AP-2γ and the Homeodomain Protein Distal-less 3 Are Required for Placental-specific Expression of the Murine 3β-Hydroxysteroid Dehydrogenase VI Gene, Hsd3b6*

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The enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) is essential for the biosynthesis of all active steroid hormones. It exists as multiple isoforms in humans and rodents, each the product of a distinct gene. Human 3β-HSD I in placenta is essential for placental progesterone biosynthesis and thus is essential for the maintenance of pregnancy. The murine ortholog, 3β-HSD VI, is the only isoform expressed in giant trophoblast cells during the first half of mouse pregnancy. This study was designed to identify the cis-acting element(s) and the associated transcription factors required for trophoblast-specific expression of 3β-HSD VI. Transfection studies in placental and non-placental cells identified a novel 66-bp trophoblast-specific enhancer element located between −2896 and −2831 of the 3β-HSD VI promoter. DNase protection analysis of the enhancer element identified three trophoblast-specific binding sites, FPI, FPII, and FPIII. Electrophoretic mobility shift assays with oligonucleotides representing the protected sequences, FPI and FPIII, and nuclear extracts isolated from human JEG-3 cells and from mouse trophoblast cells, demonstrated the same binding pattern that was distinct from the binding pattern with mouse Leydig cell nuclear proteins. Further electrophoretic mobility shift assays identified AP-2γ and the homeodomain protein, Dlx 3, as the transcription factors that specifically bind to FPI and FPIII, respectively. Site-specific mutations in each of the binding sites eliminated enhancer activity indicating that AP-2γ and Dlx 3, together with an additional transcription factor(s) that are conserved between humans and mice, are required for trophoblast-specific expression of 3β-HSD VI.

The enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) is essential for the biosynthesis of all active steroid hormones: the adrenal steroid hormones, cortisol, corticosterone, and aldosterone; the testicular steroid hormone, testosterone; and the ovarian and placental hormones, progesterone and estradiol. The 3β-HSD enzyme exists as multiple isoforms in humans and rodents, each the product of a distinct gene (1). To date, six isoforms have been identified in mouse and two in human. These isoforms are expressed in a tissue- and temporal-specific manner (1–5). In the mouse, the two major isoforms involved in steroid hormone biosynthesis are 3β-HSD I and 3β-HSD VI. 3β-HSD I is the major or only isoform expressed in gonads and adrenal glands, whereas 3β-HSD VI is the only isoform expressed in giant trophoblast cells during mid-pregnancy (3). The orthologous isoforms in human are 3β-HSD II, the isoform expressed in the gonads and adrenal glands (6), and human 3β-HSD I (7, 8), the only isoform expressed in placenta throughout pregnancy. The expression of human 3β-HSD I in placenta is essential for placental progesterone biosynthesis and, thus, is vital for maintenance of pregnancy (9). Consistent with the role of human 3β-HSD I in placental progesterone biosynthesis, we have shown that mouse 3β-HSD VI is required for progesterone biosynthesis in giant trophoblast cells between embryonic day (E) 9.5 and E10.5 (10).

Progesterone biosynthesis from cholesterol requires the activity of two enzymes, cholesterol side chain cleavage cytochrome P450 (P450sc) which catalyzes the conversion of cholesterol to pregnenolone and 3β-HSD which catalyzes the conversion of pregnenolone to progesterone. This latter step is brought about by distinct tissue-specific isoforms of 3β-HSD. Previous studies designed to identify placental-specific regulatory elements in the human placental-specific 3β-HSD promoter were unsuccessful (8). Moreover, identity of transcription factors essential for placental-specific expression of P450sc remains to be resolved (11). Therefore, the question is whether there is a unique tissue-specific transcription factor or factors required for the expression of the trophoblast-specific isoform of 3β-HSD as well as the other enzyme required for progesterone biosynthesis, P450sc, in human placenta and mouse giant trophoblast cells.

In the present study, we identify a 66-bp trophoblast-specific enhancer element located between −2896 and −2831 of the transcription start site of the murine 3β-HSD VI promoter and demonstrate the requirement for two transcription factors, AP-2γ, and the homeodomain protein, Distal-less 3 (Dlx 3), in determining trophoblast-specific expression of the 3β-HSD VI.

P450; AP-2, activator protein-2; Dlx 3, distal-less 3; TEF, transcription enhancer factor; JRE, junctional regulatory element; hCG, human chorionic gonadotropin; RACE, rapid amplification of cDNA ends; EMSA, electrophoretic mobility shift assay; tk, thymidine kinase; LUC, luciferase; SF-1, steroidogenic factor 1.

