Pi class glutathione S-transferase genes are regulated by Nrf2 through an evolutionarily conserved regulatory element in zebrafish

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Pi class GSTs (glutathione S-transferases) are a member of the vertebrate GST family of proteins that catalyse the conjugation of GSH to electrophilic compounds. The expression of Pi class GST genes can be induced by exposure to electrophiles. We demonstrated previously that the transcription factor Nrf2 (NF-E2 p45-related factor 2) mediates this induction, not only in mammals, but also in fish. In the present study, we have isolated the genomic region of zebrafish containing the genes *gstp1* and *gstp2*. The regulatory regions of zebrafish *gstp1* and *gstp2* have been examined by GFP (green fluorescent protein)-reporter gene analyses using microinjection into zebrafish embryos. Deletion and point-mutation analyses of the *gstp1* promoter showed that an ARE (antioxidant-responsive element)-like sequence is located 50 bp

upstream of the transcription initiation site which is essential for Nrf2 transactivation. Using EMSA (electrophoretic mobility-shift assay) analysis we showed that zebrafish Nrf2–MafK heterodimer specifically bound to this sequence. All the vertebrate Pi class GST genes harbour a similar ARE-like sequence in their promoter regions. We propose that this sequence is a conserved target site for Nrf2 in the Pi class GST genes.

Key words: antioxidant-responsive element (ARE), gene transcription, glutathione S-transferase (GST), green fluorescent protein (GFP), Phase 2 detoxification enzyme, Pi class glutathione S-transferase.

INTRODUCTION

The transcription factor Nrf2 (NF-E2 p45-related factor 2) induces the expression of Phase 2 detoxification and antioxidant genes in response to electrophilic insults [1,2]. The importance of Nrf2 in this induction has been demonstrated by a number of experiments using Nrf2-deficient mice [3]. The electrophile response is regulated through a *cis*-acting element called the ARE (antioxidant-responsive element) which is found within the regulatory region of each gene [4]. Nrf2 binds to the ARE sequence as a heterodimeric complex with small Maf proteins using a basic region leucine-zipper domain [5,6]. Under non-induced conditions, Nrf2 is retained in the cytoplasm by interaction with the actin-binding protein Keap1 [7,8]. The Nrf2–Keap1 interaction facilitates the degradation of Nrf2 by a proteasome-mediated mechanism [9,10]. Electrophiles cause Nrf2 to escape from Keap1, allowing Nrf2 to accumulate in the nucleus and thereby enhance the expression of target genes [7].

Recently, we demonstrated that the Nrf2–Keap1 system is conserved in zebrafish and that the Nrf2-dependent induction of Phase 2 detoxification and antioxidant genes is conserved among vertebrates [11,12]. Zebrafish Nrf2 protein shares six highly conserved domains, Neh (Nrf2-ECH homology) domains, with mammalian Nrf2 proteins, which are considered to play critical functions in Nrf2 regulation [5]. High sequence identities in each Neh domain between zebrafish and mouse Nrf2 imply a common regulatory mechanism among vertebrate Nrf2s [11]. This finding

provided us with a powerful tool to analyse the molecular basis of the Nrf2–Keap1 system in zebrafish. In particular, the use of random mutagenesis and mutant-screening techniques enables us to perform a genetic search of molecules that may regulate the Nrf2–Keap1 system. An additional advantage of the zebrafish system is the ease of detecting transactivation of endogenous genes by forced expression of Nrf2 that is difficult in cultured cells. The analysis of endogenous genes will be significant for the study gene-specific elements important for Nrf2 transactivation.

Among the known endogenous target genes of zebrafish Nrf2, the Pi class GSTs (glutathione S-transferases) showed relatively strong induction in both electrophile-treated larvae and Nrf2-overexpressing embryos [11]. GSTs are a multigene family of detoxification enzymes that catalyse the conjugation of GSH with a variety of endogenous and exogenous electrophilic compounds [13]. GSTs are widely distributed in nature, being found in all eukaryotes and in many prokaryotes. In mammals, GSTs can be divided into distinct classes: Alpha, Mu, Pi, Theta, Sigma, Zeta, Omega, Kappa and four subgroups of MAPEG (membrane-associated proteins in eicosanoid and glutathione) enzymes, according to their homologies and properties [14,15]. The sequence identity of GSTs in the same class is greater than 60 %, whereas between classes the identity is less than 30 %. Members of each class exhibit overlapping yet distinct substrate specificities.

