Co-operation of the transcription factor hepatocyte nuclear factor-4 with Sp1 or Sp3 leads to transcriptional activation of the human haem oxygenase-1 gene promoter in a hepatoma cell line

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We reported previously that the 5′-flanking region (nucleotides −1976 to −1655) of the human haem oxygenase-1 (*hHO-1*) gene enhances hHO-1 promoter activity in human hepatoma HepG2 cells, but not in HeLa cells [Takahashi, Takahashi, Ito, Nagano, Shibahara and Miura (1999) Biochim. Biophys. Acta **1447**, 231–235]. To define more precisely the regulatory elements involved, in the present study we have functionally dissected this region and localized the enhancer to a 50 bp fragment (−1793 to −1744). Site-direct mutagenesis analysis revealed that two regions were responsible for this enhancer activity, i.e. a hepatocyte nuclear factor-4 (HNF-4) homologous region and a GC box motif homologous region. Mutation in either region alone moderately decreased enhancer activity. However, mutations in both regions reduced promoter activity to the basal level. Electrophoretic mobility-shift assays demonstrated that the P5-2 fragment (−1793 to −1744) interacted with at least two nuclear factors, i.e. HNF-4 and Sp1/Sp3. Co-transfection experiments using *Drosophila* SL2 cells revealed that HNF-4 and Sp1/Sp3 synergistically stimulated the enhancer activity of the P5-2 fragment. These results indicate that co-operation of HNF-4 with Sp1 or Sp3 leads to the activation of *hHO-1* gene expression in hepatoma cells.

Key words: enhancer, GC box, gene regulation, HepG2 cells.

INTRODUCTION

Haem oxygenase (HO) is the rate-limiting enzyme in haem catabolism. This enzyme cleaves haem to form biliverdin, which is converted further into bilirubin by biliverdin reductase. Bilirubin, the final product of haem catabolism by HO, has an antioxidative capacity [1]. HO activity is derived from three isoenzymes, i.e. HO-1, HO-2 and HO-3. HO-1 is the ubiquitous and inducible form, found abundantly in the spleen, placenta and fetal liver [2–4]. HO-2 is the constitutive form found mainly in the brain and testis [2], whereas HO-3 contributes little to haem catabolism [5].

HO-1 gene expression is activated by various types of oxidative stress, such as hydrogen peroxide, UV irradiation, cadmium chloride, sodium arsenite, menadione, buthionine sulphoximine (glutathione-depleting compound), ozone inhalation, hypoxia and hyperoxia [6–11]. Recently we showed that the level of HO-1 mRNA increased rapidly in response to a rise in oxygen tension in human HepG2 hepatoma cells [12]. Studies on *HO-1* knockout mice have proved that the up-regulation of the *HO-1* gene serves as an adaptive mechanism to protect cells from oxidative damage [13].

The hepatic expression of HO-1 mRNA is developmentally regulated [3,4]. Hepatic HO-1 mRNA levels in human and rat are higher in the fetus than in the adult. On the other hand, rat brain HO-1 mRNA levels do not change significantly between day 19 of fetal gestation and the adult [4]. The physiological significance of the higher expression of HO-1 in the fetal liver is not clear at present. However, the same pattern of fetal *HO-1* gene expression in both humans and rats suggests an important function of this protein in early development and a common regulatory mechanism during development. The α-fetoprotein gene is also developmentally regulated, being expressed at a high level in the fetal liver but being dramatically repressed in the adult liver [14]. Since α-fetoprotein gene expression is high in hepatoma cells such as HepG2, the positive regulatory *trans*-factors and *cis*-elements of human *HO-1* (*hHO-1*) gene expression that function in HepG2 cells might be also active in *HO-1* gene expression in the human fetus *in vivo*.

The transcription of genes that are specifically expressed in hepatic cells, such as those encoding albumin, transthyretin, α1-antitrypsin or fibrinogen, has been shown to be regulated by liver-enriched transcription factors, such as hepatocyte nuclear factor-1 (HNF-1) [15], HNF-3 [16], HNF-4 [17] and CCAAT/enhancer-binding protein α (C/EBPα) [18]. We have demonstrated that the 5′-flanking region (nucleotides −1976 to −1655) of the *hHO-1* gene enhanced hHO-1 promoter activity in human hepatoma HepG2 cells, but not in HeLa cells. This region carries sequences similar to HNF-1 and HNF-4 binding sites [19]. In the present study, we first defined the 50 bp enhancer element responsible for the basal expression of the *hHO-1* gene. This element contains the HNF-4 binding site and confers HNF-4-

Abbreviations used: AP-1, activator protein-1; ARP, apolipoprotein regulatory protein; CBP, CREB ('cAMP-response-element-binding protein') binding protein; C/EBP, CCAAT/enhancer-binding protein; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; HIF-1, hypoxia-inducible factor-1; HNF, hepatocyte nuclear factor; (h)HO, (human) haem oxygenase; PPAR, peroxisome-proliferator-activated receptor; PRR, positive regulatory region; RAR, retinoic acid receptor; RXR, retinoid X receptor; Stat/STAT, signal transduction and activator of transcription; SV40, simian virus 40.

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mediated induction of *HO-1* gene expression. Furthermore, we demonstrated that maximal promoter activity in human hepatoma cells was dependent on co-operation between the HNF-4 site and the Sp1 site, which is located downstream of the HNF-4 site.

EXPERIMENTAL

Construction of plasmids

Hybrid genes comprising the hHO-1 promoter attached to the luciferase gene were constructed as follows (see Figure 1). Promoter fragments comprising nucleotides −58 to +24 or −263 to +24 were ligated to the pGL3-Basic vector (Promega, Madison, WI, U.S.A.) to generate phHOLUC(−58) or phHOLUC(−263) respectively [19]. phHOLUC(−58) and phHOLUC(−263) were then digested with *Kpn*I, blunted with T4 DNA polymerase, and ligated with an *Eco*RV linker (CGATATCG; Nippon Gene, Toyama, Japan) to generate phHOLUC(−58)(K/E) and phHOLUC(−263)(K/E) respectively. A pRL-TK vector (Promega) was digested with *Bgl*II and *Hin*dIII to generate a 0.8 kb fragment containing the herpes simplex virus thymidine kinase promoter. This 0.8 kb fragment was inserted into a *Bgl*II/*Hin*dIII site of the pGL3-Basic vector to generate pGL3-TK. This plasmid was digested with *Xho*I, blunted with T4 DNA polymerase, and ligated with an *Eco*RV linker (CGATATCG; Nippon Gene) to generate pGL3-TK(X/E).

phHOLUC(−1976) [19] was digested to generate a 372 bp *Kpn*I/*Bst*EII fragment containing a positive regulatory region (PRR). This fragment was blunted by treatment with T4 DNA polymerase and then cloned into a *Sma*I site of a pGL3-Promoter simian virus 40 (SV40) vector (Promega) and into *Eco*RV sites of pGL3-TK(X/E), phHOLUC(−263)(K/E) and phHOLUC(−58)(K/E), in forward and reverse orientations, to generate phHOLUCSV40/P(+), phHOLUCSV40/P(−), phHOLUCTK/P(+), phHOLUCTK/P(−), phHOLUC(−263)/P(+), phHOLUC(−263)/P(−), phHOLUC(−58)/P(+) and phHOLUC(−58)/P(−). The unique 5′ *Eco*RV site of phHOLUC(−58)(K/E) was used as the insertion site for various dissected DNA fragments in forward or reverse orientation to test their enhancer activity.

The expression vector for HNF-4α2 (pEF-BOS/HNF4) was generously provided by Dr M. Takiguchi [20]. *Drosophila* expression vectors for Sp1 and Sp3 (pPac-Sp1/flu and pPac-Sp3/flu respectively) were gifts from Dr J. M. Horowitz [21], and that for Sp4 (pPac-Sp4) was from Dr G. Suske [22]. The coding region of hHNF-4α2 was amplified from pEF-BOS/HNF4 by the PCR method, then this fragment was ligated to the *Bam*HI and *Xho*I sites of the pPac vector [21] to generate pPac-HNF-4α2. An internal control luciferase vector, pPac-Rluc, was constructed by replacing the *Sp1* gene of the pPac-Sp1/flu vector with the *Luc* gene obtained from the pRL-TK vector (Promega).

