

# RAPID COMMUNICATION

## Liver receptor homologue-1 is expressed in the adrenal and can regulate transcription of 11 $\beta$ -hydroxylase

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### ABSTRACT

Liver receptor homologue-1 (LRH-1, designated NR5A2) is a mammalian homologue of *Drosophila fushi tarazu* factor (dFTZ-F1) and structurally belongs to the orphan nuclear receptor superfamily. LRH-1 can recognize the DNA sequence 5'-AAGGTCA-3', the canonical recognition motif for steroidogenic factor 1 (SF-1). Herein, we hypothesized that LRH-1 might play a role in the regulation of human adrenal expression of steroidogenic enzymes. To test this hypothesis, LRH-1 expression in human adult and fetal adrenal glands was examined by RT-PCR analysis. The fetal and adult adrenal glands, as well as liver and pancreas, were observed to express LRH-1 mRNA using RT-PCR. The ability of LRH-1 to enhance transcription of the gene encoding human 11 $\beta$ -hydroxylase (*hCYP11B1*) was then examined using

the H295R adrenal cell line. LRH-1 co-transfection with *hCYP11B1* luciferase promoter constructs caused a 25-fold induction of luciferase activity. Furthermore, co-transfection of a *hCYP11B1* reporter construct containing a mutation in the SF-1 binding cis-element abolished the stimulatory effect of both SF-1 and LRH-1. Electrophoretic mobility shift assay (EMSA) demonstrated that LRH-1 could bind to the SF-1 response element. Taken together, our data suggested that LRH-1 is expressed in the adrenal, and can substitute for SF-1 to enhance transcription of genes encoding certain of the steroid-metabolizing enzymes. A role for LRH-1 in the regulation of adrenal or gonadal steroid hormone production should be further studied.

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### INTRODUCTION

Liver receptor homologue-1 (LRH-1), also known as  $\alpha$ -fetoprotein transcription factor (FTF), *CYP7A* promoter-binding factor (CPF), PHR-1, hB1F, and now as NR5A2 is an orphan nuclear receptor expressed in multiple tissues (Becker-Andre *et al.* 1993, Gallarneau *et al.* 1996, Li *et al.* 1998, Nitta *et al.* 1999, Repa & Mangelsdorf 1999, Nuclear Receptors Nomenclature Committee 1999). LRH-1 and steroidogenic factor 1 (SF-1) appear to be closely related proteins that have both evolved from the duplication of an ancestral gene, *Drosophila fushi tarazu* factor (dFTZ-F1). SF-1 and LRH-1 are members of the nuclear receptor superfamily that recognize the canonical recognition motif for FTZ-F1 receptors and bind to DNA as

monomers to enhance transcription of targeted genes. SF-1 and LRH-1 have different patterns of tissue expression. SF-1 is primarily expressed in steroidogenic tissues and plays a crucial role in regulating steroidogenic enzymes and is involved in the development of the gonads and adrenals (Lala *et al.* 1992, Morohashi *et al.* 1992, Luo *et al.* 1994). LRH-1 however, is expressed in liver, intestine, colon, and pancreas (Becker-Andre *et al.* 1993, Gallarneau *et al.* 1996, Li *et al.* 1998, Nitta *et al.* 1999, Repa *et al.* 1999). The ability of both transcription factors to enhance transcription through similar *cis*-elements makes it likely that they may share target genes in selected tissues. Indeed, there are recent reports of ovarian expression of LRH-1 in mouse (Repa *et al.* 1999) and equine ovaries (Boerboom *et al.* 2000). However

effects on steroidogenic enzymes were not studied. Herein, we demonstrated that LRH-1 is expressed in human adrenal tissue and is able to stimulate reporter genes prepared with the 5'-flanking region of human 11 $\beta$ -hydroxylase (*hCYP11B1*) through the same cis-element used by SF-1.

