricted to the posterior of the dorsal-mediate lips (Fig. 7d), suggesting GFP-positive cells may migrate from anterior ectoderm to the posterior neural tube. From 21-60 hpf, transgenic lines bearing the -6212/-1 segment (6k) displayed green signals in the eyes (Fig. 7h) and bones, including the basihyal, sternohyal, and palatoquadrate bones and Meckel's cartilage (Figs. 7e-7g). In lines carrying the -2937/-1 segment (2.9k), the green signals in the head were predominantly in pupils (Fig. 7i) and olfactory primordial (Fig. 7j), suggesting that pupils- and olfactory-enhancers are located within the -2937/-291 region.

Treatment with forskolin of 80k-23 embryos revealed Myf5-positive fin muscle progenitor malformation

Neumann *et al.* (1999) proposed that fin muscle progenitors differentiation is under control by *sonic hedgehog* (*shh*) signal transduction pathway. Weinberg *et al.* (1996) also demonstrated that MyoD transcripts are detected at zebrafish fin buds. In our study, we found that line 80k-23 displayed green signals in fin buds (Fig. 9a), indicating that *myf*5 is involved in fin muscle determination. We treated the 80k-23 embryos with forskolin to examine the relationship between *myf*5 and *shh* pathway during fin muscle formation. In mock control (without forskolin), the green signals of

fin buds were condensed in roundish shape at each side along the body axial (Fig. 9a). However, when embryos were treated with forskolin, although the green signals of fin buds were detected, GFP signals were wide dispersed and do not form a condense disc (Fig. 9c), indicating that *shh* signals are required for recruiting *myf*5-positive cells to their destinations for fin buds formation.

Discussion

Establishing transgenic zebrafish lines carrying a green fluorescent protein (GFP) reporter driven by regulatory elements of different sizes is a relatively simple, yet effective and reliable technique to *in vivo* study the regulation of gene expression and differentiation, and to delicately map the *cis*-regulatory elements of gene. Because transgenic line carrying -9977/-1 segment did not recapitulate endogenous expression, we develop BAC:Myf5:gfp germ-line transmission zebrafish for this study.

BAC transgenesis and Myf5 recapitulation

Myf5 is the first expression among MRFs during somitigenesis in zebrafish, understanding its regulation becomes an important issue to address. We develop a highly efficient method to generate zebrafish BAC engineering, in which as short as 42-bp homologous cassette is sufficient to drive homologous recombination. This is the first report to describe an effective method in zebrafish BAC transgenesis.

At 16-hpf, the GFP signals of BAC:Myf5:gfp transgenic lines were strongly detected mainly in the somites and segmental plates. Prominent signals occurred transiently in adaxial cells in two-parallel rows, but did not extend beyond the positive-signal somites. This expression patterns are exactly identical to what we observe with the endogenous *myf*5 mRNA transcripts from *in situ* hybridization (Chen *et al*., 2001). However, we notice that the GFP signals of BAC:Myf5:gfp transgenic

lines were also detected at early gastrula (5.5-7.5 hpf) and at some cranial- and pectoral fin-muscle precursors at larvae stage (30-hpf to 8-dpf), which were not present in whole mount *in situ* hybridization because of less sensitivity. When RT-PCR was used, the endogenous *myf*5 transcripts of wild-type embryos were detected from 5.5-hpf to 7-dpf (data not shown). Therefore, we propose that BAC:Myf5:gfp transgenic lines enable to recapitulate the endogenous *myf*5 expression.

