Journal of Animal and Veterinary Advances 11 (20): 3849-3854, 2012
ISSN: 1680-5593

Characterization of β-Actin Promoter of *Leuciscus merzbacheri*

[2]Wenge Hu, [1]Shiwei Ma, [1]Chuangfu Chen, [3]Yuanzhi Wang, [3]Yan Ren,
[3]Xudong Cao and [1]Jinliang Sheng
[1]School of Animal Science and Technology, [2]School of Life Science,
[3]School of Medicine, Shihezi University, 832003 Shihezi, China

Abstract: *Leuciscus merzbacheri* is a unique vulnerable indigenous fish which is only distributed in the Junggar Basin, Xinjiang. In this study, researchers cloned two *L. merzbacheri* β-actin promoter fragments of different length: SZ11 and SZ21 and analyzed their structural features. The mammalian expression vector pEGFP-N1 was used to construct eukaryotic expression vectors β1 pEGFP-N1-AFP III and β2 pEGFP-N1-AFP III in which fish type III antifreeze protein gene was used as the structural gene. The results showed that the cloned two fragments of β-actin promoters had the ability to drive the expression of the green fluorescent protein gene in BHK-21 cells. These data suggest that the vectors researchers constructed based on β-actin promoter could be exploited as all fish recombinant eukaryotic expression vector.

Key words: *Leuciscus merzbacheri*, promoter analysis, *β-actin* gene, green fluorescent protein, China

INTRODUCTION

Promoter is an important component of the expression vector used in creating transgenic organisms in eukaryotes which controls the expression of exogenous gene in genetically modified organisms (Ni *et al*., 2008). β-actin is the most important non-muscle or cytoplasmic isoform of actin and is expressed in the majority of non-muscle cells and undifferentiated myoblasts in eukaryotes (Pederson and Aebi, 2002). The promoters of β-actin have been isolated from carp, grass carp, blunt snout bream and have been verified to be able to drive gene expression (Liu *et al*., 1990; Robert *et al*., 1989; Williams *et al*., 1996). Different promoters of *β-actin* gene from different fish have shown different activities (Hwang *et al*., 2003). In addition, the different fragments of regulatory sequences of *β-actin* gene from the same fish have different promoter activity (Liu *et al*., 1990). Therefore, it is necessary to characterize β-actin promoter from different fish and different fragments of promoter from the same fish.

Leuciscus merzbacheri is a unique Xinjiang indigenous fish listed as vulnerable fish and its genetic resources waits to be exploited (Li *et al*., 2007). The purpose of this study is to clone and characterize the promoter of *L. merzbacheri β-actin* gene in order to construct all fish recombinant eukaryotic expression vector based on the analysis of the structural features of the promoter. The results showed that the cloned two β-actin promoters SZ11 and SZ21 had the ability to drive the expression of the green fluorescent protein gene in BHK-21 cells.

MATERIALS AND METHODS

L. merzbacheri was collected from Sailimu lake, Xinjiang. The Baby Hamster Kidney fibroblasts (BHK-21) was commercially available cell line. Plasmid vector pEGFP-N1-of AFP III containing Enhanced Green Fluorescent Protein (EGFP) reporter gene was a gift from Dr. Liangbiao Chen (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). EcoR I, Aat II and T4 DNA ligase were purcahsed from TaKaRa (Dalian, China). Lipofectamine TM 2000 was from Invitrogen. Cell culture medium DMEM was from Gibco. Primer synthesis and DNA sequencing were conducted by Sangon Biological (Shanghai, China).

PCR: Genomic DNA was extracted from *L. merzbacheri*. To amplify β-actin promoter from *L. merzbacheri* genomic DNA, three degenerate primers were designed and synthesized in reference to NCBI sequence number M24113 and M25013 as well as the primers reported earlier (Hwang *et al*., 2003). The primers were as follows: βA-P1 (Upper) 5'-GTGT/AGTGACGCT/CGGA CCAATC-3', βA-P2 (Down) 5'-CCATG/ATCA/GTCCCAG TTGGTG/CACAAT-3' and βA-P3 (Down) 5'-CCA GAGTCCATCACGATACCAGT-3'. PCR amplification

Corresponding Author: Chuangfu Chen, School of Animal Science and Technology, Shihezi University, 832003 Shihezi, China

program: initial denaturation at 94°C for 4 min; denaturation at 94°C for 50 sec, annealing at 63°C for 50 sec and extension at 72°C for 1 min, 30 cycles; extension at 72°C for 10 min. PCR products were cloned into pBS-T vector and named as pBS-SZ11-P and pBS-SZ21-P, respectively. The recombinant plasmids were verified by DNA sequencing.