## MATERIALS AND METHODS

### Reverse transcriptase PCR (RT-PCR)

Total RNA (1  $\mu$ g) prepared from human adrenal, liver and pancreas was used for reverse transcription (RT) reaction in a final volume of 20  $\mu$ l. Human adult and fetal adrenal gland use was approved by the Institutional Review Board of UT Southwestern Medical Center. Pancreas and liver RNA were obtained from Clontech (Palo Alto, CA, USA). PCR was performed using 2  $\mu$ l of RT product as template with human LRH-1 specific primers (forward primer: 5'-TGAAGCTGCTTCAGAAC TGC-3'; reverse primer: 5'-CCGTTTCAGGTGC TTGTAGTA-3'). The PCR conditions were 94 C for 20 sec, 54 C for 20 sec, 72 C for 40 sec. To insure that detected bands did not arise as a result of DNA contamination, LRH-1 primers were designed to amplify from exon 5 to 7 (across two introns) within the LRH gene. PCR product (10  $\mu$ l) of each sample was loaded and electrophoresed on 1% agarose gel.

### Preparation of *hCYP11B1* reporter construct and SF-1 and LRH-1 expression vectors

An 1102-bp fragment (pB1-1102) extending from position -2 (relative to the transcriptional start site) to -1102 of the *hCYP11B1* promoter region was cloned into the promoterless pGL3-basic luciferase reporter vector (Promega, Madison, WI, USA) (Wang *et al.* 2000). The preparation of the *hCYP11B1* reporter construct with a mutation in the SF-1 binding site (termed Ad4) was described previously (Wang *et al.* 2000). The SF-1 and LRH-1 sequences were expressed using the pcDNA 3.1 vector (Invitrogen, Carlsbad, CA, USA).

### Cell culture and transfection experiments

NCI-H295R (H295R) human adrenal tumor cells were cultured as previously described (Bird *et al.* 1993, Wang *et al.* 2000). Transfection was carried out for 6 h using 2.0  $\mu$ l Fugene 6 (Roche, Indianapolis, IN, USA) and 1.0  $\mu$ g pB1-1102 reporter plasmid DNA following the manufacturer's protocol. For co-transfection experiments, LRH-1 or SF-1 expression plasmid was included in the transfection, and the total amount of DNA was

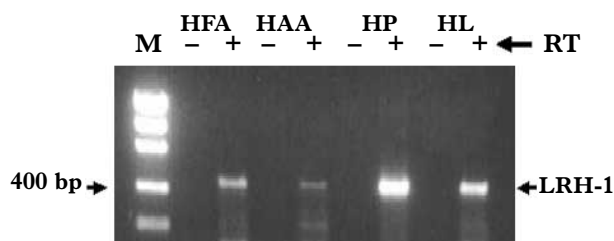


FIGURE 1. LRH-1 is expressed in the human adrenal as well as liver and pancreas. Total RNA (1  $\mu$ g) from human adult adrenal (HAA), human fetal adrenal (HFA), human liver (HL) and human pancreas (HP) were studied with (+) or without (-) reverse transcriptase (RT). Specific primers for human LRH were used to amplify a 400 bp product for 35 cycles.

kept constant at 2.0  $\mu$ g/ml by addition of pcDNA3.1 empty vector. Following transfection, the cells were incubated with low serum medium for 24 h. The cells were then lysed and the luciferase activities determined using luciferase assay (Promega).

### Electrophoretic Mobility Shift Assay (EMSA)

H295R cell nuclear extracts (NE) were prepared as previously described (Wang *et al.* 2000). Double-stranded oligonucleotide (25 pmol) containing the Ad4 *hCYP11B1* SF-1 response element (5'TGAATAATCCAAGGCTCTTGGATA3') was labeled with [ $\alpha$ -<sup>32</sup>P] dCTP by Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. The probe (40 000 c.p.m.) was incubated with 5  $\mu$ g H295R nuclear extract (NE) and 1  $\mu$ g poly (dI-dC) to block nonspecific binding. The human SF-1, LRH-1 and mouse LRH-1 were synthesized by coupled *in vitro* transcription/translation by T7 polymerase using the reticulocyte lysate system (Promega). The reactions were incubated at room temperature for 20 min. The resulting DNA/protein complexes were separated from free probe by electrophoresis on 4% polyacrylamide gel.

## RESULTS AND DISCUSSION

To study LRH-1 expression, hLRH-1 specific primers were designed to span two introns to insure specificity for mRNA. After 35 cycles of amplification, the expected 400 bp LRH-1 fragment was observed in adult and fetal adrenal RNA as well as RNA from liver and pancreas (Fig. 1). No PCR products were observed in the absence of reverse transcriptase. H295R cells were also found to express LRH-1 transcript (data not shown). These data demonstrate that the adrenal tissue and the H295R adrenocortical cells express LRH-1 mRNA.