MyoD and Myf5 control distinct cranial muscles formations

In the avian embryo, paraxial mesoderm migrates into the branchial arches and the contribution of these precursors to facial muscles is well documented (Hacker and Guthrie, 1998; Noden *et al*., 1999). In mice (Carvajal *et al*., 2001), the ontogeny of cranial myogenesis is generally equivalent to that described by Noden et al., 1999 for the chick. Although the vertebrate cranial muscles development follows the same program is speculated, few data are available of fish cranial muscle development. In zebrafish, Schilling and Kimmel, (1994) pointed out the anatomic position of occipital somite where is the original position of neural crest cells. However, no molecular marker, such as *lbx1*, *myf*5 and *myo*D are used to demonstrate that the occipital somite of zebrafish is the original compartment of cranial muscle precursors. In this study, the BAC:Myf5:gfp transgenic lines clearly reveal that Myf5- and GFP-positive cells

are detected at the occipital somite (Fig. 3c). Whole mount *in situ* hybridization experiment also reveals that the precursor cells in occipital somite are *myo*D- (data not shown) and *lbx1*-positive (Lin *et al*., unpublished data), indicating that the occipital somite of zebrafish is the original compartment of cranial muscle precursors.

MyoD and Myf5 expression in cranial muscles (especially in eye and branchial muscle progenitors) has not been studied. In this study, we found that all cranial muscles are MyoD-positive but only some of them are Myf5-positive. Injecting of the MyoD-morpholino into α-actin:rfp transgenic zebrafish showed that only Myf5-positive cranial muscles (ex: adductor hyomandibulae, adductor mandibulae, dilatator operculi, dorsal pharyngeal wall, hyohyoideus, medial rectus, sternohyoideus and superior oblique) displayed red fluorescent signals (Lin *et al*., unpublished). These results strongly suggest that Myf5 and MyoD may control different cell types during cranial myogenesis.

Fin muscle precursor

At 48-hpf, *myo*D expression slightly precedes division, revealing both this dorsal subdivision and a ventral masticatory subdivision (Fig. 9b). However, *myf*5 expressed the pectoral fin precursors that appear Myf5-positive are in round shape, indicating that *myf*5 and *myo*D might play distinct roles in pectoral fin formation. In *smoothened*-defective zebrafish mutant, *smu*$^{-}$, pectoral fins are greatly reduced,

indicating that *shh* signals are required for pectoral fin formation (Barresi *et al*., 2000). We treated the BAC:Myf5:gfp transgenic lines with forskolin to block the *shh* signals, and found that *myf*5-positive cells were still observed in fin buds, but lost their organized patterns. Unlike *myo*D which is the direct target and responses to *shh* signals, *myf*5 expression in fin buds might not be controlled by *shh*, but are maintained by *shh* signals during pectoral fin formation.

Functional analyses of *cis*-acting elements within the upstream 10-kb region

The regulatory mechanism of mouse *myf*5 is long-range (Hadchouel *et al*., 2000), heterogeneous and controlled by discrete enhancers (Summerbell *et al*., 2000; Buchberger *et al*., 2003; Hadchouel *et al*., 2003). In this study, we found that several regulatory elements are located within the upstream 80 kb region of zebrafish *myf*5 (Fig. 8).

(A) Presomotic mesoderm (PSM) specific *cis*-acting elements

In mouse embryo, endogenous myf5 transcripts cannot be seen at PSM by in situ hybridization (Summerbell *et al*., 2002; Teboul *et al*., 2003). Faint PSM expression of β-galactosidase has been observed in $Myf5^{nlacZ}$ knock-in mice, which express the reporter very strongly, and Myf5 transcripts are detectable in mouse PSM by RT-PCR (Cossu *et al*., 1996). However, the *myf*5 transcripts in zebrafish PSM are very strongly and can be easily seen (Chen *et al*., 2001). Thus, zebrafish became an excellent

animal model to study the transcription initiation of *myf*5 gene in PSM. In our study, we start to characterize the PSM-specific *cis*-elements by using germ-line transmission. The transgenic lines carrying -80 kb, -9977/-1, -6212/-1, and -2937/-1 segments displayed strong green signals in PSM (Figs. 4a-f), but the transgenics carrying -290/-1 segment displayed extremely weak signals in the PSM (Fig. 4g). These observations suggest that a PSM enhancer might locate in the -2937/-291 segment. Furthermore, no green signals were detected in the PSM of the -82/-1-injected embryos (Chen *et al*., 2001), indicating that the element responsible for minimal level expression of PSM might be located within -290/-83 segment.

