Functional Analysis of the Proximal Promoter Regions of Fish Rhodopsin and Myf-5 Genes Using Transgenesis

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Abstract: Little is known about the *cis*-acting elements controlling transcriptional activities of fish rhodopsin and myf-5 genes. Transgenic medaka and zebrafish were used to characterize promoters of carp (*Cyprinus carpio*) rhodopsin gene (*cRh*) and zebrafish (*Danio rerio*) myf-5 gene (*myf-5*), respectively. Transgenic medaka carrying different lengths of *cRh* upstream fragments fused with enhanced green fluorescence protein (EGFP) gene revealed several functional regions. Both upstream regions from –1261 to –163 bp (–1261/–163) and –163/–138 contributed to enhance the retina-specific activities of *cRh*. The cNRE (–75/–63) and CSE (–52/–46) motifs located at the *cRh* proximal promoter were sufficient to drive the transgene expressed in retinae. The –73/–68 within cNRE was also proved to be essential for the integrity of the minimal *cis*-regulatory elements. Regarding the *myf-5*, transgene expression in zebrafish embryos injected with different deletion fragments of zebrafish *myf-5* upstream sequences showed that the –2776/–2456 and –1046/–844 regions contributed to the enhancer function. The –290/–154 region might be involved in controlling the translocation of progenitor muscle cells. Moreover, the upstream –82/–1 region was identified as a minimal promoter required for somite-specific expression. The –82/–62 region was the most critical sequence for *myf-5* specificity.

Key words: *cis*-elements, medaka, *myf-5*, rhodopsin, transgenic, zebrafish.

INTRODUCTION

Rhodopsin is the photoreceptor protein in the outer segment of rod cells, which are sensitive to dim light. In nuclear runoff experiments, it has been shown that the expression of rhodopsin is primarily controlled at the transcriptional level (Treisman et al., 1988; DesJardin et al., 1993). Certain upstream *cis*-regulatory elements of rhodopsin gene (*Rh*), such as Ret1/PCE-1 (Yu et al., 1996), Ret4 (Chen and Zack, 1996), and NRE (Nrl response

element; Kumar et al., 1996; Rehentulla et al., 1996), were found in mammalian system. Compared with the extensive knowledge about the transcriptional regulation of *Rh* in mammals, little is known in fish. To address this issue, the upstream sequences of 2 types of carp *Rh* (*cRh*) (Lim et al., 1997) were compared to other known species, and some putative *cis*-acting elements were proposed (Su et al., 2000). Recently, the zebrafish *Rh* was cloned, and the transgenic zebrafish carrying 1.2-kb promoter fused with enhanced green fluorescence protein (EGFP) gene showed rod-specificity (Kennedy et al., 2001). However the functional *cis*-elements within the proximal region of fish *Rh* still remain unclear. In this study we fully dissect the up-

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stream segment to characterize the putative *cis*-elements within *cRh* in vivo.

In mice, skeletal muscle formation during myogenesis is controlled by 4 basic helix-loop-helix (bHLH) transcription factors: MyoD (Davis et al., 1987), myogenin (Braun et al., 1989a; Edmondson and Olson, 1989; Wright et al., 1989), Myf-5 (Braun et al., 1989b) and MRF4/herculin/Myf-6 (Rhodes and Konieczny, 1989; Braun et al., 1990; Miner and Wold, 1990). MyoD and Myf-5 play distinct roles during early myogenesis. Cells in epaxial myotome express *myoD*, but cells in hypaxial myotome express *myf-5* (Rudnicki and Jaenisch, 1995). The epaxial and hypaxial myotomes are finally differentiated into limbs and trunk muscles, respectively. The upstream regulatory regions of *myf-5* have been reported in mice (Hadchouel et al., 2000; Summerbell et al., 2000) and zebrafish (Chen et al., 2001). Recently, Chen et al. (2001) and Coutelle et al. (2001) demonstrated that zebrafish *myf-5* transcripts were first detectable at 7.5 hours postfertilization (hpf), increased substantially until 16 hpf, and then declined gradually to an undetectable level by 26 hpf. During somitogenesis, zebrafish *myf-5* transcripts were distributed mainly in the somites and segmental plates. Prominent signals occurred transiently in adaxial cells in 2 parallel rows, but did not extend beyond the positive-signal somites. The upstream *cis*-regulatory elements of zebrafish *myf-5* were not clearly dissected. Therefore, using the same strategy to study *cRh*, the proximal *cis*-elements of zebrafish *myf-5* were analyzed in vivo in order to elucidate the regulatory mechanisms of the *myf-5*.

