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GATA Factors and the Nuclear Receptors, Steroidogenic Factor 1/Liver Receptor Homolog 1, Are Key Mutual Partners in the Regulation of the Human 3β-Hydroxysteroid Dehydrogenase Type 2 Promoter

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The human *HSD3B2* gene encodes the 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase type 2 (3β-HSD2) enzyme that is required for steroid hormone biosynthesis. Mutations in the *hHSD3B2* gene are responsible for a form of congenital adrenal hyperplasia and male pseudohermaphroditism whereas overexpression of *hHSD3B2* has been recently associated with polycystic ovarian syndrome. Despite the importance of the *hHSD3B2* gene, the molecular mechanisms that regulate its expression remain poorly understood. Transcription factors belonging to the GATA family are emerging as novel regulators of steroidogenesis. Indeed, GATA-4 and GATA-6 are abundantly expressed in steroidogenic cells of the gonads and adrenals. We now report that the human *HSD3B2* promoter (*hHSD3B2*), which contains four consensus GATA elements, constitutes an important target for GATA factors. GATA-4 and GATA-6 by themselves are sufficient to activate transcription (up to 15-fold) from a −1073 bp *hHSD3B2* promoter fragment and blockade of endogenous GATA expression and/or activity blunts *hHSD3B2* promoter activity in steroidogenic cells. Deletion studies showed that the proximal GATA element located at −196 bp is sufficient to confer GATA responsiveness of the *hHSD3B2* promoter and is required for full *hHSD3B2* promoter activity in steroidogenic cells. Moreover, we report that GATA-4 and GATA-6 can physically interact with the nuclear receptors, steroidogenic factor 1 and liver receptor homolog 1, to synergistically activate *hHSD3B2* promoter activity in both homologous and heterologous cells. Aberrant expression of transcription factors essential for *hHSD3B2* expression might also be involved in some pathologies/syndromes associated with deregulated *hHSD3B2* expression. (*Molecular Endocrinology* 19: 2358–2370, 2005)

THE HUMAN *HSD3B2* gene encodes the 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase type 2 (3β-HSD2) enzyme that catalyzes an essential step in the formation of all classes of active steroid hormones. Expression of the *hHSD3B2* gene is almost exclusively restricted to the adrenal, ovary, and testis, which constitute the main steroidogenic tissues (1). Within the adrenals, 3β-HSD2 is detected in the three layers of the cortex (2, 3) whereas in the gonads, it is found in the main steroidogenic compartment (ovarian theca and luteinized granulosa cells, and testicular Leydig cells) (1, 4).

The requirement of sufficient and functional 3β-HSD2 enzyme in steroidogenic tissues is emphasized by the existence of human mutations that are associated with 3β-HSD2 deficiency. Mutations within the *hHSD3B2* gene lead to the classical form of 3β-HSD2 deficiency. Affected individuals suffer from congenital adrenal hyperplasia (CAH) with or without salt wasting (depending on the gravity of the mutation), a potentially life-threatening disease (reviewed in Ref. 1). Because 3β-HSD2 is also required for testosterone synthesis by Leydig cells and because testosterone is essential for proper male sex differentiation (reviewed

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Abbreviations: CAH, Congenital adrenal hyperplasia; 3β-HSD2, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase type 2; LRH-1, liver receptor homolog 1; NR, nuclear receptor; PCOS, polycystic ovarian syndrome; PVDF, polyvinylidene difluoride; SF-1, steroidogenic factor 1; siRNA, short interfering RNA; STAT, signal transducer and activator of transcription

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in Ref. 5), males with classical 3β-HSD2 deficiency are pseudohermaphrodites that exhibit female external genitalia (6). Another form of 3β-HSD2 deficiency, called nonclassical or late-onset deficiency, is observed in older females with hyperandrogenism beginning at adulthood as well as in children with premature pubarche (7). The exact causes of nonclassical 3β-HSD2 deficiency remain to be identified because *hHSD3B2* gene mutations are not involved (1, 8). In addition, overexpression of the *hHSD3B2* gene has been recently associated with polycystic ovarian syndrome (PCOS), a common endocrine disorder affecting 5–10% of women of reproductive age in which excessive androgen production by the ovary leads to hirsutism, ovulatory dysfunction, and infertility (9–12).

Despite the essential role of 3β-HSD2 activity for adrenal and gonadal steroidogenesis, very little is known about the molecular mechanisms that regulate *hHSD3B2* gene expression in these tissues. To date, five transcription factors have been shown to bind to and activate the human *HSD3B2* (*hHSD3B2*) promoter: the nuclear receptor (NR) steroidogenic factor 1 (SF-1)/NR5A1 (13), the signal transducer and activator of transcription protein 5 (STAT5) and STAT6 (14–17), LRH-1/NR5A2, an NR closely related to SF-1 (18), and the immediate early factor Nur77/nerve growth factor induced-B/NR4A1 (19–21). Consistent with their roles in regulating *hHSD3B2* expression, SF-1, LRH-1, Nur77, STAT5, and STAT6 are all present in steroidogenic tissues (14, 18, 22–30). They are, however, all weak activators on their own. Moreover, they are also expressed in other tissues that do not express *hHSD3B2*, indicating that other transcription factors are required to direct *hHSD3B2* expression to steroidogenic tissues.

The GATA family of proteins is a new group of transcription factors that are emerging as important regulators of steroidogenesis (31). Members belonging to this family, namely GATA-4 and GATA-6, are strongly expressed in the fetal and adult adrenals and gonads. In the adrenal, GATA-4 is specifically expressed during the early fetal period (32). GATA-6, however, is abundantly expressed in the adrenal cortex throughout development (33–35). Within the gonads, both GATA-4 and GATA-6 are expressed in the ovarian steroidogenic compartment (36, 37) whereas in the testis, GATA-4 is present in fetal and adult Leydig cells (38–41). More recently, several target genes for GATA factors in steroidogenic tissues have been identified and include several key enzymes/proteins involved in steroid hormone metabolism: *HSD17B1* (17β-HSD1) (42), *Cyp19* (aromatase) (43), human *HSD3B1* (3β-HSD1) (44), *Cyp17* (17α-steroid hydroxylase) (9, 34, 45, 46), *Cyp11A* (P450 side-chain cleavage) (34), *SULT2A1* (dehydroepiandrosterone-sulfotransferase) (34), and *StAR* (steroidogenic acute regulatory protein) (43, 47–49). In addition to their ability to directly stimulate transcription of target steroidogenic promoters, we have shown that GATA-4 can also regulate some of these promoters via a synergistic interaction with the nuclear NR SF-1 (43, 50). Thus, GATA factors, much like SF-1, appear to be critical regulators of steroidogenic gene expression and function.

We now report that the *hHSD3B2* promoter, which contains four consensus GATA regulatory motifs, is an important target for GATA factors. We also show that coexpression of GATA-4 or GATA-6 and the NRs SF-1 and LRH-1 leads to a striking synergistic activation of *hHSD3B2* promoter activity. Promoter deletion and mutagenesis studies revealed that proximal GATA and SF-1/LRH-1 elements are essential for maximal synergism between GATA factors and SF-1/LRH-1 on the *hHSD3B2* promoter and for full basal promoter activity in steroidogenic cells. Thus, our results provide key new data into the molecular mechanisms that regulate this critical step of steroidogenesis that might be relevant to pathological processes involving aberrant steroidogenic gene expression such as adrenal insufficiency, male pseudohermaphroditism, and PCOS.

