Synergistic activation of the Atlantic salmon hepatocyte nuclear factor (HNF) 1 promoter by the orphan nuclear receptors HNF4 and chicken ovalbumin upstream promoter transcription factor I (COUP-TFI)

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Hepatocyte nuclear factor 1 (HNF1) is a liver-enriched transcription factor that plays an important role in transcriptional networks involved in liver function. The promoters of mammalian *HNF1* genes contains a single binding site for another liver-enriched transcription factor, the nuclear hormone receptor HNF4. A transcriptional hierarchy involving HNF4-mediated activation of the HNF1 promoter has been proposed to be of crucial importance in maintaining the differentiated hepatocyte phenotype. Here we present evidence that the Atlantic salmon HNF1 promoter contains three nuclear-hormone-receptor-binding sequences. Gel-shift assays showed that these motifs are recognized with different affinities by HNF4 and the orphan nuclear receptors chicken ovalbumin upstream promoter transcription factors COUP-TFI and COUP-TFII. In hepatoma cells, the site showing highest affinity for HNF4 appears to be crucial for promoter activity. Transfection experiments in non-hepatic cells indicated that the salmon HNF1 promoter was activated by both HNF4 and COUP-TFs. We also identified a promoter fragment encompassing the two more distal nuclear-hormone-binding sites that was activated by HNF4, unaffected by COUP-TF and showed a strong synergistic activation by HNF4/COUP-TF. Results are presented detailing these interactions in relation to the salmon HNF1 promoter architecture.

Key words: gene expression, liver.

INTRODUCTION

The differentiated cellular phenotype is defined by the co-ordinate expression of specific subsets of genes. In hepatocytes this expression profile is generated by a regulatory network consisting of both liver-enriched and more globally expressed transcription factors (for review, see [1]). The liver-enriched proteins include members of the hepatocyte nuclear factor (HNF) 1, HNF3, HNF4, HNF6 and C/EBP (CAAT-enhancer-binding protein) transcription-factor families [1,2]. The genes coding for these molecules are themselves linked by various transcription-activation and -repression pathways [3,4]. These interactions are of cardinal importance in both liver development and in maintaining the differentiated hepatocyte phenotype.

HNF1 is an atypical homoeodomain protein that in adult mammals is expressed in the liver, renal tubules, intestine and stomach [1]. It is involved in the activation of a number of liver genes, including those encoding albumin, alcohol dehydrogenase and α-fetoprotein (for review, see [5]). In addition to hepatic dysfunction, homozygous *HNF1*-null mice display renal Fanconi syndrome [6], whereas mutations in the human *HNF1* gene have been linked to maturity onset diabetes of the young (MODY) type 3 [7], indicating that HNF1 plays an important role in regulatory pathways outside the liver. A closely related molecule, variant HNF1 (vHNF1), has also been identified. It displays a distinct pattern of expression, with highest levels occurring in the kidney [8]. The temporal expression of the two genes also differs, with *vHNF1* appearing before *HNF1* during embryonic development [9,10].

HNF1 expression is linked closely to the activity of another liver-enriched transcription factor, the nuclear hormone receptor HNF4. In mammals and in *Xenopus*, the HNF1 promoter contains a single HNF4-binding site [11–13] and HNF4-mediated activation of HNF1 has been suggested to constitute a transcriptional hierarchy crucial for liver function. In certain cellular contexts another nuclear hormone receptor, chicken ovalbumin upstream promoter transcription factor (COUP-TF), can co-operate with HNF4 through protein–protein interaction to synergistically activate the HNF1 promoter [14]. COUP-TF itself appears not to bind the HNF1 promoter and in the absence of HNF4 the HNF1 promoter is not activated by COUP-TF. Several promoters have been characterized in which COUP-TF and HNF4 bind to the same recognition sequence. In these promoter contexts COUP-TF acts to repress HNF4-mediated activation through competition for the common binding sites [15,16]. In contrast to HNF1, the vHNF1 promoter is activated by COUP-TF in the absence of HNF4 [17]. Although the vHNF1 promoter contains a COUP-TF recognition sequence, COUP-TF activation appears to be dependent on the presence of a proximal octamer-binding site. The mechanism of activation of the vHNF1 promoter appears to involve protein–protein interactions between COUP-TF and Oct transcription-factor family members, suggesting strongly that COUP-TF activates the vHNF1 promoter by an indirect mechanism [17].

The gene coding for HNF1 has been isolated from the Atlantic salmon [18]. Sequence alignment showed that the salmon HNF1 molecule shared roughly equal identity with rat HNF1 and vHNF1, suggesting that the gene-duplication event giving rise to

Abbreviations used: CAT, chloramphenicol acetyltransferase; C/EBP, CAAT-enhancer-binding protein; COUP-TF, chicken ovalbumin upstream promoter transcription factor; EMSA, electrophoretic mobility-shift assay; HNF, hepatocyte nuclear factor; vHNF1, variant HNF1; MODY, maturity onset diabetes of the young; RAR, retinoic acid receptor; RXR, retinoid X receptor; AF-2, activation function 2.

