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ORIGINAL ARTICLE

Genomic organization and hypoxia inducible factor responsive regulation of teleost *hsp90β* gene during hypoxia stress

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Abstract

Background The physiological significance of a large family of heat-shock proteins (HSPs), comprised of the cytosolic HSP90A and the endoplasmic reticulum component of HSPB, is evident in prokaryotes and eukaryotes. The HSP90A is believed to play critical roles in diverse physiological functions of cell viability and chromosomal stability including stress management. Heightened abundance of *hsp90β* transcript was documented in *Channa striatus*, a freshwater fish, which is capable of surviving within an extremely hypoxic environment.

Methods and results To better understand the mechanism of *hsp90β* gene expression, we investigated its genomic organization. Eleven exons were identified, including a long upstream intron with a remarkable similarity with human, but not with chicken counterpart. Dual-luciferase assays identified promoter activity in a 1366 bp 5′-flanking segment beyond the transcription initiation site. Examination detected a minimal promoter of 754 bp containing a TATA-box, CAAT-enhancer in addition to providing clues regarding other enhancer and repressor elements. The driving capability of this minimal promoter was further validated by its binding ability with TATA-box binding protein and the generation of GFP expressing transgenic zebrafish (F_2). Further, deletion of an inverted HIF (hypoxia inducible factor) motif RCGTG (upstream of the TATA-box) dramatically reduced luciferase expression in a hypoxic environment ($CoCl_2$ treated cultivable cells) and was identified as a *cis*-acting HIF responsive element, necessary for the hypoxia-induced expression.

Conclusions The results obtained herein provide an insight regarding how *hsp90β* gene expression is controlled by HIF responsive element in teleost both during hypoxia stress management and normal physiological functions, and suggested that the *hsp90β* gene promoter could be used as a potential candidate for generating ornamental and food-fish transgenics.

Keywords *hsp90β* gene · Hypoxic gene regulation · *Channa striatus* · Promoter · HIF induced expression

Introduction

Heat-shock proteins (HSPs), a large family with varying molecular weights, are termed molecular chaperones. Ample evidences suggested that HSPs play critical roles in protecting organisms from environmental stress-mediated cellular damages [1]. There are mainly three families of HSPs consisting of two high molecular weights such as HSP90 (85–90 kDa) and HSP70 (68–73 kDa), and a low molecular weight HSP (16–47 kDa). HSP90 and HSP70 are mainly associated with the critical functions linked with folding and assembly of other cellular proteins [2, 3]. They also maintain protein homeostasis pertaining to chaperon functions, minimizing apoptotic cell death, and cellular stress management [4–6].

HSP90 is a highly conserved family of proteins consisting of cytosolic HSP90A and HSPB in the endoplasmic reticulum and are commonly expressed in both prokaryotes and eukaryotes [7]. Subfamilies of HSP90A are HSP90β1, HSP90β2 and HSP90β proteins. Cytosolic HSP90 homologues are encoded by two or more genes [8]. HSP90

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constitute 1–2% of total protein in non-stressed cells and suggested to participate in normal physiological and biological functions [9]. However, its increased amount (4–6%) was documented under stress conditions [10]. HSP90β is constitutively expressed in the cytosol [11], while HSP90β1 and HSP90β2 are reported to be inducible [12]. HSP90β is believed to be responsible for cell viability. Accumulated evidences suggest that HSP90 participates in diverse physiological functions including signal transduction [13, 14], assembly/disassembly of several protein complexes [15], skeletal muscle differentiation [16] and chromosomal stability [17]. The physiological role of HSP90 during biotic and abiotic stresses has been documented [18–20]. Elevated transcript abundances were documented in goldfish (*Carassius auratus*) in summer [21] and during heat exposure in two bay scallop populations (*Argopecten irradians irradians* and *Argopecten irradians concentricus*) [22]. The hsp90 gene expression was induced following bacterial infection in Ya-fish, *Schizothorax prenanti* [23]. The hsp90b gene expression was potentiated during ammonia toxicity and bacterial infection in riverine fish, *Botia reevesae* [24]. However, functional implications of each subunit remain to be elucidated.

Aquatic organisms are exposed to various environmental biotic and abiotic stresses. Heightened expression profiles of HSPs were documented in teleosts exposed to several aspects of altered micro-environmental stress factors like that of salinity, temperature gradients, heavy metal contaminations, etc. [19, 25–29]. The fish also represent a valuable model vertebrate for undertaking experiments to elucidate the physiological functions of HSPs [21].

The *hsp90* genes of chicken [30, 31] and human [32, 33] have been cloned and characterized. The presence of common putative elements like HSE, a CAAT box, and Sp1 sites near the TATA box are documented. The information on these aspects in teleosts is very limited despite being the most diversified Kingdom. The promoter region of *hsp90* gene was cloned and sequenced in scallop (*Argopecten irradians irradians* and *Argopecten irradians concentricus*) [22]. However, in vivo promoter activity data was lacking.

Channa striatus (Family: Channidae) is a freshwater murrel fish with commercial importance and therapeutic value. Even though murrel is an air-breathing fish (having accessory respiratory organ), its natural habitat in muddy and marshy waters is well known. Interestingly, it can manage drought conditions by hibernating under hard-backed mud crust [34]. Thus, this species is very useful to undertake studies on physiological pathways associated with adaptations in severely hypoxic conditions. Adaptations to hypoxic and near anoxic conditions are linked with behavioural changes of hypo-metabolic activities [35]. The *hsp90β* gene is reported to be over-expressed during hypoxic conditions [36]. Currently, 'climate change' is a global concern and hence it is essential to better understand the mechanism of expression of each member of the HSP family during biotic and abiotic stress. Because HSP90β is involved in normal physiological functions and hypoxia stress adaptation, we intended to clone and characterize the *hsp90β* gene of murrel, including characterization of its promoter activity with an intention to understand its mechanistic expression.

Material and methods

Fish sample and genomic DNA extraction

Adult *C. striatus* (about 500 g weight) fishes were collected from the Institute's farm (ICAR-Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, India). Genomic DNA was isolated from liver following standard protocol of phenol–chloroform extraction as described earlier [37].

