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Cloning of ACTH-regulated Genes in the Adrenal Cortex

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To search for genes that are induced by ACTH in adrenocortical cells, we screened adrenal cortex cDNA libraries by a differential hybridization method using cDNA probes representing mRNAs from cells with or without ACTH stimulation. Forty clones were identified as ACTH induced (yielding a frequency of about 1/2500 plaques screened), and two clones as ACTH repressed. The cDNAs isolated and sequenced include nuclear genes for microsomal steroidogenic enzymes and novel proteins of yet unidentified functions, and mitochondrial genes encoding subunits of oxidative phosphorylation enzymes. Northern blot analysis of RNA from cells stimulated with ACTH confirmed the induction of these genes by ACTH, yet revealed important 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. The enhancement of the levels of the mRNAs could be ascribed to transcriptional activation. Since the mitochondrial genome is transcribed as a single polycistronic unit, to account for the >20-fold differences in the levels of the mitochondrial mRNAs it is necessary to invoke differential stabilities of these mRNAs. The synchronous increase in the expression of both the steroidogenic enzymes and the mitochondrial oxidative phosphorylation system subunits, provides evidence for coregulation of steroidogenic and energy producing capacities of adrenal cells to meet the metabolic needs of steroid hormone production. Suppression of β-actin gene expression may be related to changes in actin polymerization during ACTH-dependent cytoskeletal reorganization.

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INTRODUCTION

The stimulation of steroidogenic cells by trophic hormones affects both their morphology and function [1–4]. Many of these effects depend on changes in the expression of specific genes that encode structural and regulatory proteins and enzymes. ACTH and other trophic hormones have been shown to enhance the expression of protooncogenes within <60 min and of steroidogenic enzymes within hours [4–6].

The present study was initiated to examine the scope of ACTH effects on the expression of other genes in adrenocortical cells. For this purpose we screened adrenal cortex cDNA libraries by a differential hybridization method using cDNA probes representing mRNAs from cells grown with and without ACTH stimulation. The isolated cDNA clones were then sequenced to identify the encoded proteins.

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MATERIALS AND METHODS

Primary cultures of bovine adrenal cortex cells were prepared and grown to confluence in Dulbecco's modified Eagle's medium–Ham's F12 (1:1), containing 12.5% horse serum and 2.5% fetal calf serum [7]. Confluent cultures were stimulated with ACTH as described previously [7, 8]. Control cultures were treated similarly except for ACTH stimulation.

Total RNA from cultured cells was isolated by Gough's method [9], and used for cDNA probe synthesis using reverse transcriptase with oligo(dT) primers. The cDNA probes were ^{32}P-labeled using a random primer method.

For differential hybridization screening, our bovine adrenal cortex cDNA library in λgt11 [10] was plated at a density of 3500 pfu/90-mm Petri dish. The phage DNAs were blotted onto duplicate filters of nitrocellulose, and reacted with ^{32}P-labeled cDNA probes generated from total RNA samples isolated from cells stimulated by ACTH for 6 h, or from control cells. The cDNA inserts of the plaques showing a differential signal, were amplified directly from plaques by poly-

merase chain reaction (PCR). Purified double-stranded PCR products were sequenced with Sequenase (USB) using λgt11 EcoRI site primers [11].

For Northern blot analyses, total RNA from cultured adrenocortical cells was separated on 1.2% agarose-formaldehyde gel, electro-transferred to GeneScreen nylon membrane and reacted with cloned cDNAs as described previously [7, 8]. As controls the Northern blots were also reacted with previously cloned cDNAs representing glyceraldehyde 3-phosphate dehydrogenase, and mitochondrial rRNA genes. Northern blots were quantitated using a Molecular Dynamics densitometer. Each blot was repeated with RNA isolated from at least two independent experiments with essentially identical results.