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The nucleotide sequence(s) reported in this paper for the mouse 3β-HSD VI gene has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AY046511 (3256 bp promoter and exon 1) and AY046512 (1792 bp complete cDNA).

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The abbreviations used are: 3β-HSD, 3β-hydroxysteroid dehydrogenase/isomerase; P450sc, cholesterol side chain cleavage cytochrome
AP2γ and Dlx3 Determine Placenta-specific Expression of \( \beta \)-HSD

doxic gene. Dlx 3 is a member of a family comprising at least six Dlx genes sharing a homeobox sequence similar to that found in the Drosophila Distal-less gene (12). Dlx proteins are transcriptional activators which play an essential role during vertebrate development (12). AP-2γ is a member of a family of three closely related and evolutionarily conserved sequence-specific DNA-binding proteins which include AP-2α, AP-2β, and AP-2γ (13). Dlx 3 and AP-2γ are expressed in both murine and human placental trophoblast cells and are required for the placental-specific expression of a number of genes (14–18). Our studies demonstrate that these two transcription factors regulating the trophoblast-specific expression of the steroidogenic enzyme, \( \beta \)-HSD VI, are conserved in both murine and human trophoblast cells. This is the first report on the identification of at least two of the transcription factors required for placental-specific expression of \( \beta \)-HSD in humans and mice.

**EXPERIMENTAL PROCEDURES**

**Isolation of Genomic Clones**—A \( \lambda \) phage 129/STv mouse genomic library from Stratagene (La Jolla, CA) was screened with an 180-bp probe labeled with \( [\alpha-\text{P}] \) dCTP, which includes 18 bp of coding region and 9 bp of untranslated region. PCR-amplified DNA (3). Prehybridization and hybridization were performed as described (19). Two clones (402 and 602) were identified to represent \( \beta \)-HSD VI. The clones were subjected to restriction enzyme and Southern blot analysis using exon oligonucleotide probes (exon 2, 5′-CCAGAGGT- GTCCAGTGG-3′; exon 3, 5′-GACATGATGGTCTG-3′; exon 4, 5′- AGGAAAGCTCAGATTTCCA-3′).

**Preparation of Giant Trophoblast Cells**—Timed pregnant C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were killed by CO\(_2\) followed by cervical dislocation at E9.5 and E10.5, and the uterine horns were removed and placed in phosphate-buffered saline for further isolation of giant trophoblast cells as described (5). E9.5 and E10.5 implantation sites were separated from the myometrium and the embryos were removed and placed in phosphate-buffered saline for further isolation of giant trophoblast cells as described (5).

**Preparation of RNA**—Messenger RNA was isolated from E10.5 mouse giant trophoblast cells as described (5). E9.5 and E10.5 implantation sites were separated from the myometrium and the embryos were removed and placed in phosphate-buffered saline for further isolation of giant trophoblast cells as described (5). E9.5 and E10.5 implantation sites were separated from the myometrium and the embryos were removed and placed in phosphate-buffered saline for further isolation of giant trophoblast cells as described (5).

**Preparation of Nuclear Extracts**—Crude nuclear extracts from JEG-3 and MA-10 cells were prepared as described (24). Nuclear extracts from E10.5 giant trophoblast cells and E115.5 placental tissues were isolated using the 1-h minipreparation techniques (25). Total protein was quantitated by Bradford assay and normalized against extraction buffer. The extracts were aliquoted and stored at −70 °C until use for footprinting or EMSA.

**DNase I Footprinting Assay**—DNase I footprinting was carried out using Promega’s core footprinting kit with modifications. To generate the probe, the 120-bp fragment (–2916/–2800) amplified from PCR was cloned into the pBluescript-KS vector and subjected to further analysis to identify the location of exon 1 and to establish the start site of transcription.

**RAPID AMPLIFICATION OF 5′-CDNA ENDS (5′-RACE)—5′-RACE was carried out using a Marathon cDNA library from CLONTech (Palo Alto, CA) prepared from a 7-day pregnant mouse implantation site.** The sequences of some oligonucleotides for EMSAs are shown in Table I.

**Electrophoretic Mobility Shift Assay (EMSA)**—DNase I footprinting was carried out using Promega’s core footprinting kit with modifications. To generate the probe, the 120-bp fragment (–2916/–2800) amplified from PCR was cloned into the pBluescript-KS vector and subjected to further analysis to identify the location of exon 1 and to establish the start site of transcription.

