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Mitochondrial-genome-encoded RNAs: Differential regulation by corticotropin in bovine adrenocortical cells

(cytochrome oxidase/NADH dehydrogenase/ATPase/steroidogenesis)

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ABSTRACT **Differential screening of an adrenal cortex cDNA library for corticotropin (ACTH)-inducible genes led to the isolation of a group of cDNAs representing mitochondrial genes that encode subunits of cytochrome oxidase, ATPase, and NADH dehydrogenase. Northern blot analysis of RNA from cells stimulated by ACTH confirmed the induction of these genes by ACTH yet revealed major differences in the relative responses of the respective mRNAs. The levels of mRNAs for cytochrome oxidase subunit I and ATPase increased 2- to 4-fold and for NADH dehydrogenase subunit 3 increased 20-fold, whereas the levels of the mitochondrial 16S rRNA showed no change within 6 h of ACTH stimulation. These effects of ACTH on mitochondrial mRNA levels probably result from both activation of the H2 transcription unit that encodes mitochondrial mRNAs and alteration of mRNA stability. ACTH also increased the activity of cytochrome oxidase after 12 h of stimulation. Examination of the tissue specificity of expression of five mitochondrial genes showed a wide range of RNA levels among 11 tissues but high correlations between individual RNA levels, consistent with a coordinated expression of the mitochondrial genes, although at different levels in each cell type. Proportionately high levels of mitochondrial mRNAs were found in adrenal cortex, probably reflecting a stimulatory effect of ACTH *in vivo*. Overall, the results indicate that ACTH enhances the energy-producing capacity of adrenocortical cells.**

Mammalian mitochondrial DNA (mtDNA) encodes 13 proteins, 2 rRNAs, and 22 tRNAs (1–3). Four of the five complexes of the oxidative phosphorylation system include subunits encoded by mitochondrial genes. The other subunits are encoded by nuclear genes and are transferred into the mitochondria after translation in the cytoplasm (1–3). The levels of some mitochondrial mRNAs are regulated in different tissues by hormonal factors, such as thyroid hormone (4–7), androgen (8), estrogen (9, 10), glucocorticoids (11), and follicle-stimulating hormone (12).

Many central regulatory events of steroid hormone production are associated with mitochondria. Corticotropin (ACTH) and other trophic hormones initiate the transfer of cholesterol into mitochondria and also induce the synthesis of steroidogenic enzymes, including mitochondrial P450scc system that catalyzes cholesterol conversion to pregnenolone by using reducing equivalents from NADPH (13–15). *In vivo* treatment with ACTH increases the mitochondrial volume and the cristae surface area per cell (16, 17), stimulates mtDNA replication (18–20) and mitochondrial protein synthesis (16, 20–22), and elongates the half-life of mitochondria (23) and of their proteins (24). Although ACTH affects the structure of mitochondria and many associated functions, it is not known whether any of these result from changes in the transcription of mtDNA.

Our differential hybridization screening of an adrenal cortex cDNA library revealed many cDNAs representing RNAs that are induced in cultured cells after incubation with ACTH. Some of these clones represented mitochondrial genes encoding subunits of cytochrome oxidase (CO), NADH dehydrogenase (ND), and ATPase. This finding led us to examine the possible role of ACTH in the regulation of mitochondrial genome expression.

MATERIALS AND METHODS

Cell Culture. Primary cultures of bovine adrenal cortex cells were prepared and grown to confluence in Dulbecco's modified Eagle's medium/Ham's F-12, 1:1 (vol/vol), containing 12.5% (vol/vol) horse serum and 2.5% (vol/vol) fetal calf serum (25). A day before and the day of ACTH treatment the medium contained also 20 nM selenious acid (Fluka), 1 μM α-tocopherol (Sigma), and 2 mM ascorbic acid (Merck). Cells were incubated with 1 μM ACTH-(1–24) (Organon) in freshly prepared medium.

