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DAX1A (*NR0B1A*) expression levels are low compared to DAX1 (*NR0B1*) in human steroidogenic tissues

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Abstract

The orphan nuclear receptor DAX1 (dosage-sensitive sex reversal-AHC critical region on the X chromosome gene 1; *NR0B1*) is known for its role in human development, specifically sex determination and steroidogenesis. Several recent publications have described an alternatively spliced form of DAX1 called DAX1A (*NR0B1A*). DAX1A is encoded by exons 1 and 2A of DAX1, with exon 2A located within the DAX1 intron 1. DAX1A expression was observed in several tissues, including adrenal gland, ovary, and testis. Transfection studies further showed that DAX1A had an inhibitory effect on DAX1, suggesting a role for DAX1A in the regulation of adrenal and gonadal differentiation/function. However, the relative level of DAX1 versus DAX1A transcripts still remains unclear. Herein, we developed and performed quantitative real-time RT-PCR (qPCR) to measure DAX1 and DAX1A mRNA expression levels in H295R human adrenal carcinoma cell line, human adult and fetal adrenal glands, corpus luteum, testis, whole pre and post-menopausal ovaries, ovarian follicles, placenta, liver, and kidney. These mRNA expression levels were quantified using DAX1 and DAX1A standard curves. In addition, Western blotting analysis was performed to examine both DAX1 and DAX1A protein levels in H295R cells, adrenal, corpus luteum, and liver. Both DAX1 and DAX1A mRNA were detected in all samples of H295R cells, human fetal and adult adrenals, testis, ovary, ovarian follicles, and corpus luteum. However, DAX1 mRNA levels were significantly higher (>37-fold) than that seen for DAX1A (P<0.01). DAX1A mRNA expression levels were undetectable in human liver, placenta, and kidney. Western blotting analysis results demonstrated that DAX1 protein was predominantly expressed in H295R cells, human adult adrenal, and the corpus luteum. These results suggest that in comparison to DAX1A that DAX1 is, by far, the predominant mRNA isoforms found in the human adrenal gland and gonads.

Keywords

DAX1; DAX1A; adrenal; gonadal; real-time RT-PCR; Western blotting analysis

INTRODUCTION

The orphan nuclear receptor DAX1 (dosage-sensitive sex reversal-AHC critical region on the X chromosome gene 1; *NR0B1*) is known for its involvement in reproduction, embryonic development, and steroidogenesis [1, 2]. Specifically, DAX1 has been shown to contribute to the phenotypic sex differentiation in the human embryo. DAX1 is also known to act as a transcriptional repressor of several steroid hormone metabolism genes in both

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adrenal and gonadal tissues [1–9]. Mutations of DAX1 are known to cause a congenital form of adrenal hypoplasia, a disorder associated with hypogonadotropic hypogonadism [10].

DAX1 consists of two exons. One exon is composed of 1168 bp, and the other is composed of 245 bp [4]. Both exons are separated by a 3385 bp intron [4]. DAX1 encodes a protein that is 470 amino acids in length [4, 11]. Recently, an alternatively spliced form of DAX1, named DAX1A (*NR0B1A*), has been identified. DAX1A is encoded by exon 1 and exon 2A of DAX1. Exon 2A is located within the DAX1 intron 1 [3, 4]. The expression of DAX1A was observed in several tissues, including adrenal gland, ovary, and testis [3, 4]. Cell culture expression studies further showed that DAX1A has an inhibitory effect on DAX1, suggesting a role for DAX1A in the regulation of the adrenal and gonadal differentiation/function [3]. These studies also reported the relative mRNA expression levels of DAX1 and DAX1A in several human tissues; however, there seems to be discrepancies between the studies with regard to expression among the different tissues [3, 4]. Herein, we developed and performed quantitative real-time RT-PCR (qPCR) with standard curves for both DAX1 and DAX1A and measured mRNA expression levels for both transcripts to determine the expression of each transcript.

