Cloning of Factors Related to HIV-inducible LBP Proteins That Regulate Steroidogenic Factor-1-independent Human Placental Transcription of the Cholesterol Side-chain Cleavage Enzyme, P450scc*

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The cholesterol side-chain cleavage enzyme, cytochrome P450scc, initiates the biosynthesis of all steroid hormones. Adrenal and gonadal strategies for P450scc gene transcription are essentially identical and depend on the orphan nuclear receptor steroidogenic factor-1, but the placental strategy for transcription of P450scc employs cis-acting elements different from those used in the adrenal strategy and is independent of steroidogenic factor-1. Because placental expression of P450scc is required for human pregnancy, we sought factors that bind to the −155/−131 region of the human P450scc promoter, which participates in its placental but not adrenal or gonadal transcription. A yeast one-hybrid screen of 2.4 × 10^6 cDNA clones from human placental JEG-3 cells yielded two unique clones; one is the previously described transcription factor LBP-1b, which is induced by HIV, type I infection of lymphocytes, and the other is a new factor, termed LBP-9, that shares 83% amino acid sequence identity with LBP-1b. When expressed in transfected yeast, both factors bind specifically to the −155/−131 DNA; antisera to LBP proteins supershifted the LBP-9DNA complex and inhibited formation of the LBP-1bDNA complex. Reverse transcriptase-polymerase chain reaction detected LBP-1b in human placental JEG-3, adrenal NCI-H295A, liver HepG2, cervical HeLa, and monkey kidney COS-1 cells, but LBP-9 was detected only in JEG-3 cells. When the −155/−131 fragment was linked to a minimal promoter, co-expression of LBP-1b increased transcription 21-fold in a dose-dependent fashion, but addition of LBP-9 suppressed the stimulatory effect of LBP-1b. The roles of LBP transcription factors in normal human physiology have been unclear. Their modulation of placental but not adrenal P450scc transcription underscores the distinctiveness of placental strategies for steroidogenic enzyme gene transcription.

Steroid hormones regulate a wide variety of physiologic functions. Mineralocorticoids, produced by the adrenal cortex, are needed to retain sodium and maintain blood pressure (1); glucocorticoids, also produced by the adrenal cortex, raise blood sugar but also play roles in numerous physiologic processes (2); and androgens and estrogens, produced by the gonads, are required for reproduction (3, 4). In human beings, absence of mineralocorticoids leads to death in infancy, absence of glucocorticoids may lead to death during times of severe physiologic stress, and absence of sex steroids would, eventually, lead to death of the species. Thus, the regulation of steroid hormone biosynthesis is of fundamental interest. The role of steroid hormones in the fetus is less obvious, and there are important species differences among mammals. Human fetuses can develop normally, reach term gestation, undergo normal parturition, and make initial adaptations to extrauterine life in the absence of mineralocorticoids, glucocorticoids, or sex steroids (5). By contrast, normal human gestation is totally dependent on the action of progesterone to suppress uterine contractility and to maintain pregnancy (6). Progesterone is initially provided by the mother’s ovarian corpus luteum, but after about 5–10 weeks virtually all progesterone is produced by the placental syncytiotrophoblast cells, which are fetal tissue (7, 8). Therefore, placental synthesis of progesterone is essential for the initiation of human life (5).