RNA Isolation. Total RNA from cultured cells was isolated using Gough's method (26). Bovine tissues for RNA isolation were obtained within an hour after slaughter, frozen in liquid nitrogen, and ground to powder in liquid nitrogen in a Waring blender. Total RNA from the powdered tissues was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction (27). Mitochondrial RNA was isolated by phenol purification (28).

cDNA Library Screening. The bovine adrenal cortex cDNA library in λgt11 was constructed from poly(A)$^+$ RNA (29). For differential hybridization screening *Escherichia coli* Y1090 were transfected with the phages and plated at a density of 3500 plaque-forming units per 90-mm Petri dish. The phage DNAs were blotted onto duplicate filters of Biotrace NT nitrocellulose (Gelman). The blots were incubated with ^{32}P-labeled cDNA probes generated by reverse transcriptase (BRL) with oligo(dT) primers from total RNA samples isolated from control cells without ACTH stimulation and from cells stimulated by ACTH for 6 h. The plaques with differential signals were picked and rescreened.

Isolation of cDNA Inserts. The cloned cDNA inserts were amplified directly from plaques by the polymerase chain reaction (PCR) (30). Plaques were transferred with sterile toothpicks to 100 μl of PCR mixture containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, all four dNTPs (each at 0.2 mM), 100 nM forward and 100 nM reverse λgt11 *Eco*RI site primers (Biolabs), and 2.5 units of Replitherm polymerase (Epicentre Technologies). The reaction was carried out for 20 cycles in a thermal cycler (PTC-100, MJ Research) programmed for 30 sec at 95°C, 60 sec at 60°C, and 90 sec at 72°C per cycle, and a final step of

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Abbreviations: ACTH, corticotropin; CO, cytochrome oxidase; ND, NADH dehydrogenase.

300 sec at 72°C. PCR products were separated on 1% agarose gel. The fragments were isolated from agarose by electroelution in a UEA electroelutor apparatus (International Biotechnology) and ethanol-precipitated. Purified double-stranded PCR products were sequenced (31) with Sequenase (USB) using λgt11 *Eco*RI site primers. The cDNA for CO subunit IV (CO-IV) was isolated from a clone (32) generously provided by M. Lomax (University of Michigan, Ann Arbor).

Northern Blot Analysis. Total RNA was separated on a 1.2% agarose/formaldehyde gel, electrotransferred in a Trans-Blot cell (Bio-Rad) to a GeneScreen nylon membrane (New England Nuclear), and UV-crosslinked (25). Isolated cDNAs were labeled by "random-primed" method using Boehringer Mannheim kit and [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). Blot hybridization, washing, and rehybridization were carried out according to GeneScreen manual. When blots were incubated with several probes successively, the previous probe was stripped, and the removal of its signal was verified by autoradiography. The Northern blots were quantitated using a Molecular Dynamics densitometer. Each of the tissue and cultured cell blots was repeated with RNA isolated from at least two experiments with essentially identical results.

CO Assay. Partially purified mitochondria from cultured adrenocortical cells were isolated after disruption in a Dounce homogenizer (33). CO activity was assayed by the decrease in A_{550} during the incubation of crude mitochondrial preparations with 0.1 mM reduced cytochrome *c* (from bovine heart, type V, Sigma) for 1 min at 38°C in 10 mM potassium phosphate (pH 7.0). Mitochondrial protein was assayed by Pierce protein assay reagent using bovine serum albumin (Sigma) as the standard.

RESULTS

Isolation and Characterization of Cloned cDNAs. The cDNA probes for differential hybridization were synthesized from total RNA isolated in three experiments from adrenocortical cells grown with (ACTH$^+$) and without (ACTH$^-$) stimulation by ACTH. Initially, 182 plaques with even a low differential signal were picked out of ≈100,000 recombinant plaques. On subsequent screenings ≈40 clones were scored positive, yielding a frequency of ≈1/2500.

The sequences of nine cDNA inserts matched segments of bovine mtDNA (Fig. 1 and Table 1). Two of the isolated clones represented the 12S rRNA gene of mtDNA. Five clones represented the gene encoding CO-I; three of these included the Ser-tRNA sequence at the 3′ end. One clone included the overlapping coding regions for subunits 8 and 6 of ATPase [3′ end of the ATPase8 gene extends 40 nt into the 5′ end of the ATPase6 gene (1–3)], and one clone represented the gene encoding subunit 3 of ND (ND-3). Three clones corresponding to the 16S rRNA of mitochondrial ribosomes were isolated in an independent experiment.

