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# Transcription Enhancer Factor-5 and a GATA-Like Protein Determine Placental-Specific Expression of the Type I Human 3β-Hydroxysteroid Dehydrogenase Gene, *HSD3B1*

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## Abstract

The enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3βHSD) is required for the biosynthesis of all active steroid hormones. It exists as multiple isoforms in humans and rodents, each a product of a distinct gene. Two isoforms, 3βHSD I and II, are expressed in a tissue-specific manner in humans. 3βHSD I is the only isoform expressed in the placenta, where it is required for the biosynthesis of progesterone and thus essential for the maintenance of pregnancy. We recently identified two transcription factors, activating protein-2γ (AP-2γ) and the homeodomain protein, distaless-3 (Dlx-3), that are expressed in both human and mouse trophoblast cells that were shown to be required for trophoblast-specific expression of the orthologous murine 3βHSD, 3βHSD VI. Although we identified specific binding sites for AP-2γ and Dlx-3 in the distal promoter of the human 3βHSD I gene, *HSD3B1*, it was found that these transcription factors were not involved in determining placental-specific expression of human 3βHSD I. Instead, a 53-bp placental-specific enhancer element located between −2570 and −2518 of the *HSD3B1* promoter was identified. Within this 53-bp element, two potential placental transcription factor binding sites were found. EMSAs with a 20-bp oligonucleotide containing these two potential placental-specific binding sites identified one of the binding sites specific for the transcription enhancer factor (TEF)-5, which is highly expressed in human placenta and in placental choriocarcinoma-derived JEG-3 cells and the other overlapping binding site, specific for a GATA-like protein. Site-specific mutations in either the TEF-5 binding site or in the GATA binding site, each resulted in complete loss of enhancer activity. The data indicate that TEF-5 and the GATA-like protein act in a coordinate manner to determine the placental-specific expression of the human 3βHSD I enzyme and therefore are critical for placental progesterone production required for the maintenance of pregnancy.

The enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3βHSD) is required for the biosynthesis of all active steroid hormones. It exists in multiple isoforms in humans and in rodents, each a product of a distinct gene. To date, two isoforms have been identified in human: 3βHSD I, which is expressed in the placenta, skin, and breast tissue; and 3βHSD II, which is expressed in adrenal glands, ovaries, and testes (1, 2). The expression of 3βHSD I in placenta is required for placental progesterone biosynthesis and therefore is absolutely essential for maintenance of human pregnancy. During the first 6 wk of human pregnancy,



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the corpus luteum of the ovary is the source of the progesterone required for implantation and maintenance of pregnancy. After this time, there is a shift in the site of progesterone production from the corpus luteum to the placenta. Progesterone biosynthesis from cholesterol requires the activity of two enzymes: cholesterol side-chain cleavage (P450scc), which catalyzes the conversion of cholesterol to pregnenolone; and 3βHSD, which catalyzes the conversion of pregnenolone to progesterone. Previous attempts to identify placental-specific elements in the promoter of the *HSD3B1* gene have been unsuccessful (3). We recently identified two transcription factors that are expressed in both human and mouse trophoblast cells, which are required for trophoblast-specific expression of the orthologous murine 3βHSD, 3βHSD VI (4). A 66-bp trophoblast-specific enhancer element located between −2896 and −2831 of the mouse *Hsd3b6* promoter was found to specifically bind the transcription factors, activating protein-2γ (AP-2γ) and the homeodomain protein, distaless-3 (Dlx-3). Site-specific mutations in either of the sites to which these proteins bound eliminated enhancer activity (4). A GenBank search of the human *HSD3B1* promoter sequence identified an AP-2 binding site at −2857/−2848 identical with the AP-2γ binding site of the murine *Hsd3b6* promoter and an identical binding site for Dlx-3 at −2495/−2489. The current study was designed to investigate whether these two transcription factors, which are expressed in the human placenta, also determine the placental-specific expression of human 3βHSD I.

## RESULTS

### Promoter Activity and Cell-Specific Expression of the 5′ Flanking Region of the *HSD3B1* Gene

An earlier study by Guérin *et al.* (3) that sought to identify the sequences required for placental-specific expression in the *HSD3B1* promoter, examined *HSD3B1* promoter sequences between −1079 and +182. Within this promoter sequence, no placental-specific element was found. However, these investigators identified a strong positive regulatory element in the first intron that functioned in a ubiquitous manner. Previous studies from our laboratory indicated that trophoblast-specific expression of the orthologous mouse 3βHSD VI was dependent on AP-2γ and Dlx-3. A GenBank search of the human *HSD3B1* promoter sequence identified an AP-2 binding site at −2857/−2848 and a binding site for Dlx-3 at −2495/−2489 (4). We therefore examined for placental-specific transcriptional activity, a series of deletions of the *HSD3B1* promoter spanning from −2880 to +207, which included the AP-2γ and the Dlx-3 binding sites and the first exon and first intron sequences. In addition, a fragment −492/+32 that excluded the first intron also was tested for transcriptional activity. The different *HSD3B1* promoter fragments were subcloned into a promotorless reporter vector, pA3LUC (4), and transfected into human choriocarcinoma cells (JEG-3), human embryonic kidney cells (HEK293), or mouse Leydig tumor cells (MA-10). As previously reported by Guérin *et al.* (3), deletion of the first intron resulted in a marked decrease in transcriptional activity of the −492 promoter fragment when transfected into JEG-3 cells (Fig. 1A). Transcriptional activity in JEG-3 cells of promoter fragments between −492 and −2880 showed a marked decrease in transcriptional activity between −492 and −2416 with a 60-fold increase observed between −2416 and −2880. Similar transfections in MA-10 and HEK293 cells of three of the reporter constructs containing −492, −1067, and −2880 bp resulted in a very different pattern of transcriptional activity than that observed in JEG-3 cells (Fig. 1B). In both of these cell types, the highest transcriptional activity was observed with the −492 promoter fragment with decreasing activity with the longer promoter sequences. In addition, the transcriptional activity relative to the pA3LUC reporter vector was 5% in MA-10 cells and 4% in HEK293 cells for the −492 fragment and considerably less for the −2861 fragment when compared with the activity in JEG-3 cells. These results suggest that the sequence between −2416 and −2861

contains the essential element(s) for determining placental-specific expression of the 3βHSD I enzyme. Furthermore, sequences between −492 and −2416 appear to contain elements that inhibit the transcription in placental cells.

