Activation of mouse Pi-class glutathione S-transferase gene by Nrf2 (NF-E2-related factor 2) and androgen

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The Pi-class glutathione S-transferases (GSTs) play pivotal roles in the detoxification of xenobiotics, carcinogenesis and drug resistance. The mechanisms of regulation of these genes during drug induction and carcinogenesis are yet to be elucidated. Recently, *Nrf2* (NF-E2-related factor 2; a bZip-type transcription factor) knockout mice were shown to display impaired induction of Pi-class GST genes by drugs. It is known that the mouse Pi-class GST gene *GST-P1* is expressed predominantly in the male liver, and is regulated by androgen. To determine whether Nrf2 and the androgen receptor regulate *GST-P1* directly, we analysed the molecular mechanism of activation of this gene by these factors. The promoter of the *GST-P1* gene was activated markedly by Nrf2 in transient transfection analyses. Gel mobility shift assay and footprinting analyses revealed three Nrf2 binding sites: one at the proximal and two at distal elements, located at positions –59, –915 and –937 from the

cap site. The fifth intron of the *GST-P1* gene contains the androgen-responsive region. Multiple androgen receptor binding sites are clustered within a 500 bp region of this intron. The whole fragment contains a minimum of seven androgen receptor binding sites, which collectively display strong androgen-dependent enhancer activity. However, on division into small fragments containing two or three elements each, individual enhancer activities were dramatically decreased. This suggests that multiple elements work synergistically as a strong androgen-responsive enhancer. Our findings indicate that Nrf2 and the androgen receptor directly bind to and activate the mouse *GST-P1* gene.

Key words: detoxification, gene regulation, transcription, tumour marker.

INTRODUCTION

The glutathione S-transferase (GST) family consists of a group of important drug-metabolizing enzymes that catalyse the conjugation of reduced glutathione with a variety of electrophilic compounds [1,2]. The mammalian cytosolic GSTs are divided into five subfamilies (Alpha, Mu, Pi, Theta and Zeta) on the basis of similarity in primary structure [3]. The Pi-class enzyme, GST-P, is markedly elevated during rat hepatocarcinogenesis, and is an extremely reliable tumour marker for chemically induced and spontaneously derived pre-cancerous lesions and hepatocellular carcinomas [4–7]. Therefore GST-P expression may be closely related to the inherent processes of hepatocarcinogenesis. We have been studying the transcriptional mechanisms involved in the specific activation of the *GST-P* gene during hepatocarcinogenesis. The rat *GST-P* gene has a strong enhancer element (GPE1), that is 2.5 kb upstream of the transcription initiation site [8]. GPE1 consists of two TRE [PMA ('TPA')-responsive element]-like palindromic sequences [9]. This enhancer is the major control element responsible for GST-P expression in pre-cancerous lesions, as shown by analyses in transgenic rats [10,11]. However, in the mouse, the structure of

the enhancer, and consequently patterns of expression, are significantly different from those of the rat gene. The mouse Pi-class GST comprises two tandemly aligned genes (*GST-P1/-piB* and *GST-P2/-piA*) [12,13]. The strong enhancer element, GPE1, of the rat *GST-P* gene is absent from both mouse Pi-class GST genes. Although these genes are expressed similarly, only one product (GST-P1/GST-PiB) exhibits enzymic activity on the common substrate, despite a difference of only six amino acid residues in the coding regions.

In contrast with rat GST-P, mouse Pi-class GST is constitutively expressed in the normal liver of males under the control of androgen [14]. In hepatic pre-neoplastic lesions, Pi-class GST expression is increased in females, but is repressed in males, relative to that in surrounding normal liver [15]. The increased Pi-class GST in hepatic adenoma is mainly GST-P1 [16].

Targeted disruption of the *Nrf2* (NF-E2-related factor 2) gene, which encodes a bZip transcription factor, has been reported [17]. Experiments with Nrf2-deleted mice showed that the basal expression of Phase II detoxifying enzymes, such as GSTs and NAD(P)H:quinone oxidoreductase (NQO1), was not significantly different from that of wild-type mice. However, induction

Abbreviations used: ARE, antioxidant-responsive element; EMSA, electrophoretic mobility shift assay; GPE1, strong enhancer element of the rat *GST-P* gene; GST, glutathione S-transferase; GST-P1, mouse Pi-class GST; MBP, maltose binding protein; NQO1, NAD(P)H:quinone oxidoreductase; Nrf, NF-E2-related factor; RT-PCR, reverse transcription-PCR; TRE, PMA ('TPA')-responsive element.

