

# Cloning of rainbow trout (*Oncorhynchus mykiss*) histone H3 promoter and the activity analysis in rare minnow (*Gobiocypris rarus*)<sup>\*</sup>

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**Abstract** Rainbow trout histone H3 (RH3) promoter was cloned via high fidelity PCR. The cloned RH3 promoter was inserted into a promoter-lacked vector pEGFP-1, resulting in an expression vector pRH3EGFP-1. The linearized pRH3EGFP-1 was microinjected into fertilized eggs of rare minnows and the sequential embryogenetic processes were monitored under a fluorescent microscope. Strong green fluorescence was ubiquitously observed at as early as the gastrula stage and then in various tissues at the fry stage. The results indicate that RH3 promoter, as a piscine promoter, could serve in producing transgenic *Cyprinoid* such as rare minnow. Promoter activity of RH3, CMV and common carp  $\beta$ -actin (CA) were compared in rare minnow by the expression of respective recombinant EGFP vectors. The expression of pCMVEGFP occurred earlier than the following one, pRH3EGFP-1, and then pCAEGFP during the embryogenesis of the transgenics. Their expression activities demonstrated that the CMV promoter is the strongest one, followed by the CA and then the RH3.

**Keywords:** enhanced green fluorescent protein (EGFP), histone H3 promoter, transgenic fish, rare minnow.

Transgenic technology is expected to be used to generate fast-growing<sup>[1]</sup>, disease-resistant<sup>[2]</sup> and cold-resistant<sup>[3]</sup> transgenic fish for aquaculture. With the development of aquaculture, the technology will show greater potential to improve both the quantity and quality of aquatic products. In addition, compared with mammals, fish has many favorable characteristics for study of gene regulation and expression by transgenesis<sup>[4]</sup>. A transgene construct typically contains a promoter, a structural gene and transcription-termination elements. As the promoter directs the efficiency and the tissue-specific expression of foreign gene, it is necessary to seek for strong promoters for construction of recombinant vectors. However, in the early study of transgenic fish, the promoters used in transgenic vectors were commonly derived from human, bovine and rat<sup>[5,6]</sup> due to lack of piscine promoters. In 1992, Zhu put forward the concept of "all-fish" gene transgenic fish<sup>[7]</sup>, and his group obtained the "all-fish" gene transferred carp<sup>[8]</sup>. To date, the fish promoters used mainly include  $\beta$ -actin promoter<sup>[9]</sup> and anti-freeze protein promoter<sup>[10,11]</sup>.

In eukaryotes, histones, a set of basic structural proteins in chromosome, are responsible for the pack-

aging of DNA into chromatin<sup>[12]</sup>. Histone H3 and H4 are the most evolutionarily conserved ones among all the known proteins. Histone genes are expressed in all actively dividing cells<sup>[13]</sup>. It has been proved that Atlantic salmon histone H3 promoter can direct the expression of  $\beta$ -galactosidase gene in rainbow trout embryos and fry<sup>[14]</sup>. The sockeye salmon histone H3 promoter could show weak activity in human HeLa and GM637 fibroblast cells<sup>[15]</sup>. In this study, rare minnow was used as a model to study the activity of rainbow trout histone H3 promoter in *Cyprinoid*, as revealed by the expression of EGFP<sup>[16]</sup>.

## 1 Materials and methods

### 1.1 Isolation of rainbow trout DNA

Rainbow trout was from Zhengzhou Aquatic Market, Henan Province. The rainbow trout liver DNA was extracted by phenol/chloroform extraction and purified by ethanol precipitation<sup>[1]</sup>.

### 1.2 Cloning of the rainbow trout histone H3 promoter via PCR

Primers RH1 and RH2 were designed according

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to the previously described primer sequences<sup>[15]</sup> for cloning of salmon histone H3 promoter. The sequence of RH1 is 5'-ATGCGAATTCGAGAAGGCCGTCAAAGCCAA-3' and that of RH2 is 5'-ATGCGGATCCGGCGCTAGCTAGCTTCCTTC-3', in which the underlined bases indicate the additional restriction sites (*Eco*RI and *Bam*HI). Primer RH2 is located at 20 nucleotides upstream of ATG start codon. The PCR reaction contained 20~30 ng DNA, 0.2 mmol/L dNTP, 1 × Pyrobest buffer, 1U Pyrobest DNA polymerase (Takara), 0.5 μmol/L each of the primers. The reaction profile included a pre-denature at 94 °C for 2 min, and 30 cycles of 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min. The PCR products were separated on a 0.8% agarose gel by electrophoresis and visualized by a UVP system, then the target product was subcloned and sequenced.