RESULTS

GATA Factors Are Potent Activators of the *hHSD3B2* Promoter

The *hHSD3B2* gene is almost exclusively expressed in the adrenals and gonads. These two steroidogenic tissues also express high levels of the GATA transcriptional regulators GATA-4 and GATA-6. Interestingly, four consensus GATA elements are found within the first kilobase of the *hHSD3B2* promoter (Fig. 1A). As a first step to determine whether these two GATA factors could be involved in tissue-specific *hHSD3B2* gene expression, we performed transient transfection assays using two steroidogenic cell lines (mouse MA-10 Leydig cells and human H295R adrenal cells) as well as heterologous CV-1 cells. As shown in Fig. 1B, both GATA factors could activate a −1073 bp *hHSD3B2*-luciferase promoter construct in all cell lines tested. The strongest activation was observed in CV-1 cells (10- to 15-fold), followed by H295R cells (5- to 10-fold) and MA-10 cells (2- to 5-fold). The level of activation observed was inversely correlated with the level of GATA expression in the different cell types (CV-1<H295R<MA-10). CV-1 cells have very little GATA activity (43), whereas H295R and MA-10 cells weakly and strongly express GATA-4 (Fig. 1C). Thus, it was not surprising that MA-10 cells (which are already overloaded with GATA-4) yielded the weakest activations in the GATA overexpression experiments whereas CV-1 cells (which are essentially devoid of GATA activity) produced the highest.

To demonstrate that endogenous GATA factors present in steroidogenic cells do indeed contribute to *hHSD3B2* promoter activity, we used short interfering RNA (siRNA) technology to knock down GATA-4 expression in MA-10 cells and a previously described GATA dominant negative competitor (51) to block GATA activity in H295R cells (Fig. 2). As shown in Fig.

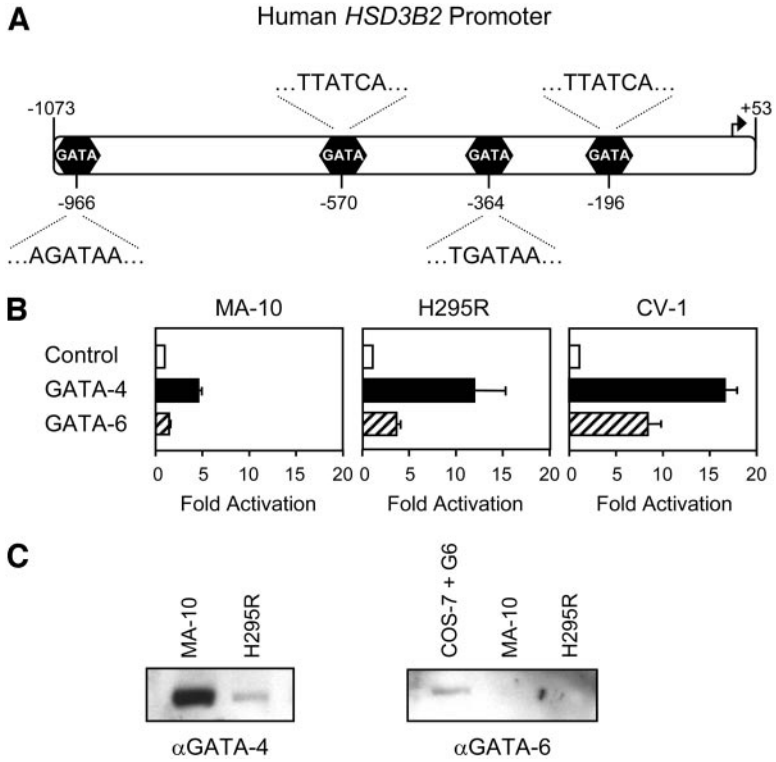

Fig. 1. Activation of the *hHSD3B2* Promoter by GATA Factors

A, Schematic representation of the human *HSD3B2* promoter highlighting the location of four consensus GATA regulatory elements. B, Mouse MA-10 Leydig, human H295R adrenal, and monkey CV-1 fibroblast cells were cotransfected with a −1073 to +53 bp *hHSD3B2*-luciferase promoter construct and either an empty expression vector (control) or expression vectors for GATA-4 (25 ng) and GATA-6 (25 ng). Results are shown as fold activation over control (±SEM). C, Western blot analysis of GATA-4 and GATA-6 expression in MA-10 and H295R cells. Ten micrograms of total protein were used per lane. COS-7 cells overexpressing recombinant GATA-6 (10 μg) were used as a positive control for the GATA-6 antiserum.

2A, the GATA dominant negative (GATA DN) blunted the activity of the −1073 bp *hHSD3B2* promoter in the adrenal H295R cell line by as much as 40%. This effect was specific because the activity of a −60 bp *hHSD3B2* reporter lacking GATA- and NR5A-regulatory elements was not significantly affected by the GATA DN competitor (Fig. 2A). A similar result was also obtained using RNA interference to silence GATA-4 expression (Fig. 2B), the predominant GATA factor present in MA-10 Leydig cells (Fig. 1C). The GATA-4 siRNA significantly decreased activity of the −1073 bp *hHSD3B2*-luciferase promoter construct by 45%, whereas a scrambled siRNA sequence used as a negative control (control siRNA) had no effect. Taken together, these results indicate that GATA factors contribute to *hHSD3B2* promoter activity in steroidogenic cells.

GATA Factors Synergize with Members of the NR5A Family of NRs (SF-1 and LRH-1) on the *hHSD3B2* Promoter

To better define the role of GATA factors in regulating *hHSD3B2* gene expression in steroidogenic tissues, we tested their ability to transcriptionally cooperate with other cell-restricted transcription factors. In addition to GATA-regulatory motifs, the *hHSD3B2* promoter contains an SF-1-regulatory element at −60 bp, and previous studies have revealed that SF-1 can activate *hHSD3B2* promoter activity through this site (13). Several other studies have also revealed the presence of another NR, named liver receptor homolog 1 (LRH-1/NR5A2), in all steroidogenic tissues (18, 22–26). LRH-1 is closely related to SF-1, and both receptors are known to bind to nearly identical regulatory elements. Moreover, Peng *et al.* (18) recently reported that LRH-1 can specifically bind to an *hHSD3B2* promoter element located at −320 bp and stimulate *hHSD3B2* promoter activity in ovarian granulosa cells. Since we previously reported that GATA factors could physically interact with SF-1 both *in vitro* and *in vivo* to synergistically activate other target genes (43, 50, 51), we tested whether synergisms between GATA-4 or GATA-6 and SF-1 or LRH-1 might also contribute to *hHSD3B2* gene transcription. As shown in Fig. 3A, SF-1 and LRH-1 by themselves are poor activators of the −1073 bp *hHSD3B2* promoter in both steroidogenic cells (MA-10 and H295R) and heterologous cells (CV-1 fibroblasts). Coexpression of these factors, however, in the presence of either GATA-4 or GATA-6 resulted in a strong synergistic activation of the −1073

