

Zebrafish HSC70 promoter to express carp muscle-specific creatine kinase for acclimation under cold condition

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Received: 2 August 2010 / Accepted: 18 January 2011 / Published online: 6 February 2011
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Abstract Zebrafish (*Danio rerio*) is used as a model system for in vivo studies. To expand the research scope of physical, biochemical and physiological studies, a cold-tolerant model of zebrafish was developed. The common carp (*Cyprinus carpio*) muscle form of creatine kinase (CK, EC 2.7.3.2) can maintain enzymatic activity at a temperature of around 15°C. However, a cold-inducible promoter of zebrafish, hsc 70 (heat shock protein 70 cognate), is able to increase the expression of gene product by 9.8 fold at a temperature of 16°C. Therefore, the carp CK gene was promoted by hsc 70 and transfected into zebrafish embryos. Resulting transgenic zebrafish survived and

could maintain its swimming behavior at 13°C, which was not possible with the wild-type zebrafish. The swimming distance of the transgenic fish was 42% greater than that of the wild type at 13°C. This new transgenic fish model is ideal for studies of ectothermal vertebrates in low-temperature environments.

Keywords Creatine kinase · Heat shock cognate · Hsc 70 · Cold · Inducible promoter · Zebrafish

Abbreviations

CK	Creatine kinase
hsc70	Heat shock protein 70 cognate
hsp	Heat shock protein
M-CK	Muscle form creatine kinase
M1- (M2-, M3-)CK	Carp muscle form creatine kinase subisoform 1 (2, 3)
CKM1 (2, 3)	Coding sequence of carp muscle form creatine kinase subisoform 1 (2, 3)
RT-PCR	Reverse transcription polymerase chain reaction
QRT-PCR	Quantitative reverse transcription polymerase chain reaction
EF-1 α	Elongation factor 1 alpha
CMV	Cytomegalovirus
CIP	Cold inducible promoter
EGFP	Enhanced green fluorescence protein
Hpf	Hour post-fertilization

Electronic supplementary material The online version of this article (doi:10.1007/s11248-011-9488-8) contains supplementary material, which is available to authorized users.

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Introduction

Every year, cold fronts sweeping across the Taiwan Strait leave a trail of damaged fish in their wake. Fish species affected include tilapia (*Oreochromis mossambicus*), milkfish (*Chanos chanos*), grouper (*Epinephelus coioides*) and cobia (*Rachycentron canadum*). Indeed, economic loss to the Taiwan economy is devastating—up to ten million US dollars annually. In the southern United States and Israel, low water temperature has been found to inhibit growth of aquaculture tropical fish and increase their mortality (Snodgrass 1991).

While considerable research has been done on the physiological, biochemical and molecular responses of various fish species to low temperature, studies on the effects of low temperature have mainly focused on one of the three following issues (Hazel 1993): metabolic compensation (Guderley 2004), homeoviscous adaptation of biological membranes (Hazel 1984), and thermal hysteresis (Kristiansen and Zachariassen 2005). Recently, a systematic analysis of cold-acclimated fish has been conducted (Gracey et al. 2004; Ju et al. 2002). Unfortunately, due to unsatisfactory genomic resources, this type of fish is probably not the appropriate model to explore the responses to and mechanisms of cold tolerance.

Potential candidates selected for studying gene response to cold stress have been mostly shock proteins, (Carmona et al. 1998; Ali et al. 2003) cell cycle regulators (Imamura et al. 2003) and transcription factors (Knight et al. 2004). In particular, heat shock protein (*hsp*) is known to be synthesized under environmental stressors such as a sudden temperature change. However, it is not clear whether promoter function and response elements of the *hsp* promoter can be induced by low temperature. *Hsc70* (*hsp70* cognate protein) is one of the *hsp70* family proteins with a highly conservative domain and similar functions (Freeman and Morimoto 1996). Both *hsc70* and *hsp70* have been reported to be induced in cold shock in different species (Neven et al. 1992; Anderson et al. 1994; Rinehart et al. 2000; Yocum 2001; Ali et al. 2003; Leandro et al. 2004). Expression of heat shock proteins is one of the adaptive mechanisms responsible for low-temperature tolerance (Storey 1997). This suggests that *hsc70* may be a potential candidate to study cold-inducible gene expression.