(B) Repressive *Cis*-acting elements

In mice, Carvajal *et al*. (2001) reported that a –58.6/-8.8 kb element is able to suppress *myf*5 expression in the dermomyotome. Summerbell *et al*. (2000) also reported that a –8.8/-6.6 kb element is able to suppress *myf*5 expression in branchial arches. In *Xenopus*, Poli and Amaya. (2002) found that a 1.2 kb element is necessary for both the activation and repression of *myf*5 expression. Recently, a novel, *cis*-acting silencer in intron 1 of zebrafish *myf*5 has been shown to have repression and orientation-specific regulatory abilities (Lin *et al*., unpublished data). Therefore, we propose that one or more repressor-rich elements are located within the -9977/-1 segment. Comparing the GFP expression of with that in line 6212-14, we found that

the later displayed more expansive expression domains than in line 9977-15. Thus, the -9977/-6213 segment seems to be a repressive *cis*-element.

(C) Bone morphogenic protein (BMP)

Transgenic line 6k-14 (bearing the –6212/-1 segment) displayed GFP signals in the bones 21-dpf (Figs. 7e and 7f), but line 2.9k-18 (bearing the –2937/-1 segment) did not. It is possible that a) the -6212/-2938 segment possesses a bone-specific element, or b) the -9977/-6213 segment possesses a silencer. Cossu and Borello (1999) reported that BMP has a repressive effect on *myf*5 regulation. Further research is needed to determine whether the -9977/-6213 segment contains a BMP response element.

(D) Notochord-specific *cis*-acting elements

Interestingly, we found that transgenic lines with segment –9977/-1, -6212/-1 or –2937/-1 (lines 9977-13, 6212-14 and 2937-18) displayed GFP signals in the notochord. A CNS-specific element was found in the -0.5/-0.1 kb region of mice *myf*5 (Summerbell *et al*., 2000).

Analysis of the putative transcription factor binding sites within the -2937/-2457 segment found two homeobox-binding sites (TAATTA)(-2904/-2899 and -2745/-2740), and one gli- (GACCACCC)(-2616/-2609), one MEF2- (CT AATTTTAG)(-2609/-2600) and one Foxh1-binding site (AATCTCC

A)(-2474/-2467). Homeobox and Foxh1 direct the notochord-specificity of *shh* gene expression (Jeong and Epstein, 2003). MEF2 is a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation (a review by Naya and Olson, 1999). Gli is capable of directing notochord- (Sasaki *et al*., 1997), and myocyte-specificity (Gustafsson *et al*., 2002). Because binding sites for these factors exist in the –2937/-2457 segment, it is reasonable to conclude that this segment directs GFP expression in the notochord and myocytes. In this study, an enhancer responsible for notochord- and myocyte-specific expression was identified by analyzing a germ-line transmitted *myf*5 promoter in zebrafish. The function of the enhancer was confirmed with an *in vivo*, transient assay of F0 individuals. Thus, germ-line transmission appears to be a reliable method for identifying the function of *cis*-elements.

Conclusion

The gene regulation of *myf*5 is delicately orchestrated. We are the first one to generate a zebrafish transgenic line carrying BAC:Myf5:gfp, in which the upstream 80 kb of *myf*5 fused GFP reporter. This transgenic line can recapitulate the endogenous *myf*5 transcription, which should provide an excellent material for studying the control mechanism of *myf*5 and for mutant screening to find novel genes involved in somitigenesis. In this study, we use this line to identify expression

domains and to characterize upstream *cis*-elements, including a repressive element, a stage-specific repressor domain, and tissue-specific enhancers for jaw and fin muscles, gills, bones, eyes, somites, olfactory organs and presomitic mesoderm. The BAC:Myf5:gfp transgenic line should also facilitate more detailed studies related to the morphogenesis of somite, presomitic mesoderm and cranial muscles.

