Hepatocytes are the major source of sex hormone-binding globulin (SHBG), a glycoprotein that transports sex steroids in the blood and regulates their access to target tissues. The human SHBG proximal promoter was analyzed by DNase I footprinting, and the functional significance of 6 footprinted regions (FP1–FP6) within the proximal promoter was studied in human HepG2 hepatoblastoma cells. Two footprinted regions (FP1 and FP3) contain binding sites for the chicken ovalbumin upstream promoter-transcription factor (COUP-TF) and hepatocyte nuclear factor-4 (HNF-4). In experiments where SHBG promoter-luciferase reporter gene constructs were co-transfected into HepG2 cells with COUP-TF and/or HNF-4 expression vectors, HNF-4 markedly increased transcription, whereas COUP-TF suppressed this probably by displacing HNF-4 from their common FP1-binding site. This COUP-TF/HNF-4-binding site within FP1 includes a TTTAA sequence, located at nucleotides −30/−26 upstream of the transcription start site, which fails to interact with human TFIID, TATA-binding protein in vitro. When this sequence was replaced with an idealized HNF-4-binding site, the transcriptional activity of the promoter increased in HepG2 cells. Taken together, these data imply that an interplay between COUP-TF and HNF-4 at a site within FP1 regulates human SHBG expression and that HNF-4 controls transcription from this TATA-less promoter by somehow substituting for TATA-binding protein in the recruitment of a transcription preinitiation complex.

Sex hormone-binding globulin (SHBG)1 is a plasma glycoprotein that transports sex steroid hormones and regulates their access to target cells (1). It is produced by hepatocytes, and blood levels of SHBG in humans are influenced by a variety of hormones, as well as several nutritional and metabolic regulators, including sex steroids (2), thyroid hormone (2, 3), insulin (4), dietary lipids (5), isoflavonoid phytoestrogens (6), and body weight (7). Low plasma levels of SHBG are associated with several sex steroid hormone-dependent diseases (2) and have been reported to be an early indicator of the onset of Type II diabetes (8) and cardiovascular disease (7).

The gene encoding SHBG is also expressed in the Sertoli cell of the testis. When the same transcription unit responsible for hepatic production of plasma SHBG is utilized in Sertoli cells, the protein product is most often referred to as the testicular androgen-binding protein. Testicular androgen-binding protein has the same primary structure and steroid-binding properties as plasma SHBG and is thought to control androgen-dependent sperm maturation events in the male reproductive tract (1, 9).

In rodents, the testis appears to be the major site of SHBG expression in adult animals, but the gene is also expressed transiently in the fetal rat liver, and this may serve to influence the differentiation of reproductive tissues during late fetal development (10).

The regulation of SHBG production has been studied using human HepG2 hepatoblastoma cells (11, 12), but little is known about the molecular control of SHBG expression in the liver. Recently, genomic DNA fragments encompassing human and rat SHBG have been introduced into the mouse genome (13, 14), and the temporal and spatial expression of these transgenes generally reflects the species-specific patterns of expression associated with these genes. In essence, a rat SHBG transgene, which included an ∼1.5-kb sequence flanking the major transcription start site in the rat testis, was expressed in the mouse testis at high levels, but no expression could be detected in the livers of adult transgenic mice (14). By contrast, a human SHBG transgene comprising the entire transcription unit encoding SHBG together with ∼0.9 kb of 5′-flanking sequence was expressed in the fetal2 and adult mouse livers, as well as in the adult mouse kidney (13). Little, if any, expression of this human SHBG transgene was detected in the testis, and differences in the cis-acting regulatory sequences within the human and rat SHBG promoters most likely contribute to the distinct tissue specificity of SHBG expression in these species.

To address this issue, and to learn more about how the gene-encoding plasma SHBG is regulated in the liver, we compared the human and rat SHBG proximal promoter sequences that flank the transcription units for human SHBG in the liver and rat androgen-binding protein in the testis, and we used mouse liver nuclear protein extracts and human hepatoblastoma cells to identify possible targets for transcriptional regulators within the human SHBG promoter. By assessing how various cis-elements identified in this way contribute to transcriptional activation, we have found that hepatocyte nuclear
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factor-4 (HNF-4) plays an important role in regulating the transcription of human SHBG in liver cells.