Cell cultures and transient expression analysis

Dulbecco's modified Eagle's medium and minimal essential medium were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum was obtained from Dainippon Pharmaceutical Co., Ltd (Tokyo, Japan). L-Glutamine and sodium bicarbonate were from Gibco BRL (Grand Island, NY, U.S.A.). Benzylpenicillin potassium was from Meiji Seika, Ltd (Tokyo, Japan), and streptomycin was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The human hepatoma cells (HepG2)

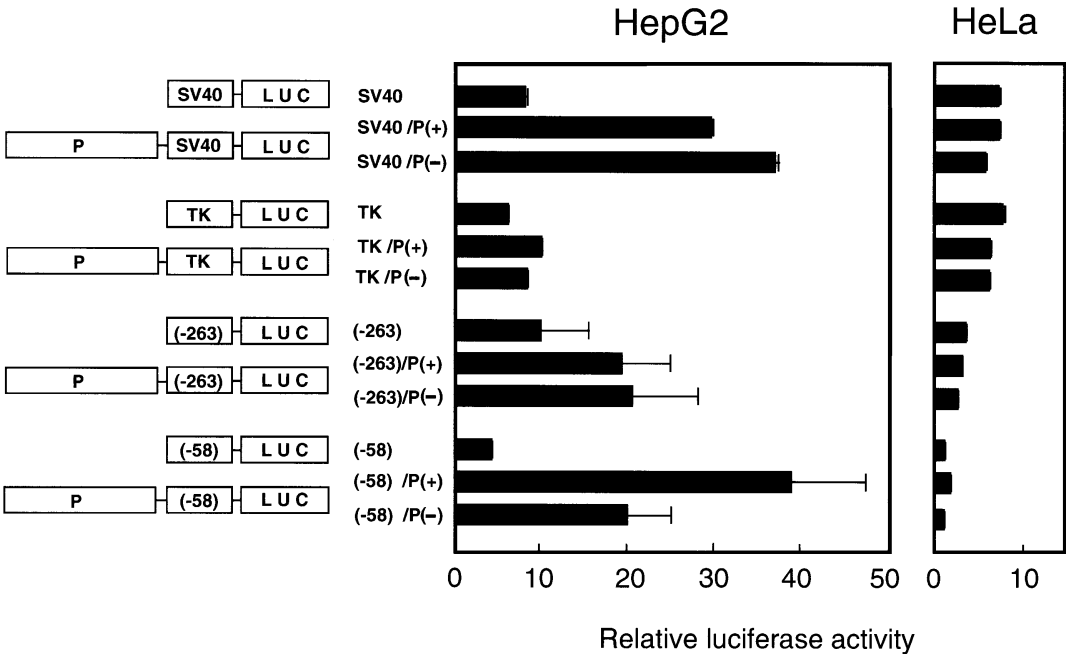

Figure 1 Cell-type-specific expression of the PRR of the *hHO-1* gene on the hHO-1 and SV40 promoters

The PRR 5′-flanking fragment (positions −1976 to −1607) of the *hHO-1* gene (P) was inserted upstream of two homologous hHO-1 promoters, phHOLUC(−263) (covering positions −263 to +24) and phHOLUC(−58) (covering positions −58 to +24), in the forward (+) or reverse (−) orientation. This sequence was also ligated to heterologous SV40 and herpes simplex virus thymidine kinase (TK) promoters. The reporter plasmids were used to transfect two cell lines, HepG2 and HeLa, as described in the Experimental section. After 48 h in culture, the cells were lysed and luciferase activities were determined. The luciferase activity of pGL3-Basic was designated as 1.0 for each cell line, and each value of luciferase activity represents the mean ± S.E.M. for four independent experiments.

and HeLa S3 cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan).

HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum, 4 mM glutamine, 250 units/ml benzylpenicillin potassium and 100 μg/ml streptomycin. HeLa cells were maintained in minimal essential medium supplemented with 10 % (v/v) fetal bovine serum, 2 mM glutamine, 250 units/ml benzylpenicillin potassium and 100 μg/ml streptomycin. Both types of cells were kept at 37 °C in CO_2 (5 %)/air (95 %) under a humidified atmosphere.

HepG2 (1×10^6) and HeLa (5×10^5) cells were plated in 60-mm dishes 1 day before transfection. A DNA solution containing 0.43 pmol of the test plasmid, 5 μl of FuGENE6 transfection reagent (Roche Molecular Biochemicals) and 95 μl of serum-free medium was added to the cultured cells. Cells were co-transfected with the Sea-pansy luciferase plasmid (0.1 μg) pRL-SV40 (Promega) as an internal control. After a 48 h incubation, the cells were lysed with passive lysis buffer (Promega). Cell lysates were used to determine luciferase activity with Lumat LB 9501 (EG and G Berthold, Badwildbad, Germany). A construct containing the luciferase reporter gene under the control of the SV40 promoter/enhancer (pGL3-Cont; Promega) was used separately as a positive control. In the co-transfection assay, 1.0 μg of reporter plasmid was used for transfection, along with 0.2–2.0 μg of modulator plasmids and 0.1 μg of an internal control plasmid, pRL-SV40. The amount of modulator plasmids was adjusted to 0.42 pmol by adding empty vector, pEF-BOS. For analysis of the P5-2 mutants, the cells were transfected with 1.0 μg of reporter plasmid, 1.0 μg of modulator plasmids and 0.1 μg of pRL-SV40 as an internal control.

Drosophila SL2 cells were maintained in Schneider's medium (Invitrogen) supplemented with 10 % (v/v) fetal bovine serum under humid air at 25 °C. On the day before transfection, the SL2 cells (2.5×10^5) were subcultured into 35 mm dishes. They were transfected with 0.5 μg of reporter gene, 0.05 μg of internal control vector pPac-Rluc and 0.6 μg of effector expression vectors, adjusting with empty pPac plasmid using FuGene6 solution. After 48 h, cells were harvested for luciferase assay.

Preparation of nuclear extracts

Preparation of nuclear extracts from HepG2 and HeLa cells was carried out using a modification of the protocol of Dignam et al. [23]. Protein concentrations were determined with a BCA Protein Assay Kit (Pierce).

Electrophoretic mobility-shift assay

Single-stranded oligonucleotides were end-labelled using [γ-^{32}P]-ATP (ICN Biochemicals, Inc., Costa Mesa, CA, U.S.A.) and T4 polynucleotide kinase, and then annealed as described previously [24]. The unincorporated label was removed by gel filtration on a Sephadex G-50 column (Amersham Pharmacia Biotech). A 10 μg aliquot of nuclear extract was incubated with 2 μg of poly(dI-dC) (Sigma) on ice for 15 min in the presence or absence of unlabelled competitor DNA (0.8 pmol), and then incubated with the end-labelled oligonucleotide (approx. 7×10^3 c.p.m./0.004 pmol) at 20 °C for 15 min. This binding reaction was carried out in a solution containing 12 mM Hepes (pH 7.9), 60 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 12 % glycerol, 1 mM dithiothreitol and 1 × Complete™ Protease Inhibitor Cocktail (Roche Molecular Biochemicals). In the supershift experiments, 2 μl of antibody was added to the above reaction mixture, which was then incubated at 20 °C for 30 min. The reaction mixtures were loaded on to a non-denaturing 4.5 % (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 29:1, w/w) containing 4 % glycerol made in 0.5 × TBE (1 × TBE contains 90 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.0) that had been pre-electrophoresed at 130 V for 2 h at 4 °C. After electrophoresis had been performed at 150 V at 4 °C for 3 h, the gels were dried and autoradiographed with an intensifying screen.

Rabbit anti-(human Sp1) polyclonal antibody was purchased from Geneka Biotechnology Inc. (Montreal, Canada). Antibodies against Sp2 (K-20), Sp3 (D-20), Sp4 (V-20), HNF-4α (C-19), chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI) (N-19), apolipoprotein regulatory protein-1 (ARP-1) (T-19), retinoid X receptor α (RXRα) (D-20), RXRα (ΔN197) and RXRγ (M-454) were purchased from Santa Cruz Biotechnology.