### 1.3 Construction of the recombinant plasmid pRH3EGFP-1

The PCR product and the plasmid pEGFP-1 were digested with *Eco*RI and *Bam*HI and separated on a 0.8% agarose gel by electrophoresis. The digested DNA fragments were purified using a glassmilk kit (MBI), then ligated to digested pEGFP-1 with T4 DNA ligase, and transformed into competent cells of *E. Coli* strain Top 10F'. The positive clones were selected on the kanamycin plate. The plasmid pRH3EGFP-1 was extracted from *E. Coli* Top 10F', and identified with *Eco*RI and *Bam*HI digestion (Fig. 1).

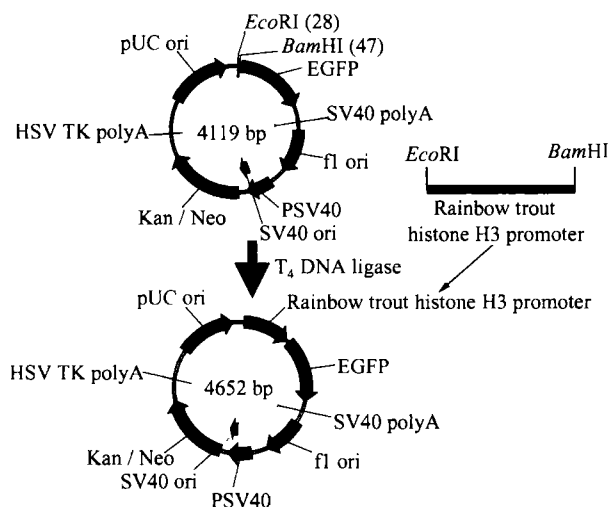


Fig. 1. Construction of the recombinant vector pRH3EGFP-1 including rainbow trout histone H3 promoter and EGFP cDNA.

### 1.4 Gene transfer

The recombinant plasmid pRH3EGFP-1 was linearized with *Eco*RI and microinjected into the fertilized eggs of rare minnow at concentrations of 50 ng/μL and 100 ng/μL. In the meantime, the linearized vectors, pCMVEGFP which contains CMV promoter and pCAEGFP which contains carp β-actin promoter, were injected into rare minnow fertilized eggs at the concentration of 100 ng/μL.

### 1.5 Detection of the EGFP expression

The expression of EGFP in transgenic fish was directly observed under a 480 nm fluorescent microscope. The activities of rainbow trout histone H3 promoter, CMV promoter and carp β-actin promoter were compared by the different brightness of green fluorescence.

## 2 Results and analysis

### 2.1 Cloning of the rainbow trout histone H3 promoter via PCR

A PCR product of about 500 bp was generated from rainbow trout genomic DNA (Fig. 2). The DNA sequence of the amplified product was aligned to the previously reported sequences of rainbow trout histone H3 promoter and salmon histone H3 promoter using the software Clustal 1.8. The cloned sequences in the present study showed 19 different nucleotides with the former and an 80.4% similarity with the latter (Fig. 3).

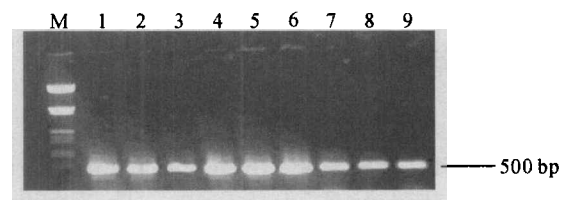


Fig. 2. The result of PCR. M, DNA marker (λDNA/*Eco*RI + *Hind*III); 1~9, Rainbow trout histone H3 promoter.

### 2.2 The activity of rainbow trout histone H3 promoter in rare minnow

The expression of EGFP started at gastrula stage. The green fluorescence was dispersed in the embryo cells (Plate I (a)). When fish embryo developed to the neurula stage, the strong green fluorescence dots were ubiquitously distributed in embryo (Plate IA (b)).