**EXPERIMENTAL PROCEDURES**

**Reporter and Expression Plasmids**—The human SHBG proximal promoter spanning nucleotides (nts) −803 to +7 (−803/+7) relative to the liver- and kidney-specific transcription start site (13) was PCR-amplified from an 11-kb genomic HindIII DNA fragment isolated from a cosmid vector (15). The forward primer spanned the human SHBG sequence from nts −810 to −792, and the reverse primer was complementary to nts +7 to +14 with a sequence (GAATGAAGCTT) for a HindIII site at its 5′-end (Fig. 1). The PCR product was digested with XhoI and HindIII and was subcloned into the promoterless pGL2 Basic luciferase reporter plasmid (Promega Corp., Madison WI). This gave a −803/+7 SHBG proximal promoter fused to the luciferase reporter gene. A −299/+7 SHBG-luciferase reporter plasmid was obtained by digesting the −803/+7 SHBG with XhoI and HindIII and inserting this into pGL2 Basic. Several 5′-deletion fragments of the SHBG promoter were also PCR-amplified using the 11-kb human SHBG HindIII fragment as the template. The 5′-primers for these constructs corresponded to the following SHBG sequences relative to the transcription start site in the liver: nts −1371 to −120, nts −941 to −77, and nts −625 to −552 (Fig. 1). In each case, a sequence GGGTTTTCAGA (XhoI site) was added to the 5′-ends of these primers for subcloning. The reverse primer was the same as that used for the −803/+7 SHBG fragment. A mutant SHBG promoter/luciferase reporter was constructed by digesting the −803/+7 SHBG-luciferase construct with SmaI followed by religation to excise nts −803 to −41.

Mutations within footprint 1 (FP1) were created by PCR amplification of the −1371/−7 SHBG region with mismatch 3′-primers spanning nts −37 to −1 and containing an additional sequence GAATGAAGCTT (HindIII site) at their 5′-ends. The region between nts −41 to −77 was amplified in an FPI mutant, in which the TTTAAC sequence was converted to TC-AGTAGC (Fig. 1). The PCR product was digested with XhoI and HindIII and subcloned into the plasmid pLEN4S, which contains the 3′-untranslated region of the human SHBG gene. A −1371/−7 SHBG promoter fragment was subcloned between the HindIII and XbaI sites of pGL2 Basic to yield a series of deletion mutants in the SHBG promoter region.

**Electrophoretic Mobility Shift Assay (EMSA)**—The following double-stranded oligonucleotides were used as radiolabeled probes and/or unlabeled competitors:

- **FP1**, tcgacCCCGGGGACATTTACCCCTCCAC
- **FP3**, tcgacTGAGGAGGTCAGATGCTGAGCTGC
- **FP4**, tcgacTGATATCGGTACTCTTGACGACTGCCCCCT
- **SP-1**, GCTGAGGGGGCGAGCGGATG

**OLIGONUCLEOTIDE FP1, FP3, FP4, and SP-1**

The lowercase letters indicate additional sequences that were filled using the Klenow fragment of DNA polymerase I and [α-32P]dCTP (16). Nuclear extracts were incubated with or without an excess of unlabeled competitor oligonucleotide in the presence of 10 mM Hepes, pH 7.6, 50 mM KCl, 2.5 mM MgCl2, 10% glycerol, 1 mM dithiothreitol, and 3 μg of poly(dI-dC). After 10 min on ice, 25 fmol of end-labeled double-stranded oligonucleotide was added for a 15-min incubation at room temperature. For antibody supershift experiments, nuclear extracts were incubated (as above) on ice for 10 min before, and 15 min after, addition of the radiolabeled oligonucleotide. Aliquots (1 μl) of antiserum or normal rabbit serum were then added and the complexes were further incubated at room temperature for 15 min. The COUP-TF antiserum (provided by Dr. M. J. Tsai) was used undiluted and the HNF-4 antiserum (provided by Dr. F. M. Sladek) was diluted 1:5. In the presence of COUP-TF and/or HNF-4 expression plasmids, the protein-DNA complexes were separated from the free probe by native 5% polyacrylamide gel electrophoresis in 0.45 M Tris borate, 1 mM EDTA. The gel was dried and exposed to x-ray film against an intensifying screen at −80°C.

