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The Proximal Promoter Region of the Gene Encoding Human 17β-Hydroxysteroid Dehydrogenase Type 1 Contains GATA, AP-2, and Sp1 Response Elements: Analysis of Promoter Function in Choriocarcinoma Cells*

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ABSTRACT

The 5′-flanking region from −78 to +9 in the HSD17B1 gene serves as a promoter, and an HSD17B1 silencer element is located in position −113 to −78. In the present studies, we have characterized three regulatory elements in the proximal 5′-flanking regions of the gene, using electrophoretic mobility shift assays and reporter gene analysis. First, nuclear factors recognized by antibodies against Sp1 and Sp3 were found to bind the Sp1 motif in the region from −52 to −43. Mutation of the Sp1-binding site decreased the promoter activity to 30% in JEG-3 cells and to 60% in JAR cells, suggesting that binding to the Sp1 motif has a substantial role in the complete functioning of the HSD17B1 promoter. Second, the binding of AP-2 to its motif in the region from −62 to −53 led to reduced binding of Sp1 and Sp3, and furthermore, mutation of the AP-2 element increased promoter activity to 260% in JEG-3 cells. The data thus implied that AP-2 can repress the function of the HSD17B1 promoter by preventing binding to the Sp1 motif. Finally, GATA factors, GATA-3 in particular, were demonstrated to bind their cognate sequence in the HSD17B1 silencer region, and mutations introduced into the GATA-binding site increased transcriptional activity to the level seen in constructs not containing the silencer element. Thus, GATA-3 seems to prevent transcription in the constructs, and hence, the GATA motif also may operate as a negative control element for HSD17B1 transcription. (*Endocrinology* **138:** 3417–3425, 1997)

THE BIOLOGICAL activity of androgens and estrogens is greatly modulated by a redox reaction at position C-17 of the steroid molecules. The reaction, interconversion of 17-ketosteroids and 17β-hydroxysteroids, involves a number of 17β-hydroxysteroid dehydrogenases (17HSDs) which are expressed in steroidogenic tissues. In addition, some of these enzymes are present in several peripheral tissues and target tissues of steroid action, thus modulating local concentrations of high- and low-activity sex steroids (1). To date, four human 17HSDs have been characterized (2–6), and the 17HSD type 1 is essential for estradiol (E2) production in human ovarian ganulosa cells and placental trophoblasts (7–9). The type 1 enzyme also participates in the regulation of E2 concentrations locally in breast epithelial (10, 11) and endometrial (12) cells. Recent findings demonstrated that cells transfected with the 17HSD type 1 expression vector respond to low-activity estrone (E1), as well as to E2, thus proving that the concentration of this enzyme plays a role in the estrogen-dependent regulation of the cell proliferation rate (13).

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17HSD type 1 is encoded by the HSD17B1 gene (14, 15) (previously also called EDH17B2) localized to loci 17q12–21 (7, 16, 17). The gene contains 2 transcription start points, about 9 and 971 nucleotides upstream from the translation initiation codon, which result in 2 transcripts, 1.3 and 2.3 kb in size, respectively (14). The latter one, whose function remains unknown, is constitutively expressed in several tissues (7, 11, 14) and cell lines with a minor relationship to the presence of the 17HSD type 1 protein (11, 18). On the other hand, the 1.3-kb messenger RNA (mRNA) is expressed in cells producing 17HSD type 1 protein, and its amount largely correlates with the concentration of the protein (11, 18). It is also the 1.3-kb mRNA whose concentration is subject to regulation by growth factors (19, 20), retinoic acids (21), and cAMP (7, 22, 23) in choriocarcinoma cells.

In a recent study, we demonstrated that a cell-specific enhancer in the region from −661 to −392 and a silencer element between the nucleotides −392 and −78 in the HSD17B1 gene participate in the regulation of its transcription. These regulatory areas are suggested to affect the transcription of the 1.3-kb mRNA of 17HSD type 1. On the other hand, the fragment −78/+9 is able to drive reporter gene expression, acting as a basal promoter (24). The region from −78 to +9 contains motifs for binding several transcription factors, such as AP-2 and Sp1 (Fig. 1). In addition, in the middle part of the area, which has been suggested to be essential for the function of the HSD17B1 silencer (24), a binding motif for GATA transcription factors is located. In this study, we characterized the function of these motifs in JEG-3 and JAR choriocarcinoma cell lines. JEG-3 cells are able

FIG. 1. Structure of the 5′-flanking region of the HSD17B1 gene. The potential binding motifs are framed. The *arrows* above the *boxes* show the orientation of each element.

to synthesize progestins and estrogens from suitable substrates and are thus commonly used for studying placental steroidogenesis. 17HSD type 1 is moderately expressed in JEG-3 cells, whereas its expression in JAR cells is scant, but detectable (20). We noticed that binding to the Sp1 motif is important for the promoter function, whereas GATA proteins repress transcription derived from the 5′-region of the HSD17B1 gene in choriocarcinoma cells. In addition, AP-2 seems to prevent binding to the Sp1 motif and thus also to decrease the transcription.

Materials and Methods

Chemicals and reagents

The isotopes [α-^{35}S]deoxy-ATP (3000 Ci/mmol), [α-^{32}P] deoxy-CTP (3000 Ci/mmol), and [^{3}H-]acetyl coenzyme A (200 mCi/mmol chloramphenicol acetyl transferase (CAT) (assay grade) were purchased from Amersham Life Science (Little Chalfont, UK) and Du Pont NEN (Boston, MA). The transfection reagent N-[(1-(2, 3-dioleoyloxy)propyl)]-N and N,-trimethyl-ammoniummethylsulfate (DOTAP) were products of Boehringer Mannheim (Mannheim, Germany). The antibodies against the transcription factors GATA-2, GATA-3, AP-2, Sp1, Sp2, Sp3, and Sp4 and the oligonucleotides for the GATA, AP-2, and Sp1-binding sites were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and the AP-2 protein and HeLa nuclear extracts and the β-galactosidase enzyme assay kit were from Promega Co. (Madison, WI). All the media, buffers, supplements, and reagents for cell culture were purchased from Gibco BRL-Life Technologics (Grand Island, NY) and Sigma Chemical Co. (St. Louis, MO). Other reagents not mentioned in the text were obtained from the Sigma Chemical Co., Boehringer Mannheim, New England Biolabs (Beverly, MA) and Merck A.G. (Darmstadt, Germany).