## Analysis of Enhancer Activity and Characterization of the Enhancer Region

To verify whether the sequence between −2880 and −2416 has the properties of an enhancer, a fragment (−2861/−2395) containing this sequence was subcloned 5′ of the heterologous thymidine kinase (TK164) promoter either in the sense or antisense direction and transfected into JEG-3 and HEK293 cells. Figure 2 illustrates the results obtained with this construct containing the 466-bp fragment in the sense direction. The fragment between −2861 and −2395 increased promoter activity almost 90-fold in either sense or antisense direction (antisense data not shown). No increase in transcriptional activity over the TK164 promoter construct was observed when this 466-bp fragment was transfected into HEK293 cells (data not shown). To determine whether the Dlx-3 and/or the AP-2γ binding sites are the determinant sites for placental-specific expression of the enhancer element, further 5′ and 3′ deletions were made involving these sites, and the resulting constructs were subcloned 5′ of the TK164 promoter and transfected into JEG-3 cells. Deletion of the sequence between −2518 and −2395, which eliminated the Dlx-3 binding site, yielding a fragment including sequences between −2861 and −2518 resulted in an approximately 3-fold increase in enhancer activity compared with the −2861/−2395 fragment. Further deletion 5′ to the Dlx-3 binding site yielding a fragment encompassing sequences between −2861 and −2570 resulted in a complete loss of enhancer activity (Fig. 2). Deletion of the AP-2γ binding site between −2861 and −2820 had no effect on enhancer activity relative to the −2861/−2518 fragment. These findings indicate that the enhancer activity was located between −2570 and −2518 (Fig. 2). To confirm that this 53-bp fragment contains the trophoblast-specific enhancer activity, a single copy and an element containing five copies of the 53-bp fragment were each cloned 5′ of the TK164 heterologous promoter and transfected into JEG-3 cells. As shown in Fig. 2, the heterologous promoter construct containing one copy of the 53-bp fragment resulted in a 90-fold increase in promoter activity, whereas the promoter construct containing five copies increased promoter activity almost 450-fold. Transfection of these two plasmids containing either one or five copies of the −2570/−2518 fragment into HEK293 cells did not result in any activity above that observed with the plasmid containing only the TK164 heterologous promoter (data not shown). These results demonstrate that sequences within this 53-bp fragment determine placental-specific expression of *HSD3B1*. A database analysis of this sequence identified two potential binding sites, **GGAATG** at −2563/−2558, which represents the core of a consensus binding site (5′**-(A/T) (A/G) (A/G) (A/T) ATG (C/T) (G/A)-**3′) for a group of four related transcription enhancer factor (TEF) transcription factors (5) and a potential GATA binding site, **TGATAG** at −2559/−2554 (6), which overlaps with the TEF-binding site. *Boldface* represents consensus sequence. To examine whether JEG-3 cells contain a protein or proteins that bind specifically to either of these potential binding sites in the *HSD3B1* enhancer region, we carried out EMSAs with nuclear extracts of JEG-3 cells and a 20-bp oligonucleotide (referred to as HSD-En in the text) comprising the sequence between −2567/−2548 (which included the potential binding site for TEF and GATA) and a series of sequential double mutations within this sequence (Fig. 3B). As shown in Fig. 3C, the JEG-3 nuclear extract forms two specific DNA-protein complexes, I and II (lane 2) when incubated with the $^{32}$P-labeled HSD-En probe as indicated by competition with 500-fold molar excess of the unlabeled oligonucleotides. Sequential double mutations of the oligonucleotide between −2567 and −2552 as represented by m1–m12 showed a loss of competition of complex II with nucleotides represented by m1–m6 (Fig. 3C, lanes 7–12), indicating that the sequence **GGAATG** from −2563 to −2558 comprised the binding site of complex II. This binding site is identical with the six core nucleotides of the placental-specific binding site,

DF-4, identified in the human chorionic sommatomammotropin-B enhancer (hCS-B) (5). Loss or markedly reduced competition for protein(s) bound to nucleotides comprising complex I was observed with the mutated oligonucleotides m5 to m10 representing the sequence **TGATAG** (Fig. 3C, lanes 11–16). This sequence (**A/T)GATA(A/G)** represents a binding site for GATA transcription factors (6).

### TEF-5 Is the Protein in Complex II of HSD-En

TEF-5 has been shown to be highly expressed in human placenta and in placental choriocarcinoma-derived JEG-3 and BeWo cells (5, 7). In addition, previous reports demonstrated that *in vitro*-translated TEF-5 specifically bound to the DF-4 binding site of the hCS-B enhancer (7). We, therefore, investigated whether an oligonucleotide representing the DF-4 binding site could compete with the radiolabeled HSD-En probe. In addition, we examined whether a nuclear extract from an 18-wk gestation placenta exhibits the same binding properties with the HSD-En probe as seen with JEG-3 cell nuclear extract. Figure 4A demonstrates that 500-fold molar excess of the DF-4 oligonucleotide competed with the protein found in complex II (lane 4), whereas the same oligonucleotide with the two GGs mutated (mDF-4) in the TEF-5 binding site (ttAATG) lost the ability to compete (lane 5). Figure 4A also demonstrates that the nuclear extract from the 18-wk gestation placenta exhibited the same binding properties as observed with JEG-3 cell nuclear extract (lanes 7–10). These data indicate that the protein that forms complex II is expressed in both JEG-3 cells and human placenta and also is the protein that binds to the DF-4 binding site of the hCS-B enhancer that was previously identified as TEF-5.

To determine whether the protein that binds to the TEF consensus sequence in the *HSD3B1* enhancer represents TEF-5, additional experiments were performed. *In vitro*-translated TEF-5 protein extracts and JEG-3 nuclear extract were incubated with the radio-labeled HSD-En or mutated HSD-En (mHSD-En) oligonucleotide probe. The recombinant TEF-5 protein forms a specific DNA-protein complex of the same mobility as seen with the JEG-3 cell nuclear extract with HSD-En (Fig. 4B, lanes 3 and 5), but not with mHSD-En (Fig. 4B, lane 4). To compare the relative affinities of the TEF-5 protein for DF-4 and HSD-En, a competitive EMSA was performed. As seen in Fig. 4C using the HSD-En as the radiolabeled probe and TEF-5 transfected JEG-3 cell extract with increasing molar concentrations of unlabeled competitor oligonucleotides, 256-fold molar excess of unlabeled HSD-En almost completely inhibited binding of the radiolabeled oligonucleotide (lane 13), whereas DF-4 even at 1024-fold molar excess did not completely inhibit the binding of the TEF-5 protein to the HSD-En oligonucleotide (lane 7). No competition was observed with mHSD-En (lanes 16–21). These results demonstrate that *in vitro*-generated TEF-5 specifically binds HSD-En and furthermore under identical conditions, HSD-En appears to have a greater affinity for TEF-5 than the well-characterized TEF-5 binding element of the hCS enhancer (5, 7).