Isolation and sequencing of 5′-flanking genomic region and intron sequences of *C. striatus hsp90β* gene

To isolate the full-length gene sequence including the 5′-upstream region of the gene with the promoter, four different restriction genomic DNA libraries were constructed using PCR based Genome Walker Universal Kit (Clontech, USA) as per manufacturer's instructions and described elsewhere [38, 39]. Briefly, Genomic DNA was extracted from *C. striatus* liver and digested with four restriction-enzymes (*Dra*I, *Eco*RV, *Pvu*II and *Stu*I). The digested product was then ligated with Genome Walker adaptor (Clontech). The adaptor-ligated genomic DNA was used as a template for the PCR amplifications. We cloned and characterized *C. striatus hsp90β* cDNA as reported earlier [36]. We designed several primer sets from our sequence as well as sequence information of other species available in the public domain. To amplify the 5′ upstream region, the restriction digested libraries were amplified independently using a gene specific reverse primer (5′-GCTTTGGTCATCCCAATTCCAGTG TCA-3′) designed from the second exon and forward adaptor primer 1 (AP1; 5′-GTAATACGACTCACTATAGGGC-3′). Fifty-fold diluted PCR products were used as the template for the second PCR amplification using GSP2 (5′-TGCGGT CAGCTTTGTTTGGGATGATG-3′) and nested AP2 (5′-ACTATAGGGCACGCGTGGT-3′). Finally, *Eco*RV restriction library generated a single band of 2.4 kb (above the ATG) that was gel-excised, purified, cloned into pGEMT-Easy vector (Promega, USA) and bi-directionally sequenced. Similarly, several primer sets were designed and synthesized to amplify Introns from these Genome-Walker libraries. Few important primers are enlisted in supplementary Table 1. All

PCR products were then cloned into pGEM-T easy vector (Promega) and sequenced bi-directionally.

For analyzing regulatory sequences in the putative promoter region, NNPP (Promoter Prediction by Neural Network), Mat Inspector tool (http://www.genomatix.de/) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) were applied together to make a comprehensive prediction.

Reporter constructs

To construct a luciferase reporter vector using the 5′ upstream region of the *hsp90β* gene, the − 1366/+ 1 genomic sequence of the gene was analyzed. A series of 5′deletions of the *hsp90β* promoter were constructed by PCR amplification and fusion to a luciferase gene of pGL4-Basic vector (Promega, USA). Five deleted constructs: HSP90β.C1, HSP90β.C2, HSP90β.C5, HSP90β.C6, HSP90β.Δhif.C5 were prepared with incorporation of desired restriction sites in the amplifying primers by base modification. The full-length reporter construct (HSP90β.C1) contained all elements of the regulatory region (− 1269/+ 1) obtained from an amplified fragment using *Eco*RV modified forward primer 5′-atgttccgtcttgga**tatc**gcaccctg-3′ and *Bgl*II modified reverse primer 5′-gtgccttctgc***aga***tctgacgcagg-3′. The HSP90β.C2 was constructed from an amplified fragment using *EcoR*V modified forward primer 5′-tccgtcttggat**atc**gcaccctg-3′ and *Bgl*II modified reverse primer 5′-tccacag**a**tc**t**agaccgagc-3′. The HSP90β.C5 reporter construct was prepared from an amplified fragment using *Xho*I modified forward primer 5′-caggtcttt**ctcgag**tcaggccca-3′ and *Bgl*II modified reverse primer 5′-gtgccttctgc**agatct**gacgcagg-3′, while the reporter HSP90β.C6 was derived from an amplified fragment using *Xho*I modified forward primer 5′-gtg**ctcgag**aaacgaagcacgccttcc-3′ and *Bgl*II modified reverse primer 5′-gtgccttctgc**agatct**gacgcagg-3′. All the amplified fragments digested with their respective restriction enzymes were inserted into the multiple cloning site of PGL4-Basic vector and transformed in DH5α competent cells. The correct orientation of the promoter was validated by sequencing.

HIF (Hypoxia inducible factor) element was deleted from the HSP90β.C5 to generate HSP90β.Δhif.C5. Briefly, PCR amplification was carried out for HSP90β.C5 DNA using upstream forward primer (5′- ctagcctcgagtcaggccca-3′) contain *Xho*I site (− 753) and reverse primer (5′- ggctccaggattacaccactgacg-3′ modified for *Pfo*I site, − 221) situated upstream to HIF element. The amplified fragment was gel-extracted and ligated to *Xho*I and *Pfo*I (located − 132 bp downstream to HIF site but upstream to TATA-box) digested larger fragment of HSP90β.C5. This resulted to generate HSP90β.Δhif.C5 lacking HIF element. For luciferase analysis the construct was transfected into *Escherichia coli* BL21 competent cells.

Cell maintenance, transient transfection and dual luciferase assay

HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, USA) with 10% FBS (Invitrogen, USA), 0.01% MEM non-essential media and 0.01% Sodium pyruvate at 37 °C in a humified CO_2 (5%) incubator (Eppendorf, New Brunswick, USA). Dual-luciferase assay was performed as reported earlier [40, 41]. To measure luciferase activity, each reporter construct (1 µg/well) along with co-reporter vector pRL (Rinella luciferase vector, Promega, USA) were transiently transfected into HEK293 cells (1×10^5 cells/well of 24-well plate) using Lipofectamine 2000 (Invitrogen, USA) as per manufacturer's protocol. Cells were harvested 48 h post-transfection, lysed with100 µl passive lysis buffer (Promega, USA), centrifuged at 12,000 rpm for 5 min to collect the supernatant. The supernatant was used to measure luciferase activity using Dual Luciferase Reporter Assay System (Promega, USA). The promoter activity was expressed by relative luciferase activity (RLA). Three independent experiments were performed with triplicate wells.

Transient transfection and reporter gene assays for validation of activity of HIF

To assay the activity of HIF, the HEK293 cells were incubated with $CoCl_2$ (Cobalt chloride) solution for hypoxia treatment. To optimize the $CoCl_2$ concentration that maintains hypoxic condition without compromising cell viability, the cells were incubated in DMEM complete media with different serial dilutions of $CoCl_2$ i.e. 150 µM, 100 µM, 50 µM, 25 µM and 0 µM for 24 h. According to the significant expression of HIF and microscopic observation of cell phenotype, $CoCl_2$ at a final concentration of 100 µM was selected to introduce hypoxia. Transfection was performed using Lipofectamine 2000 transfection reagent in a 24 well plate with 1×10^5 cells per well (500 µl per well; DMEM complete media without antibiotics) at 80–90% confluence. The vector-containing-promoter in the concentration of 1 µg was co-transfected with control vector pRL-SV40 (Promega, USA) in the molar ratio 1/50. HSP90β.C5, HSP90β.ΔC5 and HSP90β.C6 constructs were transfected in two sets—one set was treated with 100 µM $CoCl_2$ (hypoxic) and another set was without $CoCl_2$, which served as a control. The $CoCl_2$ media was treated 24 h post-transfection and incubated for 24 h in a conventional incubator (37 °C, 5% CO_2). After 48 h post-transfection, luciferase activity was measured. Luciferase activity was quantitated in a Luminometer using the Dual-Luciferase-Reporter assay kit (Promega, USA).

