行政院國家科學委員會補助專題研究計畫成果報告

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※ 魚類視覺研究：控制視網膜專一性表現的基因特性※
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計畫編號：NSC 89－2313－B－002－169－
執行期間：89 年 8 月 1 日至 90 年 7 月 31 日

計畫主持人：蔡懷楨

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執行單位：國立臺灣大學漁業科學研究所

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行政院國家科學委員會專題研究計畫成果報告

魚類視覺研究：控制視網膜專一性表現的基因特性 (2/2)

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一、中文摘要

目前對於控制魚類視紫質基因（*Rh*）表現的核酸序列所知極少。在此針對鯉魚（*Cyprinus carpio*）*Rh*（*cRh*）的啟動子在 *in vivo* 環境下進行分析。利用基因轉殖稻田魚來檢視不同長度鯉魚視紫質基因上游調控序列驅動報導基因（水母綠螢光蛋白基因）的能力，顯示出幾個具有功能區域。–1261~163 bp (–1261/–163) 以及 –163/–138 區域對於增強 *cRh* 在視網膜專一表現皆有貢獻。其中–163/–138 區域內具有一個類似 E_{opsin}-1/Ret2 element 的片段。位於 *cRh* 啟動子近端的 cNRE (–75/–63) 以及 CSE (–52/–46) 片段便足以驅動報導基因在視網膜表現. 此外，位於 cNRE 內的–73/–68 片段對於 *cRh* 最短專一性啟動子的完整性亦很重要。

關鍵詞：鯉魚、感光細胞、調控序列、基因轉殖魚、視紫質

Abstract

Little is known about the *cis*-acting elements controlling transcriptional activities of fish rhodopsin gene. Promoter of carp (*Cyprinus carpio*) rhodopsin gene (*cRh*) was characterized *in vivo*. Transgenic medaka carrying different lengths of *cRh* upstream fragments fused with enhanced green fluorescence protein (EGFP) 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 –163 /–138 contained the putative E_{opsin}-1/Ret2 element. 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 proven to be essential for the integrity of the minimal *cis*-regulatory elements.

Keywords: Carp, Photoreceptor, Regulatory elements, Transgenic fish, Rhodopsin

二、緣由與目的

Comparing to the intensive knowledge about the transcriptional regulation of rhodopsin gene (*Rh*) in mammals, extremely little is known in fish. To address this issue, the upstream sequences of two types of carp *Rh* (*cRh*)[1] were compared with other known species, and some putative cis-acting elements were proposed[2,3]. Recently, the zebrafish *Rh* was cloned, and the transgenic zebrafish carrying 1.2 kb promoter fused with enhanced green fluorescence protein (EGFP) showed rod-specificity[4]. However, the functional cis-elements within the proximal region of fish *Rh* still remain unclear. In this study, we fully dissect the upstream segment to characterize the putative *cis*-elements within *cRh in vivo*.

三、結果

Expression Patterns of Transgene Driven by Various Lengths of Rhodopsin Promoter

The EGFP expression patterns of medaka embryos injected with pDel-6k was exclusively in eyes (Fig. 1C). The expression pattern of embryos injected with pDel-138

and pDel-76 was also specific in eyes, though ectopic expression was occasionally observed. However, expression patterns and rates of transgene were with somewhat different degrees among embryos injected with different lengths of promoter-EGFP constructs. For embryos injected with promoter longer than pDel-163, the signals in eye were usually broad and strong. In contrast, the signals of embryos injected with promoter shorter than pDel-163 (pDel-138 and pDel-76) always appeared spotty and faint. The retinal expression rates of embryos injected with promoter longer than pDel-163 were apparently higher than those of embryos injected with shorter upstream fragments (Fig. 2). EGFP signals could not be detected among embryos injected with pDel-58.

Expression Patterns of Transgene Driven by Mutated Rhodopsin Promoter

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 (Fig. 2, 3). 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 (Fig. 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 at body and/or yolk sac (Fig. 1D). In addition, EGFP could also be detected in retinae among embryos injected with pCMVm at expression rate about 7.3% (Fig. 3), but the signals were usually faint and spotty. Expression patterns and the retinal expression rate of embryos injected with pCMV-CSE, which the CSE was just upstream to the TATA box of CMV promoter, were similar to embryos injected with pCMVm (Fig.3).

四、討論

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 –58/+94 (pDel-58) could not. Thus, neither the cNRE alone (pNCSE-76) nor the CSE alone (pDel-58) could drive retina-specific expression, indicating that cNRE and CSE should function together to drive EGFP expression to the detectable level. CSE is a functional homologue to the mammalian Ret4 element[3]; proteins bound at bovine NRE and Ret4 were reported to work synergistically in cotransfection experiment [5]. Thus, it is possible that proteins bound at cNRE and CSE might also work synergistically in carp retinae. 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 to its TATA box (pCMV-CSE) was similar to that of embryos injected with CMV promoter (pCMVm), suggesting that interaction between cNRE- and CSE-binding proteins might be 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. Thus, the –163/–138 region provided part of the enhancer activity. In addition, the E_{opsin}-1 element was found within the –163/–138 region suggesting that this element probably contributed to enhance the transcriptional activity both in mammalian and in fish system. Difference of retinal expression rates between embryos injected with pDel-1.2k and pDel-163 was about 40%, therefore, the –1261/–163 region also contributed to part of the enhancer activity. Surprisingly, the retinal expression rate of embryos injected with pDel-597 dropped, compared to that of embryos injected pDel-542. Whether the –597/–542 region of *cRh* contains a repressor remains to be further studied.