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cells, E9.5 or E10.5 giant trophoblast cells, or adrenal glands or testes from 50-day-old mice were prepared by homogenization in extraction buffer followed by centrifugation as described previously (3). The supernatant was subjected to SDS-PAGE and Western blot analysis. Membranes were first incubated with antiserum generated against human placental 3β-HSD and then with the horseradish peroxidase-labeled secondary antibody and exposed using the Enhanced Chemiluminescence kit (Amersham Biosciences, Inc., Arlington Heights, IL).

In Situ Hybridization—Cryosections (8 μm) of implantation sites from E9.5 and E10.5 were subjected to in situ hybridization as described (22) using a 359-bp 5′-labeled sense or antisense cRNA probe representing 45 bp from the 3′ end of the coding region and 314 bp from the 3′-untranslated region of 3β-HSD VI cDNA (3). Slides were exposed at 4 °C and developed after 4 days. The slides were stained with hematoxylin and eosin and examined under light microscopy with bright- and dark-field illumination.

RESULTS

Genomic Structure of the 3β-HSD VI Gene—To characterize the 3β-HSD VI gene, a λ phage genomic DNA library was screened with a 3β-HSD VI cDNA probe. Two phage clones, 402 and 602, were identified as encompassing the entire 3β-HSD VI gene. Restriction mapping and Southern blot analysis revealed that clone 402 contains ~10 kb of 5′-flanking region, and exon 2 through exon 4 ending at the SacI site in the 3′-untranslated region (3), whereas clone 602 contains a portion of intron 2, exon 3, exon 4, and 9 kb 3′-flanking region (Fig. 1A).

Identification of Exon 1 Sequence and Mapping of the Transcription Start Site of the 3β-HSD VI Gene—5′-RACE analysis was performed to identify the exon 1 sequence. The 3′ 340-bp sequences from the 5′-RACE PCR product matched with known cDNA sequences including 206 bp of exon 2 and 134 bp of exon 3 (3). To confirm that the new sequence from the 5′-RACE represents the exon 1 sequence, reverse transcriptase-PCR was performed using RNA isolated from E10.5 mouse giant trophoblast cells and from mouse testicular tissues with a forward primer designed from the 5′-RACE exon 1 sequence and a backward primer designed from the known sequence of exon 2 (3). A fragment of the expected size was obtained. Thus, exon 1 was identified and the size of intron 1 was established to be 3.1 kb, which differs from human 3β-HSD I or mouse 3β-HSD I (1).

The transcription start site for 3β-HSD VI was confirmed by primer extension (Fig. 1B). Using mRNA from adult mouse testes and from E10.5 mouse giant trophoblast cells, a single transcription start site was identified at a guanosine residue 315 bp 5′ of the translation initiation codon in exon 2 which defines the size of exon 1 as 252 bp.

Mouse Trophoblast-specific Expression of 3β-HSD VI mRNA and Protein—We previously reported the expression of 3β-HSD mRNA and protein in mouse giant trophoblast cells at E9.5 (5). However, the earlier study did not use isoform-specific probes for the identification of 3β-HSD. To determine the isoform-specific expression of 3β-HSD VI, in situ hybridization of sections of E9.5 and E10.5 implantation sites were analyzed using a 3β-HSD VI-specific antisense probe (see “Experimental Procedures”). Fig. 2A shows exclusive expression of 3β-HSD VI mRNA in giant trophoblast cells at E9.5 and E10.5 with considerably greater expression at E10.5. No expression of 3β-HSD VI mRNA was observed in decidua or embryo. The increase in 3β-HSD VI mRNA in giant trophoblast cells at E10.5 is accompanied by a parallel increase in 3β-HSD VI protein as determined by Western blot analysis (Fig. 2B).

SF-1 is Not Required for Expression of 3β-HSD VI in JEG-3 Cells—In gonads and adrenal glands expression of steroidogenic enzymes is dependent on steroidogenic factor 1 (SF-1) (27, 28). SF-1 null mice develop normal placental trophoblast cells that express steroidogenic enzymes (29) indicating that this factor is not involved in placental-specific expression of steroidogenic enzymes. Our studies (Table II) demonstrate that the transcriptional activity of the murine 3β-HSD VI proximal promoter transfected into JEG-3 cells that do not contain SF-1, is 22-fold greater than the transcriptional activity of the gonadal- and adrenal-specific 3β-HSD I promoter. Furthermore, co-transfection with an SF-1 expression vector resulted in a dose-dependent increase in 3β-HSD I transcriptional activity, but had no effect on 3β-HSD VI transcriptional activity. Thus, SF-1 is not required for expression of the 3β-HSD VI enzyme in the placenta.