Table 1. Coordinates of cloned cDNAs on the bovine mtDNA sequence

Gene	Coding region, bp	Clone no.	Nucleotide First	Nucleotide Last	cDNA length, bp
12S rRNA	955	426	474	1,250	777
		3605	585	1,072	488
16S rRNA	1571	411	1819	2,779	961
		3447	1524	1,993	470
		4223	1783	≈2,500	≈720
CO-I	1543	324	5992	7,300	1309
		673	5855	6,690	836
		3607	6194	7,303	1110
		3601	6368	7,308	941
		4119	6791	7,133	343
ATPase8+6	842	4112	8156	8,890	735
ND-3	346	4214	9843	10,167	325

Numbering is based on ref. 34.

To examine specificity of the cloned cDNAs, radiolabeled cDNA probes were incubated with Northern blots of RNA isolated from adrenal cortex mitochondria and 11 tissues (Fig. 2). The size of the major band recognized by each cDNA was similar to the length of the corresponding gene, as the mitochondrial genes lack noncoding regions. The CO-I probe hybridized to a single band slightly longer than CO-I gene (≈1700 vs. 1542 bp) probably because of inclusion of the flanking Ser-tRNA sequence in the mRNA, as observed in cloned cDNAs. ATPase cDNA hybridized to two bands. The main band of ≈900 bp is consistent with the 842-bp sequence of the overlapping genes encoding ATPase subunits 8 and 6. An additional weaker band at ≈1700 bp may represent the common precursor (1625 bp) for ATPase8/6 and CO-III, which are juxtaposed in the genome without punctuating tRNA genes that flank other mitochondrial genes (Fig. 1). The cDNAs for ND-3 and 12S and 16S rRNAs hybridized to single bands of ≈400, ≈1000, and ≈1600 bp, respectively, consistent with the sizes of the corresponding genes (Table 1). The cDNA probe for the nuclear-encoded CO-IV mRNA did not hybridize with the mitochondrial RNA (Fig. 2), verifying that the mitochondrial RNA preparation did not include cytoplasmic RNA.

Tissue Specificity of Mitochondrial RNA Levels. To determine whether the levels of mitochondrial RNAs vary in a tissue-specific manner, we compared the levels of the mitochondrial genome transcripts by densitometric analysis of the Northern blots (Fig. 2). The amount of the mitochondrial RNAs in the total cellular RNA widely varied among 11 tissues examined. The highest levels of all the mitochondrial RNAs were observed in the heart muscle followed by brain

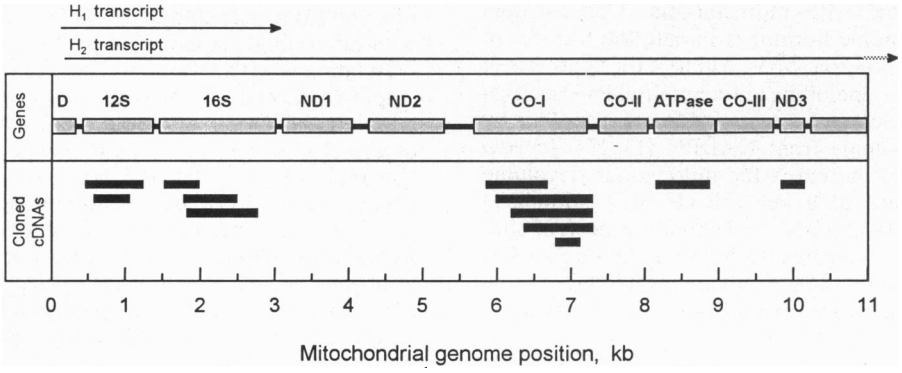

FIG. 1. Alignment of the cloned cDNAs with the mitochondrial genome. For exact coordinates see Table 1. The short unmarked solid bars encode mitochondrial tRNAs. ATPase, the overlapping genes of subunits 6 and 8 of ATPase.