### Analysis of Protein in Complex I of the HSD-En

To examine whether complex I is a specific binding site for one of the GATA-binding proteins, an initial EMSA was performed using radiolabeled HSD-En and an oligonucleotide containing two consensus GATA binding sites, GATAcw, and determined whether this oligonucleotide could compete with the radiolabeled probe HSD-En. Figure 5A demonstrates that 500-fold molar excess of the GATAcw oligonucleotide competed with the protein found in complex I (lane 4), whereas a nonlabeled HSD-En oligonucleotide with a specific mutation in the potential GATA binding site, TGcgAG (mGATA), lost the ability to compete with the protein bound in complex I, but not with the protein of complex II (lane 5). Additional EMSAs with a radiolabeled HSD-En probe and a radiolabeled GATAcw probe and JEG-3 cell nuclear extracts showed that the HSD-En /protein complex I displays

identical mobility to the GATAcw/protein complex with the JEG-3 cell nuclear extract (Fig. 5B, lanes 2 and 9). Cross-competition with the unlabeled oligonucleotides for binding to the JEG-3 protein indicated that the GAT-Acw oligonucleotide had considerably higher affinity for the JEG-3 nuclear protein than the HSD-En oligonucleotide (Fig. 5B, lanes 3–7 and 10–15). At least part of the greater affinity could be attributed to the presence of two GATA-protein binding sites in the GATAcw probe. To assess which of the known GATA proteins is found in the HSD-En/protein complex I, EMSAs were performed using the radiolabeled HSD-En or the radiolabeled GATAcw as probes, JEG-3 nuclear extract, and specific antisera to GATA 1, 2, 3, 4, and 6. Using the consensus GATAcw as a probe, Fig. 5C demonstrates that JEG-3 nuclear extracts contain GATA 2, 3, and 4 with GATA 4 being the most abundant of the proteins and GATA 2 the least abundant (lanes 11–13). With HSD-En as probe, a weak supershift was observed with the GATA-4 antiserum (Fig. 5C, lane 6). Taken together, the results suggest that the JEG-3 nuclear protein that is found in complex I may represent GATA-4 or a GATA-like protein that shows weak cross-reactivity with a GATA-4 antiserum. We also demonstrated the presence of GATA-4 protein in first trimester chorionic villi and first and second trimester cytotrophoblast cells (Fig. 5D).

### The Role of the TEF-5 and the GATA Binding Sites on Enhancer Transcriptional Activity

To determine whether the TEF-5 binding site and the GATA binding site are required for transcriptional activity, site-directed mutations were introduced in each of these sites of the −2820/−2518/TK164 promoter construct. To establish the requirement for the TEF-5 binding site, the mutation GGAATG→tctATG was introduced. The mutation of these three nucleotides does not include any of the nucleotides shown to comprise the GATA binding site. For disruption of the GATA binding site, excluding any of the nucleotides shown to comprise the TEF-5 binding site, the following mutation was introduced: TGATAG→TGAgAt. Transfection of each of these mutated enhancer-heterologous promoter constructs resulted in almost complete loss of enhancer activity (Fig. 6). A mutation in the 53-bp enhancer construct, which involved nucleotides in both of the protein binding sites GGAA**TG**ATAG (the *boldface* nucleotides represent the overlapping nucleotides) →GGAA**aG**ATAc, resulted in the complete loss of enhancer activity when transfected into JEG-3 cells (Fig. 6). These results demonstrate that both of these binding sites are required for determining placental-specific enhancer activity.

## DISCUSSION

It is a well-accepted fact that progesterone is essential for implantation and maintenance of pregnancy in all mammalian species. However, the source of the progesterone throughout pregnancy differs in humans from that found in rodents. At the beginning of pregnancy, in both humans and rodents, progesterone is synthesized by the corpus luteum. In humans, toward the end of the first trimester, there is a shift in progesterone biosynthesis from the ovary to the placenta. The biosynthesis of progesterone from cholesterol requires the action of two steroidogenic enzymes, P450scc and 3βHSD. P450scc is the product of a single gene that is expressed in gonads, adrenal glands, and placenta, whereas 3βHSD exists as two distinct isoforms in humans, 3βHSD I and 3βHSD II. 3βHSD I is the isoform expressed in the placenta. To date, no homozygous mutations in the *HSD3B1*gene have been detected. This observation supports the prediction that the product of this gene, 3βHSD I, is absolutely required for the biosynthesis of progesterone in the placenta and cannot be substituted by the action of 3βHSD II (8). Additional evidence for the absolute requirement for the expression of 3βHSD I in the human placenta after the first trimester is the observation that ovariectomy in the first trimester results in spontaneous abortion of the fetus, but ovariectomy after the first trimester does not interrupt the progress of a normal pregnancy (9). Considerable information is available on the regulation of expression of P450scc and

3βHSD II in gonads and adrenal glands, but relatively little is known regarding the factors that determine placental-specific expression of P450scc and 3βHSD I, the two critical enzymes required for the synthesis of progesterone in the placenta and thus essential for the maintenance of human pregnancy. A previous study was unsuccessful in identifying placental-specific *cis*-acting regulatory elements in the *HSD3B1* promoter (3). Guérin *et al.* (3) only examined −1079 bp of the *HSD3B1* promoter, which would account for their inability to identify the placental-specific element(s). However, these authors found that the first intron contained a strong positive regulatory element that functioned in a ubiquitous manner. The current study confirmed the presence of a strong positive element in the first intron required for basal promoter activity.

In a recent study from our laboratory on the expression of the mouse orthologous 3βHSD, 3βHSD VI, in giant trophoblast cells during midpregnancy, two transcription factors were identified that determined the trophoblast-specific expression of 3βHSD VI (4). The two transcription factors, AP-2γ and Dlx-3, were found in both mouse giant trophoblast cells and in human placental JEG-3 cells. In addition, Ben-Zimra *et al.* (10) reported that AP-2γ was the transcription factor required for mouse trophoblast-specific expression of P450scc. Thus, the identification of an AP-2γ and Dlx-3 binding site in the promoter of the human *HSD3B1* gene and the demonstration that these two transcription factor binding sites were located within an enhancer region of the *HSD3B1* promoter led us to believe that the placental-specific expression of human 3βHSD I was determined by the same transcription factors as we had found for the trophoblast-specific expression of the mouse 3βHSD VI. Moreover, the binding sites for AP-2γ at −2857/−2848 and for Dlx-3 at −2495/−2489 in the human *HSD3B1* promoter were specific as determined by EMSAs using oligonucleotides representing these two binding sites, JEG-3 nuclear extracts and specific antibodies to these two transcription factors (data not shown). However, as reported in *Results*, neither one of these two transcription factors is involved in determining the placental-specific expression of human 3βHSD I. Instead, a 53-bp placental-specific enhancer element was identified between −2570 and −2518 of the *HSD3B1* promoter. A database search of the sequence comprising this enhancer element identified a consensus binding site for a family of transcription enhancer factors, TEF. The vertebrate genome contains four of these transcription factors, TEF-1, -3, -4, and -5, all of which bind to the same consensus sequence, 5′-**(A/T) (A/G) (A/G) (A/T) ATG (C/T) (G/A)**-3′, containing a conserved ATG core (11). These transcription factors are expressed mostly in a tissue-specific manner. TEF-5 is predominantly expressed in the placenta. Human TEF-5 has been cloned from a human placental cDNA library (5, 7) and is specifically expressed in the differentiated syncytiotrophoblasts of the human term placenta with its expression up-regulated during the differentiation of cytotrophoblast to syncytiotrophoblast (11). Human TEF-5 is also highly expressed in cytotrophoblast-derived JEG-3 cells (5) and in human choriocarcinoma-derived BeWo cells (7). In the current study, we demonstrate specific binding to the *HSD3B1* enhancer element with a nuclear extract obtained from an 18-wk gestation placenta that is identical with the binding observed with nuclear extracts from JEG-3 cells. This observation demonstrates that the factor(s) required for expression of 3βHSD I are present in early second trimester placenta, a time when the expression of 3βHSD I is essential for the biosynthesis of progesterone. Previous studies have identified a placental-specific enhancer in the hCS gene referred to as hCS-B enhancer by Jacquemin *et al.* (5) and CSEn2 by Jiang *et al.* (7) as a target for TEF-5 (5, 7, 12). CSEn2 (hCS-B) is composed of multiple enhancers that act cooperatively to bring about maximal enhancer activity (5, 7).