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The nucleotide and predicted amino acid sequence of rat *Nrf2* cDNA are registered at the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under accession no. AF037350.

by electrophilic drugs was almost completely impaired in the mutant mice. Nrf2 is a heterodimer composed of a large subunit and a member of the small Maf family of proteins. Nrf2 and related factors (NF-E2 and Nrf1) recognize a 5'-TGA(C/G)TCA(T/C)-3' sequence, similar to the TRE, which is a binding site for Jun/Fos-family transcription factors [18]. This sequence is also similar to that of the ARE (antioxidant-responsive element; -GTGACTTGCA-) that is located in the genes of Phase II detoxifying enzymes, including those encoding GST-P1 and NQO1 [19].

In the present study, we analyse the activation and direct interaction of the *GST-P1* gene with Nrf2 and the androgen receptor by means of transient transfection analyses, gel mobility shift assays and DNase I footprinting analyses. The results indicate that the mouse *GST-P1* gene promoter contains at least three Nrf2/small Maf heterodimer binding sites. In addition, multiple androgen receptor binding sites were observed in the fifth intron of the mouse *GST-P1* gene. All these elements are responsible for gene activation by appropriate transcription factors.

EXPERIMENTAL

Cloning of mouse GST-P1 gene and rat Nrf2 cDNA

A mouse (Balb/c) genomic library constructed in the Charon28 phage vector was screened using mouse Pi-class GST (mouse GST-II) cDNA [20]. An *EcoRI* fragment containing the *GST-P1* gene was subcloned into a pBluescript II (Stratagene, San Diego, CA, U.S.A.) vector. Deletion constructs were prepared by digestion with exonuclease III and subjected to sequencing.

Rat Nrf2 cDNA was cloned by screening a rat liver cDNA library (Wistar strain; generously donated by Dr T. Fujita, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan), with reverse transcription-PCR (RT-PCR)-amplified Nrf2 cDNA (primers -GGTCTAGAACCATTGGATTTGATTGAC-ATC- and -GAATCCATTGTGCCTTCAG-) as a probe.

Construction of plasmids

To construct the luciferase reporter gene, each deletion construct (containing nucleotides -1188, -626 and -463 to +60 respectively, relative to the cap site) was cloned into a pGEM-luc (Promega) vector containing the luciferase gene. To construct -95/Luc and -50/Luc, the -95/+60 and -50/+60 fragments were amplified by PCR using the corresponding primers, and inserted into pGEM-luc. The appropriate restriction enzyme-digested fragments of the *GST-P1* gene were cloned into a vector containing the luciferase gene (pGVB2; Nippon Gene, Toyama, Japan) with a rat GST-P minimal promoter region (-50/+37), including GC and TATA box elements [8].

The Nrf2 expression plasmid was constructed by inserting rat Nrf2 cDNA into pA β 2, which contains the human β -actin promoter and enhancer [21].

Rat MafK cDNA was amplified from rat liver total RNA by RT-PCR (primers -GCTCTAGACCATGACGACTAATCC-CAAGC- and -CGTCATGACTAGGAGGCGGCTGAGA-AG-) [22], and inserted into the expression vector pA β 2.

Androgen receptor cDNA was amplified by RT-PCR (primers -GGGATCCAGGATGGAGGTGCAGTTAGGG- and -GGC-TCGAGTTTCCAAATCTTCACTGTGTG-) using rat testis total RNA as template, and cloned into pA β 2.

The androgen-responsive element (3 $\times$ -GATCCGAGTAC-AGCATGTTCTGC-) was fused to a luciferase gene containing a minimal rat GST-P promoter (AndRE/Luc), and used as a control.