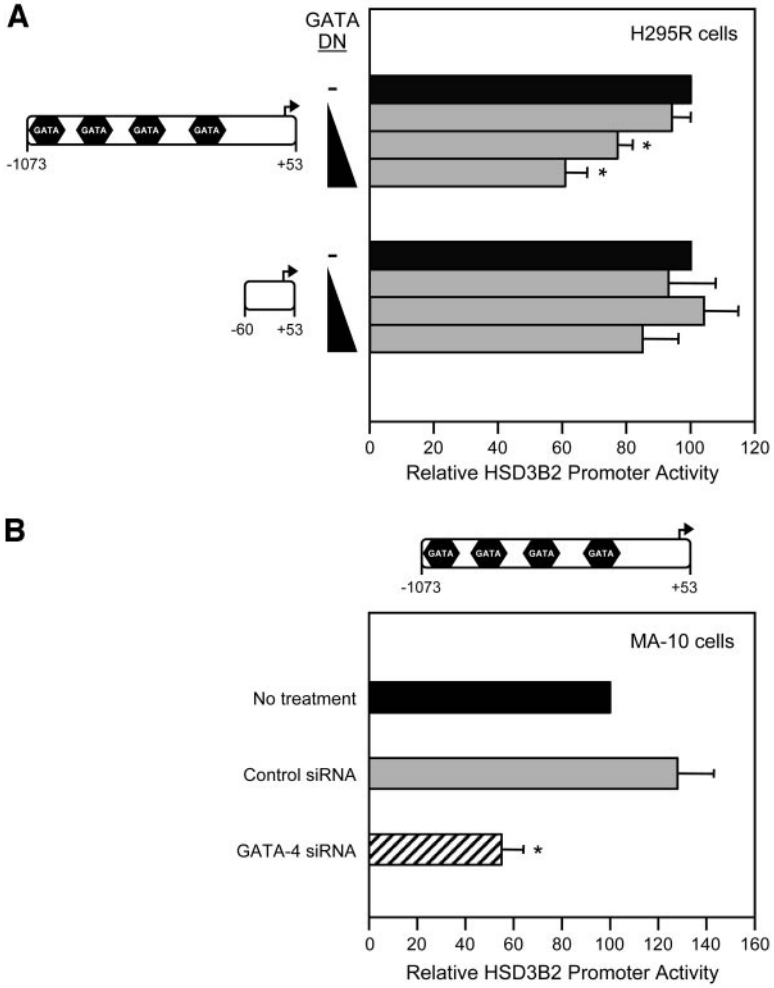

Fig. 2. Blockade of Endogenous GATA-4 Expression or Activity Blunts *hHSD3B2* Promoter Activity in Steroidogenic Cells

A, H295R cells were cotransfected with either the GATA-responsive −1073 bp *hHSD3B2* promoter or the minimal −60 bp *hHSD3B2* promoter along with increasing doses (5, 10, and 25 ng) of a truncated GATA-4 protein that acts as a dominant negative competitor of GATA activity (GATA DN). An empty expression vector (−) served as control. *hHSD3B2* promoter activity is expressed relative to the activity of each reporter cotransfected with the control expression vector (±SEM). *, Significantly different from control ($P < 0.05$; ANOVA followed by Tukey's test). B, MA-10 cells were transfected with the −1073 bp *hHSD3B2* promoter and received either no treatment or 36 nM of either control siRNA or siRNA specific for GATA-4. *hHSD3B2* promoter activities are expressed relative the untreated group (±SEM). ***,** Significantly different from both the untreated and control siRNA groups ($P < 0.05$; ANOVA followed by Tukey's test).

bp *hHSD3B2* promoter (Fig. 3B). The level of synergism was strongest in CV-1 cells (up to 80-fold), followed by H295R (up to 20-fold) and MA-10 cells (up to 10-fold). Moreover, GATA-4 and GATA-6 could synergize equally well with SF-1 or LRH-1.

The GATA Responsiveness of the *hHSD3B2* Promoter and Synergism with SF-1/LRH-1 Are Mediated through Proximal Regulatory Elements

In light of the important effect of exogenous and endogenous GATA factors on the *hHSD3B2* promoter (Figs. 1 and 2) and because the *hHSB3B2* promoter contains several consensus GATA elements (Fig. 1A), we next performed a deletion analysis of the *hHSD3B2* promoter to identify the key GATA-responsive sequences. As shown in Fig. 4A, a short (−224 bp) *hHSD3B2* promoter fragment that contains only one consensus GATA element at −196 bp and an SF-1/LRH-1 element at −60 bp was as active as its full-length counterpart (−1073 bp) in both MA-10 and H295R cells, indicating that these elements are sufficient for full *hHSD3B2* promoter activity, at least in these two steroidogenic cell types. To confirm the importance of the GATA regulatory element at −196 bp, this element was mutated by site-directed mutagenesis in context of the short −224 bp *hHSD3B2* reporter and used in transfection assays (Fig. 4B). In both MA-10 and H295R cells, mutation of this GATA element resulted in a 50% decrease in *hHSD3B2* promoter activity whereas in CV-1 cells, this same muta-

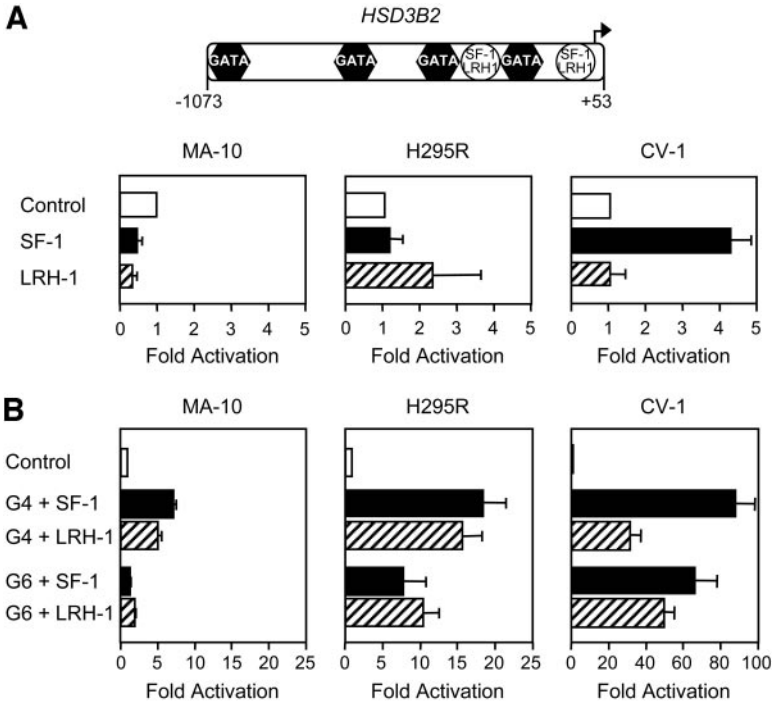

Fig. 3. Synergistic Activation of the *hHSD3B2* Promoter by GATA Factors and the Nuclear Receptors SF-1/LRH-1

A, SF-1 and LRH-1 by themselves are weak activators of *hHSD3B2* promoter activity. The −1073 bp *hHSD3B2* promoter was cotransfected in MA-10, H295R, and CV-1 cells with either an empty expression vector (control) or expression vectors for mouse SF-1 (10 ng) or human LRH-1 (10 ng). B, GATA factors (G4 and G6) synergize with NR5A family members (SF-1 and LRH-1) to enhance *hHSD3B2* promoter activity in both homologous (MA-10 and H295R) and heterologous (CV-1) cells. Cells were cotransfected with the −1073 bp *hHSD3B2* promoter and different combinations of GATA-4 or GATA-6 (25 ng) and SF-1 or LRH-1 (10 ng) as indicated. All results are shown as fold activation over the control (empty) expression vector (±SEM).

tion prevented transcriptional activation by GATA-4 and GATA-6 (Fig. 4B). An EMSA demonstrated that the GATA mutation introduced (GATA to GGTA) into the −224 bp *hHSD3B2* promoter construct does not compete GATA binding (Fig. 4C). The consensus GATA element at −196 bp is bound by recombinant GATA-4 protein (Fig. 4C, *right panel*) and by endogenous GATA-4 present in MA-10 cells as confirmed by supershift (Fig. 4C, *left panel*). Binding is displaced by unlabeled wild-type probe but not by one harboring the GATA mutation (Fig. 4C, *right panel*). Taken together, these data suggest that transactivation of the *hHSD3B2* promoter by GATA factors is likely mediated by the most proximal GATA element. We cannot exclude the possibility, however, that more distal GATA elements may also contribute to promoter activity in an *in vivo* context or in association with other transcription factors.