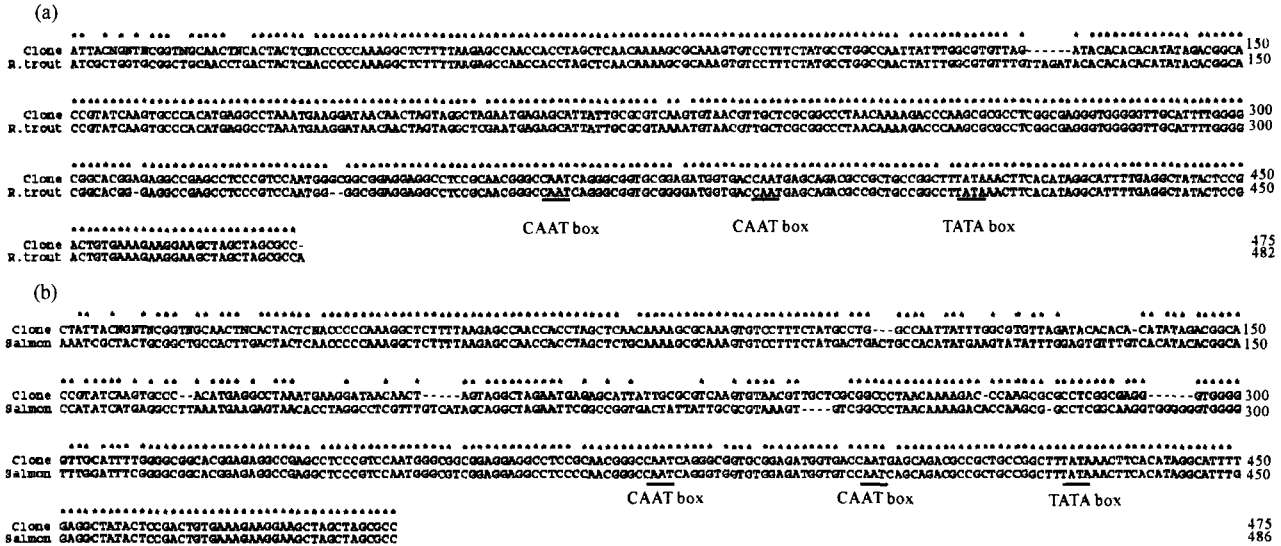


Fig. 3. Homology of the sequences. (a) Cloned rainbow trout histone H3 promoter (upper) and the sequence of the reported rainbow trout histone H3 promoter (GenBank AF416499); (b) cloned rainbow trout histone H3 promoter (upper) and the sequence of the reported Atlantic salmon histone H3 promoter (GenBank AF017147).

When the concentration of foreign gene was 50 ng/ $\mu$ L, the transgenic ratio could reach 70 %, and the expression of EGFP could be detected in 20 % of the transferred fry. When the concentration of foreign gene was 100 ng/ $\mu$ L, the transgenic positive ratio was the same but the EGFP expression ratio was 56.7 %. At fry stage, the intensive green fluorescence was detected only in a few organs, such as in brain, eye, muscle, abdomen middle plate (Plate IB). This indicates that the cloned rainbow trout histone H3 promoter has the activity in various cell types of *Cyprinoid*.

2.3 Comparison of the promoter activity among CMV, carp  $\beta$ -actin, rainbow trout histone H3 promoters

The three vectors, pRH3EGFP-1, pCMVEGFP and pCAEGFP, were respectively injected into fertilized eggs at the same concentration. The fertilized eggs developed under 14 ~ 18  $^{\circ}$ C incubation and they were observed under the fluorescent microscope (Table 1). At late-blastula stage, the fluorescent signal could be detected in one out of 80 embryos injected with pCMVEGFP construct, while no expression of EGFP was observed in the embryos injected with pRH3EGFP-1 and pCAEGFP. In eggs injected with pRH3EGFP-1, the EGFP gene was expressed at early-gastrula stage and gradually became intensive along with the development of the embryo. The strongest expression of EGFP appeared at muscular reaction

stage, and kept at a constant level at following stages. In eggs injected with pCAEGFP, the green fluorescence was also detected at early-gastrula stage, but the level of expression was very low when compared with the other two promoters. However, at the blocking blastopore stage, the expression and fluorescent intensity of pCAEGFP in eggs were higher than those directed by rainbow trout histone H3 promoter (Plate IC). Analysing the fluorescent intensity by a real time video system, it was found that the fluorescent intensity directed by rainbow trout histone H3 promoter was only one fourth of that directed by carp  $\beta$ -actin promoter.

