Human Sex Hormone-binding Globulin Promoter Activity Is Influenced by a (TAAAA)$_n$ Repeat Element within an Alu Sequence*

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Sex hormone-binding globulin (SHBG) is the major sex steroid-binding protein in human plasma and is produced by the liver. Plasma SHBG levels vary considerably between individuals and are influenced by hormonal, metabolic, and nutritional factors. We have now found that a (TAAAA)$_n$ pentanucleotide repeat, located within an alu sequence at the 5’ boundary of the human SHBG promoter, influences its transcriptional activity in association with downstream elements, including an Sp1-binding site. Furthermore, SHBG alleles within the general population contain at least 6–10 TAAAA repeats, and the transcriptional activity of a human SHBG promoter-luciferase reporter construct containing 7–10 TAAAA repeats when tested in human HepG2 hepatoblastoma cells. This difference in transcriptional activity reflected the preferential binding of a 46-kDa liver-enriched nuclear factor to an oligonucleotide containing 6 rather than 7–10 TAAAA repeats. Thus, a (TAAAA)$_n$ element within the human SHBG promoter influences transcriptional activity in HepG2 cells and may contribute to differences in plasma SHBG levels between individuals.

Plasma sex hormone-binding globulin (SHBG)$^1$ binds testosterone and estradiol with high affinity and selectivity, and regulates the access of these sex steroids to their target tissues (1). Hepatocytes are the primary site of plasma SHBG biosynthesis, and changes in the blood levels of SHBG are influenced by hormonal, as well as metabolic and nutritional status (2). Low serum SHBG levels are commonly found in women with polycystic ovarian syndrome and disorders characterized by androgen excess (3), and have been reported to be a prognostic indicator for the onset of type II diabetes and cardiovascular disease (4, 5). Low serum levels of SHBG are also inherited within families (6, 7), and associations between abnormal serum SHBG levels and disease processes may therefore be obscured by genetic differences that contribute to variations in human SHBG gene expression.

We have recently characterized two binding sites for HNF-4 and COUP-TF within the human SHBG proximal promoter, which influence its transcriptional activity in human HepG2 hepatoblastoma cells (8). In particular, binding of HNF-4 to a TA-rich sequence close to the transcription start site in liver cells appears to substitute for the TATA-binding protein in the initiation of transcription (8). The possible function of the second HNF-4 binding site in the SHBG proximal promoter is not as clear, but it may contribute to phylogenetic differences in the temporal and tissue-specific expression of SHBG because it lies within a region that is absent in the corresponding region of SHBG genes in other mammalian species (8). Apart from this obvious difference in SHBG promoter sequences between species, the human and rodent SHBG promoters show a remarkable degree of sequence conservation, which only begins to diverge at the boundary of several alu sequences located at about −700 nt from the human SHBG transcription start site (8). This likely represents a functional boundary within the promoter sequence because human SHBG gene sequences containing only 803 nt of promoter sequence are expressed in a spatially and temporally appropriate manner when introduced as transgenes into the mouse genome (9, 10). Although the significance of repetitive elements within promoter sequences is unclear, they may contain nuclear factor binding sites that contribute to the regulation of transcription (11–13).

By characterizing the upstream region of the human SHBG promoter, we have now found that a (TAAAA)$_n$ repeat within an alu sequence binds a 46-kDa liver enriched nuclear protein and acts to silence transcription. More importantly, the number of TAAAA repeats at this location is highly variable between individuals within the general population, and the transcriptional activity of the SHBG promoter and the binding of nuclear protein to this element are both directly related to the number of TAAAA repeats.