DNA immunoprecipitation

DNA immunoprecipitation was carried out by using the Immunoprecipitation Kit Dyna-bead Protein G (Invitrogen, USA) following manufactures instruction. Recombinant TATA Binding Protein (TBP) (Cloud—Clone Corp., USA) and anti-TBP antibody, mouse monoclonal (Gene Tex Inc., USA) were used for this assay. Briefly, the Dyna-bead Protein G was conjugated with anti-TBP antibody (Sigma-Aldrich) within the supplied binding buffer for 15 min at room temperature. In other reaction, 436 bp DNA fragment amplified from HSP90β.C6 (containing TATA element) was incubated with TBP protein with binding buffer at the same condition. Both incubated samples Dyna-anti-TBP conjugated protein G and TBP mixed DNA fragments were allowed to mix together and incubated for 30 min at room temperature in a rotator. Subsequently, the precipitate was washed three times by using supplied washing buffer to remove the excess unbound DNA. Rabbit IgG (in place of anti-TBP) was used as a control in a separate reaction. The final eluted products were subjected to PCR amplification (at an annealing temperature 58 °C) followed by 1.2% agarose gel electrophoresis, stained with ethidium bromide and photographed.

Transgenic zebrafish using Tol2 transposon system

The Tol2 transposon construct was prepared as described [42, 43]. Briefly, multisite recombination reactions were performed using the Tol2 kit—three entry donor vector system namely, p5E-MCS containing multiple cloning sites for ease of promoter sequence insertion, pME-EGFP middle entry vector containing an in built EGFP reporter gene and p3E-polyA SV40 signal sequence. The p5E-MCS vector was used to clone our *Xho*I/*Hind*III digested *hsp90β* promoter sequence. Multigate cloning was performed by combining these three vectors with pDestTol2pA2 destination vector for multigate recombination reactions using the LR clonase. The resultant destination vector (here after pTol2-Hsp90-eGFP) contained at its 5′-end our promoter followed by middle-EGFP and 3′polyA signal element—order was confirmed by sequencing.

Transposase RNA was synthesized using the pCS2FA-transposase plasmid as a template. DNA was linearized with *Not*I and purified using the Qiagen PCR Purification Kit. Capped RNA synthesis was performed using the mMessage mMachine SP6 kit (Ambion, Austin, TX, USA). One cell stage embryos were injected with 25 pg of pTol2-Hsp90-eGFP plasmid along with 25 pg Transposase mRNA. Embryos were raised in E3 embryonic media at 28.5 °C for 5 days and then to adult hood in a standalone zebrafish system. Bright field and fluorescent images were taken using Leica MZ16 FA stereo microscope.

Fig. 1 *hsp90β* gene structure of *Channa straitus*. **A** The 5′- upstream sequences of *C. straitus hsp90β* gene showing promoter and other regulatory elements. The + 1 indicates the transcriptional initiation site and the box shows the transcription initiation site (ATG). The putative TATA, GATA, HIF, E-Box, STAT, CAAT, E4BP4, etc. are marked by arrows. The 11 exons are underlined. **B** Schematic representations showing comparative *hsp90β* gene structure in *C. straitus*, *Homo sapiens* (GenBank Accession no. J04988), and *Gallus gallus domesticus* (GenBank Accession no. X83230)

Results

Genomic organization of *hsp90β* gene

The *hsp90β* cDNA of *C. striatus* was isolated and characterized previously, however the information on *hsp90β* gene structure and promoter is lacking [36, 41]. Gene-specific primers with complete homology were designed to perform PCR based genome walking. The 5′-flanking sequence was amplified from the *Eco*RV library using specific reverse primers, designed from the first coding-exon of *C. striatus hsp90β*, 175 bp downstream of the translational start codon. Approximately, 2.4 kb upstream sequence from ATG start codon was obtained from *Eco*RV digested library and possible matching of *cis*-regulatory elements were retrieved from the Genomatix database. Similarly, intron sequences were amplified from the PCR amplifications of libraries using several primers designed from the cDNA sequence. Alignments of cDNA and genomic DNA sequences identified multiple introns. The identified *hsp90β* gene contained 11 exons (size ranging from 98 to 334 bp) and a noncoding exon of 64 bp (Fig. 1A, B). Exon–intron boundaries were lined with a typical donor–acceptor (gt-ag) conformation. A long intron of 1022 bp was detected. Contrary to the chicken *hsp90β* gene, which contains only 7 exons, there was a remarkable similarity with the human counterpart, consisting of 11 exons and a long upstream intron (Fig. 1B).

The postulated initiating methionine codon is at the coding exon 1. The transcription initiation site designated as position +1 was determined to be 1086 bp upstream from the translation initiation site (Fig. 1A). The DNA sequence in upstream transcription site constituting of 1366 bp was further analyzed using Transfact tool. This region predicted several DNA motifs, characteristic of promoter regions. A nucleotide sequence (TATATAG) which conforms to the Goldberg-Hogness consensus TATA box (TATA(A/T)A(A/T)) at positions − 51 bp upstream of the transcription initiation site. One more putative TATA box was predicted to be positioned at − 895 bp. One predicted CAAT box was positioned at − 1103 bp, which is far away from TATA-box. Additional consensus sequence, which is known as STAT3 enhancer, was predicted to be positioned at − 133 bp. Moreover, two subunits of STAT

A

CATATAAACATACCCCAAATCAGCAGCCGTATAAAGTAATAAAATAGCTGTCAAAAGGTTGATAATTAAAAAAAATCCTCGTAATGTTCCGTCTTGGATTAGCACCCTGGCTAGTAACAGGCCTTCGAAAACATTGACGCAAGCGCTTTACGTAC -1212

CAGATTACAGGCGCGCTTGTTTTGTGTGTCGGGATGTGGCGCTGACCTGGAAATGTTAGCTGCACGAGTTTGTATTACCTAACAACACCAAAGCCACAAAGCCTTCTGCAATTATAAAGACATAAAAGAACATTTTTACAATTCCCCGAATTTA -1057

E2F E4BP4 CAAT BOX

AGAAAATCAAGCTTTCGGTTTTACTTTTAAAAAGCCTCACCTAAGTCTAAGTCATCTCTCTATATAATAATAAATACGTAAAGTACAGTAAAGTTAGAGCGTCAAAAACTTTATTATCCAAAGCAAATATATTTATTTTGCATATCTGAGGAGCA -902

SATB1

GGAACATATAAATGATAATATTTCAGGAAAAAATAACAGATGCAAATAAACATTATCAAACTGTTTGTACATTAATATTCAAGAAATCAAATAATTTTTCCAATGGTTTCAGCTCGGTCTTGATTTGTGGAAAATTCTCAGGTCTTTGTAGAGTC -747

TATA BOX GATA BOX STAT

AGGCCCAGGTTTACTTGACTTTATTCCTGTTGGGGAAATTATTAGCTAAACAAGTCTTAAAAATTATTACATTAGGATGTAACCAAGGAAGCGCTATACAGTCGTCTTTATATGTGTATATGTATATATGTATTCTTTTTCTTCTTCTTATGATT -592