Pi class GSTs are the most abundant GST family member found in many normal and malignant tissues [13]. There has been considerable interest in the properties of Pi class GSTs, particularly

Abbreviations used: ARE, antioxidant-responsive element; DEM, diethylmaleate; *ef1α*, elongation factor 1α; EMSA, electrophoretic mobility-shift assay; EST, expressed sequence tag; GPEI, Pi class glutathione S-transferase enhancer I; GST, glutathione S-transferase; MAPEG, membrane-associated proteins in eicosanoid and glutathione; Neh, Nrf2-ECH homology; Nrf2, NF-E2 p45-related factor 2; 5'-RACE, 5'-rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-PCR; TRE, PMA ('TPA')-responsive element.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AB194127 and AB194128 for *gstp1* and *gstp2* respectively.

in relationship to their role in carcinogenesis and human cancer. Pi class GSTs are highly efficient in the glutathione-conjugation of carcinogenic benzo[a]pyrene derivatives [16] and is effective in the detoxification of electrophilic α,β -unsaturated carbonyl compounds [17]. In addition, studies using rats harbouring a *GSTP1* transgene have shown that over-expression of Pi class GSTs inhibits the early phase of chemical carcinogenesis in rat liver [18]. Likewise, ablation of *GSTP1* and *GSTP2* in mice resulted in increased susceptibility to skin tumorigenesis induced by chemical carcinogens [19]. In humans, genetic polymorphisms within the *GSTP1* locus have been identified that are associated with susceptibility to bladder, testicular and prostate cancer [20]. Therefore, the expression level of Pi class GST is regarded as one of an important determinant for the protection against various chemical insults.

In mice, the administration of electrophilic agents induced expression of Pi class GSTs. This induction was shown to be drastically impaired when the Nrf2 gene is disrupted [3,21,22]. The electrophile-induction of *GSTP1* expression was also observed in the rat liver epithelial RL34 cells [23,24]. Studies in these cells identified an element known as GPEI (Pi class glutathione S-transferase enhancer I), located in a 2.5-kbp region upstream of the transcriptional initiation site, that is responsible for this regulation and shown to be a strong enhancer element for hepatocarcinogenesis [23]. GPEI contains an ARE-like sequence capable of binding Nrf2 protein [24]. The results suggest that the GPEI is a target for the Nrf2 transcription factor and that is important for the induced expression of *GSTP1* by electrophilic compounds. However, a similar GPEI has not been found in the promoter of Pi class GST genes from other species. Ikeda et al. [25] have demonstrated that Nrf2 binds to an ARE-like sequence located on the promoter region of the mouse *GSTP1* gene and that this region is important for transactivation by Nrf2. This ARE-like sequence is conserved in the promoter region of human, rat and mouse Pi class GST genes, and seems to be common target site for Nrf2 among vertebrates, although it was shown to be inactive in the rat cells [26].

As described above, Pi class GST genes are also strongly induced in zebrafish. It is of interest to identify the specific target sites for Nrf2 in the zebrafish genome and to compare the regulatory mechanism between fish and mammalian genes. In the present study, we have isolated whole genomic regions of the zebrafish Pi class GST genes and identified their transcriptional regulatory regions. The results indicate that an ARE-like sequence is also conserved in the promoter region of fish Pi class GST genes and that it is a functional element for Nrf2-dependent transactivation.

EXPERIMENTAL

Isolation of genomic DNA and full-length cDNA of zebrafish Pi class GST genes

A zebrafish EMBL3 SP6/T7 genomic library (Clontech) was screened with a probe containing a partial cDNA of zebrafish *gstp1* [11]. Probes were labelled using the AlkPhos Direct DNA labelling kit, and the positive plaques on the membrane filters were detected with CDP-Star as substrate, according to the manufacturer's instruction (Amersham Pharmacia Biotech). The DNA inserts of the positive clones were subcloned into pBluescript II SK. A 0.61-kbp fragment of genomic DNA including the *gstp2* promoter was prepared by PCR using zebrafish genomic DNA and specific primers (5'-CCGTCGACACAGCAAGAAGGTC-ACTGG-3' and 5'-GGGGATCCTCTGTGAAGTTGCTGCTCC-TGAAATGTGTAG-3'). The full-length cDNAs were isolated by

RT (reverse transcriptase)-PCR of total RNA from 4-day-old embryos treated with 100 μ M DEM (diethylmaleate) using specific primers, and subcloned into pGEM-T Easy (Promega). Total zebrafish RNA was prepared using RNazol B (TEL-TEST). The primers have the following sequences: 5'-CTAGGAGCAGC-TTTGAAACGCAC-3' and 5'-CGTTGTTGGAGAATGTTGT-ACCGACG-3' (*gstp1*); 5'-CACATTTTCAGGAGCAGCAACTT-CACAGAC-3' and 5'-CATTTGAGAACGTTGTATCAACG-3' (*gstp2*).