The synthesis of placental progesterone, and of all other steroid hormones, begins with the conversion of cholesterol to pregnenolone by mitochondrial cytochrome P450scc, which is the rate-limiting and hormonally regulated enzymatic step in steroidogenesis (9). Human P450scc is encoded by a single gene (10), formally termed CYP11A (11), that is located on chromosome 15q23-q24 (12) and is expressed in the adrenals (13), gonads (13), placenta (14), and brain (15). Because of its key role in the production of all steroid hormones, the transcription of the P450scc gene has been the subject of intensive study (reviewed in Refs. 16–18). Such studies led to the discovery of steroidogenic factor-1 (SF-1), also known as Ad4-BP, an orphan nuclear receptor that is essential for fetal adrenal and gonadal development as well as for expression of all the steroidogenic genes in these tissues (19). However, although SF-1 is expressed in many tissues (20), very little SF-1 is expressed in other tissues that contain P450scc including brain, embryonic gut, and placenta (20–22), and studies of P450scc regulation in the brain (23) and placenta (24, 25) indicate that the SF-1 sites in the P450scc promoter are not involved in P450scc transcription in these tissues. As placental P450scc expression and progesterone synthesis are mandatory for successful pregnancy...

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† The abbreviations used are: SF-1, steroidogenic factor-1; bp, base pair; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; DME-H, Dulbeco’s modified Eagle’s/Ham’s medium; TK32, minimal 32-base promoter of the thymidine kinase gene; LUC, luciferase.
and as little is known about SF-1-independent expression of genes for steroidogenic enzymes, we have studied the transcription of P450scc in human placent JEG-3 cells (24, 25). We now report the cloning and characterization of two transcription factors that modulate the human placental expression of P450scc; both factors are related to the LBP-1 family of transcription factors induced by HIV, type I infection of lymphocytes (26).

**MATERIALS AND METHODS**

**Preparation of the Yeast YM4271 Reporter Strain—**Saccharomyces cerevisiae strain YM4271 [MATa, ura3–52, his3–200, ade2–101, lys2–801, leu2–3, 112, trp1–901, tyr1–501, gal4–A538, gal80–A538, ade5::hisG (27, 28)] (CLONTECH Laboratories) was grown on standard media (29). A double-stranded oligonucleotide (see Table I) corresponding to bases –155 to –131 of the human P450scc promoter (30) was tetramerized in a head-to-tail orientation and propagated in *E. coli* propagated in their host yeast and individually transformed into *E. coli* (31), and stable, integrated transformants were identified on selective media: His marker in pLacZi (in pHISi) and LacZ (in pLacZi). After propagation in *E. coli*, these plasmids were sequentially transformed into yeast YM4271 using lithium acetate (31), and stable, integrated transformants were identified on selective media: His for pHISi and Ura for the URA3 marker in pLacZi.

**JEG-3 cDNA Library—**Both randomly primed and oligo(dT)-primed cDNA made from JEG-3 cell RNA was ligated with phosphorylated EcoRi-NotI-SaiI adapters and passed through Sephacryl S-300 to exclude DNA smaller than 300–400 bp. The cDNA was ligated to the GAL4 activation domain (32, 33) in the pLacZi. After propagation in *E. coli*, these plasmids were sequentially transformed into yeast YM4271 using lithium acetate (31), and stable, integrated transformants were identified on selective media: His for pHISi and Ura for the URA3 marker in pLacZi.

**Preparation of Total JEG-3 Cell RNA**—Total JEG-3 cell RNA (1 μg) was reverse transcribed into cDNA using 5 μl of the first strand cDNA synthesis specific for clones 1, 9, 32, human P450c17, and GAPDH (see Table I). Random primers were used for the first strand cDNA synthesises using reverse transcriptase Superscript II (Life Technologies, Inc.). PCR was done using 5 μl of the first strand cDNA product and oligonucleotides specific for clones 1, 9, 32, human P450c17, and GAPDH (see Table I).