Promoter Activity and Cell-specific Expression of the 5′-Flanking Region of the 3β-HSD VI Gene—Because of the lack of a mouse trophoblast cell line and the difficulty in obtaining mouse primary giant trophoblast cells for transfection studies, the human placental choriocarcinoma cell line, JEG-3, was chosen for the transfection studies described herein. JEG-3 cells express human 3β-HSD I (30), the tissue-specific ortholog to mouse 3β-HSD VI. To establish trophoblast-specific expression, nonplacental cell lines, both mouse Leydig tumor cells (MA-10) and monkey kidney cells (COS-7), were also used for
giant trophoblast cells at E9.5 and E10.5. A and hybridized with 35S-labeled antisense RNA to AP-2γ and Dlx3 Determine Placenta-specific Expression of 3β-HSD

### FIG. 2. Expression of 3β-HSD VI mRNA and protein in mouse giant trophoblast cells at E9.5 and E10.5. A, implantation sites from E9.5 (panels A and B) and E10.5 (panels C and D) were sectioned and hybridized with 35S-labeled antisense RNA to AP-2γ and Dlx3. Panels A and B are light and dark field views of E9.5. The dark field exposure shows hybridization only in giant trophoblast cells surrounding the embryonic cavity. Panels C and D are light and dark field views of E10.5. Panel c shows intense silver grains in the light field exposure exclusively in giant trophoblast cells. The considerably greater expression of 3β-HSD VI in giant trophoblast cells in E10.5 compared with E9.5 is observed in the dark field exposure (compare B and D). Bar, 10 μm. Data with the sense probe not shown. B, Western blot analysis of 3β-HSD proteins was carried out with protein extracts from E9.5 and E10.5 giant trophoblast cells (10 μg of protein); rVI, pCMV5.3-3β-HSD VI transfected COS cells; and rI, pCMV5.3-3β-HSD I transfected COS cells (30 μg of protein, each; T, testis from 50-day-old mouse (75 μg of protein); A, adrenal from 50-day-old mouse (1.4 μg of protein); O, ovary from 50-day-old mouse (1.5 μg of protein).

### TABLE II

**Effect of SF-1 on transcriptional activity of the mouse 3β-HSD I and VI promoters in JEG-3 cells**

| SF-1 | 3β-HSD I | 3β-HSD VI |
|------|----------|-----------|
| ng   |          |           |
| 0    | 0.7      | 15.8      |
| 200  | 3        |           |
| 400  | 4.1      |           |
| 800  | 4.9      | 17.4      |
| 1600 | 5.8      |           |

*Luciferase activity relative to pGL3-Basic.*

the promoter and enhancer analyses of the mouse 3β-HSD VI gene.

To characterize the promoter sequences involved in transcriptional regulation of the 3β-HSD VI gene in trophoblast cells, a series of 5’ deletions of the 3β-HSD VI gene, spanning from −4700 to −40 5’ of exon 1 (Fig. 3), were subcloned into a promoterless luciferase reporter vector, pA3LUC, and transiently transfected into JEG-3, MA-10, and COS-7 cells. Luciferase activity was normalized to β-galactosidase activity and expressed relative to the promoterless vector pA3LUC, whose activity is set as 1. Each value represents the mean ± S.E. of three separate transfections, each performed in triplicate. The inset illustrates the relative luciferase activity between −40 and −91 in the three cell lines (×20).

**Fig. 3. Transcriptional activity of the 3β-HSD VI promoter.** A series of 5’ deletions of the 3β-HSD VI promoter-luciferase reporter constructs, as indicated on the top, were transiently transfected into JEG-3 (JEG), MA-10 (MA), and COS-7 (COS) cells. Luciferase activity was normalized to β-galactosidase activity and expressed relative to the promoterless vector pA3LUC, whose activity is set as 1. Each value represents the mean ± S.E. of three separate transfections, each performed in triplicate. The inset illustrates the relative luciferase activity between −40 and −91 in the three cell lines (×20).
of the heterologous tk promoter contains the JEG cell-specific transcriptional element. The fragment comprising the sequence between −3004 and −1989 was subcloned in either sense or antisense orientation 5′ of the heterologous tk promoter-driven luciferase reporter vector (TK164LUC). The reporter constructs were transfected into the three cell lines and luciferase activity of each construct was expressed relative to the vector TK164LUC. Each value represents the average plus the range of two separate transfections, each performed in triplicate.