**Cell Culture and Transfection**—All cell culture reagents were from Life Technologies, Inc. (Burlington, Canada). Human HepG2 hepatocarcinoma and HeLa cervical carcinoma cells were grown initially in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Transient transfections of human SHBG promoter reporter plasmids** together with a pCMVlacZ control plasmid were carried out using LipofectAMINE™ reagent according to Life Technologies, Inc. (Burlington, Canada). Human HepG2 hepatocarcinoma and HeLa cervical carcinoma cells were grown initially in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**RESULTS**

**Human SHBG Promoter Sequence**—To map regulatory elements within the human SHBG promoter, and to identify transcription factors that regulate SHBG expression in hepatocytes, we PCR-amplified the human SHBG promoter region from nts −803 to +7 relative to the transcription start site in the liver (13). Sequence analysis of this fragment revealed differences when compared with the published SHBG sequence (15), including a 113-bp region that was missing from the GenBank™ M31651 sequence. These discrepancies were confirmed by resequencing the corresponding region from an 11-kb HindIII human SHBG genomic fragment excised from a cosmid clone (15). The additional 113-bp sequence includes an XbaI

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3 F. M. Sladek, personal communication.
site at position –299, which was previously mapped by Ger-shagen et al. (18). The revised human SHBG promoter sequence aligned with the corresponding region of rat SHBG (GenBank™ accession number M19993). Corrections to the GenBank™ accession number M31651 sequence are indicated in bold. Restriction sites used for subcloning are indicated above the human SHBG sequence. Sequences that correspond to part of an Alu sequence in human SHBG are in italics.

**Fig. 1. Partial human SHBG promoter sequence.** The human SHBG sequence from nts –810 to +106 relative to the transcription initiation site (underlined) in the liver and kidney (13) is aligned with rat SHBG promoter sequence (GenBank™ accession number M19993). Corrections to the GenBank™ accession number M31651 sequence are indicated in bold. Restriction sites used for subcloning are indicated above the human SHBG sequence. Sequences that correspond to part of an Alu sequence in human SHBG are in italics.

**Fig. 2. DNase I footprinting of transcriptional regulatory elements in the human SHBG promoter.** An end-labeled DNA fragment containing human –299/+7SHBG was incubated with increasing amounts (shown above the gel) of mouse liver nuclear extract (NE). The DNA was subjected to DNase I digestion and purified. Fragments were analyzed by denaturing polyacrylamide gel electrophoresis and exposure to an x-ray film. A Maxam Gilbert sequencing (G/A) reaction was used as a size marker (lane 1) to define the boundaries (shown in brackets) of each footprint (FP) 1–5 (lanes 3–6). Sites hypersensitive to DNase I digestion are indicated by asterisks. The labeled DNA, without the addition of DNase I or nuclear proteins, is shown in lane 2.

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SHBG promoter activity in hepatocytes, we constructed a series of 5'-deletion mutants of -803/+7SHBG fused to a luciferase reporter gene. The XhoI site in this promoter sequence was used to create a -299/+7SHBG promoter reporter construct, and both FP5 and FP6 were eliminated from it by removing the SHBG sequences -299/-137, whereas footprints 1–4 were removed individually by further 5'-deletions. The 3'-end of these deletion mutants was maintained at nt +7 relative to the transcription start site (Fig. 3). These promoter deletion constructs were transiently transfected into HepG2 cells that produce SHBG (20). The results indicate that footprints 1–4 are required for transcriptional activity in these cells and that the region upstream of nt -299 contains elements that suppress transcription, as evidenced by a 2-fold increase in promoter activity when these sequences are deleted (Fig. 3).

Each human SHBG promoter deletion fragment was also analyzed in the context of a luciferase reporter gene in HeLa cells to assess the cellular specificity of its transcriptional activity (not shown). Although the promoter was active in this cell line, the reporter gene expression was lower than in HepG2 cells and regulation at each of the footprinted regions was not observed.

Significance of the TTTAA Sequence within FP1—The footprinted region (FP1) closest to the hepatic transcription start site spans the SHBG promoter sequence from nts -41/-18 and includes a TTTAAC sequence starting at position -30. Since there is no distinct TATA box in the human or rat SHBG promoters (15, 19), we decided to investigate whether this TATA-like sequence is recognized by the transcription initiation complex. This TTTAAC sequence is also conserved between the rat (19), mouse (2), and human SHBG promoters (Fig. 1) and was therefore thought to represent an important element for basal transcription. Since a reporter construct containing only 137 bp upstream of the SHBG transcription start site had the maximal activity among the constructs tested in HepG2 cells (Fig. 3), we modified this promoter fragment to study the TTTAA sequence in FP1.