Oligonucleotides

For the gel shift competition analysis, the oligonucleotide sequence of the δEF1 site (the κE2 sequence of the immunoglobulin κ enhancer) [25], GTAATCTGGGCCACCTGCCTGCCTGGGAGGA, was used. Double-stranded oligonucleotides containing the recognition sites for transcription factors HNF-4, RXR, retinoic acid receptor (RAR), peroxisome-proliferator-activated receptor (PPAR), Stat5 (signal transduction and activator of transcription 5)/Stat6, C/EBP and activator protein-1 (AP-1) were purchased from Stratagene, and the oligonucleotide for Sp1 was obtained from Geneka Biotechnology Inc. The sequences of these oligonucleotides are: HNF-4, CTCAGCTTGTACTTTGGTACAACTA; RXR, AGCTTCAGGTCAGAGGTCAGAGAGCT; RAR, TCGAGGGTAGGGTTCACCGAAAGTTCACTCG; PPAR, CAAAACTAGGTCAAAGGTCA; Stat5/Stat6, GTATTTCCCAGAAAAGGAAC; C/EBP, TGCAGATTGCGCAATCTGCA; AP-1, CGCTTGATGACTCAGCCGGAA; Sp1, ATTCGATCGGGGCGGGGCGAGC. The sequences of the oligonucleotides for plasmid constructions and the electrophoretic mobility-shift assay were as follows: P5-1 (positions −1807 to −1769), TGGGGGACTTTATCTGCCTAGGACAACCTTTGTCCCTGT; P5-2 (positions −1793 to −1744), TGCCTAGGACAACCTTTGTCCCTGTGCGGCTCCACCTCCACCTTCCCTTA; P5-3 (positions −1768 to −1724), GCGGCTCCACCTCCACCTTCCCTTAAAGTCGGCCTTTCACCTCCA [19].

RESULTS

Cell-type-specific expression of the *hHO-1* gene on the hHO-1 and SV40 promoters

To examine the cell-type specificity of the PRR [19] of the *hHO-1* gene in detail, we introduced the constructs shown in Figure 1 into HepG2 and HeLa cells for transient expression. In HepG2 cells, insertion of the PRR upstream of the homologous promoter (−58 to +24) of phHOLUC(−58) in the forward (+) and reverse (−) orientations resulted in 9-fold and 4.6-fold increases respectively in luciferase activity. The insertion of this fragment upstream of the other homologous promoter (−263 to +24) of phHOLUC(−263) increased luciferase activity by only 1.9-fold (forward) and 2.1-fold (reverse), although the absolute luciferase activities were comparable between these promoter constructs. Furthermore, insertion of the PRR upstream of the heterologous SV40 promoter in the forward and reverse orientations resulted in 3.7- and 4.6-fold increases respectively in luciferase activity in HepG2 cells. When the PRR was inserted upstream of the thymidine kinase promoter, the increase in luciferase activity was slight. In contrast with the results in HepG2 cells, no significant activation by the PRR was observed in HeLa cells. These results indicate that the PRR exerts its effect in a cell-type-dependent

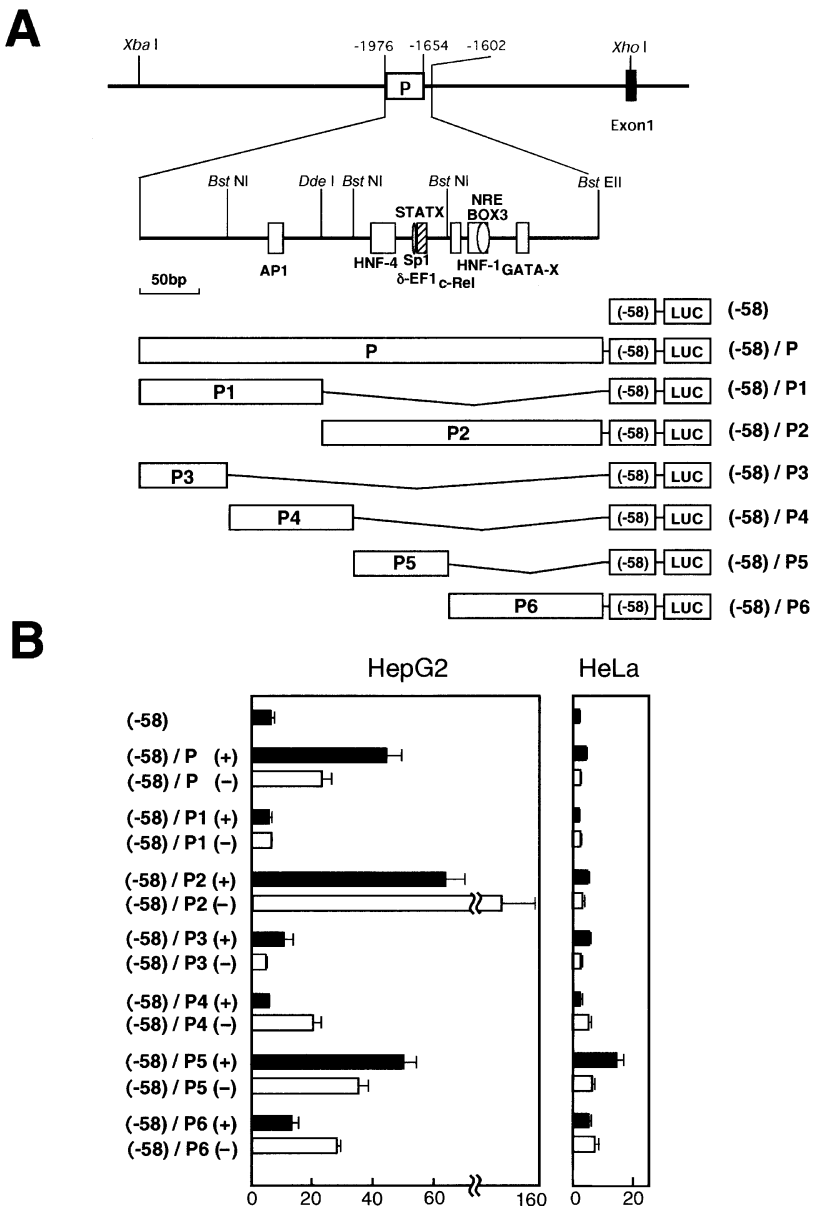

Figure 2 Localization of the core regulatory region within the PRR that confers enhancer activity

(**A**) Schematic representation of the *hHO-1* gene PRR and deletion constructs. The PRR was divided into six fragments, P1 (−1976 to −1829), P2 (−1831 to −1607), P3 (−1976 to 1905), P4 (−1905 to −1807), P5 (−1807 to −1724) and P6 (−1724 to −1607), with restriction enzymes, and cloned upstream of the hHO-1 promoter (positions −58 to +24) and the luciferase hybrid vector, phHOLUC(−58), in the forward (+) or reverse (−) orientation. The DNA sequences that resemble transcription factor elements are indicated as AP-1, HNF-4, Sp1, δEF1, STAT-X, c-Rel, HNF-1 and GATA-X, which denote the binding sites for AP-1, HNF-4, Sp1, δEF1, STAT signalling transcription factors, c-Rel, HNF-1 and GATA transcription factors respectively. NRE BOX3 denotes the sequence homologous to silencer elements of the chicken lysozyme gene. (**B**) Functional analysis of the PRR. The reporter plasmids were transfected into two cell lines, HepG2 and HeLa, and luciferase activities were determined as described in the Experimental section. The luciferase activity of pGL3-Basic was designated as 1.0 for each cell line, and each value of luciferase activity represents the mean ± S.E.M. for four independent experiments.

manner, and that the activity of the PRR depends on promoter sequences.

