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Original Article

Cloning and characterization of promoters of vascular specific genes in Zebrafish

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Abstract: Gene promoters located at the 5′ end of genes are instrumental in regulating the gene expression in a ubiquitous or tissue specific manner. The objective of the study was to identify, clone and characterize promoters involved in gene expression in specific tissues such as the blood and blood vessel of Zebrafish. Three genes, known to express in the blood and blood vessel lineage in Zebrafish, were selected viz. *Pak2a, Rac1* and *Cdc42*. Approximately 800 bp of putative promoter region of *Pak2a* and 826 bp putative promoter region of *cdc42* were cloned into plasmid vectors. This putative promoter did not show any expression in Zebrafish embryos. However, approximately 716 bp of putative promoter region of *Rac1* showed Red Fluorescent Protein expression. While study *Cmlc2* was used as a positive control.

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Introduction

The zebrafish, *Danio rerio,* is a powerful model organism to study the vertebrate biology, well suited for the genetic basis of embryonic developments and genetic analysis (Dooley and Zon, 2000) in both vertebrate and invertebrate species (Fire, 1986; Flytzanis et al., 1985; Jaenisch, 1988; Rubin, 1988). Zebrafish exhibits a rapid developmental pattern with the major organs, including a functional heart, vasculature, circulating blood cells, liver, brain etc. being developed within 30 hours after fertilization. The rapid external development and transparency of zebrafish embryos allow direct observation of developmental processes and the deployment of fluorescent reporter genes to facilitate examination of spatial and temporal gene expression patterns in living embryos (Arnaout et al., 2007; Bai et al., 2007). Attributes such as external fertilization, optically transparent embryos, high fecundity, ease of maintenance, half of the genome size as compared to humans, knowledge of whole genome sequence along with the ease of production, maintenance, analysis of mutants and transgenic lines are in a relatively short period of 3 to 4 months generation time make the zebrafish well-suited animal model of transgenic studies. Introduction of foreign genes into zebrafish genome has been found comparatively easy with respect to other vertebrate model organisms to produce transgenic zebrafish. The transgenic zebrafish is widely used to study spatio-temporal gene expression using reporter genes like RFP (Red Fluorescent Protein), and GFP (Green Fluorescent Protein). Transgenic animals are also found valuable model to study tissue/organ development in different types of mutagenesis screens (Chávz et al., 2016).

The availability of efficient techniques for manipulation of gene expression enables studies of gene function. There are different methods like: Micro injection, Electroporation, Retroviral vectors delivery etc. which are widely used to generate transgenic zebrafish. Out of these microinjection is found to be one of the most efficient techniques for gene delivery. Gene transfer, utilizing microinjection technique, in fish began in the mid 1980's (Chourrout, 1986; Dunham et al., 1987). Microinjection of DNA constructs into single celled fertilized zebrafish embryos has proven successful in the generation of transgenic zebrafish. Efficiency of transgenesis in

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zebrafish has been achieved nearly 70-100% by retroviral vectors (e.g. Pseudoretroviral vector) and transposase (e.g. Tol2, Sleeping Beauty*)* (Kawakami et al., 2007; Stuart et al., 1990).

Insertions made in the genome using Tol2 can be selected in F1 generation by outcrossing the injected fish. On an average, transgenic F1 offsprings obtained from 50-70% of the injected fish, at frequencies of 3-100%. Although the frequencies are lower than those with retroviral vectors (nearly 100%), the Tol2 transposon system has advantages over the pseudotyped retrovirus system as the handling and modification of retroviral vectors is laborious, which makes the application of psudotyped retrovirus for transgenesis method difficult. Tol2 is a transposable element obtained from Medaka fish. It is an autonomous transposon that encodes a fully functional transposase enzyme that catalyzes transposition of a transposon construct that has 200 and 150 bp of DNA from the left and right ends of the Tol2 sequence, respectively. The Tol2 transposon system has been shown to be active in all vertebrate cells, including zebrafish. Tol2 integrates as a single copy through cut and paste mechanism and it does not cause any rearrangement or modification at the target site (Kawakami et al., 2007) and transgenes created by Tol2 mediated transgenesis may not suffer from gene silencing effects.