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HNF1 and vHNF1 may have occurred after the separation of the teleost and tetrapod lineages. However, a molecule showing a high level of identity with vHNF1 has recently been isolated from zebra fish [19], which suggests that a vHNF1 homologue also exists in salmonids. The structure of the salmon HNF1 promoter differs from both mammalian HNF1 and vHNF1. The salmon gene promoter contains three elements (nt −273 to −259, site I; nt −238 to −224, site II; nt +30 to +44, site VIII) that resemble nuclear-hormone-receptor-binding sites and which interact with salmon liver nuclear extracts in gel-shift and DNase protection assays [20]. Here we present evidence that the salmon HNF1 proximal promoter has a more complex organization than the mammalian HNF1 and vHNF promoters, being recognized and activated by both HNF4 and COUP-TFs. We describe a proximal promoter region consisting of sites I and II that is activated by HNF4 but not COUP-TFI, and that displays synergistic activation by HNF4/COUP-TFI.

MATERIALS AND METHODS

Cell culture, transfection and reporter assays

Human hepatoma HepG2 cells and fibroblast-like African green monkey kidney COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were plated 1 day before transfection on 6 cm dishes at approx. 2×10^5 cells/dish. Transfections were performed in serum-free medium using lipofectamine-plus reagent (Gibco-BRL). Cells were transfected with 5 μg of luciferase reporter construct and the indicated amount of expression plasmid. In order to monitor transfection efficiency, 250 ng of pCAT-3 control plasmid (Promega) was included in each transfection. After 3 h, the serum concentration was adjusted to 10% and the cells incubated for a further 24 h before being harvested.

Luciferase assays were performed using a Luciferase Reporter Gene Assay kit (Roche) according to the manufacturer's instructions. Chloramphenicol acetyltransferase (CAT) activity was quantified using a CAT ELISA kit (Roche).

Plasmids

Defined fragments of the salmon *HNF1* gene promoter region were obtained by PCR amplification using a λEMBL3 salmon HNF1 genomic clone as template [20]. The upstream and downstream primers contained *Kpn*I and *Bgl*II recognition sequences, respectively. Amplification products were digested with *Kpn*I and *Bgl*II and inserted into *Kpn*I/*Bgl*II-cut pGL2-basic luciferase reporter plasmid (Promega). A construct in which site II was mutated was generated by overlap PCR. The sequence of the forward mutating primer was 5′-GTTTGACTACACGCATCTCACTGTTAGGGATG-3′ (base changes from the endogenous sequence are underlined). For constructs comprising the region −279 to −216, the insert sequences were synthesized directly as two complementary oligonucleotides which, when annealed, contained *Kpn*I- and *Bgl*II-compatible overhangs at their 5′ and 3′ ends respectively. Annealed oligonucleotides were ligated into *Kpn*I/*Bgl*II-digested pGL2-basic vector. All the above constructs were verified by DNA sequence analysis.

pMT2 expression vectors containing full-length HNF4 and COUP-TFI were gifts from I. Zannis (University of Crete Medical School, Heraklion, Greece) and J. Ladias (Harvard Medical School, Boston, MA, U.S.A.). An expression vector containing the COUP-TFI ligand-binding and -activation domains fused to the Gal4 DNA-binding domain (gal-COUP) was a gift from M.-J. Tsai (Baylor College of Medicine, Houston, TX, U.S.A.). An expression vector containing amino acids 1–156 of COUP-TFI has been described previously [17]. The DNA sequence coding for amino acids 1–394 of COUP-TFI was amplified by PCR using primers containing *Eco*RI sites, digested using *Eco*RI and inserted into *Eco*RI-cut pMT2 expression vector.

Electrophoretic mobility-shift assay (EMSA)

COS-1 cell lines expressing HNF4, COUP-TFI, COUP-TFII, RAR (retinoic acid receptor) and RXR (retinoid X receptor) have been described previously [17]. Nuclear extracts from these cells were prepared as described previously [21]. Mouse liver nuclear extracts were prepared using the NUN method [22].

For EMSA, 1 pmol of ^{32}P-end-labelled double-stranded oligonucleotide was incubated with 5 μg of nuclear extract for 15 min at room temperature in 1 × binding buffer [4% (v/v) glycerol/10 mM Tris/HCl, pH 7.5/1 mM $MgCl_2$/0.5 mM dithiothreitol/0.5 mM EDTA] in the presence of 1 μg of poly[d(I-C)]. In certain experiments antibodies specific for HNF4 and/or COUP-TFI or COUP-TFII were included during the incubation. These antibodies were kind gifts from J. Darnell, Jr (Rockerfeller University, New York, NY, U.S.A.), M.-J. Tsai and S. Karathanasis (Wyeth-Ayerst Laboratories, Randor, PA, U.S.A.) respectively. Following incubation samples were electrophoresed on a 6% polyacrylamide gel in 0.25 × TBE (2.5 mM Tris/2.5 mM H_3BO_3/2 mM EDTA, pH 8.5). Gels were dried and exposed to autoradiographic film at −80 °C.