Inducer treatment

Zebrafish embryos and larvae were obtained by natural mating. For the induction studies, larval and adult fish were placed in culture dishes or plastic chambers respectively, and treated with DEM.

Expression analyses by RT-PCR

Expression of Pi class GST genes was analysed by RT-PCR as described previously [11] using following primers: *gstp1*, 5'-CT-AGGAGCAGCTTTGAAACGCAC-3' and 5'-CGTTGTTGGA-GAATGTTGTACCGACG-3'; *gstp2*, 5'-CACTCTCACATACTT-CGCTATC-3' and 5'-AATATTTTCAAATGGTTTGAAGTC-3'; *efl α* (elongation factor 1 α), 5'-GCCCCTGCCAATGTA-3' and 5'-GGGCTTGCCAGGGAC-3'.

Radiation hybrid mapping

Radiation hybrid mapping using panel LN54 was performed as described in Hukriede et al. [27] using two sets of primers specific to each Pi class GST gene. Sequences of each primer are following: 5'-TTGACCAAGGAAGACGTGG-3' and 5'-CTGT-GATTGGCAGACTTGAC-3' (*gstp1*); 5'-CAACCACCTCAAA-TGTTTGAAGATG-3' and 5'-CGTTGTTGGAGAAGTTGTAC-CGACG-3' (*gstp1*); 5'-GAGAGCTGAATCAGCACTTG-3' and 5'-CAGCAACTTTGCCTGCTATG-3' (*gstp2*); 5'-CATTTGAG-AACGTTGTATCAACG-3' and 5'-GTCGTTCTTTCCATACGC-AC-3' (*gstp2*).

5'-RACE (5'-rapid amplification of cDNA ends) assays

5'-RACE assays were carried out using the 5'-RACE System (Gibco BRL) as described previously [28]. Briefly, 4 μ g each of total RNA from 4-day-old embryos treated with 100 μ M DEM was reverse transcribed using the antisense primer 5'-GCAGAT-CTTTGATGAACGC-3', which corresponds to the sequences in the sixth exon of *gstp1*. The product was further amplified using the 5'-RACE abridged anchor primer and a *gstp1* fifth exon-specific antisense primer 5'-CAGCTTTATGTACTTCAGGCG. DNA fragments of *gstp2* were also amplified using these primers. 5'-RACE products were subcloned into pBluescript II SK and their sequences were determined.

Plasmid construction

DNA fragments corresponding to the 3.5- or 0.61-kbp upstream region of the transcriptional initiation site of zebrafish *gstp1* or *gstp2* and the EGFP (enhanced green fluorescent protein) fragment of pCS2-eGFP [28] were ligated together into pBluescript IISK, and the resulting plasmids were named p3.5gstp1GFP and p0.61gstp2GFP respectively. For constructing 5'-deleted mutants, p3.5gstp1GFP was linearized with *Apa*I and *Sal*I, and incubated with *Exo*III, followed by blunting with mung bean nuclease and self-ligation. Selected constructs were sequenced and named p1.3gstp1GFP and p0.12gstp1GFP, according to the positions of their 5' ends from the transcriptional initiation site. For

Figure 1 Comparison of the vertebrate Pi class GST proteins

(A) Phylogenetic tree of Pi class GST proteins. The tree was constructed using the Clustal W program in the DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/search/clustalw-j.html>). Scale bar, genetic distance. (B) Sequence alignment of various Pi class GST proteins. Conserved amino acids among Pi class GST proteins are highlighted in grey. Closed and open circles denote G- and H-sites respectively. Abbreviations: z, zebrafish; s, salmon; e, eel; x, *Xenopus*; b, bovine; h, human; r, rat; m, mouse.

construction of p3.5gstp1m1GFP, mutations in the proximal ARE-like sequence were introduced into p3.5gstp1GFP by PCR. Other plasmids used in this study were as described previously [11,12].

Microinjection of zebrafish embryos

Reporter DNAs were linearized by digesting the vector with *SacI* (gstp1) or *SalI* (gstp2). Digested DNA was resuspended in water and injected into the blastomere of early one-cell stage embryos (see Figure 5A). Synthetic capped RNA was made with the SP6 mMESSAGE mMACHINE™ *in vitro* transcription kit (Ambion) using linearized DNA of the pCS2 derivatives described above. GFP expression was examined under a GFP Plus (480 nm excitation, 505 nm emission) filter on a MZFLIII microscope (Leica) equipped with a 600CL-CU digital camera (Pixera).