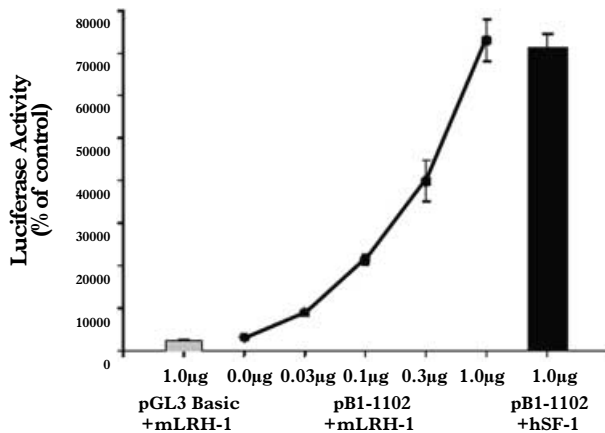


FIGURE 2. LRH-1 can transactivate the *hCYP11B1* promoter. The pB1-1102 construct (1 µg/well) was co-transfected with the indicated amounts of hSF-1 or mLRH-1 expression plasmid into H295R cells. Following recovery, cells were lysed and luciferase activity was measured. Results are expressed as a percentage of the basal reporter activity and represent the mean  $\pm$  S.E.M. of data from three independent experiments.

A previous screening of mouse tissues using Northern analysis failed to show expression of mLRH-1 in adrenal (Repa *et al.* 1999). To address this discrepancy, we studied LRH-1 expression in the human adrenal by Northern hybridization. A weak signal for LRH-1 transcript was observed using human adrenal RNA by Northern analysis (data not shown). The signal was considerably lower than that observed for the pancreas or liver positive controls suggesting that LRH-1 expression is either low in human adrenal cells or, alternatively, expressed only in certain populations of adrenal cells. LRH-1 is also expressed at high levels in the mouse and equine ovary suggesting that this transacting factor may also play a role in ovarian steroidogenesis (Repa *et al.* 1999, Boerboom *et al.* 2000).

Previous studies in our laboratory have shown that *hCYP11B1* transcription is positively regulated by SF-1 (Wang *et al.* 2000). To test whether LRH-1 could activate the transcription of *hCYP11B1*, the reporter construct pB1-1102 containing -1102 bp of *hCYP11B1* 5'-flanking DNA was transfected into H295R human adrenocortical cells. As shown in Figure 2, cotransfection of pB1-1102 with SF-1 stimulated the luciferase activity approximately 25 fold above basal levels. Co-transfection of the pB1-1102 construct with LRH-1 expression vector also stimulated the luciferase activity to the same level. The effects of

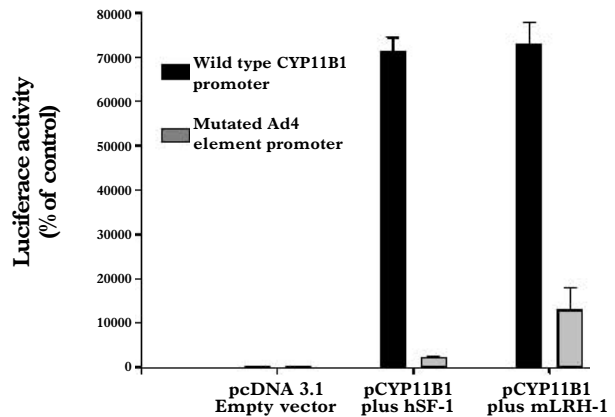


FIGURE 3. SF-1 responsive cis-elements are important for LRH-1 trans-activation. Wild type and mutated *hCYP11B1* constructs (1 µg/well) were co-transfected with pcDNA3.1 empty vector, hSF-1 or mLRH-1 expression vectors into H295R cells. Results are expressed as a percentage of the basal activity and represent the mean  $\pm$  S.E.M. of data from three independent experiments.

both SF-1 and LRH-1 on *hCYP11B1* promoter transcription were dose-dependent (only the data for LRH-1 is shown in Fig. 2). These results suggest that LRH-1 can activate transcription of *hCYP11B1*. Similar effects of LRH-1 were also observed for human 17 $\alpha$  hydroxylase (*CYP17*) reporter constructs (data not shown).