To develop a tissue specific transgenic line, gene specific promoters have been used. Promoters contain specific DNA sequences and response elements which provide a binding site for RNA polymerase and for proteins called transcription factors involved in the recruitment of RNA polymerase. Many genes promoter have been used to create transgenic fish line, e.g. Zhu et al. (2005) cloned different fragments ranging from 2.5 kb to 69 bp upstream of translation start site of *lmo2* gene in GFP vector construct and finally concluded that 174 bp proximal promoter was sufficient to recapitulate *lmo2* expression. By using *lmo2* promoter they created two transgenic lines, Tg (*lmo2*:EGFP) and Tg(*lmo2*:DsRed) both of which exhibited embryonic blood and endothelial expression. Similarly, Park et al. (2000) determined that 2.8 kb of the 5'-flanking sequence of *Huc* was sufficient to restrict GFP gene expression to neurons, the core promoter was only 251 bp. Meng et al. (1997) revealed that a 1.1 kb DNA sequence was critical for expression of *GATA-2* in neurons. Likewise promoters of many genes viz: *kdr, gata1, gata2, eno2, spi1* etc. have been characterized for their expression using reporter genes like GFP, RFP etc. Some other examples are summarized in the Table 1. Several promoter prediction programs like: BDGP (Berkeley Drosophila Genome Project), ProSOM, Promoter prediction server etc. are available which help in the promoter analysis. These bio-informatics tools provide us an idea of the existence of putative promoter region of a gene of interest, based on which required primers for the amplification of DNA region of interest can be designed by ligation to a fluorescent protein spatio-temporal expression of a particular gene can be checked. The present study is aimed at identification and characterization of promoter vascular specific genes to be employed for the generation of transgenic zebrafish species.

Materials and Methods

Sample collection: Experiments were carried out in accordance with Institutional Animal Use and Care Committee regulations and approvals. The zebrafish embryos were collected at 24 hpf (hours post fertilization), immediately frozen in liquid nitrogen and stored at -80°C for future use. The study was carried out at Zebrafish lab facility (Institute of Genomics and Integrative Biology, Delhi, India).

Isolation of Genomic DNA from zebrafish embryos: Wild type zebrafish were allowed to breed in breeding tanks and embryos were collected and frozen at 36 hours post fertilization (hpf). Extraction of DNA was performed from 50 embryos using the protocol given below and stored at -20°C. The embryos were homogenized in 0.75 ml lysis buffer using a 2 ml Dounce homogenizer. The volume was brought upto 20 ml (on ice). To this, 100 µl of proteinase K was added and incubated for 30 min at 65°C (20 mg/ml). Potassium acetate (8M) was added immediately after taking out of 65°C. The reaction mixture was

Table 1. List of primers.

S. no	Primer name	5′to 3′ sequence
1	I.D.-SSB_P1210	AAAAGATCTATTTAGCGGCCGGAATTCGC
2	I.D.- SSB_P951	AAAAGATCTGCAGGTTTAAACGAATTCGC
3	I.D.-SSB_P1195	TCCTTCTCCTTCTCCCTCAATCTC
4	I.D.-SSB_P1248	AAAAGATCTAGTACCTCGAGGCTGTGAGATT
5	I.D.-SSB_P1249	AAATCTAGAAATGATGCAATCCAGCGCCCA
6	I.D.-SSB_P1244	AAATCTAGACTTGAATATGCGCATCTTCT
7	I.D.-SSB_P1246	AAATCTAGACGTTGGTACATATTCGGAGG
8	I.D.-SSB_P1275	AAAAAGATCTGAGCAAACAGTTGAAGCGGT
9	I.D.-SSB_P1276	AAATCTAGACCGTAGCTGTATCTGTGAA

immediately incubated on ice for 30 min. It was then centrifuged at 12,000 rpm for 10 min. The supernatant obtained after centrifugation was transferred into a new tube, to which 0.6X isopropanol (13 ml) was added and was incubated for 5 minutes at room temperature. This was then centrifuged at 15000 rpm for 10 min. The supernatant was removed and pellet was washed with 100% ethanol. The air dried pellet was resuspended in 100 µl elution buffer and incubated overnight at 4°C. The optical density of DNA was quantified by spectrophotometer. The extracted genomic DNA was verified by electrophoresis on 0.8% agarose 1X TAE gel.