RESULTS

The three nuclear-hormone-recognition motifs in the salmon HNF1 promoter have different binding specificities

We had previously identified three elements (sites I, II and VIII) in the Atlantic salmon HNF1 promoter that resembled nuclear-hormone-recognition sequences and that interacted with salmon liver nuclear extracts [20]. In order to more precisely determine the binding characteristics of these sequences they were used as probes in EMSAs using nuclear extracts from COS-1 cell lines overexpressing HNF4, COUP-TFI, COUP-TFII, RAR or RXR (Figure 1). A gel shift was obtained with an oligonucleotide (−279 to −252) containing site I with extracts from COUP-TFI- and to a lesser extent COUP-TFII- and HNF4-transfected cells. An oligonucleotide (−244 to −215) encompassing site II bound preferentially to HNF4 extracts, with weaker interaction with extracts expressing either COUP-TFI or COUP-TFII. A third oligonucleotide (+25 to +51) containing site VIII produced a gel shift with COUP-TFI and COUP-TFII extracts, with no detectable binding to HNF4. None of the above oligonucleotides were shifted by extracts from RAR- or RXR-transfected cells. Mouse liver nuclear extracts formed a complex with the site II but not the site I or site VIII oligonucleotides. This is consistent with site II being recognized preferentially by the liver-enriched factor HNF4.

Site II is necessary for salmon HNF1 promoter activity in HepG2 cells

In order to determine if the binding heterogeneity exhibited by sites I, II and VIII was reflected by their capacity to direct expression in a liver cell environment, various salmon HNF1 promoter constructs were transiently transfected into the human hepatoma cell line HepG2 (Figure 2). Maximal activity was obtained with the −576 to +89 construct. Removal of the sequence from −576 to −279 resulted in a more than 3-fold reduction in activity, suggesting the presence of positively acting regulatory elements in this region. Analysis using the

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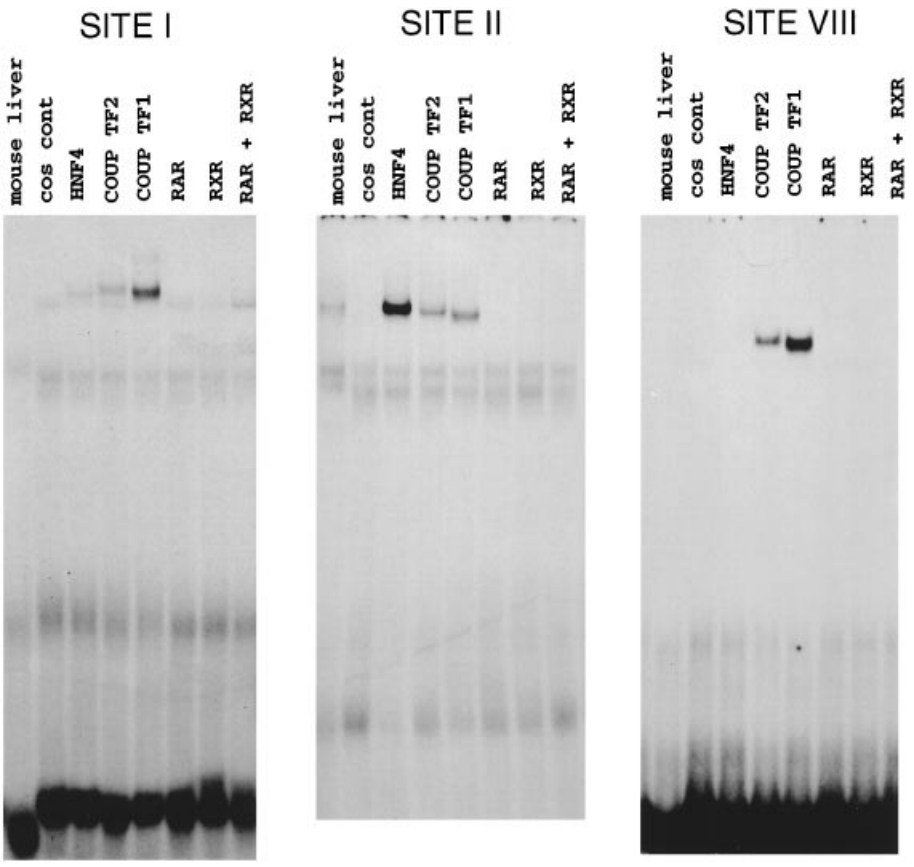

Figure 1 Gel-shift assay shows different nuclear-receptor-binding profiles for sites I, II and VIII

Oligonucleotides corresponding to site I (5′-TTGACTGCACTTTGCTCACTGTTA-3′), site II (5′-GGAGATGGGGACAAAGTTCACAGAAAGCGC-3′) or site VIII (5′-GTGTGTTTGTGACCTGTGGCCTGCGTAGT-3′) were end-labelled and incubated with nuclear extracts from mouse liver, from untransfected COS cells (cos cont) or from COS-1 cell lines expressing HNF4, COUP-TFII, COUP-TFI, RARα, RXRα or RARα and RXRα.