Acknowledgments

We are grateful to Prof. Yee-Hsiung Chen, Institute of Biochemical Sciences, National Taiwan University, for helping with cyrosectioning. We are grateful to Dr. Marc Ekker, who providing the LN54 mapping panel in this study. We are also grateful to Dr. Copeland, National Cancer Institute who providing the *E. coli* strain, DY380. This project was supported by the National Science Council, Republic of China, under grant no. NSC 91-2313-B-002-331 and NSC 92-2313-B-002-056.

References

Barresi MJF, Stickney HL, Devoto SH. 2000. The zebrafish *slow-muscle-omitted* gene product is required for Hedgehog signal transduction and the development of slow muscle identity. Development 127: 2189-2199.

Buchberger A, Nomokonova N, Arnold H-H. 2003. Myf5 expression in somites and limb buds of mouse embryos is controlled by two distinct distal enhancer activities. Development 130: 3297-3307.

Carvajal JJ, Cox D, Summerbell D, Rigby PWJ 2001. A BAC transgenic analysis of the Mrf4/ Myf5 locus reveals integrated elements that control activation and maintenance of gene expression during muscle development. Development 128: 1857-1868.

Chen YH, Lee WC, Liu CF, Tsai HJ 2001. Molecular structure, dynamic expression and promoter analysis of zebrafish (*Danio rerio*) *myf*-5 gene. Genesis 29: 22-35.

Chen YH and Tsai HJ 2002. Treatment with myf5-morpholino results in somite patterning and brain formation defects in zebrafish. Differentiation 70: 447-456.

Chen YH, Lee HC, Liu CF, Lin CY and Tsai HJ. 2003. Novel regulatory sequence -82/-62 functions as a key element to drive the somite-specificity of zebrafish *myf*-5. Dev. Dyn. 228: 41-50.

Chou CY, Horng LS, Tsai HJ 2001. Uniform GFP-expression in transgenic medaka *(Oryzias latipe*s) at the F0 generation. Transgenic Res. 10: 303-315.

Cossu G, Tajbakhsh S, and Buckingham M. 1996. How is myogenesis initiated in the embryo? Trends Genet. 12: 218-223.

Cossu G, Borello U 1999. Wnt signaling and the activation of myogenesis in mammals. EMBO J. 18: 6867-72.

Coutelle O, Blagden CS, Hampson R, Halai C, Rigby PWJ, Hughes SM 2001. Hedgehog signaling is required for maintenance of *myf*5 and *myoD* expression and timely terminal differentiation in zebrafish adaxial myogenesis. Dev. Biol. 236: 136-150.

Du, S. J., Devoto, S. H., Westerfield, M. and Moon, R. T. 1997. Positive and negative regulation of muscle cell identity by members of the hedgehog and TGF-beta gene families. *J. Cell Biol.* 139: 145-156.

Gustafsson MK, Pan H, Pinney DF, Liu Y, Lewandowski A, Epstein DJ, Emerson Jr CP. 2002. *Myf*5 is a direct target of long-range Shh signaling and Gli regulation for muscle specification. Genes Dev 16: 114-126.

Hacker A, Guthrie S. 1998. A distinct developmental programme for the cranial paraxial mesoderm in the chick embryo. Development 125: 3461-3472.

Hadchouel J, Tajbakhsh S, Primig M, Chang THT, Daubas P, Rocancourt D, Buckingham M 2000. Modular long-range regulation of Myf5 reveals unexpected heterogeneity between skeletal muscles in the mouse embryo. Development 127:

4455-4467.

Hadchouel J, Carvajal JJ, Daubas P, Bajard L, Chang T, Rocancourt D, Cox D, Summerbell D, Tajbakhsh S, Ribgy PWJ and Buckingham M. 2003. Analysis of a key regulatory region upstream of the *Myf*5 gene reveals multiple phases of myogenesis, orchestrated at each site by a combination of elements dispersed throughout the locus. Development 130: 3415-3426.

