Functional characterization of the zebrafish *gadd45αb* gene promoter and its application as a biosensor

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We report characterization of zebrafish *gadd45αb* (growth arrest and DNA damage gene) minimal promoter and its application as biosensor tool for genotoxicity studies in zebrafish. Variably sized fragments of the 5′ flanking region were generated using polymerase chain reaction (PCR). These were cloned upstream of *gfp* gene in pEGFP1 vector, transfected into human foetal fibroblasts and induced with DNA damaging agents, ultraviolet radiation (UV) and methyl methane sulphonate (MMS). The region –1064/+ 593 bp showed maximum activity after treatment with 1.5 mM MMS for 2 h or 80 mJ/cm^2 UV. The endogenous *gadd45α* expression in human fibroblast cells and zebrafish embryos in the presence of UV and MMS was confirmed by real-time PCR. When the minimal promoter linked to *dsRed2* gene was microinjected into zebrafish embryos, red fluorescence was observed in response to sub-lethal dose of UV, confirming its application as transgenic zebrafish biosensor for genotoxicity.

Keywords: Biosensor, *gadd45α*, genotoxicity, minimal promoter, zebrafish.

GENOTOXICITY is a deleterious action affecting a cell's genetic material contributing to the development of tumours or cancers[1–3]. Several genotoxic substances are known to be mutagenic and carcinogenic[4,5]. The contamination of water resources by genotoxic compounds is a worldwide problem[6–8]. The mammalian *gadd45α* (growth arrest and DNA-damage inducible) gene is known to be rapidly induced by genotoxic stressors[9–12] and functions in cell cycle arrest in response to DNA damage. Annotation of the zebrafish genome revealed that it contains two prologues of *gadd45α* genes, on 2nd and 6th chromosomes. The one present on 2nd chromosome is termed *gadd45αa* and the other on 6th chromosome is called *gadd45αb*. These genes are putative and their expression patterns are yet to be determined. However, recently an increased expression of *gadd45αb* gene in response to cadmium toxicity in a hepatocyte-like zebrafish cell line is reported[13]. Since up-regulation of *gadd45α* gene has been characterized as an important cellular response to genotoxic agents, characterization of its promoter and regulatory elements will pave way for the development of transgenic zebrafish biosensors for aquatic pollution monitoring by linking the promoter region to fluorescent reporter genes.

Zebrafish (*Danio rerio*) has been a prominent vertebrate model and has a clear potential to provide valuable insights into developmental biology, chemical toxicity and drug discovery[14]. The main benefits of using zebrafish as a toxicological model are with regards to their size, husbandry, rapid maturity, fecundity and availability of whole genome sequence[15,16]. The use of transgenics as a toxicological tool has increased in recent years owing to the improved, more successful techniques for creating stable lines of zebrafish[17]. Recently, several researchers have attempted to develop transgenic zebrafish lines expressing GFP in response to toxic chemicals for biomonitoring purposes[18–21].

Here we report the characterization of zebrafish *gadd45αb* gene (chr. 6) minimal promoter by 5′ and 3′ promoter deletion analysis using *GFP* as the reporter gene. We also report the expression of the endogenous human and zebrafish *gadd45α* genes in response to genotoxic stressor, methyl methane sulphonate (MMS) and the possibility of using the minimal zebrafish promoter to design a genotoxicity biosensor.

Material and methods

In silico analysis of the zebrafish gadd45αb promoter region

The 5′ flanking region of the zebrafish *gadd45αb* gene (www.ensembl.org/*Danio_rerio*; ENSDART00000102325)

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Table 1. Primers used in this study