EMSAs (electrophoretic mobility-shift assays)

EMSAs were carried out as described previously [12]. Zebrafish Nrf2 and MafK proteins were prepared by transcription and translation reactions *in vitro* using TNT® wheat germ extract (Promega). An oligonucleotide containing the proximal ARE-like sequence (5'-GGGGCGCGTGCATGACTCATCAAAACGCTGAG-3') was prepared by annealing synthetic oligonucleotides and labelled with using ³²P Rediprime II DNA Labelling System (Amersham Biosciences). A 40- or 100-fold molar excess of unlabelled oligonucleotide was added to the reactions for specific competitors.

Antibodies

Anti-Nrf2 antibody was produced by immunizing rabbits with N-terminal residues of mouse Nrf2 (Neh2 domain, amino acids 1–98) as an antigen (Sawady Technology, Tokyo, Japan). The Neh2 protein was purified from recombinant *Escherichia coli*

cells as described previously [29]. Anti-MafK antibody has been described previously [30].

RESULTS

Identification of two Pi class GST genes in zebrafish

We previously isolated a partial cDNA for the zebrafish Pi class GST and showed that its expression is induced in larvae by electrophilic compounds and that the induction is dependent on the Nrf2–Keap1 system [11]. To exclude the possibility that the isolated clone is a pseudogene, we tried to obtain a full-length cDNA clone of the Pi class GST gene. In the process, we found a second related Pi class GST gene in zebrafish. After carrying out RT-PCR on total RNA from DEM-treated 4-day-old larvae using primers specific to the two Pi class GST genes, we isolated full-length cDNAs for both the *gstp1* and *gstp2* genes. Primers for each gene were designed on the basis of a combination of information from the EST (expressed sequence tag) (<http://www.ncbi.nlm.nih.gov/dbEST/index.html>) and the zebrafish genomic DNA (http://www.ensembl.org/Danio_rerio/) databases and our own preliminary data on a partial clone of *gstp1*, the Pi class GST cDNA described in our previous paper [11].

The percentage identity of the coding region sequence between *gstp1* and *gstp2* is 90.4 %, and that of the encoding amino acid sequence is 87.0 %. The amino acid identities between rat GSTP1 and zebrafish GSTP1 or GSTP2 proteins are 59.0 % and 57.1 % respectively. The identity relative to other classes of rat GST proteins is less than 30 %, indicating that the isolated clones are zebrafish homologues of Pi class GST. Figure 1(A) shows a phylogenetic tree of the vertebrate Pi class GST proteins. To date, three full-length sequences of Pi class GST genes have been identified in non-mammalian animals, *Xenopus laevis* [31], *Oncorhynchus nerka* (sockeye salmon) [32] and *Anguilla anguilla* (European eel) (DDBJ, EMBL and GenBank® accession number AY530199). As

expected, zebrafish GSTP1 and GSTP2 proteins share a higher similarity with these non-mammalian proteins compared with the mammalian Pi class GST proteins. Interestingly, the similarity between zebrafish GSTP1 and GSTP2 is higher than that of other fish Pi class GSTs.

Figure 1(B) shows the multiple alignment of Pi class GST proteins. The Pi class GST protein contains two ligand-binding sites: the glutathione-binding site (G-site) and electrophilic substrate-binding site (H-site) [33–35]. The residues in the G-site (Tyr⁸, Arg¹⁴, Trp³⁹, Lys⁴⁵, Gln⁵², Leu⁵³, Gln⁶⁵ and Ser⁶⁶) are conserved in both GSTP1 and GSTP2, with the exception of Arg⁴⁵ in GSTP2 (Figure 1B, closed circles). The residues in the H-site (Tyr⁸, Phe⁹, Val¹¹, Arg¹⁴, Ile¹⁰⁵, Tyr¹⁰⁹, Asp²⁰³ and Gly²⁰⁴) are also conserved, apart from Ile¹¹ in GSTP2 (Figure 1B, open circles). It is interesting to find that residues in position 11 also vary between the mouse GSTP1 and GSTP2 proteins. In humans, variant forms of GSTP1, Ile¹⁰⁵ and Val¹⁰⁵, exist, and they have been shown to have a different substrate preference [36,37]. Isoleucine is found at position 105 in both zebrafish GSTP1 and GSTP2 proteins, suggesting that their substrate specificities are related to the Ile¹⁰⁵-type human GSTP1.