−3004 (Fig. 3), this 1700-bp fragment was also subcloned 5′ of the tk promoter and transfected into JEG-3 cells. No increase in the promoter activity was observed with this fragment (data not shown).

To further define the regulatory domains within the −3004/−1989 fragment of the 3β-HSD VI promoter, subsequent deletions were made and subcloned 5′ of the tk promoter. Fig. 5A shows the results of the deletion constructs transfected into JEG-3, MA-10, and COS-7 cells. No enhancer activity with any of the deletion constructs was observed in MA-10 or COS-7 cells. In contrast, in JEG-3 cells, deletions of the 3′ sequence from −1989 to −2500, did not change enhancer activity compared with the −3004/−1989 fragment. Further deletion from −2500 to −2723 displayed similar enhancer activity. The data suggest that the 282-bp fragment between −3004 and −2723 contains the trophoblast-specific enhancer element.

To characterize the minimal enhancer region, additional 5′ deletions within −3004/−2723 were made and subcloned 5′ of the tk heterologous promoter. The transfection results are shown in Fig. 5B. When the 5′ sequence was deleted from −3004 to −2896, the activity remained the same as the −3004/−2723 construct. However, further 5′ deletion to −2830 reduced transcriptional activity by 73%, suggesting that the enhancer element is located between −2896 and −2830. To confirm that this 66-bp fragment between −2896 and −2831 has trophoblast-specific enhancer activity, it was directly subcloned 5′ of the tk heterologous promoter. Surprisingly, this 66-bp fragment increased tk promoter activity 5-fold relative to the −3004/−2723 fragment (Fig. 5B). This finding not only provides conclusive evidence that the sequence (−2896/−2831) contains the enhancer element, but also suggests that an inhibitory element is located between −2896 and −2831. This 108-bp fragment from the −3004/−2723 region resulted in a significant increase in enhancer activity. Further 3′ or 5′ deletions of the −2896/−2831 fragment resulted in complete loss of the enhancer activity. The data suggest that the entire sequence between −2896 and −2831 is required for trophoblast-specific enhancer activity.

Potential Transcription Factor-binding Sites—To determine the corresponding protein-binding nucleotides within the 66-bp enhancer, DNase I footprinting assay was performed using an end-labeled fragment containing the sequence −2916/−2872 (including the 66-bp fragment identified above as the trophoblast-specific enhancer element). Three protected regions, FPI, FPII, and FPIII, were subcloned 5′ of the tk promoter in the TK164LUC vector and transfected into the three cell lines. Luciferase activity of each construct is expressed relative to the vector TK164LUC. Further 5′ or 3′ deletion mutants were constructed using the 282-bp fragment (−3004/−2723) identified in A as a backbone. The constructs were transfected into JEG-3 cells. Luciferase activity of each construct is expressed relative to the −3004/−2723 construct which displayed full enhancer activity. The activity of the −3004/−2723 construct was set at 1. Each value represents the average plus the range of two separate transfections, each performed in triplicate.

To determine whether the sequence representing each footprint interacts with the predicted factor present in trophoblast cells, EMSAs were performed using double-stranded oligonucleotides corresponding to each of these footprints as probes.
and nuclear extracts isolated from JEG-3 cells, E10.5 giant trophoblast cells, or MA-10 cells. The probe representing FPI or the probe representing FPIII, each gave rise to a specific DNA-protein complex. No binding activity was observed with the probe representing FPII (data not shown).

Identification of AP-2γ as the Trophoblast-specific Transcription Factor That Binds to FPI—The FPII element forms a specific DNA-protein complex (complex I) with identical mobility with nuclear extracts of either JEG-3 or giant trophoblast cells (Fig. 7B, lanes 2 and 5) which was not observed with the nuclear extract of MA-10 cells (lanes 8–10). Five hundred-fold molar excess of unlabeled FPII competed for binding of the labeled FPII probe (Fig. 7B, lanes 3 and 6), whereas an unlabeled FPII oligonucleotide containing mutations within the potential Ker1 site (mFPII) could not compete (lanes 4 and 7). This observation suggests that the FPII element may interact with the Ker1-binding protein. To test this hypothesis, labeled probes representing FPI and Ker1 were subjected to EMSAs using JEG-3 cell nuclear extract. Fig. 7C demonstrates that each of these probes yielded a DNA-protein complex with identical mobility and, furthermore, 500-fold molar excess of either unlabeled FPII or unlabeled Ker1 competed for binding of each of the probes. Binding of the JEG-3 nuclear protein to the Ker1 probe is greater than to the FPI probe (Fig. 7C, lanes 1 and 4) and competition with unlabeled FPI for binding to the Ker1 probe is somewhat less than the competition observed with unlabeled Ker1 (Fig. 7C, lanes 5 and 6). These results suggest that FPI and Ker1 bind the same trophoblast nuclear protein with different affinities.

