Human Adrenodoxin: Cloning of Three cDNAs and Cycloheximide Enhancement in JEG-3 Cells*

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Adrenodoxin is an iron-sulfur protein serving as an electron transport intermediate for two mitochondrial steroidogenic cytochromes P450. We have cloned and sequenced three human adrenal adrenodoxin cDNAs. The longest 3'-untranslated region was 131 bases long, and the coding sequences, identical in all three clones, predict a preprotein of 180 amino acids. The 3'-untranslated regions were 235, 596, and 776 bases long due to the presence of alternate polyadenylation sites. RNA transfer blots showed multiple size species of adrenodoxin mRNA consistent with finding multiple polyadenylation sites. Similar sized cross-hybridizing RNA species are found abundantly in the adrenal and testis and to a lesser degree in RNA from human fetal brain, spleen, placenta, kidney, liver, and intestine, as well as in cultured fibroblasts, suggesting the same or a very similar iron-sulfur protein is found in mitochondria of nonsteroidogenic tissues. JEG-3 cells, a transformed progesterone-producing line of trophoblastic origin, accumulate mRNAs for cytochrome P450soc (the mitochondrial cholesterol side-chain cleavage enzyme), adrenodoxin, and the fos oncogene when stimulated with 8-bromo-cyclic AMP. Addition of actinomycin D to such cultures blocked CAMP-induced accumulation of mRNAs for cytochrome P450soc and adrenodoxin. Addition of cycloheximide or puromycin to such cultures substantially reduced basal levels and markedly attenuated the CAMP-induced accumulation of mRNAs for cytochrome P450soc and adrenodoxin. Like adrenodoxin mRNA in cultured bovine adrenocortical cells, human adrenodoxin mRNA accumulation is induced by cyclic AMP. However, unlike the bovine system, cycloheximide augments, rather than eliminates, this CAMP-induced mRNA accumulation.

MATERIALS AND METHODS

The construction of our human adrenal cDNA library in Agt10 has been described (2). The library was screened as described (6) using bovine adrenodoxin clone pbAdx-1, generously provided by Dr. M. Waterman (Dallas, Texas) (5). Sequencing was done in phage M13 by the method of Biggin et al. (7) as described (2, 3, 6). JEG-3 cells (8) were grown to confluency in Dulbecco's modified Eagle's medium plus 25 mM glucose, 25 mM HEPES, 4 mM glutamine, 50 µg/ml glutamycin, and 20% (v/v) heat-inactivated fetal bovine serum on plastic dishes (Nunc, Denmark) in 5% CO₂, 95% air with changes of medium every 48 h. Treatments with 1.5 mM 8-bromo-CAMP (Sigma) and/or 20 µg/mL cycloheximide (Sigma) were done as described under "Results." Cellular RNA was extracted in 4 M guanidine thiocyanate, electrophoresed, blotted to nylon membranes, and probed, as described (9). Fetal tissues were obtained under approved protocols from deliveries by elective therapeutic cervical dilatation and evacuation. Gestational ages were estimated by fetal foot length, regardless of gestational history (10). Fetal and adult tissue RNAs were prepared, blotted, and probed as described (11). Probes used were pbAdx-6 (described below), hSCC-71 (3), and pfos-1 (12).

RESULTS

Identification and Sequence of the Three Adrenodoxin Clones—Screening of our human adrenal cDNA library was

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The abbreviations used are: HEPES, (2-hydroxyethyl)-1-piperazineethanesulfonic acid; ACTH, adrenocorticotropic; 8-Br-cAMP, 8-bromo-cAMP.
done under conditions of low stringency (6) using a bovine adrenodoxin cDNA provided by M. Waterman (Dallas, TX). Several clones were identified by probing with the 5'-half of the bovine cDNA lying on a 520-base pair *PstI/XbaI* fragment. The three clones giving the strongest hybridization signal, designated hAdx-2, -6, and -7, were plaque-purified, and their inserts were subcloned in pUC-19. The cloned cDNAs in hAdx-2, -6, and -7 were approximately 1200, 900, and 1250 base pairs, respectively, all longer than the full-length 800-base pair bovine cDNA (5). Although these clones had different restriction endonuclease mapping patterns, all possessed an *XbaI* site, as did the bovine cDNA; therefore, we sequenced all three clones. The relationship of the three clones and their sequencing strategies are shown in Fig. 1. All three clones encode full-length mature adrenodoxin, although hAdx-2 lacks the region encoding most of the prepeptide. The varying lengths of the three clones are due to the use of three alternate polyadenylation signals found 20-26 bases upstream from poly(A) stretches in each clone. Only one of these polyadenylation signals, that used in clone hAdx-7, has the AATAAA consensus sequence (13); the apparent polyadenylation signals used in hAdx-2 is ATAAA, while that used in hAdx-6 is ATTTAAA (Fig. 2). A fourth potential polyadenylation signal, ATTTAAA, found 66 bases downstream from the translational stop codon, is used in bovine adrenodoxin mRNA (5). However, no clones using this site were found, and RNA transfer blots cannot determine the presence or absence of human adrenodoxin mRNA molecules terminating at this site.