Preparation of plasmid constructs for reporter gene analyses

The nested deletion constructs, pCAT-EY-349, pCAT-EY-228, pCAT-EY-113, pCAT-EY-97, and pCAT-EY-78, were generated from the parent plasmid pCAT-EY-859 as described earlier (24), using the procedure developed by Henikoff (25) or the PCR. The overlap-extension technique described by Ho *et al*. (26) was used to introduce mutations into the AP-2 site (5′-GCCCGCAGGC-3′→5′-GC*TT*GCAGGC-3′) and the Sp1 site (5′-GGGGC*G*GGGC-3′→5′-GGGGC*TT*GGC-3′) in fragment −97/+9 to obtain the mutants (mAP-2)-97 and (mSp1)-97. The mutations into the GATA-binding site of fragment −113/+9 (5′-TTATCC-3′→5′-TTA-*A*GC-3′ and 5′-T*AT*TCC-3′) were included directly in the 5′-end primer of the PCR reaction to achieve the mutated fragments (m1GATA)-113 and (m2GATA)-113, respectively. The mutants were then inserted into the pCAT-EY vector, and the resulting constructs are called p(mAP-2)-97, p(mSp1)-97, p(m1GATA)-113, and p(m2GATA)-113. All of the plasmid constructs, both wild-type and mutated ones, were verified by sequencing, and at least two individual preparations of each plasmid were mixed together for transfection experiments.

Cell culture, transient transfection, and analysis of reporter gene expression

The human choriocarcinoma cell lines JEG-3 and JAR were obtained from the American Type Culture Collection (Rochville, MA) and were maintained according to the instructions of the supplier. For reporter gene analyses, both JEG-3 and JAR cells were plated onto 60-mm dishes (8.5×10^5 cells) 20–24 h before transfection. Then 6.0 μg of each construct were transfected into JEG-3 cells, whereas 4.0 μg of each plus 2.0 μg of the β-galactosidase control vector pCMVβ (Clontech Laboratories Inc, Palo Alto, CA) were transfected into JAR cells using the transfection reagent DOTAP (6.0 μg/ml). After 20 h, the media were replaced, and the cells were cultured for a further 52 h before collection. The harvested cells were then subjected to four freeze-thaw cycles and, further, to heat inactivation at 65 C for 20 min, after which the CAT activity of the samples was measured by fluor diffusion assay (27, 28). The β-galactosidase activities were used to normalize the transfection efficiency of JAR cells. CAT expression was assessed by comparing of the CAT activity in the samples with a CAT standard curve, and the final values were calculated as picograms of CAT protein per milligram of total protein. All the transient transfection experiments were performed in duplicate, each series was repeated independently two to three times, and the CAT activity of each sample was measured twice.

TABLE 1. Oligonucleotides for EMSAs

Oligonucleotide	Position	Sequence
HSD-GATA	-114 to -77	5′-CGCAGGAAGGCTTATCCTTGAGAT TGCGTGGGAGACAC-3′
HSD-m1GATA	-114 to -77	5′-CGCAGGAAGGCTTAAGCTTGAGAT TGCGTGGGAGACAC-3′
HSD-m2GATA	-114 to -77	5′-CGCAGGAAGGCTATTCCTTGAGAT TGCGTGGGAGACAC-3′
GATA consensus		5′-CACTTGATAACAGAAAGTGATAA CTCT-3′
βRARE01		5′-GGGTAGGGTTCACCGAAAGTTCAC TCGCTCC-3′
HSD-AP-2/Sp1	-69 to -36	5′-GGTGGGGGCCCGCAGGCGGGGCG GGGCGAAGCAG-3′
HSD-mAP-2/Sp1	-69 to -36	5′-GGTGGGGGTTCGCAGGCGGGGCG GGGCGAAGCAG-3′
HSD-AP-2/mSp1	-69 to -36	5′-GGTGGGGGCCCGCAGGCGGGGCT TGGCGAAGCAG-3′
HSD-AP-2	-69 to -49	5′-GGTGGGGCCCGCAGGCGGGG-3′
HSD-mAP-2	-69 to -49	5′-GGTGGGGTTCGCAGGCGGGG-3′
AP-2 consensus		5′-GATCGAACTGACCGCCCGCGGCCC GT-3′
HSD-Sp1	-57 to -36	5′-CAGGCGGGGGCGGGGCGAAGC-3′
HSD-mSp1	-57 to -36	5′-CAGGCGGGGGCTTGGCGAAGC-3′
Sp1 consensus		5′-ATTCGATCGGGGGCGGGGCGAGC-3′

Binding motifs have been *underlined* with *solid lines* and mutated nucleotides with *dotted* ones.

Electrophoretic mobility shift assays (EMSAs)

For the EMSAs, nuclear extracts from cultured cells were prepared by the method of Dignam *et al*. (29), as described by Ausubel *et al*. (30), and oligonucleotides were labeled by a filling-in reaction using [α-^{32}P] deoxy-CTP and Klenow fragments of DNA polymerase I. The EMSAs were performed as described previously (24), with some modifications. Briefly, 4.0–8.0 μg of nuclear extract was first incubated at RT for 10 min in 20 μl of binding buffer containing 10% glycerol, 20 mM HEPES (PH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 0.05% NP-40, and 2.0 μg poly(dI-dC). Then 0.25–0.5 ng of a probe was added to the reaction mixture, and the incubation was further continued at RT for 20 min. For the competition experiments, a 100-fold excess of the competitor was added together with the probe. Unrelated DNA, βRARE01 fragments (31), were

applied to some reactions to investigate the specificity of binding. For the supershift assays, 100 ng of appropriate antibody was added to the reaction mixture subsequent to the addition of a ^{32}P-labeled probe, and the reaction was continued at 4 C for 60 min. The sequence and position of each oligonucleotide used are illustrated in Table 1.

