

Human adrenodoxin reductase: Two mRNAs encoded by a single gene on chromosome 17cen→q25 are expressed in steroidogenic tissues

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ABSTRACT Adrenodoxin reductase is a mitochondrial flavoprotein that receives electrons from NADPH, thus initiating the electron-transport chain serving mitochondrial cytochromes P450. We have cloned and sequenced two human adrenodoxin reductase cDNAs that differ by the presence of six additional codons in the middle of one clone. The sequence in this region indicates that these six extra codons arise by alternative splicing of the pre-mRNA. Southern blot hybridization patterns of human genomic DNA cut with four restriction enzymes indicate that the human genome has only one gene for adrenodoxin reductase. Analysis of a panel of mouse-human somatic cell hybrids localized this gene to chromosome 17cen→q25. The alternatively spliced mRNA containing the six extra codons represents 10–20% of all adrenodoxin reductase mRNA. The expression of the adrenodoxin reductase gene may be stimulated by pituitary tropic hormones acting through cAMP, but its response is quantitatively much less than the responses of P450scc and adrenodoxin.

The first and rate-limiting step in the synthesis of all steroid hormones is the conversion of cholesterol to pregnenolone by a mitochondrial cytochrome termed P450scc. This cytochrome binds cholesterol and mediates three separate reactions on a single active site: 20-hydroxylation, 22-hydroxylation, and scission of the cholesterol side chain. Each of these reactions requires a pair of electrons. The electrons are transferred from NADPH to the flavoprotein adrenodoxin reductase and thence to the iron-sulfur protein adrenodoxin, which then donates them to the P450scc (1). This same electron-transport system donates electrons to another steroidogenic enzyme, P450c11 (2), to renal vitamin D 1 α -hydroxylase (3), and to hepatic 26-hydroxylase (4). The microsomal steroidogenic enzymes P450c17 (17 α -hydroxylase/17,20-lyase) (5, 6), P450c21 (21-hydroxylase) (7, 8), and P450aro (aromatase) (9) employ a different flavoprotein to transfer electrons from NADPH.

Despite this intimate functional organization, the genes encoding these three components differ greatly in structure, location, and regulation. Human P450scc is encoded by a single gene on chromosome 15 (10), and its mRNA readily accumulates in human fetal adrenal cells stimulated with cAMP or corticotropin (11, 12) or in human testicular or ovarian granulosa cells stimulated with cAMP or gonadotropins (12–14). Adrenodoxin is encoded by a single functional gene on chromosome 11q13→qter (15) that encodes several mRNAs differing in the lengths of their 3' untranslated regions (16). This mRNA also accumulates in steroidogenic

tissues stimulated with cAMP or tropic hormones (17). However, unlike P450scc mRNA, the half-life of adrenodoxin mRNA appears to be regulated posttranscriptionally (16, 18). The structure of these and other steroidogenic genes and their disease-causing lesions have been reviewed recently (19, 20).

Much less is known about adrenodoxin reductase. The recent cloning of partial-length bovine adrenodoxin reductase cDNA (21) has now permitted us to clone the full-length human cDNA[¶] so that all three components of the P450scc system may be studied. Adrenodoxin reductase is encoded by a single gene on the long arm of chromosome 17. This gene encodes two species of mRNA that apparently arise by alternative processing of the primary transcript. The accumulation of neither of these mRNAs is greatly stimulated by gonadotropins or cAMP in cultured human granulosa cells.

MATERIALS AND METHODS

The human adrenal and testicular cDNA libraries (5) were screened with the 1.6-kilobase (kb) bovine adrenodoxin reductase cDNA fragment isolated from plasmid pAR (21) by *Eco*RI cleavage and gel electrophoresis. The DNA was labeled to $>10^8$ cpm/ μ g by random-primer labeling (15) and used to screen the libraries under low-stringency conditions [$5 \times$ SCC ($1 \times$ SCC = 0.15 M NaCl/0.015 M sodium citrate)/20% (vol/vol) formamide/denatured salmon sperm DNA (100 μ g/ml)/0.1% polyvinylpyrrolidone/0.1% Ficoll/0.1% bovine serum albumin at 37°C] as described (5). Clones positive on duplicate screening were purified on DEAE-cellulose (22) and sequenced in phage M13 vectors as described (5, 10, 16) or by supercoiled DNA sequencing in pUC plasmids (23). The 30-base oligodeoxynucleotide 3'-GACCTCCGGGAGGAAAACACGGTCTCTTGC-5' complementary to the codons for amino acids 202–211, including the alternatively spliced region of the mRNA, was prepared on a commercial DNA synthesizer, end-labeled by use of [γ -³²P]-ATP, and used to probe the primary screening of both libraries in $6 \times$ SCC at 37°C as described (7).