Primer	Sequence 5′ → 3′	Length (nt)
Zgad45-F1	GGAAGATCTTTGTCCGTAGTGTATGAGTGTGTG	33
Zgad45-F2	GGAAGATCTAATGTCTGACATGGCATCACTGAG	33
Zgad45-F3	GGAAGATCTTTATTAATCCGGGGTCGCCACAGC	33
Zgad45-F4	GGAAGATCTGGTTGTGCTTAGCGTAATACCT	31
Zgad45-R1	CCGGAATTCTCATAAACTCCCACAGTGATGC	31
Zgad45-R2	CCGGAATTCGTTATCTCCACACGGTTCTTCA	31
DsRed2-F	AAAGATATCAGTTCAGCCGGAATTCACC	28
DsRed2-R	AAAAAGCTTACAGAGTGAGCCGATCCGAG	29
HgadqRT-F	GGAGAGCAGAAGACCGAAAG	20
HgadqRT-R	TGTTGATGTCGTTCTCGC	18
HactqRT-F	GCGGGAAATCGTGCGTGACATT	22
HactqRT-R	GATGGAGTTGAAGGTAGTTTCGTG	24
Zfgad-F	TGAAGAACCGTGTGGAGATAAC	22
Zfgad-R	CGTCTCATGTTGTTCACTCGC	21
Zfact-F	GGTATTGTGATGGACTCTGGTG	22
Zfact-R	AACGGAAACGCTCATTGCC	19

was analysed for the presence of DNA damage responsive elements using MOTIF search (http://motif.genome.jp). The longest promoter length was decided based on the position of these regulatory elements (Oct-1, NF-Y, AP-1, AP-4, CREB, etc.) and it was –1745 to + 593 (2.3 kb) with respect to the transcription start site (+ 1). The selected zebrafish *gadd45αb* promoter region was also aligned with the reported *gadd45α* promoter sequences of human (www.ensembl.org/*Homo sapiens*; ENSG00000116717) and mouse (www.ensembl.org/*Mus musculus*; ENSMUST00000043098) to see the homology and the extent of conservation of regulatory sites between species using ClustalW2. The seven additional deletion promoter lengths made in this study were also based on the positions of the regulatory elements (Figure 1).

Construction of deletion promoter plasmids

The full-length zebrafish *gadd45αb* promoter region (2.3 kb) was amplified from zebrafish BAC clone (DKEY–20D16) using Turbo *Pfu* polymerase. The purified polymerase chain reaction (PCR) fragment was cloned into pGEMT-Easy vector (Promega) and sequenced. The sequence was confirmed by primer walking and the recombinant plasmid was used as template to amplify all the seven deletion fragments. Four different forward primers were used to create progressively deleted 5′ ends of the promoter (Table 1). Using two reverse primers with each forward primer, two sets of deleted promoters were created, with and without the first intron. The amplified promoters were unidirectionally cloned into pEGFP-1 (with GFP reporter gene, Clontech) and pDB739-Tol2 (with DsRed2 reporter gene, Dr Ekker, Mayo Clinic, Minnesota) vectors using linker primers with *Bgl*II and *Eco*RI sites in forward and reverse primers, respectively (Table 1). The former construct series was named pZGad (1-8)-GFP, whereas the latter was named pZGad (1-8)-DsRed.

Cell culture

Primary cultures of human foetal fibroblast cells were used for *in vitro* evaluation of pZGad (1–8)-GFP construct series. Cells were grown in ADCF-DME/high modified medium supplemented with 10% foetal bovine serum and 1% penicillin–streptomycin following standard protocols by Freshney[22].

LC_{50} studies of MMS and UV

Human foetal fibroblast cells were exposed to different concentrations of DNA damaging agents, viz. MMS and ultraviolet radiation (UV), in order to determine the LC_{50}. The cells were sub-cultured in a 24-well plate for 24–48 h till the cells reached 80% confluency. Then, the media was replenished incorporating MMS at varying concentrations (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mM) and incubated at 37°C and 5% CO_2 for 2 h. After 24 h, the medium with MMS was removed and fresh growth medium was added. The cells were observed after 24 h of incubation at 37°C and 5% CO_2. The percentage of cell death was estimated by trypan blue dye exclusion test and LC_{50} was calculated. All values were calculated from triplicates. For determining the LC_{50} of UV rays, the cells were sub-cultured in a six-well plate and exposed to varying energy levels of UV rays (20, 40, 60, 80, 100, 120 and 140 mJ/cm^2) for 10 s each in a UV crosslinker and the percentage of cell death was recorded after 24 h.