GTTATTATTGAAACAATATTATTATTATTTGATGCTAGCCGGTAAGCGCTCAGTTACCATGGTGTTTTTGTTGTAGATACTCGCGCTGTTCAGCAGCTGCACCGCGAGGCCTGCGTGTGACTAGTCCGTGACGTCAGTTTAGGCGGTTTTTTTC -437

X-BOX E2a

ATGGAAAGTGCTAGAAGAAACGAAGCACGCCTTCCCTTTTAAAAATATTCTTCCATCAGAATAAAAAAAACCCACCATCGTCATGTTACAAAACACCATAAAATAAACACATGGCTTGGAGTTTTCACCGATCATATTTTTACATCGATGCAGAT -282

STAT SALL1 E-BOX

ATTTCATTGTCAGTGGTATGTTGCGGCAACTTTCAAAAATCTAGTGCGTCAGTGGTGTAATGTGGAGCCGGAGCGTTTTTGGCGGAGGCCGCCGCACGGGGGCAGCAGCGGTTCGCTGGCAAACGGCTCGCAGCTGAATTCTAGCATGTTCCAGG -127

HIF +1 STAT3

AGAGAACTTCACCGCGGGCTGGAGCGGTAGGTGGTGCACCGCGGATGGATATAGCCGCACAGTGAAACGCGTGTGTATAAAGATTCCGGTTTGTTAAAAGGAGCTCCCTTTCTATTTCCCTGCGTCAGACAGCAGAAGGCACAGTATTTTGGTTG +29

TATA BOX Non- coding Exon

CATATTATTCAAGATAAGTCAACGAAACAAATAAGGTAGGTACGCTAGCGATTTGTTTTCAGTAGAGAATGTTATGGTCTATTAATATTTGCAAGAGCGTTACTAAACCAACCGTAACTAACGTTATTTAGTGTTTCCATGGTAAGTCACGACAG +184

TAACCTTTTAGCATCCCGGCCGCCTTGCTAATGAGCAGGTAGCATTAAACGGTTTGTCCGGCCACTGCTACGCGTCGTGAAACGGTAACGTTCACTGCTCCTGCCGCTGTGATTTTGTCGTGTAACGCTGCTGAGGGCCGGTGTCTGTGAAGGGT +339

GTGAATGCGGCACAGTGCAGCGCCGTTGAGCTCGGTTTGCACCAAGGCAGGTGCGGTGAAGTTCATATCGCCGACTCGGTTTACATAACGTTATTCATAACTAGCTGTGATAATTCACACACTAACCGACTAGCTTAAACACCCCCCACCCCATT +494

ACTGACGTTAAGCAGTTACCTTTAAAACGTTTAACTTCTCTTAACGTTACGACTTAACGGTAAAATCAATTCCTCTGTCGCTCGATCGTTGGTAACGTTAGTGCTGCGGTAACGGTTGATTACGTAACTAGGGTTACGATCGATTTGTGTTACGT +649

TTATGATAAACTAGTGTCTTCCGCTTTACACGTTACCCTAAATTACAAGTGATAGCTTTAGCTTCACGTGTCGTCCTCCATCATCACCCTCTTTTTTTAATTTTTACTTTCGTTTTAACATGCAGTCCAACGTTATATGTTCGGATTGATGTTCT +804

TCAGCACGTGTTTGCGGACTCCATTGTCTCAGTCACTCGACGCCATGTTGAATAACTGCTACTTCCGCCTTTCGATAGCAACACTGTGGCAACGGTCCCGCTAATAAAAACCTTTGTGCACTTGATCAGATTGTAGTGTACTCGTACGATGAAAC +959

TCCGCCCGGGTAGCTCACTGAAAGTAGTAATAGTTTTACCATCATTGTACTAGTTATTCTTGTCTCTTAAAAGTAATAGTATTTGGAGTCAAGTTGGATTATAGCCAATTTGGTTGTTTTCTGCAGGATGCCTGAAGAAATGCACCAAGAGGAGG +1114

Exon-1

AGGCTGAGACCTTTGCCTTTCAGGCAGAGATCGCTCAGCTAATGTCCCTGATCATCAACACCTTTTATTCCAACAAAGAGATCTTTCTCAGGGAGCTCATCTCCAATGCCTCTGATGTAAGTATGCAGATGCTGTTTTCTGTGTGTAAATTGTAA +1269

ATGCCTCTAGTGTGACAGCATTTTAATTAATGTTACTTTTGATAATGGCTTTAAAGCAGCTGTTGGGAAATCTATTGTAGAGTCTTAACCTGTCAATGTTTATAGGCTCTGGACAAAATTCGCTATGAAAGCCTGACAGACCCAACCAAGCTGGA +1424

Exon-2

CAGCGGCAAAGATCTGAAAATTGACATCATCCCAAACAAAGCTGACCGCACCCTGACCCTTATTGACACTGGAATTGGGATGACCAAAGCTGACCTCATTAACAACCTGGGTACCATCGCCAAGTCTGGCACCAAGGCCTTCATGGAGGCCCTTC +1579

AGGTATTACGATAAAATGTTGTCGAGACTGCTGCATGTAGAGAATTGCAGACCACTTCCTTTGTAACCCTGAAAGATTTATGGACTGCATTTAATCATCAGGGTTTGAGACTTCATAAATAATTGTTAATAGTTAATCCTTCTAATTGTGTTTTT +1734

CTAGGCTGGAGCTGACATCTCCATGATTGGACAGTTTGGTGTGGGTTTCTACTCTGCATATCTTGTTGCAGAAAAGGTTGTTGTCATTACCAAACACAATGATGATGAGCAGTATGCCTGGGAGTCCTCTGCCGGAGGTTCATTCACAGTCAGAG +1889

Exon-3

TTGACAATGGTACGAATCTAGGCATCACTAAAACAGCTATATTTGACATTGCATTTAGATTTAGTCATTTGGGAGATGCTTTTATCCAAAGTGACTTACAAAGTGAAGCATAATCAAAGTCAAGAAGGACCTCAAAGCAAAGTGCTAAGATCCAT +2044

TAAGATTTAAGTACATGAAAGGAGCGTTTGTTTTAAGTGCATAAGGAAGATGCAGAAGAGTTTACACTCTTGATATTCTACTTATTCTATCTTTGTTTATAAAATGTAAGTCCCACATGCTGCTTCTAGGTTAGAAATGGGCAGTTTCTATAACA +2199

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CTTTCAAATGTCAAATGTCAAATTGTCTTTTTGGACAAAGCATGTGACTTTCTGTGCGTTAATGTTCACAGGTGAGCCCATTGGTCGCGGACAAAAATTATCCTGTACCTGAAGGAGGACCAGACAGAGTACATTGAGGAGAAGCGGATCAAGGAAATTGT +2509