Nrf2 can induce the expression of both *gstp1* and *gstp2*

The high level of similarity in nucleotide sequences between *gstp1* and *gstp2* suggests that the electrophile induction of zebrafish Pi class GST expression that we observed previously, by whole mount *in situ* hybridization and Northern blot analysis [11], may be the combined contribution of the two related genes. Therefore, to investigate the inducibility of the genes independently we carried out RT-PCR analysis using sets of primers specific to each gene. The specificity of each primer set was demonstrated by PCR using cloned cDNA of *gstp1* and *gstp2* as templates (Figure 2A). Total RNA from whole body of 5-day-old larvae treated with or without 100 μ M DEM for 6 h was analysed. Both *gstp1* and *gstp2* were strongly induced by DEM treatment (Figure 2B). These inductions were greatly reduced when we knocked-down the expression of Nrf2 by injecting antisense morpholino oligonucleotide (nrf2-MO). These results indicated that electrophiles induce both *gstp1* and *gstp2* genes and that Nrf2 mediates this induction. We next examined the induction of Pi class GST expression in adult gills. Electrophile-induced expression was observed for both *gstp1* and *gstp2* (Figure 2C). Next, we analysed the expression in male and female fish, since male-specific expression of *GSTP1* has been shown in mice [21,38]. As shown in Figure 2(C), we could not observe any sex-specific differences in the expression of either *gstp1* or *gstp2*. All these results were confirmed by RT-PCR using different sets of primers specific for *gstp1* and *gstp2* (results not shown). Our findings indicated that electrophiles induce *gstp1* and *gstp2* in both larvae and adults and that these two genes may be regulated by a similar molecular mechanism.

We next examined the effect of exogenous Nrf2 on the expression of *gstp1* and *gstp2*. RT-PCR was carried out using 8-h-old embryos, injected either with or without zebrafish Nrf2 mRNA at the one-cell stage. Remarkable induction of *gstp1* and *gstp2* expression was observed in the Nrf2-overexpressing embryos, but not in the control embryos (Figure 3). The induction was confirmed by RT-PCR using different sets of primers specific for *gstp1* and *gstp2* (results not shown). These results indicate that both *gstp1* and *gstp2* are endogenous target genes for Nrf2.

Identification of the genomic locus containing *gstp1* and *gstp2*

To analyse the regulatory mechanism of electrophile-induction of zebrafish *gstp1* and *gstp2* expression, genomic DNA fragments

Figure 2 Nrf2-dependent induction of *gstp1* and *gstp2* in zebrafish larvae

(A) Confirmation of the primer specificity for RT-PCR. PCR was carried out using plasmid DNAs containing *gstp1* (*gstp1*) or *gstp2* (*gstp2*) cDNA as templates. Numbers indicate reaction cycles (Cyc). (B) Effect of nrf2-MO on the DEM-induced expression of *gstp1* and *gstp2*. RT-PCR analysis of nrf2-MO (9 ng)- or mock-injected larvae, treated (+) or not treated (–) with 100 μ M DEM for 6 h at day 5. The amounts of cDNA were standardized by the expression of *ef1 α* . Each experiment was carried out at least three times and resulted in similar expression patterns. (C) Expression of the Pi class GST genes in the adult gills. RT-PCR analysis of the gills of adult male or female fish, treated (+) or not treated (–) with 100 μ M DEM for 6 h.

Figure 3 Expression of *gstp1* and *gstp2* in Nrf2-overexpressing embryos

RT-PCR analysis using total RNA isolated from embryos 8-h post-injection with or without 40 pg of Nrf2 mRNA. Amounts of cDNA were standardized by the expression of *ef1 α* .

containing these genes were cloned and characterized. For *gstp1*, a λ -phage genomic library (2×10^6) was screened using a partial cDNA of zebrafish *gstp1* [11], and two positive clones were isolated (Figure 4, #2 and #6). After characterizing these clones by restriction site mapping, a DNA fragment of the most 5'-extended clone (Figure 4, #6), containing about 11-kbp of the *gstp1* locus, was subcloned into a cloning vector for further analysis. In the case of *gstp2*, we carried out PCR on genomic DNA using a primer set whose design was based on the information from the zebrafish genomic DNA database (Figure 4, contigs NA9028 and NA575). A 0.7-kbp DNA fragment that included a 0.61-kbp region upstream of the transcriptional initiation site was isolated.

Figure 4 Structure of the zebrafish *gstp1* and *gstp2* genes

Maps of contiguated sequences and λ phage clones containing *gstp1* or *gstp2* are displayed. E, X and S indicate enzyme sites for EcoRI, XhoI and SacI respectively. Black and white boxes denote the coding and non-coding regions of the Pi class GST cDNAs respectively. Nucleotide sequence data of 3.5-kbp regions of *gstp1* and 0.61-kbp regions of *gstp2* used for the GFP-reporter constructs have been deposited in the DDBJ, EMBL and GenBank® databases with accession numbers AB194129 and AB194130 respectively.