Results

Progressive 5′-end deletion analysis of HSD17B1 promoter

We have previously demonstrated that the region from −78 to +9, with respect to the transcription start site of the 1.3-kb mRNA of 17HSD type 1, is capable of driving reporter gene expression (24). In addition, the earlier results suggested that factor(s) binding to the region between −113 and −78 may repress transcription. To further analyze the function of the 5′-flanking region of the HSD17B1 gene, a series of 5′-deletions was constructed in front of the CAT gene in promoter-testing vector pCAT-EY containing SV40 enhancer. The deletion fragments spanned from +9, the last nucleotide before the ATG codon, upstream to −349. In JEG-3 cells, shortening of the fragment from position −349 to −228 or to −113 had a minor effect on reporter gene expression, whereas further deletion of the fragment, up to position −97, resulted in an approximately 2-fold increase in CAT expression (Fig. 2). The shortest fragment, −78/+9, behaved similarly to fragment −97/+9, suggesting that the region from −113 to −97 may contain a negative control element(s). In JAR cells, on the other hand, the fragments −113/+9, −97/+9, and −78/+9 promoted similar reporter gene expression, and extending the fragment upstream to −228 and −349 decreased transcription.

Interaction of the HSD17B1 5′-flanking region with the transcription factors GATA-2 and GATA-3

The region from −113 to −97 of the HSD17B1 gene contains a consensus motif for the binding of GATA proteins at position −103 to −98 (Fig. 1). To test the capability of these putative silencer areas of the HSD17B1 gene to bind nuclear proteins, especially GATA proteins, a labeled fragment −114/−77, called HSD-GATA, was incubated together with the JEG-3 nuclear extract. As shown in Fig. 3A, HSD-GATA formed two specific DNA-protein complexes with the JEG-3 nuclear extract, and they are described as complex 1 and complex 2 in Fig. 3, A and B. The formation of these two complexes was inhibited by unlabeled HSD-GATA and GATA consensus oligonucleotide, but not by unrelated DNAs, a fragment −69/−36 called HSD-AP-2/Sp1, or βRARE01 (for complete sequences, see Table 1). As a consequence of mutations in the GATA motif (5′-TTATCC-3′→5′-TTA*AG*C-3′ and 5′-TTATCC-3′→5′-T*AT*TCC-3′), HSD-m1GATA and HSD-m2GATA were not able to prevent the formation of the two complexes either, pointing to involvement of the GATA site in the formation of the complexes (Fig. 3A).

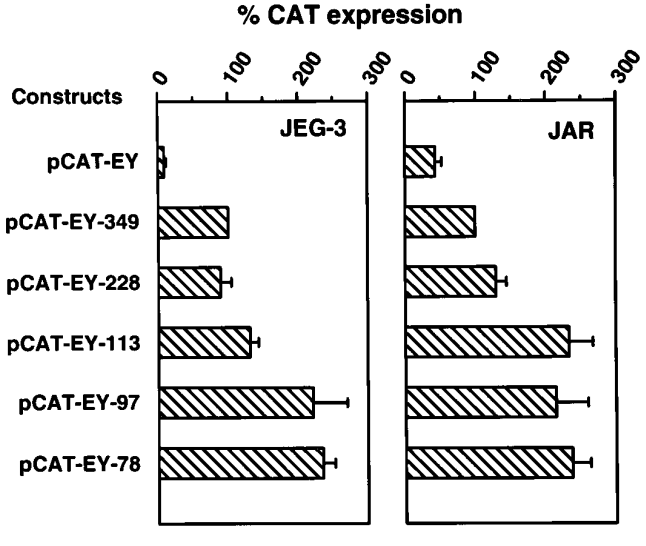


FIG. 2. Progressive 5′-end deletion analysis of the HSD17B1 basal promoter. In both *panels*, bar 1 shows the background reporter gene expression of the vector pCAT-EY, whereas bars 2–6 show the CAT expression generated by the deletion fragments. Reporter gene expression of pCAT-EY-349 is defined as 100%, and those of all the other constructs are given as relative percentages. The results represent mean ± SD from three to four independent experiments.

GATA-2 and GATA-3 have been reported to be present in JEG-3 cells (32). To investigate whether they bind to the HSD17B1 gene, antibodies against GATA-2 and GATA-3 were added to the EMSA reaction. When anti-GATA-3 was included in the binding reaction together with the HSD-GATA fragment and the JEG-3 nuclear extract, the lower complex (complex 1) vanished and a new complex (Supershift 1) appeared (Fig. 3B). In addition to the JEG-3 nuclear extract, HSD-GATA formed similar DNA-protein complexes (complexes 1 and 2) with nuclear extracts prepared from JAR and T47D cells, and the Supershift 1 complex was generated when anti-GATA-3 was present in the binding reaction. Adding antibody against GATA-2 to the binding reaction created a weak complex (Supershift 2), which was detected in the sample containing a nuclear extract from JEG-3 cells. Although Supershift 2 was not observed with JAR nuclear extract, probably because of low concentration of GATA-2 in JAR cells, the intensity of complex 2 was reduced to some extent by the addition of anti-GATA-2. The nuclear extract from BT-20 cells resulted in two DNA-protein complexes with HSD-GATA fragments, but they differed from the ones formed with nuclear extracts from JEG-3, JAR, and T47D cells. Furthermore, they were not supershifted by anti-GATA-2 or anti-GATA-3 (Fig. 3B).