In vitro promoter analysis

Human foetal fibroblast cells with 80% confluence were transfected with the appropriate plasmid construct using Effectene® Transfection Reagent (Qiagen) following the manufacturer's instructions. The deletion constructs were transfected into the cells under optimized conditions

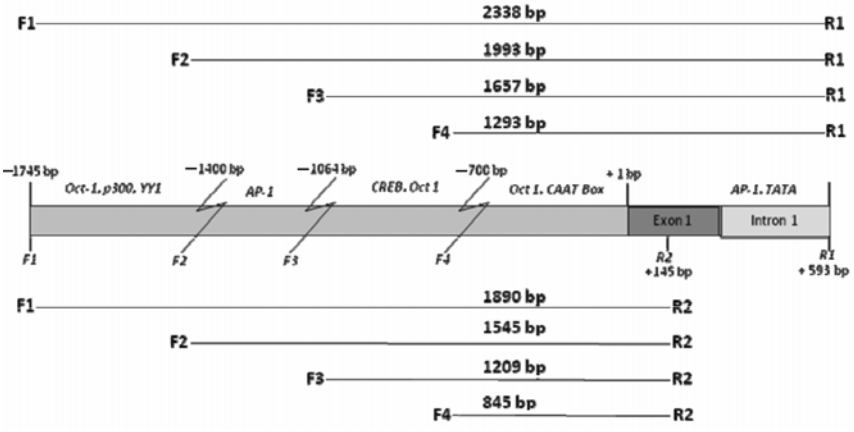

Figure 1. Schematic diagram of the zebrafish *gadd45α* gene promoter deletion analysis using different primer sets. Transcription factor binding sites present in each fragment are marked.

(0.30 µg of plasmid DNA and 1 : 35 DNA to Effectene ratio), which were standardized in a pilot study (data not shown). After 24 h of transfection, the cells were treated with either 1.5 mM MMS for 2 h or 80 mJ/cm^2 UV light for 10 s to induce expression from the *gadd45αb* promoter. The induction was done in triplicate and un-induced cells were used as controls. The cells were checked for the GFP expression under a fluorescence microscope 24 h after treatment. To quantify GFP in terms of relative light units (RLU), cells were harvested and washed twice with phosphate buffered saline (PBS) containing 1% v/v penicillin–streptomycin. Cell count was performed using haemocytometer and aliquots containing equal number of cells were drawn from all the treatments, pelleted and suspended in PBS buffer. An aliquot of each cell suspension was used to quantify the GFP expression using LB Mithras Multilabel Reader following manufacturer's instructions. For GFP fluorescence detection, excitation wavelength of 485 nm and emission at 535 nm was used. The readings were recorded directly on a computer using Microwin 2000 software.

Real-time PCR

Plasmid copy number in the cells transfected with deletion promoter constructs was estimated through absolute quantification by real-time PCR in order to normalize GFP fluorescence against plasmid copy number. In brief, the plasmid copy number in the control plasmid, pGad8-EGFP (enhanced green fluorescent protein; 40 ng/µl) was determined as 7.3×10^9 copies/µl. The original stock was diluted 10^4, 10^5 and 10^7 times to get 7.3×10^6, 7.3×10^5 and 7.3×10^3 copies respectively. These dilutions were used to construct a standard graph from which the exact copy number in the transfected cells was determined. The plasmid copy number was expressed as number of copies/ng of total DNA. Induction of the endogenous *gadd45α* gene expression in both human fibroblast cells and zebrafish larvae (48 hpf) exposed to MMS and UV was estimated by relative quantification of the *gadd45α* mRNA using real-time PCR (ABI 7500). *β*-actin was used as internal control and the experimental triplicates were run. Comparative C_t method was used to estimate the relative expression of *gadd45α* mRNA. Fold change in expression was calculated by $2^{-\Delta\Delta}$ C_t method.