At low temperature, physiological properties have to be enhanced for acclimation. Muscle twitch (Bernal

et al. 2005) and muscle contraction (Johnston et al. 1985) change in an acclimation-temperature-dependent manner. Ion channel (Krumshabel et al. 1997) and proton pump (Itoi et al. 2003) are also mentioned as a regulation target at low temperature. In order to offer high demand of energy for these physiological responses, muscle forms of creatine kinase (CK, EC 2.7.3.2), which is one of the enzymes keeping cellular energy homeostasis and maintaining high ATP/ADP and ATP/AMP ratios in vertebrate (Wallimann et al. 1992), have been studied. When demanding a higher energy supplement, a more efficient creatine kinase to catalyze phosphocreatine and ADP to creatine and ATP is needed. In a previous study on the common carp, the M3-CK sub-isoform of muscle creatine kinase was found to shift its activity at temperatures at 20–15°C at the common carp intracellular pHs (Sun et al. 1998; Wu et al. 2008). As expected, M3-CK dimerizes at lower temperature. Its dimerization region is located at the N-terminal, and a specific residue, lysine 3, changes the side chain orientation to enhance the electronic force of inter-molecular binding (Sun et al. 2002).

Zebrafish is popular in biological research, as this fish is easy to breed and maintain. However, zebrafish, as a stenothermal teleost, cannot survive water temperatures below 12°C or feed when temperature is below 16°C (Chou et al. 2008). The purpose of this study was to develop a transgenic zebrafish model with cold tolerance that could be used to study the biochemical and physiological responses of stenothermal teleosts to cold temperature. To trigger the expression of carp M-CKs at lower temperature, a suitable promoter is needed. In this study, the carp M3-CK isoenzyme was used to maintain the normal physiological function of M-CKs that would enhance cold tolerance in vivo. *Hsc70* promoter was integrated in the expression control of M3-CK. The physiological role of the *hsc70* promoter triggered carp M3-CK in transgenic zebrafish was evaluated by assessing their swimming performance, balance and orientation at low temperature.

Materials and methods

Maintenance of zebrafish and temperature shock treatment

Adult zebrafish A/B strain were obtained from Oregon State University and maintained in our own

fish facility with a controlled light cycle of 14 h light/10 h dark at 28°C. They spawned soon after the onset of light and fertilized eggs were collected at the single-cell stage according to standard methods (Westerfield 1995).

Three to five zebrafish that had undergone cold-shock treatment were put in a 500 ml glass container filled with 400 ml of freshwater at 28°C. After one to 2 h, the glass container was transferred to a tank with a temperature controller at different temperature. The temperature inside the glass container was then measured. The temperature controller was kept within a $\pm 0.5^\circ\text{C}$ range in each temperature. The 0 h cold-shock fish samples were collected after 30 min while the water temperature was kept equilibrated. Whole fish samples were frozen immediately and stored in liquid nitrogen for further analysis.

Expression analysis of native zebrafish *hsc70*

In order to detect *hsc70* expression of wild-type zebrafish during cold-shock treatment, RT-PCR and QRT-PCR were performed using whole fish total RNA extracted by RNeasy (Qiagen, Crawley, UK) from various treatment samples. β -actin was used as an internal control in which the primer pair was 5'-catcagcattgctctgctgtatgg-3' and 5'-gacttgctcagtgtacagagacacct-3'.

The RT-PCR program was one cycle of 55°C for 20 min and 94°C for 2 min, followed by PCR amplification with 35 cycles of 94°C for 15 s, 55°C for 45 s and 68°C for 1 min. Finally, the product was extended to one cycle of 72°C for 7 min (Thermo Electron Corporation PCR sprint Thermal Cycler). The primer pair was 5'-ctacgtcgtttcacggacaccga-3' and 5'-tttccttgcctccagggtaccg-3' which produce a 1.7 kb fragment. The RT-PCR products were applied onto 1% agarose TBE gel (Lonza, USA) for electrophoresis. BioRad ChemiDoc XRS was used for image analysis, the software Quantity One of BioRad was used for product density analysis. Results have been normalized by the β -actin.