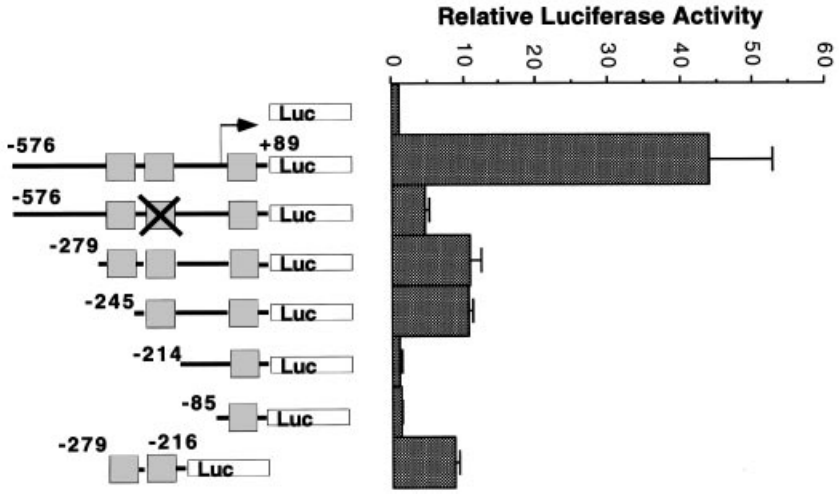

Figure 2 Deletional analysis of the promoter of the salmon *HNF1* gene

A series of salmon HNF1 promoter/luciferase constructs were prepared and transfected into HepG2 cells as described in the Materials and methods section. The line graph is a schematic representation of the regions of the salmon HNF1 promoter included in each construct. The pGL2 basic vector is represented at the top. Numbering is relative to the major transcription start point (+1). The three boxed areas correspond to (from left) sites I, II and VIII. The luciferase activity of each construct was normalized to CAT activity and expressed as fold luciferase activity compared with the pGL2 basic vector. The histograms represent the mean ± S.E.M. from four independent transfections.

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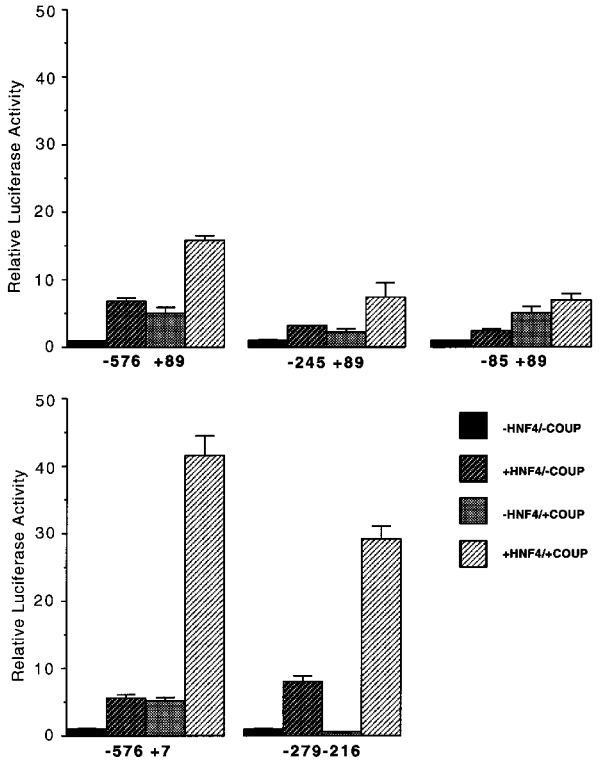

Figure 3 Salmon HNF1 promoter fragments are activated by HNF4 and COUP-TFI in transient transfection assays

COS-7 monolayer cells were transfected with 5 μg of the HNF1 promoter/luciferase construct indicated and 500 ng of expression plasmids for HNF4 or COUP-TFI or 500 ng of HNF4 and 500 ng of COUP-TFI. Plasmid dosage was normalized by the addition of empty expression vector. Cells were harvested 48 h after transfection and the luciferase activity normalized to CAT activity. Histograms denote fold activation where in each case the activity of the construct co-transfected with empty expression vector has been arbitrarily assigned a value of 1. Results represent the means $\pm$ S.E.M. from four independent transfections.