Promoter sequence analysis: Software DNAman 5.2.2 and NCBI online blastn were used to compare SZ11 and SZ21 sequence with homologous sequences from carp and grass carp. Software GenScan (http://genes.mit.edu/GENSCAN.html) was used in combination with sequence alignment to predict intron and exon splice junction of the cloned promoters. Matinspector software and Match program were used to predict the structural characteristics of the cloned promoters. In order to ensure the specificity of the transcriptional regulatory elements, the binding sites in the core area with the correlation coefficient >0.9 were selected.

Construction of pEGFP-N1- AFP III vectors: Eukaryotic expression vector pEGFP-N1-AFP III was digested by Aat II to destroy its CMV promoter and was named as pEGFP-N1-AFP III (CMV dead). Aat II restriction site GACGTC was introduced into 5' end of upstream primer βA-P1 (labeled as SZ1A-U 5'-CGGACGTCGTGAGTGA CGCTGGACCAATC-3'). PCR products were cloned into pBS-T vector and then subjected to Aat II/EcoR I digestion to release the SZ11 and SZ21 fragments which were then cloned into pEGFP-N1-AFP III (CMV dead) and named as β1 pEGFP-N1-AFP III and β2 pEGFP-N1-AFP III. The recombinant plasmids were verified by DNA sequencing.

Cell culture and transfection: BHK-21 cells were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum, 100 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. pEGFP-N1-(CMV dead)-AFP III was linearized by Vsp I, β1 pEGFP-N1-AFP III and β2 pEGFP-N1-AFP III were linearized by Aat II and transfected into BHK-21 cells grown to 75-80% confluence using lipofectamine TM 2000 (Invitrogen). About 24 h after transfection, BHK-21 cells were examined under the fluorescence microscope to observe the expression of green fluorescent protein. When green fluorescent cells were observed, cells were screened with 400 μg mL^{-1} G418 until the green fluorescent cell clones appeared.

RESULTS

Analysis of β-actin promoter of *L. merzbacheri*: The size of promoter regions amplified from *β-actin* gene of *L. merzbacheri* was 1870 bp (SZ11) and 2398 bp (SZ21) and they showed 60.46 and 58.72% homology, respectively with the homologous sequences of carp (NCBI sequence number M24113). The sequences of SZ11 and SZ21 were shown in Fig. 1 although, they had the same 5 'end sequence, SZ21 was 528 bp longer than SZ11 in the 3' end.

Both promoter fragments contained 104 bp of 5' flanking proximal promoter and the 5' flanking sequence contained 3 core transcription closely related elements present in most fishes: CAAT-box, CArG motif and the TATA-box which were located at -89, -59, -26 upstream of the transcriptional start site (+1). In splicing intron, the splice sites were all GT AG. The 1766 bp long open reading frame of SZ11 promoter region contained partial sequences of exon 1-3 while the 2294 bp long open reading frame of SZ21 promoter region contained partial sequences of exon 1-4. In SZ21 promoter region, G + C contents of intron 1-3 exon were 39.3, 29.4, 40.9 and 56%, respectively consistent with previous results that non-coding region of fish genes had high A + T contents (Liu *et al*., 1998).

Furthermore, researchers analyzed the potential regulatory elements present in β-actin promoter of *L. merzbacheri*. The results showed that SZ21 promoter region contained many important transcriptional regulatory elements including one CAAT-box, 2 CC (A/T), 6 GG-boxs, 10 TATA-boxs, 1 MEF2, 2 SATBs, 1 CHRF, 1 INRE, 1 MTEN, 2 E-boxs, 1 RU49, 2 ZBPF, 3 CREBs, 1 enhance region, 7 CEBPs (except for MEF2 and MTEN, the correlation coefficients for all elements were >0.9). Compared to SZ11, SZ21 promoter region contained one more Caat binding site (2140-2154 bp) in the third intron and one more zinc binding protein binding site (2206-2228 bp) in the fourth exon (Table 1).