The protein that specifically binds to Ker1 has previously been identified as AP-2γ (31). The AP-2 family consists of three distinct proteins, AP-2α, AP-2β, and AP-2γ. AP-2γ is the predominant AP-2 family member expressed in mouse placenta and giant trophoblast cells (15). Both AP-2α and AP-2γ are expressed in JEG-3 cells and in human placenta (16, 17). To identify whether the protein that specifically binds to FPI is a member of the AP-2 transcription factor family and, furthermore, to distinguish between AP-2α and AP-2γ, EMSAs were performed with the FPI probe and nuclear extracts of JEG-3 cells, of E10.5 giant trophoblast cells, or of E15.5 mouse placentas in the presence or absence of polyclonal antisera to AP-2α or AP-2γ. As shown in Fig. 8A, mouse placental nuclear proteins formed a DNA-protein complex (lane 4) with identical mobility to complex I as shown in Fig. 7 for JEG-3 cell and mouse giant trophoblast cell nuclear extracts. This complex was displaced by the addition of antiserum to AP-2γ (lanes 3, 6, and 10) but not by the addition of AP-2α antiserum (lanes 7 and 11) or by normal rabbit serum (NRS, lane 8). The results shown in Fig. 8B illustrate that the JEG-3 cell nuclear protein that specifically binds to the Ker1 probe also is AP-2γ and not AP-2α. The data presented in Fig. 8 establish that the human and murine trophoblast-specific protein that interacts with FPI is the transcription factor AP-2γ.

Identification of Distal-less 3 (Dlx3) as the Trophoblast-specific Transcription Factor That Binds to FPIII—As described above, a database search for transcription factors involved in producing FPIII in the DNase I protection assay (Fig. 6) indicated two potential binding sites, GATA or Nkx2-5. These two potential sites overlapped. To characterize binding of JEG-3 and giant trophoblast nuclear proteins to the FPIII sequence, EMSAs were performed using a radiolabeled probe comprising the entire protected sequence as well as an FPIII mutant as competitor (Fig. 9A). A specific DNA-protein complex (complex III) with identical mobility was observed with nuclear extracts of both JEG-3 and giant trophoblast cells (Fig. 9A, lanes 2 and 5), which was distinct from the complex formed with MA-10 cell nuclear extract (Fig. 9A, lane 8). This finding indicates that the FPIII-binding nuclear protein is trophoblast-specific. The competition assay with mFPIII which contains mutations comprising the entire GATA site, as well as the 5′ nucleotides of the Nkx2-5-binding site, resulted in loss of competition with the FPIII probe (Fig. 9A, lanes 4 and 7). To delineate the recognition sequence for the trophoblast nuclear protein within the FPIII element, a series of FPIII oligonucleotides with sequential double mutations were tested for their ability to compete with the FPIII probe in an EMSA (Fig. 9B). As illustrated in Fig. 9B, oligonucleotides containing mutations involving the nucleotides as represented by m3, m4, m5, m7, m8, and m9 lost or markedly reduced the capacity of these mutants to compete with the wild type FPIII probe indicating that TAATTG from −2848 to −2843 was the critical binding site for the FPIII-binding protein. The TAATTG sequence is identical to the binding site for the murine homeodomain protein, Nkx-2.5, or the current nomenclature, Nkx2-5 (34, 35), and to the binding site of another homeodomain protein Dlx 3. Roberson et al. (18) recently demonstrated that Dlx 3 is the transcription factor that binds to the junctional regulatory element (JRE) of the human glycoprotein hormone α (hCGα) subunit gene and is required for basal placental-specific expression of this gene (18) (Fig. 10A). This observation suggests that Dlx 3 may be the human and murine trophoblast-specific nuclear protein that binds to FPIII. To examine whether Dlx 3 is the FPIII-binding protein, EMSAs were carried out with radiolabeled probes FPIII and JRE (18) and JEG-3 cell nuclear extract (Fig. 10B). The FPIII-protein complex (lane 1) displayed identical mobility to the JRE-protein complex with JEG-3 cell nuclear extract (lane 8). In addition, both unlabeled FPIII and JRE oligonucleotides had similar self- and cross-competition with each of the probes for binding to the trophoblast nuclear protein (lanes 2–7 and 9–14). These results are consistent with Dlx 3 being the transcription factor that forms complex III. To establish that the trophoblast-specific protein that binds to FPIII is Dlx 3, EMSAs were performed with the FPIII probe and the JRE probe, nuclear extracts of JEG-3 cells (Fig. 10C) or of mouse placentas,
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Fig. 7. Specific binding activity of FPI with trophoblast cell nuclear proteins. A, sequences in the oligonucleotides representing FPI, Ker1, an AP-2 binding element in the human k14 keratin gene (31) and the mutated FPI (mFPI) which represents a five-nucleotide substitution mutation in the potential Ker1 site within the FPI sequence. The consensus nucleotides for AP-2 binding are illustrated in bold. B, EMSA with 10 μg of nuclear protein isolated from JEG-3 cells, or E10.5 mouse giant trophoblast cells (GTC), or MA-10 cells and radiolabeled probe FPI. Lane 1 is free of nuclear extract. C, EMSA with JEG-3 nuclear extract and with probes FPI or Ker1.