RESULTS AND DISCUSSION

Isolation and characterization of cloned cDNAs

The cDNA probes for cDNA library screening were synthesized from total RNA isolated in three experiments from adrenocortical cell cultures incubated with fresh medium for 6 h with or without ACTH. In the first round 182 plaques with even a low differential signal were picked out of about 100,000 recombinant plaques. After final plaque hybridization purification about 40 clones were scored as ACTH induced positive (a frequency of about 1/2500), and 2 clones as ACTH repressed.

Among the cDNAs sequenced, 7 showed identity with the known cDNA sequences of microsomal steroidogenic enzymes, cytochrome *P*450c17 (1 clone), and 3β-hydroxysteroid dehydrogenase (3β-HSD) (6 clones). The sequences of 9 cDNA inserts matched bovine mitochondrial genome encoded oxidative phosphorylation system subunits: subunit 1 of cytochrome oxidase (CO-1), subunits 8 and 6 of ATPase (3′ end of the ATPase8 gene extends 40 nucleotides into the 5′ end of the ATPase6 gene), and subunit 3 of NADH dehydrogenase (ND-3) [12–14]. One of the ACTH repressed clones matched bovine cytoplasmic β-actin. Two of the cDNA sequences had no significant match in the EMBL sequence database.

Time course of ACTH-regulation of mRNA levels

To determine the time course of ACTH-dependent changes in the levels of the mRNAs represented by the cloned cDNAs, we assayed the levels of specific RNAs by Northern blot analysis of total RNA from ACTH stimulated cells. The results confirmed that the RNAs recognized by the cDNAs are indeed regulated by ACTH (Figs 1–3). However, the responses of different RNAs to ACTH varied greatly.

Among the steroidogenic enzymes the highest induction of nearly 14-fold was observed for *P*450c17 mRNA. The time course of induction of this mRNA paralleled the response of the nuclear mRNAs encoding mitochondrial *P*450 system enzymes (Fig. 1). The 3β-HSD mRNA showed at most a 3-fold rise in 24 h. The sizes of the mRNA bands recognized by the *P*450c17 and 3β-HSD cDNAs were consistent with previous observations [15, 16].

Although the entire mitochondrial genome is transcribed as one polycistronic unit, the responses of the processed mRNAs representing CO-1, ATPase, and ND-3 subunits also showed major differences (Fig. 2). The highest induction of nearly 40-fold was observed for ND-3, after 12 h of ACTH stimulation. 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.

To assure that there are equal amounts of total RNA in each lane, generally Northern blots were also probed with control cDNAs for RNAs that are presumed to have little or no response to treatment in various systems. The control probes commonly used include cDNAs for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPD), cytoplasmic β-actin, and mitochondrial 16S rRNA. Among these three cDNAs employed in this study only the 16S rRNA showed no significant change during the first 36 h of ACTH stimulation and could serve as a control

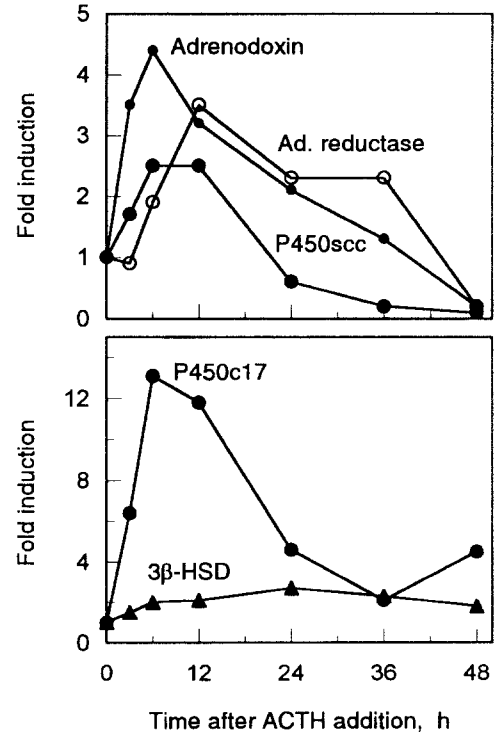

Fig. 1. ACTH regulation of steroidogenic enzyme mRNA levels in bovine adrenocortical cells. Primary cultures were incubated with ACTH for the number of hours shown for each point. The RNA gel including 20 μg of RNA per lane was prepared and blotted as described previously [7, 8]. The quantitation is based on laser densitometric analysis of the Northern blot reacted successively with the respective cDNA probes. The upper panel shows the results for the mitochondrial *P*450 system from Hanukoglu *et al.* [7].