Characterization of promoter activity of SZ11 and SZ21 regions: To characterize the promoter activity of SZ11 and SZ21 regions cloned from β-actin promoter of *L. merzbacheri*, researchers made CMVΔ, β1, β2 vectors and transfected them into BHK-21 cells. The results showed that 24 h after transfection, scattered green fluorescent cells appeared in β1 and β2 group but none of the green fluorescent cells appeared in the CMVΔ and blank group, 36 h after transfection, the number of green fluorescent cells in β1 and β2 groups increased but still no green fluorescent cells were detected in the blank group and CMVΔ group (Fig. 2 A-D). After G418 selection, a

Fig. 1: The sequences of β-actin promoter of *Leuciscus merzbacheri*. Shown were the sequence of SZ21 fragment and SZ11 fragment contained the sequence from -104~ to 1766. +1 indicated transcriptional ignition site

larger number of green fluorescent cell clones appeared in β1 and β2 group and after several passages, green fluorescent protein continued to be expressed in BHK-21 cells (Fig. 2E and F).

These results demonstrated that both SZ11 and SZ21 regions exhibit promoter activity that could drive the expression of exogenous genes in eukaryotic cells. SZ11 and SZ21 regions are integrated into the genome of

Table 1: Predicated regulatory elements in SZ21 region of β-actin promoter of *L. merzbacheri*

Regulatory elements	Position (5'~3', bp)	Core score	Matrix score	Character and function
CAAT box	-89~-85	1.000	1. 000	CAAT box in core promoter
CC(A/T)GG box	-59~-50//1176~1194[a]	1.000	1.000	CArG basic sequence in core promoter
TATA box	-26~-22// 105~121[a]//116~132[a]//186~202// 450~466[a]//453~467[a]//834~850[a] //893~909//1176~1192[a]// 1372~1388[a]	1.000	1.000 >0.9	TATA box in core promoter
MEF2[b]	273~295	1.000	0.773	Muscle cell specific enhancer binding factor
SATB	244~258[a]//247~261	1.000	>0.9	binding protein of AT rich DNA sequence
CHRF	300~312	1.000	>0.9	Regulator of cell cycle
INRF	429~439	1.000	>0.9	Initiator element of core promoter
MTEN[b]	513~533[a]	1.000	0.801	basic sequence and element of core promoter
E-box	1306~1318//1467~1481	>0.9	>0.9	E-box binding factor
RU49	1721~1727	1.000	>0.9	Zinc transcriptional factor
ZBPF	73~85//2206~2228	1.000	>0.9	Zinc binding protein factor
CREB	275~295//443~463//967~987	1.000	>0.9	cAMP responsive element binding protein
Enhance region	1175~1225	1.000	0.941	Enhance region
CEBP	771~785//1008~1022//1093~1107 //1094~1108[a]//1395~1409// 1564~1578[a]//2140~2154			Ccaat/enhancer binding protein

[a]Represented the antisense sequence; [b]Due to the importance of the role of MEF2 and MTEN although their matrix scores were only 0.773 and 0.801, they were listed