Cell culture, transfection and reporter assays

HepG2 human hepatoma cell lines were maintained in Dulbecco's modified minimal essential medium (Nissui, Tokyo, Japan) with 10% (v/v) fetal bovine serum. At 24 h before transfection, cells were plated at a density of 5×10^5 cells/60 mm plate. A total of 4 μ g of DNA, including 1 μ g of reporter plasmid, 0.5 μ g of β -galactosidase expression plasmid (pSV- β -gal; Promega), with or without 1 μ g of effector gene expression plasmid (Nrf2, MafK or androgen receptor) and pUC18 DNA, was transfected into HepG2 cells, according to the procedure of Chen and Okayama [23]. After a 45 h incubation, cells were harvested and extracted with 100 μ l of lysis buffer (Nippon Gene) by repeated freezing and thawing. Cell extracts were assayed for luciferase activity using a luciferase assay kit (Nippon Gene), and for β -galactosidase activity [24]. When treated with steroid hormone, cells were seeded into Phenol Red-free minimal essential medium (Nissui) containing charcoal-treated serum (10%, v/v), 24 h before transfection. Following overnight transfection, cells were washed, and fresh Phenol Red-free charcoal-treated medium was added with or without 10 nM synthetic androgen R1881 (methyltrienolone; Sigma), and incubated for a further 24 h. Cells were harvested, and luciferase and β -galactosidase activities were determined as described above. Luciferase activities were standardized to β -galactosidase activities, and all experiments were repeated at least twice.

Electrophoretic mobility shift assay (EMSA) and DNase I footprinting analysis

The Nrf2 cDNA fragment corresponding to the DNA binding bZip domain (amino acids 318-598) was fused in-frame to the gene encoding *Escherichia coli* maltose binding protein (MBP) of the pMalc2 expression vector (New England BioLabs). The entire MafK cDNA was fused to the MBP gene of pMalc2. Fusion proteins were expressed in *E. coli* and purified by amylose affinity column chromatography, according to the manufacturer's instructions (New England BioLabs). The cDNA corresponding to the DNA binding domain of the androgen receptor (amino acids 524-648) was amplified by PCR (primers CCGGATCCAGGGACCATGTTTTACCC and GGGTCGACATT-CATAGCCTTCAATG), and cloned into the GST expression vector pGEX-2T (Pharmacia, Stockholm, Sweden). The GST fusion protein was synthesized in *E. coli* and purified by glutathione affinity chromatography, according to the supplier's protocol. Probes for EMSA and DNase I footprinting analyses were prepared by filling in the 5'-overhang end of appropriate restriction enzyme fragments of the *GST-P1* gene with Klenow DNA polymerase I (Takara, Kyoto, Japan) and [α - 32 P]dCTP. A double-stranded oligonucleotide of the rat NQO1 ARE sequence (-TCTAGAGTCACAGTGACTTGCGAAATCTGA-) [19] was synthesized and labelled using T4 polynucleotide kinase (Takara) and [γ - 32 P]ATP.

EMSA and footprinting analyses were performed essentially as described previously [25]. Approx. 50 ng of fusion protein expressed in *E. coli* was preincubated with 0.5 μ g of poly(dI/dC) (Pharmacia) at room temperature for 15 min in 10 μ l of binding buffer (20 mM Hepes, pH 7.9, 20 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 4 mM MgCl₂, 15% glycerol and 100 μ g/ml BSA). 32 P-labelled DNA probe (approx. 2×10^4 c.p.m.) was added to the binding mixture and incubated for another 15 min. Protein-DNA complexes were analysed by 4% (w/v) polyacrylamide gel electrophoresis in 0.5 $\times$ TBE (25 mM Tris/borate, 0.5 mM EDTA, pH 8.2). For footprinting analysis, the DNA-protein binding reaction was performed as for EMSA, but on a 5 $\times$ larger scale (50 μ l volume) with five times the protein con-

centration. Aliquots (50 μ l) of 5 mM $MgCl_2$, 5 mM $CaCl_2$ and 20 ng of DNase I (Takara) were added to the reaction and incubated for 2 min at room temperature. The reaction was terminated by the addition of 100 μ l of 20 mM EDTA/1% SDS/0.2 M NaCl. After Proteinase K treatment (100 μ g/ml for 15 min), DNA was extracted and analysed on a 6% (w/v) polyacrylamide gel containing 8 M urea. Adenine and guanine bases of the same probes were modified, digested by the Maxam–Gilbert method, and loaded as markers.