Identification of the core regulatory region within the PRR that confers cell specificity

To localize the core regulatory region within the PRR fragment, we divided the PRR into six fragments, i.e. P1, P2, P3, P4, P5 and P6, using restriction enzymes as shown in Figure 2(A), and cloned them upstream of the hHO-1 promoter (positions −58 to +24) of phHOLUC(−58) in the forward or reverse orientation. We employed the region encompassing positions −58 to +24 as the test promoter in this assay, since the PRR strongly activated phHOLUC(−58) by up to 9-fold (Figure 1), in contrast with the weaker 2.1-fold enhancement of phHOLUC(−263). HepG2 and HeLa cells were transfected with each test plasmid, and luciferase activities were then measured (Figure 2B). In HepG2 cells, the full enhancer activity of the PRR resided in the P2 fragment (10-fold increase). Further dissection showed that nearly comparable activity resided in the P5 fragment, the activity of which was

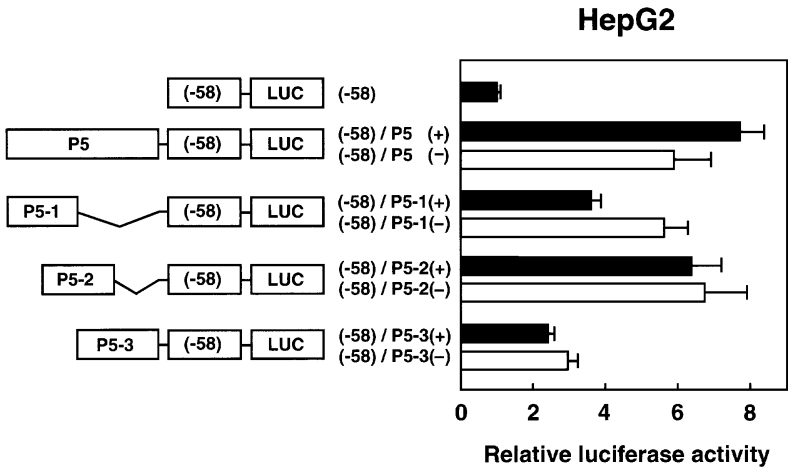

Figure 3 Sublocalization of the core regulatory region within the P5 fragment containing the *cis*-regulatory element that confers enhancer activity

The P5 fragment was divided into three fragments, P5-1 (−1807 to −1769), P5-2 (−1793 to −1744) and P5-3 (−1768 to −1724), and cloned upstream of the hHO-1 promoter (positions −58 to +24) in phHOLUC(−58). HepG2 cells were transfected with reporter plasmids, and luciferase activities were determined as described in the Experimental section. The luciferase activity of phHOLUC(−58) was designated as 1.0, and each value of luciferase activity represents the mean ± S.E.M. for four independent experiments.

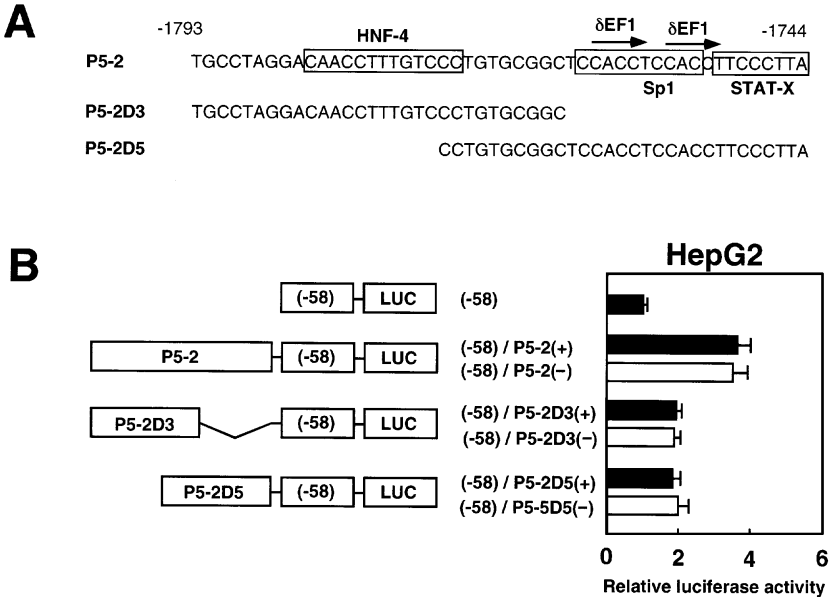

Figure 4 Identification of the core regulatory region within the P5-2 fragment containing the *cis*-regulatory element that confers enhancer activity

(**A**) Nucleotide sequence of the P5-2 fragment. The DNA sequences that resemble transcription factor elements are indicated as HNF-4, Sp1, δEF1 and STAT-X. The P5-2 fragment was divided into two overlapping fragments, P5-2D3 and P5-2D5, representing deletion of the 3′-end and the 5′-end respectively. (**B**) Functional analysis of the P5-2 fragment. The reporter plasmids were used to transfect HepG2 cells, and luciferase activities were determined as described in the Experimental section. The luciferase activity of phHOLUC(−58) was designated as 1.0, and each value of luciferase activity represents the mean ± S.E.M. for four independent experiments.

approx. 79% that of the P2 fragment when located in a forward orientation in relation to the hHO-1 promoter. The P2 fragment displayed high activity (23-fold activation) when inserted in the reverse orientation. The P4 and P6 fragments showed greater activation in the reverse than in the forward orientation. These results indicate that the P5 fragment contains mainly positive elements. Surprisingly, when HeLa cells were transfected with these plasmids, although the P2 fragment did not activate the HO-1 promoter, the P5 fragment was able to activate the HO-1 promoter to an extent comparable with that in HepG2 cells (5.5-fold). This may result from unidentified negative regulatory elements that potentially act only in HeLa cells.

In order to localize the positive regulatory element within the P5 fragment, we segmented P5 into three overlapping fragments, i.e. P5-1, P5-2 and P5-3. These fragments were ligated upstream of the HO-1 promoter of phHOLUC(−58), to generate phHOLUC(−58)/P5-1, phHOLUC(−58)/P5-2 and phHOLUC(−58)/P5-3, as shown in Figure 3. The positive regulatory activities of these vectors were assayed in HepG2 cells. The activity of the P5-2 fragment was comparable with that

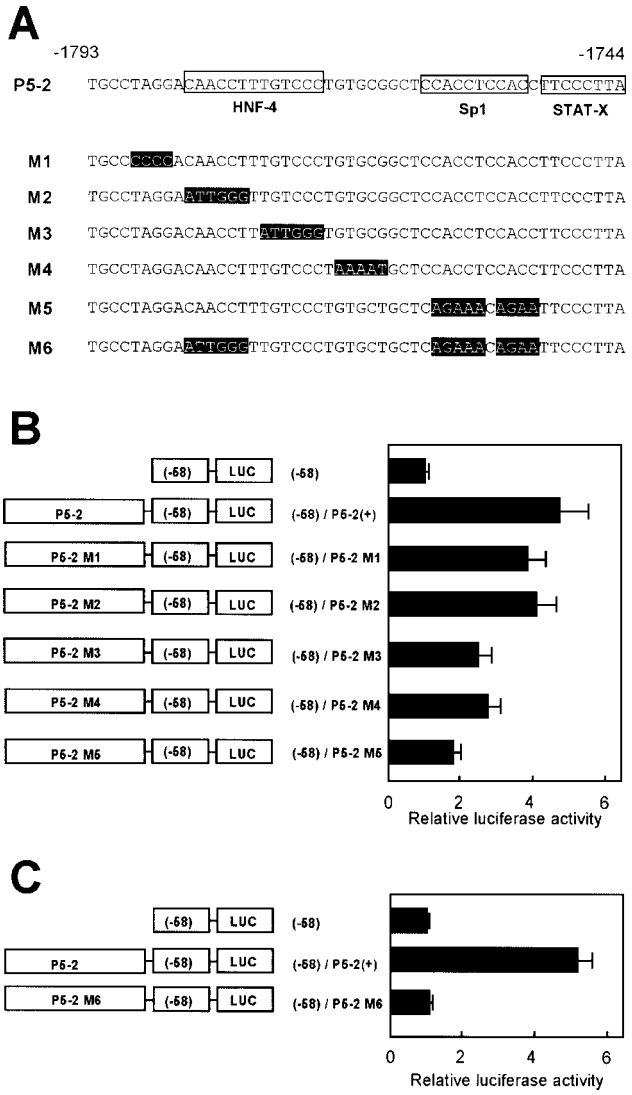

Figure 5 Effect of mutation of the P5-2 fragment on transcriptional activity

(**A**) Sequences of P5-2 and its mutants. The original (P5-2) and mutated (M1–M6) fragments are shown (top-stranded sequence). Altered bases are highlighted. (**B**, **C**) Enhancer activity of P5-2 and its mutants. HepG2 cells were transfected with the reporter plasmids, and luciferase activities were determined as described in the Experimental section. The luciferase activity of phHOLUC(−58) was designated as 1.0, and each value of luciferase activity represents the mean ± S.E.M. for at least six independent experiments.