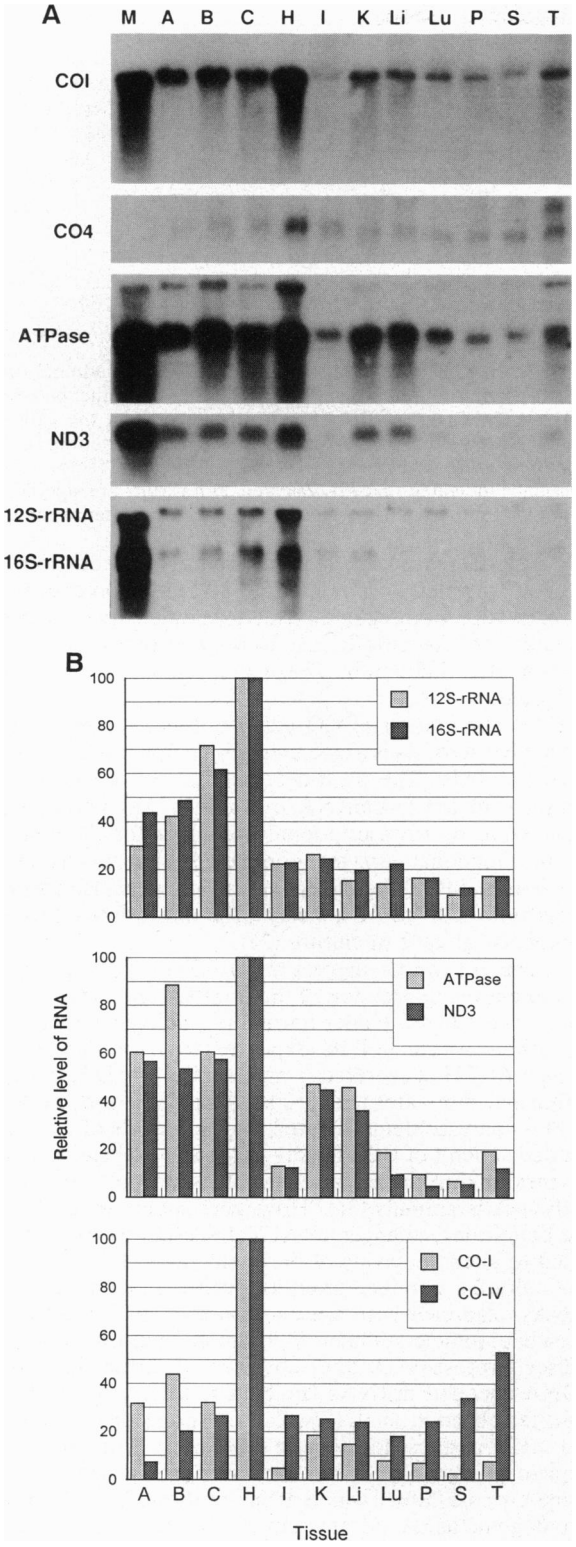

FIG. 2. Levels of the mtDNA-encoded RNAs in various tissues. (*A*) Autoradiograms of total RNA blot prepared and incubated with the indicated cDNA probes. The amount of total RNA per lane was 5 μg for mitochondrial RNA and 20 μg in other lanes. M, adrenal cortex mitochondria; A, adrenal cortex; B, brain; C, corpus luteum; H, heart; I, intestine; K, kidney; Li, liver; Lu, lung; P, pituitary; S, spleen; T, testis. (*B*) Quantitative presentation of the Northern blot results based on densitometry.

and steroidogenic tissues. The levels of the 12S and 16S rRNAs were highly correlated across all tissues ($r = 0.98$; Fig. 2). The levels of CO-I, ATPase, and ND-3 mRNAs also correlated highly with each other across tissues ($r > 0.89$). The tissue levels of mRNA for the nuclear-DNA-encoded CO-IV subunit showed a lower correlation ($r = 0.69$) with the levels of CO-I mRNA and even lower with other mitochondrial mRNAs.

ACTH Effects on Mitochondrial RNA Levels. Since most of the cloned cDNAs were isolated by differential hybridization using $ACTH^-$ vs. $ACTH^+$ probes, the corresponding RNAs were expected to be induced in adrenocortical cells stimulated with ACTH. To examine the time course of ACTH-induced changes, we isolated total RNA from cells treated with ACTH for various times and assayed the levels of specific RNAs by Northern blot hybridization.

The Northern blot results confirmed that all the RNAs identified by differential hybridization are indeed induced by ACTH (Fig. 3). However, the responses of different RNAs to ACTH greatly varied. While the levels of the CO-I and ATPase mRNAs and 12S rRNA increased 2- to 3-fold after 6 h of ACTH stimulation, ND-3 mRNA levels rose 20-fold. After induction, all the mRNA levels declined at various rates and dropped below the control levels by 48 h after the start of the experiment. The 16S rRNA showed no significant change during the first 36 h and could serve as a control that there are equal quantities of RNA on each lane of the blot. The glycolytic enzyme glyceraldehyde-3 phosphate cDNA probe, used as an additional control, showed a pattern of regulation similar to CO-I mRNA (data not shown).