**TABLE I**

| Sense (s) and antisense (as) primers for RT-PCR experiments |
|------------------------------------------------------------|
| **Helper primers** | **Sense (s)** | **Location** |
| NH-1s-4 | ATGGGCTGGTGCTCAA | 175–191 |
| NH-1as-1 | GCCAGGCTGCTGTA | 848–864 |
| NH-9s-1b | AGCTGCAAGAAGACCT | 269–287 |
| NH-9as-2 | TCTCGAGACATCCAGCA | 1103–1121 |
| NH-32s-1b | GATCTAGTCGAGATG | 794–811 |
| NH-32as-2 | AATGACATCTGCTGCGT | 1632–1650 |
| c11-as-12 | CGTGCTGCTGCTGCTGA | 53–72 |
| c17-as-810 | AGCCATTATCTGAGTTCATCTT | 849–870 |
| cccs-a1 | CTCGAGAATTCGAGTTGACAG | 747–766 |
| SCC-as-1 | CACCTGCGGCCGTCTCCGCT | 1478–1497 |
| GAPDH-s-1 | GTATCGGTGGAAGACCTAT | 566–584 |
| GAPDH-as-1 | TATCCCTCGTGAGGCAGATG | 1049–1067 |

**RT-PCR**—Total RNA was isolated from JEG-3 cells, NCI-H295A cells, HeLa cells, HepG2 cells, COS-1 cells, and human adrenal tissue. Random primers were used for the first strand cDNA synthesises using reverse transcriptase Superscript II (Life Technologies, Inc.). PCR was done using 5 μl of the first strand cDNA product and oligonucleotides specific for clones 1, 9, 32, human P450c17, and GAPDH (see Table I).

**Tissue Culture and Transfections—**Human placent JEG-3 cells (35) (American Type Culture Collection, Manassas, VA) were grown at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s/Ham’s 21 medium (DME-H21) with 50 μg/ml gentamycin, 5% fetal bovine serum, and 5% horse serum. JEG-3 cells were grown to 60–80% confluence on 6-well tissue culture plates and transfected by calcium phosphate precipitation for 6 h with 3 μg of plasmid DNA for each well. After aspirating the calcium phosphate–DNA precipitates, the cells were washed with 3 ml of DME-H21 medium and incubated for an additional 36 h in regular medium. Human adrenal NCI-H295A cells (36), an adherent sub-line of NCI-H295 cells (37, 38), were grown in 50% DME-H16, 50% DME-F12 (RPMI 1640), and 2% fetal bovine serum supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, and antibiotics/antimycotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin). Full-length cDNAs for clones 1, 9, and 32 were re-cloned into pBSG5 (39) for transfection of mammalian cells. The human P450scc cDNA constructs 1xwt–155/–131/TK32LUC and 1xmt–155/–131/TK32LUC were described previously (25). As an internal control, 100 ng of pRL-CMV vector (Promega) was co-transfected for each well. Luciferase assays were performed with Dual-Luciferase™ assay system (Promega).
using a Monolight 1500 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

Preparation of Protein Extracts—Nuclear extracts were prepared from JEG-3 cells and NCI-H295A cells as described (24). Protein concentrations were determined by Bradford assay. Yeast transformed with HIS multicopy vector YEp90 expressing the cDNA for clones 1, 9, or 32 were grown in 50 ml of SD selective media overnight at 30 °C, transferred into 300 ml of YPD media, and incubated for 3 ha t 30° C (40). Cell pellets were collected by centrifugation, washed once with H2O, and then resuspended in 1.5 ml of 20 mM Tris-HCl, pH 7.5, 20% glycerol, 0.4 M KCl, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride with protease inhibitors (Roche Molecular Biochemicals). The yeast were vortexed vigorously for 2 min with an equal volume of 450–600-μm glass beads (Sigma), and the debris was pelleted at 4 °C for 15 min at 13,000 rpm. The protein concentration of the supernatant was determined, and aliquots of these yeast extracts were stored at −70 °C.