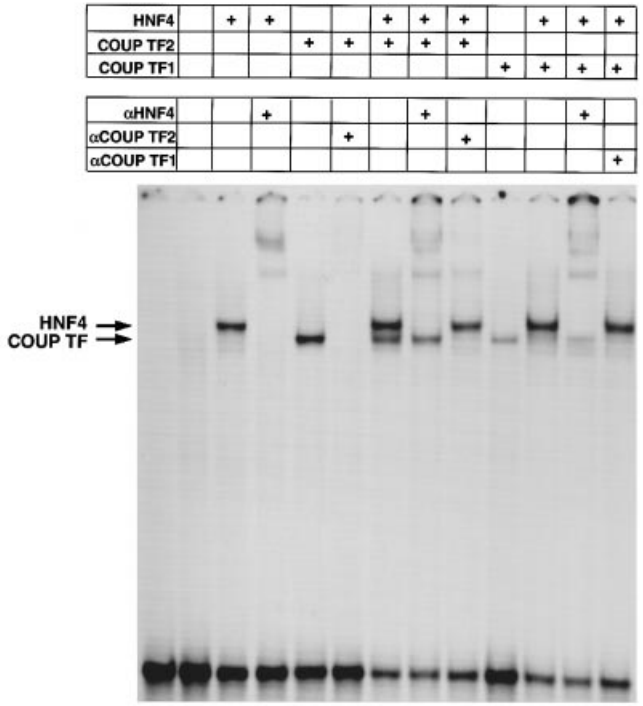

Figure 4 Gel-shift assays show no evidence of HNF4/COUP-TF protein–protein interactions occurring on an oligonucleotide encompassing sites I and II from the salmon HNF1 promoter

Nuclear extracts from COS-1 cell lines expressing HNF4, COUP-TFI or COUP-TFII were incubated with an oligonucleotide corresponding to the region from -279 to -216 of the salmon HNF1 promoter (5′-GTTTGACTGCACTTTGCTCACTGTTAGGGATGGACGGAGATGGGGA-CAAAGTTCACAGAAAGCG-3′) as described in the Materials and methods section. Antibodies against HNF4, COUP-TFI or COUP-TFII were included in the incubation as indicated in the lower grid.

MatInspector program [23] revealed the presence of a consensus HNF3-binding site (5′-GCATATTTATTT-3′) between nucleotides -407 and -396. This motif is a candidate positive regulatory element, as HNF3 has been shown to activate the mammalian HNF1 promoter [8]. The further removal of site I had no significant effect on the promoter activity (construct -279 to $+89$ compared with construct -245 to $+89$). In contrast, the removal of site II reduced activity to just above background levels (construct -245 to $+89$ compared with construct -214 to $+89$). When site II was mutated in the context of the -576 to $+89$ construct, activity was reduced by approx. 10-fold. Constructs in which site VIII was the only nuclear-hormone-recognition sequence showed no activity. Rather surprisingly, a construct consisting only of the region encompassing sites I and II (-279 to -216) had an activity approx. 6-fold that of background. These results indicate that, in hepatic cells, the most important site for promoter activity is the HNF4-binding site.

The salmon HNF1 promoter is synergistically activated by HNF4 and COUP-TFI

It has been reported previously that HNF4-mediated transactivation of the rat HNF1 promoter in COS-1 cells can be increased approx. 15-fold by the addition of COUP-TFs [22]. Using salmon HNF1 promoter constructs containing sites I, II and VIII (-576 to $+89$), sites II and VIII (-245 to $+89$) and site VIII only (-85 to $+89$), we performed co-transfection experiments in COS-1 cells using vectors expressing full-length HNF4 and COUP-TFI. As shown in Figure 3, the -576 to $+89$ construct was activated approx. 7.5-fold by HNF4, 5-fold by COUP-TFI and 16-fold when both HNF4 and COUP-TFI were co-transfected. The -245 to $+89$ construct was activated 3.5-fold by HNF4, 3-fold by COUP-TFI and 8-fold by HNF4 and COUP-TFI transfected together. The corresponding values for the -85 to $+89$ construct were 2-, 5- and 7-fold respectively. We then tested a construct (-576 to $+7$) that contained sites I and II but which terminated just 5′ of site VIII. This construct displayed synergistic activation by HNF4 and COUP-TFI, being activated approx. 6-fold when they were transfected individually and over 40-fold when both were transfected together. We also tested the responsiveness of the -279 to -216 construct that displayed basal activity in HepG2 cells (Figure 2). This construct was activated approx. 8-fold by HNF4, showed no reactivity to COUP-TFI, and was activated over 30-fold by COUP-TFI and HNF4 transfected simultaneously. A construct (-463 to -344) corresponding to a region of the salmon HNF1 promoter that contained no nuclear-hormone-receptor-binding sites was also tested. Its activity was unaffected by HNF4 and COUP-TFI (results not shown).

We were interested to determine if the synergistic activation of the -279 to -216 HNF1 promoter fragment by HNF4 and COUP-TFI could be correlated to the formation of HNF4–