EXPERIMENTAL PROCEDURES

In Vitro Footprinting—In vitro footprinting templates of human SHBG promoter upstream regions were produced by digesting a human SHBG fragment (14) with XhoI and XbaI. This released a 504-base pair region of the SHBG promoter corresponding to −803/−299 nt relative to the transcription start site in the liver (8). This fragment was further digested with HaeIII or HindIII, and the products (−803/−656 nt XhoI-HaeIII, −735/−587 nt HindII-HindIII, −587/−362 nt HindII-HindIII, −541/−298 nt HaeIII-XbaI) were cloned into the EcoRV site of pBluescript (Stratagene, La Jolla, CA) in the correct orientation to permit labeling of the sense strand after digestion with HindIII. The HindIII-digested constructs were end-labeled with the Klenow fragment of DNA polymerase I in the presence of [α-$^{32}$P]dCTP and purified using a NICK™ column (Amersham Pharmacia Biotech, Baie d’Urfe, Quebec, Canada). Radiolabeled probes were released from the plasmids by digestion with EcoRI and purified by 6% polyacrylamide gel electrophoresis (PAGE).
The DNase I footprinting reactions with mouse liver nuclear extracts were carried out as described previously (8). Hydroxy-radical footprinting with the same extracts was also performed using the ~803/656 nt XhoI-HaeIII region of the human SHBG promoter (15, 16). Mouse liver nuclear extracts were used in these and other experiments to characterize and study the regulation of human SHBG promoter activity because human SHBG transgenes are expressed efficiently in mouse hepatocytes postnatally (9, 10).

**Reporter Plasmids—** Human SHBG promoter deletion constructs were generated by amplifying a region from an SHBG fragment (14) in a polymerase chain reaction (PCR). This was done using a common reverse primer containing an XhoI site (~299) and forward primers (containing an XhoI site) corresponding to various positions in the 5′ promoter region (Table I). These PCR products were digested with XhoI and XhoI and then subcloned into a pSP2 vector (Promega Corp., Madison, WI) containing the ~299/+60 nt region of the human SHBG proximal promoter. The entire promoter sequences were then excised with HindIII and XhoI and inserted into a pG2 Basic luciferase reporter plasmid (Promega).

Site-directed mutagenesis was accomplished using a pSELECT vector containing the ~803/299 nt region of the human SHBG promoter, according to the Altered Sites manual (Promega). Mutated sequences in pSELECT were removed by XhoI and XhoI digestion and subcloned into the pSP2 vector containing the ~299/+60 nt region of the human SHBG promoter, and the entire promoter sequences were then inserted into pG2 Basic, as described above. All PCR-generated and mutated sequences were confirmed by DNA sequencing using a commercially available kit (Amersham Pharmacia Biotech).

**Cell Culture and Transfection—** All cell culture reagents were from Life Technologies, Inc. (Burlington, Canada). Human HepG2 hepatoblastoma cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and were transiently transfected with human SHBG promoter-pG2GL2 reporter constructs (1.2 μg) and pCMV LacZ (0.2 μg) using LipofectAMINE reagent (8). Cells were harvested 48 h following transfection, and cell extracts were prepared by three cycles of freezing and thawing for analysis of luciferase and β-galactosidase activity. Luciferase units were divided by readings obtained from a β-galactosidase assay to correct for efficiency of transfection.

**Electrophoretic Mobility Shift Assay (EMSA)—** Mouse liver nuclear protein extracts (4 μg) were prepared (17), and then incubated in EMSA buffer (2.5 mM HEPEs, pH 7.6, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 6.25% glycerol, and 3 μg of poly(dI-dC)) on ice for 10 min in the presence or absence of double-stranded competitor oligonucleotides (Table II). The corresponding end-labeled oligonucleotide probes were then added, and the binding reaction was allowed to proceed for 15 min at room temperature. For antibody supershift assays, radiolabeled probe was incubated with nuclear extract on ice for 15 min prior to addition of either normal rabbit serum, or an antisem specific to SP1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Reactions were then incubated for 15 min at room temperature before electrophoresis. Nuclear proteins bound to radiolabeled oligonucleotides were separated from free probe by 5% PAGE, and the gel was dried and exposed to Biomax MR film (Eastman Kodak Co., Rochester, NY) against an intensifying screen at ~80 °C.

**Analysis of Nuclear Factor Binding to TATAA Repeats by UV Cross-linking—** Binding reactions of nuclear protein extracts to double-stranded oligonucleotides comprising various TATAAA repeats (Table II) were carried out under the same conditions used for EMSSAs. After a 15-min incubation at room temperature, samples were exposed to UV light (302 nm) at a distance of 10 cm for 15 min at 0 °C. Equal volumes of sodium dodecyl sulfate (SDS) loading buffer were added, and samples were heated at 95 °C for 5 min and subjected to SDS-PAGE with 4 and 10% acrylamide in the stacking and resolving gels, respectively. Gels were dried and exposed to x-ray film, as described above.