MATERIALS AND METHODS

Constructs for Promoter Analysis of Carp Rhodopsin Gene

The upstream -597/+94, -542/+94 and -418/+94 regions of type I *cRh* gene were amplified using the forward primers 5'-TAGAGCACTTGTTAGGGATT-3', 5'-TTCTCCTCAGGCGTATACCC-3', and 5'-AGCTTGCGTATTGAATCACT-3', respectively, with reverse primer 5'-ATTGGATCCGGCTGCGGTTGGATGT-3' (EPA-B, nucleotides +79/+94 with an extra *Bam*HI site) by polymerase chain reaction (PCR). The -597/+94 and -418/+94 fragments were cloned into the shuttle vector pGEM-T Easy (Promega, Madison, WI.) and the -542/+94 fragment was cloned into pGEM-3zf(+) (Promega). The sequences of the cloned fragments were confirmed.

The fragments were subsequently excised by *Eco*RI/*Bam*HI digestion and then ligated into the corresponding sites of pEGFP-1 (Clontech, Paloalto, CA.), which contained an EGFP gene. All isolated clones, surprisingly, contained backbones of shuttle vectors in the 5' end of *cRh* promoter. These constructs were *Eco*RI-digested and the ~5-kb fragments were extracted from the gel and used for microinjection. Purified DNA fragments containing -597/+94, -542/+94, and -418/+94 regions with EGFP gene were designated as pDel-597, pDel-542, and pDel-418, respectively. pDel-6k, pDel-1.2k, pDel-641, pDel-163, pDel-138, pDel-76, and pDel-58 contained different lengths (-6k, -1.2k, -641, -163, -138, -76, and -58 bp) of *cRh* upstream regions fused with EGFP gene, pNCSE-76 contained -76/+94 region but without the CSE sequence (Ma et al., 2001). While constructing the pDel-76, one clone, pFD-76 (folded dimer), containing head-to-head dimer of the -76/+94 sequence followed by EGFP gene was obtained. The -73/-71 region within pDel-76 was transversely mutated from CTG to AGT using mutated forward primer and primer EPA-B to construct pSpM2. In addition, the -70/-68 region within pDel-76 was mutated from ACA to CAC to construct pSpM3.

Four primers, 5'-GTGGAATTCGTTACATAACTTAC-3', 5'-CACAGTACTCGCTACCGCCCAT-3', 5'-GCGAGTACTGTGGGAGGCTAT-3', and 5'-GTTGGATCCTAGTGAGCTCGTCG-3' were used in sequential PCR-mediated mutagenesis (Ausubel et al., 1995) to introduce an additional *Sca*I cutting site upstream of the TATA box of cytomegalovirus (CMV) promoter-enhancer within pGreen Lantern-1 (Gibco BRL, Rockville, MD.). The mutated CMV promoter-enhancer was excised and inserted into pEGFP-1 to construct pCMVm. The double-stranded oligonucleotide CSE (Ma et al., 2001) was inserted into the *Sca*I site of pCMVm to construct pCMV-CSE. The orientation of CSE within pCMV-CSE was the same as in the *cRh* promoter.

Constructs for Promoter Analysis of Zebrafish Myf-5 Gene

A forward primer, 5'-AGATCTGTGAACATTTTAATTGTA-3', and a reverse primer, 5'-GGATCCGATTGGTTTGGTGTG-3', were used during PCR amplification to introduce an additional *Bam*HI site into the upstream sequence. The PCR product with molecular mass of 809 bp was ligated to pEGFP-1 producing pZMYP759Et. Then a 2.9-kb *Hind*III-*Apa*I fragment from pZMYP3.3 (Chen et al., 2001) was cloned

into pZMYP-759Et to construct pZMYP-2937E, and a 3.3-kb *Xba*I fragment from pZMYP6.6 (Chen et al., 2001) was introduced into pZMY-2937E to construct pZMYP-6212E.