A QRT-PCR analysis of the *hsc70* expression profile was performed. Reverse transcription was reacted within 5 μg total RNA, 1 μl random primer adjust RNAase-free water to 11 μl and heated at 70°C for 5 min. 4 μl 5X RT buffer, 2 μl dNTP, 1 μl MMLV RT enzyme, 2 μl RNAase-free water were added, mixed well and heated at 42°C for 90 min. Finally, the sample was heated at 70°C for 10 min. Using 0.5 μl

cDNA, 1 μl primer pairs (5'-ctacgtcgtttcacggacaccga-3' and 5'-tttccttgcctccagggtaccg-3' for *hsc70*; 5'-tgccttgcctcccaattcag-3' and 5'-taccctccttgcgctcaatg-3' for EF-1 α) 10 μl 2X SYBR Green master mix and 8.5 μl RNAase-free water were added. The temperature cycles were one cycle of 50°C for 2 min and 95°C for 10 min, followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. The normalized relative expression level of Q-PCR results was calculated by the $\Delta\Delta\text{Ct}$ method as described previously (Kvamme et al. 2004). In the QRT-PCR experiment, all chemicals were purchased from Roche and the thermal cycler was a Roche Lightcycler 480.

Hsc 70 promoter and plasmid construction

The pEGFP-1 (Clontech, US) was double digested by XhoI and EcoRI and ligated with *hsc70* promoter, which was amplified by PCR with the primers (up) 5'-ctcagtagataactgaggctccgaaaagcggtg-3' (*XhoI* underlined) and (down) 5'-gaattcctctggactcctgtgtgtttatagcgc-3' (*EcoRI* underlined) from zebrafish muscle genomic DNA. The plasmid was sequenced and named pCIP-EGFP (CIP: cold inducible promoter).

To amplify the cytomegalovirus (CMV) promoters, PCR was performed with the primer pairs (up) 5'-ctcagtagataactgatagttataatagtaataacacggggtc-3' (*XhoI* underlined) and (down) 5'-gaattcgcgctagcggatctgacg-3' (*EcoRI* underlined). The CMV promoter and CIP were both digested by XhoI and EcoRI and ligated with pEGFP-C1 (Clontech, US). These two plasmids were named pEGFP-C1-CMV and pEGFP-C1-CIP. The M3-CK gene was amplified by the primers (up) 5'-gtcagaccgcccattgggtatgcttctcgaaacacca-3' (*SalI* underlined) and (down) 5'-ggatccttactctggcagggatcat-3' (*BamHI* underlined). Whereas the M3-CK template was M3-CK-pET28a (Sun et al. 1998), the amplified M3-CK gene was ligated to pEGFP-C1-CMV and pEGFP-C1-CIP. The final plasmids were named pEGFP-C1-CMV-M3CK and pEGFP-C1-CIP-M3CK. The construction maps of the plasmids are shown in Fig. 1.

Transgenesis of target genes into zebrafish

Five vectors, pEGFP-C1, pEGFP-C1-CMV, pEGFP-C1-CIP, pEGFP-C1-CMV-M3CK and pEGFP-C1-CIP-M3CK, for the cold-shock treatment assay were prepared by transfection of *E. coli* DH5 α . After

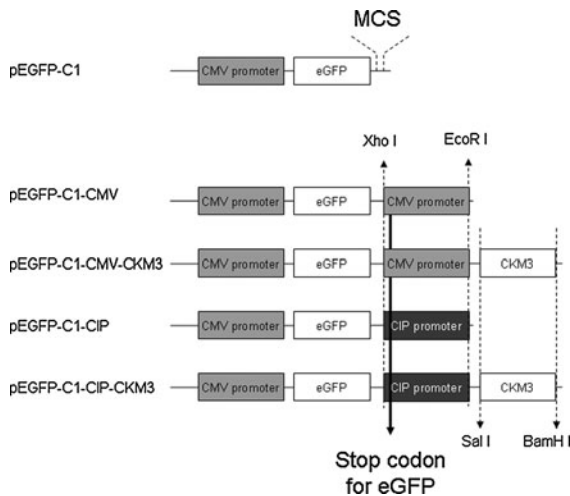


Fig. 1 Construction maps of plasmids. The construction maps of the plasmids as described in “Materials and methods”