RESULTS

Cloning and sequencing of a gene encoding mouse Pi-class GST

In the mouse, the two genes encoding Pi-class GSTs are arranged in tandem (5'-GST-P2-GST-P1-3'). Of the two gene products, only GST-P1 (GSTPiB) displays catalytic activity towards common substrates (e.g. 1-chloro-2,4-dinitrobenzene), and this enzyme is predominantly increased during hepatocarcinogenesis. We have cloned a 4.8 kb fragment encompassing the region between

the sixth exon of the *GST-P2* gene and the fifth intron of the *GST-P1* gene. This clone includes the entire 5'-flanking sequence and possibly the regulatory region of the *GST-P1* gene. The cloned gene is displayed schematically in Figure 1. The nucleotide sequence of this clone is almost identical with that of the published sequences [12,13]. As described previously [12,13], a TATA box, and possible GC and CCAAT boxes (at positions -26, -46 and -69 respectively), are present in the promoter region. A TRE, similar to a Maf recognition element and an ARE, is located at position -59.

Cloning and sequencing of rat Nrf2 cDNA

Rat Nrf2 cDNA was cloned from the rat liver cDNA library and sequenced. The longest clone, prNrf2, contained 2307 nucleotides, encoding a 598-amino-acid open reading frame. A possible methionine initiation codon surrounded by the Kozak consensus sequence [26] is located 16 amino acids upstream from the initiation codon predicted for the human or mouse cDNA [27,28]. The amino acid sequence of rat Nrf2 shares 80.3% and

Figure 1 Schematic representation of mouse Pi-class GST genes

The boxes represent exons.

Figure 2 Activation of the *GST-P1* promoter by Nrf2

Transient transfection assay of the *GST-P1* promoter. (A) Deletion constructs were transfected into HepG2 cells with or without an Nrf2 expression vector. The arrow indicates the transcription start site. (B) The indicated fragments from the *GST-P1* 5'-flanking region were fused to the luciferase gene with a minimal promoter, and analysed as for (A).

Figure 3 Identification of Nrf2 binding sites

(A) MBP fusion proteins of Nrf2, MafK and MafB were incubated with an *NQO1* ARE probe (left panel) or *GST-P1* 5'-flanking fragments, as indicated (right panel). Approx. 50 ng of fusion protein was mixed with approx. 2×10^4 c.p.m. of the indicated probe. Probes employed in this experiment are indicated in the upper panel. (B) Footprinting analysis of Nrf2/MafK with the *GST-P1* promoter. Portions of approx. 1 μ g of Nrf2 and MafK fusion proteins were mixed and incubated with approx. 2×10^5 c.p.m. of probe, digested with DNase I and analysed, as described in the Experimental section. The same probe, modified by the Maxam–Gilbert method, was used as a marker (M). Thick and thin vertical bars indicate protection regions with strong and weak affinities respectively. The arrows specify the direction of Nrf2 binding consensus sequences. (C) Alignment of Nrf2/MafK binding sites.

98.4% identity with the corresponding proteins of human and mouse respectively.

Activation of the *GST-P1* gene by Nrf2

To determine whether the *GST-P1* gene is activated directly by Nrf2, transient transfection analyses were performed. A series of deletion constructs of the *GST-P1* 5'-flanking region fused to the luciferase gene were transfected into HepG2 cells with or without an Nrf2 expression plasmid. Figure 2(A) shows the results of luciferase assays of the 5'-deletion constructs. The -1188/Luc construct exhibited strong promoter activity, directing more than 100-fold higher luciferase activity than the negative plasmid control (pGEM-luc). Progressive deletion of the 5'-end (-1188 to -626) resulted in a slight increase in promoter activity (Figure 2A, open bars). This suggests the existence of negative element(s) in this region. Promoter activity was decreased gradually upon further deletion to position -50.