In order to determine if LRH-1 stimulated the transcriptional activity of the *hCYP11B1* reporter construct through the SF-1 binding site, termed Ad4, we mutated the Ad4 site 5'-ATCCAAGGC TCT-3' (-242/-234) to 5'-ATCgAAttCTCT-3'. Co-transfection of the mutated *hCYP11B1* reporter construct with either SF-1 or LRH-1 expression vector reduced luciferase activity to a level similar to that of the pGL3-basic vector (Fig. 3). This result suggested that LRH-1, like SF-1, stimulated *hCYP11B1* transcriptional activity through an interaction with the Ad4 cis-regulatory element.

To determine if LRH-1 could interact with the Ad4 site, a synthetic oligonucleotide probe encompassing this site was prepared and used for EMSA (Fig. 4). In the presence of H295R nuclear extracts, two major DNA/protein complexes (C1 and C2) were formed. C1 could be completely displaced by adding 100-fold molar excess of non-radiolabeled Ad4 oligonucleotide. However, C1 was not displaced when 100-fold molar excess of non-radiolabeled oligonucleotide containing a CRE sequence (Ad1) was added to the reaction. Both *in vitro* synthesized human SF-1 and human LRH-1 protein caused formation of a complex that

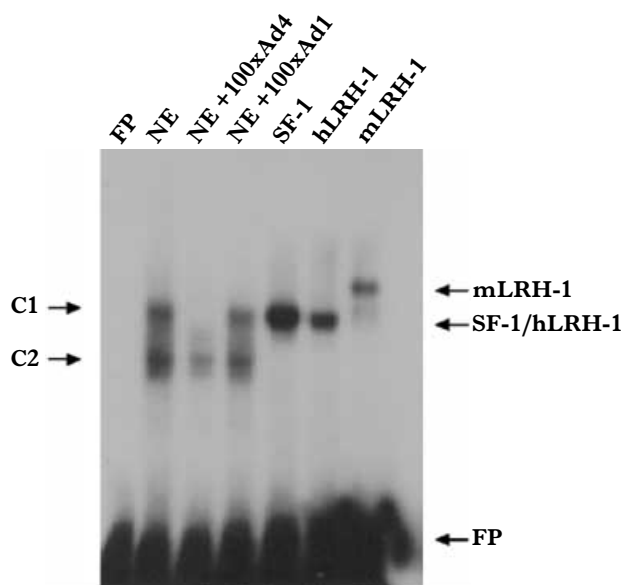


FIGURE 4. LRH-1 can bind to SF-1 consensus sequences in electrophoretic mobility shift assay. The labeled oligonucleotide was incubated with 5.0  $\mu$ g H295R cell nuclear extract (NE) or with *in vitro* synthesized human SF-1 (SF-1), human LRH-1 (hLRH-1), and mouse LRH-1 (mLRH-1) protein. Non-radiolabeled competitor DNA was added to a 100-fold molar excess to identify non-specific protein/DNA interactions (NE+100xAd4 for specific, NE+100xAd1 for non-specific). The resulting DNA/protein complexes (shown by arrows) were separated from free probe (FP) by electrophoresis.

migrated in parallel with C1; this complex was also observed using H295R nuclear extract. In contrast, the *in vitro* synthesized mouse LRH-1 protein caused a retarded DNA/protein complex that migrated more slowly than the human LRH-1. This may be due to the difference in protein size between the human and mouse LRH-1. However, these data suggest that both SF-1 and LRH-1 can recognize and bind to the SF-1 consensus sequence (Ad4), which is an important regulator for *hCYP11B1* transcription.

In summary, LRH-1 is expressed in human fetal and adult adrenals as well as liver and pancreas. Further, LRH-1 can interact with an SF-1 response element, termed Ad4, and enhance *hCYP11B1* gene transcription. These data suggest that LRH-1 could work in parallel or independently of SF-1 to control the expression of steroid-metabolizing enzymes. A role for LRH-1 in the regulation of adrenal and/or gonadal steroid hormone production should be further studied.

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