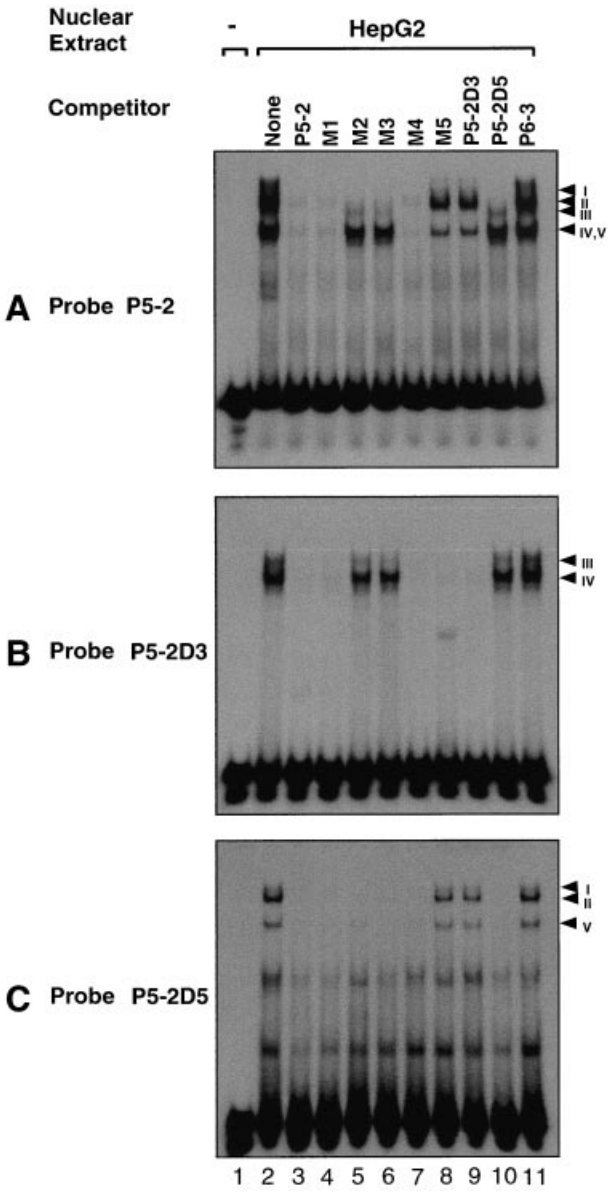

Figure 6 Gel shift competition analysis using P5-2 and its mutants

Nuclear extract of HepG2 cells (10 μg) was incubated with 0.004 pmol of ^{32}P-labelled probes: P5-2 (**A**), P5-2D3 (**B**) and P5-2D5 (**C**). In a competition assay, a 200-fold molar excess of unlabelled competitors was added to the reaction mixture. Lane 1 contains no extract. Lane 2 shows the binding of the HepG2 nuclear factors to the ^{32}P-labelled wild-type P5-2, P5-2D3 or P5-2D5 fragment without competitor. Lanes 3–8 show competition of nuclear factor binding by a 200-fold molar excess of unlabelled mutant oligonucleotides (M1–M5). Lanes 9–11 show competition with unlabelled probes P5-2D3, P5-2D5 and P6-3 (unrelated sequence in the 5′-upstream region of the *hHO-1* gene; −1712 to −1687) respectively. The resulting complexes were resolved by electrophoresis in non-denaturing 4.5% (w/v) polyacrylamide gels. Arrowheads indicate novel DNA–protein complexes.

of the original P5 fragment, while those of P5-1 and P5-3 were less than half of the activity of P5.

Consequently, the P5-2 fragment was dissected further into two overlapping fragements, P5-2D3 and P5-2D5, which are 3′ and 5′ end-deletion products respectively of the parental P5-2 fragment (Figure 4A). The promoter activities of P5-2D3 and P5-2D5 were approximately half that of P5-2, in both orientations (Figure 4B). These results indicate that the core PRR is localized primarily in the P5-2 fragment, and that full activity of the PRR requires both the 5′- and 3′-regions of the P5-2 fragment.

Inspection of the sequence of P5-2 using TFSEARCH [26] revealed that this region contains several putative binding sites for a number of known transcription factors, such as HNF-4, Sp1, δEF1 and STAT-X (Figure 4A). To define further the core sequences within the P5-2 fragment, we synthesized a series of base-substitution mutants of this fragment (Figure 5A), and examined their effects on hHO-1 promoter activity (Figure 5B). Mutations in the HNF-4 site (−1777 to −1772; mutant M3 in Figure 5A) reduced promoter activity to a level lower than that observed for wild-type P5-2. Mutations in the Sp1 site (−1761 to −1752; M5) resulted in an even greater decrease in promoter activity. Mutations in the region −1770 to −1766 (M4) had a weaker effect on promoter activity than mutants M3 or M5. The

base-substitution mutants (M3, M4 and M5) moderately reduced the HO-1 promoter activity. This prompted us to test whether these authentic sequences co-operate to activate gene expression. When a double mutation was introduced into the P5-2 fragment (mutant M6), enhancer activity was reduced to the basal level shown by the minimal promoter construct phHOLUC(−58) (Figure 5C).

Gel shift competition analysis of factors binding to P5-2, P5-2D3 and P5-2D5 fragments

To identify nuclear factors that bind to the regulatory elements in the P5-2 fragment, we performed gel shift analysis using end-labelled P5-2 (Figure 6A), P5-2D3 (Figure 6B) and P5-2D5 (Figure 6C) fragments as probes. Incubation of the probes with nuclear extracts prepared from HepG2 cells produced at least five DNA–nuclear-factor complexes (I–V; Figure 6, lane 2). These complexes represented sequence-specific interactions of nuclear factors with the P5-2, P5-2D3 and P5-2D5 fragments, since an excess (200×) of unlabelled P5-2 (lane 3), P5-2D3 (lane 9) or P5-2D5 (lane 10) fragments competed with the probes, whereas an unrelated fragment [P6-3, which is located in the 5′-upstream region of the hHO-1 promoter (−1712 to −1687); lane 11] did not compete with P5-2 and its deletion probes.

Next, we tested whether the binding of nuclear factors to the P5-2, P5-2D3 and P5-2D5 fragments would correlate with transcriptional activation. For this purpose, P5-2 mutant sequences were examined as competitors for the binding of nuclear factors to the P5-2, P5-2D3 and P5-2D5 fragments in the gel shift assay (Figure 6, lanes 4–8). When P5-2D3 was used as a labelled probe, mutants M2 and M3 failed to compete for the formation of DNA–nuclear-factor complexes III and IV. This result indicates that the M2 and M3 regions are critical for the binding of nuclear factors to the P5-2D3 fragment, although the M2 and M3 mutants showed moderately reduced enhancer activity compared with the wild-type sequence, as shown in Figure 5. When we used P5-2D5 as a probe, only mutant M5 showed impaired binding of nuclear factors. This loss of competition of M5 was comparable with the reduced transcription activity observed in Figure 5. As for the M4 mutant, although this mutation did not have any retarding effects on the formation of DNA–nuclear-factor complexes, it resulted in moderately reduced transcriptional activity compared with the wild-type sequence, suggesting that the M4 mutation could affect productive *trans*-factor binding to *cis*-DNA elements or protein–protein interactions *in vivo*, but such effects could not be detected using an *in vitro* gel shift competition assay. These results indicate that nuclear factors in complexes III and IV bind to the HNF-4 site, and those in complexes I, II and V bind to the Sp1 site.

Binding of HNF-4 and Sp1/Sp3 to the PRR of the *HO-1* gene

To characterize the factors that bind to the P5-2 fragment, we carried out gel shift competition analysis using double-stranded oligonucleotides containing binding sites for HNF-4, RXR, RAR, PPAR, Stat5/Stat6, δEF1, AP-1 and C/EBP (Figure 7), and Sp1 (Figure 8). The P5-2 fragment contains sequences equivalent to binding sites for HNF-4, Sp1, Stat5/Stat6 and δEF1 (Figure 4A). When the P5-2D3 fragment was used as a DNA probe, the HNF-4-binding oligonucleotide efficiently competed with the probe for the formation of complex IV (Figure 7B, lane 4). When the P5-2 fragment was used as a DNA probe, the competition with the HNF-4 oligonucleotide apparently reduced the formation of complex IV (Figure 7A, lane 4). Since it has