ACTH Effects on CO Activity. The RNA blot experiments suggested that ACTH may regulate the activity of the oxidative phosphorylation system. To examine this we studied the effect of ACTH on CO activity in mitochondria isolated from adrenocortical cells (Fig. 4). ACTH had no significant acute effect on CO activity within the first few hours, but it increased the activity nearly 2-fold after 12 h of stimulation. Thus, the activity rose with an ≈6-h delay after the peak of CO-I mRNA level (cf. Figs. 3 and 4). The assay of total mitochondrial protein per plate showed no significant change during the first 36 h and a 30% increase at 48 h.

DISCUSSION

Differential screening of an adrenal cortex cDNA library for ACTH-inducible genes led to the isolation of a group of cDNAs representing mitochondrial genes that encode subunits of CO, ATPase, and ND. Northern blot analysis of RNA from cells stimulated by ACTH confirmed the induction of these genes by ACTH and yet revealed major differences in the relative responses of the respective mRNAs. The time courses showed the major increase in the initial 6 h and a decline after 24–36 h. Examination of the tissue specificity of expression of five mitochondrial genes demonstrated a wide range of RNA levels among 11 tissues but high correlations between individual RNA levels consistent with a coordinated expression of the mitochondrial genes, although at different levels in each cell type.

The ACTH-dependent increase in mRNA levels may stem from effects on three levels: mtDNA replication, mtDNA transcription, and the stability of the RNAs. A 2- to 20-fold increase in mRNA levels within 6 h (Fig. 3) could not result from increased mtDNA synthesis, since ACTH stimulates mtDNA replication much more slowly after 2 days of treatment (19).

The transcription of the H strand of mtDNA is initiated at two adjacent promoters in the D region (Fig. 1). The H_1 transcript includes the two rRNAs. The H_2 transcript encompasses the whole length of the H strand and includes the two rRNAs and 12 of the 13 protein-encoding genes of mtDNA (1–3). This precursor is processed into mature RNAs by endonuclease cleavage generally at the junctions between the coding regions (35).

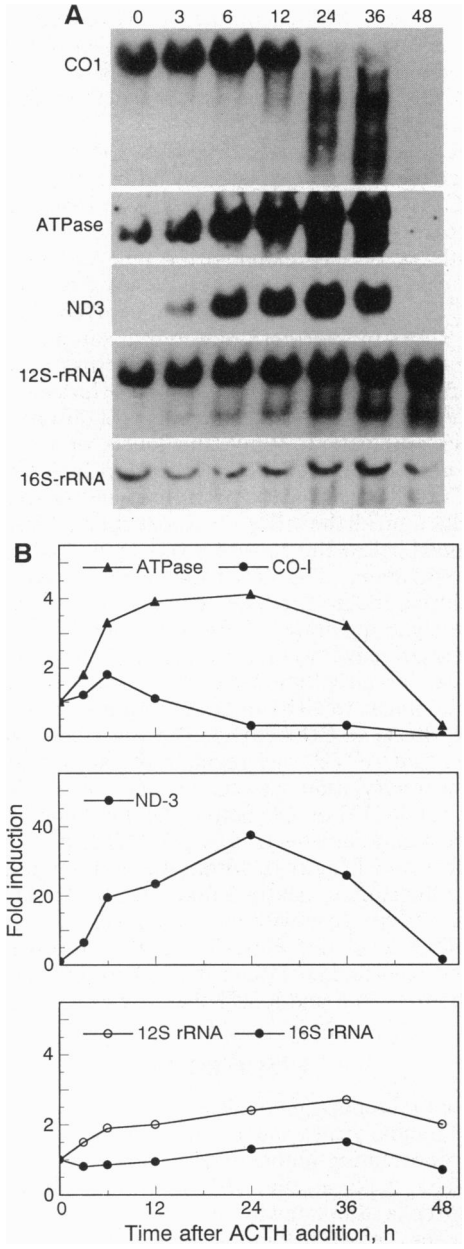

FIG. 3. ACTH regulation of the levels of the mtDNA-encoded RNAs in adrenal cortex cells. Primary cultures were incubated with ACTH for the number of hours shown above each lane. The amount of total RNA was 20 μg in all lanes. (*A*) Autoradiograms of the RNA blot hybridized successively with the indicated cDNA probes. (*B*) Quantitative presentation of the Northern blot results based on densitometry. The experiment was repeated twice yielding essentially identical results.