MATERIALS AND METHODS

Human Tissues and Cell Lines

Human adult adrenal, corpus luteum, testis, whole pre and post-menopausal ovary, ovarian follicles, placenta, liver, and fetal kidney were obtained through the Cooperative Human Tissue Network (Philadelphia, PA). Human fetal adrenal was obtained from Advanced Bioscience Resources (Alameda, CA). The use of these tissues was approved by the Institutional Review Boards of both the University of Texas Southwestern Medical Center (Dallas, TX) and the Medical College of Georgia (Augusta, GA). We also used the H295R human adrenal carcinoma cell lines [11].

RNA Extraction

Total RNA was extracted from human tissues and H295R cell line using TRIzol Reagent (Invitrogen, Carlsbad, CA). The purity and integrity of the RNA were assessed using NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

cDNA Synthesis

2 μg of Deoxyribonuclease I (Ambion, Inc., Austin, TX) treated total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). cDNA was incubated at 25°C for 10 min and 37°C for 2 h. The synthesized cDNA was diluted 1:10 and stored at −20°C.

Plasmids

The DAX1A plasmid was constructed via a nested PCR using the adult adrenal cDNA template that was made as described above. DAX1A was initially PCR amplified using 5 μM of forward primer 5′-GGTACCATGGCGGGCGAGAAC-3′, 5 μM of reverse primer 5′-TCAAGTAATTAGCAGTTGACCACTTGGTT-3′, 10X High Fidelity PCR Buffer (Invitrogen), 10 mM dNTP mixture (Invitrogen), 50 mM magnesium sulfate ($MgSO_4$) (Invitrogen), 5% sterile tissue culture quality dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO), High Fidelity Taq Polymerase (Invitrogen), and 50 ng of total cDNA template. The mixture was placed in a Biometra thermocycler and amplified with a denaturing temperature of 94 °C, annealing temperature of 54 °C, and an extension temperature of 72 °C for 1 min. Cycles were repeated 41 times. The nested PCR was set up by using 2 μL of product to amplify DAX1A between DAX1 forward primer 5′-GGAGGAGCGGCCTGAGGGC-3′

and 5 μM of DAX1A reverse primer 5′-CAATCATTTTCCTTCCCTTTTCC-3′. All PCR reagents and PCR cycling conditions were the same as described above. 20 μL of sample was electrophoresed on an 8% agarose gel at 150 V for 60 min, and the 489 bp band was excised from the gel and purified using a S.N.A.P. Gel Purification Kit (Invitrogen). The DAX1A insert was then trioctylphosphine oxide (TOPO) ligated into pCR 2.1-TOPO (Invitrogen). The DAX1 plasmid standard was previously used in our laboratory and was described in detail [7].

qPCR Analysis

Primers for the amplification of the target sequences were based on published sequences for the human DAX1 and DAX1A [2]. The primer and probe sequences are given in Table 1. A DAX1/DAX1A common probe was designed near the 3′ end of exon 1. PCR reactions were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems), with a total volume of 30 μL per reaction. This total volume consisted of TaqMan Fast Universal PCR Master Mix (2X) (Applied Biosystems), 300 nM of each primer and probe, and 5 μL of each first-strand cDNA sample. Standard curves were prepared using human DAX1 and DAX1A vectors, which are described above. Negative controls contained water instead of first-strand cDNA. Samples were normalized on the basis of 18S ribosomal RNA content (μg). The quantification of the 18S in each sample was performed using a TaqMan Ribosomal RNA Reagent Kit (Applied Biosystems).

Western Blotting Analysis

Total protein from H295R cells was prepared using 1X Mammalian Protein Extraction Reagent (Pierce Chemical Co., Rockford, IL). Total proteins from frozen adult adrenal, corpus luteum, and liver were prepared by first homogenizing tissue in 1X Mammalian Protein Extraction Reagent. Polyacrylamide gel electrophoresis (PAGE) was carried out using NuPAGE 4–12% Bis-Tris gels (Invitrogen). After transfer, the membranes were blocked for 1 h at room temperature with a 5% milk solution and incubated overnight at 4 °C with DAX1 antibody (K-17) (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200. The DAX1 antibody is directed against the N-terminus of DAX1, which is common to both DAX1 and DAX1A proteins. Immunoreactive bands were visualized using the ECL plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ) and a G: BOX image analyzer system (Syngene, Cambridge, UK).