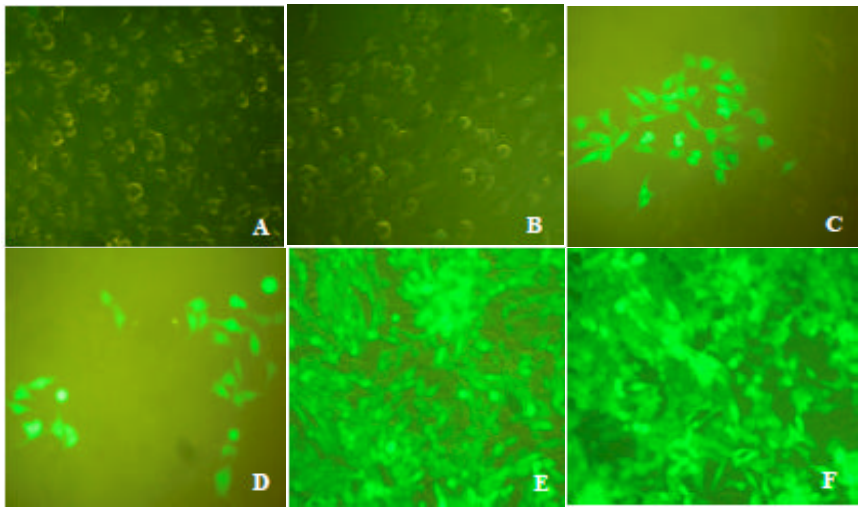

Fig. 2: Fluorescent imaging of BHK-21 cells. About 36 h after transfection, no green fluorescent cells were detected in; A) the blank liposome transfected cells; B) CMVΔ transfected cells while a number of green fluorescent cells were detected in; C) β1 vector transfected cells and D) β2 vector transfected cells. In addition, almost all cells were fluorescent in; E) stable BHK-21 cells transfected by β1 vector and F) transfected by β2 vector. Magnification fold: 400x

BHK-21 cells. PCR analysis showed that the expected bands with the size of 1870 and 2398 bp were detected in β1 and β2 transfected stable BHK-21 cells, respectively while no bands were detected in the control CMVΔ group and blank group (Fig. 3). These results indicated that the promoter regions SZ11 and SZ21 were integrated into stable BHK-21 cells researchers selected by G418.

The regulatory sequences of fish *β-actin* gene are widely used in transgenic studies in fish (Hwang *et al*., 2003). Elements implicated in the downregulation of transcription are mainly located in the 3' untranslated region and promoters of *β-actin* gene without these elements are constitutively expressed in a variety of cell types including muscle cells (Kato *et al*., 2007). The promoter of β-actin contains the typical 5' flanking regulatory elements CAAT box, the TATA box and the CArG motif. The activity of β-actin promoter is regulated by 5' untranslated region in addition to 5' flanking regulatory elements. It has been confirmed that the first

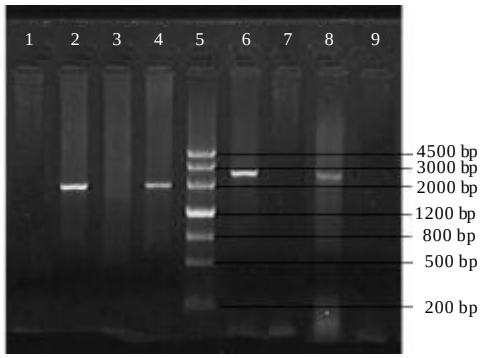

Fig. 3: PCR amplification of SZ11 and SZ21 from stable transfected BHK-12 cells. 1) pEGFP-N1-AFPβ (negative control); 2) β1 pEGFP-N1-AFPβ (positive control); 3) CMVΔ transfected cells; 4) β1 transfected cells; 5) DNA Marker; 6) β2 pEGFP-N1-AFPβ (positive control); 7) CMVΔ transfected cells; 8) β2 transfected cells; 9) pEGFP-N1-AFPβ (negative control)

intron of the *β-actin* gene contains enhancer (DePonti-Zilli *et al*., 1998). In addition, human *β-actin* gene contained the 5' flanking sequence and its first intron had stronger initiating transcriptional activity than the SV40 early promoter (Robert *et al*., 1989). The cloned promoter regions of *L. merzbacheri β-actin* gene SZ11 and SZ21 contained proximal promoter elements CCAAT box, the CC (A/T) 6GG box, TATA box and the enhancer sequence in the first intron but did not contain the 3'untranslated down regulatory sequence. The proximal promoter element CC (a/T) 6GG sequence was located at -59 to -50 upstream of the transcription starting point and at 1176~1194 in the antisense sequence of the first intron. The TATA box was located at 26 to -22 upstream of the transcription starting point and at multiple sites in the sense and antisense sequences of the first intron. These data suggest that the first intron of *L. merzbacheri β-actin* gene plays an important role in initiating transcription of β-actin.