Activation of the *GST-P1* promoter by Nrf2 was determined using these deletion constructs. Nrf2 binds to DNA as a heterodimer in conjunction with a small Maf protein, e.g. MafK [22]; see Figure 3A). However, on co-transfection of the Nrf2 and MafK expression vectors, reporter activity was markedly decreased [29,30]; results not shown). This suggests that small Mafs are ubiquitously expressed [31], and that excess amounts

inhibit transcription of Nrf2-responsive genes by competing at the binding site to form homodimers that do not contain a transcription activation domain [32]. Nrf2 directly activated the *GST-P1* gene promoter, up to the -95 deletion position. The highest levels of activation were observed with -95/Luc, which contained the TRE/ARE sequence. To better define the responsive region for activation by Nrf2, various fragments of the *GST-P1* promoter were fused to the luciferase gene with a rat *GST-P* minimal promoter sequence containing TATA and GC box elements [8]. Figure 2(B) shows that fragments A (-1188/-777) and E (-95/+60) were strongly activated by Nrf2. Our data suggest that the *GST-P1* promoter contains at least two Nrf2-responsive regions.

Identification of Nrf2/MafK binding sites on the *GST-P1* gene promoter

To identify the binding site for Nrf2 on the *GST-P1* gene promoter, EMSA and footprinting analyses were performed using various fragments derived from the *GST-P1* promoter. The bZip DNA binding domain of Nrf2 fused to MBP was employed for these studies, as described in the Experimental section. As a control experiment, we used the ARE sequence of the *NQO1* gene promoter as a probe. Nrf2 alone did not bind to the *NQO1* ARE, but bound this sequence strongly as a heterodimer with

MafK (Figure 3A). A homodimer of MafK and MafB (a large Maf) binds this probe with weaker affinity. We divided the 5'-flanking region of the *GST-P1* gene into six fragments and analysed binding to the Nrf2/MafK heterodimer. Of the six fragments, fragments A-2 and E bound the Nrf2/MafK heterodimer proteins strongly, while fragments B and D bound weakly. A competition experiment using the same probe or the *NQO1* ARE confirmed specific binding (results not shown).

To further identify the binding sequence, footprinting analyses were performed. Fragments B and D, with no Nrf2-dependent enhancer activity (Figure 2B), bound Nrf2/MafK weakly, with no clear footprints (results not shown). In contrast, fragments A-2 and E displayed clear footprints, as shown in Figure 3(B). The A-2 fragment exhibited two relatively weak footprinting regions, while one strong binding region was observed for fragment E. Nucleotide sequences of the binding sites are aligned in Figure 3(C). The Nrf2 binding sequence of the proximal region (fragment E) is nearly identical with the Nrf2 consensus sequence. The two distal regions (fragment A-2) contain five conserved nucleotides of an eight-nucleotide consensus sequence. This suggests that the 5'-TGA-sequence may be important for binding to the Nrf2/MafK heterodimer. As expected, transcription enhancement of the proximal region by Nrf2 was greater than that of the distal region (Figure 2B).

Activation by androgen and responsive elements of its receptor

In contrast with rat GST-P, mouse GST-P1 is strongly expressed in adult male liver, whereas expression in the livers of female mice is approx. 10% of that in males. Hatayama et al. [14] reported that expression was decreased upon castration in males, while that in females increased upon administration of testosterone. These results clearly indicate that the gene encoding Pi-class GST in the mouse is strongly regulated by the androgen.

To determine whether androgen activates the *GST-P1* gene directly, reporter transfection analyses were performed. The 5'-upstream region of the *GST-P1* gene (up to position -1188) fused to the luciferase reporter was co-transfected with an androgen receptor expression vector into HepG2 cells. However, this region of the *GST-P1* gene was not strongly activated by androgen (4–5 fold; Figure 4A). Next, we examined the region further downstream from the cap site. The androgen-dependent activities of various constructs comprising the *GST-P1* gene (including introns) fused to a luciferase gene with a minimal promoter sequence were determined. Androgen-responsive activity was located in the fifth intron. As shown in Figure 4(A), androgen strongly activated the transcription of the reporter gene fused to a 700-nucleotide fragment (*SmaI*–*EcoRI*) of the fifth intron of the *GST-P1* gene. The 5' and 3' halves of this fragment (*SmaI*–*DraI* and *DraI*–*EcoRI*) also separately exhibited androgen-induced activity, although activation levels were lower. The fragment containing the fifth intron was further divided into four segments and analysed. Individual fragments exhibited androgen responsiveness with significantly lower activities (Figure 4B). These results indicate the presence of multiple androgen-responsive elements in the fifth intron of the *GST-P1* gene. The androgen-dependent activation of the smaller fragments was lower than that of the large fragment, suggesting a synergistic effect of multiple androgen-responsive elements.