Data Analysis and Statistical Methods

Statistical analyses for qPCR were done by unpaired t-test for comparisons between two groups. Significance was accepted at the 0 – 0.01 level of probability ($P<0.01$).

RESULTS

qPCR for DAX1 and DAX1A

In this study, we performed qPCR reactions with standard curves for both DAX1 and DAX1A and determined mRNA expression levels (Figures 1A and 1B). We confirmed that both the DAX1 and DAX1A primers designed in this study did not cross-react with each other (data not shown). Both DAX1 and DAX1A mRNA were detected in all samples of H295R cells, human fetal and adult adrenals, testis, ovary, ovarian follicles, and corpus luteum. DAX1 mRNA was detected in all samples of human adult adrenal (6.60 ± 2.94 amoles/μg 18S), fetal adrenal (3.79 ± 1.56 amoles/μg 18S), and H295R cells (2.33 ± 0.81 amole/μg 18S) (Figure 2 and Table 2). DAX1A mRNA was detectable in all samples of H295R cells (0.06 ± 0.03 amole/μg 18S), human adult adrenal (0.03 ± 0.02 amoles/μg 18S), and fetal adrenal (0.08 ± 0.05 amoles/μg 18S) (Figure 2 and Table 2). DAX1 mRNA levels

were significantly higher (>50-fold) than DAX1A in H295R cells and adult and fetal adrenal sample ($P<0.01$) (Figure 2 and Table 2). DAX1 mRNA was also detected in all samples of human testis (2.30 ± 0.75 amoles/μg 18S), pre-menopausal ovary (3.38 ± 0.99 amoles/μg 18S), post-menopausal ovary (1.94 ± 0.45 amoles/μg 18S), ovarian follicles (0.79 ± 0.21 amoles/μg 18S), and corpus luteum (2.22 ± 1.22 amoles/μg 18S) (Figure 3 and Table 2). DAX1A mRNA was detectable in all samples of testis (0.03 ± 0.02 amoles/μg 18S), pre-menopausal ovary (0.04 ± 0.03 amoles/μg 18S), post-menopausal ovary (0.01 ± 0.00 amoles/μg 18S), ovarian follicles (0.006 ± 0.003 amoles/μg 18S), and corpus luteum (0.02 ± 0.01 amoles/μg 18S) (Figure 3 and Table 2). DAX1 mRNA levels were significantly higher (>37-fold) than seen for DAX1A in testis and ovary ($P<0.01$) (Figure 3 and Table 2). On the other hand, neither of the two isoforms were detectable in human liver, placenta, and fetal kidney (data not shown).

Expression of DAX1 and DAX1A Protein

We also evaluated the expression of the endogenous DAX1 and DAX1A protein in H295R cells, adult adrenal, corpus luteum, and liver. The DAX1 antibody specifically recognized a band corresponding to DAX1 protein (approximately 60 kDa) in the extracts from H295R cells, adult adrenal, and corpus luteum (Figure 4). We also examined whether there were any distinct bands for DAX1A protein, for Hossain *et al.* reported a DAX1A band at approximately 35 kDa [3]. We could detect faint bands that ran between 20 and 40 kDa in H295R cells and corpus luteum but not in adult adrenal (Figure 4). However, based on previously reported data, the 30 kDa band did not correspond to the DAX1A band (Figure 4) [3]. No DAX1 or DAX1A was detected in liver. These data support the concept that the DAX1 protein is the predominant isoform in these tissues.

DISCUSSION

While two studies have shown the existence of a DAX1 splice variant termed DAX1A, significant disparities existed between the sequence of the DAX1A and the level of its expression [3, 4]. The relative expression of DAX1 versus DAX1A would represent an indicator of the role of DAX1A in cellular functions. Herein, we demonstrated that DAX1A mRNA is detectable in steroidogenic cells but it is at least 37-times lower than the DAX1 isoform. These results suggest that DAX1 is the predominant isoform found in steroidogenic tissues.