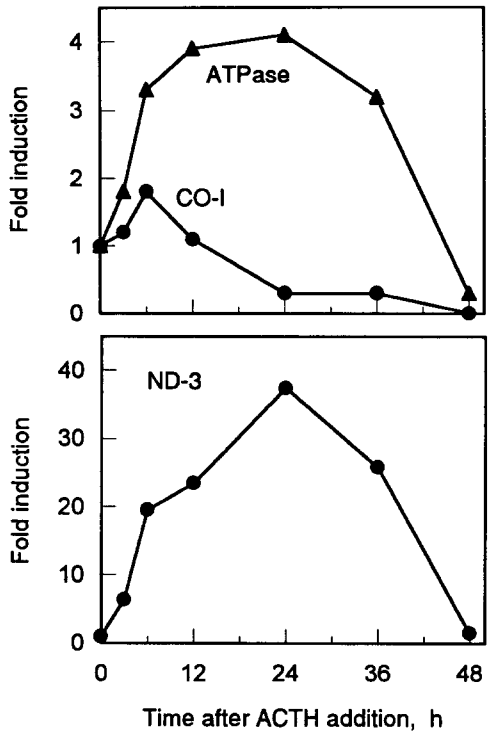

Fig. 2. ACTH regulation of the levels of the mtDNA encoded RNAs in adrenal cortex cells. The data were taken from Raikhinstein and Hanukoglu [8]. For experimental details see Fig. 1.

that there are equal quantities of RNA on each lane of the blot. The GAPD probe showed a pattern of regulation similar to CO-1 mRNA (Figs 2 and 3). The β-actin mRNA levels showed a continuous decline (Fig. 3), consistent with the fact that the cDNA was isolated with an ACTH repressed signal.

ACTH effects on the synthesis and stability of the RNAs

Changes in RNA levels within a few hours of ACTH stimulation may stem from effects on gene transcription, and the stability of the RNAs. The half lives of the mRNAs encoding steroidogenic enzymes examined here are >12 h [17]. Thus, the many fold rises within <12 h in mRNA levels observed for the steroidogenic enzymes (Fig. 1) depend on enhanced RNA transcription, and cannot be ascribed to changes in RNA stability.

The mitochondrial DNA is transcribed in two distinct units. 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 also 12 of the 13 protein encoding genes of mtDNA [12–14]. This precursor is processed into mature RNAs by endonuclease cleavage generally at the junctions between the coding regions. The observed rapid increases in the levels of all the mRNAs examined (Fig. 2) are compatible with specific activation of the H_2 transcription unit. 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. The decreased levels after prolonged incubation with ACTH may reflect termination of ACTH action, and possibly activation of a regulatory response mechanism, such as activation of a specific RNAse to prevent over-accumulation of RNA after induction. These mechanisms remain to be elucidated.

Functional significance of the ACTH-regulated genes

The findings noted above showed that ACTH enhances in parallel the transcription of mitochondrial genes encoding oxidative phosphorylation enzyme subunits, and the nuclear genes encoding steroidogenic enzymes, while suppressing the cytoplasmic β-actin gene. Measurement of CO activity showed that the rise in mRNA amounts is associated with an increase in enzyme activity [8]. The levels of the mitochondrial *P*450 system enzymes were similarly observed to rise with a 6–12 h delay after the mRNA amounts [7]. However, the drop in these mRNAs was not followed by a decrease in enzyme levels, as the enzyme half-lives are longer than that of the mRNAs [7]. It remains to be determined whether the levels of other mitochondrial enzymes identified in this study and the β-actin follow those of their mRNAs.