purification by the QIAgene mini DNA prepared kit (QIAGEN), the vectors were digested with EcoRI and *SalI*. A control plasmid pEGFP (Clontech) containing the BGH poly-A tail drove the CMV promoter. DNA solution containing 100 ng/μl plasmid in 250 μM KCl and 0.1% phenol red was used for injection. Approximately 200 pl of DNA solution was injected into one- or two-cell zebrafish embryos with a glass micropipette. At 96 hpf (hour post-fertilization), fish were examined using fluorescence microscopy and GFP-expressing fish were saved. The founder fish was selected and crossed to create the stable line of transgenic fish. The fish were cultured to 2 month for further assays.

Quantification of endogenous and transfected genes

The muscle tissues of zebrafish maintained at 13°C for 5 h were isolated for RNA extraction (PureLink Micro-to-Midi Total RNA Purification System) and RT-PCR (SuperScript™ III One-Step RT-PCR with Platinum® Taq) was performed. The following RT-PCR primer pair were designed for the detection of the foreign carp M3-CK gene: 5′-aagctgaagtctctctgaggaggagtac-3′ and 5′-ttactctgggcagggatcatgcatc gat-3′. The following primer pairs were used for the detection of the wild-type zebrafish CK genes: 5′-ccactggattcactgtggatgatgcatc-3′ and 5′-ttactctggg-cagggatcatgctctc gat-3′; 5′-aagatgaagttgctgtggatgagg

agttt-3′ and 5′-ttactctgggcagggatcatgcatc gat-3′. The zebrafish β-actin primers were used as an internal control. The RT-PCR program was one cycle of 55°C for 20 min and 94°C for 2 min, followed by PCR amplification with 35 cycles of 94°C for 15 s, 55°C for 45 s and 68°C for 1 min. PCR products were applied onto 1% Agarose TBE gel for electrophoresis. Muscle proteins from zebrafish maintained at 13°C for 5 h were separated by 12.5% polyacrylamide gel (Amesco) electrophoresis. Separated proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 3% non-fat dried milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.6; 137 mM NaCl) for 2 h at room temperature with agitation, followed by 1 h incubation in the presence of the primary antibody (antisera M1-CK: 1:5000 dilution; antisera M3-CK: 1:5000 dilution), as described in a previous study (Sun et al. 2002). After washing in TBST (TBS buffer with 0.1% Tween-20, Sigma), goat anti-rabbit IgG conjugated to alkaline phosphatase (1:5000 dilution, Zymed) was added for 30 min at room temperature, followed by development with AP Development Buffer (Pierce). Chemicals unmarked were purchased from Sigma.

Determination of swimming speeds

Swimming ability was filmed using a system that recorded 30 frames per second. The camera was placed directly above a filming cylindrical tank (diameter: 20 cm, height: 20 cm) filled with water from the aquaria. The water temperature was controlled by external temperature controller to the experimental temperature within ±0.5°C. Swimming distance was measured on the monitor. The relationship between the known length and its monitor length was used to convert monitor speeds into real speeds.

Statistical analyses

Quantitative data were expressed as the mean ± standard deviation. The effects of temperature change on all experimental variables was compared using a one-tailed Student’s t-test ($\alpha = 0.05$ and $n \geq 3$). Swimming speeds in the transgenic and wild-type zebrafish at different temperatures were also compared using a one-way ANOVA. The null hypothesis was rejected whenever the p value was <0.05.

Results

Hsc70 gene was up-regulated in low temperature

To confirm that the zebrafish *hsc70* is a cold-inducible gene, wild-type zebrafish were assayed after exposure to different temperatures for different time periods. The semi-quantitative RT-PCR results indicated that the *hsc70* gene expression level was 9.8-fold higher in the cold shock treated (16°C for 5 h) wild-type. At 24°C and 20°C, the *hsc70* gene slightly increased by two-fold after 2 and 5 h of cold-shock treatment (Fig. 2a). From the results, it is clear that the zebrafish *hsc70* was up-regulated under cold-shock condition.