Table 1. Expression of pRH3EGFP-1, pCMVEGFP and pCAEGFP in transgenic rare minnow

Recombinant	No. of expression <sup>a)</sup>				
	Late-blastula stage (11 h)	Early-gastrula stage (14 h)	Blocking blastopore stage (23 h)	Muscular reaction stage (48 h)	Fry stage (110 h)
pRH3EGFP-1	0/55	10/36	9/28	15/28	17/30
PCMVEGFP	1/80	20/60	50/52	50/53	40/43
PCAEGFP	0/74	5/40	24/36	28/35	21/28

a) positive/tested

3 Discussion

In rainbow trout, the histone genes are arranged in the order H4-H2B-H1-H2A-H3<sup>[17]</sup>. The cloned 529 bp DNA region contains the histone H3 promoter and the last 20 nucleotides of the histone H2A coding region. The resulting sequence was aligned to the re-

ported rainbow trout histone H3 promoter sequence, which exhibited 19 different nucleotides between them. However, because the samples were received from different places, we could not explain what caused this difference now. The homology comparison showed that the cloned histone H3 promoter was 80.4% homologous to Atlantic salmon histone H3 promoter, suggesting that the nucleotide sequence of histone H3 promoter is quite conserved.

About 300 transgenic rare minnow were obtained by microinjection of the foreign genes. When using an increased concentration of foreign gene (100 ng/ $\mu$ L) for microinjection, the expression percentage of the transgenic rare minnow increased a lot but more abnormal fish fry was produced.

Though all of the rainbow trout histone H3 promoter, CMV promoter and carp  $\beta$ -actin promoter could direct the EGFP gene to express in rare minnow embryo, their expression time and intensity were quite different. Along with the embryo development, all the promoters began to show activities at late-blastula stage. At early-gastrula stage, weak fluorescence appeared in all transgenic embryos. After gastrula stage, the expression of EGFP gene gradually increased and the green fluorescence gradually enhanced. The strong green fluorescence was ubiquitous at blocking blastopore stage. At this time, the percentage (66.7%) of foreign gene expression directed by carp  $\beta$ -actin promoter was higher than the percentage (32.1%) of rainbow trout histone H3 promoter. It was found that the fluorescent intensity directed by rainbow trout histone H3 promoter was only one fourth of that directed by carp  $\beta$ -actin promoter. The results demonstrated that the activity of carp  $\beta$ -actin promoter is stronger than rainbow trout histone H3 promoter after the gastrula stage. As a viral promoter, the CMV promoter was almost not influenced by rare minnow genome, so it showed its activity earliest among the three promoters and 33.3% of the embryos transferred with CMV promoter exhibited the EGFP expression at early-gastrula stage. Rainbow trout histone H3 promoter started to show its activity from gastrula stage. The percentage of EGFP gene expression directed by carp  $\beta$ -actin promoter reached the peak at muscular reaction stage. In conclusion, the expression of pCMVEGFP was earlier than pRH3EGFP-1 and pCAEGFP, CMV promoter was the most active one among the three promoters, and rainbow trout histone H3 promoter was the least active one.

Zhao et al.<sup>[18]</sup> reported that CMV promoter and carp  $\beta$ -actin promoter directed the expression of EGFP gene at late-gastrula stage. They also proved that the integration of foreign gene took place at blastula stage. The present study showed that the expression of pCMVEGFP and pCAEGFP was earlier in rare minnow since the green fluorescence could be detected at early-gastrula stage. Nevertheless, the translation regulation of early embryos depends on the molecular factors coming from the maternal partner, so that the transcription and translation of zygote itself do not occur until the beginning of zygotic genome activation. From late-blastula to gastrula stage, the embryonic cells begin to get differentiation and the factors for transcription and translation are gradually produced, thus it is the time for the expression of foreign gene to start and gradually enhance.

