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Global Profiles of Gene Expression Induced by Adrenocorticotropin in Y1 Mouse Adrenal Cells

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ACTH regulates the steroidogenic capacity, size, and structural integrity of the adrenal cortex through a series of actions involving changes in gene expression; however, only a limited number of ACTH-regulated genes have been identified, and these only partly account for the global effects of ACTH on the adrenal cortex. In this study, a National Institute on Aging 15K mouse cDNA microarray was used to identify genome-wide changes in gene expression after treatment of Y1 mouse adrenocortical cells with ACTH. ACTH affected the levels of 1275 annotated transcripts, of which 46% were up-regulated. The up-regulated transcripts were enriched for functions associated with steroid biosynthesis and metabolism; the down-regulated transcripts were enriched for functions associated with cell proliferation, nuclear transport and RNA processing, including alternative splicing. A total of 133 different transcripts, *i.e.* only 10% of the ACTH-affected transcripts, were represented in the categories above; most of these had not been described as ACTH-regulated previously. The contributions of protein kinase A and protein kinase C to these genome-wide effects of ACTH were evaluated in microarray experiments after treatment of Y1 cells and derivative protein kinase A-defective mutants with pharmacological probes of each pathway. Protein kinase A-dependent signaling accounted for 56% of the ACTH effect; protein kinase C-dependent signaling accounted for an additional 6%. These results indicate that ACTH affects the expression profile of Y1 adrenal cells principally through cAMP- and protein kinase A-dependent signaling. The large number of transcripts affected by ACTH anticipates a broader range of actions than previously appreciated. (*Endocrinology* 147: 2357–2367, 2006)

THE PRINCIPAL function of the adrenal cortex is to produce glucocorticoids and mineralocorticoids—hormones essential for survival. These adrenal hormones regulate a broad range of physiological processes including: ionic balance and pH, cellular metabolism, immune system function, and the response to stress. The pituitary hormone ACTH, a master regulator of adrenocortical function, acutely regulates the synthesis of the corticosteroids—especially the glucocorticoids—by mobilizing cholesterol and delivering it to the inner mitochondrial membrane where it serves as the substrate for CYP11A1 (the cholesterol side chain cleavage enzyme), the first enzyme in the pathway of corticosteroid synthesis. In addition, ACTH regulates cholesterol availability, the synthesis of the enzymes required for steroidogenesis, and the size and structural integrity of the adrenal cortex through a series of actions involving changes in gene expression that occur over many hours. Despite the global effects of ACTH on adrenal structure and function, only a relatively small number of ACTH-regulated transcripts have been identified (1).

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Abbreviations: BLAT, BLAST (basic local alignment and search tool)-like alignment tool; CYP11A1, cholesterol side chain cleavage enzyme; FDR, false discovery rate; GO, Gene Ontology; GO-BP, GO Biological Process; GO-MF, GO Molecular Function; MGI, Mouse Genome Informatics; PMA, phorbol 12-myristate 13-acetate; SAM, Statistical Analysis of Microarrays.

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The signaling pathways that mediate the actions of ACTH have been studied extensively in a variety of *in vitro* systems including cell suspensions and primary cell cultures from human, bovine, rat, and mouse adrenal glands and cell lines from minimally deviated tumors such as the mouse Y1 adrenal cell line (2). The consensus of these studies is that the cAMP/protein kinase A signaling cascade mediates most of the effects of ACTH; however, this conclusion is not without debate and other signaling pathways including protein kinase C, Ca^{2+}, cGMP, and MAPK have been implicated as primary or secondary mediators of the steroidogenic actions of the hormone (3–5). The effects of ACTH on adrenal cell proliferation are especially complex and poorly understood. ACTH added to adrenal cells proliferating *in vitro* inhibits cell cycle progression, DNA synthesis and mitosis via a cAMP/protein kinase A-mediated process. The growth-inhibitory effects of ACTH appear to mask an underlying proliferative effect, which can be elicited by adding the hormone as a pulse to cells in the early G1 phase of the cell cycle. The proliferative effect of ACTH is not mediated by the cAMP/protein kinase A pathway; rather it appears to act via a protein kinase C-activated MAPK (Erk1/2) signaling cascade (6).

The identification of only a limited number of ACTH-regulated transcripts and the persistent debate over the central importance of cAMP and protein kinase A in ACTH action prompted us to undertake a global assessment of ACTH action using the Y1 mouse adrenocortical tumor cell line as a model system. Probing the National Institute on Aging (NIA) 15K mouse cDNA microarray (7) for genome-

wide changes in mRNA expression, we demonstrate that ACTH affected the accumulation of 1275 annotated transcripts on the array. Most of these transcripts had not been identified as ACTH responsive previously. Transcripts up-regulated in response to ACTH included a subset with functions in steroid biosynthesis and metabolism; transcripts that were down-regulated in response to ACTH included subsets with functions in RNA processing and cell proliferation. These subsets, however, accounted for only a small fraction of the global response to ACTH. The majority of the effects of ACTH on RNA accumulation could be accounted for by activation of the cAMP/protein kinase A pathway, whereas the protein kinase C pathway made a much smaller contribution. Approximately 38% of the ACTH-affected transcripts could not be ascribed to either pathway and are thus candidates for regulation via other signaling pathways.

Materials and Methods

Cells, cell culture, and RNA isolation

The origin and properties of the ACTH-responsive Y1 mouse adrenocortical tumor cell line used in this study (clone Y1BS1) have been described previously (2). Clone Kin8 is a mutant derivative of Y1BS1 that harbors a dominant inhibitory point mutation in the regulatory subunit of the type 1 enzyme, which renders protein kinase A resistant to activation by cAMP (8, 9). Clone Kin8Cev is a derivative of Kin8 with restored cAMP-responsive protein kinase activity as a consequence of transfection with an expression vector encoding the catalytic subunit of protein kinase A (10). Cells from each clone were plated at a density of 2×10^6 cells/100-mm tissue culture dish, propagated for 3 d in αMEM as described (11) and treated with $ACTH_{1-24}$ (Organon Inc., West Orange, NJ) or other pharmacological agents for 24 h as specified. Cells were harvested by scraping in a guanidine thiocyanate buffer and total RNA was isolated by centrifugation through a cushion of 5.7 M CsCl (12). Because there was no evidence of DNA contamination of the RNA in RT-PCR experiments, deoxyribonuclease treatment was considered unnecessary.