DNA was isolated from human lymphocytes and from mouse-human somatic cell hybrids, cleaved with restriction endonucleases, and analyzed as described (10, 15).

Human granulosa cells were prepared and cultured with or without hormonal stimuli (13) and used as a source of RNA for gel-transfer hybridization analysis (11, 12).

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^{¶¶}The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03826).

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RESULTS

Identification of Human Adrenal and Testicular Adrenodoxin Reductase cDNA Clones. Hybridization of the bovine adrenodoxin reductase cDNA to our human adrenal cDNA library yielded 49 positively hybridizing plaques. These were pooled in groups of 3 without plaque purification, and the DNA was cleaved with *EcoRI*, electrophoresed through agarose, and analyzed by Southern blot (24) hybridization with the bovine probe, thus identifying the pool containing the longest adrenodoxin reductase cDNA insert (5). These three clones were then analyzed in the same fashion individually, and the one containing the longest insert, λ haAR-1, was plaque-purified. Sequencing of this cDNA in phage M13 showed that it was about 1600 base pairs long, lacking about 200 bases from the 5' end (Fig. 1). We therefore cloned the 272-base fragment from the 5' *EcoRI* cloning linker to a *Sma* I site and used this to screen a human testis cDNA library, identifying 97 clones. These were screened by identifying the one bearing the longest *EcoRI*-*Sma* I fragment that hybridized to the adrenal 272-base *EcoRI*-*Sma* I fragment. The longest fragment detected was slightly less than 500 bases and was included in a 1.8-kb cDNA. This clone, designated λ htAR-1, appeared to be full-length and was sequenced in its entirety by supercoil and M13 sequencing.

Sequence of Human Adrenodoxin Reductase cDNA Clones. The nucleotide sequence and encoded amino acid sequence of the adrenal clone λ haAR-1 corresponded very well with the sequence of the bovine adrenal clone pAR used as the initial probe (I.H. and T. Gutfinger, unpublished data). This nucleotide sequence also corresponded well with a preliminary report of a full-length bovine adrenodoxin reductase cDNA (25). The sequence of our adrenal clone λ haAR-1 was confirmed by the sequence of the testicular clone λ htAR-1. That testicular cDNA corresponds to the complete coding sequence, the complete 3' untranslated sequence, and 20 bases of the 5' untranslated region, a total of 1829 bases excluding the poly(A) region (Fig. 2).

The most notable feature of the λ htAR-1 sequence is the presence of 18 additional nucleotides encoding 6 amino acids not found in the human adrenal clone. This extra region, encoding amino acids 204–209 in Fig. 2, contains an abundance of pyrimidines and ends with CAG (encoding Gln-209). Since pyrimidine-rich sequences ending with CAG constitute the canonical 3' splice donor sites of eukaryotic introns (26),