Sequences that deviate from the canonical TATA element have been shown to bind TFIID, and mutations within such elements generally abolish the activity of a given promoter (21–25). We therefore produced two different mutations by PCR amplification of the -137/+7SHBG region with mismatch 3'-primers, and we analyzed these mutated promoter fragments in the context of a luciferase reporter gene. When the TTTAAC sequence was altered to TCCCAC, the promoter activity was reduced by 80% in HepG2 cells (Fig. 4A). The same sequence was also converted into an ideal TATA sequence, TATAAA, because this was expected to increase the affinity of TFIID for this sequence and to thereby increase the transcriptional activity of the promoter (26). However, this mutation again reduced the activity of the SHBG promoter by approximately 24% (Fig. 4A), and this suggested that a factor other than TFIID recognizes the TTTAA sequence within FP1 in hepatoblastoma cells. This was confirmed by DNase I footprinting wild type and mutated -137/+7SHBG fragments with the TBP of TFIID, which showed that the wild type SHBG sequence between nts -30/-25 is very poorly protected by TBP when compared with the mutant that contains an optimal TATA sequence in this position (Fig. 4B).

The two FP1 mutants were also tested in HeLa cells (Fig. 4). In these cells, an optimal TATA sequence at -30 increased the promoter activity by almost 3-fold, and these data suggest that TFIID can recognize a TATA box in this promoter context in HeLa cells but not in HepG2 cells.

CUP-TF and HNF-4 Bind FP1 and FP3 in the Human SHBG Promoter—Although a sequence within FP1 (GGGCAACCTTTAACCCT) also resembles a binding site for the liver-enriched transcription factor, HNF-1, an oligonucleotide corresponding the HNF-1 site in the rat albumin promoter (27) failed to displace complexes from an FP1 probe in a competition EMSA, and this indicated that the protein that binds FP1 in the human SHBG promoter was not related to HNF-1 (data not shown). Further analysis using MatInspector (28) Release 2.1 revealed some similarities between a sequence within FP1 (CAACCTTTAACCCT) and the consensus HNF-4-binding site (29). By contrast, a consensus binding site (GGGTCAAGGGTCA) for CUP-TF (30), which is known to share
binding sites with HNF-4 in several promoters (29, 31, 32), was readily identified within FP3.

To analyze the protein-DNA interactions at these elements, we performed EMSAs with mouse liver nuclear proteins and double-stranded oligonucleotides as radiolabeled probes. Fig. 5 shows the complexes that form between nuclear proteins and FP1 (nts 241/218) and FP3 (nts 288/266). The specificity of these DNA-protein interactions was demonstrated by preincubating the nuclear extract with an excess of unlabeled oligonucleotides, which compete the nuclear proteins from their respective complexes (lanes 3 and 4, and 13 and 14), whereas unrelated sequences (SP-1 consensus or human SHBG FP4) are unable to do this (lanes 7–10 and 17–20). Oligonucleotides specific for FP1 and FP3 also compete with each other (lanes 5 and 6 and 15 and 16), and this indicates that the same DNA-binding protein(s) recognize both elements. This competition assay also indicates that FP3 has a higher affinity for these factors than does FP1 (see Fig. 5, lanes 5 and 6 versus 15 and 16).

Antisera specific for COUP-TF and HNF-4 were used to confirm the identities of proteins that bind to FP1 and FP3 in the human SHBG promoter. In an EMSA, antisera against mouse COUP-TF or rat HNF-4 partially supershift the complexes, and the supershifts are essentially complete when these antisera are used in combination (Fig. 6). Thus, these two sites, which are separated by only 23 bps in the human SHBG promoter, are recognized by both COUP-TF and HNF-4 in liver nuclear extracts. From these data, it also appears that FP1 is occupied preferentially by COUP-TF (Fig. 6, lane 3 versus lane 4), whereas FP3 is bound by COUP-TF and HNF-4 at essentially similar levels when the same nuclear extract is used (Fig. 6, lane 8 versus lane 9).

Function of the COUP-TF/HNF-4 Sites in the Human SHBG Promoter—Site-specific mutations that interfere with the binding of COUP-TF and HNF-4 to FP1 and FP3 in the human SHBG promoter were used to analyze these DNA-protein interactions in greater detail. The alteration of the TTTAAC sequence to either TCCCAC or TATAAA within FP-1 reduced the transcriptional activity of the -137/+7SHBG promoter in HepG2 cells (Fig. 4A), and the same alterations within an FP1 oligonucleotide abolished any protein binding in an EMSA (not shown). When these mutations (Fig. 7A) were introduced either individually or together into the -299/+7SHBG promoter, the altered FP1 and FP3 were not protected from DNase I digestion.
in an in vitro footprinting assay (Fig. 7B). Moreover, these mutations appear to disrupt protein binding to FP1 and FP3 without disturbing the binding of other nuclear proteins to adjacent promoter elements (Fig. 7B) and demonstrate that COUP-TF/HNF-4 interactions with FP1 and FP3 occur independently of each other.