Exon-4

CAAGAAGCACTCCCAGTTCATTGGCTACCCCATCACCCTCTTTGTAAGCATGGAATTTCAGTAGATGACAATATTAAGTTTAGGCTAGTTTAAACTGTTTGGGCCACTGTAGCATTAACAACTAGTAATCTTTGATTGCAGGTAGAGAAGGAGCG +2664

Exon-5

TGACAAGGAGATCAGTGATGATGAGGCAGAGGAGGAAAAGACAGAGAAGGAGGATAAGGAAGAGAAGGAAGAAGGTGAGGACAAGCCAAAAATTGAGGATGTGGGCTCTGACGATGAGGAAGACTCAAAAGACAAGACAAGAAGAAGAAAAAGA +2819

AGATCAAGGAAAAGTACATCGACCAGGAAGAGCTGAATAAGACCAAGCCCATCTGGACCAGAAACCCTGATGACATCACAAACGAGGAGTACGGAGAGTTCTACAAGAGTCTGACTAATGACTGGGAGGATCACCTGGCTGTAAAGGTAAATGAT +2974

GAATAGAAAGAGAGTGGTTGCATGTAGCTTTCTACTAAATGTGGTCCATCGGTTTTAATTTTCCTTGTTGATTTCTGGCTTATTGCCCAGAGACGACCCTGCCCCAACTCCTTTCTTCATTTCTTCATTTGCATCTAAAATGTCCCAGCATAAGC +3129

TGCATTTATAAATTAGCATAAGCTGCATTTATAAATAAGTGAGCACACTTGTTGTTTTGGACAGTGCTTACTGATCTGCATGGGGGTGTATGTGTGTTCCACTGAAAAAGCATATTCCAGCTCTAAATTGTCAGGGCCCACTGGCTAAAAACTTA +3284

ATTGTACTCTCTTTTGACAGCACTTCTCAGTGGAGGGTCAACTTGAATTCCGGGCCCTGCTCTTTATTCCCCGCCGTGCACCTTTTGACCTCTTTGAGAACAAGAAAAAGAAGAATAACATCAAGCTGTACGTCAGGAGGGTTTTCATCATGGAC +3439

Exon-6

AACTGTGAAGAGCTCATCCCAGAGTACCTAAGTAAGTTGACTGTCTCCCACAAAATAAGTACCATAGCTCTTGCATTTAGCATTAGCTCACAGGCACTGCTTAAATGTAATGTTAAAATATGTTTGACTAAACTGTAATTAAAATTTGTTCCAGA +3594

Exon-7

CTTTGTGCGTGGTGTAGTGGACTCTGAGGACCTGCCCCTCAACATCTCCAGAGAAATGCTGCAGCAGAGCAAGATTCTCAAGGTCATACGCAAGAACATCGTCAAGAAGTGTCTAGAGCTCTTTGCTGGACTGGCTGAGGACAAGGAGAACTACA +3749

AGAAATTCTATGAAGCCTTTTCCAAAAACATCAAGGTGATGAAAAATGTGCAATTACAACAGTTAAAGTGTGTTTGCTTTAGACCACATTGGACCATAATTGTGTGTGAATGACAAGCAAAATAGTGAAGGACTTTGAATAAAAACTCTTAAGTG +3904

GCCATTTAATATGCATTTACCCAAAGAACAGCTGTAGTTCTGCACTGTTCTAAAAACATCAGATTGATATGCCAAGGTTTCCAGAAGCTGGTGTTCATTGTCAGCCTTCAGTTTGACATAAGACAAATGCTTAAGGGCAGTGGGCTATAAAGACC +4059

AGGTTTGGAAACCTTGGTCAGTTGTGCATAATTTGGAGTACGGAGTTCCAAAGCTTAAACTAAGTTGAAATGGTAACTCAACTTGTACAGTTGTGCCAGATTATTGTTGCTTGTGAAATGTATTTGTTTAATAGGGCCTAACTAGCCATCATAAT +4214

TCTTTTCAGGAAGCTGCTTTAACCCATGAACTGATTAGTGGCAATTAACCTTGTTTGGAATTCTATTGTGTTTTAACAATTGTCCATTATTGTCTCTGTAGCTGGGAATTCATGAGGATTCTCAAAACCGCAAGAAGCTCTCTGAACTGCTTCGT +4369

Exon-8

TATCACAGCTCCCAGTCTGGAGATGAGACAACTTCCCTCACAGAGTACCTTTCCCGCACAAAGGAGAACCAGAAGTCAATCTACTACATTACTGGTGAGTCCATAGAATCAATGTTTCCAGTAACACTAGAGCTTAAAAACTTCTAAGTCATTCA +4524

TAAAAGCTCACAAATTTGCACATAAGGAAAATTGTAATTGCATAAATTTGTATTTCATTTTCTTTTTTTAATCTCTAGGTGAGAGCAAGGATCAAGTGGCCAACTCTGCTTTTGTTGAGCGTGTCCGCAAGCGCGGCTTTGAGGTCCTGTACATG +4679

Exon-9

ACAGAGCCCATTGACGAGTACTGTGTCCAGCAGTTAAAGGAGTTTGATGGCAAGAGCCTGGTCTCAGTCACCAAAGAGGGCCTGGAACTGCCAGAGGATGAGGAGGAGAAGAAAAAGATGGAGGAAGACAAGGCCAAATTTGAGAACCTCTGCAA +4834

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Exon-10

CTCAGGGATAACTCTACCATGGGCTACATGATGGCTAAGAAGCATCTGGAGATCAACCCTGACCACCCCATTGTGGATACACTCAGACAGAAGGCTGAAGCTGACAAGAACGACAAGGCTGTGAAGGACCTCGTCATCCTGCTGTTTGAGACTGC +6229

CCTGCTGTCCTCAGGTTTCTCCCTGGATGACCCACAGACCCACTCCAACCGCATCTACAGAATGATTAAACTCGGACTGGGTAAGCACACACAGCAAATGCATATTTATATACTTGCAACATGCGGGTTGGTTCCATTGCTTTGTCAACTGACAT +6384

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Exon-11

AGATTCCAAACACTCCAGCCTCACTTTCAATTGTTCATCTTAAAACTGCAGTAACTGCAACACCAATAGTTGTTCATATTGTGTGGTGGACCAATGTTGCTCTTGTGTCTAGAGTATTTACTGCGAGACCTTTAAAAGCAGTTTTGGTTTTTCCT +6694