**Southwestern Blot Analysis—** Nuclear proteins extracted from MSC-1 mouse Sertoli cells and human HeLa cervical cancer cells (18), as well as mouse liver (17), were fractionated by SDS-PAGE and transferred by electrophoresis onto a Hybond™ ECL™ nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was washed three times for 45 min in a buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 2.5% Nonidet P-40, 5% milk powder, and 0.1 mM DTT. It was then rinsed twice in binding buffer (10 mM Tris, pH 7.5, 40 mM NaCl, 1

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**Table I**

Sequences of oligonucleotides used for deleting (A) or mutating (B) human SHBG promoter sequence in the context of luciferase reporter gene constructs

| Description | PCR oligonucleotides for promoter deletions |
|-------------|-------------------------------------------|
| <strike>–299 reverse</strike> | 5′-GAGGCTCTAGAAGACATGCCCTCCTCGGTT |
| <strike>–696 forward</strike> | 5′-TGAATTCGAGTCGAGGATGGGGTA |
| <strike>–670 forward</strike> | 5′-GAATTCGACTAGCTTGGTGCCGCT |
| <strike>–536 forward</strike> | 5′-GAATTCGCTAGGCGGAGGTCCTTAAAT |
| <strike>–552 forward</strike> | 5′-GAATTCGCTAGGGCGGACTTGGA |
| <strike>–509 forward</strike> | 5′-GAATTCGAGATGGCCAGCATTGCTGTC |
| <strike>–458 forward</strike> | 5′-GAATTCGAGACCCGTAAAGATTTTTC |

**Table II**

Sequences of oligonucleotides used in South-western blotting, UV cross-linking experiments, and electrophoretic mobility shift assays (EMSSAs)

Lowercase letters indicate additional sequences that were filled using the Klenow fragment of DNA polymerase I and (α-32P)dCTP (17). Mutations (in bold) introduced into FP12 (~536/~510) were based upon a previous mutation that disrupts an SP1 recognition sequence (39). Double-stranded oligonucleotides representing SP1 (40) and HNF-4/COUP-TF (8) binding sites were used as reported by others.

| Description | EMSA oligonucleotides |
|-------------|-----------------------|
| 6xTAAA | 5′-GTC(TAAA)GTTG |
| 7xTAAA | 5′-GTC(TAAA)TGGT |
| 8xTAAA | 5′-GTC(TAAA)TGGT |
| 9xTAAA | 5′-GTC(TAAA)TGGT |
| 10xTAAA | 5′-GTC(TAAA)TGGT |
| FP12 | 5′-GAAAGCTGGGGAGGTCAGAC |
| FP12 mutant | 5′-CTCGAGGAGGAGGTCAGAC |
| HNF4-COUP-TF | 5′-GAATTCGAGACCCGATGAGG |

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The DNase I footprinting reactions with mouse liver nuclear extracts were carried out as described previously (8). Hydroxy-radical footprinting with the same extracts was also performed using the ~803/656 nt XhoI-HaeIII region of the human SHBG promoter (15, 16). Mouse liver nuclear extracts were used in these and other experiments to characterize and study the regulation of human SHBG promoter activity because human SHBG transgenes are expressed efficiently in mouse hepatocytes postnatally (9, 10).

**Control of Human SHBG Transcription**

Underlined sequences denote restriction enzyme recognition sites used for creation of human SHBG promoter constructs. Δ6TAAA AAA oligonucleotide designed to remove the TAAA sequence. Mutations introduced into the FP12 SP1 binding site (~536/~510) were based upon a previous mutation in an SP1 consensus sequence (39).
mm EDTA, 1 mM DTT, 8% glycerol, and 0.125% milk powder). A radio-
labeled double-stranded oligonucleotide (250,000 cpm/ml) spanning the
(TAAA)_n sequence in the SHBG upstream promoter (Table II) was
added to the binding solution containing 5 mM MgCl_2 and 5 μg/ml poly(dI-dC),
and was incubated with the membrane for 16 h at room
temperature. The membrane was then washed in 50 mM Tris, pH 7.5,
150 mM NaCl and exposed to Biomax MR film, as described above.