Microinjection and fluorescence microscopy

One/two-celled zebrafish embryos were microinjected with 8.3 ng/µl of the pTol2-Gad-DsRed2 series of constructs and 100 ng/µl of Tol2 transposase mRNA in a 3-nl injection volume. The contents were delivered at the blastoderm/yolk interface (14). Thirty microinjected embryos per construct were dechorionated carefully 24 h post-fertilization (hpf) avoiding damage and treated with UV light. An energy dose of 30 mJ/cm^2 was administered in a UV crosslinker. The dose was determined after exposing the uninjected embryos to a series of UV dosages and determining the LD_{50} value at 24 h in a pilot study (data not shown). Post-treatment, the embryos were rinsed thrice with water and incubated at 28.5°C. One set of embryos served as control. For dsRed2 detection, Zeiss Axioscope 2 compound microscope with a rhodamine filter set (excitation and emission wavelengths of 546 ± 10 and 570 ± 10 nm respectively) was used. Zeiss Axiocam digital camera was used in conjunction with Zeiss Axiovision software to document the expression of fluorescent reporters in the transgenic zebrafish.

Results

In silico analysis of zebrafish gadd45αb promoter region

Several transcription factor binding sites responsive to DNA damage were identified based on the available

Table 2. Regulatory sites in the 5′ flanking region of the *gadd45α* gene of three species

	Human		Mouse		Zebrafish	
Motif	No. of sites	Positions	No. of sites	Positions	No. of sites	Positions
Oct-1	3	–431 to –443 –355 to –368 –99 to –92	2	–485 to –504 –60 to –75	3	–464 to –479 –243 to –255 –38 to –52
AP-1	2	–746 to –757 +478 to +487	2	–909 to –918 +340 to +351	3	–914 to –924 –493 to –504 +395 to +404
AP-4	2	–830 to –840 –297 to –307	1	–289 to –299	1	–869 to –879
CREB	2	–1215 to –1225 –182 to –190	1	–1207 to –1217	2	–1418 to –1428 –1182 to –1191
CAAT Box	1	–225 to –237	1	–40 to –52	1	–58 to –70
CEBP	2	–908 to –921 –582 to –594	2	–654 to –667 –85 to –96	3	–806 to –820 –594 to –612 –122 to –138
STRE	1	–384 to –392	2	–154 to –162 –369 to –377	1	–1318 to –1326

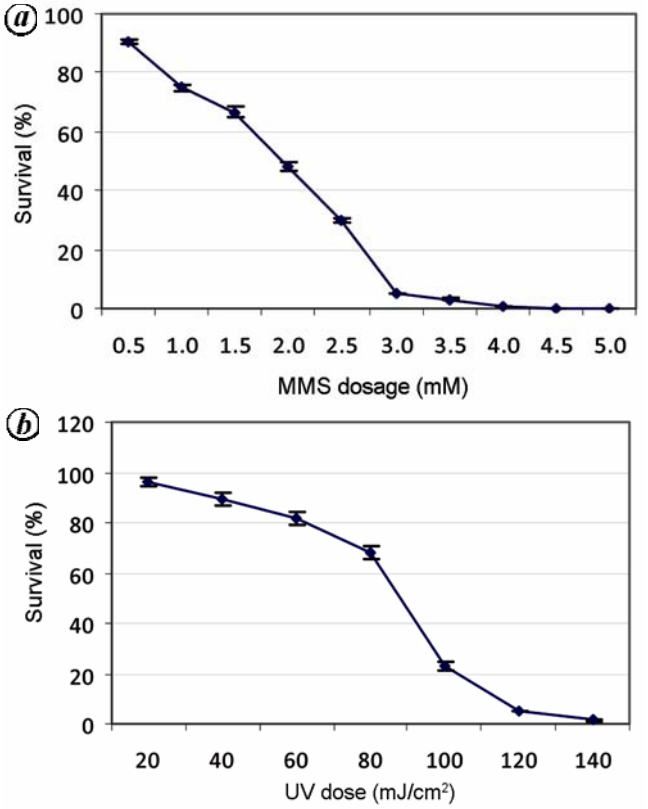

Figure 2. ***a***, Viability of cultured human fibroblast cells exposed to MMS for 2 h; ***b***, Viability of cultured human fibroblast cells exposed to UV.