pZMY-2937E, containing the upstream 2937-bp region of zebrafish *myf-5* and EGFP gene, was purified via Plasmid Miniprep Kit (Viogene). Various deletions of the zebrafish *myf-5* upstream region were obtained using the Double-Stranded Nested Deletion Kit (Pharmacia Biotech, Piscataway, N.J.). Approximately 5 µg of template DNA, pZMY-2937E, was completely digested by *Xba*I and *Pst*I. After digestion, salt and template DNA concentrations were adjusted in 1× exonuclease III buffer. The reaction was carried out at 33°C with 2-minute intervals. After agarose gel electrophoresis, deletions of different sizes were selected and their sequences were confirmed using a primer specific for pEGFP-1 (5'-CCCTGATTCTGTGGA-TAACCGTA-3').

Experimental Fish and Microinjection

The breeding and microinjection of medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) were described previously (Chen et al., 2001; Chou et al., 2001). *cRh*-related constructs were linearized by either *Xho*I or *Eco*RI, and medaka were used as the model fish. The *myf-5*-related constructs were linearized by *Stu*I and zebrafish were used instead.

Fluorescence Detection and Microscopy

Transgenic medaka embryos developed from stage 35 to 39 (Iwamatsu, 1994) were examined under a fluorescence stereomicroscope (MZ FLIII, GFP-Plus filter set, Leica). Transgenic zebrafish embryos were observed especially from 9 to 96 hpf. Expression rates of transgenic zebrafish embryos were categorized into 4 groups: (1) total expression rate or proportion of survival embryos with EGFP-expression in any cells; (2) somite-specific expression rate or proportion of survival embryos that have EGFP-expression in somite exclusively; (3) somite specificity and translocation rate or proportion of survival embryos that have EGFP-expression in somite exclusively and also have translocation of green signal; and (4) nonspecific expression rate or proportion of surviving embryos with somite expression that have expression in other cells. Photographs were taken with a MPS 60 camera (Leica) and FUJI 400 ASA film.

RESULTS

Expression Patterns of Transgene Driven by Various Lengths of Rhodopsin Promoter

The EGFP expression pattern of medaka embryos injected with pDel-6k was exclusively in the eyes (Figure 1, C). The expression pattern of embryos injected with pDel-138 and pDel-76 was also specific to the eyes, though ectopic expression was occasionally observed. However, patterns and rates of transgene expression were somewhat different among embryos injected with different lengths of promoter-EGFP constructs. For embryos injected with a promoter longer than pDel-163, the signals in eyes were usually broad and strong. In contrast, the signals of embryos injected with a promoter shorter than pDel-163 (pDel-138 and pDel-76) always appeared spotty and faint. The retinal expression rate of embryos injected with promoter longer than pDel-163 were apparently higher than those of embryos injected with shorter upstream fragments (Figure 2). EGFP signals could not be detected among embryos injected with pDel-58. Retinal expression rate of embryos injected with pFD-76, a head-to-head dimer of the -76/+94 region, was about 40%, which was dramatically higher than that of pDel-76-injected embryos (Figures 2 and 3). However, embryos injected with pSpM2 (mutated -73/-71), pSpM3 (mutated -70/-68), and pNCSE-76 (not containing CSE) totally lost their retina-driving ability, suggesting the crucial roles of the -73/-68 and CSE motifs (Figure 3). Expression patterns of embryos injected with pCMVm were quite different from the specific patterns of embryos injected with pDel-76 or longer constructs. Almost all embryos injected with pCMVm expressed EGFP in the body or yolk sac (Figure 1, D). EGFP could also be detected in retinae of embryos injected with pCMVm at an expression rate of about 7.3% (Figure 3), but the signals were usually faint and spotty. The retinal expression patterns and expression rates of embryos injected with pCMV-CSE, in which the CSE was just upstream of the TATA box of the CMV promoter, were similar to those of embryos injected with pCMVm (Figure 3).

