IV. Results

1. Construction of plasmid

(1). Isolation of 5'-Flanking Region from Carp (*Cyprinus carpio*) M3CK Genomic DNA

We have got a 1.6kilo bases 5' untranscriptional region of carp M3CK and sequencing by Mission Biotech. The resulting plasmid was named pM3CKPro (Fig. 6A, Fig. 7 and Table. 8). After sequencing, the pM3CKPro used computational analysis to analyze the potential transcriptional elements.

(2). Promoter expression analysis

We analyze the potential transcriptional elements by paper searching on PubMed in NCBI and MatInspector database in Genomatix (http://www.genomatix.de/) (Fig. 8). There have some muscle specific transcription factors binding domains such as MEF, MEF2, E-box and Muscle TATA box (Shanti K & Kanungo MS, 2004; de La Serna *et al*. 2005; McGee *et al.* 2005; Wang *et al.* 2005; Tang *et al.* 2006) on the M3-CK promoter region and a cold inducible transcription factor binding domain, C/EBPβ (CCAAT/enhancer-binding protein β) or named inverted CCAAT box (Valadao *et al.*, 2002) on the M3-CK promoter region. The inverted CCAAT box transcriptional activity has regulated by p38 MAPKs in the absence of environmental stress (Ambrosino *et al*. 2006; Ghosh, A. K. 2002; Goldberg *et al.* 1992).

Expression vector construction was named pM3CKPro-EGFP (Fig. 8). At 48 h post injection, fish were examined using fluorescent microscopy and the GFP expressing fish (Fig. 9) were saved for isolating different kind of tissues for RNA extraction (PureLink Micro-to-Midi Total RNA Purification System) and RT-PCR (SuperScript™ III One-Step RT-PCR with Platinum® Taq).

Form the RT-PCR performance (Fig. 10) we can see clearly the expression of GFP in skeletal muscle and heart. We can make sure that the M3-CK 5'-flanking region (M3CKpro) from Carp is a promoter that can drive gene expression in M3-CK expressed tissues.

(3). Plasmid construction

After PCR reaction, we have got the two genes (M1-CK and M3-CK) and ligated the PCR product into the pGEM-T Easy Vector System I (Promega, Madison, WI) for amplification and sequencing. The resulting plasmids were named pGEMT-M1CK and pGEMT-M3CK and used *Not*I digested to check the insert size (Fig. 6B).

We constructed a dual functional expression vector that constitutively expressed the transgene and a GFP marker gene. The CMV promoter drove the GFP marker and the CMV promoter, cold-inducible promoter and carp M3-CK promoter drove the carp MCK transgene, respectively (Fig. 10-12).

We finished the nine kinds of constructs: pEGFP-C1-CMV, pEGFP-C1-CMV-M1, pEGFP-C1-CMV-M3, pEGFP-C1-CIP,

pEGFP-C1-CIP-M1, pEGFP-C1-CIP-M3, pEGFP-C1-M3CKP, pEGFP-C1-M3CKP-M1, and pEGFP-C1-M3CKP-M3. After acquiring the vector that we need, the nine kinds of constructs digested to check the insert size by *Xho*I, *Eco*RI, *Bam*HI, *Sal*I, and *Pst*I (Table. 6). Then sequencing the nine plasmids used primers: EGFP-C, CIP1605, MP1321, and BamHI-MCK (Table. 4).

2. Generation of transgenic zebrafish

(1). Microinjection

When we finished the nine kinds of constructs, we injected fertilized zebrafish eggs with purified plasmid through a microinjection gene transfer system.

After injected, the one-cell stage embryos were taken care in 90mm petri dish that was filled with Embryo Medium (Westerfield M, 1994) until 48 hours post-fertilization (hpf).

(2). Green fluorescence protein (GFP) expression analysis

At 48 hpf, newly hatched larvae were examined under fluorescence microscope and those larvae that expressed GFP were collected for further characterization (Fig. 13).