Huang R, Zhi Q, Izpisua-Belmonte JC, Christ B, Patel K. 1999. Origin and development of the avain tongue muscles. Anat. Embryol. 200: 137-152.

Hudson TJ, Stein LD, Gerety SS, Ma J, Castle AB, Silva J, Slonim DK, Baptista R, Kruglyak L, Xu SH, Hu X, Colbert AME, Rosenberg C, Reeve-Daly MP, Rozen S, Hui L, Wu X, Vestergaard C, Wilson KM, Bae JS, Maitra S, Ganiatsas S, Evans CA, DeAngelis MM, Ingalls KA, Nahf RW, Horton Jr. LT, Anderson MO, Collymore AJ, Ye W, Kouyoumjian V, Zemsteva IS, Tam J, Devine R, Courtney DF, Renaud MT, Nguyen H, O'Connor TJ, Fizames C, Faure S, Gyapay G, Dib C, Morissette J, Orlin JB, Birren BW, Goodman N, Weissenbach J, Hawkins TL, Foote S, Page DC, Lander ES. 1995. An STS-based map of the human genome. Science 270: 1945-1954.

Jeong Y and Epstein DJ. 2003. Distinct regulators of *Shh* transcription in the floor plate and notochord indicate separate origins for these tissues in the mouse node.

Development 130: 3891-3902.

Jessen RJ, Meng A, McFarlane RJ, Paw BH, Zon LI, Smith GR, Lin S. 1998. Modification of bacterial artificial chromosomes through Chi-stimulated homologous recombination and its application in zebrafish transgenesis. Proc. Natl. Acad. Sci. USA 95: 5121-5126.

Lin GF, Geng X, Chen Y, Qu B, Wang F, Hu R and Ding X. 2003. T-box binding site mediates the dorsal activation of *myf*-5 in *Xenopus* gastrula embryos. Dev. Dyn. 226: 51-58.

Lee EC, Yu D, de Velasco JM, Tessarollo L, Swing DA, Court DL, Jenkins NA, and Copeland G. 2001. A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting abd subcloning of BAC DNA. Genomic 2001: 56-65.

Mackenzie S, Walsh FS, Graham A. 1998. Migration of hypoglossal myoblast precursors. Dev. Dyn. 213: 349-358.

Mei W, Yang J, Tao Q, Geng X, Rupp RAW and Ding X. 2001. An interferon regulatory factor-like binding element restricts *Xmyf*-5 expression in the posterior somites during *Xenopus* myogenesis. FEBS Lett 505: 47-52.

Naya FJ, and Olson E. 1999. MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation. Curr. Opin. Cell Biol. 11:

683-688.

Neumann CJ, Grandel H, Gaffield W, Schulte-Merker S, Nusslein-Volhard C. 1999. Transient establishment of anteroposterior polarity in the zebrafish pectoral fin bud in the absence of sonic hedgehog activity. Development 126: 4817-4826.

Noden DM, Marcucio R, Borycki AG, and Emerson Jr. CP. 1999. Differentiation of avian craniofacial muscles: I. Patterns of early regulatory gene expression and myosin heavy chain synthesis. Dev. Dyn. 216: 96-112.

Polli M and Amaya E. 2002. A study of mesoderm patterning through the analysis of the regulation of *Xmyf*-5 expression. Development 129: 2917-2927.

Pownall ME, Gustafsson MK, Emerson, Jr. CP. 2002. Myogenic regulatory factors and the specification of muscle progenitors in verterbrate embryos. Annu. Rev. Cell Dev. Biol. 18: 747-783.

Rescan PY 2001. Regulation and functions of of myogenic regulatory factors in lower vertebrates. Comp. Biochem. Physiol. 130B: 1-12.