Expression Patterns of Transgene Driven by Various Lengths of *myf-5* Promoter

To identify the *cis*-acting elements of zebrafish *myf-5* promoter, we isolated 15 deletion clones that encompassed

Figure 1. EGFP expression in transgenic medaka embryos. **A:** Bright field, untreated control embryo. **B:** Fluorescent light, untreated control embryo. The yellow and light green colors resulted from endogenous fluorescence. **C:** Fluorescent light, pDel-6k-injected

embryo, EGFP was expressed specifically and exclusively in the eyes. **D:** Fluorescent light, pCMVm-injected embryo. Green fluorescent signals appeared in the trunk and yolk sac. Arrows indicate the location of the eyes in stage 38 embryos. Scale bars indicate 0.2 mm.

Figure 2. Retinal expression rates of transgenic embryos. **A:** Schematic illustrations of *cRh* promoter-EGFP fusion constructs. Black boxes indicate EGFP gene; white boxes indicate the upstream region of *cRh*. **B:** Retina-specific expression of transgene in medaka embryos. After microinjection, embryos were examined between stages 35 and 39. The retinal expression rate is the percentage of all surviving injected embryos that exhibited EGFP expression in their eyes. The number of embryos examined is indicated above each bar.

Figure 3. Retina-specific expression rates of embryos injected with mutated *cRh* promoter-EGFP fusion constructs. pFD-76 contains a head-to-head dimer of the -76/+94 sequence. pSpM2 and pSpM3 contains mutated -73/-71 (CTG to AGT) and mutated -70/-68 (ACA to CAC) within the -76/+94 region, respectively. pNCSE-76 reflects deleted CSE core sequence (-52/-46) within the -76/+94 region. pCMVm contains CMV promoter/enhancer and EGFP gene, whereas pCMV-CSE contains CSE (-58/+32) upstream of the TATA box of CMV promoter/enhancer. Retinal expression rates were calculated as percentages of positive embryos among embryos examined. White boxes indicate the upstream region of *cRh*; hatched boxes indicate the CMV promoter/enhancer; black boxes indicate EGFP gene; black bars within *cRh* promoter indicate the mutated regions.

different lengths of the upstream regulatory sequences fused with EGFP gene. These constructs were microinjected into zebrafish fertilized eggs. Embryos injected with DNA fragments containing the upstream sequences of -82, -154, -290, -526, -702, -843, -1045, -1345, -1815, -2456, -2775, -2937, and -6212 bp (pZYMP-82E, -154E, -290E, -526E, -702E, -843E, -1045E, -1345E, -1815E, -2456E, -2775E, -2937E, and -6212E, respectively) displayed EGFP-positive signals in the somites at 10 hpf and remained detectable until 60 hpf. We noticed that

Figure 4. EGFP expression patterns in transgenic zebrafish embryos. One-celled fertilized eggs were microinjected with upstream -6 kb of zebrafish *myf-5* (pZMYP-6212E). Embryos at 60 hpf were photographed under fluorescent light. EGFP signals appeared in somite as a spot (arrowhead) and bar (arrows). Higher magnification of the white rectangle in the top panel is shown in the bottom panel. Scale bars: top panel, $200\ \mu\text{m}$; bottom panel, $30\ \mu\text{m}$.

GFP-positive signals appeared in spot- and rod-like shapes in somites (Figure 4), and the latter patterns were defined as EGFP translocation. As the total expression rates (Figure 5), the somite-specific expression rates of the above groups were high (from 47% to 91.5% of survival embryos) and the nonspecific expression rates were low (from 0% to 22.5%).

It was noticeable that the -82 -bp upstream sequence was capable of driving somite-specific expression of zebrafish *myf-5*. However, embryos injected with DNA fragments containing less than 82 bp (pZMYP-62E and -22E) did not give EGFP-positive signals (Figure 5), even when the concentration of injected DNA was increased to $50\ \text{ng}/\mu\text{l}$ (date not shown). On the basis of this evidence, we suggest that the proximal $-82/-1$ sequence of zebrafish *myf-5* is a minimal *cis*-element for controlling specific expression.

DISCUSSION

The *Rh* genes of bony fish lack introns (Fitzgibbon et al., 1995), making them ideal for studying the upstream regulatory regions that control tissue-specific expression of photoreceptor genes. The $-76/+94$ region (pDel-76) of *cRh* promoter could direct exogenous reporter gene expressed specifically in transgenic medaka embryos, whereas both the $-76/+94$ region lacking the $-52/-46$ sequence (CSE core region) and the $-58/+94$ (pDel-58) region could not.