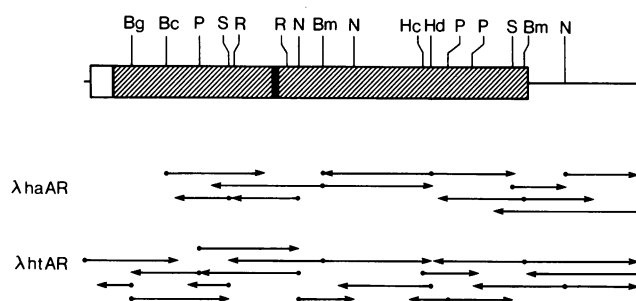


FIG. 1. Schematic diagram of the two human adrenodoxin reductase cDNAs. The upper diagram shows the protein-coding regions of the clones as boxes; the open region corresponds to the mitochondrial leader peptide, the hatched regions correspond to mature adrenodoxin reductase, and the solid region indicates the six additional amino acids resulting from alternative splicing of the mRNA precursor. The 5' and 3' untranslated sequences are indicated with a solid line. λ haAR, partial-length human adrenal cDNA; λ htAR, full-length human testicular cDNA. Only the restriction endonuclease sites mentioned in the text and used in the sequencing strategy are shown. Bg, *Bgl* II; Bc, *Bcl* II; Bm, *Bam*HI; Hc, *Hinc*II; Hd, *Hind*III; N, *Nae* I; P, *Pst* I; R, *Rsa* I; S, *Sma* I. The sequencing strategies are shown by arrows representing lengths of subclones and direction of sequencing.

we propose that the presence or absence of these 18 extra nucleotides is based on the alternative use of potential 3' donor sites during the splicing of intronic RNA in this region.

Frequency of Alternative Splicing in Human Adrenodoxin Reductase. We sequenced one cDNA from adrenal and one from testis and found two alternatively spliced sequences. To determine whether this splicing might be tissue-specific, we synthesized a 30-base oligonucleotide corresponding to the complement of the mRNA in this region and used this to reprobe the plaque "lifts" from the primary screenings of both the adrenal and testis cDNA libraries. The adrenal library contained 2 clones that hybridized to the 30-mer, whereas the testicular library contained 18. Thus, both tissues contained this alternatively spliced form of adrenodoxin reductase mRNA.

To determine the frequency with which this alternative splicing event occurs, we needed to know the number of adrenodoxin reductase cDNA clones lacking this 18-base region. Since the region is about 1150 bases from the poly(A)-addition site, and since the libraries might contain partial-length cDNA clones not extending to this region, we could not compare the number hybridizing to the 30-mer to the total number of positives initially identified with pAR. Therefore, we reprobed the plaque lifts from the primary screening with a 188-base-pair *Rsa* I-*Rsa* I fragment encompassing the 18-base region (extending from codon 160 to codon 220, Figs. 1 and 2). This probe identified 23 adrenal clones and 77 testicular clones. Therefore, 2 of 23 adrenal clones (9%) and 18 of 77 testicular clones (23%) contain this 18-base region.

Number of Adrenodoxin Reductase Genes. An alternative explanation for the presence of these two types of adrenodoxin reductase mRNAs might be that they are encoded by two different genes. To determine the number of adrenodoxin reductase genes in the human genome, we digested human leukocyte DNA with four different restriction enzymes and probed Southern blots of this DNA with λ haAR-1 (Fig. 3). DNA digested with *Kpn* I and *Xba* I produced single hybridizing bands indicating the presence of a single gene, as previously reported (21). Digestion with *Hind*III produced two bands of 5.8 and 6.4 kb, whereas digestion with *Pst* I produced four bands of 2.8, 1.8, 0.8 and 0.6 kb, thus totaling about 6 kb. As the cDNA is about 2 kb long and contains a single *Hind*III site, this hybridization pattern indicates that the single adrenodoxin reductase gene is fairly small, between 6 and 12 kb.

Chromosomal Location of the Human Adrenodoxin Reductase Gene. To determine the location of this unique gene, we probed *Hind*III- or *Pst* I-digested DNA from a panel of mouse-human somatic cell hybrids having karyotypically determined complements of human chromosomes. Correlation of the hybridization pattern and the karyotypic data (Table 1) indicated that the human adrenodoxin reductase gene lies on chromosome 17. We then examined additional hybrids of human cells with partial deletions of chromosome 17, localizing the adrenodoxin reductase gene to 17cen-q25 (Fig. 4).

Hormonal Regulation of Adrenodoxin Reductase. Human granulosa cells, if cultured *in vitro* for 8–12 days and then stimulated with gonadotropins or cAMP, will accumulate mRNAs for both P450_{scc} and adrenodoxin (12–14, 16, 17). Transfer blots of human granulosa cell RNA probed with λ haAR-1, however, showed that the same protocol of culture and hormonal stimulation has little effect on the abundance of adrenodoxin reductase mRNA (Fig. 5). Thus, the regulation of expression of the adrenodoxin reductase gene in this tissue differs greatly from the temporal pattern of P450_{scc} and adrenodoxin regulation.