Microarray analyses

Transcription profiles were determined by probing cDNA microarrays with Cy3-and Cy5-labeled cDNA prepared from total RNA using a two-color dye hybridization protocol (13). The microarrays and the protocols for labeling of transcripts were obtained from The University Health Network Microarray Centre (Toronto, Ontario, Canada), details of which are available at www.microarrays.ca. Essentially, total RNA (50 μg) was reverse-transcribed using Superscript II according to the supplier's instructions (Invitrogen Canada, Burlington, Ontario, Canada) in a reaction (40 μl) that included oligo-dT_{20}-dVdN (150 pmol) as primer and Cy3-deoxy (d)-CTP or Cy5-dCTP (25 μM; Invitrogen Canada) as labeling reagent. The fluorescently labeled cDNAs from mock-treated and agonist-treated cells were combined, purified by precipitation with isopropanol, resuspended in 5 μl of nuclease-free water, and used immediately for microarray analysis. A cocktail containing 100 μl digoxygenin Easy Hyb solution (Roche Diagnostics, Laval, Quebec, Canada), 5 μl yeast tRNA (10 mg/ml; Invitrogen Canada) and 5 μl salmon sperm DNA (10 mg/ml; Sigma/Aldrich Canada, Oakville, Ontario, Canada) was added to the fluorescently labeled cDNAs and aliquots of this solution (85 μl) were applied to the microarray slides under coverslips. Slides then were incubated overnight at 37 C in a sealed humidified chamber, washed successively with 1× saline sodium citrate/0.1% sodium dodecyl sulfate at 50 C (three times), 1× SCC at room temperature (twice) and 0.1× SCC (once) and dried by centrifugation. Cy3 and Cy5 signals on the array were quantitated using an Axon GenePix 4000A laser scanner and Axon GenePix software (Molecular Devices, Downingtown, PA). On-slide data analyses, including the assignment of the Cy3 and Cy5 signals to specific cDNAs on each array and the application of the Lowess smoothing function to correct for global differences in Cy3 and Cy5 incorporation were performed with GeneTraffic software (Stratagene, La Jolla, CA). Annotations for the cDNAs spotted on the arrays were retrieved from the Stanford Online Universal Resource for Clones and ESTs (S.O.U.R.C.E.) database (http://genome-www5.stanford.edu/cgi-bin/SMD/source/sourceSearch) in March 2005; only those changes affecting well-annotated transcripts are reported here.

Quantitative RT-PCR

Transcripts were quantitated by RT-PCR using the 7300 Real Time PCR System with resident software (Applied Biosystems, Foster City CA). Total RNA (5 μg) was reverse transcribed as above in 20-μl reactions containing oligo-dT_{18} primer (100 pmol). Aliquots of the RT reaction (1 μl) were amplified over 40 cycles in 25-μl reactions containing gene-specific forward and reverse primers (5 pmol each; Table 1) and the double-stranded DNA binding dye SYBR Green I as provided in the Platinum SYBR Green qPCR SuperMix UDG system (Invitrogen Canada). In each amplification cycle, samples were heated at 95 C for 15 sec to denature the template and incubated at 60 C for 60 sec for 40 cycles to anneal and extend the gene-specific primers. The identity and purity of the resultant PCR products were confirmed by the uniformity of dissociation curves obtained from the amplified products after the quantitative PCR cycles and by electrophoresis of the amplified product before and after digestions with diagnostic restriction endonucleases. Transcript concentrations were determined by comparing the threshold cycle obtained for each transcript to the threshold cycles obtained with cDNA standards and normalizing the data to the levels of transketolase RNA; as determined by quantitative RT-PCR, the levels of transketolase did not change under these conditions of treatment (data not shown).

Statistical analyses

For microarray data, the effects of treatment on the relative abundance of individual transcripts were evaluated using a two-class (unpaired) analysis which compared the ratios of Cy3 to Cy5 signals obtained using RNA from replicate treated and control cells, respectively, to the ratios of signals obtained using RNA samples from a group of independent control cells (n = 6) separately labeled with Cy3 and Cy5. The two-class analysis controls for gene-specific variances in the data—*e.g.* variances caused by preferential labeling of transcripts with either the Cy3 or Cy5 dye (14). In experiments where hypothesis testing involved responses to single agonists, significantly affected transcripts (up-regulated and down-regulated) were identified using the SAM (Statistical Analysis of Microarray) algorithm (15) with a false discovery rate (FDR) cutoff at 0.05. In those experiments where hypothesis testing involved responses to multiple agonists and different cell lines, *e.g.* identification of cAMP/protein kinase A-regulated transcripts, *P* values for each transcript under the different test conditions were combined using the multiple univariate combination method of Fisher (16), and the FDR was determined using the Benjamini-Hochberg procedure (17).

The set of transcripts significantly affected by ACTH in microarray experiments (FDR < 0.05) was tested for enrichment in specific categories relative to the representation of these categories on the NIA 15K mouse cDNA array using Fisher's exact test. Transcripts were categorized according to Gene Ontology (GO) Biological Process (BP), GO Molecular Function (MF), and tissue enrichment based on data compiled previously (18, 19). The GO hierarchy was downloaded from the Gene Ontology database (20), and mouse gene annotations were downloaded from Mouse Genome Informatics (MGI). The gene symbols corresponding to the annotated genes on the NIA 15K mouse cDNA array were mapped to MGI IDs downloaded from the MGI Database in June 2005 (18). The MGI GO annotations were augmented to include all those implied by annotations in a more specific category. In the GO analysis, categories with fewer than ten genes were excluded based on a statistical power analysis, and those with more than 200 genes were excluded because they were not specific to distinct physiological processes or molecular functions. Probes assigned XM IDs in the Zhang *et al.* study (19) were mapped to MGI marker IDs in two steps. First, the 60-oligomer probe sequences were mapped to the GenBank sequences associated with each MGI marker using BLAT [BLAST (basic local alignment and search tool)-like search tool] (21). A FASTA file containing the GenBank sequences and a database report associating MGI marker IDs with these sequences were downloaded from MGI in June 2004. A probe was deemed a hit to a GenBank sequence if BLAT reported a match between

TABLE 1. Gene-specific primers used for quantitative RT-PCR

Gene symbol	NCBI accession no.	Forward primer	Reverse primer	Position	Exons
Cyp11a1	NM_019779	AAGGTACAGGAGATGCTGCG	AGTGTCTCCTTGATGCTGGC	1068–1183	6[a]
Nr5a1	AF511594	TGGACTATTCGTACGACGAGG	GACTGTGCGCTTGAAGAAGC	33–154	2/3
Mc2r	NM_008560	CAGTGCTCACCTTCACATCG	AAGGATGGTTAGTGTCATGGC	777–928	3
Sat1	NM_009121	GATGGCTTTGGAGAACACC	CCTCTGTAATCACTCATCACG	371–546	3/5
Ptp4a3	BC066043	GACTGGATGCCATGCTTAGC	ACAGCACACTGTGACAACCG	527–631	1
Inha	NM_010564	AGCTGTGGTTCCACACAGG	TCAGCAGAGGGAAAGCAGAC	563–742	2
Alas1	NM_020559	TGTGATGAGTTGATGACCAGG	AATGTGGCTTCAGTTCCAGC	1726–1909	11/12
Hmox1	NM_010442	TGAATCGAGCAGAACCAGC	ATGAACTCAGCATTCTCGGC	89–239	1/2
Ftl1	NM_010240	GGAGAAGAACCTGAATCAGGC	GAGGTTGGTCAGATGGTTGC	514–661	1
Tfrc	NM_011638	GCTGAGAATGATGGATCAAGC	GCCTTCATGTTATTGTCGGC	43–210	2/3
Alad	NM_008525	ACATGGACTTGGCAACAGG	AGGAGGCAGCTGATAACAGC	830–962	7/9
Maoa	NM_173740	ACAGCAACACAGTGGAGTGG	GAACATCCTTGGACTCAGGC	1322–1458	13/15
Avpr1	NM_016847	CGCATGCATCTCTTCATCC	CATCACCACCAGCATGTAGG	559–741	1
Fech	NM_007998	CATGAAGTGGAGCACAATCG	TCGCTTCTCTTCTCCTCTGG	751–861	6/7
Hmox2	NM__010443	ATTCAAGCAGTTCTACCGCG	TCTCTTGCTAGCATGGAGCC	752–901	4/5
Hmbs	NM_013551	GGAATGCATGTATGCTGTGG	TCACTGTATGCACTGCTACGG	773–957	3/5
UroS	NM_009479	AGCTGTCTCATCCTGAAGGC	AACCACGTACACAGACTTGGC	365–516	3/5
UroD	NM_009478	AGGACTTCTTCAGCACCTGC	ACCATGGTCACCTCTATGCC	439–583	3/5
Cpox	NM_007757	CCAAGAATCCTTATGCGCC	ATGCTGATCGCAAGCTTCC	847–1022	3/4
Ppox	NM_008911	CGCAAGTTATCATCTGATCCG	AAGATCGCACCATCTGATCC	345–458	2/3
Fth1	NM_010239	TTCTCCACCAATCTCATGAGG	CACACTCCATTGCATTCAGC	334–477	2/3
Tkt	NM_009388	TTGCTAACATCCGAATGCC	CGAGAAGGTGGAATTCTTGG	1019–1170	7/8