Functional analysis of the GATA motif in the HSD17B1 gene by reporter gene analysis

The role of the GATA motif in the function of the HSD17B1 promoter was further investigated by reporter gene analysis, in which intact and mutated −113/+9 fragments were analyzed. In JEG-3 cells, two GATA mutation constructs, p(m1GATA)-113 (5′-TTATCC-3′→5′-TTA*AG*C-3′) and p(m2GATA)-113 (5′-TTATCC-3′→5′-T*AT*TCC-3′), resulted in increased CAT expression, about 170% and 160%, compared with that generated by intact pCAT-EY-113 (Fig. 4). In fact, the mutation in the constructs raised reporter gene expression to the level driven by the shorter fragment −78/+9. The result confirmed that the reduced promoter activity of fragment −113/+9 compared with fragment −78/+9 is caused by the binding of GATA proteins or related factors to the GATA-binding motif at a position from −102 to −98. In

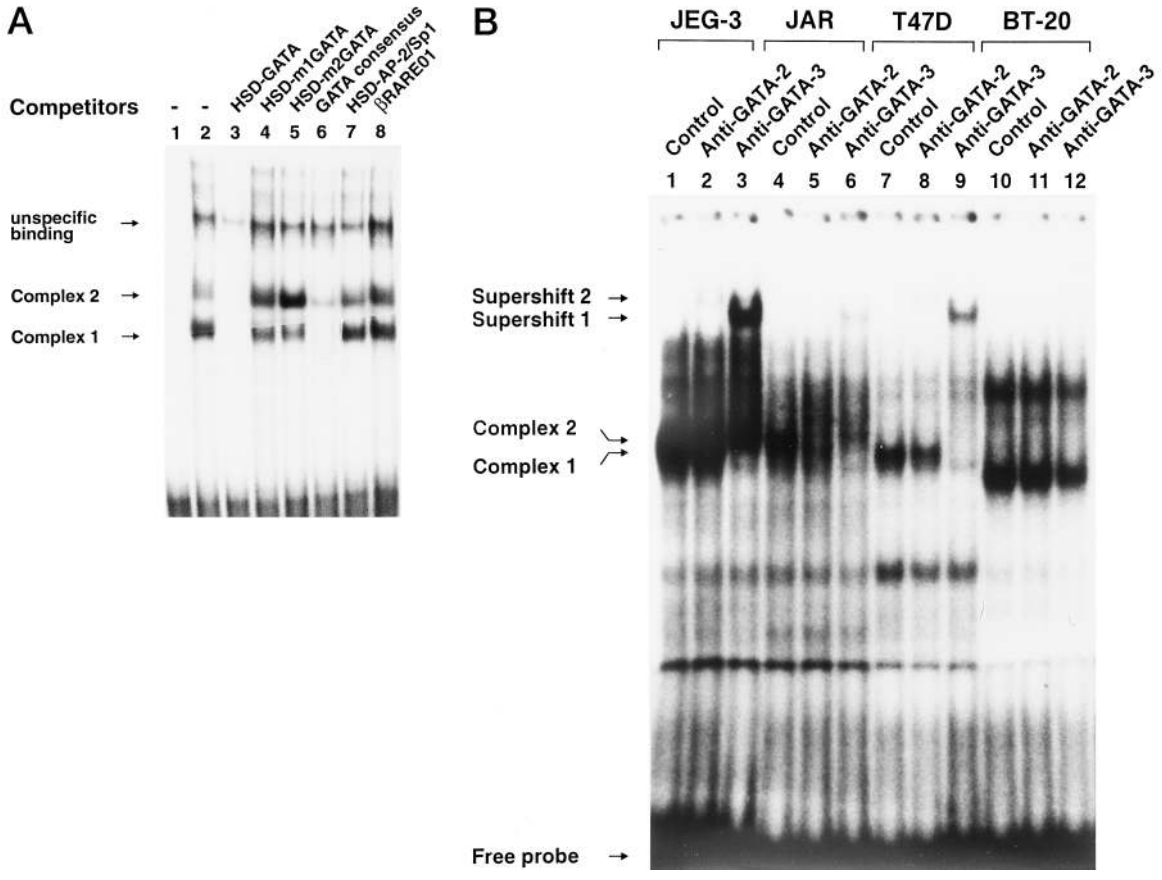


FIG. 3. Interaction of GATA-2 and GATA-3 with the HSD17B1 proximal promoter. A, Interaction of HSD-GATA fragment with the JEG-3 nuclear extract. Lane 1 shows a free probe, whereas lane 2 shows binding between the probe and the JEG-3 nuclear extract. As indicated in lanes 3–8, unlabeled oligonucleotide competitors were included in the binding reactions in 100-fold molar excesses over probe. Complete sequences of the oligonucleotides used are listed in Table 1. The positions of the binding complexes, which have been separated from each other in 6% polyacrylamide gel, are indicated by *arrows* on the *left*. B, Supershift analysis of HSD-GATA using antibodies against GATA-2 and GATA-3. Lanes 1–3 show interaction of HSD-GATA with the JEG-3 nuclear extract in the absence of antibody, and in the presence of anti-GATA-2 and anti-GATA-3. Similarly, lanes 4–6, lanes 7–9, and lanes 10–12 show the interaction of HSD-GATA with nuclear extracts prepared from JAR, T47D, and BT-20 cells, without and with antibodies. *Arrows* on the *left* indicate the positions of the binding complexes, supershifted complexes, and free probe, which have been separated from each other in 5% polyacrylamide gel.

addition, the results verified that the difference between the activities of the fragments −113/+9 and −78/+9 is not a result of dissimilar distances between the SV40 enhancer and the HSD17B1 promoter in the corresponding constructs. In JAR cells, mutations of the GATA response element only slightly raised the promoter activity of fragment −113/+9 (Fig. 4), which was in line with the fact that there was no notable difference in the promoter activities of the fragments −113/+9, −97/+9, or −78/+9 (Fig. 2).