Analysis of the (TAAA)_n Repeat in the SHBG Promoter—Genomic DNA was isolated from peripheral blood leukocytes of healthy human
volunteers (19). Amplification of the TAAA repeat region was accom-
plished using PCR with a forward primer (5'-CTTTGACTGAGAGAG-
GCAG) within an alu sequence in the human SHBG promoter (14), and
a reverse primer (5'-CAGGGCTTACAACGTCTAGCAGT) correspond-
ing to a sequence at −651/−673 nt within the upstream promoter
sequence. Amplified products were separated by 10% PAGE followed by
digestion in the presence or absence of nuclear protein, lies
between FP16 and FP17 (Fig. 1). One of these footprints
(FP17) contains a sequence (5'-TGAATAGGGAAAGAC), which
resembles a CEBPβ binding site when examined by homology
searches using MatInspector (20) release 2.1. In addition, a
sequence (5'-GGGGCAAGGACT) within FP12 (nt −527/−517)
resembles a consensus SP1 binding site (5'-GGGCGGGGC(T/H)) by analysis using the TRANSFAC data base (21). A region
containing six TAAA repeats, which is resistant to DNase I
digestion in the presence or absence of nuclear protein, lies
between FP16 and FP17 (Fig. 1), and the boundary of the
footprint within the TAAA repeat region was confirmed by
hydroxy-radical footprinting with a mouse liver nuclear protein
extract (Fig. 2).

Interaction between SP1 and a double-stranded oligonucleo-
tide that included FP12 (nt −536/−510) was confirmed by EMSA
(Fig. 3A). The specificity of this interaction was demonstrated by
inhibition of DNA-protein complexes using an oligonucleotide
containing a known SP-1 binding site (Table II). In addition,
the major DNA-protein complex formed in an EMSA reaction was
supershifted with an SP1-specific antibody (Fig. 3B).

Sequences in the Human SHBG Upstream Promoter Silence
Transcription—The activity associated with the nuclear factor
binding sites identified by DNase I footprinting (represented by
boxes in Fig. 4A) was investigated by adding them sequentially
to a human SHBG proximal promoter-luciferase reporter gene
construct (8). These constructs were used for transcriptional
activity measurements in a human hepatoblastoma cell line
HepG2 that produces SHBG (22). This revealed that up-
stream sequences markedly reduce transcriptional activity
when compared with the activity of the proximal promoter (Fig. 4A).
In particular, addition of sequences that include FP16, as
well as a putative CEBPβ-binding site within FP17 and the
intervening (TAAA)_n sequence resulted in progressive and
significant reductions of promoter activity (Fig. 4A). The reduc-
tion of transcription by sequences at the 5' boundary of the
upstream promoter appeared to be associated specifically with the
(TAAA)_n sequence because a 6-fold increase in transcrip-
tion was observed when it was removed from the full-length
promoter (Fig. 4B). It was also noted that mutation of the SP1
binding site (Table II) within FP12, which prevents its inter-
action with liver nuclear extracts in an EMSA (data not
shown), also resulted in a similar increase in the transcriptional
activity of the full-length promoter (Fig. 4B). However,
recovery of the transcriptional activity of the full-length pro-
moter was not further enhanced by removal of TAAA pen-
tanucleotide repeat in combination with a mutant SP1 binding
site within FP12 (Fig. 4B). It is also important to note that the
presence of FP12 appears to have a negative effect on transcrip-
tion only in the context of upstream sequences within the
full-length promoter (Fig. 4A).

Nuclear Factor Binding to a (TAAAA)₆ Sequence in the Human SHBG Promoter—When tested in an EMSA, a radiolabeled oligonucleotide comprising the (TAAAA)₆ sequence interacted with nuclear factor extracted from mouse liver and could be competed for by unlabeled oligonucleotides spanning this repeat region, but not with an unrelated DNA sequence (Fig. 5). To further characterize the nuclear factor(s) binding at this pentanucleotide repeat, a radiolabeled (TAAAA)₆ repeat oligonucleotide sequence (Table II) was used as a probe for a Southwestern blot (Fig. 6). The radiolabeled probe recognized a nuclear protein with an apparent molecular mass of 46 kDa, which is enriched in mouse liver nuclear extracts when compared with nuclear extracts from mouse MSC-1 Sertoli cells or HeLa cells (Fig. 6). By contrast, a nuclear protein with a molecular mass of ~67 kDa was recognized in all nuclear extracts and likely represents a ubiquitous nuclear factor, which binds to this sequence (Fig. 6).