Jacquemin *et al.* (13, 14) defined two distinct sites in the hCS-B enhancer, DF-3 and DF-4, which determine placental-specific expression. DF-3 contains two sites I and II arranged as a tandem repeat that specifically bind recombinant human TEF-5. Mutations in either of these binding sites that disrupt TEF-5 binding results in almost complete loss of enhancer

activity when transfected into JEG-3 cells (5). DF-4 contains a single active site, T**GGAATG**TG, the six core nucleotides, **GGAATG**, are identical with the six nucleotides identified in the *HSD3B1* enhancer. EMSA studies with *in vitro*-translated TEF-5 protein demonstrated that oligonucleotides representing the HSD-En and the hCS-B DF-4 enhancer, each formed a specific DNA-protein complex with an identical migration rate (Fig. 4C). Cross-competition studies with these two enhancer elements confirmed that HSD-En had a greater affinity for TEF-5 than the DF-4 (Fig. 4C). Point mutations in the **GGAATG** core sequence of HSD-En prevented the formation of the protein/DNA complex (Fig. 4B) and resulted in loss of competition with the wild-type HSD-En (Fig. 4C) and loss of enhancer activity when transfected into JEG-3 cells (Fig. 6). In addition, we demonstrated that a nuclear extract from an 18-wk gestation placenta formed a DNA/protein complex with the HSD-En oligonucleotide with identical properties as observed with nuclear extract of JEG-3 cells and the TEF-5 protein (Fig. 4, A and B). Together, these findings provide convincing evidence that TEF-5 is an essential factor in determining the placental-specific expression of 3βHSD I.

Experiments designed to demonstrate that TEF-5 can transactivate the *HSD3B1*-53-bp enhancer by co-transfection of the enhancer TK164 plasmid and a TEF-5 expression vector (7) into JEG-3 cells, BeWo cells, or into HEK293 cells did not result in increased transcriptional activity (data not shown). Jacquemin *et al.* (5) also reported that overexpression of TEF-5 in JEG-3 or in HeLa cells did not result in increased transactivation of the hCS enhancer. However, Jiang *et al.* (7) reported that the coexpression of a plasmid containing the CSEn2 (hCS) enhancer element and the TEF-5 expression vector in BeWo cells resulted in increased transcriptional activity. The most likely explanation for the different results obtained in the current study to that reported by Jiang *et al.* is that TEF-5 had a much higher affinity for the HSD-En element than the DF-4 of the hCS-B enhancer, suggesting that the TEF-5 binding site in HSD-En was already saturated with JEG-3 or BeWo endogenous TEF-5, thus the addition of more TEF-5 in the cotransfection experiment would not result in higher transcriptional activity. [The amount of TEF-5 expressed in JEG-3 or in BeWo cells is not significantly different (Jiang, S.-W., unpublished data).] The ineffectiveness of showing transactivation of the *HSD3B1*-53-bp enhancer in HEK293 cells can be explained by the absence in HEK293 cells of the protein(s) needed in addition to TEF-5 for mediating enhancer transcriptional activity.

The binding site comprising complex I of the HSD-En represents a binding site for GATA proteins. We demonstrated competition with a GATA oligonucleotide that binds all of the known GATA proteins. In EMSA studies using HSD-En as a probe and all of the GATA antisera available against human GATA proteins, we observed a weak supershift with the GATA-4 antiserum. This finding suggests that the nuclear protein in complex I represents GATA-4. GATA-4 was also shown to be the most abundant GATA protein in JEG-3 cells, and furthermore, GATA-4 protein is present in first trimester chorionic villi and first and second trimester cytotrophoblast cells. Mutations, specific in the GATA binding site, resulted in marked reduction in placental-specific transcriptional activity indicating that GATA-4 or a GATA-like protein is essential in addition to TEF-5 in determining placental-specific expression of 3βHSD I. From our studies, we cannot exclude the possibility that the relatively weak supershift represents an as-yet-unidentified GATA protein that shows weak cross-reactivity with the GATA-4 antiserum. Wang and Melmed (15) identified a specific binding site for a GATA-like protein in the placental-specific enhancer of the human leukemia-inhibitory receptor. However, none of the GATA (1–4) antisera resulted in a supershift of the GATA-like protein in an EMSA with the oligonucleotide representing the human leukemia-inhibitory receptor GATA binding site (15). It has been reported that GATA-2 and GATA-3 proteins are involved together with at least three other proteins in conferring placental-specific expression of the human chorionic gonadotropin-α subunit

gene (16). As can be concluded from the current study and the reports by Wang and Melmed (17) and by Steger *et al.* (16), GATA factors act in conjunction with other transcription factors to confer placental-specific expression.

Although the tissue-specific expression of mouse 3βHSD VI and human 3βHSD I are very similar, it may not be too surprising that the placental-specific expression is regulated by different transcription factors in the two species. Placental progesterone production in humans, which requires 3βHSD I, is absolutely essential for maintenance of pregnancy, whereas we have shown that mouse trophoblast cells during mid-pregnancy have the capacity for progesterone production (4). There is no evidence to date that trophoblast-generated progesterone is required for maintenance of mouse pregnancy. The difference in the transcription factors that determine the placental-specific expression does not appear to be the result of the absence of expression of the factors identified that mediate human or mouse trophoblast-specific expression. As discussed above, AP-2γ and Dlx-3 are present in human placental JEG-3 cells as well as in mouse trophoblast cells. TEF-5 (11), GATA-2, and GATA-3 (18) are expressed in mouse giant trophoblast cells. Whether GATA-4 or other GATA-like proteins are expressed in mouse trophoblast cells is not currently known. A somewhat analogous finding to our study has been reported for the *CYP19* aromatase gene. Kamat *et al.* (19) reported that a 501-bp of DNA flanking the 5′ end of the human placental-specific *CYP19* exon I.1 introduced as a fusion gene into transgenic mice resulted in labyrinthine trophoblast cell-specific expression, although endogenous aromatase is not expressed in the mouse placenta. Human and rodent trophoblast cells differ in the expression of other steroidogenic enzymes. Rodent trophoblast cells express CYP17 (cytochrome P450 17α-hydroxylase/C17–20 lyase) (20, 21), which is not expressed in human placental cells. From these findings, it can be concluded that although the mouse and human placental cells express the same transcription factors, this property does not necessarily indicate that these transcription factors act in an identical manner in regulating the tissue-specific expression of steroidogenic enzymes of the different species.