The activities of the −299/+7SHBG promoter mutants were also assessed in the context of a luciferase reporter gene in a transient transfection assay. When compared with the activity of the wild type −299/+7SHBG promoter in HepG2 cells, mutations within FP1 and FP3 resulted in 56 and 31% decrease in transcriptional activity, respectively (Fig. 7C). The activity of the SHBG promoter, in which both COUP-TF/HNF-4-binding sites were disrupted, was reduced by 73%. These results indicate that both COUP-TF/HNF-4-binding sites in the human SHBG promoter are functional in hepatoblastoma cells and provide additional evidence that they act independently of each other.

**HNF-4 Trans-Activates the Human SHBG Promoter**—To test further the function of FP1 and FP3, we co-transfected COUP-TF and HNF-4 expression vectors together with a −299/+7SHBG promoter-luciferase reporter plasmid into HepG2 cells. As shown in Fig. 8, exogenous HNF-4 causes a 15–20-fold induction in human SHBG promoter activity, whereas overexpression of COUP-TF has virtually no effect. In this experiment, the COUP-TF and HNF-4 expression vectors were under the control of different promoters, and we cannot be certain that these transcription factors were produced at equivalent levels. However, the co-transfection of a COUP-TF expression vector clearly repressed the trans-activation by HNF-4 in these experiments, and this is probably due to competition for the same response element, as has been reported in the context of several other promoters (29, 31, 32). The fact that overexpression of COUP-TF by itself does not alter the activity of the promoter supports the idea that FP1 is essentially fully occupied by COUP-TF in the cell nucleus, and this is consistent with the in vitro data indicating that FP1 has a preference for COUP-TF.

The role of HNF-4 in the regulation of the human SHBG promoter was confirmed by a co-transfection experiment in HeLa cells (Fig. 8), which do not contain HNF-4 (32). In these cells, the human SHBG promoter was also up-regulated by HNF-4, but the 7-fold increase in activity was much lower than that observed in hepatoblastoma cells, and this may reflect a difference in the amounts of COUP-TF and/or other proteins that might interact with HNF-4 in these cell types. This experiment also reinforces the concept that displacement of COUP-TF from FP1 by HNF-4 results in a marked change in transcriptional activation.

The relative contributions of the HNF-4-binding sites within FP1 and FP3 to human SHBG promoter activity were assessed in the same experiment by using reporter constructs containing the mutant −299/+7SHBG promoters shown in Fig. 7. Maximal trans-activation by HNF-4 was obtained when FP1 and FP3 were both intact, whereas the promoter in which both FP1 and FP3 were mutated responded poorly to exogenous HNF-4. Although HNF-4 clearly exerts its effects independently through FP1 and FP3, the mutation within FP1 reduced the trans-activation by HNF-4 much more effectively than the mutation within FP3, in both cell lines (Fig. 8). These data confirm that HNF-4 functions primarily through the sites we have identified within FP1 and FP3.

To obtain additional support for the concept that HNF-4 binding within FP1 plays a pivotal role in controlling transcription of what appears to be an example of a TATA-less promoter, an idealized HNF-4-binding site (GGGTCAGGTTACA) was introduced into FP1 so that it substituted for the region (AAC-
CTTTAACCCT containing a potentially atypical TATA box, and this increased the transcriptional activity of the promoter in HepG2 cells (Fig. 9).

**DISCUSSION**

Despite the fact that rodents express SHBG in their livers only during fetal life (10), human SHBG transgenes comprising...
only ~0.9 kb of promoter sequence are transcribed actively in mature mouse livers (13). To gain insight into why SHBG is expressed so differently in hepatocytes between species, we compared the human and rodent SHBG promoter sequences. When this was done, regions of sequence similarity were obvious throughout the first 600 bp of the human and rat SHBG promoters, but there are two regions within the first 220 bp from the human and rat SHBG transcription start sites where major differences exist. In both cases, the human SHBG proximal promoter contains additional sequences that are not present in the corresponding regions of rat (19) or mouse SHBG promoters. This directed our attention to sequences within the SHBG proximal promoter, and when this region was examined by DNase I footprinting at least six potential DNA-binding sites for mouse liver nuclear proteins were identified. One of these sites (FP4) is clearly absent in the rat promoter, and this gap in the two sequences may extend into the region encompassed by FP3. The only other region in the human SHBG promoter where relatively large stretches of DNA are lacking in the corresponding rodent sequences is centered around FP6, but this region does not exert any obvious regulatory influence on the transcriptional activity of the human SHBG proximal promoter in liver cells.