The first paper to describe the DAX1A sequence was published by Hossain and colleagues [3]. However, a subsequent article by Ho *et al.* reported a sequence that included an additional G at the 3′ end of exon 1 in ***NR01B*** and 5 more nucleotide bases in ***NR01BA*** exon 2A [4]. Both studies used RT-PCR to examine the expression pattern of DAX1A in several steroidogeinic and nonsteroidogenic tissues. We were unable to replicate the qPCR data reported by Hossain *et al.* because the primer sequences provided in the manuscript were identical for both DAX1 and DAX1A [3]. While the study by Ho *et al.* did not attempt to quantify the relative expression of DAX1 or DAX1A, a nested primer strategy had to be used to detect the expression of the DAX1A transcript—suggesting low expression. To help clarify this issue, the current study was done using qPCR with primers and probe sets for both DAX1 and DAX1A that were designed based on Ho's report [4]. We developed and performed qPCR using standard curves for both DAX1 and DAX1A and measured mRNA expression levels for both transcripts in human tissues. For qPCR, two approaches of data analysis are commonly used in basic and clinical research—the comparative C_T (cycle threshold) method (also known as $\Delta\Delta C_T$) and the standard curve methods [13–15]. Both methods have advantages and certain limitations, as discussed by Schefe and colleagues [13]. However, in this study, we utilized a standard curve method to account for differences

in PCR efficiencies between DAX1 and DAX1A [13]. When designing the qPCR primers and probe set, we thoroughly examined the current literature to determine the proper sequence of DAX1A [4].

Based on our findings, DAX1 and DAX1A mRNAs were detected in all the samples of human adrenal and gonadal tissues. However, the levels of both isoforms were undetectable in human liver, placenta, and fetal kidney. Using standard RT-PCR methods, these results are consistent with qualitative data reported by Ho *et al.* [4]. Interestingly, except for Hossain's report, DAX1 has not been studied previously in human tissues using qPCR methods although its expression in human testis, ovarian follicles, corpus luteum, and adrenal are well established [16–20]. We found that the relative expression levels for DAX1 mRNA were similar among human adrenal and gonadal samples. This is quite different from the distribution seen in the mouse, in which the gonadal expression of DAX1 is much higher than is seen in adrenal [21]. This is likely due to the fact that mice express relatively low levels of DAX1 in adrenal gland, most of which is in the zona glomerulosa [22]. This adrenal expression pattern contrasts sharply with the human adrenals situation, where DAX1 is found throughout the adrenal cortex [23]. We also demonstrated that DAX1A mRNA levels were significantly lower than DAX1 mRNA levels in human adrenal and gonadal tissues. Through SYBR Green qPCR analysis, Hossain *et al.* reported that DAX1A expression was higher than DAX1 in human adrenal gland and ovarian tissue [3]. We were unable to replicate these findings because the primer sequences included in their manuscript were the same for DAX1 and DAX1A. Data from Ho *et al.* reported DAX1 as the predominant isoform in human adrenal and gonadal tissues, though they did not perform qPCR for evaluation [4]. As described above, we designed DAX1 and DAX1A primers and probe sets based on Ho's sequence information [4]. In this study, we used TaqMan Fast Universal PCR Master Mix (2X) and DAX1/DAX1A primers/probe sets for analysis to increase the specificity of the data obtained, which may account for the difference in results reported by Ho *et al.* [3, 4]. Our data agree with Ho *et al.* in that DAX1 is, by far, the major transcript present in steroidogenic tissues.

We also evaluated the expression of the endogenous DAX1 and DAX1A protein in H295R cell, adult adrenal, and corpus luteum and confirmed DAX1 protein was the predominant isoform in these tissues. DAX1 protein is previously reported to be abundant in H295R cells and human adrenal cortex [7, 23]. In addition, Sato *et al.* demonstrated DAX1 protein is abundantly expressed in ovarian follicles and corpus luteum [17]. Our results are compatible with these findings and are supportive for qPCR data demonstrated above. We do not have an explanation for the observation by Hossain *et al.*, where the DAX1A isoforms were abundant in testis.

