as a distinct precursory phenomenon related to the occurrence of earthquakes with a magnitude $M > 4$ and $M < 5$. However, distinct variation has been observed at the same time of origin of the earthquakes, which can be considered as co-seismic activity. Detailed data analysis is in progress to locate precursory phenomena, if any, related to earthquakes.

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Expression analysis of β-actin promoter of rohu (*Labeo rohita*) by direct injection into muscle

The cytosolic β-actin is expressed ubiquitously and abundantly in almost all eukaryotic undifferentiated and differentiated cells[1]. The β-actin promoter of several fish species has been cloned, characterized and subsequently used for generating transgenics[2–6]. The use of 'autotransgene' construct, where both the promoter/regulatory element and targeted structural gene derived from the same species, was demonstrated to be beneficial in transgenesis[7]. This requires isolation of species-specific functional promoter capable of driving either ubiquitous or tissue-specific expression of the gene of interest. The Indian major carp, *Labeo rohita*, popularly known as rohu, is an economically important freshwater fish in India as well as other Asian countries[8]. Isolation of rohu β-actin promoter will have potential use in expressing desired genes of interests for generating transgenic rohu. The transgenic approach is a rather time-consuming process for verification of functional activity of the isolated promoter. Muscular injection of naked plasmid DNA has proved to be a simple, efficient and quicker method of functional validation[4,9–12]. In the present study, we have isolated rohu β-actin 5′-upstream region and identified promoter/regulatory regions. Functional validation of the promoter was carried out by direct injection into the rohu skeletal muscle.

Genomic DNA was isolated from the liver of rohu as described earlier[8]. The β-actin 5′-flanking region containing promoter, untranslated exon1 and intron1 was derived using GenomeWalker Universal Kit (Clontech) according to the manufacturer's instructions. The gene-specific reverse primers GSP1 (5′-GGGAGCATCATCTCCAGCGAATCCGGCTTTGC-3′) and nested GSP2 (5′-CACATACCGGATCCGTTGTCAACAACCAGTGC-3′) were designed from the coding exon2 sequences of β-actin available for rohu and other species in the GenBank. The upstream adaptor primers AP1 and nested AP2 were supplied along with the kit. Four independent *Dra*I, *Eco*RV, *Pvu*II and *Stu*I digested genomic DNA libraries were generated. The cycling parameters for touch-down PCR were five cycles at 94°C for 25 s and 72°C for 3 min followed by 20 cycles of 94°C for 25 s and 69°C for 3 min. A 1.6 kb fragment amplified from the *Stu*I library was cloned into a pGMET-easy vector (Promega) and bidirectionally sequenced in an automated ABI 310 genetic analyser (Applied Biosystem). The sequence data were submitted to GenBank (accession no. GU338376). The sequence data analysis by MatInspector 7.4.3 of Genomatix Inc. (http://www.genomatix.de/products/MatInspector/index.html) revealed that the 5′-flanking region contained exon1 and intron1, including consensus

Table 1. Sequence comparison of rohu β-actin promoter and regulatory regions with other related species

Species	Percentage of homology	Accession number
Catla (*Catla catla*)	96	AF415205.1
Common carp (*Cyprinus carpio*)	85	M24113.1
Grass carp (*Ctenopharyngodon idella*)	88	M25013.1
Mud carp (*Cirrhinus molitorella*)	94	DQ241809.1
Zebrafish (*Danio rerio*)	93	EF026002.1

TATA- and CAAT-box elements. The sequence variation was analysed by comparing with other carp species (Table 1). Maximum homology of rohu β-actin regulatory region with that of catla could be due to their genetic closeness[8].

Sequence analysis also revealed that it contains ten putative E-boxes (CANNTG), two CC(A/T)$_6$GG (CArG box or serum-response element) and three potential MEF2 (myocyte enhancer factor 2) binding sites (Figure 1). The CAAT and CArG boxes in the proximal promoter are essential for optimal transcriptional activity of human and common carp β-actin promoters[13,14]. Both bHLH and MEF2 families of transcription factors are essentially involved in activating muscle-specific genes[15]. The *Sma*I (present in the adopter sequence) and *Age*I-digested fragment of 400 bp comprising the TATA, CAAT, CArG boxes, four E-boxes and two MEF2 elements was chosen as promoter to clone into a pAcGFP1-1 reporter vector (Clontech) devoid of any promoter element. The direction of the insert was verified by sequencing.