Interaction of HSD17B1 proximal promoter with transcription factors binding to AP-2 and Sp1 motifs

In addition to the GATA-binding site, consensus sequences for binding of the AP-2 and Sp1 are present in the HSD17B1 proximal promoter at positions from −62 to −53 and from −52 to −43, respectively (Fig. 1). As shown in Fig. 5A, the ^{32}P-labeled fragment, termed HSD-AP-2/Sp1, covering the region from −69 to −36 in the HSD17B1 gene, formed a pronounced complex (complex 3) with the JEG-3, JAR, and T47D nuclear extracts and the AP-2 extract. The HeLa extract formed with the fragment a major complex

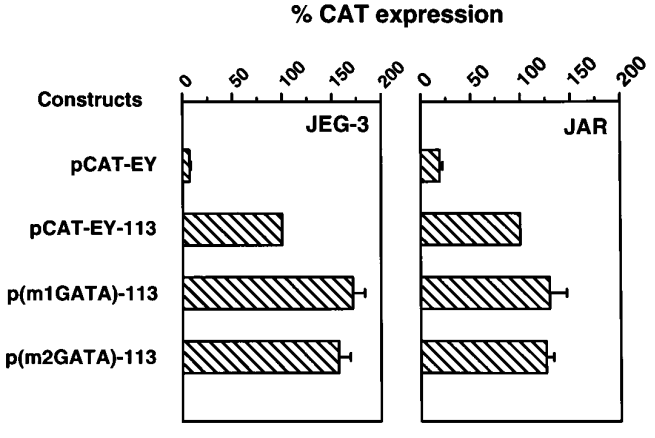
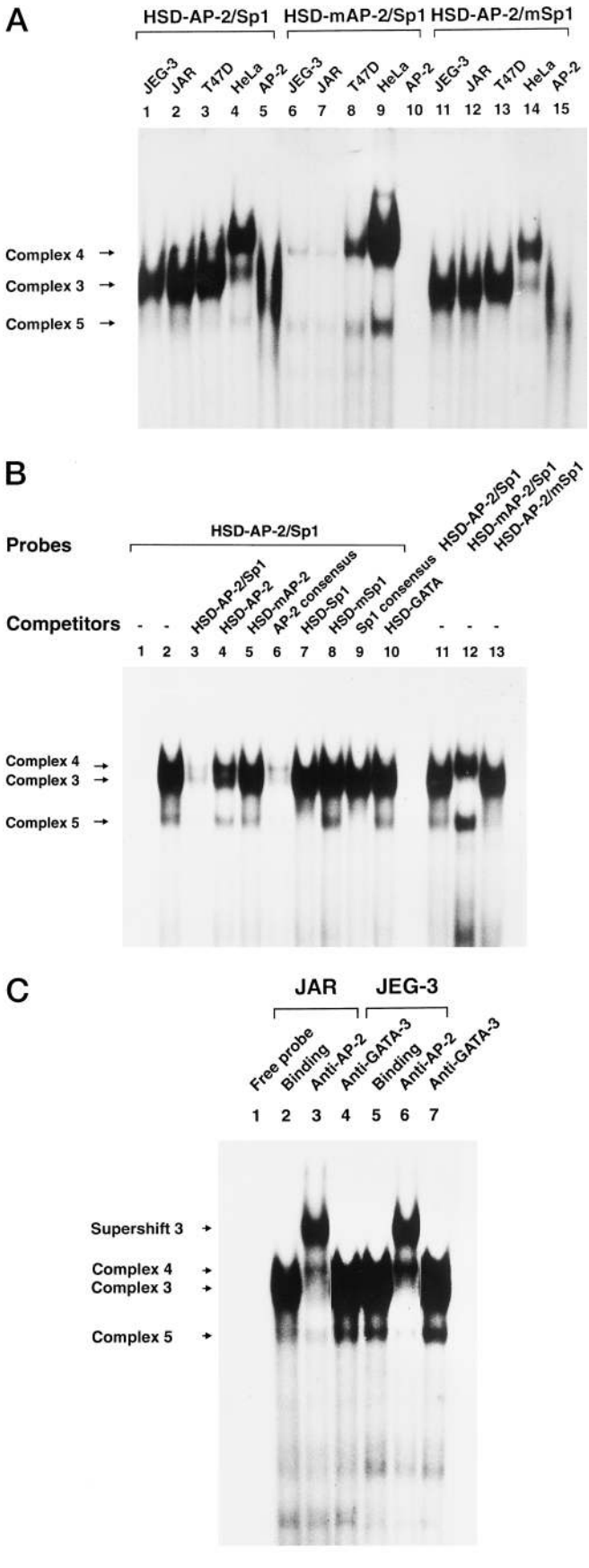


FIG. 4. Functional analysis of the GATA motif in the HSD17B1 proximal promoter. In both *panels*, bar 1 shows the background CAT activity of the vector pCAT-EY. Bars 2–4 show the reporter gene expression of the wild-type, m1GATA mutant and m2GATA mutant of fragment −113/+9, respectively. The CAT expression of pCAT-EY-113 is defined as 100%, and those of all the others are given as relative percentages. The results represent mean ± SD from four independent experiments with duplicate samples in each.



(complex 4) and two weak complexes, one of which drifted similarly to complex 3, whereas the other was termed complex 5. Mutation of the AP-2 consensus site in the fragment resulted in a disappearance of complex 3 and in a strengthening of the complexes 4 and 5 in all the nuclear extract tested, including JEG-3, JAR, and T47D (Fig. 5A, lanes 6–9). Instead, mutation of the Sp1 consensus binding site inhibited or reduced remarkably the formation of the complexes 4 and 5 (Fig. 5A, lanes 11–14). To sum up, the AP-2 motif was likely to be involved in the formation of complex 3, and the Sp1 motif in the formation of the complexes 4 and 5.