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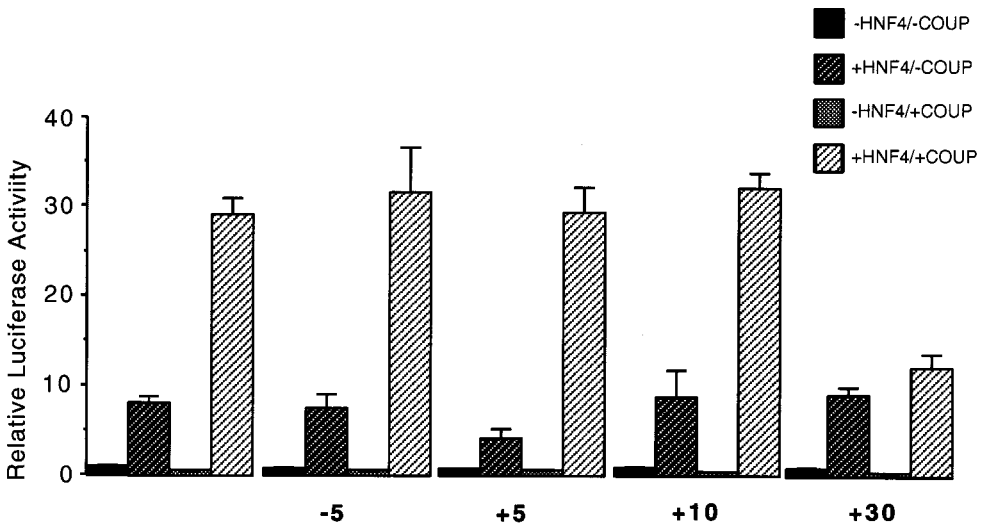

Figure 5 Effect of adding or removing sequence between sites I and II on the reactivity of constructs with HNF4 and COUP-TFI

A series of constructs spanning the region −279 to −216 were prepared in which 5 bp of the endogenous sequence was removed or 5, 10 or 30 bp of pUC18-derived sequence inserted at nt −248. Histograms denote fold activation where in each case the activity of the corresponding construct co-transfected with empty expression vector has been arbitrarily assigned a value of 1. Results represent the means ± S.E.M. from four independent transfections.

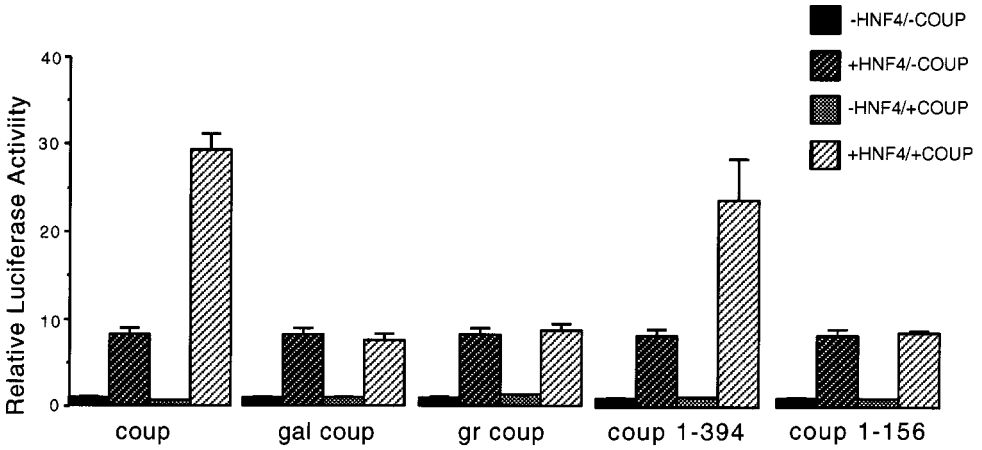

Figure 6 COUP-TFI requires a functional DNA-binding domain to potentiate HNF4-mediated transactivation of the region spanning sites I and II

COS-7 cells were transfected with 5 μg of the −279 to −216 construct and 500 ng of expression plasmids for HNF4, 500 ng of the COUP constructs indicated (see the Materials and methods section) or 500 ng of HNF4 plus 500 ng of the indicated COUP construct. Histograms denote fold activation where in each case the activity of the −279 to −216 construct co-transfected with empty expression vector has been arbitrarily assigned a value of 1. Results represent the means ± S.E.M. from four independent transfections. gr coup, recognizes glutocorticoid-receptor-binding sequences; gal coup, COUP-TFI ligand-binding and -activation domains fused to the Gal4 DNA-binding domain.

COUP-TF complexes on sites I and II. We therefore performed EMSA using an oligonucleotide corresponding to this region as a probe. As shown in Figure 4, HNF4, COUP-TFI and the closely related molecule COUP-TFII all interacted with the site I–II oligonucleotide. When both HNF4 and either COUP-TFI or COUP-TFII were included in the reaction, two shifted bands were produced that had the same mobility as the bands produced by HNF4 and COUP-TF individually. When antibodies specific for HNF4, COUP-TFI or COUP-TFII were included, only the band corresponding to the protein recognized by the antibody was supershifted. The same pattern was obtained when oligonucleotides encompassing sites I and II separated by either 10 or 30 bp were used (results not shown). These results indicate that HNF4 and COUP-TFs bind independently to sites I and II respectively.

Up to 30 bp can be inserted between sites I and II without altering the response to HNF4 and COUP-TFI

In the salmon HNF1 promoter, sites I and II are separated by approx. two helix turns. To determine the importance of the spacing and stereospecific relationship of sites I and II in mediating the response to HNF4 and COUP-TFI, we tested constructs in which 5 bp of sequence was removed from between the two sites, or 5, 10 or 30 bp of pUC-derived DNA inserted. As shown in Figure 5, the removal of 5 bp or the insertion of

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5 or 10 bp did not affect reactivity to HNF4 and COUP-TFI. The construct containing 30 bp of inserted DNA was activated by HNF4 but the HNF4/COUP-TFI synergism observed using the above constructs was eliminated.