Thus neither the cNRE alone (pNCSE-76) nor the CSE alone (pDel-58) (Figure 6, A) could drive retina-specific expression, indicating that cNRE and CSE should function together to drive EGFP expression to a detectable level. CSE is a functional homologue to the mammalian Ret4 element (Ma et al., 2001); proteins bound at bovine NRE and Ret4 were reported to work synergistically in cotransfection experiments (Chen et al., 1997). Thus it is possible that proteins bound at cNRE and CSE might also work synergistically in carp retinæ. In this study we injected a dimer of cNRE and CSE (pFD-76) fragment, and results showed that the retinal expression rate dramatically increased. The retinal expression rate of embryos injected with CMV promoter fused with CSE upstream of its TATA box (pCMV-CSE) was similar to that of embryos injected with CMV promoter (pCMVm), suggesting that cNRE-binding and CSE-binding proteins might interact in a specific manner.

The retinal expression rate of embryos injected with pDel-163 was apparently higher than that of embryos injected with pDel-138. Obviously, the $-163/-138$ region provides part of the enhancer activity. The nucleotide position of the carp $-163/-138$ region is close to the rat E-opsin-1 box (Ahmad, 1995), but they are not overlapping (Figure 6, A). Thus, whether the $-163/-138$ region can be designated as an E-opsin-1 box of fish, which is bound by Mash-1 in the rat system (Ahmad, 1995), remains to be determined. The difference in retinal expression rates between embryos injected with pDel-1.2k and pDel-163 was

Figure 5. Expression rates of transgenic zebrafish embryos injected with upstream region of *myf-5* gene. DNA fragments containing different lengths of the upstream zebrafish *myf-5* were injected and analyzed. pZMYP-6212E, -2937E, -2776E, -2456E, -1816E, -1343E, -1046E, -844E, -702E, -526E, -290E, -154E, -82E, -62E, and -22E represented the upstream regions extending from -1 to corresponding numbers of upstream positions. Calculations of total expression rate (black bars), somite-specific expression rate (white bars), expression rate of somite-specificity and translocation (crossed bars), and nonspecific rate (hatched bars) are described in “Materials and Methods.”

about 40%; therefore, the -1261/-163 region also contributes to part of the enhancer activity. Surprisingly, the retinal expression rate of embryos injected with pDel-597 dropped, compared with that of embryos injected pDel-542. Further study is necessary to determine whether the -597/-542 region of *cRh* contains a repressor.

Recently, zebrafish *Rh* was cloned and the 1.2-kb upstream region was injected into zebrafish embryos (Kennedy et al., 2001). Results showed the EGFP expression rate to be as low as 6%. Nevertheless, in our study the specific expression rate of medaka embryos injected with *cRh* 1.2-kb promoter (pDel-1.2k) was over 50%. In fact, the nucleotide sequences at the proximal region of *Rh* between zebrafish and carp are similar. Thus we believe that expression of EGFP in fish eyes might be more detectable using the medaka system than using the zebrafish system. Owing to the mosaic expression of the transgene, one should examine the pigmented eyes of transgenic embryos from every possible angle under the fluorescence microscope, so that the EGFP signals will not be missed. However, when the zebrafish egg is oriented, the embryo usually rotates within the chorion, making it difficult to be examined; in contrast, the medaka embryo will not rotate. In addition, observations of transgene expression patterns in retinæ of nonhatchingout medaka embryos (8 dpf) are

Figure 6. Alignments of the 5' flanking regions of rhodopsin and *myf-5* genes. **A:** Alignment of rhodopsin proximal promoters from different known species. The defined rat and bovine *cis*-elements are underlined. The CSE is boxed, and the carp NRE is boxed with dashed lines. Question marks represent elements that need to be confirmed in the fish system. Sequence comparison was based on DNASIS software with default setting (Hitachi). **B:** Sequences identities of the -82/-63 motif between zebrafish and mouse *myf-5* genes. Nucleotide sequences identical to zebrafish sequences are represented by dots. Dashes represent gaps created to maximize the degree of identity.

obviously much easier and more accurate than those of hatching-out, free-swimming fry (3 dpf).