Human placental-specific expression of CYP11A, P450scc, the other steroidogenic enzyme required for progesterone biosynthesis, is yet to be established. Huang and Miller (22) identified two transcription factors related to human immunodeficiency virus-inducible LBP proteins, LBP-1b and LBP-9, which were found to modulate expression of P450scc initiated by other placental-specific transcription factors that as yet have not been identified. Because the expression of P450scc and 3βHSD I is essential for the biosynthesis of progesterone, one would assume that their expression should be coordinated. It will be of interest in future experiments to investigate whether TEF-5 and GATA-proteins also determine the human placental-specific expression of P450scc.

## MATERIALS AND METHODS

### Construction of Plasmids

The plasmids comprising the *HSD3B1* promoter region up to −2880 from the start site of transcription plus the first exon and intron (GenBank accession no. AL121995) were generated from human genomic DNA by the PCR using the primers listed in Table 1. All of the primers had *Kpn*I site added at the 5′ end. To characterize the promoter region of the human *HSD3B1*, a series of promoter fragments that included the first exon and intron (Fig. 1) were subcloned into a promoterless luciferase reporter vector pA3LUC at the *Kpn*I site (4). To make the heterologous constructs, the different fragments within the −2861 to −2395 enhancer region were subcloned into a heterologous thymidine kinase promoter driven luciferase reporter vector TK164LUC (4) at the *Kpn*I site. Site-directed mutations were introduced into the potential TEF-5 and/or GATA-like binding sites using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) after the instructions given in the

manual. PCR fidelity, orientations of inserts, introduced mutations were verified by restriction enzyme digestion and sequencing. Construction of the TEF-5 expression plasmid was as described previously (7).

### Cell Culture and Transient Transfection

Human choriocarcinoma cells (JEG-3) and human embryonic kidney 293 cells (HEK293) were obtained from American Type Culture Collection (Manassas, VA), and mouse Leydig tumor cells (MA-10 cells) were a gift from Dr. Mario Ascoli. JEG-3 and HEK293 cells were cultured in DMEM with 50 μg/ml gentamycin supplemented with 10% fetal bovine calf serum (Invitrogen Life Technologies, Carlsbad, CA). MA-10 cells were grown in Waymouth's MB752/1 medium containing 15% horse serum. JEG-3 cells and MA-10 cells were transfected by calcium phosphate-DNA precipitation (23). HEK293 cells were transfected using Lipofectamine reagent (Invitrogen). Cells were plated at a density of $0.5 \times 10^5$ cells/20-mm well. After 20 h of culture, the test plasmid (~0.4 μg DNA) and $pSV_2\beta$-Gal (24) (0.1 μg DNA) were cotransfected in triplicate. All transfections were normalized with carrier DNA to equal 0.7 μg DNA/well. After transfection (42 h), cells were lysed using Reporter Lysis Buffer (Promega Corp., Madison, WI). The activities of luciferase and β-galactosidase were measured according to the manufacturer's description. The luciferase activity of each construct was normalized to the cotransfected β-galactosidase activity. JEG-3 cells used for transfection of the TEF-5 expression plasmid (pDR2-TEF-5) (7) were plated in 10-cm dishes and grown to 50% confluency before transfection. Lipofectamine-mediated transfection was performed with 30 μg of TEF-5 expression plasmid or 30 μg of empty vector pDR2 (Stratagene) control DNA. After 28 h, cells were washed three times with cold PBS and collected for preparation of cell extracts.

### Tissue and Cell Preparation of Nuclear Extracts

Preparation of crude nuclear extract from nontransfected JEG-3 and HEK293 cells were prepared as described (25). Human placentas were collected under a protocol approved by the Stanford University Panel on the Use of Human Subjects in Medical Research, after informed consent. Placentas were obtained at elective termination of genetically normal pregnancies. Chorionic villi and purified cytotrophoblasts cells from first and second trimester human placentae were prepared as previously described by Fisher *et al.* (26). Trophoblast cells, which included a mixed population of synciotrophoblasts and cytotrophoblasts, were obtained as described (27). Nuclear extracts from these placental trophoblast cells were isolated using a 1-h minipreparation technique as described by Deryckere and Gannon (28). Cell extracts of the TEF-5 and the pDR2 transfected JEG-3 cells were subjected to a micro-scale method for the isolation of the DNA-binding proteins (29).

### *In Vitro* Translation

TEF-5 protein was generated *in vitro* by transcription-translation reaction with TNT Coupled Rabbit Reticulocyte Lysate (Promega) as described previously (7). Standard *in vitro* transcription-translation reaction was performed at 30 C for 2 h with 25 μl TNT, 2 μl reaction buffer, 1 μl $T_3$ RNA polymerase, 1.5 μl ribonuclease inhibitor (RNasin, 40 U/μl; Roche Diagnostics Corp., Indianapolis, IN), 1 μl amino acid mixture, and 1 μg of pDR2-TEF-5 DNA.

### EMSA

EMSAs were performed essentially as described previously (4, 7). The binding reaction contained the appropriate radio-labeled probes, 5–10 μg of crude nuclear extract, or 4–5 μg of TEF-5 transfected JEG-3 cell extract or *in vitro* generated TEF-5 protein as indicated in

the legends. The sequences of the double-stranded oligonucleotides used for EMSAs are listed in Table 2. For competition assays, the indicated fold molar excess of unlabeled oligonucleotides was added to the binding reaction. For supershift assays, 2 μg of polyclonal antisera to GATA 1, 2, 3, 4, or 6 (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the mixture before the addition of probe. The binding reactions were resolved on a 5% nondenaturing polyacrylamide gel.

### Western Blot Analysis

Chorionic villi and cytotrophoblast cells were prepared by treatment with 10% sodium dodecyl sulfate in 50 mM sodium phosphate buffer. The supernatant was subjected to 10% SDS-PAGE and Western blot analysis. Membranes were hybridized with a 1:500 dilution of polyclonal antisera to GATA 4 (Santa Cruz Biotechnology) and then with horseradish peroxidase-labeled secondary antibody and exposed using the Enhanced Chemi-Luminescence kit (Amersham Biosciences, Inc., Arlington Heights, IL).