COUP-TFI requires an intact DNA-binding domain in order to potentiate HNF4-mediated activation of the salmon HNF1 promoter

It has been reported previously that enhancement of HNF4-mediated activation of a mammalian HNF1 promoter by COUP-TF is not dependent on DNA binding by COUP [15]. To test if this applied to the salmon HNF1 promoter, co-transfections were performed on the −279 to −216 construct using HNF4 and various COUP-TFI deletion mutants that differed in their abilities to bind to COUP recognition sequences. The results are shown in Figure 6. A construct consisting of amino acids 1–147 of the Gal4 DNA-binding domain fused to the putative ligand-binding domain of COUP-TFI did not potentiate HNF4-mediated activation of the reporter, and neither did a construct in which three amino acids in the COUP-TFI P-box were replaced by the corresponding residues from the glucocorticoid receptor (Gr-COUP). The resulting protein is structurally conserved but no longer recognizes COUP-TF-binding sequences (results not shown). A third construct (1–394) that was partially functional in terms of DNA binding but which was missing the activation function 2 (AF-2) activation domain was able to increase HNF4-mediated activation approx. 3-fold. A construct (1–156) that contained a functional DNA-binding domain was unable to potentiate activation by HNF4, indicating that regions in the C-terminal region of COUP-TFI are also required for this activity.

DISCUSSION

The promoters of several liver-expressed genes are synergistically activated in co-transfection experiments by the nuclear hormone receptor HNF4 in conjunction with another transcription factor [14,24–26]. In most cases this synergism requires the presence of recognition sequences for both HNF4 and the second protein [24–27]. In this sense the promoter of the mammalian *HNF1* gene is atypical in that COUP-TFs increase HNF4-mediated activation without actually binding to the HNF1 promoter themselves [14]. We have shown that the salmon HNF1 promoter contains three sites that are recognized with different affinities by COUP-TFs (Figure 1). The structure of the salmon HNF1 promoter is therefore different in terms of the arrangement of nuclear-hormone-binding sites from either the mammalian *HNF1* or *vHNF1* gene promoters. Our results have delineated a fragment (−279 to −216) in the salmon HNF1 promoter that displays transcriptional activity in HepG2 cells. This region contains two sites that are recognized by both COUP-TFs and HNF4. Site I is bound preferentially by COUP-TFs, and in a hepatoma cell line it does not appear to play a significant role in the regulation of the salmon *HNF1* gene (Figure 2). Site II interacts more strongly with HNF4 and mutation of this site markedly reduces the activity of salmon HNF1 promoter constructs in HepG2 cells. In COS cells, HNF4-mediated activation of the −279 to −216 fragment is increased approx. 4-fold by the addition of COUP-TFI. Experiments performed using COUP-TFI deletion mutants suggest that the presence of a functional COUP-TFI DNA-binding domain is necessary but not sufficient for this synergism to occur (Figure 6).

The simplest model for synergistic activation involves co-operative binding of transcription factors to DNA through protein–protein interaction [28]. We could observe no evidence of HNF4/COUP-TF co-operative binding in the context of gel-shift experiments performed using an oligonucleotide encompassing sites I and II (Figure 4). We were able to insert up to 20 bp between sites I and II without disrupting the synergy seen in COUP-TFI/HNF4 co-transfections, and at least 30 bp while still observing the maximal level of HNF4-mediated activation (although losing the HNF4/COUP-TFI synergy). We also inserted or removed 5 bp, therefore changing the spatial relationship of the two sites by half a turn of the helix, without changing the pattern of activation (Figure 5). These results argue against the above mechanism of synergistic activation through co-operative binding applying in this case. A similar situation is observed in the synergistic activation of the apolipoprotein A1 promoter by HNF3 and HNF4 [24] and of the apolipoprotein B promoter by C/EBPα and HNF4 [26], which are not dependent on the spatial or stereospecific relationship of the two cognate sites involved. Recent models have suggested that binding of factors to adjacent recognition sequences may be intrinsically co-operative in certain cases [29,30]. Nucleosome remodelling or destabilization of histone–DNA contacts mediated by the binding of one factor could facilitate the binding of the other without protein–protein interactions occurring [31]. Recognition-sequence spacing is likely to be less critical in this model than in a mechanism based on physical interactions between transcription factors.