Electrophoretic Mobility Shift Assays—Double-stranded wild-type and mutant probes corresponding to bases 2155/2131 of the human P450scc promoter (see Table I) were end-labeled by [γ-32P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (New England BioLabs) and purified through a G-50 column. About 10 fmol of labeled probe (20,000 cpm) were incubated at room temperature for 15 min with 5–8 μg of nuclear extracts or yeast cell extracts and various competitor oligonucleotides in a final volume of 15 μl 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 M EDTA, 4% glycerol, 5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin with 1 μg of poly(dI-dC) added as a nonspecific competitor. For supershift experiments, 1 μl of undiluted rabbit anti-human LBP-1 antiserum was added following formation of the DNA–protein complex and was incubated for an additional 15 min at room temperature. DNA–protein complexes were analyzed by electrophoresis in 4% native polyacrylamide gel in 50 mM Tris base, 38 mM glycine, 2 mM EDTA, pH 8.0, and 0.35% -mercaptoethanol at 20 °C for 90 min at 230 volts.

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**FIG. 1. Sequences of clones NH1 (panel A), NH9 (panel B), and NH32 (panel C).** Clones NH9 and 32 were completed by 5'-RACE. GenBank™ accession numbers for these sequences are: LBP-1b (NH1), AF198487; LBP-9 (NH9), AF198488; LBP-32 (NH32), AF198489.

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**FIG. 1—continued**
showed that the multicopy clones NH1, NH9, and NH32 were all related to LBP-1; these clones were then sequenced in their entirety (Fig. 1).

Sequences of LBP-related Clones—The insert in clone NH1 was 3843 bp long, had a long 3'-untranslated region, and encoded a protein of 540 amino acids with a predicted molecular weight of 62 kDa. The encoded amino acid sequence of NH1 was 98% identical to human LBP-1b. Because the differences between the published LBP-1b amino acid sequence and the sequence encoded by clone NH1 were mainly confined to one region, we considered whether these were alternately spliced products of the same gene. Therefore, we used RT-PCR to amplify the cDNA regions that were different between NH1 and LBP-1b, using total RNA from JEG-3 and NCI-H295A cells and primers flanking the variant region. Sequencing of two JEG-3 and two NCI-H295A clones showed the sequence found in the NH1 cDNA, suggesting that the NH1 sequence is the correct LBP-1b sequence and that the reported sequence of LBP-1b (26) has a short, framematched, incorrect amino acid sequence (Fig. 2). Hence we refer to the NH1 cDNA sequence as LBP-1b.

The insert in clone NH9 was 4777 bp long, had a long 3'-untranslated region, and had an open reading frame of 1395 bp without an initiating methionine in a Kozak sequence. Therefore we performed 5'-RACE (42) using JEG-3 cell RNA reverse-transcribed into cDNA using the specific primer 9-GSP1 (Table I) followed by nested PCR. The 591-bp double-stranded RACE/PCR product was cloned and sequenced, showing complete sequence overlap with the 5'-end of NH9, indicating that it was an accurate RACE product. After appropriate ligation of this RACE product, the full-length clone NH9 was 4909 bp long and encoded a protein of 479 amino acids with a predicted molecular weight of 55 kDa that had 83% amino acid sequence identity to our corrected sequence of LBP-1b and hence is hereafter termed LBP-9.

The insert in clone NH32 also appeared to be less than 6 x 10^6 unique Agt11 clones with radiolabeled 155/2 DNA. Since we sought to determine whether the expression of clones LBP-1, LBP-9, and LBP-32 was detectable in other human tissues, we performed RT-PCR to amplify the cDNA regions that were different between NH1, NH9, and NH32 and some transcription factors found in the placenta but not in the adrenals (24, 25). Therefore we performed 5'-RACE (42) using JEG-3 cell RNA reverse-transcribed into cDNA using the specific primer 9-GSP1 (Table I) followed by nested PCR. The 591-bp double-stranded RACE/PCR product was cloned and sequenced, showing complete sequence overlap with the 5'-end of NH9, indicating that it was an accurate RACE product. After appropriate ligation of this RACE product, the full-length clone NH9 was 4909 bp long and encoded a protein of 479 amino acids with a predicted molecular weight of 55 kDa that had 83% amino acid sequence identity to our corrected sequence of LBP-1b and hence is hereafter termed LBP-9.