Since the expression of EGFP gene can be directly observed under the fluorescent microscope, it is a simple and direct method to use the EGFP gene as a reporter for analysing the foreign gene expression. When the pRH3EGFP-1 transgenic embryos developed to fry, the expression patterns of foreign EGFP gene appeared fairly diverse among fry. Some fry did not express EGFP gene at all, some fry expressed EGFP gene in some tissues, and some fry expressed EGFP gene in all tissues. This revealed that the cloned rainbow trout histone H3 promoter contained the necessary regulation elements to direct EGFP to express in various tissues of rare minnow. Therefore, the rainbow trout H3 promoter could be used in the transgenic research of *Cyprinoid* (such as rare minnow) as an active promoter.

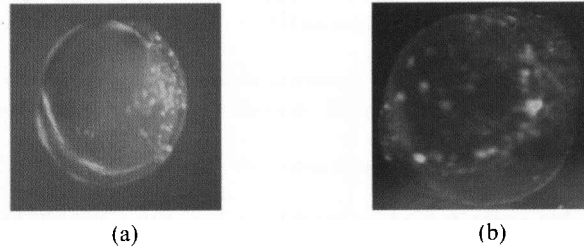
## References

- 1 Zhu, Z. et al. Novel gene transfer into the fertilized eggs of goldfish (*Carassius auratus* L. 1758). *Angew Ichthyol*, 1985, 1: 31.
- 2 Fletcher, G. L. et al. Antifreeze peptides confer freezing resistance to fish. *Can. J. Zool*, 1986, 64: 1897.
- 3 Zhong, J. et al. Introduction of the human lactoferrin gene into grass carp (*Ctenopharyngodon idellus*) to increase resistance against GCH virus. *Aquaculture*, 2002, 214: 93.
- 4 Alestrom, P. et al. Zebrafish, a vertebrate model for transgene expression and biological function. *Anim. Biotechnol.*, 1994, 5: 147.
- 5 Zhu, Z. et al. Biological effects of human growth hormone gene microinjected into the fertilized eggs of loach *Misgurnus anguillicaudatus* (Cantor). *Chinese Science Bulletin*, 1986, 31(4): 988.
- 6 Iyengar, A. et al. Regulation and expression of transgenes in fish. *Transgen. Res.*, 1996, 5: 147.
- 7 Zhu, Z. Generation of fast growing transgenic fish: methods and mechanisms. In: *Transgenic Fish*. Singapore: World Scientific Publishing Co. Pte. Ltd, 1992, 106~116.