The observed increases in RNA levels are compatible with specific activation of the H_2 transcription unit. This would affect the levels of all mRNAs encoded by this transcript. Indeed, our results show an increase in the levels of all the mRNAs examined (Fig. 3).

Since the H strand is transcribed as a single unit, the differences in the levels of the mRNAs cannot result from differential transcription but must reflect differential stability of the mRNAs. It is noteworthy that the Northern blots revealed degradation products for CO-I 24–36 h after ACTH stimulation (Fig. 3). Moreover, as compared to the other two mRNAs, the levels of CO-I mRNA showed a much lower increase. These findings suggest that CO-I mRNA may be more rapidly degraded than the other mRNAs examined here. The decreased levels after prolonged incubation with ACTH may reflect termination of ACTH action and, possibly, activation of a regulatory feedback mechanism, such as activation of a specific RNase to prevent overaccumulation of RNA after induction. These mechanisms remain to be elucidated.

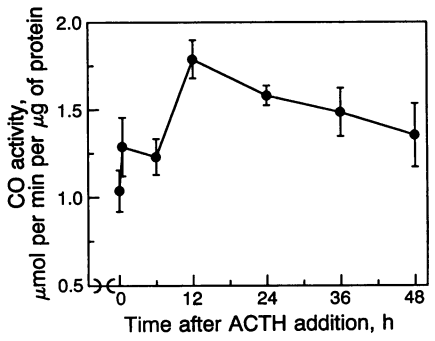

FIG. 4. ACTH regulation of the CO activity in adrenal cortex cells. Primary cultures of adrenal cortex cells were incubated with ACTH. CO activity of mitochondria isolated from the cells was assayed. Each point is the mean of two determinations of two incubations. The error bars represent the SEM. One factor analysis of variance indicated that 12-, 24-, and 36-h results are significantly different from the control value at 0 h at 95% confidence limit.

ACTH stimulation of CO activity demonstrates that the rise in mRNA levels is associated with a functional change in enzyme activity. The ≈6-h delay in the rise of CO activity, after the peak in CO-I mRNA levels (Figs. 3 and 4), probably results from posttranscriptional processes for the constitution of functional enzyme complexes. The levels of the mitochondrial P450 system enzymes were similarly observed to rise with a 6- to 12-h delay after the mRNA levels in adrenocortical cells in culture (25).

The present results suggest that ACTH may be necessary to maintain the expression of the mtDNA-encoded enzymes at an induced level. Under normal physiological conditions, their levels would not be expected to vary significantly, because ACTH is secreted from the pituitary in a circadian rhythm and stimulates the adrenal gland daily. Indeed, Western blot analyses demonstrated that the levels of a nuclear-encoded subunit of CO and key mitochondrial steroidogenic enzymes in the bovine adrenal cortex do not vary significantly across animals (36). However, under stress and disease conditions, changes in ACTH secretion would be expected to alter the levels of these enzymes (14).

In cultured porcine granulosa cells, the mitochondrial mRNAs examined here are expressed at high basal levels, and while follicle-stimulating hormone treatment induces P450scc expression, it does not cause a further increase in mtDNA-encoded mRNAs (F. Hatey, M.R., and I.H., unpublished observations). Hence, the expression of mitochondrial mRNAs does not depend on trophic hormonal stimulation in all types of cultured steroidogenic cells. Yet, in bovine corpora lutea, which at maturation can be as highly steroidogenic as the adrenal cortex, the levels of a nuclear-encoded CO subunit and the mitochondrial P450scc system enzymes are correlated (36). Similarly, during the mouse estrous cycle, increased ovarian progesterone secretion during diestrus is accompanied by an increase in CO activity in the ovary (37). Based on the current results, these changes in enzyme activity may be hypothesized to result from transcriptional induction of the respective genes.

Steroidogenesis is an energy-dependent and NAD(P)H-consuming process (13, 38). Enhancement of the energy-producing capacity of steroidogenic cells by trophic hormones is probably essential to meet the metabolic needs of steroid hormone production. Currently, we do not know

whether the much higher induction of the ND subunit is of physiological significance. This enzyme is assumed to participate in succinate-dependent pathway of reducing equivalent supply to the P450scc system (38). Thus, its dramatic induction by ACTH may be related to its role in NADPH generation, in addition to oxidative phosphorylation.

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