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tal JEG-3 cells, at very low levels in non-steroidogenic cells, and were not detected in human adrenal NCI-H295A cells or in human adrenal tissue. P450c17, a steroidogenic enzyme expressed in the adrenals, gonads, and brain but not the placenta (13, 15) was found in NCI-H295A cells and human adrenal tissue as predicted but not in JEG-3 cells or the non-steroidogenic cell lines, demonstrating the specificity of this RT-PCR experiment.

Binding of LBP-1b, -9, and -32 to the P450scc Promoter—The clones expressing LBP-1b, -9, and -32 had been identified through trans-activation of selectable markers, presumably through specific binding to the incorporated tetramer of the 28155/28131 sequence of the human P450scc promoter. Therefore we sought to determine whether the proteins encoded by these three clones would bind this DNA in vitro. The inserts of each clone were sub-cloned into the multicopy vector YEp90, expressed in yeast, and yeast protein extracts were prepared; these yeast extracts and JEG-3 cell nuclear extracts were then used in electrophoretic mobility shift assays. As shown in Fig. 4, JEG-3 nuclear extracts created two protein-DNA complexes with end-labeled 28155/28131 double-stranded DNA. Complex B appeared to be nonspecific, as it was not competed by a 100-fold molar excess of unlabeled oligonucleotide; by contrast, complex A was readily competed by a 100-fold excess of unlabeled probe. Bkg, background.

Fig. 2. Amino acid sequence alignments of LBP-1b, -9, and -32. The published amino acid sequence of LBP-1b* (26) and our revised sequences of LBP-1b, LBP-9, and LBP-32 were aligned with the MacVector Clustal W program. The amino acids that differ in the two LBP-1b sequences are shown in **bold letters** and *underlined*.

Fig. 3. Tissue distribution of expression of LBP-1b, -9, and -32. Random primers were used to prepare cDNA from 1 μg of total RNA from each cell line or tissue as indicated, and 5 μl of the cDNA were then amplified for 30 cycles to yield fragments of 690, 862, 856, and 820 bp, respectively, for LBP-1b, -9, and -32 and human P450c17; a similar procedure was used to amplify a 502-bp GAPDH cDNA fragment as control. The PCR products were resolved on 1% agarose gel, blotted, and probed with the corresponding LBP-1b, -9, and -32, human P450c17, and GAPDH cDNA as indicated. Only 1/25 of the cDNA product was loaded. Negative control: 5 μl of cDNA synthesis reaction without reverse transcriptase was used as template for PCR amplification. Positive control: 5 ng of plasmid DNA for LBP-1b, -9, and -32, P450c17, or GAPDH, respectively, was used as template for PCR amplification.

Fig. 4. Bandshift and antibody supershift experiments. JEG-3 cell nuclear extract or yeast-expressed LBP-9 or LBP-1b were incubated with radiolabeled 15155/15131 double-stranded probe in the absence (−) or presence (+) of a 100-fold molar excess of the unlabeled probe as a competitor. LBP-1 antibody was added to the protein-DNA complex and incubated for an additional 15 min at 25 °C. LBP-9 appears to be responsible for JEG-3 cell complex A, but complex C formed by yeast-expressed LBP-1b was not detected in JEG-3 cells in this experiment. Bkg, background.
Luciferase activity is expressed relative to the TK32LUC construct without additional upstream sequences. Values represent the average of two independent experiments, each performed in triplicate after normalization for the internal control (renilla luciferase activity of pRL-CMV). Left column, activity of the wild-type or mutant −155/−131/TK32LUC co-transfected with 250 ng of empty pSG5 vector; middle column, co-transfection with 250 ng of pSG5-based vector expressing LBP-1b; right column, co-transfection with 250 ng of pSG5 vector expressing LBP-9. 100 ng of pRL-CMV (Promega) was included in each transfection as internal control.