Sasaki H, Hui C, Nakafuku M, and Kondoh H. 1997. A binding site for Gli proteins is essential for *HNF*-3 β floor plate enhancer activity in transgenics and can respond to Shh in vitro. Development 124: 1313-1322.

Schilling TF, and Kimmel CB. 1994. Segment- and cell-type restricted lineages during pharyngeal arch development in the zebrafish embryo. Development 120:

483-494.

Schilling TF, and Kimmel CB. 1997. Musculoskeletal patterning in the pharyngeal segments of the zebrafish embryo. Development 124: 2945-2960.

Seamon KB, Day JW. 1981. Forskolin: a unique diterpene activator of cyclic AMP-generating systems. J. Cyclic Nucleotide Res. 7: 201-224.

Shizuya H, Birren B, Kim UJ, Mancino V, Slepak T, Tachiiri Y, Simon M. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc. Natl. Acad. Sci. USA 89: 8794-8797.

Summerbell D, Ashby PR, Coutelle O, Cox D, Yee SP, Rigby PWJ 2000. The expression of Myf5 in the developing mouse embryo is controlled by discrete and dispersed enhancers specific for particular populations of skeletal muscle precursors. Development 127: 3745-3757.

Teboul L, Summerbell D, Rigby PWJ. 2003. The initial somatic phase of Myf5 expression requires neither Shh signaling nor Gli regulation. Genes Dev. 17: 2870-2874.

Wang TM, Chen YH, Liu CF, Tsai HJ. 2002. Functional analysis of the proximal promoter regions of fish *rhodopsin* and *myf*-5 gene using transgenesis. Mar. Biotechnol. 4: 247-255.

Weinberg ES, Allende ML, Kelly CS, Abdelhamid A, Murakami T, Andermann P, Doerre OG, Gruwald DJ, Riggleman B 1996. Developmental regulation of zebrafish MyoD in wild-type, *no tail* and *spadetail* embryos. Development 122: 271-280.

Yu D, Blis HM, Lee EC, Jenkins NA, Copeland NG, Court DL. 2000. An efficient recombination system for chromosome engineering in *Escherichia coli.* Proc. Natl. Acad. Sci. USA 97: 5978-5983.

Figure Legends

Figure 1. Plasmid constructs for germ-line transmission in zebrafish. (a) Strategy for constructing a *myf*5-BAC clone containing GFP reporter. Myf5 gene contained 3 exons (E1, E2, and E3) and 2 introns (I1 and I2). The cross hatched boxes and dotted boxes represented zebrafish *myf*5 promoter and the desired regions of *myf*5 for homologous recombination. pZMYP-BAC80E was the *myf*5-BAC clone containing GFP reporter. The primers ZMFP-117F, GFP-R, Kan-F and ZMF-1000R were used for checking recombinants. (b-g) Plasmid constructs for promoter analysis. Plasmid pZMYP-2456E was described by Wang *et al*., 2002. Thick lines and crossed boxes represented plasmid vectors and *myf*5 promoters, respectively. Numbers above boxes indicated the nucleotide positions of the zebrafish *myf*5. GFP, green fluorescent protein; ITR, inverted terminal repeats of adeno-associated virus; SVpA, polyadenylation signal of SV40.

Figure 2. The expression patterns of transgenic GFP and endogenous *myf*5 transcripts in somites during somitogenesis. (a-c) GFP expression in the 10.5-, 16- and 28-hpf embryos of transgenic line carrying BAC: Myf5: gfp. (d) Cryosection of 28-hpf embryos of transgenic line carrying BAC: Myf5: gfp. (e) Whole-mount *in situ* hybridization of wild-type embryos using *myf*5 probe at 16-hpf. (f) Transsient GFP expression in the 28-hpf embryos injected with BAC: Myf5: gfp construct.