Hossain *et al.* reported that highly concentrated DAX1A is capable of binding to steroidogenic factor 1 (SF-1) and to DNA but is unable to repress SF-1-mediated transcriptional activation of the steroidogenic acute regulatory (StAR) and 17α-hydroxylase (CYP17) reporter genes [3]. In addition, DAX1A acts as an antagonist to DAX1 under non-physiologically high concentrations [3]. Thus the interactions between the DAX1 and DAX1A isoforms could impact the activities of SF-1 and its many target genes that include StAR and CYP17. However, based on our observation that DAX1A mRNA is expressed at quite low levels, further studies will be needed to determine if DAX1A plays a role in adrenal or gonadal tissues.

In summary, we have determined the relative expression levels of DAX1 and DAX1A mRNA in human adrenal and gonadal tissues. The results provide strong evidence that DAX1A is expressed at low levels compared to DAX1. Therefore, the determination of a

role for the DAX1A isoform in human adrenal or gonadal physiology will need further study.

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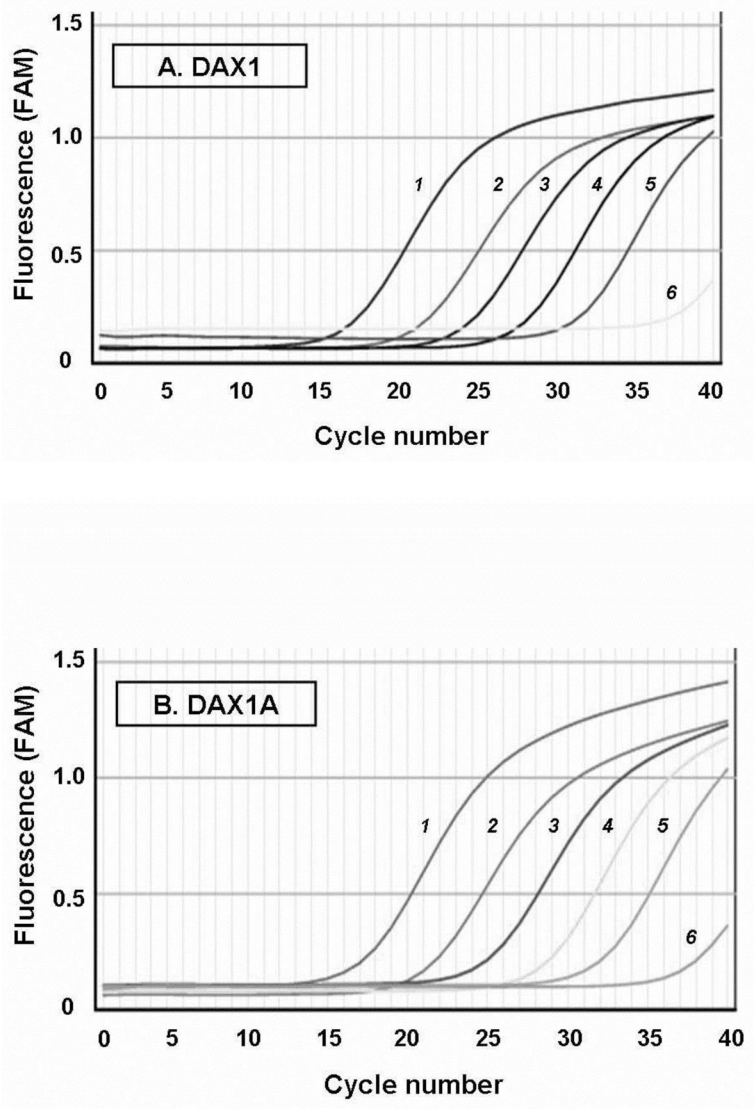

FIGURE 1.

The standard curves for DAX1 (Panel A) and DAX1A (Panel B) mRNA expression levels. These standard curves were prepared using plasmids containing both human DAX1 and human DAX1A. These curves correspond to 50,000 fg (line 1), 5000 fg (line 2), 500 fg (line 3), 50 fg (line 4), and 5 fg (line 5) of the vector DNA and the negative control (line 6).