As shown in Figure 4, the information of the zebrafish genomic DNA database suggests that *gstp1* and *gstp2* are arranged in tandem on the same chromosome. We carried out the genomic PCR around the junction site of *gstp1* and *gstp2* and confirmed this tandem arrangement (results not shown). Furthermore, we mapped *gstp1* and *gstp2* on the linkage map by using a LN54 hybrid panel [27], and demonstrated that both genes mapped to a linkage group 14, 124.37 cR (centiray) from the most terminal marker. The approximate sizes of the introns for both genes were determined by PCR, and it was shown that *gstp1* and *gstp2* span about 6.8- and 7.5-kbp respectively (Figure 4). These gene lengths are quite large in comparison with the mammalian Pi class GST genes such as mouse *GSTP1* and *GSTP2* [39,40]. To investigate the transcription initiation sites, a 5'-RACE assay was performed. The results indicated that the transcriptional initiation site of both *gstp1* and *gstp2* are about 60 bp upstream of translation initiation sites. These positions are quite similar to the mammalian Pi class GST genes [40–42].

Isolation of gene regulatory regions of *gstp1* and *gstp2*

To identify the gene regulatory regions, we prepared GFP reporter constructs that are fused to DNA fragments containing the 3.5- and 0.61-kbp regions of the transcriptional initiation sites of *gstp1* and *gstp2* respectively (Figure 4, 3.5gstp1GFP and 0.61gstp2GST). An examination of promoter activity was made using zebrafish embryos. These constructs were microinjected into one-cell-stage embryos and expression of the GFP reporter genes monitored using a fluorescence microscope (Figure 5). Only low GFP expression was observed during development in the case of both genes, suggesting that basal promoter activities are quite weak (Figure 5B, –Nrf2). We next tested whether Nrf2 can transactivate the activity of these reporter genes. GFP-reporter DNAs with synthetic capped RNA of zebrafish Nrf2 were co-injected into zebrafish embryos at the one-cell stage (Figure 5A) and the GFP activity was examined at the 8-h stage. GFP expression was dramatically enhanced in embryos over-expressing Nrf2 compared with embryos injected with the reporter gene alone (Figure 5B, +Nrf2). These results suggest that the Nrf2-respon-

Figure 5 Induction of the GFP-reporter genes by Nrf2 overexpression

(A) Scheme showing the analysing method. (B) GFP expression of the 3.5gstp1GFP and 0.61gstp2GFP constructs. 3.5gstp1GFP (20 pg) or 0.61gstp2GFP (100 pg) was injected with or without 40 pg of Nrf2 mRNA into blastomere of one-cell stage embryos. GFP expression was analysed in 8-h embryos. Values indicate the percentages of embryos that showed more than ten GFP-positive cells. The number of embryos observed for each construct are indicated in parentheses.

sive element is present in both the 3.5- and 0.61-kbp regions of *gstp1* and *gstp2* respectively.

A proximal ARE-like sequence is essential for Nrf2 transactivation

To determine the exact target sites for Nrf2 in the 3.5-kbp region of *gstp1*, a series of deletion constructs of 5'-flanking region fused to the GFP gene were prepared and injected into one-cell embryos along with Nrf2 mRNA. The induction of GFP expression by Nrf2 was maintained despite deleting the promoter to a region only –122 bp upstream of the transcription initiation site (Figure 6A, 0.12 k). This result indicated that the target site for Nrf2 is located within a 122-bp region. Examination of this region identified an

Figure 6 Difference in GFP induction by Nrf2 among various *gstp1* constructs

(A) Transcriptional activity of deletion and mutation constructs. GFP expression was analysed in 8-h embryos that were injected with 40 pg of each construct and 40 pg of Nrf2 mRNA. More than 30 embryos were examined for each construct. (B) Structure of the proximal ARE-like sequence in the *gstp1* promoter and its mutation construct. Numbers indicate nucleotides from the transcriptional initiation site.

ARE-like sequence (TGACTCATC), located between basepairs –53 and –45, which contains a single mismatch from the core ARE (TGAC/GnnnGC) [43]. To determine whether this ARE-like sequence is essential for the transactivation, we introduced point mutations into the ARE-like sequence of the 0.12*gstp1*GFP construct (Figure 6B). The mutation eliminated GFP induction activity (0.12*gstp1*m1), suggesting that the ARE-like sequence is essential for induction by Nrf2 (Figure 6A).

Other than the ARE-like sequence in the promoter region, five sequences containing the core ARE can be identified in the remaining 3.5-kbp region as shown in Figure 6(A). It is possible that some of these core ARE regions can substitute for the activity of the proximal ARE-like sequence. To characterize the promoter activity of these additional ARE motifs, we generated the construct 3.5*gstp1*GFPm1, a derivative of 3.5*gstp1*GFP with point mutations in the proximal ARE-like sequence. The mutation completely eliminated the GFP induction activity of Nrf2, confirming that the proximal ARE-like sequence, but not other ARE motifs, in the 3.5-kbp regions is required for Nrf2-dependent activation (Figure 6A).