Gene-specific primers were designed based on sequences provided under the accession numbers listed in the National Center for Biotechnology Information (NCBI) database. The sequences of forward and reverse primers, the position of the amplified fragment within the transcript, and the exon locations of the forward and reverse primer are noted.

[a] Inasmuch as the Cyp11a1 primers were derived from a region of the transcript that doesn't match the current (Build 34, version 1) annotation of the genomic sequence, their exonic locations were deduced from the genomic sequence provided under NCBI accession no. AC158996.

them at least 20 bp in length, with no insertions or deletions and no more than one mismatching nucleotide. A probe was deemed a match to a MGI marker if it satisfied three criteria: it hit at least one of the GenBank sequences associated with the marker, it hit no sequence associated with a different marker, and it was the only probe that satisfied the first two criteria for the marker. A list of the XM ID to MGI marker ID matches can be found on our web site at http://www.utoronto.ca/schimmer/supplements/supplements.html.

For quantitative RT-PCR experiments, statistical significance among group means was determined using Peritz's multicomparison F test (22).

Results

ACTH regulated transcripts in the Y1 mouse adrenocortical cell line

Transcripts isolated from Y1 mouse adrenocortical cells stimulated with $ACTH_{1–24}$ (20 nM) and from mock-treated controls were differentially labeled with Cy3- and Cy5-dCTP and compared by microarray analysis to identify ACTH-induced changes in RNA accumulation. A representative experiment (Fig. 1) demonstrates the effects of ACTH on the expression profile of Y1 adrenal cells. Cy3- and Cy5-labeled cDNA pools from mock-treated Y1 cells produced signals at 5412 of the 5655 unique annotated cDNAs on the NIA 15K mouse cDNA array that were, for the most part, tightly clustered around a $\log_2$ ratio of 0. The greatest variance in ratios of Cy3- and Cy5-labeled transcripts from the control set was seen among weakly labeled genes where signal to noise ratios were lower and variations were random and not gene specific. In contrast, the $\log_2$ signal intensity ratios obtained comparing labeled cDNAs from ACTH-treated and untreated Y1 cells deviated from 0 for a subset of genes regardless of labeling intensity, reflecting transcripts that either increased or decreased after treatment. The results of replicate experiments (n = 6) were analyzed for ACTH-induced changes using the SAM algorithm from Stanford University (15) with a FDR cut-off set at 0.05. ACTH significantly affected the expression of 1275 of these annotated transcripts—*i.e.* 23.5% of the transcripts represented on the array—of which 588 increased in response to the hormone and 687 decreased. Responses to ACTH ranged from 8.7-fold to 0.46-fold relative to unstimulated controls (supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Among the ACTH-regulated transcripts were 19 that had been identified previously as ACTH-responsive using other methods (Table 2).

Quantitative analysis of ACTH-regulated transcripts

Several ACTH-regulated transcripts and several transcripts that were not significantly affected by ACTH on the microarrays were selected for further examination by quantitative RT-PCR (Table 3). These experiments confirmed that ACTH increased the accumulation of transcripts encoding Cyp11a1, Nr5a1, Mc2r, Sat1, Ptp4a3, Inha, Alas1, Hmox1, Ftl1, and Tfrc and decreased the levels of Alad, Avpr1, Fech, and MaoA. Except for the larger fold-changes that seemed dampened on the microarrays, the changes in transcript levels determined using the microarrays correlated with the changes measured by quantitative RT-PCR ($P \leq 0.003$ overall). For some of these transcripts, the maximal effects of ACTH were seen within 4 h of stimulation; for others, 24 h of stimulation was required to achieve a statistically significant effect. An exception was Hmox2, which showed no response to ACTH when assayed by quantitative RT-PCR, but which exhibited a 29% increase after ACTH treatment when tested against the 15K mouse cDNA array. This disparity may have resulted from a false-discovery error or from cross-hybridization of Hmox1 transcripts to regions of sim-

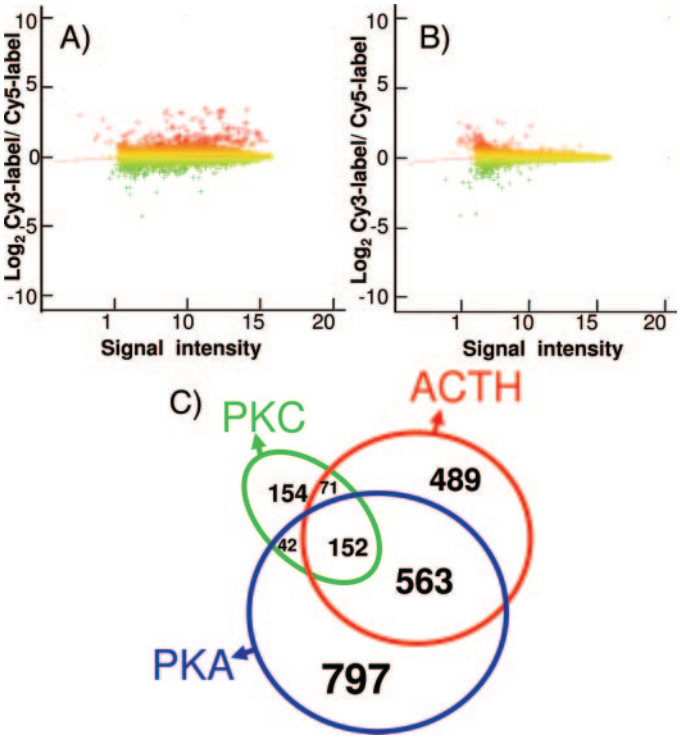

FIG. 1. Effects of ACTH on transcript accumulation in Y1 adrenal cells. A and B, Total RNA was isolated from mock-treated Y1 mouse adrenocortical tumor cells and from Y1 cells treated with $ACTH_{1–24}$ for 24 h (A) or from two different batches of mock-treated Y1 cells (B) and reverse transcribed in the presence of Cy5- and Cy3-dCTP, respectively. The labeled cDNAs were combined and hybridized to the NIA 15K mouse cDNA microarray and quantitated for dye incorporation as described in *Materials and Methods*. Results are expressed as the average $\log_2$ ratio of the intensities of the Cy3 and Cy5 signals for each transcript *vs.* the $\log_2$ product of the intensities/2, a measure of the intensity of the combined signals. The *red line* indicates the best fit of the data after normalization using the Lowess smoothing function; the *red* and *green crosses* indicate transcripts that increased and decreased after ACTH treatment respectively. C, Comparisons of ACTH-affected transcripts with transcripts corresponding to signatures of the cAMP/protein kinase A- and protein kinase C-dependent signaling pathways. Results were compiled from data in supplemental Tables 1–3; the numbers correspond to the number of affected transcripts in each category. PKA, Protein kinase A; PKC, protein kinase C.

ilarity in the arrayed Hmox2 cDNA. Inasmuch as the microarray data included several ACTH-regulated transcripts linked to heme and iron metabolism, quantitative RT-PCR was used to evaluate several other transcripts involved in heme metabolism or iron homeostasis that seemed unaffected by ACTH on the microarrays. Quantitative RT-PCR confirmed that ACTH did not affect the levels of the other transcripts with functions in heme biosynthesis, *i.e.* Hmbs, Urod, Uros, Cpox, and Ppox, nor did it affect the levels of Fth1.