literature[23–25]. These include Oct-1 (octomer-binding site-1), AP-1 (activation protein-1), AP-4, CREB (cyclic AMP-responsive element-binding site), C/EBP (CAAT-enhancer binding protein) and NF–Y (also termed CBF–CCAAT binding factor). Based on this analysis, the coordinates of the longest promoter (2.3 kb) were fixed as – 1745 to + 593 bp, with respect to the transcription start site (+1). An AP-1 binding site was identified in the first intronic sequence and a CAAT box was located 83 bp upstream to the transcription start site. However, unlike humans *gadd45α* promoter, zebrafish lacks the NFkB transcription factor-binding site in the *gadd45αb* 5′ regulatory region.

Multiple sequence comparison

Sequence comparison of the zebrafish *gadd45αb* gene revealed 44.1% and 41.5% similarity with mouse and human genes respectively in the 2 kb proximal promoter region while there was 52% similarity between human and mouse *gadd45α* promoter region. Apart from this, a greater similarity has been observed in several regions of 15–20 bp. A list of transcription factor binding sites and regulatory elements along with their position in all the three species is given in Table 2. It shows a more or less similar number of sites across the species, although the positions are quite different.

Dose standardization of genotoxic agents

Both genotoxic agents inhibited cell growth in a dose-dependent manner as shown in Figure 2. Two-hour exposure of MMS was found to be lethal beyond 4.0 mM dosage with cell death occurring within 24 h post-treatment while there was gradual but significant ($P < 0.01$) inhibition of cell growth up to 3.0 mM MMS. Fifty-two

per cent loss of cell viability was observed after 24 h of 2.0 mM, 2 h MMS exposure (LD_{50} at 24 h). Hence, a lower dose of 1.5 mM MMS that caused 33% mortality was selected for subsequent induction. Cell viability decreased on exposure to increasing dosages of UV. From the cell viability curve (Figure 2 *b*), the 24 h LD_{50} value of UV was estimated as 85 mJ/cm^2. Exposure to 140 mJ/cm^2 UV irradiation for 10 s was completely lethal within 24 h. A dose of 80 mJ/cm^2 UV irradiation for 10 s that caused 32% mortality was used for induction studies.

In vitro promoter analysis

The absolute quantification of the plasmid copy number showed some variation among various transfected constructs, ranging from 1.73×10^6 to 6.5×10^5 copies/ng. The increase in normalized EGFP expression over the un-induced control cells was found to be maximum in cells transfected with pGad3–EGFP construct (Figure 3). It was also observed that EGFP expression was significantly higher ($P < 0.01$) in the set of promoter constructs that included first intron sequence.

Relative quantification of human and zebrafish gadd45α mRNA

Induction of *gadd45α* gene in human fibroblast cells and zebrafish larvae (48 hpf) at toxic levels of MMS (1.5 mM) and UV (80 mJ/cm^2) was confirmed by real-time PCR using SYBR green chemistry and appropriate replicates. The results showed that there is 28-fold and 3.5-fold increase in *gadd45α* transcript in zebrafish and human respectively after exposure to 1.5 mM MMS (Figure 4). UV exposure resulted in 11-fold and 4-fold increase in *gadd45α* transcript in zebrafish and human respectively. In comparison to human fibroblasts, the fold change in *gadd45α* transcript is found to be higher in zebrafish embryos after exposure to DNA damaging agents (MMS and UV).