Involvement of the AP-2 and Sp1 motifs in the formation of the complexes was further supported by the data showing that the formation of complex 3 was reduced or prevented by the short HSD17B1 fragment containing the AP-2-binding site or by the AP-2 consensus fragment (Fig. 5B, lanes 4 and 6), whereas the fragment with the mutated AP-2 motif was not cabable of such reduction (Fig. 5B, lane 5). The formation of the complexes 4 and 5 was correspondingly inhibited by HSD-Sp1 and Sp1 consensus fragments (Fig. 5B, lanes 7 and 9) but not by the mutated form of HSD-Sp1, HSD-mSp1 (lane 8). The formation of complex 4 was not clearly detectable when binding to the AP-2 motif was effective, but again, mutation of the AP-2 site led to enhanced formation of complex 4, as well as complex 5 (Fig. 5B, lane 12). Unlabeled HSD-AP-2/Sp1 abolished the formation of all complexes, whereas unrelated DNA, HSD-GATA, did not affect the formation of any of the complexes, showing the specificity of the binding reactions (Fig. 5B, lanes 3 and 10). Finally, antibodies against AP-2 were used in EMSAs to confirm the binding of the AP-2 factor to its motif in the HSD17B1 promoter. Complex 3, formed from both JAR and JEG-3 nuclear extracts, was supershifted by anti-AP-2 (Supershift 3), whereas the formation of the complex was not influenced by anti-GATA-3 used as a negative control (Fig. 5C).

Because it was obvious that the binding of AP-2 to its motif prevented the binding of factors to the Sp1-motif (Fig. 5, A and B), a HSD17B1 gene fragment from −69 to −36 with a mutated AP-2 motif (HSDmAP-2/Sp1) was further incubated with the nuclear extracts JAR, JEG-3, and HeLa (Fig. 6, lanes 2, 7–9). As a result, three complexes, marked 4a, 4b, and 5, could be detected in a high-resolution gel. The formation of all the three complexes was prevented by the HSD-Sp1

FIG. 5. EMSA for the HSD-AP-2/Sp1 fragment. A, Interaction of the HSD-AP-2/Sp1 fragment and its mutated forms with JEG-3, JAR, T47D, HeLa, and AP-2 extracts. B, Effect of various fragments on binding between the HSD-AP-2/Sp1 fragment and the JAR nuclear extract. Lane 1 is for free probe, whereas lane 2 shows binding between the probe and the JAR nuclear extract. Lanes 3–10 demonstrate complexes formed when unlabeled oligonucleotide competitors were included in the binding reactions in 100-fold molar excesses over probe. Interactions of proteins in the JAR nuclear extract with the intact and mutated HSD-AP-2/Sp1 fragments are illustrated in lanes 11–13. C, Supershift analysis of HSD-AP-2/Sp1 using antibodies against AP-2. Lane 1 shows free probe, whereas lanes 2–4 represent interaction of probe with the JAR nuclear extract in the absence of antibody and in the presence of antibodies against AP-2 and GATA-3. Similarly, lanes 5–7 show interaction of the probe with the JEG-3 nuclear extract without and with antibodies. The binding complexes, marked by *arrows* on the *left*, have been segregated in 4% polyacrylamide gels in panels A and B, and in 5% gel in panel C.

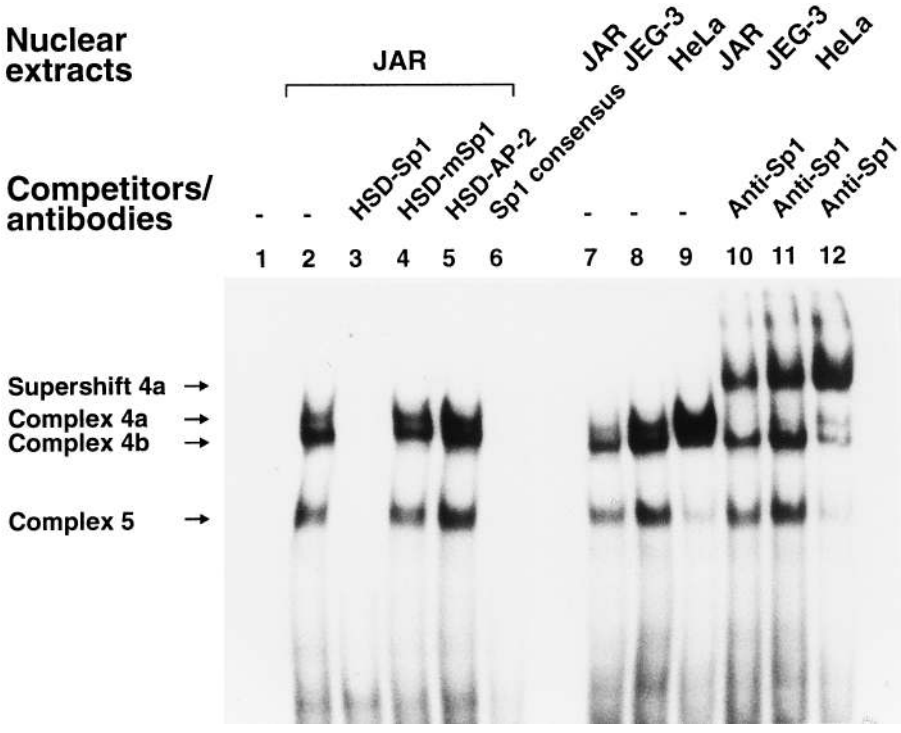


FIG. 6. Effect of the mutation of the AP-2 motif on the binding of nuclear proteins to the HSD-AP-2/Sp1 fragment. Lane 1 is for free probe, whereas lanes 2 and 7–9 show binding between the HSD-mAP-2/Sp1 fragment and JAR, JEG-3, and HeLa nuclear extracts, as indicated above. The influence of unlabeled competitors on the binding of JAR proteins is illustrated on lanes 3–6, whereas the effects of the Sp1 antibody on the formation of complexes between the HSD-mAP-2/Sp1 fragment and the JAR, JEG-3, and HeLa nuclear proteins are demonstrated on lanes 10–12. Four-percent polyacrylamide gel was used to separate the complexes formed.

fragment, as well as by the Sp1 consensus fragment, but not by HSD-mSp1 or HSD-AP-2 (Fig. 6), the latter demonstrating that none of the complexes formed were caused by the binding of AP-2 to the mutated fragment.