Fig. 8. Identification of the FPI-binding protein in trophoblast cells as AP-2γ. EMSA was performed with 10 μg of nuclear extracts of JEG-3 cells, E15.5 mouse placenta, or E10.5 mouse giant trophoblast cells (GTC) and specific antisera to AP-2α or AP-2γ or normal rabbit antiserum (NRS). A, FPI as the probe (see Fig. 7A). Lanes 2 and 5 represent 500-fold molar excess of cold competitor FPI. B, Ker as the probe (see Fig. 7A). Lane 2 represents 500-fold molar excess of Ker1.

DISCUSSION

Progesterone biosynthesis in the human placenta is essential for maintenance of pregnancy (9). Although progesterone is required for maintenance of pregnancy in the mouse, the source has not been unequivocally established. The biosynthesis of progesterone from cholesterol requires two steroidogenic enzymes, P450scc and 3β-HSD. P450scc is the product of a single gene that is expressed in human gonads and adrenal glands (36), the human placenta (37), mouse decidua, and giant trophoblast cells (5). Unlike P450scc, 3β-HSD exists in multiple isoforms and the isoform expressed in human and mouse trophoblast cells is distinct from the isoform expressed in the gonads and adrenal glands, with one exception, mature mouse Leydig cells express 3β-HSD VI in addition to the major gonadal isoform, murine 3β-HSD I (Fig. 2B and Refs. 3 and 4). Both P450scc and 3β-HSD VI are expressed in a tissue- and temporal-specific manner during the first half of mouse pregnancy (Fig. 2) (5). Several investigations have been undertaken during the past few years in the search for a factor or factors which determine placental-specific expression of steroidogenic enzymes. Guerin et al. (8) were unsuccessful in their attempt to identify a placental-specific element in the promoter of human 3β-HSD I. They did identify a strong positive regulatory element in the first intron which functioned in a ubiquitous man-

All Three FP Elements within the 66-bp Enhancer Are Essential for 3β-HSD VI Transcriptional Activity in JEG-3 Trophoblast Cells—To determine whether the binding sites within each FP are functional, site-directed mutations were introduced into the heterologous enhancer-tk construct, 2866/2831 (Fig. 5D). To determine the requirement for the AP-2γ-binding site identified in FPI, the mutation, TAGGCA → GGTACC (mFPI), which had been shown to disrupt binding of AP-2γ was introduced (Fig. 7A). For disruption of the Dlx 3-binding site in FPIII, TGATAA → AAGCTT (mFPIII) was introduced into the enhancer construct and the potential TEF-binding site in FPII was mutated (GCATTC → AAGCTT) (Table I). Transfection into JEG-3 cells of each of the mutated 66-bp enhancer-tk constructs resulted in complete loss of enhancer activity (Fig. 11). These results indicate that all three proteins, AP-2γ, FPII-binding protein, and Dlx 3 homeodomain protein, are essential for determining trophoblast-specific enhancer activity. The results do not eliminate the possible requirement for additional proteins.