To determine whether the androgen receptor binds these fragments directly, the DNA binding domain of the androgen receptor fused with GST was expressed in *E. coli* and used for EMSA and footprinting analyses, as described in the Experimental section. Consistent with the results of the reporter

Figure 4 Androgen-responsive elements in the *GST-P1* gene

(A) Various DNA fragments of the *GST-P1* gene were fused to the luciferase gene with a minimal promoter sequence and co-transfected into HepG2 cells with an androgen receptor expression vector, with (+) or without (–) synthetic androgen R1881 (10 nM). The *GST-P1* gene and fragments used for constructs are represented schematically in the upper panel. The numbers in parentheses indicate approximate fold induction by R1881. (B) Androgen-dependent enhancer activities of small fragments of the fifth intron of the *GST-P1* gene. *HaeIII* fragments (as shown in upper panel) of the fifth intron were fused to the luciferase vector and analysed for activation by androgen, as in (A).

transfection assay, all the fifth-intron fragments examined bound to the DNA binding domain of the androgen receptor (Figure 5B). Multiple retarded bands were observed for all fragments, suggesting that each part is composed of multiple receptor binding sites [33]. In footprinting analyses, at least three clear footprints were observed, at positions 219 (site 2), 333 (site 4) and 632 (site 7) from the 5'-end of the fifth intron (Figure 5C). These regions contain sequences similar to the androgen-responsive element consensus sequence (Figure 6). In addition, several weak binding sites were observed, between nucleotides 183 and 197 (site 1), 306 and 328 (site 3), 514 and 535 (site 5), and 549 and 612 (site 6) (Figure 5C). Figure 6 depicts part of the nucleotide sequence of fifth-intron and footprinting sites.

Androgen-responsive elements are usually composed of derivatives of palindromes or direct repeats containing -TGTTCT-. As shown in Figure 6, -TGTTCT- or similar sequences were observed in footprinting regions, but the other halves of the palindrome or repeat sequences were difficult to identify. Similar results have been obtained with a number of previously reported androgen-responsive elements, in that, while one half of the element was relatively conserved, the other half was divergent from the consensus sequence [34]. The androgen receptor binds as a dimer to a palindromic or direct-repeat DNA element. It is hypothesized that the first half of the receptor dimer binds half

Figure 5 Identification of androgen receptor binding sites in the fifth intron

(A) Schematic representation of the probes used for EMSA and footprinting analyses. (B) EMSA of various DNA fragments of the fifth intron of the *GST-P1* gene with the androgen receptor (AR). The DNA binding domain of the androgen receptor fused to GST (10–50 ng) was mixed with the indicated DNA fragments (2×10^4 c.p.m.), and analysed as described in the Experimental section. (C) Footprinting analyses of androgen receptor (AR) binding to the fifth intron of the *GST-P1* gene. Approx. 0.2 and 1.0 μ g (probes A and B) or 1.0 μ g (Probe H3) of fusion protein was bound to the indicated probe (2×10^5 c.p.m.) and digested by DNase I, as described in the Experimental section. G and A residues were chemically modified, cleaved and used as markers (M). Thick and thin vertical bars indicate protection regions with strong and weak affinities respectively.

Figure 6 Nucleotide sequence of the fifth intron of the *GST-P1* gene and androgen receptor binding sites

(A) Nucleotide sequence of the fifth intron of the *GST-P1* gene. The number from the 5'-end of the fifth intron is indicated on the left side of the sequence. Thick and thin lines above the sequence indicate strong and weak binding sites respectively. (B) Alignment of androgen receptor binding sites. Consensus sequences are underlined.

of the consensus sequence (-TGTTCT-) with high affinity, which promotes co-operative binding of the dimeric partner to the other half of the repeat sequence [34]. The presence of multiple androgen receptor binding sites in the fifth intron suggests a similar mechanism to explain their co-operative function.