## Abbreviations

| | |
|---|---|
| **AP-2γ** | Activating protein-2γ |
| **Dlx-3** | the homeodomain protein, distaless-3 |
| **3βHSD** | 3β-hydroxysteroid dehydrogenase/isomerase |
| **hCS** | human chorionic somato-mammotropin |
| **HEK** | human embryonic kidney |
| **JEG-3** | human choriocarcinoma cells |
| **MA-10** | mouse Leydig tumor cells |
| **mHSD-En** | mutated HSD-En |
| **P450scc** | cholesterol side-chain cleavage |
| **TEF** | transcription enhancer factor |
| **TK** | thymidine kinase |

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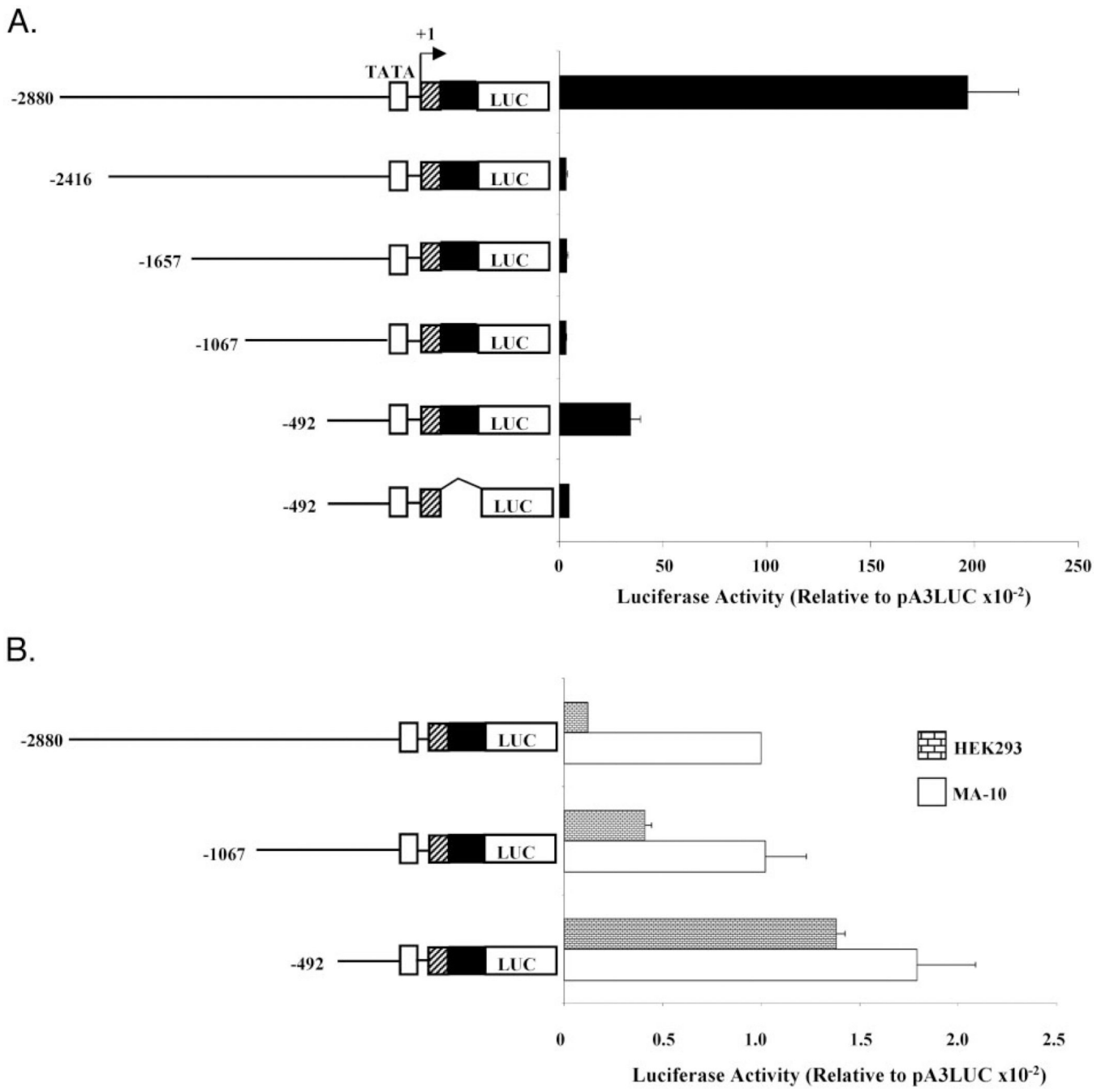



**Figure 1. Transcriptional Activity of the *HSD3B1* Promoter**

A series of 5′ deletions of the *HSD3B1* promoter-luciferase reporter constructs, as indicated on the *right*, were transiently transfected into (A) JEG-3 cells or (B) HEK293 or MA-10 cells. Luciferase activity was normalized to β-galactosidase activity and expressed relative to the promotorless vector pA3LUC, whose activity is set at 1. Each value represents the mean ± SE of three separate transfections, each performed in triplicate, except for the transfections in HEK293 cells, which represent the average of two separate transfections, each performed in triplicate. (Note the difference in scale in panels A and B.)

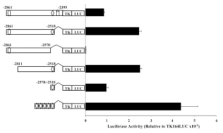
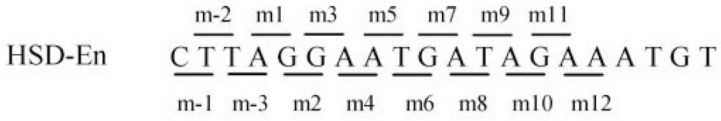


**Figure 2. Identification of the Placental-Specific Minimal Enhancer Element**

Deletions (5′ or 3′) within the sequence −2861 and −2395, which contained the placental-specific element(s) identified in Fig. 1 were subcloned 5′ of the *tk* promoter in the TK164LUC vector and transfected into JEG-3 cells. ▫ Dlx-3 binding site; □ AP-2γ binding site; ◊, represents the 53-bp placental-specific enhancer element. Luciferase activity of each construct is expressed relative to the TK164LUC vector. Each value represents the mean ± SE of three separate transfections, each performed in triplicate.

A.




B.

| | m-1 | m-2 | m-3 | m1 | m2 | m3 | m4 | m5 | m6 | m7 | m8 | m9 | m10 | m11 | m12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| wt | CT | TT | TA | AG | GG | GA | AA | AT | TG | GA | AT | TA | AG | GA | AA |
| mt | ag | gg | gc | ct | ta | tc | cc | cg | gt | tc | gc | cg | ct | tc | gc |

C.