Recently, zebrafish *Rh* was cloned and the 1.2 kb upstream region was injected into zebrafish embryos[4]. Results showed that EGFP expression rate was 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 proximal region of *Rh* between zebrafish and carp are similar. Thus, we believe that detection of EGFP expressed in fish eyes might be more accessible using medaka system than using zebrafish system. Owing to the mosaic expression of the transgene, it should be more convincible that one examines 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. In addition, observations of transgene expression patterns in retinae of non-hatching out medaka embryos (8 dpf) are obviously much easier and more accurate than those of hatching out, free-swimming embryos (3 dpf).

The *Xenopus* system provides a powerful complementary approach for the analysis of cell-specific promoters[6]. However, the mechanism involved in the transcriptional activation of *Xenopus Rh* seems somehow different from those of mammal and fish systems[3,7]. Moreover, the Ret4 element, which is bound by Crx transcription factor in mammal[5] and in carp retinal nuclear extracts[3], was not protected clearly in footprint experiment using *Xenopus* retinal nuclear extract and recombinant mammalian Crx[6]. Accordingly, the mechanisms regulating *Rh* transcription in carp and mammal are more similar than those of *Xenopus* and mammal, although the upstream sequences of carp and mammal *Rh* are much less similar than the upstream sequences of carp and *Xenopus Rh*[2,7].

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Figure 1. EGFP expression in transgenic medaka embryos. (A) Bright field; (B) to (D) fluorescent light. (A) and (B), untreated control embryos. The yellow and light green colors resulted from endogenous fluorescence. (C), pDel-6k-injected embryos. EGFP was expressed specifically and exclusively in the eyes. (D), pCMVm-injected embryos. 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. The retinal expression rates of transgenic embryos. (A), Schematic illustrations of *cRh* promoter-EGFP fusion constructs. Solid boxes indicate EGFP cDNA; empty boxes indicate the upstream region of *cRh*. (B), The 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 survival injected-embryos that exhibited EGFP expression in eyes. The number of embryos examined is indicated above the bar.

Figure 3. Retina-specific expression rates of

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

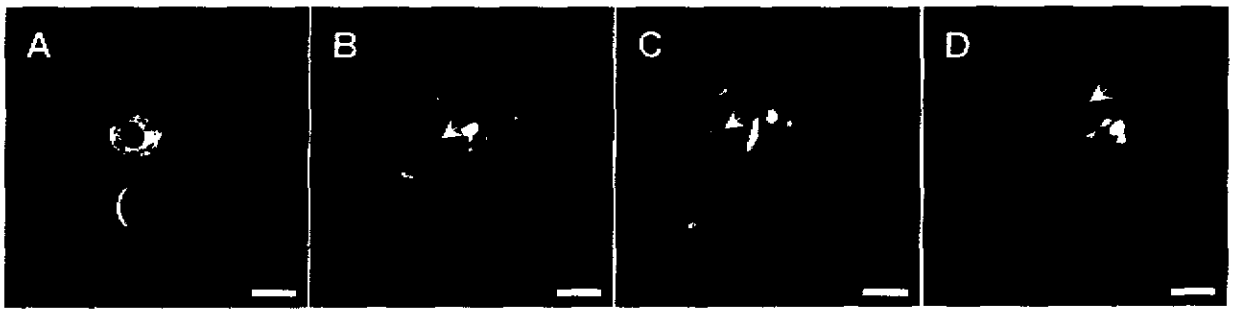

Figure 1

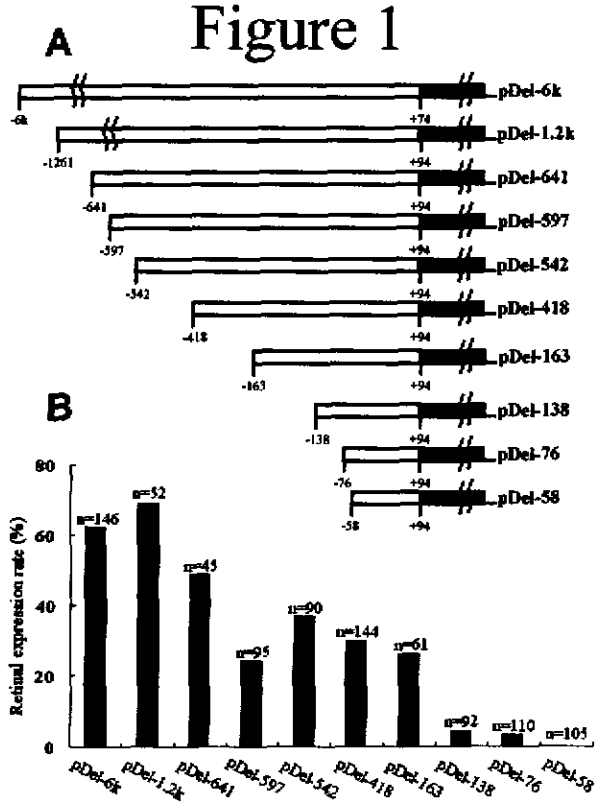

Figure 2

Constructs	Retinal Expression rate (%)	Embryos examined
pFD-76	39.7	58
pSpM2	0	115
pSpM3	0	96
pNCSE-76	0	100
pCMVm	7.3	41
pCMV-CSE	3.8	53

Figure 3