**Tissue Distribution and Size of Adrenodoxin mRNA—** Human adrenal, testis, and placenta all synthesize steroid hormones and contain P450scc mRNA (11, 14); hence, these tissues are also expected to be rich sources of adrenodoxin mRNA. An RNA gel transfer blot (Fig. 3) shows that adrenodoxin mRNA is most abundant in the testis and adrenal but that appreciable quantities are found in the placenta. Furthermore, some adrenodoxin mRNA is also detected in each of the other six tissues examined. While some steroidogenic activity has been ascribed to these tissues, especially in the fetus (15), no P450scc mRNA was detected in similar RNA gel transfer experiments (11). Thus, it is unlikely that the encoded adrenodoxin in these nonsteroidogenic tissues participates in electron transfer to P450scc. Our hAdx-6 cDNA may be cross-hybridizing with mRNA for a different but structurally related iron-sulfur protein. Alternatively, it is possible that the same adrenodoxin iron-sulfur protein mediates electron transport to other nonsteroidogenic mitochondrial cytochrome P450 enzymes in these other tissues. Since the RNA transfer blot was probed and washed under highly stringent conditions, it is most likely that all these tissues contain the same adrenodoxin mRNA.

All tissues contained the same pattern of adrenodoxin mRNAs ranging from 1.0 to 1.7 kilobases. These three species of RNA are poorly distinguished in the blot in Fig. 3, since large amounts of RNA were loaded, and some degradation of

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**Fig. 1.** Schematic diagram of the three human adrenodoxin cDNAs. The upper diagram shows the protein-coding regions of the clones as boxes; the hatched region corresponds to the mature adrenodoxin found in mitochondria while the open regions correspond to the additional amino acids found in the prepro-protein. The 5'- and 3'-untranslated regions are indicated as a solid line. The 5'-ends of clones hAdx-2, -6, and -7 are designated by lines going to the numbers 2, 6, and 7, respectively. The 3'-ends of these clones are designated by the open arrowheads below the numbers 2, 6, and 7. Only the restriction endonuclease sites used in sequencing are shown: B, *BamHI*; S, *Sau3A1*; X, *XbaI*. The sequencing strategies for each clone are shown below.

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**Fig. 2.** Sequences of human adrenodoxin cDNAs. The corresponding regions of the three clones were identical at all nucleotides except for two bases in the 5'-untranslated region of clone hAdx-6 shown below the composite sequence. The cleavage sites of mature adrenodoxin from the prepro-protein are between amino acids 56 and 57, and between amino acids 170 and 171. The apparent polyadenylation signals used by hAdx-6, -7, and -2 (reading from 5' to 3') are indicated in *bold boxes*, and the ATTTAAA sequence used in the polyadenylation of bovine adrenodoxin mRNA is indicated in a *light box*. The sites of poly(A) addition are shown by *arrowheads*.
RNA often occurs in fetal tissues before they are frozen. However, bands of these three sizes are seen in RNA from JEG-3 cells (Figs. 4 and 5) and also from primary cultures of human granulosa cells (9), and from primary cultures of human fetal adrenal or testicular cells (16). An additional band of about 3.8 kilobases is also seen in most blots. The nature of this RNA is unknown. It may represent an unprocessed nuclear precursor or another species of adrenodoxin mRNA having a very long 3′ untranslated region. However, examination of Southern blots and multiple genomic clones indicates there is only one functional adrenodoxin gene; hence, this RNA does not arise from another related gene.

Regulation of Expression of Adrenodoxin—The principal hormonal stimulators of steroid hormone synthesis are ACTH in the adrenal and the gonadotropins, luteinizing hormone and follicle-stimulating hormone, in the gonad. All work through cell-surface receptors to stimulate intracellular cAMP, which in turn stimulates accumulation of mRNA for P450scc (15, 17), mediated principally by increased gene transcription (18). Cyclic AMP also stimulates steroidogenesis and accumulation of P450scc mRNA in the placenta (3, 15, 19). We examined the hormonal regulation of adrenodoxin and P450scc mRNAs in the transformed cytotrophoblast tumor cell line JEG-3.

JEG-3 cells accumulate mRNA for both P450scc and adrenodoxin for up to 48 h while in the presence of 1.5 mM 8-Br-cAMP. By contrast, mRNA encoded by the fos oncogene accumulates very rapidly, reaching its maximal value by 30 min and then diminishing steadily thereafter (Fig. 4), a time course similar to that seen in activated fibroblasts (20); however, densitometric scanning of the data in Fig. 4 shows that 6 h after stimulation the amount of fos mRNA is 5-fold greater than control. Incubation of JEG-3 cells with 20 μg/ml cycloheximide for 30 min before adding 8-Br-cAMP had varying effects on the mRNA for P450scc, adrenodoxin, and fos measured 6 h later (Fig. 5). Cycloheximide quickly reduced basal P450scc mRNA to undetected amounts and reduced the cAMP-stimulated P450scc mRNA to much less than baseline amounts. By contrast, both cycloheximide and 8-Br-cAMP had stimulatory effects on adrenodoxin mRNA; when administered together these effects were additive as shown by laser densitometric quantitation of the data in Fig. 5. Both cycloheximide and 8-Br-cAMP also stimulated accumulation of fos mRNA, though to a greater extent than their stimulation of adrenodoxin mRNA. When administered together, laser densitometry shows that the stimulatory effects of cycloheximide and 8-Br-cAMP on fos are multiplicative. Thus, cycloheximide and 8-Br-cAMP appear to exert at least three different classes of effects on JEG-3 cell mRNA. These effects of cycloheximide on P450scc and adrenodoxin mRNAs could be duplicated by incubating the cells with 200 μM puromycin for 30 min before adding the 8-Br-cAMP. By contrast, adding 2 μg/ml actinomycin D 30 min before stimulating with 8-Br-cAMP blocked the increases in P450scc and adrenodoxin (not shown). Thus, two drugs inhibiting protein synthesis by different mechanisms stimulate accumulation of adrenodoxin mRNA, while inhibiting the accumulation of P450scc mRNA.