Protein interactions with trophoblast cell nuclear extracts were determined by supershift or disruption of the DNA-protein complex (Fig. 10, C and D). Like AP-2γ, Dlx 3 and Nkx2-5 proteins specifically bind to the JEG-3 nuclear extract containing an Nkx2-5-binding site yielding a DNA-protein complex with identical mobility to the DNA-protein complex formed with the FPIII probe (data not shown). The addition of antisera to Nkx2-5 did not result in either a supershift or disruption of the DNA-protein complex (Fig. 10, C and D). Lane 1 represents 500-fold molar excess of cold competitor FPI. B, Ker as the probe (see Fig. 7A). Lane 2 represents 500-fold molar excess of Ker1.
expression of murine 3\(\beta\)-HSD VI is the same in humans and mice. Human placenta and JEG-3 cells express both AP-2\(\alpha\) and AP-2\(\gamma\) (15, 16), while AP-2\(\gamma\) is the predominant member of the AP-2 family expressed in mouse trophoblast cells and its expression is restricted to the trophoblast lineage (15). Shi and Kellem (15) suggest that AP-2\(\gamma\) may be one of the key transcription factors regulating gene expression in trophoblast cells. Thus, the finding in this study demonstrating that AP-2\(\gamma\) is required for trophoblast-specific expression of 3\(\beta\)-HSD identifies one of the important target genes whose product is required for progesterone biosynthesis and thus essential for maintenance of pregnancy.

The protein that specifically binds to FP III was identified as the homeodomain protein, Dlx 3. Dlx 3 was recently identified as a placenta-specific transcriptional activator of the hCG\(\alpha\) subunit gene (18). This transcription factor is expressed in human placenta as well as in JEG-3 cells (18) and in the trophoblast cell lineage that forms the placenta in mice (14). Targeted deletion of the Dlx 3 gene resulted in embryonic death between E9.5 and E10.5 due to placental defects (14). Whether mutation in the human Dlx 3 gene would lead to loss of the fetus during human pregnancy is not known at present. Immunocytochemical studies on sections of first trimester human chorionic villus samples showed that expression of Dlx 3 was found primarily in the trophoblast cell layer surrounding the villus and expression was restricted to the nucleus (18).

EMSA studies using nuclear extracts of either JEG-3 or giant trophoblast cells and an oligonucleotide representing the FPII sequence identified by DNase I protection analysis did not result in the formation of a DNA-protein complex, suggesting that the binding of a nuclear protein to FPII requires the prior binding of either AP-2\(\gamma\) or Dlx 3 or both. Although we were unable to show binding of a nuclear protein to the FPII oligonucleotide within the 66-bp enhancer element, binding of a specific protein is essential for trophoblast-specific transcriptional activity as demonstrated by the loss of transcriptional activity when site-directed mutations were introduced separately into each of the FP-binding sites of the heterologous enhancer-tk construct (Fig. 11). This study also demonstrates that AP-2\(\gamma\), FPII-binding protein, and Dlx 3 are required for trophoblast-specific expression of the murine 3\(\beta\)-HSD VI gene.

A GenBank\textsuperscript{TM} search of the human 3\(\beta\)-HSD I promoter sequence (accession number AL121995) identified an AP-2-binding site at \(-2857\) to \(-2848\) identical to the FPI core-binding site of murine 3\(\beta\)-HSD VI and a binding site for Dlx 3 at \(-2495\) to \(-2489\) identical to the FPIII-binding site of murine 3\(\beta\)-HSD VI (Table III). The placental-specific function of these potential binding sites for AP-2\(\gamma\) and Dlx 3 in the human 3\(\beta\)-HSD I promoter needs to be established.

The identification of a trophoblast-specific enhancer in the murine 3\(\beta\)-HSD VI promoter and the demonstration that the two transcription factors, AP-2\(\gamma\) and Dlx 3, which are required for the cell-specific expression of this gene, are expressed in both human placenta and mouse trophoblast cells, and the identification of binding sites for these two transcription factors in the human 3\(\beta\)-HSD I promoter, suggests that AP-2\(\gamma\) and Dlx 3 are the placental nuclear proteins that determine the cell-specific expression of human 3\(\beta\)-HSD I whose product is required for placental progesterone production. It is of interest to note that these two transcription factors have been shown to be required for placental-specific expression of the hCG\(\alpha\) subunit gene (16, 18). Another similarity between the cell-specific expression of the hCG\(\alpha\) subunit and the expression of 3\(\beta\)-HSD is the fact that the nuclear factor SF-1 determines pituitary-specific expression of the LH/hCG subunit and adrenal- and gonad-specific expression of 3\(\beta\)-HSD (27). It is intriguing to
speculate that AP-2γ and Dlx3 are the SF-1 analogous nuclear transcription factors that coordinate trophoblast-specific expression of the placental hormones and enzymes required for maintenance of pregnancy.
AP-2γ and the Homeodomain Protein Distal-less 3 Are Required for Placental-specific Expression of the Murine 3 β-Hydroxysteroid Dehydrogenase VI Gene, \( \text{Hsd3b6} \)

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