Using 5′-deletion constructs of the *hHSD3B2* promoter in CV-1 cells, we observed that synergisms between GATA-4 or GATA-6 and SF-1 or LRH-1 were unaffected by deletions up to −340 bp (Fig. 5). Synergisms were decreased when the region between −340 bp and −224 bp was removed, and completely abrogated with a deletion to −60 bp that removes all regulatory elements (Fig. 5). Interestingly, the region between −340 bp and −224 bp does not contain any GATA element and, as expected, deletion of this region did not abolish GATA-dependent activation of the *hHSD3B2* promoter (Fig. 5). As previously mentioned, this region contains an LRH-1/SF-1 binding site at −320 bp. Thus, these results indicate that although this element contributes to the synergism between GATA factors and SF-1 or LRH-1, it is not required, and that other elements located within the −224 bp fragment are sufficient to sustain GATA/SF-1 or GATA/LRH-1 synergisms (Fig. 5).

The −224 bp *hHSD3B2* promoter fragment contains one GATA element (at −196 bp) and one SF-1/LRH-1 element (at −60 bp). Mutation of the GATA element in context of the −224 bp reporter not only decreased *hHSD3B2* promoter activity in MA-10 and H295R cells but also severely blunted the synergism between GATA factors and SF-1 or LRH-1 in heterologous CV-1 cells (Fig. 6A). Because this mutation completely abrogated activation by GATA-4 and GATA-6 (Fig. 4B), the remaining synergistic activation was likely due to the direct interaction between GATA factors and DNA-bound SF-1 or LRH-1 to the −60 bp SF-1/LRH-1 regulatory element. The fact that we have previously described a similar mechanism for GATA/SF-1 synergism on the *MIS* promoter supports this hypothesis (50, 51). The critical requirement of the −60 bp SF-1/LRH-1 element was demonstrated by site-directed

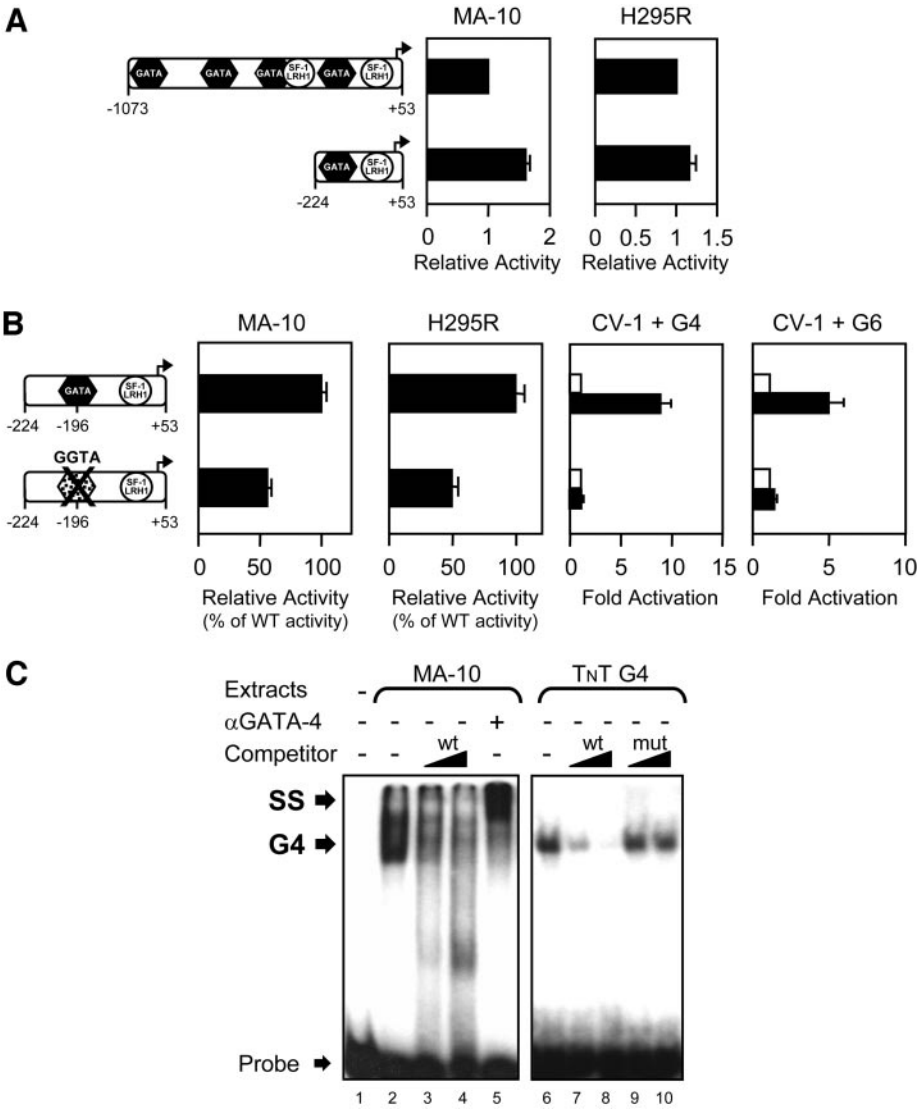

Fig. 4. Mapping of the GATA-Responsive Element in the *hHSD3B2* Promoter

A, Regulatory elements present within the proximal *hHSD3B2* promoter (−224 bp) are sufficient for full activity in homologous cells. To identify the GATA motifs that contribute to the GATA responsiveness of the *hHSD3B2* promoter, MA-10 and H295R cells were first transfected with the −1073 bp construct containing four consensus GATA elements and a −224 bp construct deleted of all but the most proximal GATA element. Data are expressed as promoter activity relative to the −1073 bp construct. B, The GATA element at −196 bp contributes to *hHSD3B2* promoter activity in MA-10 and H295R cells and is essential for GATA responsiveness in heterologous CV-1 fibroblasts. Cells were transfected as described in *Materials and Methods* using the wild-type (WT) or GATA mutated −224bp *hHSD3B2* reporter and where indicated, either in the presence of GATA-4 (25 ng) or GATA-6 (25 ng). For MA-10 and H295R cells, promoter activities are expressed relative to the WT construct and as fold activation (±SEM) over the control (empty) expression vector for the experiments in CV-1 cells. C, EMSA was used to assess the binding of either endogenous GATA-4 (MA-10 cells) or *in vitro* produced GATA-4 to a double-stranded ^{32}P-labeled oligonucleotide corresponding to the proximal GATA element (at −196 bp) of the *hHSD3B2* promoter. For the MA-10 cells, GATA binding (G4) was supershifted (SS) with a GATA-4 antiserum (lane 5). Using recombinant GATA-4 protein, GATA-4 binding was specifically competed by an excess (2× and 5×) of unlabeled oligonucleotides (lanes 7 and 8), but not by mutated oligonucleotides in which the GATA element had been changed to GGTA (lanes 9 and 10).