The recombinant vector DNA containing β-actin promoter element (β·4pAcGFP1-1) was directly injected into the skeletal muscle, below the dorsal fin of rohu of 15 cm length. A total of 10 fishes were injected with this construct. The injection volume was maintained at 100 µl containing 50 µg supercoiled vector DNA as described earlier[11]. The slides were prepared with thin layers of the injected muscles to observe the green fluorescence protein expression under fluorescence microscope. An efficient expression of the reporter gene surrounding the site of injection was detected, thus validating its transcription activity (Figure 2). The geometry of *cis*-acting transcriptional elements comprising only one CArG box positioned between the CAAT and TATA boxes is capable of *in vivo* transcriptional activation in rohu. All the injected fishes expressed green fluorescence in the muscle fibres. In sections from non-injected fish muscles, no green colour expression was detected (Figure 2). The levels of gene expression were observed 3 days after injection, which remained stable and stronger for about eight weeks in all the fishes examined. Maximum level was observed 15 days after injection. The expression was strong at the site of injection. We also observed expression of fluorescence protein in the muscle far from the site of injection (data not presented), in agreement with the observations in zebrafish[16]. It could be possible that the injected DNA accesses other tissues through circulation immediately after injection. There was no apparent variation with reference to the duration of fluorescence expression in the injected fishes.

Our results of functional validation of rohu β-actin regulatory promoter/regulatory regions in rohu, demonstrate its potential applications in vaccination and gene therapy programmes, in addition to the generation of transgenics. The DNA sequence for leader polypeptide linked to disease resistance or other genes can be transcribed under the regulatory control of muscle-specific promoter for fish vaccination. Direct injection-mediated transgene expression driven by the β-actin promoter offers the possible opportunity of nonviral gene therapy approach. The retroviral-mediated gene therapy in humans creates a major apprehension due to the integration of exogenous elements into the human genome[17]. The technology of 'direct injection method' appears to be safer and hence could be an alternate approach to conventional retroviral-mediated gene therapy.

The reported cross-species commonality of β-actin promoter[18] also widens the scope of utilizing it in several other fishes and animals. The feature of ubiquitous expression patterns of β-actin in almost all cells of the body will be beneficial in generating transgenic rohu and other closely related Indian major carps for the targeted traits such as growth, disease resistance, stress tolerance, etc. Though conventional selective breeding approach has proven to genetically upgrade rohu[19], it is a rather time-consuming process. Traditional selective breeding approach in combination with

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                                                        -194
CCTTGTTCTTCAAATAATCTAGCTTCCCCTTCTTTCACTCTC***CAGTTG***C  -146
AAGAAG***CAAGTG***TAGCAATGTGCAACGCGACAGCCGGGTGTGTGAC    -100
GCTAGA**CCAAT**CAGAACGCAGAGCTCCGAAAGTTTA**CCTTTTATGG**   -54
CTAGAGCCGGG***CATATG***CCGTCG**TATAAAA**GAGCGCGCCCAGCTTTT  -7
      +1
CAGCTTCACTTTGAGCTCCTCCACACGCAGCTAGTGCGGAATAT***CATC***   +41
***TG***CTTGTAACCCATTCTCTTAAGTCGACAAACCCCCAAACCTAAGGTG   +89
AGTTGATTTTAAAGCCTTTTATTACATGTTTAATTGGTTAATATTAACT        +138
CAAATATTTAATTAAAAAGTACAAATAAAGCTACATTAAGTTATTAG          +185
        AgeI
GCTCAGTTA**CCGGT**CTTTTTG***CAGTTG***ACGTTATAAACTTGGCTTAAA  +233
AACTCTAGCTATTCTTCATTAACATGCGATTGTGGTTGTTTTTTTTTTT        +282
AAATAAATGTCTGATTTGTCTTCATAACTCTAAATGTATGAATTGGAA         +330
GAGACCGTTGGAAGTGATGGTTATCGTTGTAGGCACGACATTGAATG          +377
GGCCGGTGTGAAATAAGCGTTCAGTCCTTTTAACCTCAAGATGTTCTC         +425
TAGTTAACAGTGATTTAACAGCTATGAACGTGATTGTACAGTTTTCAA         +473
GATTAGTGAGCCATGTTGCA***CACTTG***ATGGATAGCCGGCATGGGAAG    +520
TTCTTTGTGCAGGCAGTGCTGCAGCAGGGTGTGACCTAGTTTAGCTAG         +568
CCGGCTAACCAGCATT***CATCTG***CTGTAAACCTGATGAAAATAACTTTA    +616
GACACTTTTAGTGGAAAAATTGCGATTTATATCGCAAGTCAGTGTGC          +663
AGCCCTTCAGTCTTAAAAGCTGAGTGAGATTATTAAGGTGTTTTATAT         +711
TCACTATTAAAATGTTATAATTTAAGGTAGTCAATTTTAATAGCTGTT         +759
GAAACGTTTCTGGATAGATTTTAAATGGTGGTAATTGTGTAGTATGAA         +807
ACAATTAAAATAAATAATTAAAGCTTATTGATTCAAAGGCTGTGTTG          +854
CAAAATGAATGGGCTGAGTAAGATGGTTCA***CAAGTG***CTTTAGTGTGA    +901
AGTCTGCTCACAAGGAGTCACTGAAGTGACTGCAGATCTGTGACGCA          +948
GTAATTTCGGGCAGACACCCGTTAAAATTCGGTTGTGTAATTGATACC         +996
AGGCGAGGATCGAAAAGGATGTAAAACTT***CATTTG***TGTAGAATTTAG    +1043
GGAGTGGCCCCTGGCGTGATGAATGTCGAAATCTGTTCCTTTTTACTG         +1091
AACCCTACGACTCTGGCTGAGTGCCACACCGCCGGCAGCCGCAAAGC          +1138
GGCTCAAACCATTG**CCTTTTATGG**TAATAATGAGAGAATGCAGAGG       +1148
GACTTCCTTTGTCTGGCATATCTGAGGCGCGCATTGTCACTCTAGCAC         +1232
CCACTAGCGGTCAGACTATAGAATGCAGCACGAAACAGGAAGTTGA           +1278
CTC***CACATG***GTCACATGTTCTTCCGTGGCAGCGGTGCACTTCTAAACC    +1326
GTTTTCTCTTTCTTTTACAGTTCAGCCATGGATGATGAAATTGCC            +1371
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Figure 1. Nucleotide sequence of the 5′-flanking region of *Labeo rohita* β-actin gene. Numbering of the nucleotide sequence is given at the right. The CAAT, CArG and TATA boxes are shown in underlined bold letters. E-boxes are shown in bold italics and shaded sequences indicate MEF2 elements. +1 indicates the first transcription initiation site.