Steroidogenesis is an energy-dependent and NAD(P)H-consuming process [3, 18–21]. Hence, the

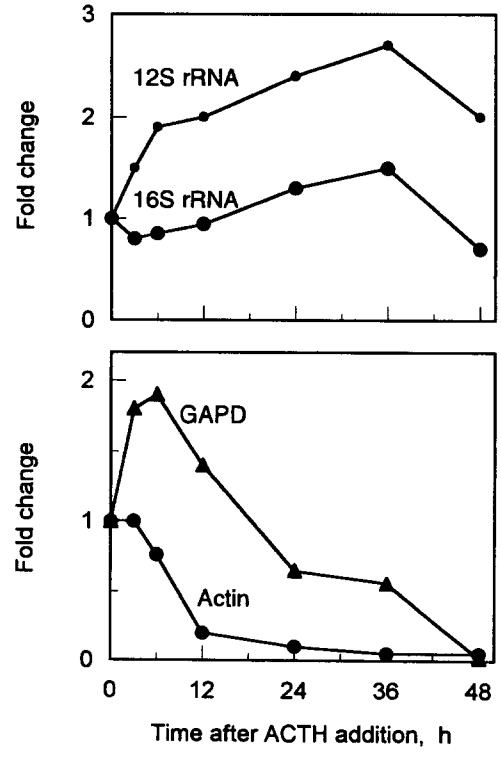

Fig. 3. ACTH regulation of the levels of the RNAs that were used as putative controls in Northern blots. For experimental details see Fig. 1.

synchronous increase in the expression of steroidogenic enzymes and the mitochondrial oxidative phosphorylation system subunits provides evidence for coregulation of steroidogenic and energy producing capacities of adrenal cells to meet the metabolic needs of steroid hormone production.

NADPH that supplies electrons to the mitochondrial *P*450 systems may be generated by several alternative routes [18–21]. The synthesis of NADPH may be regulated in the short-term at the level of the activity of the enzymes involved in the routes of NADPH biosynthesis. Our studies indicated that NADPH generation may also be regulated at the level of the expression of the enzymes that take part in NADPH production. Currently, we do not know whether the high induction of the NADH dehydrogenase subunit is of physiological significance. This enzyme is assumed to participate in succinate dependent pathway of reducing equivalent supply to the *P*450scc system [20]. Thus, its dramatic induction by ACTH may be related to its role in NADPH generation, in addition to oxidative phosphorylation.

Steroidogenesis requires the transfer of cholesterol from cytosolic lipid droplets into mitochondria where the first step of steroid synthesis is catalyzed by cytochrome *P*450scc [2–4]. This process involves the movement of intracellular organelles associated with cytoskeletal elements [2]. The morphological changes that are observed after trophic hormonal stimulation of cultured steroidogenic cells probably reflect these cytoskeletal changes under the artifactual conditions of culture. The suppression of β-actin gene expression revealed by the present study, may result from changes in actin polymerization state during cytoskeletal reorganization [2]. Studies on gonadotropin stimulated granulosa cells also revealed suppression of actin expression [1]. Thus, there appears to be a general pattern of actin gene suppression in trophic hormone stimulated steroidogenic cells from both the ovary and the adrenal.

At present the functions of the proteins encoded by the cloned cDNAs that had no match in the sequence database are not known. Northern blot analysis revealed that one of these cDNAs recognizes a single mRNA band in the corpus luteum and adrenal cortex but not in other peripheral organs (data not shown). The steroidogenic tissue-specific expression of this novel protein suggests a special role in these tissues. The full effect of ACTH in adrenocortical cells probably involves additional genes that were not identified in the present study. The identity and function of these genes remain to be examined by further similar studies.

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