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GCTAATCATTGGGATTCTGACTTTAAGTTGGTTCAGGTTCTTTCATGTG +7053

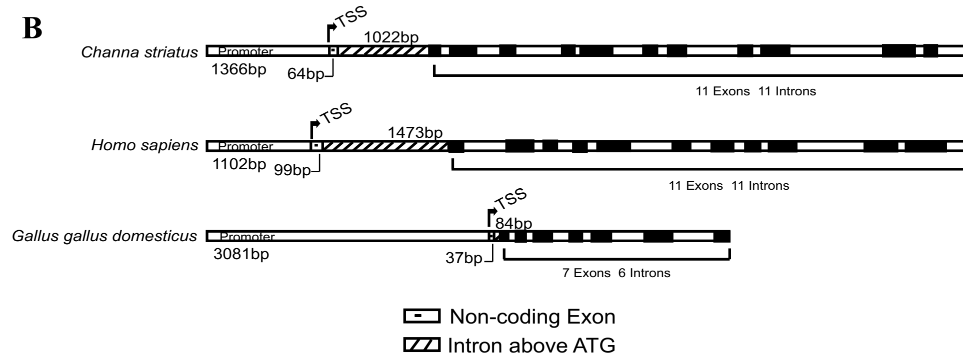

Fig. 1 (continued)

binding elements positioned at − 439 and − 880 bp, which are known to be involved in signal transduction and transcriptional activation, were identified. Two core consensus E-boxes (5′-CANNTG-3′) were also identified at position − 329 and − 498 bp (Fig. 1A). An E2F activator found at -1202. Enhancer GATA- was also detected at − 888 bp position (Fig. 1A). A special AT-rich region (termed) was predicted at − 881 bp and E4BP4 motif in negative orientation was found at −1130. A transcriptional repressor known as SALL1 (at − 376 bp) was also identified. Importantly, a putative HIF, defined as having a consensus core sequence (5'-RCGTG-3'), was predicted to be positioned at − 184 to − 187 bp upstream from the transcription initiation site. X-box is present at − 543 bp. These results indicated the presence of promoter and regulatory regions, which are responsible to drive *hsp90β* expression.

Promoter activity analysis of *hsp90β* gene

To identify core promoter along with other regulatory elements of the *hsp90β*, in vivo luciferase assays were performed using five different deleted constructs designated as HSP90β.C1 (1269 bp), HSP90β.C2 (492 bp), HSP90β.C5 (754 bp), HSP90β.C6 (436 bp) and HSP90β.Δhif.C5 (665 bp) and cloned into pGL4 luciferase plasmid (Fig. 2A). The constructs were transfected into the HEK293 cell line and the dual luciferase assay data is depicted in Fig. 2A. The highest luciferase activity (in the tune of about 40-fold as compared to control) was detected in cells transfected with HSP90β.C5 construct containing a TATA-box (spanning − 51 to − 46 bp). Contrary to this, the distantly positioned TATA-box containing HSP90β.C2 construct failed to drive luciferase expression. These findings confirmed that the minimal promoter (HSP90β.C5) element is positioned immediate upstream of the transcription initiation site. A portion of DNA containing predicted enhancer elements (X-box, E-box, STAT, etc.) was deleted from HSP90β.C5 to prepare the HSP90β.C6 construct. The driving capability of HSP90β.C6 construct was dramatically reduced to about tenfold as compared to ~ 40-fold as detected in HSP90β.C5. This demonstrated that the putative enhancer elements like X-box, E-box, STAT, etc. are present in HSP90β.C5. The least promoter activity (about fivefold) was detected in HSP90β.C1, which contained an entire 5′-flanking region (full-length) including sequences of HSP90β.C5 and HSP90β.C6. This indicated the presence of strong repressor element(s).

In vitro DNA immunoprecipitation assay was performed to examine its binding affinity with purified TATA-box binding protein (TBP). A 436 bp long DNA fragment containing the TATA-box domain of HSP90β.C5 could bind with TBP followed by immunoprecipitation with anti-TBP-agarose beads. Bound DNA was subjected to PCR amplification. As shown in Fig. 2B, TBP was immune-precipitated with DNA fragments, which were detectable with 20 PCR-cycles as compared to non-detectable control (using non-specific IgG). The amplified unbound DNA was also proportionately reduced in comparison to control reaction. These findings demonstrated/confirmed that a putative TATA-element, which is positioned in HSP90β.C5, is responsible for the above promoter activities and thus capable of luciferase reporter gene expression.

Further, the identified promoter activity of HSP90β.C5 was validated by raising transgenic zebrafish. Tol2 construct with GFP reporter, as delineated in materials and methods, were co-injected with transposase mRNA into fertilized eggs. The injected embryos showed expression of GFP and were grown to adulthood. These adults were crossed with wild type to check for germline transmission of the transgene and to establish stable transgenic lines by in crossing the progenies. The GFP expression driven by *hsp90β* promoter could be seen in the 1 cell stage embryos and remained strongly expressed in a non-spatially restricted