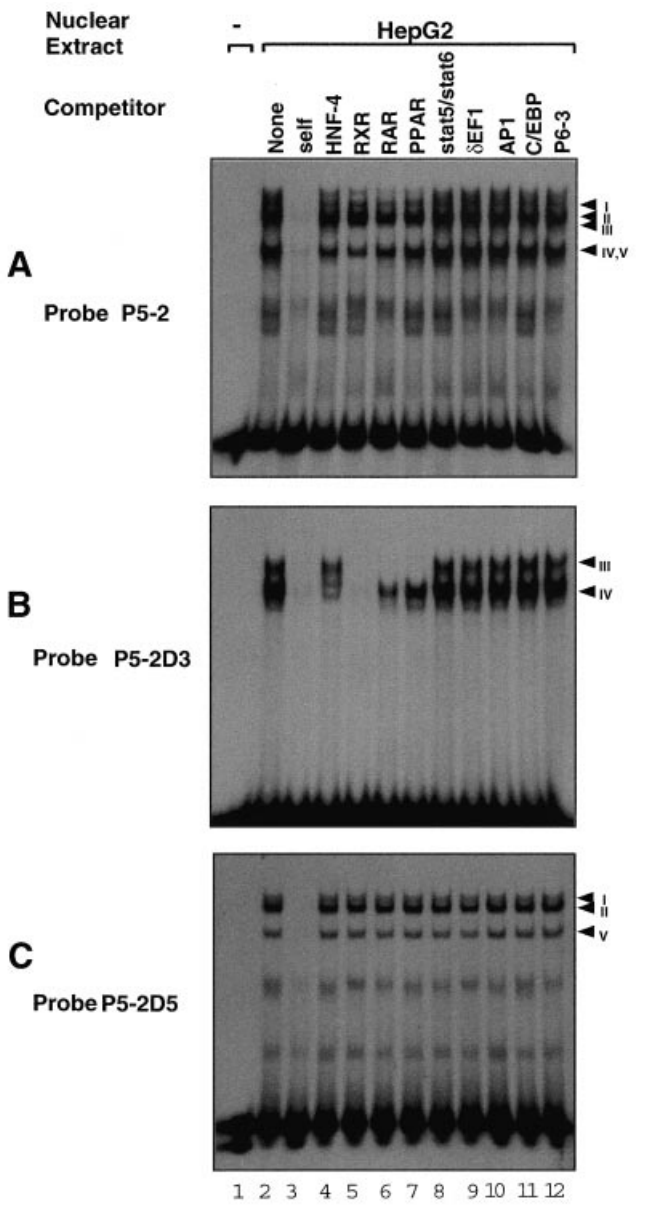

Figure 7 Gel shift analysis of P5-2 and its deletion fragments in the presence of exogenous competitors

Nuclear extract of HepG2 cells (10 μg) was incubated with 0.004 pmol of ^{32}P-labelled probes: P5-2 (**A**), P5-2D3 (**B**) and P5-2D5 (**C**). In a competition assay, a 200-fold molar excess of unlabelled P5-2, P5-2D3 or P5-2D5 ('self'; lane 3), P6-3 (unrelated sequence in the 5′-upstream region of the *hHO-1* gene; lane 12), or various double-stranded oligonucleotides containing binding sites for known transcription factors (lanes 4–11, HNF-4, RXR, RAR, PPAR, Stat5/Stat6, δEF1, AP-1 and C/EBP respectively) were added to the reaction mixture. The resulting complexes were resolved by electrophoresis in non-denaturing 4.5% (w/v) polyacrylamide gels. Lane 1 contains no extract. Arrowheads indicate novel DNA–protein complexes.

been demonstrated that one nuclear receptor-binding sequence shows cross-reactivity with those for other different nuclear receptors [27], oligonucleotides with which the nuclear receptors RXR, RAR and PPAR associate were tested as competitors. RXR, RAR and PPAR oligonucleotides competed with the P5-2 and P5-2D3 probes, although their affinities for the nuclear

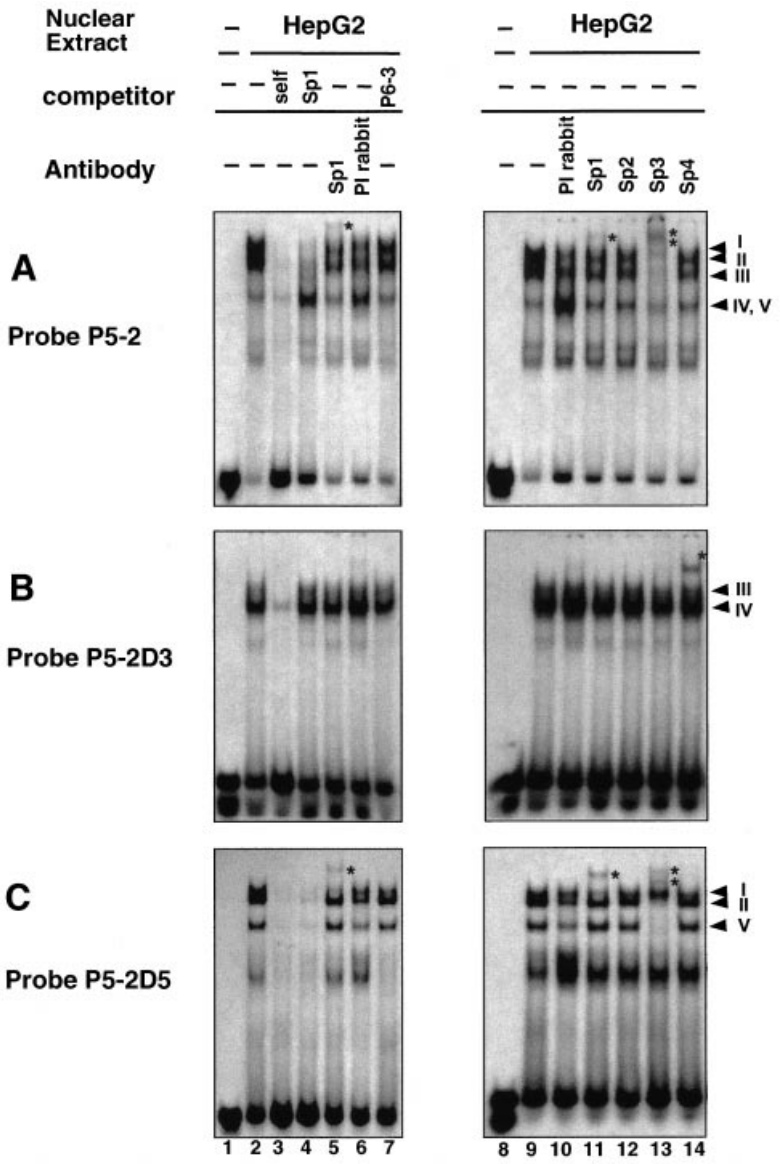

Figure 8 Gel shift analysis of P5-2 and its deletion fragments using Sp1 competitor and Sp-family antibodies

Nuclear extract of HepG2 cells (10 μg) was incubated with 0.004 pmol of ^{32}P-labelled probes: P5-2 (**A**), P5-2D3 (**B**) and P5-2D5 (**C**). In a competition assay, a 200-fold molar excess of unlabelled P5-2, P5-2D3 or P5-2D5 fragment ('self'; lane 3), P6-3 (unrelated sequence in the 5′-upstream region of the *hHO-1* gene; lane 7) or Sp1 double-stranded oligonucleotide containing the binding site for the Sp transcription factor (lane 4) was added to the reaction mixture. In a supershift assay, nuclear extracts were incubated with the ^{32}P-labelled P5-2, P5-2D3 or P5-2D5 probe alone (lanes 2 and 9) or with preimmune serum (PI rabbit; lanes 6 and 10) or antibodies against Sp1 (lanes 5 and 11), Sp2 (lane 12), Sp3 (lane 13) or Sp4 (lane 14). The resulting complexes were resolved by electrophoresis in non-denaturing 4.5% (w/v) polyacrylamide gels. Lanes 1 and 8 contain no extract. Arrowheads indicate novel DNA–protein complexes. The stars indicate novel complexes of reduced mobility generated in the presence of anti-Sp-family antibodies.

factors in complexes III and IV seemed to be different from each other (Figures 7A and 7B, lanes 5–7). When the P5-2D5 fragment was used as a DNA probe, only the Sp1 oligonucleotide competed with the probe for the formation of the nuclear-factor–DNA complexes (Figure 8C, lane 4). Addition of the unlabelled Sp1 oligonucleotide (100 ×) to the assay clearly competed with the P5-2 probe for formation of the retarding complex (Figure 8A, lane 4), concomitant with an enhanced HNF-4 shift. The oligonucleotides for Stat5/Stat6, δEF1, AP-1 (the ubiquitous factor), C/EBP (the typical liver-enriched factor) and P6-3 (an unrelated oligonucleotide) failed to block the formation of the complexes (Figure 7, lanes 7–9).