Next, researchers wondered whether SZ11 and SZ21 have the ability to initiate the transcription of green fluorescent protein in eukaryotic cells. Researchers successfully constructed a promoter absent vector pEGFP-N1-of AFP III (CMV dead) based on eukaryotic expression vector pEGFP-N1-AFP III. The CMV promoter in the vector pEGFP-N1-AFP III was located at ~589 bp position where 4 Aat II restriction sites were located. Researchers removed the 126~447 bp nucleotide in the CMV promoter region by cutting the vector with Aat followed by self-ligation to destroy the CMV promoter. In order to confirm that CMV dead pEGFP-N1-AFP III (CMVΔ) loses the ability to drive the expression of green fluorescent protein, researchers lineralized CMVΔ and transfected it into HK-21 cells. Researchers could not detect green fluorescent cells after transfection, confirming that CMV promoter missing 322 bp nucleotide sequence lost its promoter activity. Based on CMVΔ vector we constrcuted the recombinant vector β1 and β2 pEGFP-N1-AFP which had following characteristics: Enhanced Green Fluorescent Protein gene (*EGFP*) as the reporter gene, neo gene as downstream screening marker, fish *AFP III* gene located is between SZ11 or SZ21 and EGFP. To examine the ability of promoters SZ11 and SZ21 to drive transcription of exogenous gene, researchers linearized the vectors and transfected them into HK-21 cells. Researchers detected green fluorescent cells as expected. Because CMV promoter was inactivated in the vectors due to the deletion, the expression of EGFP was driven by promoter SZ11 or SZ21. To further confirm that the expression of EGFP is initiated by SZ11 and SZ21, researchers selected stable BHK-21 cells by G418 and extracted genomic DNA from green fluorescent BHK-21 cells and performed PCR analysis.

CONCLUSION

The results demonstrated the existence of the target fragment, confirming that the promoter regions SZ11 and SZ21 were integrated into stable BHK-21 cells. Taken together, researchers characterized the structural and functional characteristics of β-actin promoter of *Leuciscus merzbacheri* and the vectors researchers constructed based on β-actin promoter could be exploited as all fish recombinant eukaryotic expression vector for transgenic fish research.

ACKNOWLEDGEMENTS

This research was supported by grants from the 973 Program (2010CB530200) and Projects of Cultivating New Varieties by Transgenic Technology (2009ZX08005-003B). Wenge Hu and Shiwei Ma contributed equally to this research.

REFERENCES

DePonti-Zilli, L., A. Seiler-Tuyns and B.M. Paterson, 1998. A 40-base-pair sequence in the 3'end of the *β-actin* gene regulates β-actin mRNA transcription during myogenesis. Proc. Natl. Acad. Sci., 85: 1389-1393.

Hwang, G.L., M. Azizur Rahman, S. Abdul Razak and F. Sohm *et al*., 2003. Isolation and characterisation of tilapia β-actin promoter and comparison of its activity with carp β-actin promoter. Biochim. Biophys. Acta, 1625: 11-18.

Kato, K., M. Takagi, Y. Tamaru, S.I. Akiyama, T. Konishi, O. Murata and H. Kumai, 2007. Construction of an expression vector containing a β-actin promoter region for gene transfer by microinjection in red sea bream Pagrus major. Fish. Sci., 73: 440-445.

Li, D.L., C. Fu, W. Hu, S. Zhong, Y. Wang and Z. Zhu, 2007. Rapid growth cost in all-fish growth hormone gene transgenic carp: Reduced critical swimming speed. Chin. Sci. Bull., 52: 1501-1506.

Liu, Z., Z. Zhu, K. Roberg, A.J. Faras, K.S. Guise, A.R. Kapuscinski and P.B. Hackett, 1998. The *β-actin* gene of carp (*Ctenopharyngodon idella*). Nucleic Acids Res., 17: 5850-5850.

Liu, Z.J., B. Moav, A.J. Faras, K.S. Guise, A.R. Kapuscinski and P.B. Hackett, 1990. Functional analysis of elements affecting expression of the *β-actin* gene of carp. Mol. Cell Biol., 10: 3432-3440.

Ni, J., K.J. Clark, S.C. Fahrenkrug and S.C. Ekker, 2008. Transposon tools hopping in vertebrates. Brief Funct. Genomic. Proteomic., 7: 444-453.

Pederson, T. and U. Aebi, 2002. Actin in the nucleus: What form and what for. Struct. Biol., 140: 3-9.

Robert, M.F., R.M. Monette and I. Aikichi, 1989. 5'flanking and first intro sequences of the human *β-actin* gene required for efficient peomoter activity. Nucleic Acids Res., 17: 254-270.

Williams, D.W., F. Muller, F.L. Lavender, L. Orban and N. Maclean, 1996. High transgene activity in the yolk syncytial layer affects quantitative transient expression assays in zebrafish (Danio rerio) embryos. Transgenic Res., 5: 433-442.