To identify proteins involved in the formation of the complexes 4a, 4b, and 5, antibodies against Sp1, Sp2, Sp3, and Sp4 were added to the binding reactions. Antibodies against Sp1 supershifted complex 4a (Supershift 4a) (Fig. 6, lanes 10–12; Fig. 7), whereas antibodies against Sp3 prevented the formation of the complexes 4b and 5 and/or slowed down their migration (Supershift 4b/5) (Fig. 7A). Finally, Fig. 7B shows the binding reactions between the nonmutated HSDAP-2/Sp1 fragment and the JAR, JEG-3, and HeLa nuclear extracts, together with the Sp-factor antibodies. Altogether, the results strongly suggest that both Sp1 and Sp3 can bind to the HSD17B1 promoter, especially in the absence of the AP-2 factor.

Functional analysis of AP-2 and Sp1 motifs in HSD17B1 proximal promoter by reporter gene analysis

The roles of the AP-2 and Sp1 motifs in the function of HSD17B1 promoter were analyzed by reporter gene analysis (Fig. 8). Mutation of the Sp1-binding site in fragment −97/+9 decreased CAT expression to 30% in JEG-3 cells and to 60% in JAR cells, compared with that generated by the intact fragment. In contrast, mutation of the AP-2-binding site in fragment −97/+9 increased promoter activity to 260% in JEG-3 cells, indicating that transcription can be suppressed by AP-2.

Discussion

In human placenta, 17HSD type 1 catalyzes the reduction of low-activity E1 to E2, subsequent to aromatization of androgens to E1 by aromatase. Recent results have demonstrated that the 1.3-kb transcript of the HSD17B1 gene, rather than 2.3-kb mRNA, is mainly translated to 17HSD type 1 protein (11, 18). Thus, knowledge of the structure and function of the promoter for the 1.3-kb mRNA is critical for understanding the mechanisms controlling 17HSD type 1 expression.

The HSD17B1 gene fragment from −78 to +9 is extensive enough to drive reporter gene expression in connection with either HSD17B1 or SV40 enhancer (24), and it contains consensus binding sites for transcription factors Sp1 and AP-2, for example. In the present study we showed that mutation of the Sp1 motif considerably decreased the promoter activity of fragment −97/+9 and that the intact motif was bound by two member of the Sp transcription factor family, Sp1 and Sp3, particularly when the adjacent AP-2 motif was mutated. Both Sp1 and Sp3 are widely distributed transcription factors that bind to the GC-rich Sp1 motif with identical affinities (33). Sp1 activates a wide array of cellular and viral promoters, and it can interact also with several regulatory factors and, consequently, mediate cell- and gene-specific effects on the target promoter (34–36). For instance, Sp1 is necessary for both basal and enhancer-mediated transcription of the human chorionic somatomammotropin gene (37), a gene which, similarly to HSD17B1, is expressed in JEG-3 cells and in placental syncytiotrophoblasts. Instead, Sp3 has been demonstrated to repress Sp1-mediated transcriptional activation by competing with Sp1 for their common binding site (38–40) and to be able to increase transcription in only a few cases (40, 41). It has been suggested that some genes have evolved the capacity both to be activated by Sp1 and to be repressed by Sp3, to control the levels of their gene expression more stringently (39). Our present results indicate that binding to

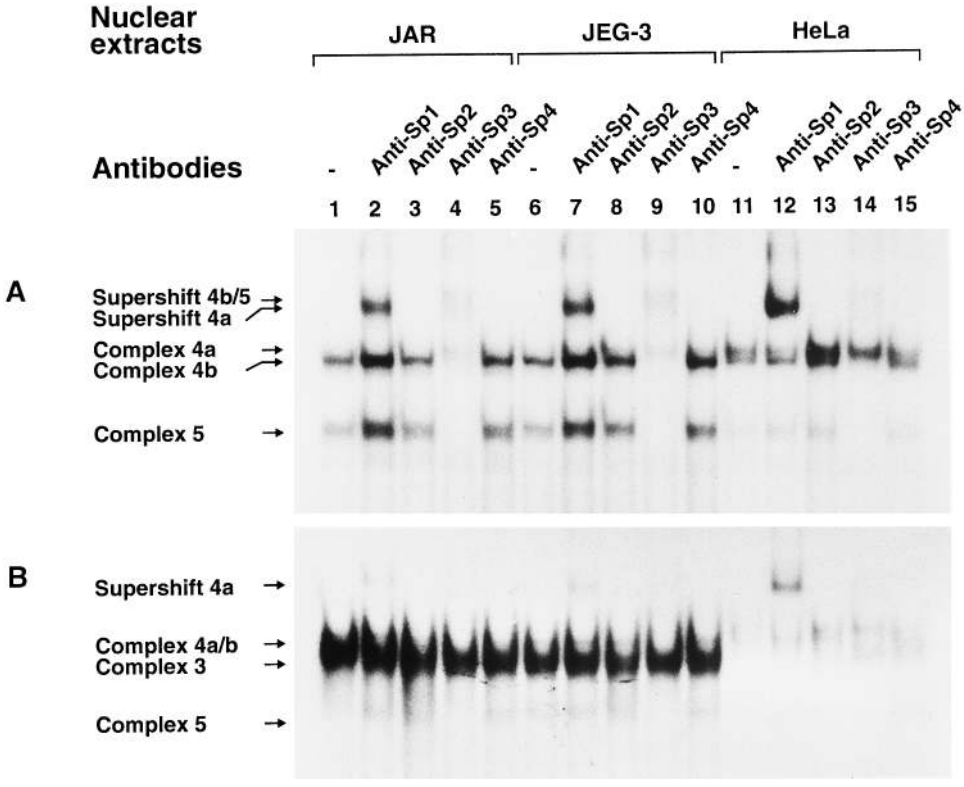


FIG. 7. Identification of the factors binding to the Sp1-binding site in the HSD17B1 promoter. Interaction of the HSD-mAP-2/Sp1 fragment (A) and the intact HSD-AP-2/Sp1 fragment (B) with JEG-3, JAR, and HeLa nuclear extracts in the absence (lanes 1, 6, and 11) and presence of antibodies against Sp1, Sp2, Sp3, and Sp4. The binding complexes and their supershifted forms were separated from each other in 4% polyacrylamide gel.