mutagenesis, also in context of the −224 bp reporter (Fig. 6A). This mutation (TCAAGGTAA into TCAATTTAA), which completely prevents SF-1 and LRH-1 binding (Fig. 6B), markedly reduced *hHSD3B2* promoter activity in both MA-10 and H295R cells and GATA-4/SF-1 and GATA-6/SF-1 synergisms on the *hHSD3B2* promoter in heterologous CV-1 cells (Fig. 6A). Taken together, these results indicate that the proximal SF-1/LRH-1 element (at −60 bp) is the cornerstone for synergism between SF-1 or LRH-1 and GATA factors and that the distal SF-1/LRH-1 element (at −320 bp) can amplify this synergistic activation.

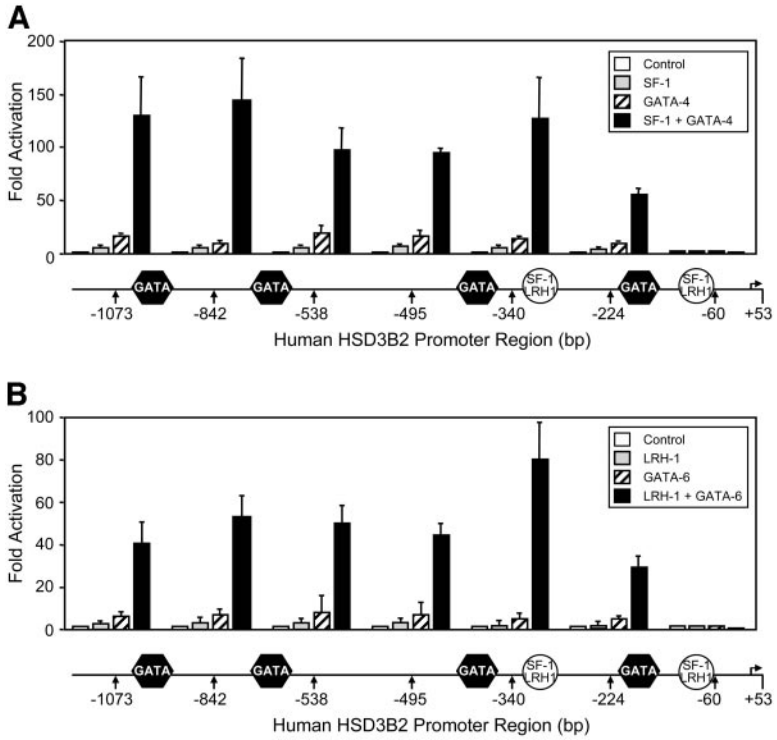

Fig. 5. Mapping of the Minimal *hHSD3B2* Promoter Region Required for Synergism between GATA Factors and SF-1/LRH-1

CV-1 cells were cotransfected with various 5′-deletion constructs of the *hHSD3B2* promoter (the 5′-end point of each construct is represented by *arrows under the graph*) with either an empty expression vector (control, *open bars*) or expression vectors for GATA-4 (25 ng) and SF-1 (10 ng) alone or in combination (A); or GATA-6 (25 ng) and LRH-1 (10 ng) either alone or in combination (B). Similar results were obtained when GATA-4 was used in combination with LRH-1 and GATA-6 with SF-1. The positions of the four consensus GATA elements (*hexagons*) and the two previously described SF-1/LRH-1 binding sites (*circles*) are shown. Results are shown as fold activation over the empty (control) expression vector (±SEM).

LRH-1 Is a New Transcriptional Partner for GATA Factors

In the gonads, a major transcriptional partner for GATA factors is SF-1 (50). Because the gonads also express LRH-1, this factor might be a new partner for GATA proteins in gonadal cell types, such as granulosa cells of the ovary, that are known to strongly express GATA factors (GATA-4 and GATA-6) but not necessarily SF-1. Indeed, we have reported here that cooperation between LRH-1 and GATA-4 or GATA-6 leads to a strong synergistic activation of the *hHSD3B2* promoter in both homologous and heterologous cells (Fig. 3B). We previously reported that transcriptional synergism between GATA-4 and SF-1 is the result of a direct physical interaction between the two proteins (50). We now show that LRH-1 can also physically interact with GATA-4 or GATA-6 (Fig. 7). As shown in Fig. 7A, LRH-1 could be coimmunoprecipitated with GATA-4 in HeLa cells overexpressing both factors, indicating that both proteins can interact within a cellular context. Unfortunately, the lack of a good (high affinity) GATA-6 antibody prevented us from performing similar experiments between LRH-1 and GATA-6. As shown in Fig. 1C, the commercial GATA-6 antibody used could only weakly detect recombinant GATA-6 protein, making it unsuitable for immunoprecipitation studies. To circumvent this problem and to demonstrate that GATA-6 can at least physically interact with GATA-6, *in vitro* pull-down experiments were performed using a bacterially produced 6xHIS-tagged LRH-1 fusion protein and *in vitro* translated ^{35}S-labeled GATA-4 or GATA-6 proteins (Fig. 7B). Both GATA-4 and GATA-6 were specifically retained by the immobilized HIS-LRH-1 fusion protein, demonstrating that GATA-6, much like GATA-4, can physically interact with LRH-1. No labeled protein was retained when a HIS-LacZ fusion was used as control, indicating that the GATA/LRH-1 interaction was not mediated by the HIS moiety of the fusion protein. The fact that the physical and transcriptional synergisms observed were not limited to specific GATA factors or to either SF-1 or LRH-1 suggests that this mechanism might contribute to *hHSD3B2* transcription in both the adrenals and gonads despite the differential expression of GATA factors, SF-1, and LRH-1 in these tissues.

DISCUSSION

The 3β-HSD2 enzyme plays an essential role in the biosynthesis of all classes of active steroid hormones.