Functional enrichment of the ACTH-regulated transcripts

The ACTH-affected transcripts identified by microarray analysis were analyzed for specific biological processes and molecular functions using GO-BP and GO-MF annotations (20) assigned to these transcripts by MGI (18). Of the 1275 affected transcripts, 1065 had GO-MF annotations; 999 of these also had GO-BP annotations (supplemental Table 1). The ACTH-regulated transcripts were deemed enriched for a specific function when the number of transcripts associated with each function was greater than the number that might be expected from random chance. Considered as a whole, the 1275 transcripts were not significantly enriched for any GO-BP category relative to their representation on the array. When the up-regulated and down-regulated transcripts were considered separately, the transcripts that increased in response to ACTH were significantly enriched (FDR < 0.05) only for those with functions in steroid biosynthesis and metabolism. Table 4 lists the 14 transcripts from the ACTH-regulated set that were assigned to these pathways on the basis of GO-BP annotations. An additional nine ACTH-regulated transcripts involved in steroid biosynthesis and metabolism but not identified as such in the Gene Ontology database are also included in Table 4. The transcripts down-regulated in response to ACTH were significantly enriched (FDR < 0.05) for those with functions in DNA replication and aspects of cell division including cell cycle regulation and for those with functions in of RNA processing, including mRNA processing and RNA splicing and nuclear transport (Table 5). A total of 133 different transcripts were represented in the categories above. None of the up-regulated transcripts were enriched for functions in GO-MF categories. The down-regulated transcripts were enriched for functions in the GO-MF category structural constituent of ribosome (GO:0003735), likely a manifestation of the growth-inhibiting effect of ACTH (23); and for functions in the GO-MF category isomerase activity (GO:0016853), particularly those associated with protein folding. A total of 55 different transcripts were represented in these GO-MF categories (data not shown).

Comparison of the set of ACTH-regulated transcripts with transcript profiles characteristic of 55 different mouse tissues

Transcripts characteristic of each of 55 different mouse tissues, including the adrenal gland, were defined as transcripts that were more abundant in the tissue queried than in any of the 54 other mouse tissues surveyed based on a tissue-profiling study of mouse gene expression (19). We queried the set of transcripts affected by ACTH in Y1 adrenal cells against the profiles of the 55 different tissues represented in the NIA 15K mouse cDNA array and found that the up-regulated set was significantly enriched ($P < 0.0001$, Fisher's exact test) for transcripts most abundant in the adrenal gland but not for transcripts most abundant in any of the other 54 tissues. Of the well-annotated transcripts on the NIA 15K mouse cDNA array, 41 were considered to be characteristic of the adrenal gland by these criteria; 14 of these were up-regulated by ACTH and are listed in Table 6. The transcripts that were down-regulated in response to ACTH contained a subset of 130 that were characteristic of embryonic tissues at different stages of development and a subset of 13 that were characteristic of bone marrow. A large proportion of these (45%, $P \leq 0.00025$) were associated with the categories represented in Table 5—including DNA replication, cell division, and cell cycle regulation, RNA processing and

TABLE 2. ACTH-induced changes confirmed by microarray analysis

Symbol	Gene name	$\log_2$ Fold change	q (%)	PKA	PKC	Reference
Por	P450 (cytochrome) oxidoreductase	0.44	0.08	+	+	(52)
Mc2r	Melanocortin 2 receptor	0.72	0.69	+	+	(53)
Star	Steroidogenic acute regulatory protein	1.55	0.08	+	−	(54)
Acls4	Acyl-CoA synthetase long-chain family member 4	0.40	0.08	+	−	(55)
Nr5a1	Nuclear receptor subfamily 5, group A, member 1	0.85	0.08	+	−	(56)
Gstm1	Glutathione S-transferase, μ 1	−0.71	0.08	+	−	(57)
Hnrpab	Heterogeneous nuclear ribonucleoprotein A/B	−0.23	0.08	+	−	(40)
Scarb1	Scavenger receptor class B, member 1	1.94	0.08	+	−	(58)
Bzrp	Benzodiazepine receptor, peripheral	1.01	0.08	+	−	(59)
Hsd3b1	Hydroxysteroid dehydrogenase-1, δ<5>-3-β	0.62	0.08	+	−	(60)
Abcb1b	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	0.65	0.08	+	−	(61)
Fdxr	Ferredoxin reductase	0.30	0.40	+	−	(62)
Ldlr	Low-density lipoprotein receptor	0.43	0.40	+	−	(63)
Hmgcr	3-Hydroxy-3-methylglutaryl-CoA reductase	0.55	0.64	+	−	(64)
Hmox1	Heme oxygenase (decycling) 1	0.40	1.47	+	−	(65)
Plat	Plasminogen activator, tissue	0.99	3.68	+	−	(50, 66)
Odc1	Ornithine decarboxylase, structural 1	1.01	0.08	−	+	(30, 50)
Inha	Inhibin α	0.78	0.15	−	−	(67)
Cyp11a1	Cytochrome P450, family 11a, polypeptide 1	0.44	0.48	−	−	(68)

Listed are ACTH-affected transcripts identified using the NIA 15K mouse cDNA microarray that had previously been identified as ACTH responsive in adrenal cells, together with the effects of ACTH on transcript accumulation [fold changes (n = 6) and the lowest FDR at which each transcript was called significant (q) were extracted from data in supplemental Table 1]. The assignment of each transcript to the protein kinase C (PKC)- and cAMP/protein kinase A (PKA)-dependent signaling pathways (+) is based on data presented in supplemental Tables 2 and 3. CoA, Coenzyme A.

RNA splicing—possibly reflecting the proliferative activities inherent in these tissues (data not shown).