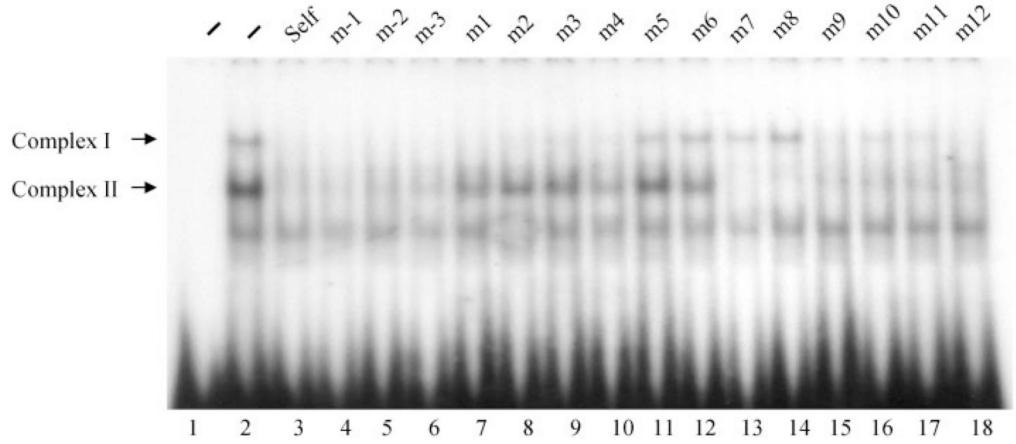



**Figure 3. Analysis of the Sequence between −2567 and −2548 (HSD-En) for JEG-3 Cell-Specific Nuclear Protein Binding Sites**

A, The sequential double mutations in the HSD-En oligonucleotide as indicated with *lines above* or *below* the sequence. B, List of base pairs mutated in each of the competitor oligonucleotides. C, EMSA was performed with 6 μg of nuclear extract of JEG-3 cells and HSD-En as the radioactive probe and 500-fold molar excess of the indicated mutated oligonucleotide. Lane 1 represents the absence of nuclear extract.

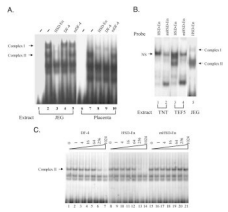


**Figure 4. TEF-5 Is the Likely Protein that Forms Complex II**

A, EMSA was performed with 8 μg of nuclear extract of JEG-3 cells (lanes 1–5) or 11 μg of nuclear extract of human placental trophoblast, 18-wk gestation (lanes 6–10), and HSD-En as the radiolabeled probe. Lanes 1 and 6, No nuclear extract; lanes 2 and 7, indicated nuclear extract; lanes 3 and 8, 500-fold molar excess of HSD-En; lanes 4 and 9, 500-fold molar excess DF-4; lanes 5 and 10, 500-fold molar excess mutant DF-4 (Table 2). B, EMSA was performed with 6 μg of TNT *in vitro* generated TEF-5 protein (lanes 3 and 4) or 6 μg TNT Coupled Rabbit Reticulocyte Lysate (lanes 1 and 2), or 4 μg of JEG-3 nuclear extract (lane 5) and radiolabeled HSD-En (lanes 1, 3, and 5) or radiolabeled mHSD-En (lanes 2 and 4). NS, Nonspecific binding. TNT represents the coupled transcription/translation reticulocyte lysate system. TNT-TEF-5 represents *in vitro*-generated TEF-5 protein prepared with the reticulocyte lysate system (see *Materials and Methods*). C, Relative affinities of TEF-5 protein for HSD-En and DF-4. EMSA was performed with 4 μg of TEF-5 transfected JEG-3 cell extracts as described in *Materials and Methods* and HSD-En as the radiolabeled probe. Indicated unlabeled competitors were added in increasing amounts (1- to 1024-fold molar excess). DF-4, Lanes 2–7; HSD-En, lanes 9–14; mHSD-En, lanes 16–21 (Table 2).