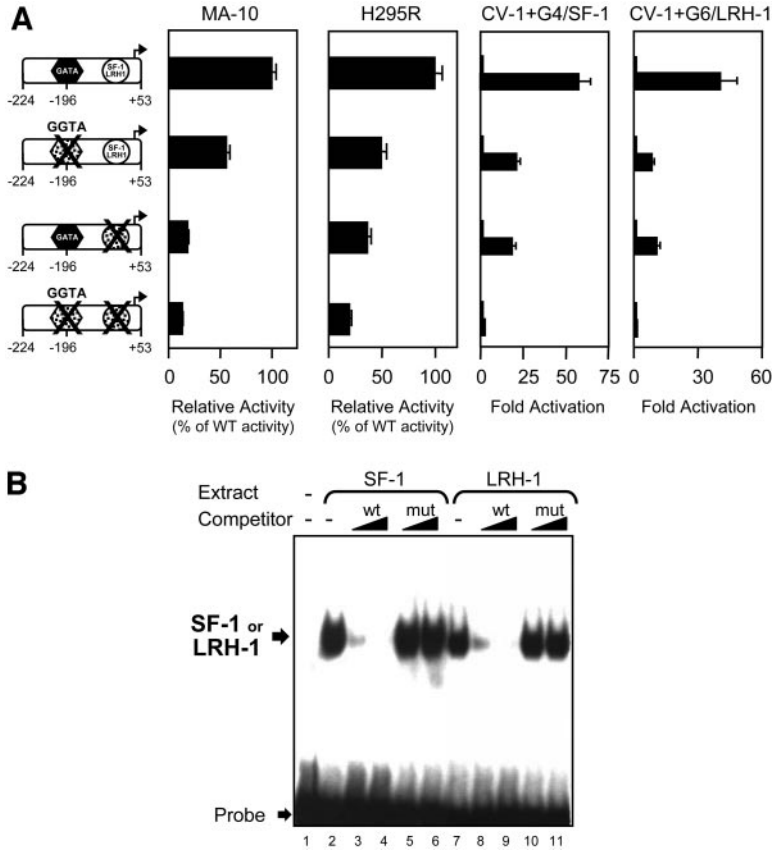

Fig. 6. Synergism between GATA Factors and NR5A Family Members on the *hHSD3B2* Promoter Requires Intact GATA and SF-1/LRH-1 Regulatory Elements in Its Proximal Promoter Region

A, To assess the binding site requirements for synergism between GATA factors and SF-1/LRH-1, several *hHSD3B2* promoter constructs were used: −224 bp wild-type (WT) (containing intact GATA and SF-1/LRH-1 elements), −224 bp mutant (mut) GATA, −224 bp mut SF-1/LRH-1, and −224 double mut (in which both the GATA and SF-1/LRH-1 elements were mutated). In MA-10 and H295R cells, both elements are required for full *hHSD3B2* promoter activity. In heterologous CV-1 cells, synergisms between GATA-4 and SF-1 and between GATA-6 and LRH-1 are markedly decreased when either the GATA or SF-1/LRH-1 elements are mutated. Similar results were obtained when GATA-4 was used in combination with LRH-1 and GATA-6 with SF-1. Cells were transfected as described in *Materials and Methods* using GATA-4 or GATA-6 (25 ng) and SF-1 or LRH-1 (10 ng) as indicated. For MA-10 and H295R cells, promoter activities are expressed relative to the WT −224 bp construct and as fold activation (±SEM) over the control (empty) expression vector for the experiments in CV-1 cells. B, EMSA was used to assess the binding of *in vitro* produced SF-1 and LRH-1 to a double-stranded ^{32}P-labeled oligonucleotide corresponding to the proximal SF-1/LRH-1 site (at −60 bp) of the *hHSD3B2* promoter. Binding of both proteins (lanes 2 and 7) was equally and specifically competed by increasing doses (*black triangles*; molar excesses of 2× and 5×) of unlabeled oligonucleotides (lanes 3/4 and 8/9) but not by oligonucleotides in which the SF-1/LRH-1 element has been mutated from TCAAGGTAA to TCAATTTAA (lanes 5/6 and 10/11).

The importance of this enzyme is underscored by the numerous human pathologies and/or syndromes, such as CAH, male pseudohermaphroditism, and PCOS, that are associated with deregulated *hHSD3B2* expression (6, 7, 9–12). Therefore, identifying the factors involved in controlling proper spatiotemporal *hHSD3B2* expression is key to our understanding of these endocrine disorders. Unfortunately, our knowledge of the transcriptional regulation of the *hHSD3B2* gene has been limited. So far, only a few transcription factors, which function as weak activators, have been shown to play a role in the regulation of *hHSD3B2* promoter activity (13–20). In the present study, we now report that the human *HSD3B2* promoter is also a target for members of the GATA family of transcription factors.

The *hHSD3B2* Gene Is an Important Target for GATA Factors in Steroidogenic Tissues

Although the *hHSD3B2* gene was isolated more than a decade ago (52), surprisingly little is known about the transcriptional mechanisms that control its expression in steroidogenic tissues. Previous studies of the *hHSD3B2* promoter have identified one binding site for the STAT5 and STAT6 factors (14–16) and three regulatory elements that are recognized by members of the NR5A (SF-1 and LRH-1) and NR4A (Nur77) families

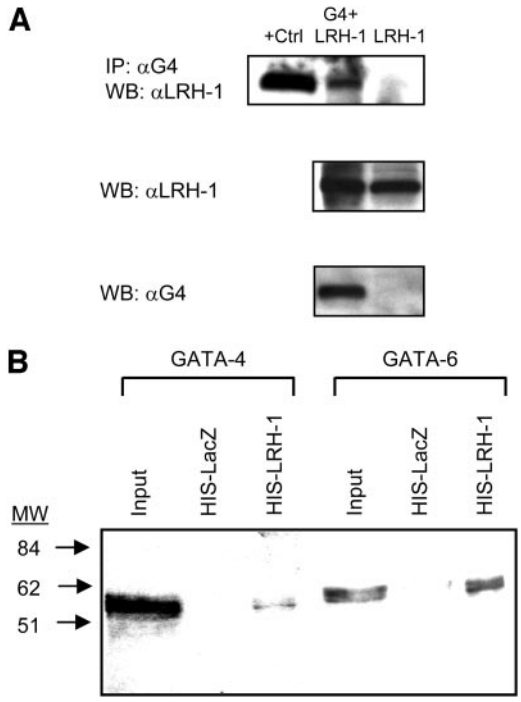

Fig. 7. LRH-1 interacts with both GATA4 and GATA6

A, Coimmunoprecipitation of GATA-4 and LRH-1. HeLa cells were transfected with expression vectors for GATA-4 and LRH-1 or LRH-1 alone. Aliquots (80 μg) of nuclear extract were immunoprecipitated (IP) with a GATA-4 antiserum. A 10-μg aliquot of nuclear extract from HeLa cells overexpressing LRH-1 was directly loaded onto the gel to reveal the position of the LRH-1 protein (+Ctrl). The IPs were immunoblotted [Western blot (WB)] with a LRH-1 antiserum to reveal a GATA4/LRH-1 interaction. The nuclear extracts used in the IPs were also directly immunoblotted (10 μg per lane) to control for the specificity of the GATA-4 and LRH-1 antisera used. B, *In vitro* pull-down (protein-protein) assays were used to determine whether LRH-1 could physically interact with GATA-4 and/or GATA-6. Pull-down assays were performed using an immobilized, bacterially produced histidine (HIS)-tagged fusion proteins HIS-LRH-1 or HIS-β-galactosidase (HIS-LacZ; negative control) and either *in vitro* translated ^{35}S-labeled GATA-4 or GATA-6 proteins. Inputs represent 10% of the total ^{35}S-labeled protein used in each assay. Protein molecular mass (MW) markers in kilodaltons are indicated on the *left*. As shown, LRH-1 can physically interact with both GATA-4 and GATA-6. Ctrl, Control.

of nuclear receptors (13, 18–21). Consistent with a role in tissue-specific *hHSD3B2* expression, STAT5, STAT6, SF-1, LRH-1, and Nur77 are all strongly expressed in steroidogenic tissues (27–30, 53–55). However, these factors are also expressed in tissues such as the pituitary, liver, and pancreas in which *hHSD3B2* is not expressed (53, 56, 57). Thus, the participation of other factors is required to restrict *hHSD3B2* expression to the adrenals and gonads *in vivo*. Our present data suggest that GATA-4 and GATA-6, which are potent regulators of *hHSD3B2* promoter activity, might be two of these factors.