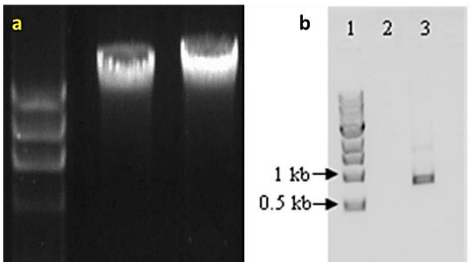
Isolation of Putative promoters, cloning and sequencing: Putative promoter regions of selected genes involved in RhoGTPase pathway like *Pak2a*, *cdc42* and *Rac1* were amplified using the primer pairs as given in Table 1. To amplify 800 bp putative promoter region of *Pak2a* gene forward primer I.D.-SSB_P1248 with *Bgl*II site and reverse primer I.D.-SSB_P1249 with *Xba*I *site* was used. To amplify 826 bp putative promoter region of *cdc42* forward primer I.D.-SSB_P1244 with *Xba*I site and reverse primer I.D.-SSB_P1246 with *Xba*I site. 716 bp putative promoter region of *Rac1* gene was amplified using following primer pairs: forward primer I.D.-SSB_P1275 with *Bgl*II *site* and reverse primer I.D.-SSB_P1276 with *Xba*I *site* (Table 1). These putative promoter fragments were cloned in vector pSS536 by ligating at the appropriate site. The ligated product was transformed in *E. coli* DH5α, clones were checked by PCR using vector back bone specific primers and insert specific primers. The positive clones were sent for sequencing to TCGA (The Centre for Genomic Application, Delhi, India).

Expression analysis: 3 nl of the DNA (vector construct + insert) sample were microinjected into the 1-2 celled fertilized eggs, which were incubated at 29°C in dishes containing low concentration of methylene blue solution. Dechorionated embryos were euthanized using tricaine. The Red Fluorescent Protein (RFP) in transgenic embryos were observed in vivo using fluorescence microscope (Carl Zeiss, Germany). Images were captured at 2-3 days post fertilization with a Zeiss Axiocam camera using the Zeiss Axiovision software (Carl Zeiss, Germany). Promoter of *cmcl2* gene was used as positive control (Huang et al., 2003).

Results

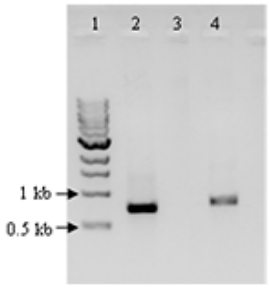
Putaive promoters isolation from zebrafish genomic DNA: The isolated genomic DNA from zebrafish embryos were checked on 0.8% TAE gel, showed the presence of heavy band just near to wells, no RNA contamination was found and 260/280 ratio was nearly 1.9 (Fig. 1a). The zebrafish genomic DNA was used as template to amplify putative promoters related to blood vasculature. The *Pak2a* putative promoter amplification was observed just below 1 kb as expected (promoters of vascular specific genes). The *Rac1* and *cdc42* gene putative promoter amplified fragments were also observed at the expected size (Fig. 2). For validating a promoter fragment in zebrafish, the strategy shown in Figure 3 was followed.

Cloning and sequencing of putative promoters: The amplified putative promoter region of three genes i.e. *Pak2a, Rac1* and *cdc42* were cloned in TOPO-TA



Lane	Sample	Expected	Observed
1	1 kb ladder		
3	Pak2a	800	just below 1 kb

Figure 1. (a) Gel shows genomic DNA isolated from zebrafish embryos, M is the λ Hind III marker, lane 1 and 2 is the genomic DNA and (b) Gel picture showing PCR amplification of *Pak2a*. Genomic DNA was used as template DNA.



Lane	Sample	Expected	Observed
1	1 kb ladder		
2	Rac1	716	b/w 1 & 0.5 kb
4	Cdc42	826	Just below 1 kb

Figure 2. Gel picture showing PCR amplification of *Rac1* and *cdc42* Genomic DNA was used as template DNA.