The Number of TAAAA Repeats in the Human SHBG Upstream Promoter Varies and Influences Its Activity—In view of reports that the number of TAAAA repeats in the promoters of other human genes varies among individuals (23), we amplified the region of the SHBG upstream promoter containing the TAAAA repeat sequence from eight healthy male volunteers. The sizes of the PCR products indicated that the number of TAAAA repeats is highly variable both within and between individuals (Fig. 7A), and we demonstrated that the repeat number ranges from 6 to 10 by sequencing them (Fig. 7B). The serum SHBG concentrations in these individuals (with respect to their (TAAAA)₆ allele genotypes) were as follows: 13 nmol/liter (6/10), 14 nmol/liter (6/6), 15 nmol/liter (7/7), 25 nmol/liter (7/8), 24 nmol/liter (6/8), 24 nmol/liter (6/7), 15 nmol/liter (6/8), and 23 nmol/liter (9/7). Although there was no obvious relationship between serum SHBG concentrations and the number of TAAAA repeats on each allele, an individual with two (TAAAA)₆ containing SHBG alleles had a relatively low serum SHBG level. However, the number of individuals we have studied is small, and no attempt was made to control for factors that might otherwise influence serum SHBG levels, such as body mass index.

The influence of this polymorphism on transcription was tested in the context of the full-length human SHBG promoter. We found that the activity of human SHBG promoter-luciferase reporter constructs in HepG2 cells increased 5-fold as the number of TAAAA repeats was increased from six to seven. As additional repeat sequences were added, little if any further increases in promoter activity were observed (Fig. 8). These increases in promoter activity associated with increasing repeats is strikingly similar to the increase observed when the (TAAAA)₆ sequence is removed from the full-length promoter (Fig. 4B). Taken together, these data suggest that the silencing activity is associated specifically with the presence of only six TAAAA repeats. This was confirmed by performing similar experiments using full-length SHBG promoter sequences containing only four or five TAAAA repeats, which also provided ~5-fold higher levels of transcriptional activity when compared with the promoter sequence containing six repeats (Fig. 8).

Nuclear Factor Binding to Various TAAAA Repeats—To further examine nuclear factor binding in relation to the number of TAAAA repeats, we performed a UV cross-linking experi-
FIG. 4. Analysis of human SHBG promoter activity in HepG2 cells. A, a series of human SHBG promoter deletion constructs was generated and transiently transfected into HepG2 cells. Regions of the human SHBG promoter protected from DNase I digestion by mouse liver nuclear proteins (represented as boxes) were sequentially removed, and the effect of these nuclear factor binding sites on promoter activity was assessed. B, the transcriptional silencing activity of the (TAAAA)\textsubscript{6} repeat appears to require the presence of an SP1 site within FP12. Mutation of FP12 to prevent SP1 binding (X) relieves the transcriptional silencing of the (TAAAA)\textsubscript{6} repeat. Transcriptional silencing is also relieved when the (TAAAA)\textsubscript{6} repeat is removed (X), irrespective of an intact SP1 site within FP12. The activities of the human SHBG reporter constructs are expressed relative to that of the promoterless pGL2 Basic luciferase reporter plasmid (Luc). In panel A, statistically significant differences were \( p < 0.05 \) (*) and \( p < 0.001 \) (**) when compared with the activity of the −299/+60 human SHBG promoter. In panel B, statistically significant differences were \( p < 0.001 \) (***) when compared with the full-length (−803/+60) human SHBG promoter. Data are represented as means ± S.E. from at least three experiments.