GATA-4 and GATA-6 are coexpressed with *hHSD3B2* in the adrenals and gonads beginning early in development and lasting throughout adulthood (33–41). In these tissues, they are known to regulate transcription of a variety of genes through consensus WGATAR regulatory elements found in their respective promoter regions (9, 31, 34, 42–49). Similarly, we found that the *hHSD3B2* promoter contains four consensus GATA elements (Fig. 1A). It is only the most proximal one, however, located at −196 bp, that is sufficient to confer GATA responsiveness of the *hHSD3B2* promoter in heterologous cells and that is required for full *hHSD3B2* promoter activity in steroidogenic cells (Fig. 4B). Our data in MA-10 and H295R cells (Fig. 4B) are consistent with the drop in promoter activity in granulosa cells, which Peng *et al.* (18) had observed in an *hHSD3B2* promoter deletion that happened to remove the GATA element at −196 bp. Thus, the *hHSD3B2* gene is an important target for GATA factors in steroidogenic cells.

GATA Factors and SF-1/LRH-1 Are Mutual Cofactors

Much like STAT5, SF-1, and LRH-1, GATA factors are too broadly expressed for them alone to account for the specificity of *hHSD3B2* expression. As previously mentioned, however, the *hHSD3B2* promoter also contains two consensus SF-1/LRH-1 regulatory elements (13, 18). Deletion or mutation of either of these elements resulted in decreased *hHSD3B2* promoter activity in steroidogenic H295R adrenal cells (Fig. 6A and Ref. 13) and in ovarian granulosa cells (18). Because we previously reported that GATA-4 and GATA-6 could cooperate with SF-1 to synergistically activate transcription from several gonadal promoters (43, 50), we surmised that GATA/SF-1 synergism might also contribute to the transcription of the *hHSD3B2* gene. Indeed, we found that coexpression of GATA factors (GATA-4 or GATA-6) and NR5A family members (SF-1 and LRH-1) led to a striking synergistic activation (up to 100-fold) of the *hHSD3B2* promoter. Interestingly, the level of activation we observed between GATA-4 or GATA-6 and SF-1 in heterologous CV-1 fibroblasts was similar to what has been previously reported for SF-1 alone on the *hHSD3B2* promoter in HeLa cells (13). Because HeLa cells are well known to express GATA factors (58, 59), the strong *hHSD3B2* promoter activation attributable to SF-1 in the study of Leers-Sucheta *et al*. (13) was likely the result of synergism between endogenous GATA factors expressed by HeLa cells and the exogenously expressed (transfected) SF-1.

In addition to SF-1, we now report for the first time a similar transcriptional cooperation between GATA factors and the nuclear receptor LRH-1 (Fig. 3B). As previously reported for SF-1 (50, 51), synergism between GATA (GATA-4 or GATA-6) and LRH-1 is also the result of a direct physical interaction between the two proteins (Fig. 7). The synergism between GATA

factors and nuclear receptors that bind DNA as monomers appears to be specific to the NR5A family (SF-1 and LRH-1) because no synergism was observed between GATA factors and the NR4A1 (Nur77) orphan nuclear receptor (data not shown). Thus, specific synergisms between GATA factors and NR5A nuclear receptors appear to be an important contributing mechanism for the tissue- and cell specificity of a number of genes expressed in steroidogenic tissues.

Gene Dosage and Its Implication for *hHSD3B2* Gene Expression in Both Health and Disease

Mutations in the *hHSD3B2* gene leading to 3β-HSD2 deficiency are responsible for some cases of CAH and male pseudohermaphroditism. Alternatively, mutations and/or aberrant expression of transcription factors essential for *hHSD3B2* expression might also be involved in some pathologies/syndromes associated with deregulated *hHSD3B2* expression. For instance, two human *SF-1* gene mutations, a dominant *de novo* heterozygous G35E mutation and a recessive homozygous R92Q mutation, have been shown to be responsible for adrenal insufficiency and male pseudohermaphroditism (60, 61). We found that one of these SF-1 mutants (SF-1 G35E), failed to synergize with GATA factors on the *hHSD3B2* promoter (data not shown), thus providing an additional molecular basis for the phenotype associated with the SF-1 G35E mutation. As another example, overexpression of the *hHSD3B2* gene in ovarian theca cells has been associated with another human syndrome, PCOS (9–11). Although the exact cause of aberrant *hHSD3B2* expression in PCOS remains to be elucidated, a recent differential genetic screen of PCOS ovaries have identified that the GATA-6 transcription factor is also upregulated in theca cells (9). Because we have shown that GATA-6 can directly activate the *hHSD3B2* promoter (Fig. 1) and can interact and transcriptionally cooperate with both SF-1 and LRH-1 (Figs. 3 and 7), it is tempting to speculate that deregulated expression of GATA-6 in theca cells might be a contributing factor for *hHSD3B2* overexpression in PCOS. Thus, our data not only highlight the importance of GATA and SF-1/LRH-1 transcriptional cooperation for the tissue- and cell-specific regulation of the *hHSD3B2* gene but also provide new insights in which modulation of transcriptional cooperation between GATA and SF-1/LRH-1 factors might be linked to pathological conditions in humans.

MATERIALS AND METHODS

Plasmids

The −1073 bp to +53 bp *hHSD3B2* promoter fragment was obtained by PCR using human genomic DNA as template along with a forward primer containing a *Bam*HI (shown in *italics*) cloning site (5′-*CGGGATCC*GGAAAATATAGGAATAAAGTGGG-3′) and a common reverse primer containing a *Kpn*I (shown in *italics*) cloning site (5′-*GGGGTACC*CGTAACTTAGATTGTTAAAAGCTGG-3′). Deletions of the *hHSD3B2* promoter to −842 bp, −538 bp, −495 bp, −340 bp, −224 bp, and −60 bp were obtained by PCR using the −1073 bp *hHSD3B2* promoter as template, along with the same reverse primer described above and the following forward primers: −842 bp, 5′-*CGGGATCC*CCTTGTAATGCCCAGATTACATC-3′; −538 bp, 5′-*CGGGATCC*TCCATTAGGAACCCAGAGCTCTCC-3′; −495 bp, 5′-*CGGGATCC*GGTTTTGGATATATTGGGTGGAAAAG-3′; −340 bp, 5′-*CGGGATCC*CCAGGTGGATTTACTGTACAAGGAC-3′; −224 bp, 5′-*CGGGATCC*CTGTTAAGGCTAAAGCCAAGAC-3′; −60 bp, 5′-*CGGGATCC*GGTAATAAGGGCTGAGACACAAGC-3′. The −224 bp *hHSD3B2* construct containing a mutation of the GATA element at position −196 bp (−224 bp mut GATA) was obtained by PCR using the wild-type −224 bp reporter as template along with the common reverse primer described above and the forward primer (the mutation is *underlined*) 5′-*CGGGATCC*TGTTAAGGCTAAAGCCAAGACTCTTTACCACACTGTGGC-3′. The −224 bp reporter containing a mutation of the SF-1/LRH-1 element at −60 bp was obtained by site-directed mutagenesis of the −224 bp wild-type reporter using the QuikChange XL mutagenesis kit (Stratagene, La Jolla, CA) and the following pair of oligos (the mutation is *underlined*): sense: 5′-GAGTATGTGGCAGGAGTTCAATTTAATAAGGGCTGAGACACAAG-3′, antisense: 5′-CTTGTGTCTCAGCCCTTATTAAATTGAACTCCTGCCACATACTC-3′. The −224 bp construct harboring mutations in both the GATA and SF-1/LRH-1 elements (−224 bp double mut) was obtained as described above but using the −224 bp mut GATA construct as template. The various promoter fragments were cloned into the corresponding site of a modified pXP1 luciferase reporter plasmid (43, 50) and verified by sequencing. Expression vectors for full-length rat GATA-4, rat GATA-6, the GATA dominant negative competitor (GATA DN), and mouse SF-1 have also been previously described (43, 51, 62). The human LRH-1 expression vector (63) was kindly provided by Dr. Luc Bélanger (Centre de recherche en cancérologie de l'Hôtel-Dieu de Québec, Université Laval).