For further analysis, *hsc70* was induced at 12°C. The QRT-PCR analysis at 12°C, compared to those at 20 and 28°C, showed that the amount of *hsc70*

mRNA was increased by 3.2 folds after 3 h (Fig. 2b). Compared with the semi-quantitative RT-PCR and QRT-PCR at 20°C, the *hsc70* gene was up-regulated to a maximum after 2 to 3 h followed by a decrease. However, at 16 or 12°C, the *hsc70* gene expression was found to continuously increase with time, thus demonstrating that *hsc70* is indeed a cold-induced gene containing a cold-inducible promoter.

Isolation and identification of *hsc70* 5'-flanking sequence

According to the Sanger Institute Zebrafish Database, the *hsc70* gene of zebrafish is located on the 15th chromosome of the genome. There are 9 exons, and the first exon is not translated. A 2.0 kb zebrafish *hsc70* 5'-flanking sequence from zebrafish genomic DNA is 99.89% identical to the Sanger Center Zebrafish Genome EST Database. This promoter region was named CIP.

Construction, microinjection and in vivo expression of the CIP-EGFP, CMV-M3-CK and CIP-M3-CK

In order to assay the promoter activity of CIP at low-temperature, the zebrafish CIP expression vector pCIP-EGFP was used. For in vivo study of the promoter, transgenic zebrafish were produced by microinjection of the pCIP-EGFP expression vectors at the single-cell stage of zebrafish embryos. The injected embryos were pre-selected at 48 h post-fertilization by fluorescence microscopy. The expression profile of pCIP-EGFP transgenic zebrafish treated at 16°C was estimated (Fig. 3a). The mRNA after 5 h of cold shock was found to be two-fold higher than that at 0 h. The pCIP-EGFP transgenic larva fish expressed green fluorescence during 16°C cold-stress for 5 h (Fig. 3b). This result shows that the CIP was functional in vivo.

The carp M3-CK transgene was driven by the CMV promoter or CIP, and the green fluorescence protein gene was driven by CMV promoter in each vector. Then, the vectors were injected into fertilized zebrafish eggs with purified plasmid using a microinjection gene transfer system. At 96 h post-fertilization, newly hatched larvae were examined under the fluorescence microscope. Those larvae that expressed GFP were collected for further

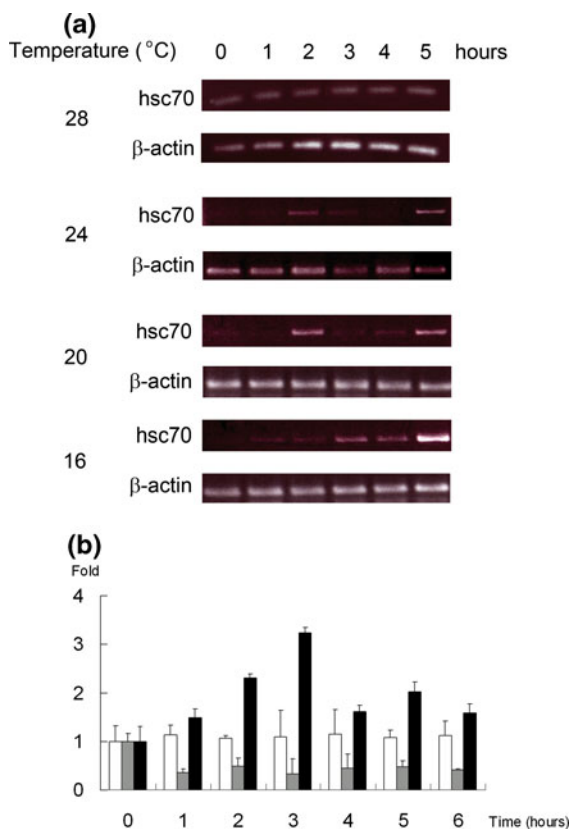


Fig. 2 *Hsc70* gene expression profile over time at different temperatures. **a** The semi-quantitative RT-PCR results of wild-type (Wt) zebrafish at 28, 24, 20 and 16°C for 0–5 h. **b** The QRT-PCR results of Wt zebrafish at 28 (white), 20 (grey) or 12°C (black) for 0 to 6 h