**DISCUSSION**

The sequence of mature human adrenodoxin is highly homologous to the bovine protein (105 of 114 amino acids, 3242 Human Adrenodoxin

![Fig. 3. Gel blot of total cellular RNA probed with hAdx-6.](image)

![Fig. 4. RNA transfer blots. Confluent JEG-3 cells were exposed to 1.5 mM 8-Br-cAMP for the number of hours indicated below panel C, then harvested for RNA preparation, gel electrophoresis (15 μg of total RNA/lane), blot transfer, and probing with the three probes indicated: upper panel, hSCC-71; middle panel, phAdx-6; lower panel, v-fos. The autoradiographs shown are from one of two experiments yielding the same result.](image)

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appears to be due to enzymes other than those functioning in the adrenals and gonads, as the mRNAs for P450scc, P450c17, and P450c21 could not be detected in transfet blots of RNA from human fetal ovary, kidney, muscle, liver, intestine, or spleen (11). Therefore, detecting adrenodoxin mRNA in these tissues was unexpected. As these tissues lacked detectable P450scc mRNA, it is likely that the adrenodoxin in these tissues is functioning as an electron transport intermediate for other mitochondrial cytochromes P450. Thus, the term “adrenodoxin” may be inappropriately parochial for this widely distributed protein.

Cycloheximide-mediated accumulation of fos mRNA has been described by several groups (24–26). However, the stimulatory effect of cycloheximide on adrenodoxin mRNA in JEG-3 cells stands in sharp contrast to earlier studies of adrenodoxin in primary cultures of bovine adrenocortical cells. In the bovine adrenal system, the mRNAs for P450scc and adrenodoxin (as well as for the microsomal steroidogenic enzymes P450c17 and P450c21) always respond in parallel: ACTH and cAMP stimulate these mRNAs, and cycloheximide ablates that stimulation (5, 18). These observations suggested the presence of a rapidly turning over cycloheximide-sensitive “steroid hormone-inducing protein” (18). While a 30-amino acid “steroidogenesis activator polypeptide” has recently been isolated (27, 28), the relationship of steroidogenesis activator polypeptide to the hypothetical steroid hormone-inducing protein, if any, is unknown. However, the parallel stimulation of P450scc and adrenodoxin mRNAs seen in bovine adrenal cells is clearly not a generalized phenomenon among steroidogenic tissues. Normal human cytotrophoblasts as well as transformed trophoblastic cells respond to cAMP with accumulation of adrenodoxin mRNA.3 Similarly, cycloheximide does not inhibit the accumulation of P450scc and adrenodoxin mRNAs in primary cultures of human ovarian granulosa cells stimulated with 8-Br-cAMP (9). The responses of the JEG-3 cells employed in our present study differ. Unlike the human granulosa cells but like the bovine adrenocortical cells, cycloheximide inhibits the accumulation of P450scc mRNA in response to cAMP. Unlike the bovine adrenal cells, cycloheximide stimulates accumulation of adrenodoxin mRNA and promotes a further additive increase in adrenodoxin mRNA in cells stimulated with 8-Br-cAMP. Thus, the differences among the bovine adrenocortical system and human systems we have studied appear to involve multiple factors including species, cell type, cell transformation, and hormonal pretreatment. One possible mechanism for cycloheximide’s stimulatory effect on adrenodoxin mRNA might involve a rapidly turning over cycloheximide-sensitive nuclease specific for AU-rich regions. AU-rich sequences increase mRNA turnover when incorporated into the 3′-untranslated regions of otherwise stable mRNAs in transformed cells, and cycloheximide treatment of such cells increases accumulation of these modified mRNAs (29). The 3′-untranslated region of adrenodoxin mRNA is 72% AU. In cultured human granulosa cells, cycloheximide similarly stimulates accumulation of mRNA for the low density lipoprotein receptor, which also has an AU-rich 3′-untranslated region (30). While these data do not rule out the existence of rapidly turning over cycloheximide-sensitive steroid hormone-inducing proteins, they indicate that cellular strategies for the control of steroidogenesis are varied and complex and are unlikely to fit into a single common overall scheme.

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3 L. Kao, T. Golos, and J. F. Strauss III, unpublished results.
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