Cell Culture and Transfections

Mouse Leydig MA-10 cells (64), provided by Dr. Mario Ascoli (University of Iowa, Iowa City, IA), were grown in Waymouth's MB752/1 medium supplemented with 20 mM HEPES, 15% horse serum, and 50 mg/liter of gentamycin and streptomycin sulfates at 37 C and 5% CO_2. Human adrenal H295R cells were obtained from ATCC (Manassas, VA) and grown in DMEM:Ham's F12 (1:1) medium supplemented with 15 mM HEPES, 1.2 g/liter of $NaHCO_3$, 2.5% NuSerum, and 10 ml/liter of ITS+ Premix (BD Biosciences, Mississauga, Canada) at 37 C and 5% CO_2. All transfections were done in 12-well plates using the Lipofectamine 2000 method (Invitrogen Canada, Burlington, Canada) as previously described (20). HeLa cells were grown in DMEM/F12 containing 10% fetal bovine serum. African green monkey kidney CV-1 fibroblast cells were grown in DMEM supplemented with 10% newborn calf serum at 37 C and 5% CO_2. Transfections of CV-1 and HeLa cells were performed using the calcium phosphate precipitation method (65) as previously described (43, 66). For the GATA-4 siRNA experiment, MA-10 cells were seeded in 12-well plates and transfected in serum- and antibiotic-free medium using 10 μl of Lipofectamine reagent (Invitrogen Canada) along with 1.5 μg of *hHSD3B2*-luciferase reporter and 36 nM of either control siRNA (catalog no. SC-37007, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or mouse GATA-4 siRNA (catalog no. SC-35454, Santa Cruz Biotechnology, Inc.). The next morning, the medium was changed and the cells were incubated for an additional 24 h and then finally harvested and analyzed for luciferase activity. The phRL-TK *Renilla* luciferase vector (10 ng) was used as internal control and the Dual Luciferase Assay System (Promega Corp., Mad-

ison, WI) was used to measure luciferase activities. For all experiments, the data reported represent the average of at least three experiments, each done in duplicate.

DNA-Binding Assays

Recombinant GATA-4, GATA-6, SF-1, and LRH-1 proteins were *in vitro* translated using the T_7 QuickCoupled TnT system (Promega Corp.). MA-10 nuclear extracts were prepared by the procedure outlined by Schreiber *et al.* (67). DNA binding assays were performed as described previously (38) using 5 μg protein. When working specifically with *in vitro* translated proteins, 2-μl aliquots of protein and 100 ng of polydeoxyinosinic-deoxycytidylic acid were used. GATA-4 binding in MA-10 cells was supershifted by adding a 1-μl aliquot of GATA-4 antibody (catalog no. SC-1234X; Santa Cruz Biotechnology). The ^{32}P-labeled double-stranded oligonucleotides used as probes were as follows: *hHSD3B2* GATA element (shown in *bold*) at −196 bp (sense: 5′-GATCCGACTCT**TTATCA**CACTGTA-3′ and antisense: 5′-GATCTACAGTG**TGATAA**AGAGTCG-3′); *hHSD3B2* SF-1/LRH-1 element (shown in *bold*) at −60 bp (sense: 5′-GATCCGGAGT**TCAAGGTAA**TAAGAA-3′, antisense: 5′-GATCT TCTTA**TTACCTTGA**ACTCCG-3′). For the competition experiments, double-stranded oligonucleotides corresponding to mutated versions of the GATA and SF-1 elements were used. The sequences of the oligonucleotides are the same as those described above except that the GATA element was mutated into GGTA, and the SF-1 element was changed from TCAAGGTAA to TCAATTTAA.

Coimmunoprecipitation, Western Blot, and *in Vitro* Protein Interaction Assays

Coimmunoprecipitation. Nuclear extracts were prepared from HeLa cells transfected with expression vectors for LRH-1 or a combination of GATA-4 and LRH-1. An 80-μg aliquot of each extract was then immunoprecipitated overnight using a GATA-4 polyclonal antiserum (Santa Cruz Biotechnology) in lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Igepal (Sigma-Aldrich Canada, Oakville, Canada)] supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich Canada), 1.0 μg/ml pepstatin A (Sigma-Aldrich Canada), and 1.0 μg/ml aprotinin (Sigma-Aldrich Canada), and 1.0 μg/ml BSA (Sigma-Aldrich Canada) at 4 C. Immune complexes were then collected by incubation with 10 μl of protein G-Sepharose (Amersham Biosciences, Baie-D'Urfé, Canada) beads at 4 C for 1 h. The beads were washed three times in lysis buffer and twice in PBS. Immune complexes were eluted by boiling for 5 min with 1×Laemmli buffer. After centrifuging, the supernatants were separated by SDS-PAGE and then electrotransferred to Hybond polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). LRH-1 proteins were detected using a commercially available LRH-1 polyclonal antiserum (catalog no. SC-25389, Santa Cruz Biotechnology) and a VECTASTAIN-ABC-Amp Western blot detection kit (Vector Laboratories Canada, Burlington, Canada).

Western Blot. Aliquots (10 μg) of nuclear extracts from MA-10 and H295R cells were separated by SDS-PAGE and then electrotransferred to Hybond PVDF membranes (Amersham Biosciences). GATA-4 and GATA-6 proteins were detected using commercially available GATA-4 and GATA-6 polyclonal antisera (Santa Cruz Biotechnology) and a VECTASTAIN-ABC-Amp Western blot detection kit.

***In Vitro* Pull-Down Assay.** The 6×HIS (histidine)-tagged LRH-1 fusion construct (HIS-LRH-1) was made by subcloning the human LRH-1 cDNA in frame with the 6×HIS using the commercial pRSET expression vector (Invitrogen Canada). The 6×HIS-β-galactosidase (HIS-LacZ) fusion protein was provided by the pRSET vector itself. Fusion proteins were produced in the *Escherichia coli* strain BL21/pLysS (DE3) after induction with isopropyl-β-D-thiogalactopyranoside. Bacterial cultures were lysed by sonication, and the fusion proteins were purified using the BD TALON metal affinity resin (BD Biosciences) as outlined by the manufacturer. The HIS-LRH-1 fusion protein was used in *in vitro* pull-down experiments (direct physical interactions) using *in vitro* translated ^{35}S-labeled full-length GATA-4 or GATA-6 proteins which were produced using the TnT *in vitro* transcription/translation kit (Promega). Protein-protein interaction assays performed done using 500 ng of fusion protein and 5.0 μl of *in vitro* translated ^{35}S-labeled protein as previously described (50). Bound complexes were separated by SDS-PAGE and transferred onto PVDF. Retained proteins were revealed by autoradiography.

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