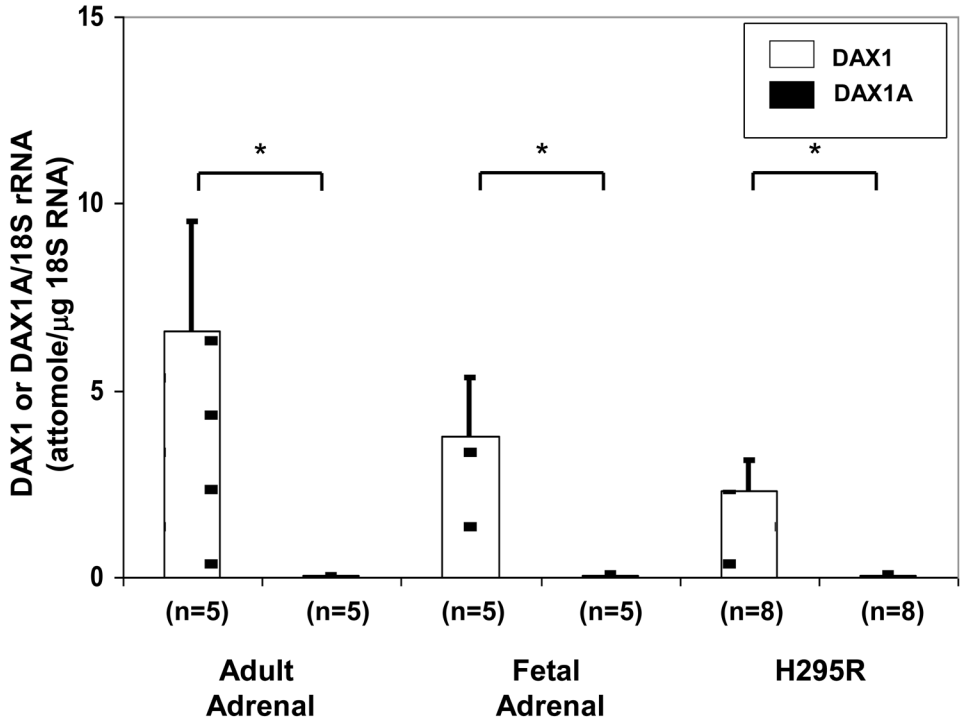

FIGURE 2.

Quantification of DAX1 and DAX1A transcript levels in human adrenal tissues. Real-time RT-PCR was performed to quantify the level of these mRNA in human adult and fetal adrenal glands (n=5 for each sample) and H295R cells (n=8). Data points are expressed as mean ± standard deviation (SD) amoles/μg 18S (*, $P<0.01$).