DISCUSSION

Nrf2 directly binds and activates the mouse *GST-P1* gene

The present study demonstrates that the mouse *GST-P1* gene contains a minimum of three Nrf2 binding sites, and that Nrf2 activates this gene directly. However, Nrf2 mRNA is significantly expressed in normal liver, and levels are not affected by drug treatment. Itoh et al. [35] reported that Keap1 (an Nrf2 binding protein) binds Nrf2 in the cytoplasm and inhibits its transportation to the nucleus. Electrophilic compounds activate Keap1 and release Nrf2 into the nucleus. In *Nrf2* knockout mice, the pattern of expression of the *GST-P1* gene in the normal state was similar to that of wild-type mice, although induction of expression by xenobiotics was almost completely inhibited [17]. Therefore the basal expression of this gene must be activated by other factor(s). A computer search for transcription factor binding consensus sequences in the promoter region revealed a number of candidate elements: XRE (xenobiotic-responsive element), Ah receptor/Arnt binding site (-544), C/EBP (CAAT enhancer binding protein; -979 and -963) and CRE (cAMP-responsive

element; -976). The sequence of the Nrf2 binding site was similar to those of the TRE and Maf recognition elements. The Jun/Fos heterodimer binds the upstream Nrf2 binding site (-59), but not the distal sites (results not shown). Using transient transfection assays, we additionally confirmed that Jun/Fos and large Maf (c-Maf and MafB) activated the -59 element (results not shown). Jun, Fos and large Maf are promising candidates as a transcription factor responsible for the expression of this gene in normal mouse liver.

Androgen activation of the *GST-P1* gene

Hatayama et al. [14] clearly demonstrated regulation of this gene by androgen. In the present study, we have identified multiple androgen-responsive elements in the fifth intron of the *GST-P1* gene. None of these elements was completely identical with the consensus binding sequences reported so far. Although the androgen-dependent enhancer activity of the fifth intron was comparable with that of the three-time-repeats consensus element (-AGTACAGCATGTTCT-; Figure 4A), the binding of these elements to the receptor was weak in comparison with that to the androgen binding consensus sequence, as determined by EMSA analyses (results not shown). However, androgen-dependent enhancer activity was decreased drastically when the fifth-intron region was divided into small fragments. These results suggest that these relatively weak binding sites function as multiple androgen-responsive elements that activate transcription in a synergistic manner. Kasper et al. [36] reported that two androgen-responsive elements of the probasin ('prostate-specific protein regulated by androgen') gene bind the androgen receptor cooperatively and are mutually dependent on each other. The probasin gene contains two androgen-responsive elements approx. 100 nucleotides apart, which show strong androgen-dependent activity. However, upon fragmentation, the enhancer activity of each fragment was almost completely abolished, and binding affinity to the androgen receptor decreased. A similar situation was observed for the androgen-responsive elements of the *GST-P1* gene. In addition to the fifth intron, another androgen receptor binding sequence has been identified in the 5'-upstream region (-1175). The site displays a rather clear footprint, despite little androgen-dependent enhancer activity (Figure 4A). Therefore this element alone may not be sufficient for strong enhancer activity. Indeed, most androgen-regulated genes reported previously have multiple responsive elements [34]. Since a similar situation appears to exist for oestrogen-responsive genes [37,38], it may be a common feature of steroid-dependent gene regulation.

The expression of genes encoding Pi-class GSTs in the rat and mouse is surprisingly different. Rat GST-P is almost completely suppressed in normal liver, but highly expressed during hepatocarcinogenesis. Although expression of mouse GST-P1 is also increased in hepatic transformation, the extent of expression is much less than that in rat liver. A part of the reason for this discrepancy may be the absence of a strong enhancer element, GPE1, from the mouse gene. No androgen-dependent expression is observed for the rat *GST-P* gene, although the nucleotide sequence of the fifth intron is 75% identical with that of the mouse gene. The differences in expression patterns of the genes encoding Pi-class GSTs in the rat, mouse and human require detailed clarification.

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