To determine whether LBP-1b or LBP-9 influenced this basal transcription via the P450scc promoter, we assessed the regulation of human P450scc gene transcription, we assessed the basal transcription from the TK32LUC promoter (Fig. 5A). When the −155/−131 sequence linked to the TK32LUC reporter was co-transfected with increasing amounts of the vector expressing LBP-1b and increasing amounts of vector expressing LBP-9 (5, 50, and 500 ng as indicated) were co-transfected together with 1 μg of −155/−131/TK32LUC reporter, LUC activity was increased threefold, but not complex B. Thus JEG-3 nuclear extract contains a factor that can substitute for the essential role of LBP-1b.

DISCUSSION

The orphan nuclear receptor SF-1 is required for the production of steroid hormones in the adrenals and gonads but not in the placenta, brain, or other “extra-glandular” tissues. SF-1-independent transcription of P450scx has been demonstrated in the human placenta (24) and rat brain (23), prompting a search for factors that can substitute for the essential role of SF-1. Some candidate factors have been identified. SF-1-independent transcription of the rat gene for steroid 17α-hydroxylase (P450c17) can be regulated by two factors operationally termed Stf-IT-1 and Stf-IT-2 (45) and by ku autoantigen (46). Stf-IT-1 has recently been identified as the oncoprotein SET (47), a factor not previously known to be a transcriptional regulator. Thus it appears that an unexpectedly broad array of proteins can regulate the transcription of the genes for the steroidogenic enzymes. We have now added factors related to the LBP group of transcription factors to this growing family. The LBP family of transcription factors was initially characterized as a single cellular factor that bound to two different sites in the HIV, type I promoter (48–51). Later work showed that there are two related LBP genes, each of which encodes
two alternately spliced transcripts, so that LBP-1a and LBP-1b arise from one gene, and LBP-1c and LBP-1d arise from a second gene (26). LBP-1c is identical to the α-globin transcription factor CP2 (52), and proteins in the LBP family are all related to Elf-1/NTF-1, which is essential for Drosophila embryogenesis (43, 44). Thus the LBP proteins represent an evolutionary ancient family of transcription factors that participate in development. Sequences related to retrovirus are found throughout the human genome (53, 54) and frequently regulate expression of adjacent cellular genes, especially those expressed in the placenta (55, 56). We find no evidence for the insertion of a retroviral regulatory sequence as has been described for placentational expression of human plieotropin (57), although sequences similar to −155/−131 are not found in the bovine or rat P450scc promoters. Hence, whereas it may not be surprising to find LBP-related proteins participating in transcriptional regulation in the placenta, to our knowledge this is the first report of an action of LBP-1b on an endogenous, non-viral promoter or of any LBP-related factor participating in the transcriptional regulation of a gene involved in steroid hormone biosynthesis.

Our mobility shift data show that LBP-1b and LBP-9 bind to the −151/−131 segment of the human P450scc promoter. In comparison with our previous studies of the P450scc promoter in JEG-3 cells, it appears that LBP-9 forms what was previously called Complex IV, LBP-1b forms a previously undetected complex, and neither LBP-1b or LBP-9 forms what was previously called Complex VII (25). Our previous data suggested that a 55-kDa protein forming Complex VII was required for basal, placental-specific expression of P450scc, whereas Complex IV appeared to be involved in modulating P450scc expression (25). Our present data are consistent with those earlier observations, suggesting that LBP-1b and LBP-9, respectively, amplify or diminish the level of expression initiated by other factors. This action of LBP-related proteins to function as quantitative “volume controls” rather than as basal “on/off switches” is consistent with their similar action to modulate the level of HIV, type I transcription and is similar to the reciprocal action of Sp1 and Sp3 on numerous genes (58, 59) including adenral expression of bovine P450scc (60). Thus LBP proteins are important newly discovered modulators of placental P450scc expression, but other, as yet uncharacterized proteins are probably required for basal placental-specific expression of this gene.

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