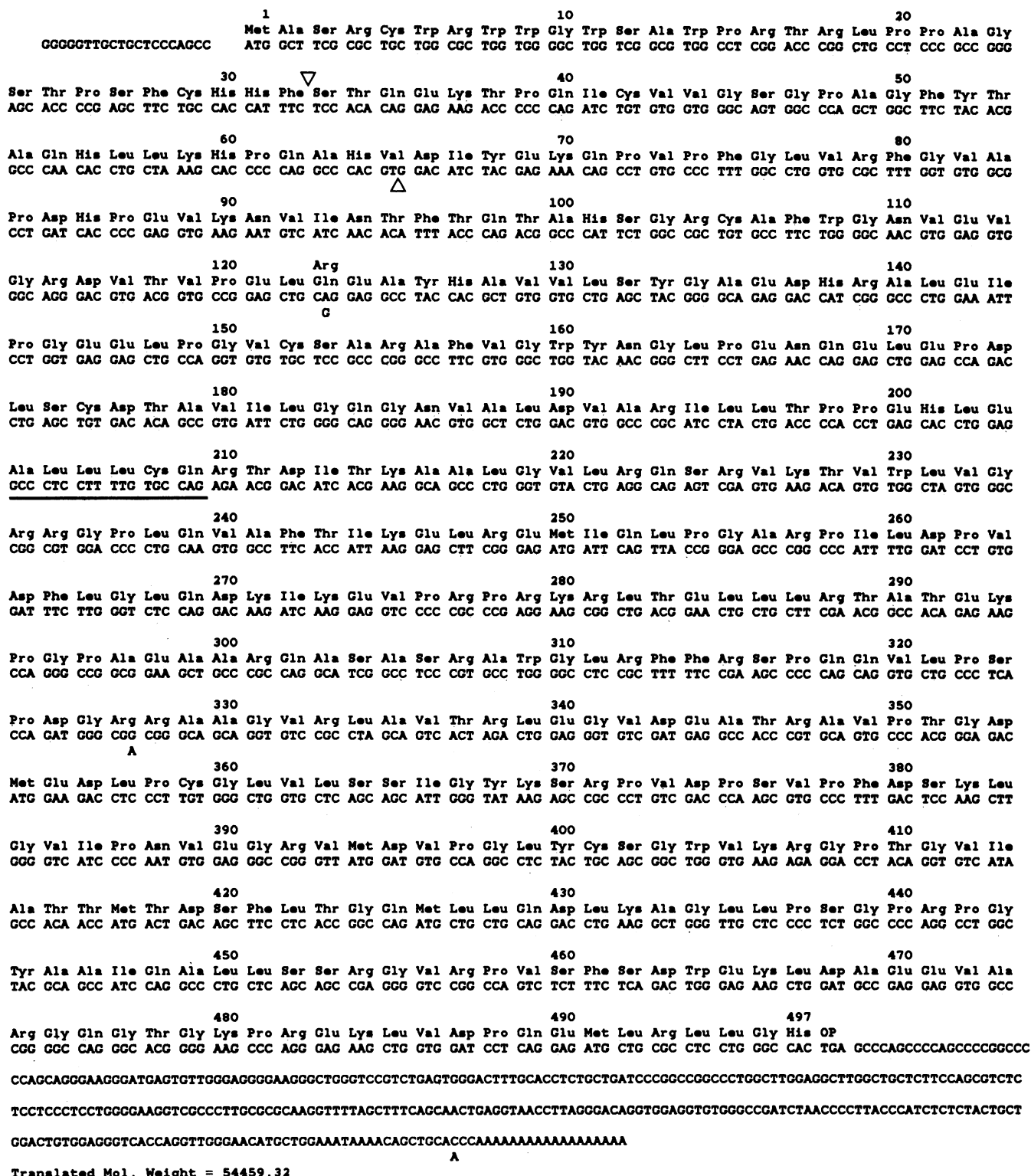


FIG. 2. Sequence of λ htAR-1 cDNA. The downward-pointing arrowhead between amino acids 32 and 33 indicates the cleavage site of the enzyme precursor as it traverses the mitochondrial membrane. The upward pointing arrowhead between the second and third bases of codon 65 (Val) indicates the 5' limit of λ htAR-1 cDNA. Three nucleotide differences between λ htAR-1 and λ haAR-1 are indicated below the sequence, a C-to-A transversion near the end of the 3' untranslated region, a G-to-A transition in codon 327, and an A-to-G transition in codon 123, changing Glu to Arg. The presence of this allelic variation was proven experimentally; λ htAR-1 could be cleaved with *Pst* I at this location but λ haAR-1 could not, since the A-to-G transition eliminates this site.