Protein kinase C-regulated transcripts

To identify protein kinase C-affected changes in RNA accumulation, Y1 adrenal cells were treated with the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) (0.1 μM, n = 6), with 4-α-PMA (0.1 μM, n = 4), a PMA epimer that does not activate protein kinase C or with PMA in the presence of the protein kinase C inhibitor GF109203X (2 μM, n = 4; Ref. 24). These concentrations of activator and inhibitor maximally affected protein kinase C activity in Y1 adrenal cells (Wang, L., and B. P. Schimmer, unpublished observations). The effects of the various treatments on transcript accumulation were analyzed using the NIA 15K mouse cDNA microarray and significant changes were calculated

TABLE 3. Measurements of ACTH effects on transcript accumulation by quantitative RT-PCR

Symbol	Gene name	Time (h)			Microarray ratio[a]	Pathway	
		0	4	24		PKA	PKC
Sat1	Spermidine/spermine N1-acetyl transferase 1	0.22 ± 0.03 (4)	0.28 ± 0.03 (4)	0.48 ± 0.09 (4)[b]	0.94	+	+
Tfrc	Transferrin receptor	0.04 ± 0.01 (8)	0.07 ± 0.02 (8)[b]	0.08 ± 0.05 (8)[b]	0.53	+	+
Alas1	Aminolevulinic acid synthase 1	1.88 ± 0.79 (5)	6.10 ± 1.80 (4)[b]	13.72 ± 10.2 (10)[b]	1.39	+	−
Nr5a1	Nuclear receptor subfamily 5, group A, member 1	0.18 ± 0.08 (8)	0.47 ± 0.25 (8)[b]	0.34 ± 0.07 (10)[b]	0.85	+	−
Mc2r	Melanocortin 2 receptor	0.03 ± 0.01 (4)	0.04 ± 0.02 (4)	0.06 ± 0.01 (4)[b]	0.72	+	−
Ptp4a3	Protein tyrosine phosphatase 4a3	0.03 ± 0.01 (4)	0.03 ± 0.01 (4)	0.05 ± 0.01 (4)[b]	0.50	+	−
Hmox1	Heme oxygenase (decycling) 1	0.24 ± 0.12 (8)	0.39 ± 0.21 (8)[b]	0.69 ± 0.37 (8)[b]	0.40	+	−
Ftl1	Ferritin light chain 1	3.40 ± 0.32 (4)	3.76 ± 0.34 (4)	4.63 ± 0.33 (4)[b]	0.34	+	−
Alad	Aminolevulinate, δ-, dehydratase	2.59 ± 0.53 (8)	2.63 ± 0.62 (4)	1.91 ± 0.37 (9)[b]	−0.74	+	−
Maoa	Monoamine oxidase A	1.64 ± 0.36 (4)	n.d.	0.99 ± 0.26 (4)[b]	−0.49	+	−
Fech	Ferrochelatase	3.04 ± 0.40 (5)	2.93 ± 0.61 (4)	1.59 ± 0.81 (10)[b]	−0.38	+	−
Cyp11a1	Cytochrome P450, family 11a, polypeptide 1	4.20 ± 0.30 (4)	5.5 ± 0.80 (4)[b]	6.80 ± 0.70 (4)[b]	0.44	−	−
Inha	Inhibin α	0.13 ± 0.02 (4)	0.25 ± 0.08 (4)[b]	0.99 ± 0.21 (4)[b]	0.78	−	−
Hmox2	Heme oxygenase (decycling) 2	0.07 ± 0.02 (4)	0.09 ± 0.06 (4)	0.08 ± 0.01 (4)	0.37	−	−
Avpr1	Arginine vasopressin receptor 1A	0.04 ± 0.01 (4)	0.02 ± 0.01 (4)[b]	0.02 ± 0.01 (4)[b]	−0.56	−	−
Hmbs	Hydroxymethylbilane synthase	0.29 ± 0.26 (7)	n.d.	0.19 ± 0.11 (7)	n.s.	n.s.	n.s.
UroS	Uroporphyrinogen III synthase	0.46 ± 0.19 (7)	n.d.	0.33 ± 0.15 (7)	n.s.	n.s.	n.s.
UroD	Uroporphyrinogen decarboxylase	0.33 ± 0.13 (7)	n.d.	0.26 ± 0.08 (7)	n.s.	n.s.	n.s.
Cpox	Coproporphyrinogen oxidase	0.92 ± 0.6 (7)	n.d.	1.35 ± 0.39 (7)	n.s.	n.s.	n.s.
Ppox	Protoporphyrinogen oxidase	2.21 ± 1.11 (7)	n.d.	2.49 ± 0.98 (7)	n.s.	n.s.	n.s.
Fth1	Ferritin, heavy polypeptide 1	1.84 ± 0.18 (4)	1.67 ± 0.05 (4)	1.81 ± 0.14 (4)	n.s.	n.s.	n.s.

Total RNA was isolated from Y1 adrenal cells stimulated with ACTH for 0 h, 4 h or 24 h as indicated and assayed for specific transcripts by quantitative RT-PCR. Results are expressed as average *pg/pg* transketolase RNA ± SD of n replicates. The assignment of each transcript to PKA- and PKC-dependent signaling pathways is described in Table 2. n.d., Not determined; n.s., not significant by microarray analysis.

[a] The $\log_2$ fold-change in transcript accumulation induced by ACTH after 24 h as determined by microarray analysis.

[b] Significantly different from the untreated control ($P \leq 0.05$).

TABLE 4. Functional annotations of up-regulated transcripts

Symbol	Gene name	$\log_2$ Fold change	q (%)	Functional category		Pathway	
				Steroid metabolism [GO:0008202]	Steroid biosynthesis [GO:0006694]	PKA	PKC
Hsd17b12	Hydroxysteroid (17-β) dehydrogenase 12	0.29	0.08	MGI	MGI	+	+
Sc4 mol	Sterol-C4-methyl oxidase-like	0.48	0.08	MGI	MGI	+	+
Por	P450 (cytochrome) oxidoreductase	0.44	0.08	(Ref. 69)	(Ref. 69)	+	+
Mc2r	Melanocortin 2 receptor	0.72	0.64	(Ref. 70)	(Ref. 70)	+	+
Prkar1a	Protein kinase, cAMP- dependent regulatory, type I, α	0.26	3.68	(Ref. 9)	(Ref. 9)	+	+
Star	Steroidogenic acute regulatory protein	1.55	0.08	MGI	MGI	+	−
Nr5a1	Nuclear receptor subfamily 5, group A, member 1	0.85	0.08	(Ref. 71)	(Ref. 71)	+	−
Acsl4	Acyl-CoA synthetase long-chain family member 4	0.40	0.08	(Ref. 55)	(Ref. 55)	+	−
Hsd3b1	Hydroxysteroid dehydrogenase-1, δ<5>-3-β	0.62	0.08	MGI	MGI	+	−
Bzrp	Benzodiazepine receptor, peripheral	1.01	0.08	(Ref. 72)	(Ref. 72)	+	−
Sqle	Squalene epoxidase	0.58	0.08	(Ref. 73)	(Ref. 73)	+	−
Scarb1	Scavenger receptor class B, member 1	1.94	0.08	(Ref. 74)	(Ref. 74)	+	−
Abcb1b	ATP-binding cassette, subfamily B (MDR/TAP), member 1B	0.65	0.08	(Ref. 75)	(Ref. 75)	+	−
Osbpl6	Oxysterol binding protein-like 6	0.80	0.15	MGI		+	−
Dhcr7	7-Dehydrocholesterol reductase	1.04	0.20	MGI	MGI	+	−
Fdft1	Farnesyl diphosphate farnesyl transferase 1	0.42	0.31	MGI	MGI	+	−
Fdxr	Ferredoxin reductase	0.30	0.40	MGI	(Ref. 69)	+	−
Ldlr	Low-density lipoprotein receptor	0.43	0.40	MGI	(Ref. 76)	+	−
Hmgcr	3-Hydroxy-3-methylglutaryl-CoA reductase	0.55	0.64	MGI	MGI	+	−
Lss	Lanosterol synthase	0.52	1.11	MGI	MGI	+	−
Idi1	Isopentenyl-diphosphate δ isomerase	0.48	2.87	MGI	MGI	+	−
Acbd3	Acyl-CoA binding domain containing 3	0.26	0.31	MGI	MGI	−	−
Cyp11a1	Cytochrome P450, family 11a, polypeptide 1	0.44	0.48	MGI	MGI	−	−