Although protein–protein interactions between HNF4 and COUP-TFs have been demonstrated *in vitro* [14], the formation of COUP-TFI/HNF4 heterodimers with a greater activation potential than HNF4 homodimers seems unlikely, as HNF4 has been shown to bind DNA exclusively as a homodimer [32]. COUP-TF-mediated activation of the vHNF1 promoter occurs independently of DNA binding by COUP-TF, but nonetheless requires the presence of a DNA-binding domain. In this case the COUP-TF DNA-binding domain acts as an interface for interactions between COUP-TF and Oct proteins [17]. We cannot exclude the possibility that although COUP-TFI is capable of binding to elements in the salmon HNF1 promoter, the potentiation of HNF4-mediated activation may be due entirely to protein–protein interactions that nonetheless require the presence of an intact COUP-TFI DNA-binding domain. However, the inability of Gr-COUP, which recognizes glucocorticoid-receptor-binding sequences due to the substitution of three amino acids in the DNA-binding domain, to further increase the HNF4-dependent activity of the −279 to −216 promoter argues that in this case the HNF4/COUP-TFI synergism requires DNA binding by COUP-TFI. A construct expressing the Gal4 DNA-binding domain (amino acids 1–147) fused to the putative ligand-binding/activation domains of COUP-TFI was also unable to increase HNF4-mediated activation of this promoter fragment. The ability of a deletion mutant comprising amino acids 1–394 of COUP-TFI to potentiate HNF4-dependent activation of the −276 to −216 promoter fragment was comparable with that of full-length COUP-TFI. This construct contains an intact DNA-binding domain (amino acids 85–156) but lacks the AF-2 activation domain. In contrast, a deletion mutant comprising amino acids 1–156 of COUP-TFI did not increase the activation potential of HNF4 (Figure 6). These results suggest that that the synergism observed may involve COUP-TFI binding in proximity to HNF4 in conjunction with a stabilization event or the recruitment of accessory factors that is dependent on the presence of domains occurring between amino acids 156 and 394 of the COUP-TFI molecule.

Constructs that contained site VIII (nt +30 to +44), including a construct (−85 to +89) in which site VIII appeared to be the only nuclear-hormone-receptor-binding site, were activated

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by COUP-TFI in the absence of HNF4 (Figure 3). Interestingly, COUP-TFI was found to activate transcription of the mouse mammary tumour virus promoter through a high-affinity binding sequence located 3′ to the transcription start point [33]. The salmon *HNF1* gene is TATA-less and lacks an initiator sequence [20], so it is possible that COUP-TFI binding to site VIII may act to recruit components of the basal transcription machinery directly to the promoter in the absence of TFIID binding. A similar mechanism has been proposed for the TATA-less promoter of the human sex-hormone-binding globulin gene, which is activated by HNF4 through a sequence that overlaps with a non-functional TFIID-binding site [34]. The binding of COUP-TFI to site VIII may also explain the relative lack of COUP-TFI/HNF4 synergy observed in transfections performed using the −576 to +89 construct (Figure 3). If a significant amount of the co-transfected COUP-TFI is bound to site VIII, although it can contribute to the activation of the promoter construct, it is also sequestered and unable to participate in the site I–II-mediated synergistic activation with HNF4. The relevance of this observation to the regulation of salmon HNF1 expression *in vivo* is not clear, as the levels of COUP-TFI in salmon liver are unknown. Another construct (−576 to +7) that terminated about 20 bp upstream of site VIII showed a greater synergistic response to COUP-TFI/HNF4. It was also activated by COUP-TFI in co-transfection experiments (Figure 3). The −576 to +7 construct contains a consensus Sp1-recognition sequence (nt −211 to −197, site III). Recent studies have shown that COUP-TFs and Sp1 are capable of physically interacting and that COUP-TFs can activate gene transcription through Sp1 sites [35,36]. We tested a construct (−279 to −185) that contained only the region spanning sites I–III for reactivity to COUP-TFI in the absence of HNF4. This construct was activated approx. 3-fold by COUP-TFI (results not shown). It is possible that COUP-TFI may enhance expression of the salmon HNF1 promoter by mechanisms involving direct binding to site VIII and Sp1-mediated interaction with site III.

Our results have demonstrated that the salmon HNF1 promoter has a markedly different structure to either the mammalian HNF1 or vHNF1 promoters. We have also shown that elements of the salmon HNF1 promoter can be upregulated by HNF4, COUP-TFI and synergistically by HNF4/COUP-TFI. This pattern of reactivity to nuclear receptors overlaps that of mammalian HNF1 and vHNF1 [14,17]. A molecule likely to correspond to the zebra fish vHNF1 orthologue has been isolated recently [19], suggesting that vHNF1 also exists in salmonids. It therefore seems unlikely that the structure of the salmon HNF1 promoter can be explained in terms of salmon HNF1 fulfilling the overlapping but distinct functions of mammalian HNF1 and vHNF1. It should also be noted that the strong HNF4/COUP-TF synergy was localized to a fragment of the salmon HNF1 promoter and that the salmon *HNF1* gene *in situ* may not react with HNF4/COUP-TF in the same manner. Nevertheless the site I–II region provides an interesting context in which to study HNF4/COUP-TF-mediated synergistic activation. These interactions have recently been shown to have a physiological consequence. A form of MODY type 1 has been linked to a mutation in the *HNF4* gene E domain that leads to impaired synergy with COUP-TFII on the human HNF1 promoter [37].

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