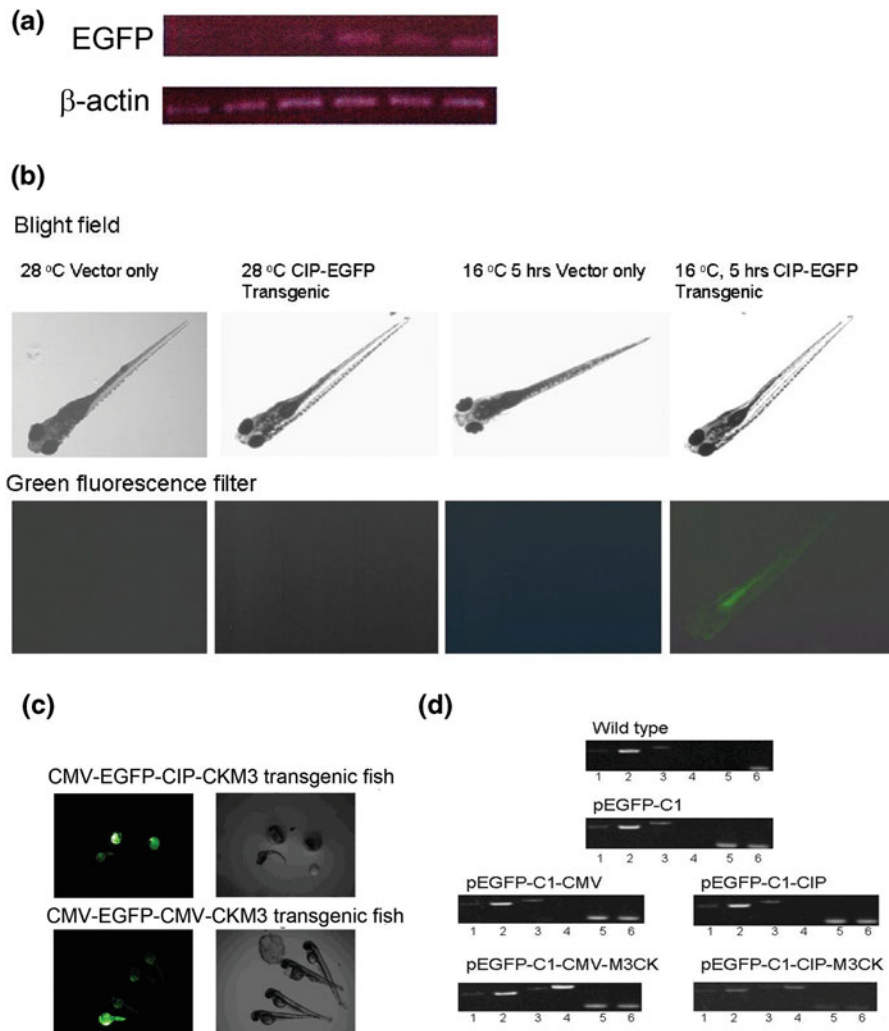


Fig. 3 Cold-shock induced CIP-EGFP fluorescence and CIP-M3-CK and CMV-M3-CK of zebrafish **a** the semi-quantitative RT-PCR of CIP induced EGFP expression. *Upper*: the expression level of EGFP; *Bottom*: the expression level of β -actin. **b** Zebrafish was treated at 28 or 16°C for 5 h. Bright field images are shown in the *upper panel*, and *green* fluorescence images are shown in the *lower panel*. **c** Screening of transgenic

zebrafish embryo by GFP fluorescence. *Green* fluorescence could be detected in transgenic zebrafish. **d** Semi-quantitative analysis of the wild type, transgenic pEGFP-C1-CMV, pEGFP-C1-CIP, pEGFP-C1-CMV-CKM3 and pEGFP-C1-CIP-CKM3 expression patterns at 13°C. *Lane 1*: maker (1 kb); *lane 2*: zebrafish M-CK1; *lane 3*: zebrafish M-CK2; *lane 4*: M3-CK; *lane 5*: EGFP; *lane 6*: β -actin. The bar is 1 mm

characterization (Fig. 3c). When the GFP-positive larvae reach their sexual maturity, they were back-crossed with the wild type in order to establish a stable transgenic founder with germ-line transmission. However, phenotypic screening by fluorescence microscopy did not reveal any progeny showing the expression of the transgene.