Induction studies in zebrafish embryos

LD_{50} of UV dosage was determined by exposing the 24 hpf zebrafish embryos to 10, 20, 30, 40, 50 and 60 mJ/cm^2 of UV irradiation for 10 s. It was found that 50% of the embryos died after 24 h when exposed to 30 mJ/cm^2, and this dose was subsequently used for inducing the microinjected embryos. UV-induced expression of the *zGad3-DsRed2* reporter gene in live larvae was assessed 24 h after UV (30 mJ/cm^2) exposure to allow for maximal red fluorescent protein (RFP) accumulation. DsRed2 fluorescence was detected in the eye, yolk and tail region (Figure 5), whereas no expression was observed in the unexposed controls.

Discussion

Zebrafish genome consists of two *gadd45α* paralogues, one on 2nd chromosome (*gadd45αa*) and the other on 6th chromosome (*gadd45αb*) with four exons and three introns. The *gadd45αb* gene present on chr 6 has two transcripts of length 1193 and 896 bp giving rise to products of 156 and 163 aa in length respectively. Multiple sequence alignment of the peptide sequences of these two transcripts revealed that they differ only in first exonic region. These two transcripts are believed to be the result of alternative splicing. Recently, Sandrini *et al.*[13] reported the up-regulation of *gadd45αb* gene large transcript in response to cadmium toxicity. However, the expression pattern of *gadd45αa* and small transcript of *gadd45αb* is yet to be determined. In the present study, the promoter region of *gadd45αb* gene has been characterized and the

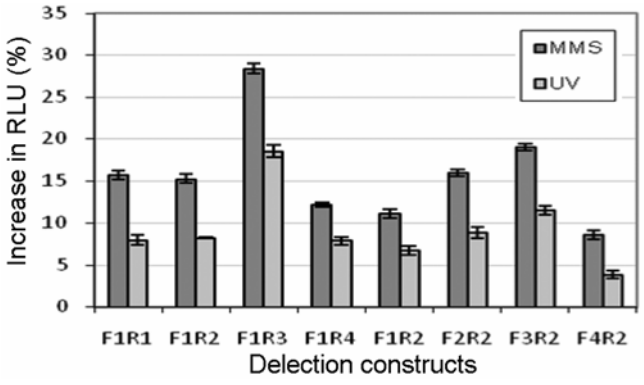

Figure 3. GFP fluorescence of MMS and UV-induced transfected cells as measured in relative light units.

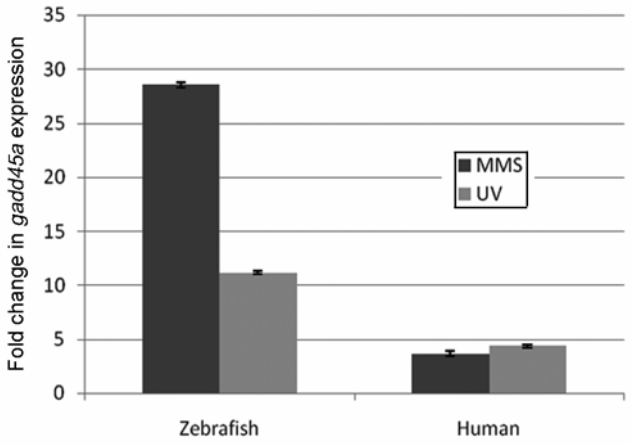

Figure 4. Fold change in gadd45α mRNA expression following induction with MMS (1.5 mM) and UV (30 mJ/cm^2). Fold change was calculated over un-induced controls using real-time PCR. β-actin was used as reference gene.

Figure 5. Red fluorescence detected in the eye, yolk and tail region of embryos injected with pTol2-ZGad3-DsRed2 construct after UV exposure. Unexposed pTol2-Gad3-DsRed2 served as control.

possibility of using it to develop transgenic zebrafish genotoxicity biosensor has been shown.