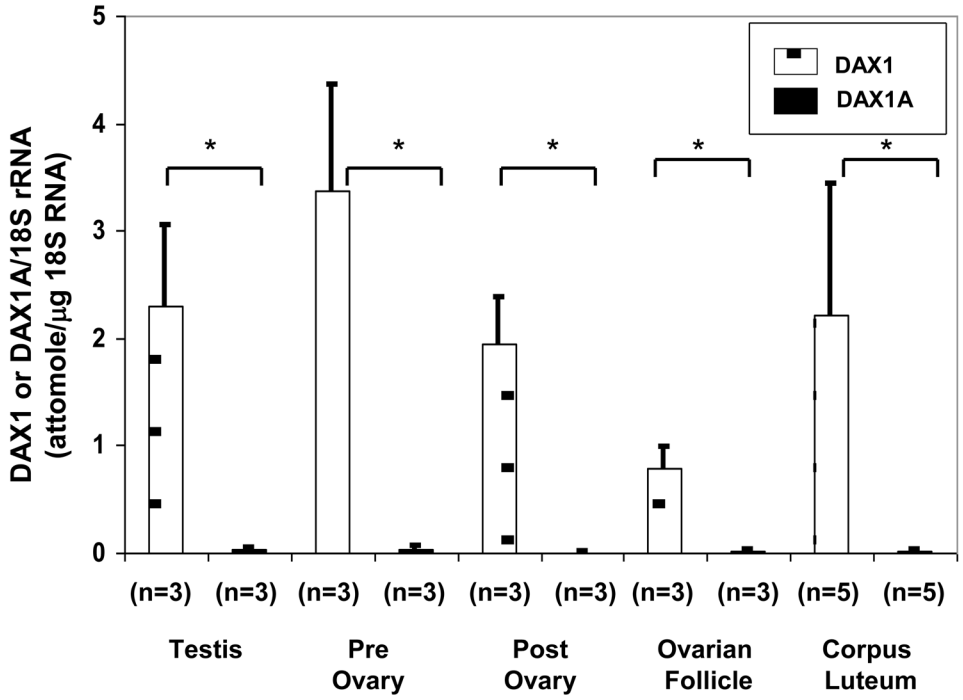

FIGURE 3.

Quantification of DAX1 and DAX1A transcript levels in human gonadal tissues. Real-time RT-PCR was performed to quantify the level of these mRNA in human testis, whole pre and post-menopausal ovary (**Pre ovary and Post ovary**, respectively), ovarian follicles (n=3 for each tissue), and corpus luteum (n=5). Data points are expressed as mean ± standard deviation (SD) amoles/μg 18S (*, $P<0.01$).

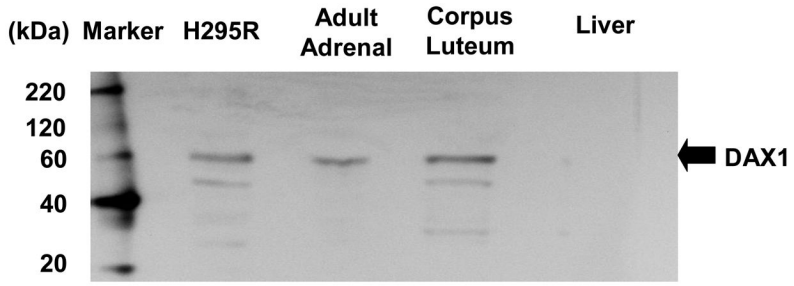

FIGURE 4.

Western blotting analysis for endogenous expression of DAX1 and DAX1A proteins in H295R cells, human adrenal, corpus luteum, and liver. The DAX1 antibody specifically recognized a band corresponding to DAX1 protein (approximately 60 kDa) in all lysates except for the liver. 30 μg of cell or tissue lysate protein was loaded for each lane.

TABLE 1

Primer and probe sequences used for real-time RT-PCR to distinguish DAX1 and DAX1A mRNA expression in human tissues.

Gene	Forward Primer	Reverse Primer	Probe
DAX1	CCAAG GAGTACGCCTACCTCAAG	TGAAGTACATTCAGGGACTCCAGT	AAG GGG ACC GTG CTCTTT AAC
DAX1A	CAGTACCAAG GAGTACGCCTACCT	GGAAAAGGGAAGGAAAATGATTG	AAG GGG ACC GTG CTCTTT AAC

TABLE 2

Quantification of DAX1 and DAX1A transcript levels in human adrenal and gonadal tissues. Data points are expressed in attomoles/μg 18S as the mean ± standard deviation (SD).

Tissue	DAX1	DAX1A	P value
Adult adrenal (n=5)	6.60 ± 2.94	0.03 ± 0.02	$P<0.01$
Fetal adrenal (n=5)	3.79 ± 1.56	0.08 ± 0.05	$P<0.01$
H295R cells (n=8)	2.33 ± 0.81	0.06 ± 0.03	$P<0.01$
Testis (n=3)	2.30 ± 0.75	0.03 ± 0.02	$P<0.01$
Pre-menopausal ovary (n=3)	3.38 ± 0.99	0.04 ± 0.03	$P<0.01$
Post-menopausal ovary (n=3)	1.94 ± 0.45	0.01 ± 0.00	$P<0.01$
Ovarian follicles (n=3)	0.79 ± 0.21	0.02 ± 0.01	$P<0.01$
Corpus luteum (n=3)	2.22 ± 1.22	0.02 ± 0.01	$P<0.01$