Transcripts up-regulated in response to ACTH were assigned to the GO-BP functional categories of steroid metabolism and steroid biosynthesis based on evidence within the GO database (MGI, Ref. 18) or as extracted from published reports. The effects of ACTH on transcript accumulation and the assignment of transcripts to the PKA- or PKC-dependent signaling pathway were determined as described in Table 2. CoA, Coenzyme A.

using the SAM algorithm. PMA affected the levels of 514 annotated transcripts on the array. More than 80% of these—*i.e.* 419 (328 up-regulated, 91 down-regulated)—fulfilled our additional experimental criteria for a protein kinase C-regulated data set—*i.e.* unaffected by 4-α-PMA and unresponsive to PMA in the presence of the protein kinase C inhibitor GF109203X (supplemental Table 2).

cAMP-regulated and protein kinase A-dependent transcripts

To identify transcripts regulated via the cAMP/protein kinase A pathway, Y1 adrenal cells were stimulated with forskolin (10 μM, n = 6) or 8BrcAMP (3 mM, n = 6), two different effectors of the cAMP pathway, and global changes in transcript levels were assessed using the NIA 15K mouse cDNA array. Similar experiments (n = 4) were carried out with a protein kinase A-defective mutant derivative of the Y1 cell line (clone Kin8) and a Kin8 transformant with restored cAMP-responsive protein kinase A activity (Kin8Cev) to test the dependence of these changes on protein kinase A. The results obtained under each experimental condition were combined and used to construct a set of transcripts affected at a FDR ≤ 0.05 (see *Materials and Methods*). This analysis identified 1569 transcripts that responded to agonists of the cAMP/protein kinase A pathway in parent Y1 cells and in the Kin8Cev transformant. Of these, 99%—*i.e.* 1554 transcripts (922 up-regulated and 632 down-regulated)—were resistant to forskolin in mutant Kin8 cells and thus fulfilled our criteria of a cAMP/protein kinase A-regulated pathway (supplemental Table 3). Included in this list were 194 transcripts (12.5%) that were similarly affected by protein kinase C-dependent signaling (Fig. 1C).

Contributions of the cAMP/protein kinase A and protein kinase C signaling pathways to ACTH action

The 1275 transcripts affected by ACTH included 563 that only met the criteria for cAMP/protein kinase A-dependent regulation, 71 that only met the criteria for protein kinase C-dependent regulation and 152 that were common to both signaling pathways (Fig. 1C). Approximately 38% of the ACTH-regulated transcripts were not assignable by these criteria and are candidates for regulation via other signaling pathways. The global effects of ACTH thus appeared to overlap to a much greater extent with the cAMP/protein kinase A pathway than with the protein kinase C pathway, and this preferential association of ACTH action with cAMP/protein kinase A-dependent signaling was mirrored in the subsets of ACTH-regulated transcripts linked to specific biological processes (Tables 4 and 5) and to transcripts most abundantly expressed in the adrenal gland (Table 6).

Discussion

In vivo, the continuous exposure of the adrenal cortex to ACTH and other proopiomelanocortin-derived peptides has a tonic effect on gene expression that is essential for the maintenance of its steroidogenic machinery, steroidogenic capacity, cellular architecture, and size (25, 26). In isolated adrenal cells and in adrenal cell cultures maintained in the absence of ACTH, this tonic effect is manifested by the coordinate induction of the enzymes and other proteins required for steroidogenesis upon addition of the hormone. In this study, the global actions of ACTH and other agonists on RNA accumulation were assessed in the Y1 mouse adreno-

TABLE 5. Functional annotations of down-regulated transcripts

Gene symbol	log_2 Fold change	q (%)	PKA	PKC	Gene symbol	log_2 Fold change	q (%)	PKA	PKC	Gene symbol	log_2 Fold change	q (%)	PKA	PKC
DNA replication [GO:0006260]					Cell division [GO:005130] and mitotic cell cycle [GO:0000278]					RNA processing [GO:0006397] and RNA splicing [GO:0000375]				
Chaf1b	−0.47	0.08	+	−	Cetn3	−0.45	0.08	+	+					
Msh6	−0.38	0.08	+	−	Tubb5	−0.27	1.11	+	+	Ncl	−0.42	0.08	+	+
Nfix	−0.45	0.08	+	−	Bub1b	−0.74	0.08	+	−	Magoh	−0.45	0.08	+	−
Orc6l	−0.36	0.08	+	−	Ccna2	−0.92	0.08	+	−	Dhx15	−0.27	0.08	+	−
Rbbp4	−0.30	0.08	+	−	Incenp	−0.43	0.08	+	−	Ewsr1	−0.38	0.08	+	−
Rpa2	−0.67	0.08	+	−	Smc2l1	−0.62	0.08	+	−	Gemin6	−0.32	0.08	+	−
Rpa3	−0.71	0.08	+	−	Smc4l1	−0.45	0.08	+	−	Hnrpab	−0.23	0.08	+	−
Rrm1	−0.62	0.08	+	−	Mad2l1	−0.92	0.08	+	−	Hnrpf	−0.25	0.08	+	−
Mcm3	−0.49	0.15	+	−	Anln	−0.69	0.08	+	−	Rbmxrt	−0.40	0.08	+	−
Pole2	−0.30	0.15	+	−	Cdca7	−0.67	0.08	+	−	Sfrs2	−0.42	0.08	+	−
Rpa1	−0.47	0.15	+	−	Top2a	−0.81	0.08	+	−	Sfrs7	−0.38	0.08	+	−
Mcm6	−0.38	0.20	+	−	Ppp1cc	−0.32	0.15	+	−	Fmr1	−0.29	0.40	+	−
Lig1	−0.45	0.48	+	−	Cdca5	−0.47	0.20	+	−	Hnrpa2b1	−0.60	0.08	+	−
Rfc3	−0.49	0.48	+	−	Smc1l1	−0.25	0.40	+	−	Pcbp2	−0.27	0.08	+	−
Prim1	−0.23	1.78	+	−	Pttg1	−0.60	0.48	+	−	Exosc8	−0.43	0.08	+	−
Msh2	−0.42	0.08	−	−	Lig1	−0.45	0.48	+	−	Snrpb2	−0.42	0.20	+	−
Set	−0.23	0.08	−	−	Chc1	−0.22	0.48	+	−	Ncbp2	−0.43	0.64	+	−
Nfia	−0.27	0.48	−	−	Bub1	−0.49	0.64	+	−	Hnrpr	−0.14	1.47	+	−
Trp53	−0.27	0.82	−	−	Cdk2	−0.15	0.64	+	−	Snrpb	−0.23	1.47	+	−
Cdc6	−0.56	1.11	−	−	Cdc2a	−0.30	0.82	+	−	Snrp116	−0.17	1.78	+	−
Top1	−0.22	1.11	−	−	Cks2	−0.54	1.11	+	−	Pspc1	−0.18	2.28	+	−
Mcm5	−0.30	1.47	−	−	Ccng1	−0.30	1.47	+	−	Slbp	−0.18	2.28	+	−
Dut	−0.32	2.28	−	−	Cdc5l	−0.14	1.78	+	−	Cpsf6	−0.15	3.68	+	−
Dnajc2	−0.22	3.68	−	−	Ccnb2	−0.42	3.68	+	−	Rbm14	−0.20	3.68	+	−
Orc2l	−0.15	3.68	−	−	Cdc20	−0.40	3.68	+	−	Hnrpk	−0.43	0.08	−	+
					Rad21	−0.23	3.68	+	−	Rbm9	−0.15	3.68	−	+
Nuclear transport					Tfdp1	−0.45	0.08	+	−	Hnrpa3	−0.23	0.31	−	+
Ipo9	−0.43	0.08	+	−	Fbxo5	−0.76	0.08	−	−	Ssb	−0.20	0.08	−	−
Khdrbs1	−0.34	0.08	+	−	Bccip	−0.25	0.31	−	−	Crnkl1	−0.27	0.08	−	−
Kpnb1	−0.32	0.08	+	−	Calmbp1	−0.67	0.48	−	−	Elavl1	−0.47	0.08	−	−
Magoh	−0.45	0.08	+	−	Cdc6	−0.56	1.11	−	−	Sfrs10	−0.23	0.08	−	−
Cse1l	−0.38	0.20	+	−	Pard3	−0.17	1.11	−	−	Nol5	−0.38	0.08	−	−
G3bp	−0.34	0.15	+	−	Anapc7	−0.29	1.47	−	−	Mki67ip	−0.20	0.08	−	−
Tacc3	−0.67	0.31	+	−	Cdc25a	−0.34	1.78	−	−	Rbm16	−0.29	0.08	−	−
Fmr1	−0.29	0.40	+	−	Kntc2	−0.38	1.78	−	−	Tardbp	−0.43	0.20	−	−
Ncbp2	−0.43	0.64	+	−	Tnfsf5ip1	−0.23	1.78	−	−	Sf3a3	−0.27	1.11	−	−
Rpl23	−0.18	1.11	+	−	Stag1	−0.18	2.87	−	−	Syncrip	−0.12	1.11	−	−
Ipo11	−0.27	2.87	+	−	Cables1	−0.18	3.68	−	−	Ddx20	−0.23	1.47	−	−
Nol5	−0.38	0.08	−	−	Dnajc2	−0.22	3.68	−	−	Hnrpc	−0.17	1.78	−	−
Ssb	−0.20	0.08	−	−						Ptbp2	−0.32	2.28	−	−
Phax	−0.22	0.20	−	−										
Trp53	−0.27	0.82	−	−										
Kpna3	−0.20	1.78	−	−										