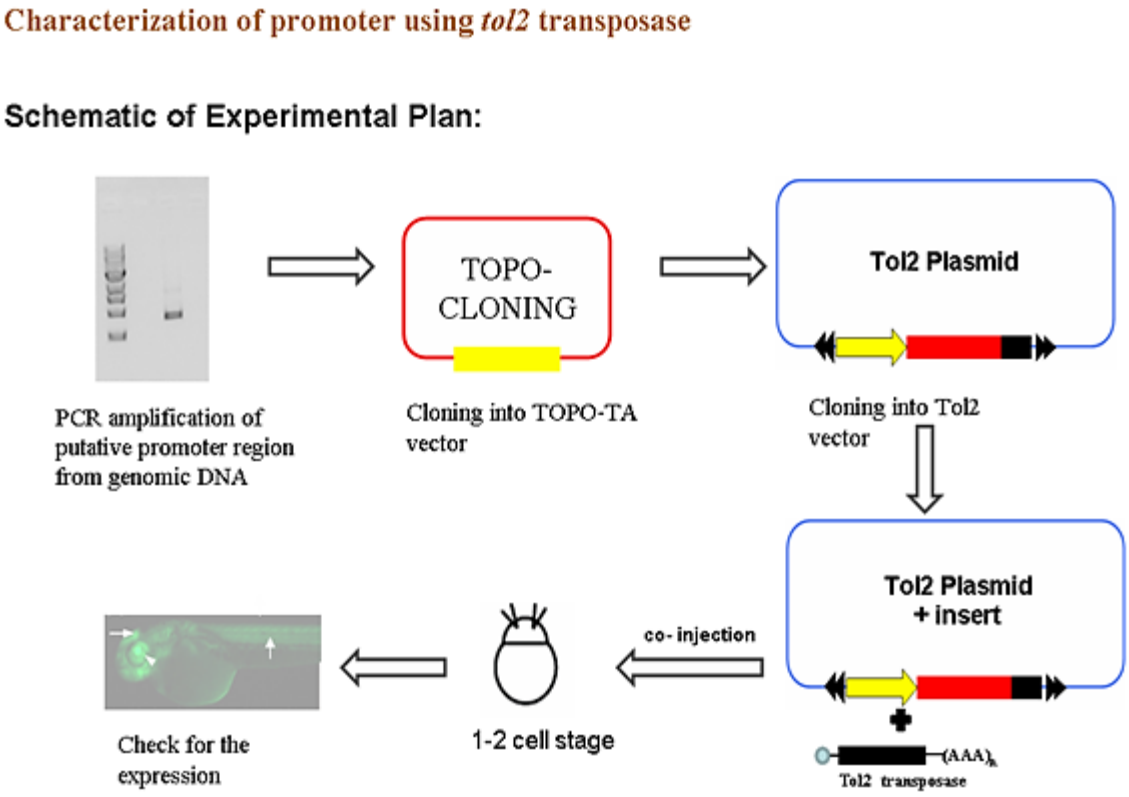


Figure 3. Basic Strategy to clone the putative promoter region in TOl2 plasmid. The TOPO-TA step was skipped for *Pak2a*, *Rac1* and *cdc42* putative promoter region.

vector (Invitrogen, USA) as per manufacturer's instructions. After cloning, these constructs were sent for sequencing to TCGA by using primers T7 and SSB_P1195. The sequencing results were analyzed using BLAST-N (www.blast.ncbi.nlm.nih.gov.in) and assembled using CAP3 assembler software tool. The sequencing results, alignment and BLAST results are shown in supplementary file (Figs. S1-S8). The alignment showed 97, 97 and 96% similarity for *Pak2a*, *Rac1* and *cdc42,* respectively. The putative

Table 2. Expected and observed expression of different constructs.

Construct	Expected expression	Observed
Tg(*Cmlc2*:DsRed)	Heart specific	Heart specific
Tg(*Pak2a*:DsRed)	Ubiquitous	No expression
Tg(*Rac1*:DsRed)	Ubiquitous	Ubiquitous
Tg(*Cdc42*:DsRed)	Ubiquitous	No observation

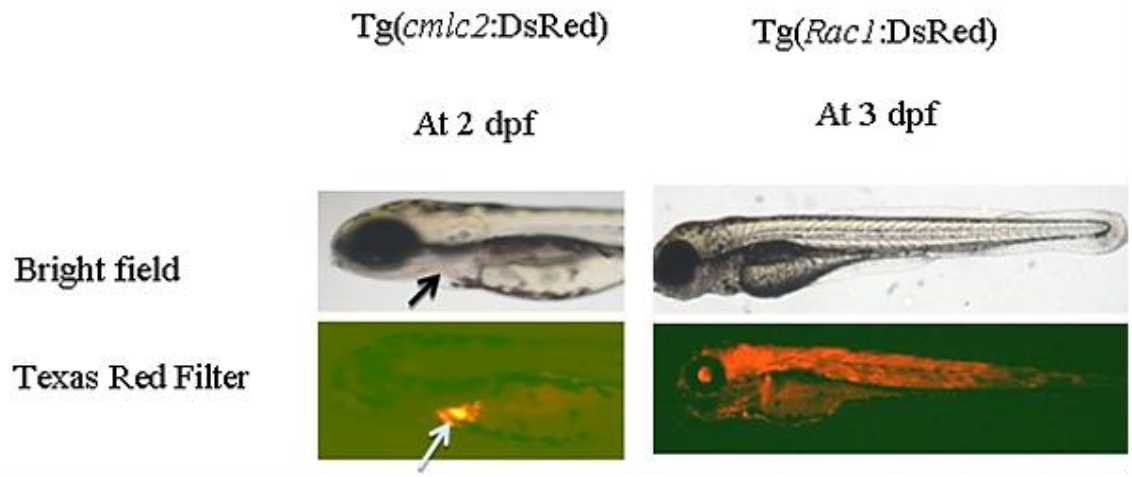


Figure 4. The heart specific expression for *cmlc-2* and ubiquitous expression for the *Rac1* gene.