DISCUSSION

Blood levels of SHBG in humans are highly variable even between healthy individuals (6). To determine if this variability can be explained by a genetic polymorphism, we sequenced the first 300 nt of the human SHBG promoter from normal individuals and patients with various reproductive and endocrine disorders in a separate study, but found no significant deviations between sequences.\textsuperscript{2} By contrast, our studies of human SHBG promoter activity in HepG2 hepatoblastoma cells alerted our attention to a (TAAAA)\textsubscript{6} repeat located within an \textit{alu-Sx} sequence close to the 5′ boundary of the transcription unit expressed in hepatocytes (9). This particular pentanucleotide repeat has been found to vary in number within the human cholesterol side-chain cleavage enzyme \textit{CYP11A1} promoter (23), and there is a strong association between alleles with this polymorphism and total serum testosterone levels in patients with polycystic ovarian syndrome and hyperandrogenism (24). Since this (TAAAA)\textsubscript{6} repeat appears to have a silencing effect on SHBG promoter activity, we focused our attention on the possible factor(s) that might bind to it, and how it might function in concert with other elements in the upstream region of the human SHBG promoter to influence transcription.

The upstream region of the human SHBG promoter silences transcription in HepG2 cells, and most of this activity is associated with a region between FP16 and FP17, which includes the (TAAAA)\textsubscript{6} repeat element. We have demonstrated that the (TAAAA)\textsubscript{6} repeat is responsible for this silencing activity by removing it or by increasing the number of repeats according to the number observed in the general population. It is also clear that this activity is dependent on downstream promoter elements and appears to involve an SP1 nuclear factor-binding site within FP12. Although SP1 is generally considered to be an activator of transcription, it can participate in transcriptional silencing through interactions with other transcriptional regulators in a context-dependent manner (25, 26). This might explain why FP12 only appears to regulate SHBG promoter activity in association with upstream elements (FP16-FP17) within the promoter. Although our EMSA supershift data indicate that SP1 binds to FP12, we also observed that a second protein complex forms with a FP12 oligonucleotide, and this complex does not appear to be supershifted with an SP1-specific antisemur. It is therefore possible that FP12 interacts with another SP1-related factor, such as SP3 (27, 28), and this

\textsuperscript{2} G. L. Hammond, unpublished data.
might be relevant because SP3 often acts as a negative regulator of transcription (29, 30). Furthermore, competition between SP3 and other SP1-related factors for a common site within promoter sequences alters their transcriptional activity (28).

Variations in the number of polynucleotide repeats within several other promoters have been reported to modulate transcription (31, 32) and have been linked to disease states (24, 31, 32). Although the number of (TAAAA)\textsubscript{n} repeats in the human SHBG promoter is closely associated with serum testosterone levels and might therefore reflect variations in the expression of the gene (24), it is not known whether this is due to an effect at the level of transcription. Differences in the number of an inverted (TTTTA)\textsubscript{n} repeat in the apolipoprotein(a) gene (APO(a)) promoter have also been associated with individual variations in plasma Lp(a) levels (33, 34). Furthermore, an APO(a) promoter containing nine TTTTA repeats is associated with low plasma Lp(a) levels, and has a 5-fold lower transcriptional activity in HepG2 cells, when compared with a promoter sequence containing eight TTTTA repeats from an individual with relatively high plasma levels of Lp(a) (35). The (TAAAA)\textsubscript{n} repeat found within the 5’ region of the human SHBG promoter occurs frequently within the human genome (36) and is a common feature of repetitive elements such as alu sequences (37) and LINE elements (36). Close inspection of the pentanucleotide repeats in the CYP11A1 and APO(a) promoters by RepeatMasker (www.genome.washington.edu/uwgc/analysis/tools/repeatmasker.htm) indicates that they also flank Sf/p/q and Sg subfamilies of human alu sequences, respectively, and differences in the number of these repeats between individuals likely re-

| Competitor (position in promoter) | TAAAA (-730/-692) | SP1 (-536/-510) |
|-----------------------------------|-------------------|------------------|
| DNA:protein complex               |                   |                  |
| Free probe                        |                   |                  |

![DNA:protein complex](image1.png)

**Fig. 5.** A (TAAAA)\textsubscript{n} repeat in the human SHBG promoter interacts with a protein from mouse liver nuclear extract. Radiolabeled oligonucleotides spanning the (TAAAA)\textsubscript{n} repeat in the human SHBG promoter were incubated with 4 μg of adult mouse liver nuclear extract in the absence (lane 2) or presence of excess unlabeled competitor oligonucleotides (lanes 3–6). Free probe was separated from DNA-protein complexes by non-denaturing polyacrylamide gel electrophoresis. Lane 1 represents the radiolabeled probe in the absence of nuclear protein. A double-stranded oligonucleotide spanning the SP1 site in the human SHBG promoter (FP12, -536/-510) was used as an unrelated competitor to demonstrate specificity.