To further characterize nuclear factor binding to the P5-2 fragment, we performed a supershift assay using antibodies against HNF-4α, COUP-TFI, ARP-1 (COUP-TFII), RXR and RAR (Figure 9), and Sp1, Sp2, Sp3 and Sp4 (Figure 8). The anti-HNF-4α antibody supershifted complex IV with the probes P5-2 and P5-2D3, but not with the P5-2D5 probe (Figure 9, lane 5). Antibodies against Sp1 and Sp3 also interacted with the retarding complex I with the DNA probes P5-2 and P5-2D5, but not with P5-D3 (Figure 8, lanes 5 and 11). Anti-Sp3 antibody supershifted complexes II and V with the DNA probes P5-2 and P5-2D5, but not with P5-D3 (Figure 8, lane 13). Anti-Sp4 antibody interacted with the retarded band with the P5-2D3 DNA probe (Figure 8B,

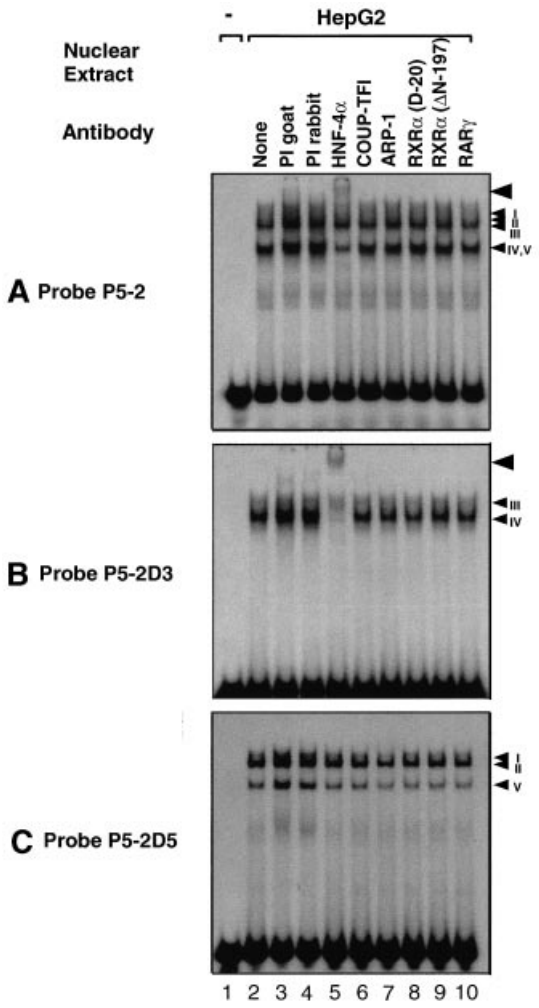

Figure 9 Supershift analysis of factors binding to the P5-2, P5-2D3 and P5-2D5 fragments

Nuclear extract of HepG2 cells (10 μg) was incubated with 0.004 pmol of ^{32}P-labelled probes: P5-2 (**A**), P5-2D3 (**B**) and P5-2D5 (**C**). Lane 1 contains no extract. Nuclear extracts prepared from HepG2 cells were incubated with the ^{32}P-labelled P5-2, P5-2D3 or P5-2D5 probe alone (lane 2), or with preimmune serum (PI; lanes 3 and 4) or polyclonal antibodies (lanes 5–10) as indicated. Anti-RXRα antibody (D-20) is specific for RXRα. Anti-RXRα antibody (ΔN-197) is reactive with RXRα, RXRβ and RXRγ. Anti-RARγ reacts with RARγ, RARβ and RARα. Numbered arrowheads indicate novel DNA–protein complexes. The large arrowheads indicate novel complexes of reduced mobility generated in the presence of anti-HNF-4α antibody.

lane 14). Antibodies against COUP-TFI, ARP-1, RXR, RAR and Sp2 did not interact with the DNA–protein complexes. Thus it appears that HNF-4α, Sp1 and Sp3 are modulators of the expression of the *HO-1* gene in HepG2 cells.

Synergistic activation of the HO-1 promoter by HNF-4 and Sp1/Sp3

The reporter gene assay and supershift analysis demonstrated the binding of HNF-4, Sp1 and Sp3 to the HO-1 promoter. To obtain further insight into the actions of HNF-4 and Sp1/Sp3 on *HO-1* gene expression, we performed co-transfection experiments using HepG2 cells and *Drosophila* Schneider SL2 cells. SL2 cells were used because they show no endogenous expression of Sp1 and Sp3. When HepG2 cells were co-transfected with the HNF-

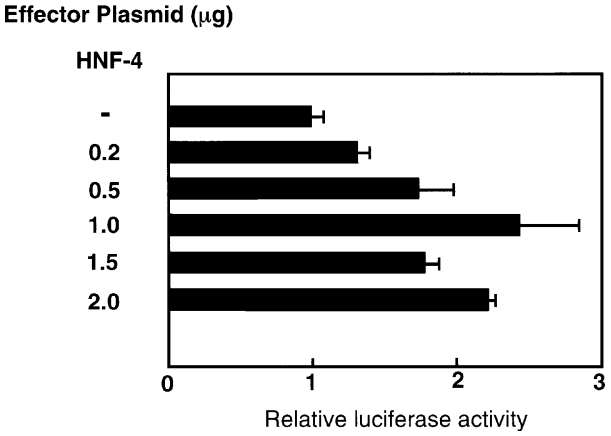

Figure 10 Activation of the hHO-1 promoter by HNF-4 in a co-transfection assay

HepG2 cells were co-transfected with various amounts of the HNF-4α2 expression plasmid pEF-BOS/HNF-4 and 1.0 μg of the reporter plasmid phHOLUC(−58)/P5-2(+) containing the hHO-1(−58) promoter. Luciferase activities were determined as described in the Experimental section. The activity of the control expression vector pEF-BOS was designated as 1.0, and each value of luciferase activity represents the mean ± S.E.M. for four independent experiments.

4α2 expression vector and the P5-2 Luc reporter, promoter activity was enhanced in a dose-dependent manner, reaching up to 2.5-fold increased expression (Figure 10). However, the mutant promoter/reporter, P5-2 M6 Luc (Figure 5), was not activated by co-transfection with the HNF-4α2 expression vector (results not shown).

Synergistic effects of Sp1/Sp3 and HNF-4α2 were demonstrated using SL2 cells (Figure 11). The P5-2 reporter was co-transfected into SL2 cells with Sp1/Sp3 and HNF-4α2 expression vectors, alone or in combination. Co-transfection of the reporter with the HNF-4α2 expression vector alone resulted in an approx. 2.5-fold increase in promoter activity in comparison with the control. This enhancement observed in SL2 cells was comparable with that in HepG2 cells. The transient expression of Sp3 in SL2 cells elevated reporter activity to 7-fold that of the control. The expression of Sp3 and HNF-4α2 in combination caused 24-fold up-regulation. Synergistic elevation was also demonstrated with the combined expression of Sp1 and HNF-4α2, whereas Sp4 expression with HNF-4α2 did not result in any synergism.

DISCUSSION

In recent years, a number of studies have been published on the *HO-1* gene promoter. *HO-1* gene expression is activated by stresses, such as heat shock, heavy metals, UV irradiation, lipopolysaccharide, hypoxia and hyperoxia, and the elements responsive to these stresses have been identified [28]. The stress-induced activation of the *HO-1* gene is mediated through heat-shock elements [29], hypoxia-response elements [9], stress-response elements [30] and cadmium-responsive elements [31]. Many physiological and environmental stresses have been shown to induce *HO-1* gene expression via the stress-response element, to which nuclear factor-E2-related factors bind [32]. HO-1-deficient cell lines are hypersensitive to oxidative stress induced by sub-lethal levels of hemin and H_2O_2. *HO-1* knockout mice, having no HO-1 activity, are not resistant to a sub-lethal lipopolysaccharide injection [13]. This indicates that HO-1 activity is very important in defence against the oxidative burden. It has also been proposed that a polymorphic DNA sequence of