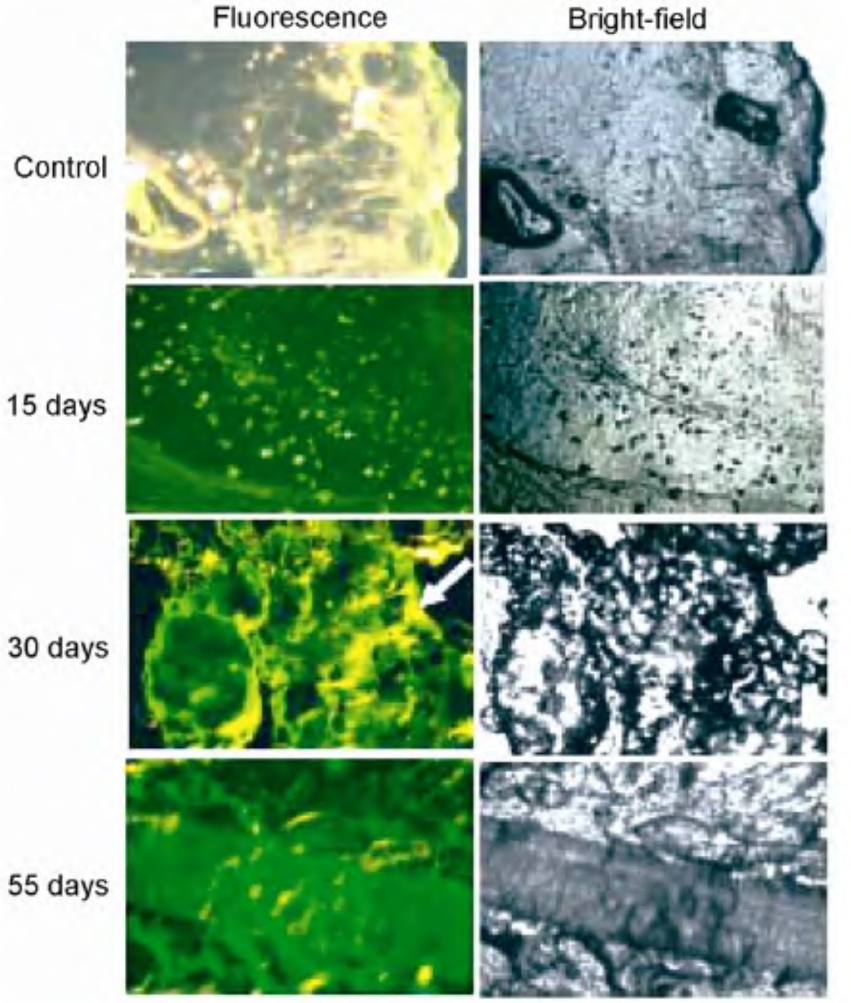

Figure 2. Green fluorescence protein expression in muscle at the site of injection at 15, 30 and 55 days. Control is the muscle of non-injected fish. Images are taken under fluorescence (left) and bright-field (right). Arrow indicates autofluorescence.

transgenesis can bring about faster improvement of selected traits.

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