![Southwestern blotting of nuclear factors binding to the (TAAAA)\textsubscript{n} repeat element](image2.png)

**Fig. 6.** Southwestern blotting of nuclear factors binding to the (TAAAA)\textsubscript{n} repeat element. Nuclear proteins from adult mouse liver, a mouse Sertoli cell line (MSC-1), and HeLa cells were separated on a denaturing SDS-PAGE gel, followed by transfer to a nitrocellulose membrane. The blot was incubated with a radiolabeled double-stranded oligonucleotide spanning the six TAAAA pentanucleotide repeats in the 5’ region of the human SHBG promoter. Migration of standards of known molecular mass is shown on the left.

**Fig. 7.** Demonstration of a (TAAAA)\textsubscript{n} polymorphism in the human SHBG promoter. A illustrates the degree of polymorphism in the TAAAA repeat element in the 5’ region of the human SHBG promoter within DNA samples from four individuals. This was accomplished by PCR amplification of this sequence using a forward primer within the alu sequence together with a reverse primer within the human SHBG promoter, and gives rise to an amplified product of 160 base pairs in samples with six repeats. B, the number of TAAAA repeats in SHBG alleles was confirmed by sequencing the resultant PCR products (T/A reactions are shown) obtained from 4 individuals (panel A). The sequence of the allele containing seven repeats on the right of the figure appears to contain an additional polymorphism in the A lane, but repeat sequencing has indicated that this is an artifact due to compression of the sequencing products.
flect an inherent instability of *alu* repetitive elements (38).

Unlike the (TA)<sub>n</sub> dinucleotide repeat in the UDP-glucurono-
syltransferase 1 (*UGT1A1*) promoter, where increasing numbers of repeats are associated with decreased promoter activity (32), the activity of the human *SHBG* promoter is not linear with respect to TAAA repeat number. Differences in the number of TTATA repeats in the *APO(a)* promoter influence its transcriptional activity, but this has not been studied in any great detail (35). In particular, there is no information concerning the identity of proteins that might interact with these types of repeats, or how they influence gene transcription. In experiments presented here, silencing of the human *SHBG* promoter was only observed in the presence of six TAAA repeats, and this correlated with the preferential binding of a liver-enriched 46-kDa nuclear factor to the (TAAA)<sub>6</sub> repeat element. Although alleles containing less than six TAAA repeats were not observed in the limited group of individuals we examined, the activities of *SHBG* promoters containing four or five TAAA repeats also lacked the silencing properties associated with the presence of six repeats. These data suggest that the 46-kDa factor that binds six TAAA repeats with high affinity acts in concert with downstream elements within the human *SHBG* promoter to alter its transcriptional activity.

In summary, a (TAAA)<sub>n</sub> repeat polymorphism within the human *SHBG* promoter has a marked effect on its transcriptional activity *in vitro* in HepG2 cells. This could contribute to individual differences in plasma SHBG levels and thereby influence the access of sex steroids to their target tissues. Furthermore, variations in the number of TAAA repeats within regulatory regions of other human genes may contribute to inter-individual differences in gene expression. The genomic DNA samples we examined were from healthy male volunteers of various ethnic backgrounds, and the number of TAAA repeat elements ranged from 6 to 10 in this limited group of subjects. Although there was no obvious relationship between the number of TAAA repeat elements and the serum SHBG concentrations, the number of individuals examined is too small to draw any conclusions, especially as almost all of them were all bi-allelic for different numbers of TAAA repeats. It is, however, important to appreciate that the ability of the (TAAA)<sub>n</sub> polymorphism to influence the transcriptional activity of the human *SHBG* promoter as naked DNA in transient transfection experiments may not necessarily reflect its activity in the context of genomic DNA within a chromatin structure. It will therefore be important to conduct a carefully controlled clinical study to determine whether this polymorphism is associated with differences in plasma SHBG levels, and to determine whether specific alleles are associated with sex steroid hormone-dependent diseases and/or correlate with responses to various hormone treatments that influence plasma SHBG levels.

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