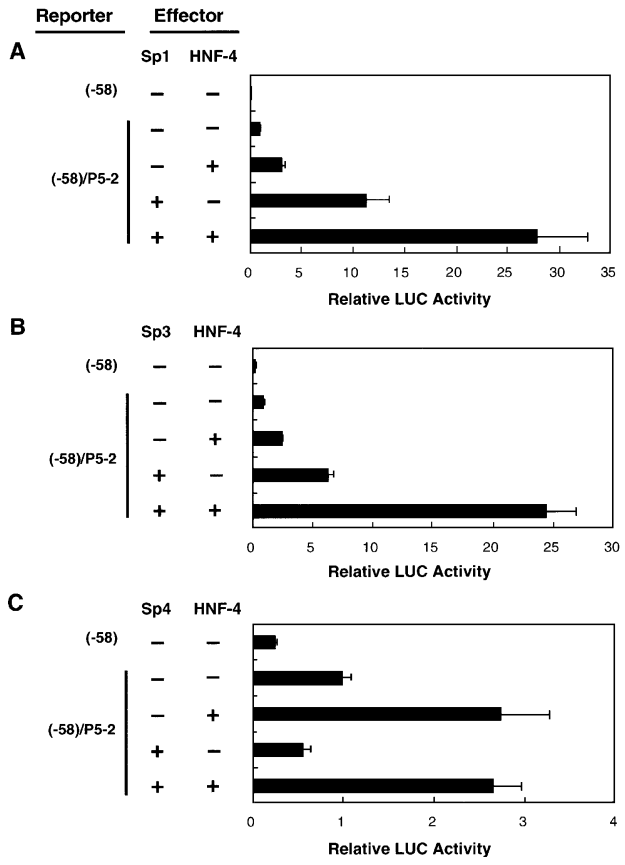

Figure 11 Synergistic activation of the hHO-1 promoter by HNF-4 and Sp1/Sp3

Drosophila SL2 cells were transfected with the reporter plasmid phHOLUC(−58)/P5-2(+) containing the hHO-1(−58) promoter and with Sp1, Sp3, Sp4 and HNF-4α2 expression vectors (pPac-Sp1, pPac-Sp3, pPac-Sp4 and pPac-HNF-4α2 respectively), alone or in combination as indicated. Luciferase activities were determined as described in the Experimental section. The activity of the control expression vector pPac was designated as 1.0, and each value of luciferase (LUC) activity represents the mean ± S.E.M. for four independent experiments.

the HO-1 promoter can modulate the degree of vulnerability to stress. A positive correlation between the length of the $(GT)_n$ repeat in the HO-1 promoter and susceptibility to the development of chronic pulmonary emphysema has been reported [33]. The long $(GT)_n$ repeat could attenuate the *HO-1* expression that may be induced by reactive oxygen species in cigarettes, thereby resulting in the development of chronic pulmonary emphysema. The PRR in the *HO-1* gene exhibited strong enhancer activity (9-fold activation) in the context of a minimal HO-1 promoter (positions −58 to +24) which did not contain the $(GT)_n$ repeat; in contrast, only low activity was observed with a $(GT)_n$-repeat-containing larger HO-1 promoter (−263 to +24) (Figure 1). This promoter dependency of the enhancer activity of the PRR may be partly attributable to the $(GT)_n$ repeat.

In the present study, deletion analysis of the cell type-specific promoter of the *hHO-1* gene identified the HNF-4 binding site in the 5′-flanking region. This direct repeat (5′-CAACCTTTGT-CCC-3′) is the consensus DNA sequence for HNF-4 binding [34]. Mutations at this site resulted in a partial reduction in the basal expression level of the gene. Similar phenomena were also observed for coagulation factor VII and the hepatic *CYP2D6* gene promoters [35–37]. This moderate reduction in basal expression caused by the M2 mutation was extended to full reduction by an additional mutation at the M5 site, whereas the M5 mutation alone caused only half reduction. The same reducing effect of a single mutation at the M2 or M5 site was demonstrated in the HNF-4 cotransfection experiments (data not shown). On the other hand, gel shift analysis revealed that the mutations in the HNF-4 site (M2 and M3) abolished the binding capacity for trans-acting factors. These contradictory results obtained from transfection experiments and gel shift assays imply that the binding affinity between M2 or M3 mutant and DNA-binding factors may be so weak that the each mutant does not efficiently compete with the wild-type sequence in the gel shift analysis. But these affinities could be strong enough to promote transcription in the reporter gene assay.

HNF-4 plays a critical role in the tissue-specific and hypoxia-inducible expression of the erythropoietin gene, whereas COUP-TF has a negative modulatory role [38]. Expression of HNF-4 in HeLa cells enhanced the hypoxic induction of an erythropoietin gene reporter, and mutation of the HNF-4 site diminished HNF-4 enhancement of the hypoxic response in HeLa cells from approx. 11-fold to 3-fold [38]. Hypoxia is known to induce *HO-1* gene expression via a hypoxia-response element that is located approx. 8 kbp upstream of the *hHO-1* gene and is bound by hypoxia-inducible factor-1 (HIF-1), although the physiological significance of the hypoxic induction of the *HO-1* gene is not clearly understood [9]. Hypoxia *in vivo* also induces *HO-1* expression in rat tissues. A marked increase in the HO-1 mRNA level was observed in the lung, liver, heart and aorta. Hypoxic induction in the liver is greater than in other tissues [9]. This high responsiveness in the liver might be due to the co-expression and association of HIF-1 and HNF-4 in hepatic cells under hypoxic conditions.

Expression of the *HO-1* gene is developmentally regulated. The level of mRNA for HO-1 in the rat liver is higher in the fetus than in the adult. In the human fetal liver, the enzyme activity was 8 times higher than that detected in the adult liver [3,4]. The HO-1 mRNA level in the placenta was comparable with that in the spleen, the major organ for HO-1 synthesis in the adult [4]. These changes seem to occur in response to the hypoxic environment that is maintained in the fetus. Taken together with the finding that cells overexpressing HO-1 exhibited remarkable resistance to high oxygen toxicity [9], these results suggest that the high-level expression of antioxidative HO-1 at late gestation may function in protection of the newborn against the sudden oxygen surge after birth.

Sp1 is a sequence-specific DNA-binding protein that plays a role in the transcription of many genes that contain a GC or GT box (KRGGMGKRRY) in their promoters [39,40]. Additional members of the Sp gene family, i.e. Sp2, Sp3 and Sp4, have been identified, and they all have a highly conserved zinc-finger DNA-binding domain [41]. Sp1, Sp3 and Sp4 can all bind to the same recognition sequences. Sp1 and Sp3 are ubiquitous factors, but Sp4 is expressed mainly in neural cells. This expression profile is consistent with our results showing the interaction of Sp1 and Sp3 with the P5-2 fragment of the *HO-1* gene in HepG2 cells (Figure 8). Double transfection of Sp1 and HNF-4 into *Drosophila* SL2 cells resulted in synergistic activation of the P5-2-derived *HO-1* gene promoter. Sp3 transfection also activated HO-1 transcription synergistically with HNF-4 (Figure 11). In contrast, transcriptional repression of Sp3 has been reported in several genes using various types of cells [42,43]. Therefore these results suggest that roles of Sp3 in transcription may depend on the cellular context.

Synergism of HNF-4 and Sp1/Sp3 in up-regulating *HO-1* gene expression was demonstrated in SL2 cells. Co-ordination of

HNF-4 and Sp1 has been reported in apolipoprotein CIII gene expression [44,45]. The molecular mechanism behind the synergistic up-regulation of target genes by HNF-4 and Sp1/Sp3 is unclear. It has been demonstrated that a co-activator, CREB ('cAMP-response-element-binding protein') binding protein (CBP), stimulated interaction of Sp1 with the target DNA, whereas DNA binding by Sp1 inhibited the interaction of Sp1 with CBP [46]. HNF-4 and Sp1 were shown to stimulate the histone acetyltransferase activity of CBP [47]. Moreover, acetylated HNF-4 also showed higher affinity for the target DNA than the unacetylated protein [48]. Studies are ongoing in our laboratory to determine the interaction of HNF-4 and Sp1/Sp3 proteins, and the role of acetylation of these transcription factors, in *HO-1* gene expression.

In conclusion, we have identified the cell-type-dependent positive regulatory region of the *hHO-1* gene and have localized its core elements to which a cell-type specific factor, HNF-4, and general transcription factors, Sp1/Sp3, bind. Furthermore, co-operation between HNF-4 and Sp1/Sp3 induces synergistic transcriptional activation of the *hHO-1* gene promoter in SL2 cells. The physiological function and detailed mechanism of the co-operation between HNF-4 and Sp1/Sp3 in gene expression will be investigated in future studies. Moreover, studies on the regulatory elements of the *HO-1* gene and identification of the individual sequence patterns of these elements should be applicable to protection against diseases caused by physiological and environmental oxidative stresses.

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