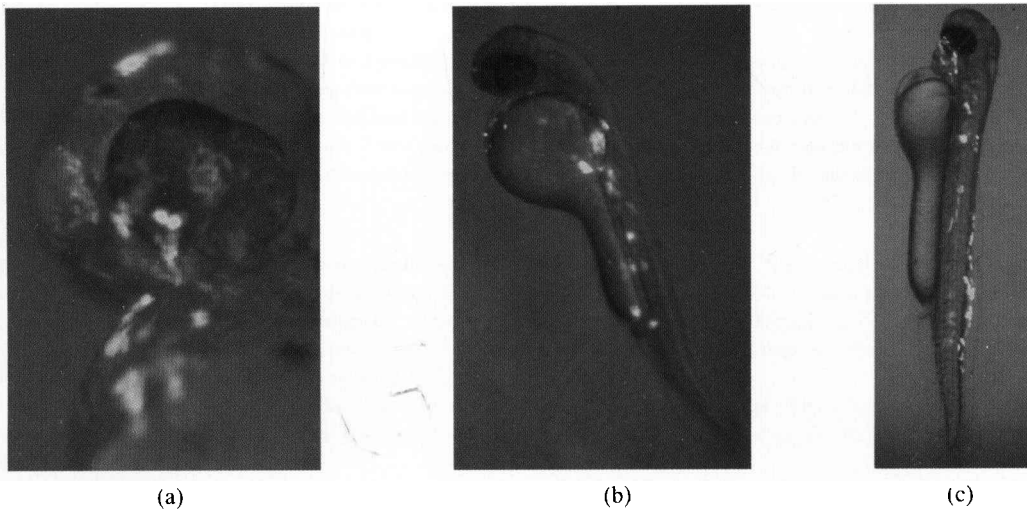
- 8 Wang, Y. et al. "All-fish" growth hormone gene transferred Yellow River carp(*Cyprinus carpio*) and the genetic analysis of its F1 generation. Chinese Science Bulletin, 2001, 46(3): 226.
- 9 Liu, Z. et al. Isolation and characterization of  $\beta$ -actin gene of carp (*Cyprinus carpio*). DNA Sequence, 1990, 1: 125.
- 10 Devlin, R. H. et al. Production of germline transgenic Pacific salmonids with dramatically increased growth performance. Can. J. Fish. Aqua. Sci., 1995, 52: 1376.
- 11 Du, S.J. et al. Growth enhancement in transgenic Atlantic salmon by the use of an all fish chimeric growth hormone gene construct. Bio/Technology, 1992, 10(2): 176.
- 12 Kornberg, R. The structure of chromatin. Annu. Rev. Biochem., 1977, 46: 931.
- 13 Heintz, N. The regulation of histone gene expression during the cell cycle. Biochim. Biophys. Acta., 1991, 1088: 327.
- 14 Hanley, S. et al. Isolation and functional analysis of the histone H<sub>3</sub> promoter from Atlantic salmon(*Salmo salar L.*) Molecular Marine Biology and Biotechnology, 1998, 7(3): 165.
- 15 Chan, W. K. et al. Polymerase chain reaction amplification and functional characterization of sockeye salmon histone H3, metallothionein-B, and protamine promoters. Mol. Mar. Biol. Biotechnol., 1993, 2(5): 308.
- 16 Cormack, B.P. et al. FACS-optimized mutants of the green fluorescent protein (GFP). Gene, 1996, 173(1): 33.
- 17 Connor, W. et al. Organization and nucleotide sequence of rainbow trout histone H2A and H3 genes. J. Mol. Evol., 1984, 20(3~4): 236.
- 18 Zhao, H. et al. Time course of foreign gene integration and expression in transgenic fish embryos. Chinese Science Bulletin, 2000, 45(8): 734.

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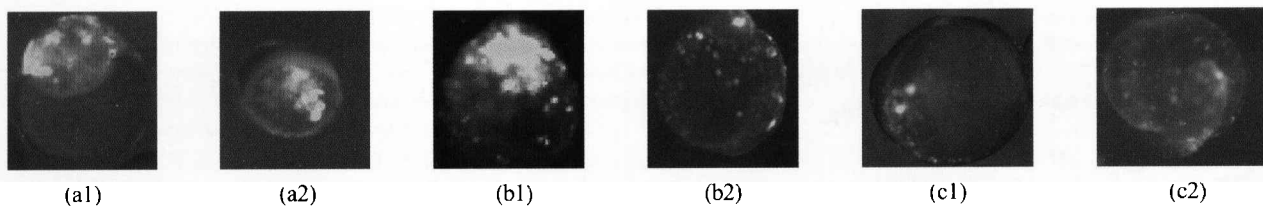
Plate I



A. Observation of the gastrula of the transgenic rare minnow under the fluorescent dissector. (a) EGFP expression in the embryo cells. (b) EGFP expression at the neurula stage.



B. Observation of the fry of transgenic rare minnow. (a) Expression of EGFP in brain, eye, abdomen middle plate of the transgenic rare minnow, (b) Expression of EGFP in muscle of the transgenic rare minnow, (c) Expression of EGFP in dorsal fin of the transgenic rare minnow.



C. Observing the expression of EGFP directed by the three promoters in the transgenic rare minnow under the fluorescent dissector. (a1). Directed by CMV promoter in the late blastula of the transgenic rare minnow, (a2). Directed by CMV promoter in the early gastrula of the transgenic rare minnow, (b1). Directed by carp  $\beta$ -actin promoter in the late gastrula of the transgenic rare minnow, (b2). Directed by carp  $\beta$ -actin promoter in the blocking blastopore of the transgenic rare minnow, (c1). Directed by rainbow trout histone H3 promoter in the middle gastrula of the transgenic rare minnow, (c2). Directed by rainbow trout histone H3 promoter in the blocking blastopore of the transgenic rare minnow.