Human and rodent SHBG promoters lack a canonical TATA box (15, 19), but a relatively broad region of DNA (FP1), centered around an AT-rich sequence between 26 and 30 bp upstream of the human SHBG transcription site in the liver, was protected by nuclear protein in our DNase I footprinting experiments. This TTTAA sequence was therefore investigated initially to determine whether it functions as an atypical TATA box. First, to assess the relative importance of this sequence in the overall activity of the SHBG promoter, it was converted to TCCCA, and this caused a marked reduction in transcription. It was also converted into an optimal TATA sequence because this change permits binding of the TBP subunit of TFIID and was therefore expected to allow it to engage the transcription initiation complex at this site (26), but it also reduced transcription. When considered together, the results of these functional assays and DNase I footprinting experiments all imply that the TTTAA sequence within FP1 is an important component of a cis-element which does not involve a direct interaction with TBP, and we therefore sought to identify the factor(s)

FIG. 8. HNF-4 trans-activates the human SHBG promoter. HepG2 and HeLa cells were transfected with a wild type −299/+7SHBG luciferase construct or equivalent reporter constructs with mutated footprints 1 and 3, as shown in Fig. 7A, together with plasmids expressing COUP-TF and/or HNF-4 and a pCMVlacZ control plasmid. trans-Activation is indicated as fold of induction over the base line of the wild type promoter (mean ± S.D. of four experiments). In the schematic, each mutated footprint is marked with an X.

FIG. 9. Effect of substituting the region containing the TATAAA sequence within FP1 with an idealized HNF-4-binding site. Wild type and mutated −137/+7SHBG promoter luciferase reporter constructs were transiently transfected into HepG2 cells together with a pCMVlacZ control plasmid. Positions of footprints 1–4 are indicated by shaded boxes, and the mutated nucleotides within FP1 are shown above each schematic. The transcriptional activity (mean ± S.D. of four experiments) of the FP1 mutated promoters is indicated relative to the wild type SHBG promoter activity.
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HNF-4 and COUP-TF are capable of interacting with FP1 and that mutations within its TTTAA sequence completely abolished these interactions. These experiments suggested that COUP-TF or HNF-4 might serve to recruit the transcriptional machinery directly to the promoter in a manner analogous to that originally reported for the YY1 transcription factor (33). This is not inconceivable, because several members of the steroid hormone receptor superfamily, including COUP-TF (34) and HNF-4 (35), interact directly with TFIIB, and HNF-4 has been shown to be capable of activating transcription by facilitating assembly of a preinitiation complex intermediate via direct physical interactions with TFIIB (35). Like most steroid hormone receptors, HNF-4 has two trans-activation domains, AF-1 and AF-2, which interact with other nuclear proteins to modulate transcriptional responses (36). In the context of the apoA1 promoter (35), the recruitment of TFIIB to the promoter complex by HNF-4 is not dependent on the presence of an AF-2 domain, but AF-2 is required for transcriptional activation. Thus, under these circumstances, it has been proposed that HNF-4 serves as a transcriptional activator by recruiting TFIIB to the promoter complex and by influencing entry of preinitiation complex components that act downstream of TFIIB via an AF-2-dependent mechanism (35).

The finding that a COUP-TF/HNF-4-binding site resides within FP1 was unexpected, but a consensus binding site for COUP-TF was immediately recognizable within FP3. Our EMSA experiments indicated that both proteins interact with the same sites in FP1 and FP3 in vitro, but co-transfection experiments in HepG2 cells demonstrated that it is HNF-4, rather than COUP-TF, that enhances transcription via FP1 and that this is not influenced appreciably by the interaction of either protein with FP3. Furthermore, in the absence of a functional HNF-4-binding site in FP1, both HNF-4 and COUP-TF had equivalent and relatively modest effects on the transcriptional activity of the human SHBG promoter. Similar effects were seen in HeLa cells, which contain COUP-TF (34) but not HNF-4 (32), but the magnitude of the responses was smaller.