promoter regions were cloned in a promoter less vector pSS536 for expression analysis. The constructs were named as Tg(*Rac1*:DsRed), Tg(*Pak2a*:DsRed) and Tg(*cdc42*:DsRed).

Promoter expression analysis in Zebrafish through fluorescence microscopy: Three constructs Tg(*Rac1*:DsRed), Tg(*Pak2a*:DsRed) and Tg(*cdc42*:DsRed) were injected in zebrafish embryos (2-4 celled stage). Tg(*cmlc2*:DsRed) was used as positive control (for heart specific expression analysis), *Rac1* putative promoter region was able to drive the expression of RFP in zebrafish embryos and it was observed throughout the body while *Pak2a*, *cdc42* putative promoters were not able drive any expression (Fig. 4). Based on the literature, the expected expression and observed pattern of RFP in zebrafish embryos has been shown in Table 2.

Discussion

Rho GTPases are Guanine nucleotide binding proteins that cycle from the active GTP bound state and inactive GDP-bound state (Buchner et al., 2007). They are known to play key roles in the modulation of a wide range of cellular processes like proliferation, apoptosis, cell migration, membrane trafficking, cytoskeletal rearrangements etc. (Vidal et al., 2003). There are 32 zebrafish Rho genes representing one or more homologs of 17 of the 23 predicted Rho genes in human. In zebrafish, most of the Rho genes are expressed in wild type healthy adults, but only a subset is expressed early in zebrafish development and *Rac1* is one of them, this might represent that it plays most critical roles before and around epiboly gastrulation. It has been shown that Rho genes are conserved from lower to higher vertebrates. In general, central coding exons are the most conserved region and the Rac family members are amongst the most conserved genes between zebrafish and humans (other genes are *Rho* and *Cdc42*) (Dumont et al., 2009). The overall protein similarity between human and zebrafish Rac family members is 98.4% (Liu et al., 2007; Vidal et al., 2003). This degree of conservation indicates that their functions are important in all vertebrates. Different RhoGTPases are known to participate in normal animal development. However, it is also known that the deregulation of their activities contributes to the generation of different human pathologies, including cancer progression, neurodegenerative disorders and infectious diseases. The transgenic zebrafish Tg(*Rac1*:DsRed) may help

us to study the importance of molecular RhoGTPase signaling pathway and can also form the basis of research to treat some of the lethal human diseases like: cancer progression, neurodegenerative disorders etc.

Tg(*Cmlc2*:DsRed) construct was used as positive control, the cardiac myosin light chain (cmlc) has been identified as a major contractile component of cardiac and other striated muscles; cmlc-2 is a regulatory light chain and potential modulator of contractile activity in heart and skeletal muscle cells (Rottbeaur et al., 2006). Chen et al. (2008) used zebrafish to identify the *cmlc2* as the main RLC (Regulatory Myosin Light Chain) orthologue. They further showed that in *cmlc2* morphants the sarcomere length is shorter and mutation in the genes encoding for RLCs have been casually linked to ~1% human cardiomyopathies (Hsu et al., 2004; Huang et al., 2003). The zebrafish is a powerful vertebrate model used to dissect molecular pathways of cardiovascular development and disease. Because fundamental electrical properties of the zebrafish heart are remarkably similar to those of the human heart, the zebrafish may be an appropriate model for studying human inherited arrhythmias. Heart disease is currently one of the most common causes of human death. A large number of researchers are currently searching for a simple animal model to assist in finding either a cure for heart disease or a novel gene that regulates heart development (Mingjun et al., 2008). Zebrafish is viewed as having advantages for heart related research. Heart specific transgenic zebrafish will be valuable as research model for tracing the development the for the fate of heart cells, finding new heart specific genes and functions, establishing biological indices of environmental pollutants, and study the efficacy of therapeutic drugs (Leskow et al., 2006).

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