Semi-quantitative RT-PCR confirms the expression of carp M3-CK isoform in the transgenic

zebrafish (Fig. 3d). The expression of carp M3-CK isoenzyme in transgenic zebrafish was determined by western blotting analysis using M3-CK specific antiserum. This analysis showed that carp M3-CK was uniquely found in the transgenic fish. Therefore, we conclude that the transgenesis was successful and that the carp M3-CK isoenzyme, driven by the CIP or CMV promoter, was expressed in the cold-treated transgenic zebrafish.

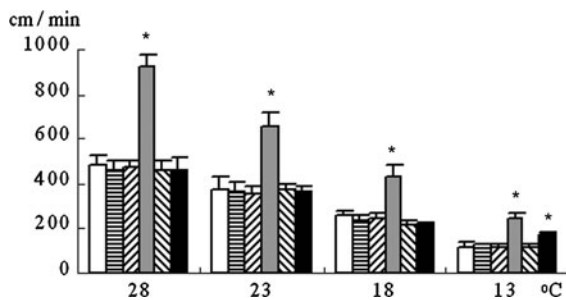


Fig. 4 Swimming ability of transgenic zebrafish The swimming ability of zebrafish with CMV and CIP driven M3-CK. Error bars indicate the standard deviation. □: wild type; ▨: pEGFP-C1 vector only; ▩: pEGFP-C1-CMV; ■: pEGFP-C1-CMV-M3CK; ▤: pEGFP-C1-CIP; ▥: pEGFP-C1-CIP-M3CK. *is the significant difference in swimming distance using one-way ANOVA ($p < 0.05$)

CIP-M3-CK transgenic zebrafish had higher swimming ability at low temperature than wild type zebrafish

When water temperature dropped below 13°C, cold-shock changed the swimming behavior of wild-type zebrafish—including imbalance, disorientation and inversion (Supplemental material 1). The wild-type fish failed to swim normally at this unfavorable temperature. They struggled to maintain balance, but eventually proved to be unsuccessful. They swam for a short distance while remained disoriented before succumbing to the adverse environment. In contrast, the transgenic fish were unaffected by cold shock and swam with normal turns and reversing direction at low temperature (Supplemental material 2).

The physiological significance of carp M3-CK in these transgenic fish was assessed by their swimming ability and behavior at 28°C to 13°C. The movement of both the transgenic and wild-type zebrafish was observed and recorded at different water temperature. An acute change in the surrounding water temperature did not induce any visible changes in the swimming ability of the transgenic fish. The swimming speed of the pEGFP-CIP-M3CK transgenic zebrafish was 42% higher than that of the wild-type at 13°C, and 108% higher than that of the wild-type at 28°C (Fig. 4). The results in Fig. 4 reveal a statistically significant difference in swimming velocity between the wild-type and transgenic zebrafish at temperatures of 28, 23, 18 and 13°C.

Discussion

Function of *hsc70* is different in different species. In arachnid, *hsc70* was reported to remain unchanged during cold shock (Shim et al. 2006, Sonoda et al. 2006, Rinehart et al. 2007). However, *hsc70-2* in carp could be induced ten-fold by cold shock (Ali et al. 2003). The molecular chaperone function of *hsc70* at low temperature has been evaluated in plants (Zhang and Guy 2006). The fluorescence of the *hsc70* promoter driven GFP expression in cold-shock transgenic zebrafish in the present study, and the improvement of their swimming ability by M3-CK expression demonstrates the cold-induced function of the *hsc70* promoter. These findings indicate that the *hsc70* gene is a cold-induced gene, and that cold stress is able to increase the expression of *hsc70*.

Temperature change is a common stress of poikilothermal animal. There are molecular responses to counter environment changes. Hsc70 is a well-known chaperon correlated with stress-induced apoptosis through its conjugation with BAG-1 (Takayama et al. 1998; Hohfeld 1998). The regulatory mechanisms of heat shock protein in bacteria were reviewed in 1999, and the sigma factor (σ^{32}), which positively controls *hsc70*, was reported to transiently increase after an elevation of temperature. The stability of σ^{32} is controlled by ambient temperature and correlated proteases (Narberhaus 1999). Heat-shock stress was found to be the most important factor that increased the expression of *hsc70* (Lim et al. 2008). Moreover, it was found that the *hsc70* gene was downregulated by NF- κ B in mammalian cells (Lim et al. 2008) and upregulated by Kruppel-like factor 4 (KLF4) (Liu et al. 2008).