GFP-positive larvae were raised to their sexual maturity stage and backcrossed 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 transgene. In general, DNA integration is a rare and random event whereby only a fraction of the germ-line cells posses the integrated DNA. On the other hand, the over expression of M3-CK could result in certain undesirable effects such as lethality or developmental interruption and retardation during zebrafish embryogenesis, so we used cold-inducible promoter and carp M3-CK promoter to drive the carp M-CK. Therefore, it may be necessary to screen for a larger pool of embryos to identify transgenic products. Unexpectedly, we established F0 transgenic founder lines expressed the transgene but failed to generate F1 stable transgenic line from those founders. Therefore, we were compelled to analyze the transient expression of carp M-CK sub-isoenzyme in the transgenic fish.

(3). mRNA expression analysis

We isolate different kinds of tissues for RNA extraction (PureLink Micro-to-Midi Total RNA Purification System) and RT-PCR (SuperScript™ III One-Step RT-PCR with Platinum® Taq).

Form the RT-PCR performance, detection of transgenic Carp CK and endogenous zebrafish gene from adult wild type, only vector control and transgenic zebrafish (Fig.14).

(4). Immunoblot analysis

The expression of carp M3-CK isoenzyme in transgenic zebrafish was determined by western blotting using M3-CK specific monoclonal antibody. The specific antibody could specifically distinguish between the

transgenic zebrafish-expressed carp M3-CK protein from the wild type. Western blotting analysis showed that the carp M3-CK was uniquely found in the transgenic fish whereas the M1-CK or M2-CK proteins were indiscriminately detected in transgenic and wild type zebrafish. We concluded that the transgenesis was successful and carp muscle-specific M3-CK sub-isoenzyme driven by the CMV promoter, cold-inducible promoter and M3CK promoter were expressed well in transgenic zebrafish (Fig. 15).

(5). Determination of swimming speeds and statistical analysis

The physiological significance of carp M-CK in these transgenic fish was assessed by their swimming ability and behavior at an otherwise intolerable water temperature. The movements of the transgenic and wild type zebrafish were observed and recorded at different water temperatures. In the acute temperature change experiment, wild type and only vector transgenic zebrafish almost lost their swimming ability instantly (Fig. 16A). In contrast, an acute change in surrounding water temperature did not evoke any visible changes in the swimming ability of the M3CK transgenic fish (Fig. 16B). Statistically, the results in Table 7 revealed significant differences in the swimming velocity between the wild type and the transgenic zebrafish at the lower temperature (Fig. 18).

When water temperature dropped to below 13℃, cold shock incurred changes in swimming behavior such as imbalance, disorientation and inversion in wild type zebrafish (Fig. 17A, B). These fishes failed to

swim normally at this unfavorable temperature. They would struggle to balance themselves and to restore swimming, but their efforts were in vain and unsuccessful. They swarm for a short distance and remained disoriented most of the time before they succumbed to the adverse environment (Fig. 17C). In counterpart, the transgenic fish were unaffected by cold shock and resumed swimming by making normal turns and reverses at the lower temperature (Fig. 17D).

3. Conclusions

In our lab, we analyze the potential transcriptional elements by paper searching on PubMed in NCBI and MatInspector database in Genomatix (http://www.genomatix.de/) (Fig. 8). We observed GFP by fluorescence microscopy and extracted total RNA from 30 Days M3CKpro-EGFP transgenic zebrafish to do RT-PCR. The result demonstrated that some important transcription factor binding domains such as MEF, MEF2, E-box and Muscle TATA box (Shanti K & Kanungo MS, 2004; de La Serna *et al*. 2005; McGee *et al.* 2005; Wang *et al.* 2005; Tang *et al.* 2006) on the M3-CK promoter region and a cold inducible transcription factor binding domain, C/EBPβ (CCAAT/enhancer-binding protein β) or named inverted CCAAT box (Valadao *et al.*, 2002) on the M3-CK promoter region.

RT-PCR and western blotting determined the expression of carp M-CK isoenzyme in transgenic zebrafish. Confirmed the specific carp M-CK expression, we determinates swimming speeds of transgenic zebrafish and wild type. Statistically, the results in Table 7 revealed significant differences in the swimming velocity between the wild type and the transgenic zebrafish at the lower temperature. The M3-CK transgenic zebrafish and M1-CK driven by M3CKpro transgenic zebrafish have significant effect (Fig. 18).