The *Xenopus* system provides a powerful complementary approach for the analysis of cell-specific promoters (Mani et al., 2001). However, the mechanism involved in the transcriptional activation of *Xenopus Rh* seems somehow different from those in mammal and fish systems. In transgenic medaka carrying a 6-kb *cRh* promoter, the transgene was detected in cones (Ma et al., 2001); in transgenic mice carrying a 2.1-kb bovine *Rh* promoter, the transgene was expressed in cones and rods (Zack et al., 1991; Gouras et al., 1994); and in transgenic mice carrying a 4.4-kb mouse *Rh* promoter, transgene expression also in both rods and cones (Lem et al., 1991). Nevertheless, the 0.5-kb *Xenopus Rh* upstream fragment was reported to drive expression of the transgene exclusively in the rods (Knox et al., 1998). Accordingly, the mechanisms regulating *Rh* transcription in carp and mammals are more similar than those regulating *Xenopus* and mammals, although the upstream sequences of carp and mammal *Rh* are much less similar than the upstream sequences of carp and *Xenopus Rh* (Figure 6, A).

Using the same strategy, we also found that the –6-kb upstream region of zebrafish *myf-5* (pZMYP-6212E) directed transgenic EGFP expressed in the peripheral layer of the neural tube during the pharyngula stage (24 to 48 hpf) (data not shown). In mouse, 2 elements of *myf-5* upstream region are defined: one located between –96 and –63 kb, which was required for later *myf-5* expression in head and many trunk muscles; the other located between –58 and –48 kb, which is capable of directing *myf-5* expression in the myotome, the limbs, and the central nervous system (Hadchouel et al., 2000). Furthermore, the –23 kb upstream of mouse *myf-5* was capable of directing expression in the epaxial dermomyotome, intercalated myotome, and branchial arches. Regarding zebrafish *myf-5*, it is worthwhile to further study whether the proximal upstream regulatory region within –6 kb contains an element for controlling neuron- or limbs-specific expression.

Some EGFP signals shown in the somite also have a tendency that allows their green fluorescent signals to translocate and form a linear shape during the pharyngula stage. Interestingly, we found that the somite-specific expression rates of embryos declined significantly from 68.4% to 43.3% when embryos were injected with DNA fragments of –2776/–1, compared with embryos injected with –2456/–1. The same tendency was observed among embryos injected with –1046/–1 and –844/–1: somite-specific expression rate decreased from 61.9% to 41.2%. By TFSEARCH (Heinemeyer et al., 1998) prediction, we found that both regulatory elements contained MEF2 (myocyte enhancer factor 2), HSF (heat shock protein), and CdxA binding sites (CTAATTTTAG, AGAAAT, and ATTTATA). It is noteworthy that MEF2, HSF, and CdxA might be involved in the regulation of zebrafish *myf-5*.

Embryos injected with *myf-5* upstream fragments ranging from –6212/–1 to –82/–1 did display somite-specific expression. However, in embryos injected with DNA fragments shorter than –82/–1 (pZMYP-62E and –22E), no EGFP signals were observed. Comparing the –82/–62 motif with the relative fragment of mouse *myf-5*, we found that they displayed 60% sequence identity (Figure 6, B). On the basis of this observation, we propose –82/–1 to be the minimal promoter of zebrafish *myf-5*, and the –82/–62 motif to be an important player in directing somite-specific expression.

There are several ways to functionally analyze the transcriptional regulation of fish genes of interest. Among them, transgenesis provides an approach to study gene

regulation *in vivo*. The transparency of embryos in transgenic model fish (medaka and zebrafish) allows us to observe the dynamic expression patterns of transgenes during embryogenesis. Techniques used in transgenic fish are simpler, cheaper, and more efficient than those used in transgenic mammalian systems. Moreover, transient expression of transgenic F₀ embryos could somewhat recapitulate the expression patterns of endogenous genes, as reported for intronic enhancer of sonic hedgehog gene (Müller et al., 1999), promoter of myosin light chain 2 gene (Xu et al., 1999), and promoters of α - and β -actin genes (Chou et al., 2001; Hsiao et al., 2001). Therefore, transgenic fish present an excellent system not only for analyzing the promoters of fish genes in detail but also for screening the *cis*-regulatory elements.

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