DISCUSSION

The cloning of human adrenodoxin reductase cDNA now completes our genetic knowledge of the human cholesterol side-chain-cleavage system. There is only one functional gene for each of the three components, P450scc (10), adreno-

doxin (15), and now, adrenodoxin reductase. Despite this genetic simplicity, the P450scc system is differentially regulated in the adrenal by corticotropin, acting through cAMP, and angiotensin II, acting through calcium (27). Furthermore, kinetic studies indicate different enzymologic parameters for various lipoidal derivatives of cholesterol (28). Thus, there

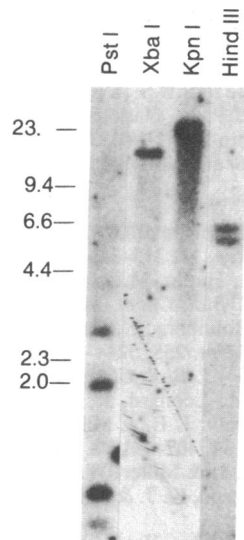


FIG. 3. Southern blot of human genomic DNA probed with λ haAR-1. The enzymes used to digest the DNA from one individual are shown above the corresponding lanes. Size markers (in kb) show positions of fragments produced by *Hind*III digestion of bacteriophage λ DNA.

appear to be multiple additional layers of control of the cholesterol side-chain-cleavage system, in addition to transcriptional control. Such variations in the behavior of a single enzyme often reflect posttranslational modification (glycosylation, phosphorylation, etc.) or the presence of small molecules acting as competitors or facilitators. The finding of alternative splicing of adrenodoxin reductase mRNA sug-

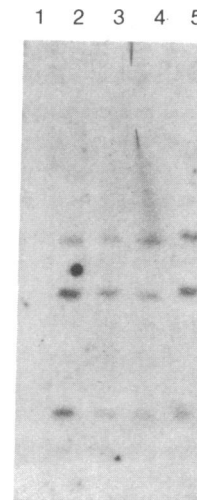


FIG. 4. Southern blot of *Pst* I-cut genomic DNA from mouse-human somatic cell hybrids probed with λ haAR-1. Lane 1: DNA from cell line CF 11-4, which lacks human chromosome 17 (third cell line from right in Table 1). Lane 2: DNA from cell line CF 80-8, which contains human chromosome 17pter \rightarrow q25. Lane 3: same as lane 2. Lane 4: DNA from cell line CF 17-24/7, which contains the entire long arm of chromosome 17. Lane 5: DNA from cell line CF 84-11/5, which contains human chromosome 17 but no other human chromosomes (last cell line on right in Table 1).

gests the possibility of yet another level of control of the cholesterol side-chain-cleavage system.

Both alternatively spliced forms of adrenodoxin reductase mRNA were found in the testis, which has only a single steroidogenic mitochondrial P450 enzyme, P450_{sc}. Thus, it

Table 1 Correlation of human chromosomes in hybrid cell lines and hybridization patterns