The *gadd45α* promoter has been fully characterized in human and hamster[26–28]. Sequence comparison of the 2 kb proximal promoter region of zebrafish *gadd45αb* gene revealed 44.1% and 41.5% similarity with mouse and human respectively. However, a high similarity was observed in the type and number of regulatory elements present in the promoter (Oct-1, AP-1, AP-4, CEBP, STRE and CREB), which suggests that these *gadd45α* genes are transcriptionally regulated in a similar manner in response to DNA damage. This is proven by the induction of zebrafish *gadd45αb* promoter-driven GFP expression in human foetal fibroblasts as shown here. Further, simultaneous induction of the endogenous *gadd45α* gene in human fibroblasts indicates that the two promoters are similarly regulated. Hollander *et al.*[26] have shown that the hamster and human promoters are functional in rodent cells suggesting that both regulatory sequences within the *gadd45* genes and trans-acting factors involved in induction are conserved in mammalian cells. Here, we show that the induction mechanism is similar across taxa.

In vitro promoter analysis carried out in human fibroblast cells showed that the promoter length of 1657 bp (–1064 to +593) triggered the highest expression of GFP as measured in terms of RLU. This region includes the first intron along with three sites of Oct-1, 3 of AP-1 (one in first intron), 1 of AP-4 and 3 of CEBP. Based on mutation analysis of the human *gadd45α* promoter, it has been proved that the Oct-1 site between –99 and –92 bp relative to the transcription start site is involved in the transcriptional activation of the *gadd45α* promoter in response to UV irradiation[24]. In addition, the AP-1 binding site in the 1st intron is highly conserved in mouse, human and zebrafish indicating its significance in transcriptional regulation of the *gadd45α* gene. It was therefore, not surprising that better promoter performance requires the first intronic region. The lower activity of the full length zebrafish promoter compared to the minimal promoter could be attributed to the presence of CCAAT displacement protein (CDP) repressor binding site. CDP, coded by the *Cutl1* gene has been implicated as a transcriptional repressor in diverse processes such as terminal differentiation, cell cycle progression and the control of nuclear matrix attachment regions[29].

Induction of the endogenous human and zebrafish *gadd45α* promoters at the same MMS and UV concentration was observed by real-time PCR. The results show a higher response (28-fold) to MMS toxicity in zebrafish and indicate the possibility of using the zebrafish *gadd45αb* promoter as a genotoxicity pollution indicator. To test this possibility, the minimal promoter was linked to *dsRed2* gene (pTol2-zGad3-dsRed2) that produces red fluorescence. The microinjected zebrafish embryos showed red fluorescence in response to sub-lethal dose of UV. Red fluorescence was observed in eyes, yolk, skin epithelium and tail region. Quantitative estimation of genotoxic chemical can be done by measuring the amount of RFP produced. However, this requires sophisticated techniques such as real-time PCR and Western blotting. Therefore, the biosensor can be best employed as a primary screening tool to detect the presence of genotoxic chemicals in the aquatic ecosystems. Earlier, transgenic zebrafish embryos expressing an Aryl hydrocarbon receptor (AhR)-dependent GFP reporter gene have been utilized to identify 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-susceptible target tissues[18]. Transgenic zebrafish have also been used as an *in vivo* system that uses *hsp70* gene activation (with an eGFP reporter) as a measure of cadmium toxicity[19]. Seok *et al.*[20] reported that transgenic zebrafish containing hsp70 promoter–EGFP construct can be used as an indicator of arsenite toxicity in mosaic transgenic zebrafish. Wu *et al.*[21] had developed a heat shock inducible transgenic zebrafish using hsp27 promoter and *GFP* gene. The present study concludes that 1657 bp (–1064/+593 bp) region of the zebrafish *gadd45αb* promoter can be used to develop transgenic zebrafish genotoxicity biosensor as a primary screening tool to monitor genotoxic pollutants in aquatic environment.

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ACKNOWLEDGEMENTS. We thank Dr W. S. Lakra, Director, CIFE and Dr Dilip Kumar, former Director, CIFE for providing necessary facilities. Funding by Department of Biotechnology, New Delhi to carry out research work and financial support by Indian Council of Agricultural Research, New Delhi to the first three authors is gratefully acknowledged.

Received 6 February 2012; revised accepted 4 July 2012