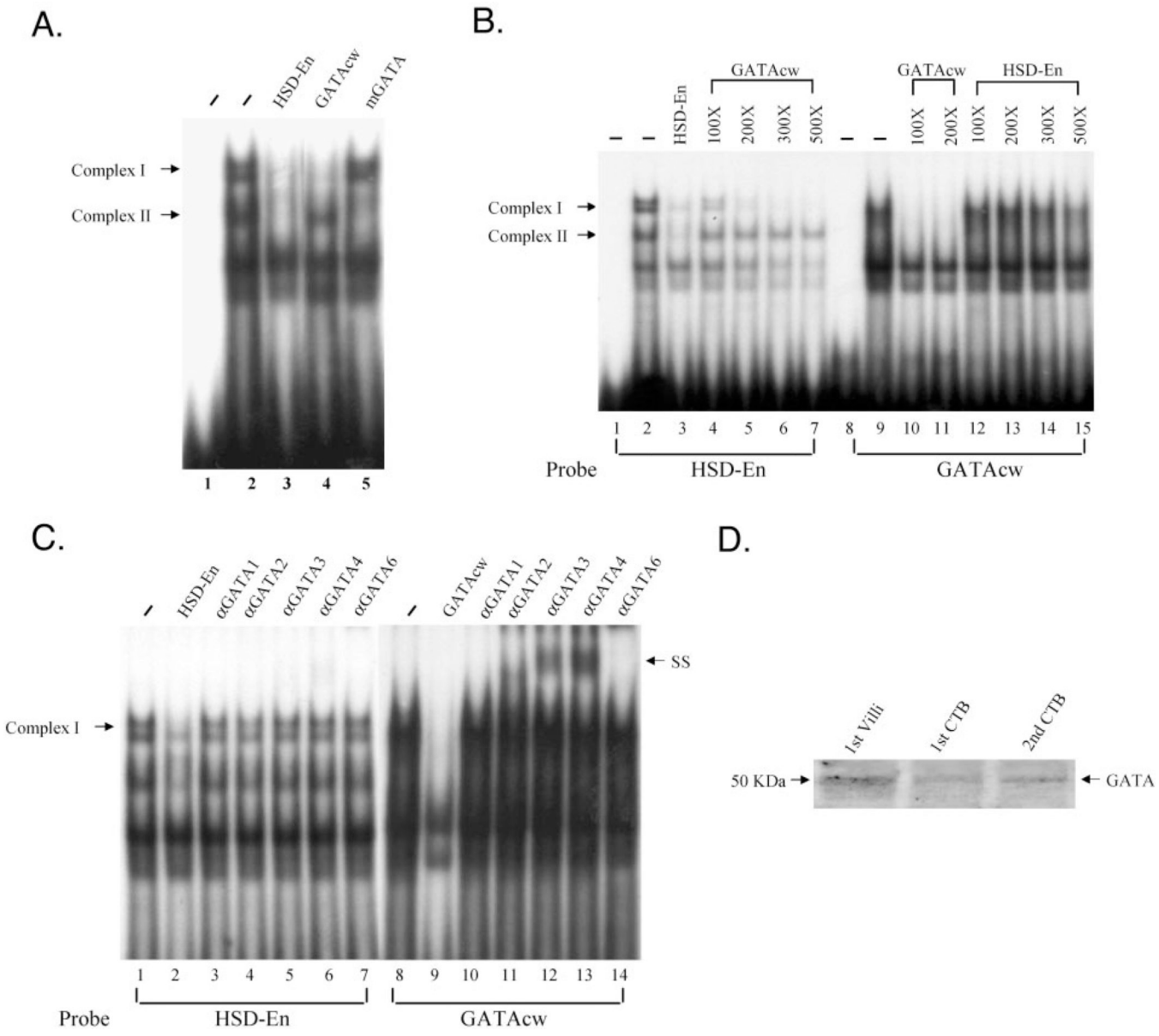



**Figure 5. Analysis of HSD-En GATA-Like Binding Site**

A, EMSA was performed with 8 μg of nuclear extract of JEG-3 cells and HSD-En as the radiolabeled probe. Lane 1, No nuclear extract; lane 2, nuclear extract only; lane 3, 500-fold molar excess of HSD-En; lane 4, 500-fold molar excess of a synthetic GATA oligonucleotide (GATAcw) containing two GATA-binding sites (Table 2); lane 5, 500-fold molar excess of mGATA (HSD-En with two nucleotides mutated in the GATA-specific region leaving the TEF site intact; Table 2). B, EMSA was performed with 8 μg of nuclear extract of JEG-3 cells and HSD-En as the radiolabeled probe, lanes 1–7 or radiolabeled GATAcw probe, lanes 8–15. Lanes 1 and 8, No nuclear extract. Indicated unlabeled competitors as shown above the gel were added in increasing amounts (100- to 500-fold molar excess). C, EMSA was performed with 8 μg of JEG-3 nuclear extract and HSD-En as the radiolabeled probe, lanes 1–7 or radiolabeled GATAcw probe, lanes 8–14. Lanes 1 and 8, Nuclear extract only; lane 2, 200-fold molar excess of HSD-En; lane 9, 200-fold molar excess of GATAcw. Lanes 3–7 and 10–14 represent incubations with HSD-En and

GATAcw as probes, respectively, and JEG-3 nuclear extract in the presence of antisera to the indicated specific GATA proteins. D, Western blot analysis of GATA-4 protein in cell extracts of first trimester placental chorionic villi (1st Villi) and first (1st CTB) and second (2nd CTB) trimester cytotrophoblast cells.

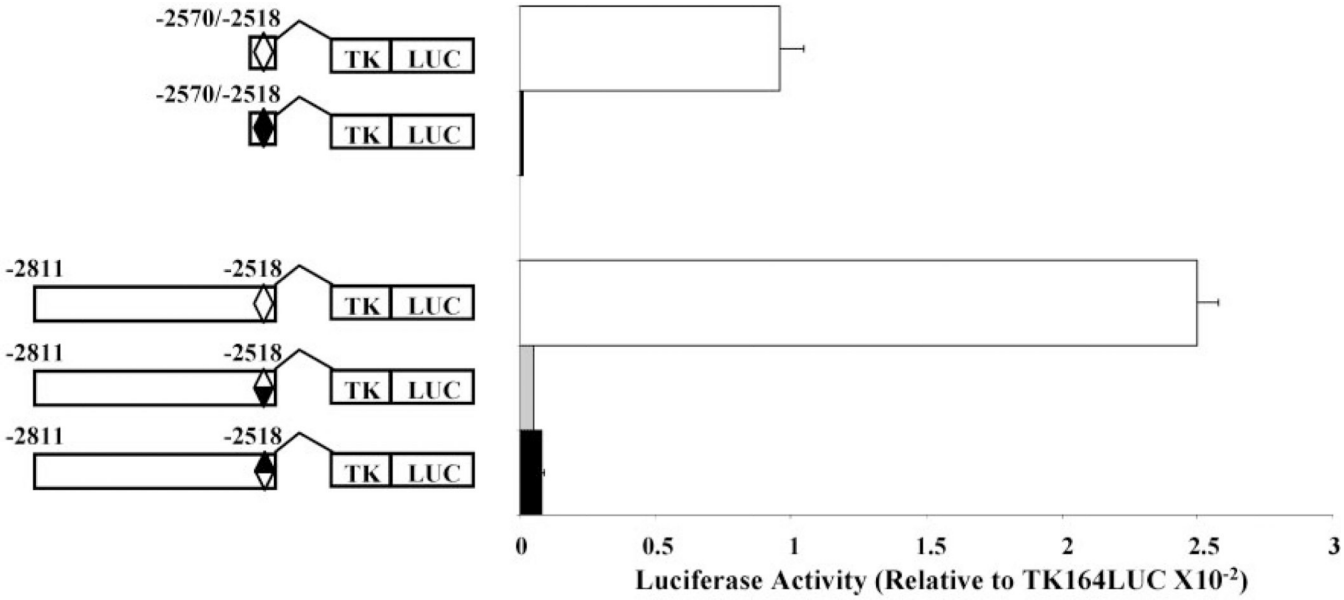



**Figure 6. Effect of Specific Mutations in the TEF-5 and/or the GATA-Like Binding Sites on Placental-Specific Enhancer Activity**

The indicated TK164LUC-enhancer plasmids were transfected into JEG-3 cells as described in *Materials and Methods*. Luciferase activity of each construct is expressed relative to the TK164LUC vector. Each value represents the mean ± SE of three separate transfections, each performed in triplicate. The following mutations were introduced: ♦, CTTAGGAA**a**GTA**ct**AATGT; ⬙, TEF-5-specific: CTTAT**ct**ATGATAGAATGT; and ⬘, GATA-site-specific, CTTAGGAATGA**g**A**tct**ATGT.

**Table 1**

Primers for *HSD3B1* Promoter and Enhancer Plasmids

| **Promoter Primers** | **Sequence (5′→3′)** |
|---|---|
| −2880/−2861 | AATTACCCAGGCATGGTGGC |
| −2416/−2395 | GTTGGTAGTATGGTTCCCAATC |
| −1657/−1638 | GTTGGGTACAGCTCACACAG |
| −1067/−1048 | TCCAGAACCACAGAGATCTT |
| −492/−473 | TTTCAGATATATTGGGTAGG |
| +207/+187 | GATTCTAGGGTGACCCTGGA |
| +/32/+8 | CTGGACAGAAGAGTGGACGTACTT |
| Enhancer Primers | |
| −2861/−2839 | CAGGTGCCTGGAGGCTGAGGCA |
| −2395/−2416 | GATTGGGAACCATACTACCAAC |
| −2518/−2537 | TGTGGACCATCACTGTAATC |
| −2570/−2589 | ATCCCTCGTGCTCATCAGTC |
| −2811/−2792 | GTTGAAGTGAGCCAACATCG |
| −2570/−2551 | TTTCTTAGGAATGATAGAAATG |

**Table 2**

Oligonucleotides

| Oligo Name | Sequence (5′→3′) |
|---|---|
| HSD-En | CTTAGGAATGATAGAAATGT |
| mHSD-En | CTTAGGAActATAGAAATGT |
| DF-4 | ATTAGACT**GGAATG**TGGTCC |
| mDF-4 | ATTAGACT**ttAATG**TGGTCC |
| GATAcw | CACT**TGATAA**CAGAAAG**TGATAA**CTCT |
| mGATA | CTTAGGAATGcgAGAAATGT |

*Line below*, Potential TEF-binding site; *line below in bold*, TEF-binding site; *line above*, potential GATA-binding site; *line above in bold*, consensus GATA binding site; *lower case letters*, mutant nucleotides.