The effect that HNF-4 exerts on SHBG promoter activity via its interaction with FP1 can be blocked by COUP-TF, irrespective of whether the COUP-TF-binding site in FP3 is intact. Both HNF-4 and COUP-TF bind to the same sequence within FP1, and COUP-TF has been shown to actively repress or modulate the effects of HNF-4 on the transcription of several other genes in the liver (29, 31, 32). Heterodimerization between COUP-TF and other members of the steroid hormone receptor superfamily (37) can result in the trans-repression of activated transcription (38), but this is unlikely to account for our observations because HNF-4 appears to function exclusively as a homodimer (39), and these orphan receptors therefore probably simply compete for the same DNA-binding site within FP1. Like HNF-4 (35), COUP-TF also binds to TFIIB (34), and its overexpression could block any interaction between TFIIB and HNF-4. Even if this occurs, our data imply that any association between COUP-TF and TFIIB in the context of FP1 is relatively non-productive in hepatoblastoma cells, and the fact that HNF-4 specifically enhances transcription when bound to FP1 may reflect a unique ability of its AF-2 domain to influence the entry of preinitiation complex components downstream of TFIIB (35, 36). However, it is also generally acknowledged that members of steroid hormone receptor superfamily may contribute to assembly of the preinitiation complex by interacting directly with TBP or the TBP-associated factors (TAFs) that comprise the TFIID complex (40), as well as a variety of adaptor or “co-activator” proteins that mediate interaction between their activation domains and the general transcription factors (41, 42). Furthermore, it is also possible that HNF-4 interacts with the TAFs or other components of a recently identified multi-protein complex (TFTC) that lacks TBP (43) but which is capable of directing transcription mediated by RNA polymerase II.

It remains to be seen whether these interactions are physiologically relevant, but alterations in the concentrations or conformational states of HNF-4 and COUP-TF may determine their relative abilities to occupy a common binding site within FP1, and this could provide a mechanism for the control of SHBG expression. As members of the steroid hormone receptor superfamily, the conformational states of COUP-TF and HNF-4 and their ability to interact with other nuclear proteins may be influenced by ligand binding. A ligand for COUP-TF has not yet been identified, but it has recently been reported that long chain fatty acyl-CoA thioesters bind HNF-4 and enhance its ability to dimerize and bind DNA (44). Although it has been suggested that this may explain the relationships between the actions of HNF-4 and the expression of several genes that respond to metabolic regulators, it is not yet clear how these ligands influence the ability of HNF-4 to function as a transcriptional regulator in the context of specific gene sequences.

The orphan receptor HNF-4 is responsible for the constitutive expression of a wide variety of genes in the liver (45). Several isoforms of HNF-4 exist that are encoded by two separate genes (46). The HNF-4a gene is expressed in the liver, kidney, pancreas, intestine, colon, and testis, whereas the newly discovered HNF-4γ gene is expressed in all of these tissues except the liver (46). Disruption of HNF-4-binding sites in the promoters of specific genes can result in disease. For instance, a single nucleotide change in the 5′-flanking region of factor IX gene prevents HNF-4 binding and decreases its transcription in liver cells and accounts for the very low plasma levels of factor IX in individuals with hemophilia B (47). Recently, a mutation that leads to maturity-onset diabetes of the young was mapped to the HNF-4α locus, and it was proposed that a decrease in the amount of HNF-4 indirectly causes pancreatic β cell dysfunction (48). Given the critical role that HNF-4 seems to play in maintaining SHBG expression in liver cells, any deficiency in either its levels or activity in the liver may explain the low plasma levels of SHBG observed in many pre-symptomatic individuals with Type II diabetes (8).

Our functional analysis of the human SHBG proximal promoter has relied primarily on the use of HepG2 cells that resemble fetal hepatocytes in terms of their ability to express many liver-specific genes (49). It should therefore be appreciated that the interactions between COUP-TF and HNF-4 and a common binding site in FP1 may play an important role in the expression of SHBG in mammals during fetal development, especially as the sequence of FP1 is perfectly conserved in SHBG promoters between species, some of which only express this gene in the liver during fetal life. It is also important to appreciate that the SHBG proximal promoter we have examined is defined relative to the major transcription start site mapped by primer extension of SHBG mRNA extracted from adult liver (13), and the sequence surrounding this site does not conform to a transcriptional initiator sequence which plays a key role in directing the transcription of many mRNA-encoding genes with TATA-less promoters (50). However, the COUP-TF/HNF-4-binding site within FP1 is located at a minor transcription start (13) and resembles the loose initiator sequence (51).
If this region acts as an initiator sequence, the fact that TBP does not interact with FP1 raises the possibility that HNF-4 functions as an initiator binding protein, which recruits TAFs and/or TBP to this site in a manner similar to that proposed by studies of the mouse terminal deoxynucleotidyltransferase gene promoter (52). This hypothesis is supported by the fact that the introduction of a consensus HNF-4-binding site at this position enhances the transcriptional activity of the human SHBG proximal promoter in hepatoblastoma cells.