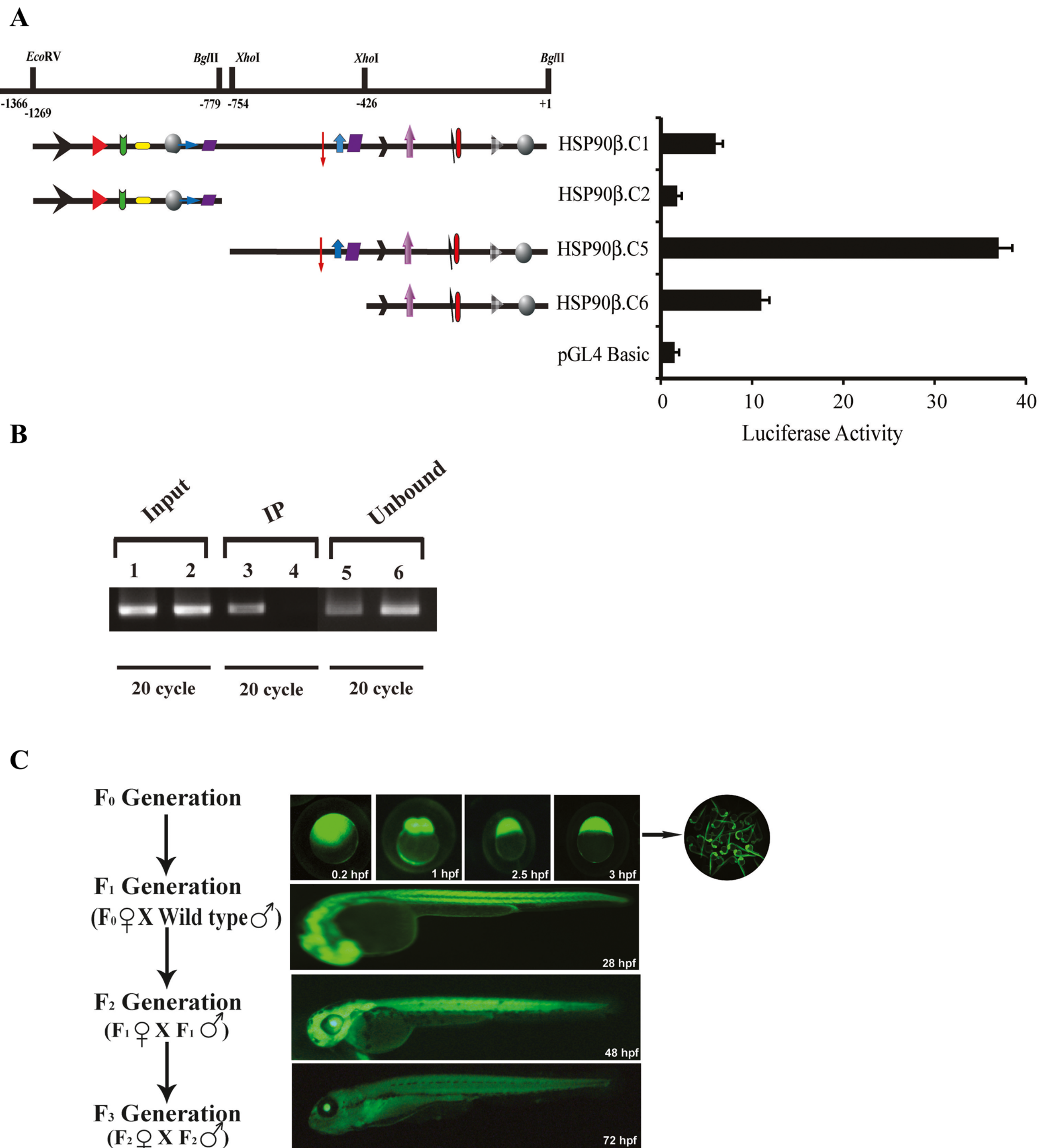

Fig. 2 Mechanism of *hsp90β* gene expression. **A** Relative luciferase activity of different 5′—serially deleted *hsp90β* promoter-reporter constructs transfected to HEK293 cell line. The putative TATA box shown in gray ( ) color, E2F activator in black ( ), E4BP4 motif in red ( ), CAAT box in green ( ), special AT- rich region in yellow ( ), GATA box in light blue ( ), STAT binding elements in violet ( ), X-box in red ( ), E2a element in blue ( ), SALL1 repressor element in black ( ), E-box element in purple ( ), AP1 element in black ( ), HIF binding factor in red ( ), STAT3 box in gray ( ) color. The results are expressed as the mean of three different experiments ± S.E. Top-panel shows the restriction enzyme sites used to generate constructs in pGL4- Basic. On left, a schematic representation of all the 5′–deleted constructs used for transfection is depicted. Approximate locations of transcription factor binding sites are shown. **B** DNA immunoprecipitation assay depicting TBP binds with TATA box. Immunoprecipitated DNA was detected by PCR analysis (20 cycles). Lane: 1, 3, 5 with anti-TBP conjugated agarose beads. Lane: 2, 4, 6 with IgG conjugated agarose beads (control). DNA immune precipitation with TBP (Lane 3) as compared to unbound DNA with IgG conjugated agarose beads (Lane 4). Relative unbound DNAs are shown in lanes 5 and 6. **C** Stable lines of transgenic zebrafish (F_2) expressing GFP driven by *hsp90β* minimal promoter (derived from HSP90β.C5). (Color figure online)

manner throughout the development Fig. 2C. This clearly validated that the identified promoter is functional and very strong, which is capable of driving massive GFP expression.

Identification of HIF responsive *hsp90β* gene promoter

Our bioinformatics analysis identified a possible inverted HIF element/motif RCGTG spanning − 187 to − 184 bp [44, 45]. It is positioned upstream of the TATA-box (HSP90β.C5). HIF is believed to play an adaptive role during oxygen limitation for increased expression of HSP90β gene. The predicted HIF element (spanning − 221 to − 132 bp) was deleted (HSP90β.ΔC5) to validate this HIF responsive element. Hypoxic conditions-can was imposed with $CoCl_2$ treatment as reported earlier [45, 46]. To verify the tolerance level (dose) of $CoCl_2$, HEK293 cells were first treated with various doses as described in materials and methods. As shown in Fig. 3A, it is evident that cells remain healthy with proliferating capability when exposed up to 100 µM $CoCl_2$, as compared to distorted cells treated with 150 µM $CoCl_2$. As shown in Fig. 3B, $CoCl_2$ treatment phenomenally heightened luciferase expression driven by HSP90β.C5 construct to the tune of more than 150-fold as compared to non-treated transfected cells. Contrary to this, HSP90β.ΔC5 failed to drive heightened luciferase expression in the presence of $CoCl_2$. HSP90β.C5 construct was also capable of driving increased luciferase reporter expression in the presence of $CoCl_2$. From these data, it was possible to identify a HIF responsive element, *cis*-acting elements necessary for the hypoxia-induced expression of the *hsp90β* gene.

Discussion

HSP90β is believed to play critical roles in maintaining regular physiological functions and in mitigating various abiotic and biotic stresses [1, 21]. Adaptation to hypoxia is likely to be mediated by a complex and finely tuned