Nrf2 can bind the proximal ARE-like sequence

To investigate whether Nrf2 has the capability of binding the proximal ARE-like sequence, we carried out EMSA experiments using an oligonucleotide containing the region –68 to –40 of *gstp1* gene as a probe (Figures 6 and 7). To bind DNA, it is necessary for Nrf2 to form a heterodimeric complex with the small Maf family proteins [5]. Therefore, we prepared zebrafish Nrf2 and MafK proteins using the wheat germ *in vitro* translation system. MafK is one of four zebrafish small Maf proteins that are known to heterodimerize with Nrf2, and thereby activate its DNA binding and transactivation activity [12]. As shown in Figure 7, a binding complex was observed when we added both Nrf2 and MafK to the reaction mixture (lane 4, arrowhead), but not when the proteins were supplied individually (lanes 2 and 3). Since addition of anti-Nrf2 or anti-MafK antibodies resulted in a gel super-

Figure 7 Binding of Nrf2–MafK heterodimers to the ARE-like sequence

In vitro-translated Nrf2 and MafK proteins were incubated with the ³²P-labelled oligonucleotide probe containing the ARE-like sequence in *gstp1*. Arrowheads indicate the positions of shifted bands containing the Nrf2–MafK heterodimer, which were super-shifted (arrow) or reduced with the addition of anti-Nrf2 (lane 5) or anti-MafK (lane 6) antibodies (Ab). Shifted complexes, including Nrf2–MafK heterodimers, were specifically competed by the addition of a 25- or 100-fold molar excess of unlabelled oligonucleotide (lanes 8 and 9). Mutations (Mut) in the ARE-like sequence, as shown in Figure 6(B), eliminated the competition (lanes 10 and 11).

shift or reduction of the binding complex (Figure 7, lanes 5 and 6), we concluded that the complex is an Nrf2–MafK heterodimer.

To verify the importance of the ARE-like sequence for the binding of the Nrf2–MafK complex to the DNA probe, competition assays were performed. Unlabelled oligonucleotides containing the wild-type ARE-like sequence or its mutation were added to the reaction mixture. As shown in Figure 7 (lanes 7–11) wild-type, but not mutant, competitor reduced the formation of the shifted complex by the Nrf2–MafK heterodimer. These results indicated that Nrf2 specifically binds to the proximal ARE-like sequence.

DISCUSSION

Nrf2 targets the proximal ARE-like sequence

In this study, we have demonstrated that Nrf2 binds directly to a proximal ARE-like sequence located –50 bp upstream of the transcription initiation site of the zebrafish *gstp1* gene, and that Nrf2 activates gene expression through this element in zebrafish embryos. The ARE-like sequence harbours a ‘TC’ in place of a ‘GC’ motif previously reported to form the core ARE sequence (TGAC/GnnnGC). Though the GC dinucleotide is an important motif in the ARE for the interaction to the extended homology region of small Maf proteins [44], we speculate that a TC sequence is functionally equivalent to GC for the following reasons: (i) Nrf2–MafK heterodimer can specifically bind the proximal ARE-like sequence (Figure 7); (ii) Kataoka et al. [45] have shown that chicken Nrf2 has the ability to bind the GATGACTCATC which has the sequence TC instead of GC; and (3) Nioi et al. [46] mutated every nucleotide across the ARE of the mouse *NQO1* [NAD(P)H:quinone oxidoreductase] gene and produced clear evidence that mutation of 5′-TGAGTCCGC-3′ to 5′-TGAGTCGTC-3′ did not result in loss of ARE-driven induction by sulphoraphane or transactivation by mouse Nrf2.

Figure 8 Alignment of various Pi class GST promoters

ARE-like sequences and the TATA box are highlighted in grey. Numbers indicate nucleotides from the transcriptional initiation site.

In the upstream region of *gstp1*, in addition to the proximal ARE-like sequence, five distinct core ARE sequence can be identified. Reporter analysis showed that these distal AREs are dispensable for the transactivation, even though they all comprise GC motifs. This raises the question of what differences exist between the proximal ARE-like sequence and the distal AREs. The Nrf2–MafK heterodimer may prefer to bind the proximal ARE-like sequence. It is important to remember that AREs are also the target sites for small Maf homodimers, which are known to negatively regulate transcription [47,48]. To distinguish heterodimer- and homodimer-favouring ARE sequences, we recently developed a new method to predict binding sequences for Nrf2–small Maf heterodimer using surface plasmon resonance imaging technique (T. Yamamoto, H. Motohashi and M. Yamamoto, unpublished work, and [49]). According to this prediction, the proximal ARE-like sequence was classified into preferable ARE sequences for the Nrf2–small Maf heterodimer. Indeed, it is of note that zebrafish MafK homodimers could not bind to a probe containing the proximal ARE-like sequence (Figure 7, lane 2). An alternative hypothesis is that an increase of distance between ARE sequences and the core promoter region can reduce the transactivation ability of Nrf2. Numerous studies have mapped a functional ARE to within 700 bp of the transcriptional initiation site. An ARE located far from the core promoter may be inefficient, although some functional AREs have been found to exist more than 1 kbp upstream of the transcriptional initiation sites.