The COUP-TF/HNF-4-binding site within FP3 of the human SHBG promoter is not as well conserved among species and is not essential for trans-activation by these orphan receptors in HepG2 cells. This might suggest another role for HNF-4 in human SHBG regulation that may be operative later in development, and in more specific situations. In support of this, FP3 has a higher affinity for HNF-4 than does FP1 when nuclear extract from adult mouse liver is used, and HNF-4 could therefore act as an accessory factor in the regulation of SHBG by an extracellular stimulus, as reported for the glucocorticoid regulation of hepatocyte phosphoenolpyruvate carboxykinase gene (53). Binding sites for HNF-4 and a ubiquitous helix-loop-helix transcription factor, known as the upstream stimulator factor (USF), are located in close proximity within the proximal promoter of the L-type pyruvate kinase gene, and they cooperate in mediating the effects of glucose on the expression of this gene in the liver (54). It is debatable as to whether USP plays a general role in the carbohydrate regulation of liver-specific genes (55–57), but this may be relevant in the context of SHBG because FP4 in the human SHBG promoter contains a binding site for USP, and this entire region is absent in rat and mouse SHBG promoters. Given the close proximity between this site in FP4 and the HNF-4-binding site in FP3, a functional interaction between these two unrelated transcription factors could explain why the low plasma SHBG levels are linked to abnormalities in nutritional and metabolic status that are associated with obesity, diabetes, and cardiac diseases (7, 8).

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REFERENCES

1. Hammond, G. L. (1993) in Steroid Hormone Action: Frontiers in Molecular Biology (Parker, M. G., ed) pp. 1–25, IRL Press at Oxford University, Oxford

2. Anderson, D. C. (1974) Clin. Endocrinol. 3, 69–96

3. Sarne, D. H., Refetoff, S., Rosenfield, R. L., and Farriaux, J. P. (1988) Mol. Endocrinol. Metab. 66, 712–725

4. Reed, M. J., Cheng, R. W., Simmonds, M., Richmond, W., and James, V. H. T. (1989) Mol. Cell. Biol. 9, 2332–2340

5. Mukai, S., and Karathanasis, S. K. (1996) Mol. Cell. Biol. 16, 1468–1478

6. Slenk, F. M. (1993) Receptor 3, 223–232

7. Drewes, D., Tenkel, S., Holewa, B., and Ryffel, G. U. (1996) Mol. Cell. Biol. 16, 925–931

8. Rejneke, M. J., Sladek, F. M., Bertina, R. M., and Reitsma, P. H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6300–6303

9. Yamagata, K., Furuta, H., Oda, N., Kaisaki, P. J., Menzel, S., Cox, N. J., Fujii, T., Signorini, S., and Bell, G. I. (1996) Nature 384, 458–461

10. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M. J., Sladek, F. M., Bertina, R. M., and Reitsma, P. H. (1992) J. Biol. Chem. 267, 17161–17163

11. Malik, S., and Karathanasis, S. K. (1996) Mol. Cell. Biol. 16, 1824–1831

12. Shihata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

13. Malik, S., and Karathanasis, S. K. (1996) Mol. Cell. Biol. 16, 1824–1831

14. Shihata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

15. Malik, S., and Karathanasis, S. K. (1996) Mol. Cell. Biol. 16, 1824–1831

16. Shihata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

17. Malik, S., and Karathanasis, S. K. (1996) Mol. Cell. Biol. 16, 1824–1831

18. Shihata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

19. Malik, S., and Karathanasis, S. K. (1996) Mol. Cell. Biol. 16, 1824–1831

20. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M. J., Sladek, F. M., Bertina, R. M., and Reitsma, P. H. (1992) J. Biol. Chem. 267, 17161–17163

21. Serke, D., Blank, M., Nachtsheim, J., and Tschop, M. (1996) Nature 384, 458–461

22. Myohga, J., Furuta, H., Oda, N., Kaisaki, P. J., Menzel, S., Cox, N. J., Fujii, T., Signorini, S., and Bell, G. I. (1996) Nature 384, 458–461

23. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

24. Malik, S., and Karathanasis, S. K. (1996) Mol. Cell. Biol. 16, 1824–1831

25. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

26. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

27. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

28. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

29. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

30. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

31. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

32. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

33. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

34. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163

35. Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, J. M., and O’Malley, B. W. (1992) J. Biol. Chem. 267, 17161–17163