Transcripts down-regulated in response to ACTH were assigned to GO-BP functional categories of DNA replication, the overlapping categories cell division and mitotic cell cycle, RNA processing, including mRNA processing and RNA splicing *via* transesterification reactions, and nuclear transport as described in Table 4. The effects of ACTH on transcript accumulation and the assignment of transcripts to the PKA- or PKC-dependent signaling pathway were determined as described in Table 2.

cortical cell line, a widely used experimental system that shares many features associated with normal cells from the mouse adrenal cortex (2). Cells were stimulated with 20 nM ACTH, and effects were measured after a 24-h stimulation. This concentration of ACTH maximally activates cAMP and MAPK signaling (27, 28) and produces maximum effects on steroidogenesis (28), proliferation (29), and Odc1 induction (30); the 24-h time period was chosen because the hormone is known to have a delayed inductive effect on most genes involved in steroidogenesis and because much of the work published previously has used similar time points of induction (*e.g.* Ref. 25). As determined by quantitative RT-PCR (Table 3), the ACTH-induced changes reported here included both intermediate- and late-onset effects.

In the Y1 adrenal cell line, ACTH affected the levels of 1275 different transcripts, a number limited in part by the annotated genes represented on the NIA 15K mouse cDNA array. For example, Cyp11b1, Cyp11b2, and Cyp21a1, ACTH-regulated transcripts required for corticosteroid biosynthesis in the mouse, were not part of the annotated set on the array and thus were not examined in this study. Although previous studies of ACTH action, for the most part, have focused on up-regulated transcripts (25), our experiments indicate that approximately half of the affected transcripts decreased in concentration after treatment with the hormone. Whether these global effects of ACTH are due to changes in gene expression or RNA stability cannot be distinguished on the microarrays. Most of the 1275 affected transcripts have not been documented as ACTH-responsive previously and their contributions to hormone action merit

TABLE 6. Transcripts up-regulated by ACTH that are characteristic of the mouse adrenal gland

Symbol	Gene name	$\log_2$ Fold change	q (%)	PKA	PKC
Mod1	Malic enzyme, supernatant	0.79	0.08	+	+
Abhd2	Abhydrolase domain containing 2	0.12	0.08	+	−
Fads1	Fatty acid desaturase 1	0.72	0.08	+	−
Nr5a1	Nuclear receptor subfamily 5, group A, member 1	0.85	0.08	+	−
Star	Steroidogenic acute regulatory protein	1.55	0.08	+	−
Alas1	Aminolevulinic acid synthase 1	1.39	0.08	+	−
Bzrp	Benzodiazepine receptor, peripheral	1.01	0.08	+	−
Hsd3b1	Hydroxysteroid dehydrogenase-1, delta<5>-3-β	0.62	0.08	+	−
Pank3	Pantothenate kinase 3	0.42	0.20	+	−
Prss15	Protease, serine, 15	0.84	0.48	+	−
Abhd4	Abhydrolase domain containing 4	0.35	0.64	+	−
Rerg	RAS-like, estrogen-regulated, growth inhibitor	0.26	1.47	+	−
Btbd7	BTB (POZ) domain containing 7	0.25	2.28	−	−
Robo1	Roundabout homolog 1 (*Drosophila*)	0.84	2.28	−	−

ACTH-regulated transcripts characteristic of the mouse adrenal gland are listed. The effects of ACTH on transcript accumulation and the assignment of transcripts to the PKA- or PKC-dependent signaling pathway were determined as described in Table 2.

further investigation. For a vast majority of affected transcripts, the ACTH-induced changes detected by microarray analysis were 2-fold or less (Table 3 and supplemental Table 1). Part of this is due to the documented tendency of the microarray analysis to underestimate the fold-changes in transcript accumulation (31, 32). Nonetheless, the data suggest that an appreciable portion of ACTH action may involve relatively small changes in transcript accumulation that are usually overlooked in other types of experiments but that have been shown to be of physiological significance in other settings (*e.g.* Ref. 33).