A proximal ARE-like sequence is conserved among vertebrates

The positions and sequence of the proximal ARE-like motif are conserved among vertebrate Pi class GST genes (Figure 8) [40,41,50], all being located immediately upstream of the TATA box. Since amino acid sequences of the basic DNA-binding domains (RDIRRRGKKNKVAQNCRKRK) [5] are completely identical between zebrafish Nrf2 and its vertebrate orthologues, it is reasonable to assume all the vertebrate Nrf2 proteins will bind the conserved ARE-like sequence. In mice, Nrf2 was demonstrated to target this proximal ARE-like sequence and thereby activate gene expression [25]. Taken together, these previous reports and our present data suggest that the proximal ARE-like sequence is a conserved target site for Nrf2 among vertebrates. It has been reported that in case of the rat *GSTP1*, the proximal ARE-like sequence does not function as an Nrf2 target site [26]. We suppose that this discrepancy arises from the existence of a GPEI in the rat *GSTP1*, which may have a much higher affinity for Nrf2 in comparison with the proximal ARE-like sequence. Indeed, Kawamoto et al. [24] have demonstrated that a response element to Phase 2 detoxification inducers is localized in a region between –140 and +59 bp of the rat *GSTP1* which contains the

proximal ARE-like sequence but not GPEI, suggesting that GPEI is not the only target site for Nrf2 in the rat gene. We could not find GPEI-related regions in the zebrafish Pi class GST genes, and consider that the proximal ARE-like sequence is the major target sites for Nrf2 in zebrafish.

Interestingly, the TRE [PMA ('TPA')-responsive element] sequence (TGAG/CTCA) embedded in the proximal ARE-like sequence is completely conserved among vertebrate Pi class GST genes, implicating the Jun and Fos family of transcription factors in the universal regulation of Pi class GSTs. Indeed, members of the Jun and Fos family have been shown to bind this conserved TRE sequence [51,52]. In many human cell lines, however, the TRE sequence in human *GSTP1* is unresponsive both to phorbol esters and to forced expression of Jun and Fos proteins [53,54]. This is a similar situation in zebrafish larvae in which PMA treatment did not induce expression of *gstp1* or *gstp2* (Y. Takagi, M. Kobayashi and M. Yamamoto, unpublished work). Of particular note, Borde-Chiché et al. [52] have demonstrated PMA-induced *GSTP1* expression in human K562 leukaemia cells and importance of the conserved TRE sequence in this induction. Relationship between Jun/Fos proteins and Nrf2 on Pi class GST regulation should be elucidated in future.

Identification of *gstp1* and *gstp2*

We isolated two zebrafish Pi class GST genes, *gstp1* and *gstp2*, and revealed that they are arranged in tandem in the chromosome. Their close proximity and high degree of similarity suggest that they arose as a consequence of a recent gene duplication event. Intriguingly, two mouse genes of Pi class GST also exist whose protein products differ by only 6 amino acids. However, no such duplication of Pi class GST genes has been observed in rat [41]. Similarly, identification of multiple forms of Pi class GST proteins in crab-eating macaque (*Macaca fascicularis*) [55] suggested that some monkeys harbour two Pi class GST genes, unlike humans which have a single gene [50]. Since all previously identified Pi class GST genes in fish seems to be single genes, we suggest that the duplications of Pi class GST genes of zebrafish, mouse and monkey occurred independently. Phylogenetic analysis of vertebrate Pi class GST proteins (Figure 1A) supports this idea. The homology between salmon Pi class GST [32] and zebrafish GSTP1 and GSTP2 proteins (68% and 62% respectively) is lower than that between the zebrafish GSTP1 and GSTP2 proteins (87%). The result indicates that gene amplification of zebrafish Pi class GST occurred after divergence of Euteleostei. Furthermore, we found a Pi class GST gene from catfish in the EST database (DDBJ, EMBL and GenBank® accession number CK405822) whose homology with zebrafish GSTP1 or GSTP2 was lower than 87% (71% and 65% respectively). Catfish belongs to Ostariophysi, the same superorder as zebrafish, suggesting that

gene amplification of *gstp1* and *gstp2* took place after emergence of cypriniformes.

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