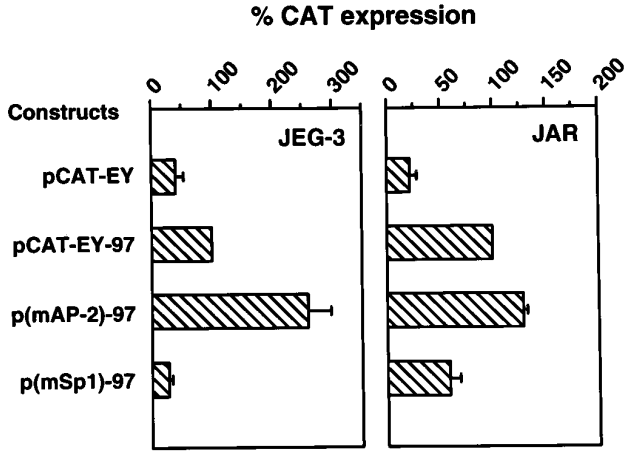


FIG. 8. Functional analysis of AP-2 and Sp1-binding sites in HSD17B1 proximal promoter. In both *panels*, bar 1 shows the background CAT activity of the vector pCAT-EY. Bars 2–4 show the reporter gene expression of the wild-type, mAP-2 and mSp1 mutants of fragment −97/+9, respectively. The CAT expression of pCAT-EY-97 is defined as 100%, and those of all the others are given as relative percentages. The results represent mean ± SD from three independent experiments.

the Sp1 motif is needed for full activation of the HSD17B1 promoter. Mutation of the motif prevented effectively the binding of both Sp1 and Sp3, which means that specific roles of Sp1 and Sp3 in controlling the transcription of the HSD17B1 gene and their possible mutual competition in the HSD17B1 gene regulation remain to be clarified.

Sp1 and Sp3 were able to bind their cognate sequence in the HSD17B1 promoter more efficiently when the adjoining AP-2 motif was mutated. The intact motif was bound by AP-2, or an immunologically and functionally similar protein, but in contrast to the Sp1-binding site, mutation of the AP-2 motif increased the transcriptional activity of the reporter gene construct. The results hence suggest that the binding of AP-2 decreases the HSD17B1 promoter activity, which is, at least partially, caused by the prevention of binding of the Sp factor(s). The AP-2 factor is activated by several signaling pathways, such as protein kinase C and cAMP (42), but no further activation/inactivation of the factor apparently is needed for binding of AP-2 to the HSD17B1 promoter, because neither phorbol 12-myristate 13-acetate nor cAMP treatment significantly affected transcriptional activity of the promoter in JEG-3 and JAR cells (data not shown). Therefore, the AP-2 site at the position −62 to −53 may not be involved in mediating the effects of protein kinase C and cAMP pathways on HSD17B1 gene expression, reported previously (7, 20–23). It seemed that the formation of Sp complexes with intact HSD17B1 promoter was scant in JEG-3, JAR, and T47D nuclear extracts, compared with that in HeLa cells containing a greater abundance of the Sp1 factor in respect to the AP-2 factor. Low amount of Sp factor(s), compared with AP-2 concentration, might explain why fragment −78/+9 alone led to only low reporter gene expression in these cell lines (24).

We previously showed that the promoter fragments −113/+9 and −78/+9, when connected with either HSD17B1 or SV40 enhancer, drove reporter gene expression. Interestingly, however, the promoter activity of the shorter fragment was significantly greater than that of the longer one in JEG-3 cells, as well as in PC-3 prostate cancer and CV-1 kidney cells (24). In the present study, we demonstrated that

the GATA-binding motif, which is present in the region between −113 and −78, was able to bind transcription factors GATA-2, and in particular, GATA-3 in JEG-3 cells. Mutation of the motif decreased binding of the GATA proteins and led to increased promoter activity. This suggests that GATA-2 and GATA-3 can repress function of the HSD17B1 gene, at least in the constructs used, and the motif may thus be an essential part of the silencer localized previously (24). In JAR cells, the GATA complexes were formed less than in JEG-3 cells, and deletion or mutations of the GATA site generated only minor increases of promoter activity, which may be caused by low expression of GATA proteins in this cell line.

The GATA family currently comprises six members, GATA-1 to GATA-6, which recognize the GATA motif WGATAR (see Ref. 43). GATA-1 is mainly present in erythroid cells, in which it regulates erythroid-specific gene expression (reviewed in Refs. 43 and 44), whereas other members of the GATA family are more widely distributed (see Ref. 43). In placental trophoblasts, GATA-2 and GATA-3 are expressed and are required to direct trophoblast-specific expression of gonadotropin α-subunit and placental lactogen I genes (32, 45), for example, and it has been suggested that these transcription factors may play a crucial role in trophoblast cell differentiation (45). GATA factors are thus important regulators of placental gene expression. Our present results suggest that GATA factors may limit the function of the HSD17B1 gene in trophoblast like cells, thus increasing the number of target genes under the control of the GATA family in placental tissue.

Altogether, we have identified three binding motifs in the promoter of the HSD17B1 gene that are specifically bound by nuclear factors recognized by antibodies against Sp1, Sp3, AP-2, GATA-2, and GATA-3. Binding to Sp1 motif resulted in increased gene transcription, whereas AP-2 and GATA proteins had an opposite effect. These findings point to complicated regulation of HSD17B1 gene expression, in which a cell-specific enhancer containing several interacting subunits (24), a silencer element with GATA motif, a proximal promoter region with competing Sp1 and AP-2 sites, and possibly still unidentified regions, participate. Mutual interactions of the factors binding to these elements, their concentrations, and activation/inactivation of them finally control the tissue specific expression of HSD17B1 gene.

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