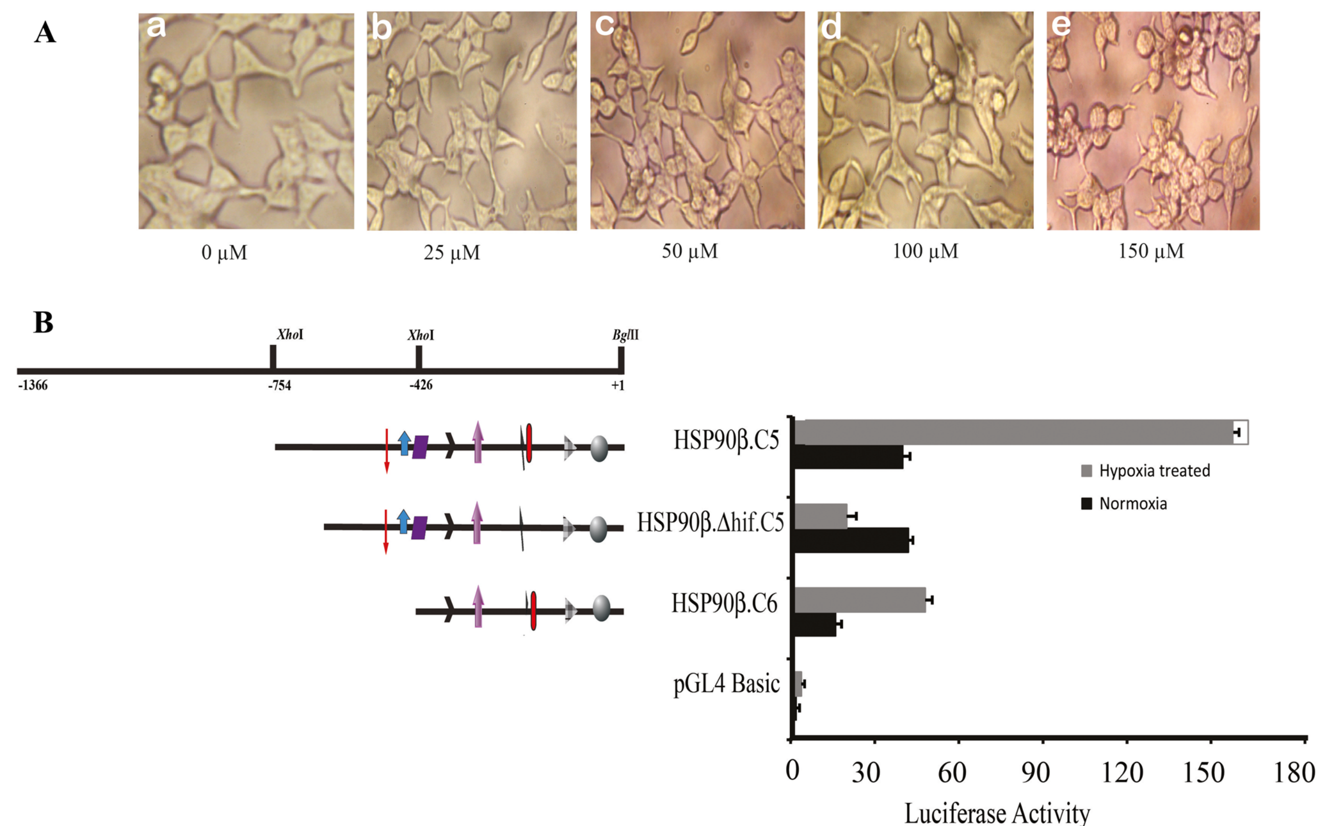

Fig. 3 Delineation and functional validation of HIF induced *hsp90β* gene promoter activity under hypoxic condition. **A** Dose dependent analysis of impact of $CoCl_2$ at various concentrations on HEK293 cell line showing healthy cells using up to 100 µM for 24 h. **B** Luciferase expressions between normal minimal promoter (HSP90β.C5) and HIF deleted minimal promoter (HSP90β.Δhif.C5) showing HIF induced heightened luciferase expression during oxygen limitation (hypoxic treated with $CoCl_2$) in HSP90β.C5 as compared to mutant HSP90β.Δhif.C5, while luciferase expression remained constant in both the constructs in the presence of oxygen (normoxic). The symbols of regulatory elements are same as depicted in Fig. 2A

mechanism involving multiple factors. We have shown that HSP90β plays a significant role in mitigating challenges of hypoxia faced by *C. striatus* in its natural habitat [36, 47]. Little information is available regarding the molecular mechanisms involved in adaption to extreme environmental stressors. Since *hsp90β* gene expression is activated during its prolonged exposure to hypoxic environment knowledge on genomic organization and mechanisms regulating *C. striatus hsp90β* gene expression is essential to understrand the role of this gene in hypoxia response [36].

Here, we present the genomic organization and mechanistic expression of *hsp90β* gene from hypoxia tolerant *C. striatus*. Like that of human, fish *hsp90β* gene is transcribed from 11 exons. Every reported species contains an untranslated region (UTR) upstream of the start codon. This is the first evidence of *hsp90β* gene structure in any teleost fish. HSP90 chaperon proteins are highly conserved [7, 36] across eukaryotes, it appears that the gene structure of *hsp90β* to differ from species to species. A longer intron in *C. striatus* relative to *hsp90β* genes of other vertebrates [30, 32] might be linked with major regulatory functions, since *C. striatus* is capable of adapting against environmental stressors better than other reported vertebrates.

The minimal promoter region (HSP90β.C5), containing TATA-box, positioned at − 51 bp upstream of the transcription initiation site, was identified by in vivo luciferase assay. This is in line with the previously identified chicken *hsp90β* gene promoter, where the TATA-box was located at − 26 bp from the transcription initiation site [30, 31]. In vitro DNA immunoprecipitation assay revealed that TBP was efficiently bound with this promoter region. Strong driving capability of this promoter in transgenic zebrafish confirmed the notion that the *hsp90β* gene is ubiquitously expressed throughout the body for various biological needs/functions starting from post-fertilization. We also observed that GFP expression was induced by stress, while observing transgenic zebrafish under microscope (data not presented). This supported the notion that *hsp90β* gene expression is linked with normal biology and stress physiology. When the predicted X-box, E2a and STAT consensus elements were deleted from HSP90β.C5, to derive HSP90β.C6; its promoter activity was reduced dramatically. This is indicative of the fact that the deleted elements are enhancers. This is possible because STAT transcription factor binding sites are known to act as enhancers, which are mediated by interleukins for *hsp* genes [3]. Similarly, X-box and E2a are reported to potentiate promoter activities [48, 49]. Surprisingly, the entire 5′-flanking region (HSP90β.C1); containing TATA-box and a distantly positioned CAAT-enhancer (− 1103 bp) including other possible enhancers like E2F (− 1202), GATA-1 (− 888 bp), etc.; failed to drive reporter gene expression as compared to HSP90β.C5 and HSP90β.C6. This provided a clue that there must be strong repressor motif(s). The repressor functions could be mediated by a special AT-rich region (− 881 bp) and a negatively oriented E4BP4 (− 1137 bp) elements as reported earlier [50]. A putative domain known as SALL1 (at − 371 bp) was identified as a possible strong transcriptional repressor [51]. It would be interesting to examine these possible repressive functions. Nevertheless, we confirmed that the *hsp90β* gene is induced by hypoxic stress and an inducible HIF element was functionally linked with hypoxia stress management.

In conclusion, the genomic organization and HIF inducible expression of the *hsp90β* gene of hypoxia tolerant freshwater fish (*C. striatus*) were presented. These findings, along with established laboratory-based stress treatment protocol, demonstrated that *C. striatus* has the potential to become a candidate model fish (large-body) for studies investigating hypoxia stress adaptation. The HIF inducible promoter of this gene successfully drove reporter gene (GFP) expression in zebrafish. This could have immense implications in the future to generate transgenic food fishes, such as commercially valuable farmed carps, for desirable traits of interests [52]. This is possible since we had already established gene integration protocol via homologous recombination using CRISPR/Cas9 nuclease in carps [53]. It is likely that transgenic carp, expressing desired gene driven by inducible *hsp90β* gene promoter, could be reared in higher density (over-crowding) that may limit oxygen supply for maximum expression of desirable traits. Thus, it might be possible to implement cage-culture practice, which has proven to be not effective in carps. Our findings also provide an avenue to generate color-variant ornamental transgenic freshwater fish using the HIF inducible *hsp90β* gene promoter of *C. striatus*.

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Author contributions HKB: overall designing and execution of the project, manuscript writing. SDM, SM, MMS and KP: gene characterization experiments, Writing—original draft, reviews and editing. VC, BM, RKS: transgenic experiments, maintenance of zebrafish and manuscript editing.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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