The zebrafish *hsc70* gene was highly expressed in 12 h-old larvae. The amount remained stable during zebrafish development, but was not detected at the gastrula embryonic stage (Yeh and Hsu 2002). The promoter analysis using Genomatix tools showed that there are four Fkh-domain factor FKHL1 (*FOXO*) binding sites. One binding site controlled calorie restriction in a mouse model (Murakami 2006); two heat-shock factor 1 (*HSF1*) binding sites directly contributed to heat-shock response of *hsc70* (Park et al. 1994); and two CCAAT/enhancer binding protein beta induced different transcription levels by different transcription-factor binding activity (Hung et al. 1998; Chang et al. 1999). These transcription

elements suggest a possible regulatory mechanism for the *hsc70* promoter in response to cold shock.

Light and temperature cycles are the most important synchronizers of biological rhythms in nature. The thermal cycle is dominant over the light cycle in zebrafish (Lopez-Olmeda et al. 2006). Temperature changes affect membrane properties, ion homeostasis, calcium influx and other signal cascades (Rensing and Ruoff 2002). Muscle contractions at low temperature rely on maximum capacity of chemical energy supplemented by the creatine pool and the balance of ATP and phosphocreatine, which is influenced by M-CK. Indeed, the significant improvement in cold tolerance in the transgenic zebrafish at otherwise intolerable low temperature suggests that the carp *M3-CK* gene was functionally integrated into the energy metabolism of the transgenic fish. Physiologically, the carp M3-CK enzyme exhibits low-temperature preference by altering the endogenous energy metabolic pathways to sustain muscle contraction, as depicted in the phenotypic expression of the transgenic zebrafish.

According to previous study of in vitro analysis of carp muscle form creatine kinase (Wu et al. 2008), the carp M1-CK was supposed to be the most active subisoform. The M1-CK transgenic zebrafish has been raised. However, the M1-CK with either CMV or CIP promoter transgenic zebrafish did not grow to maturity and mate. Too high energy activity of creatine kinase might disturb the homeostasis of energetic compounds and result in the death of the transgenic zebrafish. The present findings suggest that the constitutively-expressed carp M3-CK is well adapted in transgenic zebrafish. Moreover, the M3-CK enzyme might be more suitable to compensate for a rapid energy demand in muscle at low temperature by transferring high-energy phosphate from phosphocreatine to ATP (Roussel et al. 1998). Thus, carp M3-CK supports active swimming behaviour of transgenic fish at low temperature. This is the first demonstration of enhancing the motility and survivability of zebrafish under hypothermic conditions by introducing a heterologous carp *M3-CK* gene that is coupled to energy metabolism in fish muscle. Furthermore, this transgenic model represents an important first step towards enhancing cold tolerance in subtropical fish.

The swimming ability of a fish is strongly correlated with muscle contraction during thermal

acclimation (Swank and Rome 2001). This process depends heavily on energy metabolism, which is tightly regulated by CK isoenzymes. Elevated expression of M3-CK was detected in transgenic zebrafish, indicating that the enhanced swimming ability of transgenic zebrafish under hypothermic condition could be sustained by this transgene. The creatine pool, along with ATP concentration and the activity of CK, regulate the homeostasis of the muscle cell, which in turn maintains the energy reserve for muscle contraction (Boutilier et al. 1997). Application of the *hsc70* promoter to drive the carp *M3-CK* was beneficial to the swimming ability of zebrafish at low temperature. This is the first application of a vertebrate cold-inducible promoter that triggers functional gene to overcome cold stress and maintains normal activity. The establishment of a cold-tolerant model fish can be broadly used in physiological research and economic aquaculture. Thus, the active swimming behaviour of the transgenic fish at low temperature can be maintained.

Acknowledgments This work was supported by grants from Academia Sinica and the Council of Agriculture, Taiwan (Project No. 9521011700060106F125).

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