Figure 3. Dynamic GFP expression patterns in cranial muscles of transgenic line carrying BAC:Myf5:gfp. GFP express except in the somite of the transgenic line 80k-23 were observed at 30-hpf (a-b): GFP signals were detected in pectoral fin muscle (pm), dorsal anterior muscle (dam) and hypixial muscle (hy); at 42-hpf (c-e): GFP signals were first appeared in occipital somite (os) and some cranial muscles, such as superior oblique (so) and adductor mandibulae (am); at 60-hpf (g-i): GFP signals were detected in adductor hyomandibulae (ah), am, dilatator operculi (do), dorsal pharyngeal wall 1-5 (dpw1-5), medial rectus (mr), sternohyoideus (sh) and so; at 72-hpf: GFP signals were expressed persistently (f), and MyoD signals were detected at all cranial muscles (j). hh : (hyohyoideus), ima (intermandibularis anterior), imp: (intermandibularis posterior), ih (interhyal).

Figure 4. GFP expression patterns in the presomitic mesoderm of five transgenic lines. Green fluorescent signals were detected in the presomitic mesoderm of transgenic embryos harboring -80 kb (line 80k-23, a and b), –10 kb (line 10k-13, d), -6 kb (line 6k-16R, e) or –2.9 kb (line 2.9k-18R, f) segments. Wild-type embryo developed at 16-hpf was treated with polyclonal antiserum against mouse Myf5 to detect the expression patterns of endogenous Myf5. (c). ad: adaxial cells; n: notochord; psm: presomitic mesoderm; s: somite.

Figure 5. GFP expression patterns in the notochord of three transgenic lines.

Green fluorescent signals were detected in the notochord of transgenic embryos harboring –10 kb (line 10k-13, a-b), -6 kb (line 6k-16R, c-d), –2.9 kb (line 2.9k-18R, e-h) or -0.3 kb (line 0.3k-14R) segments.

Figure 6. Cassette -2937/-2457 is able to direct notochord specificity. Upper left: Schematic illustration of microinjected plasmids pEGFPmTATA, pEGFPm(2937/2457) and pEGFPm(2457/2937). Right: The calculation of total expression rates, notochord- and myocyte-specific expression rates, and non-specific expression rates are described in the Materials and methods sections. Bottom: Embryos were photographed under fluorescent light. In pEGFPm(2937/2457)-injected zebrafish, EGFP signals appeared as bars with sharp edges (myocyte-specific) and squares (notochord-specific).

Figure 7. GFP expression patterns in the spinal cord, bones, eyes and olfactory-pits of different transgenic lines. Green signals appeared at 2-dpf, especially along the dorsal-medial lips (a). Green signals on the dorsal-medial lips extended toward the caudal region 3-dpf (b). Cross-sections (c and d) of a 3-dpf embryo along the planes indicated by c and d on panel (b). Green signals were located in the dorsal-medial lips, which are part of the spinal cord (SC). GFP expression was observed in bones at 21-dpf (e and f), and in eyes (h) and bones 60-dpf (g and h). am: adductor mandibulae; bh: basihyal; cv: carinatus ventralis; mc: Meckel's cartilage; n: notochord; pq: palatoquadrate; sc: spinal cord. Green fluorescent signals were

observed in embryos from transgenic lines (2.9k-18R and –92R), which were derived from embryos injected with the -2937/-1 segment. The GFP gene was expressed strongly both in eyes and olfactory pits at 7-dpf (i) and 24-hpf (j). (k): bright field of (j).

Figure 8. Schematic illustration of the proposed *cis*-regulatory elements located at the upstream 80-kb of zebrafish *myf*5. Thick lines, crossed boxes, and dotted boxes represent plasmid vector, *myf*5 upstream region and untranslated regions, respectively. Numbers above crossed boxes indicate the nucleotide positions of zebrafish *myf*5. EGFP, enhanced green fluorescent protein.

Figure 9. Forskolin treatment of embryos of line 80k-23. Embryos of transgenic line 80k-23 developed at 48-hpf were treated without (a) or with forskolin (c). (b) Whole-mount in situ hybridization of wild-type 48-hpf embryo by using MyoD riboprobe.