Chromosome	Cell line																	Discordant, no. (<i>Pst</i> I bands)	
	2	7	21	25	20	26	4	34	27	39	3	5	37	30	116	11	35		11
1	+	-	-	-	-	+	+	-	-	-	+	-	-	-	+	-	-	-	13
2	+	-	-	-	+	-	+	-	+	-	+	+	-	-	+	-	-	-	11
3	-	+	-	-	+	+	+	-	-	+	+	+	-	+	+	-	+	-	8
4	+	+	-	+	+	+	+	+	-	-	+	-	+	+	+	+	-	+	6
5	(+)	-	-	+	(+)	+	-	-	+	-	-	+	+	(+)	+	-	-	-	9
6	+	+	-	+	-	+	+	+	+	-	+	(+)	+	+	+	-	+	-	5
7	+	(-)	+	-	+	+	+	+	-	+	+	-	+	-	-	-	+	-	6
8	+	(+)	-	+	+	+	+	+	+	+	+	+	(+)	-	+	-	+	-	4
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	17
10	-	-	+	+	-	(+)	+	+	-	-	+	-	-	+	-	-	-	-	9
11	(-)	+	-	+	+	-	-	+	+	-	-	-	+	-	-	-	-	-	10
12	+	(+)	+	+	+	+	-	+	-	-	+	-	+	(+)	+	-	+	-	7
13	-	-	-	-	+	+	+	-	-	-	+	(+)	-	+	-	-	-	-	10
14	+	+	+	-	+	+	+	+	-	-	+	+	+	+	-	-	+	-	4
15	+	-	-	-	(-)	+	+	-	+	+	+	+	-	-	+	-	+	-	9
16	+	-	-	-	+	(+)	-	-	-	-	-	-	-	-	+	-	-	-	14
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	0
18	+	-	-	-	+	+	+	-	+	-	+	-	-	+	+	-	+	-	9
19	+	-	+	(+)	-	+	+	(+)	-	-	+	+	-	-	+	-	-	-	9
20	+	+	+	-	+	(+)	+	+	+	-	+	-	+	(-)	+	-	-	-	7
21	-	-	+	+	+	+	-	-	+	-	-	+	+	+	+	-	-	-	9
22	+	-	-	-	+	+	-	(+)	-	-	-	+	+	+	+	-	-	-	10
X	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	16
Y	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14

Presence of human chromosomes in mouse-human somatic cell lines is scored as follows: present in >30% of analyzed cells, +; 10-30%, (+); 5-9%, (-); not detected, -. Number of discordances with the *Pst* I (or *Hind*III) hybridization pattern detected on Southern blots probed with λ haAR-1 cDNA appears at right.

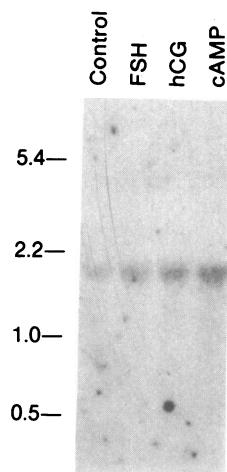


FIG. 5. Transfer blot of RNA from cultured human granulosa cells. Molecular size (kb) markers were *Hind*III fragments of phage PM-2. Each lane contained 30 μ g of total RNA. Control: cells cultured 12 days without hormonal stimulus. FSH: Duplicate cells grown 10 days without treatment and 2 days with human follicle-stimulating hormone (100 μ g/ml). hCG: Duplicate cells grown 10 days without treatment and 2 days with human choriogonadotropin (100 μ g/ml). cAMP: Duplicate cells grown 10 days without treatment and 2 days with 8-bromo-cAMP (1 mM).

is clear that the two different forms of adrenodoxin reductase are not specific for various mitochondrial P450s such as P450scc and P450c11. Since the three-dimensional structure of adrenodoxin reductase is unknown, we cannot predict whether the alternative splicing event alters the molecule significantly. The six additional amino acids could conceivably alter the binding of NADPH to take up electrons or the binding of adrenodoxin to give them up. The alternatively spliced form of adrenodoxin reductase contains an additional cysteine residue (no. 208) that, in the reducing environment of the mitochondrial matrix, should exist in the free sulfhydryl form, thus changing the redox properties of the protein. It will be of considerable interest to compare the redox properties of the two forms of adrenodoxin reductase prepared by bacterial expression of the cDNAs.

The regulation of adrenal steroidogenesis is complex. Two different tropic hormones, corticotropin and angiotensin II, act through different intracellular second messengers. Both appear to activate cholesterol side-chain cleavage activity acutely, possibly by phosphorylation of cholesterol esterase, and both stimulate steroidogenesis chronically, by stimulating transcription of the P450scc and adrenodoxin genes (29). In addition to these two mechanisms, evidence is accumulating rapidly that the abundance of adrenodoxin mRNA is regulated posttranscriptionally by changes in mRNA half-life (16, 18). Our finding of alternative splicing of the mRNA encoding adrenodoxin reductase suggests that yet another class of regulation is involved in this crucial biological system.

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