One way to predict functions of transcripts within large data sets is to identify clusters that are associated with specific biological processes or molecular functions (20), an approach that uses the coordinate expression of functionally related transcripts to predict pathway-specific regulatory networks (*e.g.* Ref. 34). Not surprisingly, the set of transcripts that increased in response to ACTH included transcripts with functions in steroid biosynthesis and metabolism, including cholesterol synthesis, cholesterol mobilization, and steroid hormone biosynthesis. Some of these had been described as ACTH-responsive previously (Table 2); most, however, had not, thus indicating that ACTH plays a more extensive role in maintaining adrenal steroidogenic capability than previously appreciated. A second approach is to identify transcripts that are abundant in specific tissues because they tend to be enriched for functions typically associated with those tissues (19). The transcripts up-regulated in response to ACTH included a cluster of 14 that was most abundantly expressed in the adrenal gland compared with 54 other tissues examined (Table 6). Whereas adrenal gland-specific transcripts might be derived from both the steroidogenic and nonsteroidogenic portions of the gland, the 14 up-regulated by ACTH are likely to be specific to the adrenal cortex and, by inference, important for steroidogenesis. Indeed, the contributions of four members of this group—Nr5a1, Star, Bzrp, and Hsd3b1—to steroidogenesis have already been documented (Table 4). Although the contributions of the remaining 10 members to adrenal function have not been investigated, it is possible to speculate on the roles of a few. Alas1 is the rate-limiting enzyme in heme biosynthesis and may serve to maintain a supply of heme for the prosthetic groups of the steroidogenic cytochrome P450s that are induced in response to ACTH. This hypothesis is supported by previous observations indicating that heme availability limits adrenal corticosteroid biosynthesis (35). Fads1 (36), Pank3 (37), and Mod1 (38) are all involved in aspects of intermediary metabolism, and in this general capacity may generate reduced nicotinamide adenine dinucleotide phosphate, a cofactor required for the mixed function oxidase activities of the steroidogenic cytochromes.

The transcripts that decreased in response to ACTH had functions that clustered to aspects of cell proliferation, including DNA replication, mitotic cell cycle, and cell division, and to aspects of RNA processing and nuclear transport. The down-regulation of transcripts associated with cell proliferation seems consistent with the known growth-inhibitory actions of ACTH on cells of the adrenal cortex (*e.g.* Ref. 8); however, the extent to which these changes were directly responsible for the growth-inhibitory effects of the hormone is uncertain. The effects of ACTH on transcripts with functions in RNA processing including RNA splicing are especially intriguing. Alternative splicing of precursor mRNA is thought to affect between one and two thirds of mouse genes and to greatly expand the number of proteins encoded by the mouse genome. Alternative splicing can influence protein function, protein-protein interactions, cell proliferation and other aspects of cell regulation independent of gene expression (39). Although effects of ACTH on alternative splicing have not been demonstrated directly, recent findings that ACTH down-regulates hnrpb1 (one of the pre-mRNA binding proteins involved in alternative splicing) at the protein level in adrenocortical cells (40) lends additional support to this hypothesis.

The ability of ACTH to stimulate cAMP accumulation and activate protein kinase C in Y1 adrenal cells and in adrenocortical cells from a variety of species has been documented (41, 42). In this study, the contributions of the cAMP/protein kinase A and protein kinase C pathways to ACTH action were evaluated using pharmacological probes of each pathway. Together, the ability of ACTH to activate each pathway and the ability of pharmacological agonists to mimic the effects of ACTH are criteria classically used to assess the contributions of second messengers to hormone action (re-

viewed in Ref. 41). Forskolin, a well-characterized activator of most isoforms of adenylyl cyclase including those expressed in the Y1 cell line (43), and the active cAMP analog 8BrcAMP were used to probe the cAMP/protein kinase A pathway. The protein kinase C-dependent signaling cascade was probed using the phorbol ester PMA, a well-characterized activator of most protein kinase C isoforms, including the isoforms (α and ϵ) that are most abundant in Y1 adrenal cells (44). Although these reagents are commonly used activators of their respective pathways, each has other activities (45–47) that prompted the inclusion of several additional criteria to validate their modes of action. Accordingly, protein kinase A-specific effects of forskolin were further evaluated in a protein kinase A-defective Y1 mutant (clone Kin8) and in a Kin8 transformant with cAMP-responsive protein kinase activity restored. Protein kinase C-specific effects of PMA were assessed with 4-α-PMA, an epimer that has the same physicochemical properties as PMA but is unable to activate protein kinase C, and GF109203X, an inhibitor that affects the actions of PMA on protein kinase C but not on other PMA receptors (47). By these criteria, activators of the cAMP/protein kinase A signaling pathway affected a much larger number of transcripts than did activators of the protein kinase C pathway in the adrenal cell line and signaling via the cAMP/protein kinase A pathway accounted for over 55% of the global effects of ACTH on gene expression, whereas signaling via the protein kinase C-dependent pathway was much less important. Indeed, only 6% of ACTH action could be assigned specifically to the protein kinase C signaling pathway (Fig. 1C).

Despite the ability of ACTH to activate the cAMP/protein kinase A-signaling cascade, the hormone affected only 45% of the transcripts comprising the signature of the cAMP/protein kinase A pathway (Fig. 1C). The most likely explanation for the inability of ACTH to reproduce the complete cAMP/protein kinase A signature may be related to the inability of ACTH to sustain the cAMP/protein kinase A signal. After a relatively brief exposure to ACTH, desensitization sets in and cAMP levels fall due to an uncoupling of the ACTH receptor from the adenylyl cyclase system. In contrast, pharmacological agonists of the cAMP/protein kinase A pathway act downstream of this step and have activities that are more sustained (48).

Together, the cAMP/protein kinase A and protein kinase C pathways appeared to account for approximately 60% of the effects of ACTH. It is possible that we have underestimated the contributions of these two pathways to ACTH action due to limitations of the pharmacological probes in these studies as noted above or by the restrictive criteria used to identify each signature. For example, the cAMP/protein kinase A signature is derived from a statistical analysis that is designed to reduce type 1 errors when combining the results of the four different tests used to define this pathway. While providing a level of certainty regarding the cAMP/protein kinase A signature, this approach may underestimate the total number of transcripts assigned to this pathway. In addition, differences in the mechanisms of action of forskolin and 8BrcAMP, phenotypic variations between Y1 and Kin8Cev cells and residual cAMP-dependent protein kinase activity in the Kin8 mutant (8) may lead to the inappropriate exclusion of some transcripts from the cAMP/protein kinase A signature. Two such transcripts, Cyp11a1 and Odc1, had markedly compromised levels of basal and cAMP-stimulated expression in the Kin8 mutant in Northern blot experiments (49, 50) but were excluded from the cAMP/protein kinase A signature on the basis of a residual response in the Kin8 mutant that was detectable in the microarray experiments. Alternatively, the possibility that other signaling pathways mediate the actions of ACTH on selected transcripts cannot be excluded.

Because Y1 adrenal cells do not express *Cyp21a1* (51), they do not synthesize corticosterone and instead produce the physiologically inactive metabolites 20α-hydroxyprogesterone and 11β-20α-dihydroxyprogesterone in response to ACTH (reviewed in Ref. 2). Thus, it is unlikely that these steroid products contribute to the effects observed here. It is nonetheless possible that other tertiary messengers act downstream of the cAMP/protein kinase A pathway to mediate the effects of ACTH as reviewed elsewhere (3–5). Although our studies have not addressed the contributions of tertiary messengers to these global actions of ACTH, our ACTH-regulated data set includes a number of transcripts involved in transcription control and in cell signaling (supplemental Table 1), some of which may act down-stream of ACTH to influence the expression profile of these cells.

The results presented here provide a reference set of ACTH-affected transcripts and reference signatures of the cAMP/protein kinase A and protein kinase C signaling pathways. These should provide useful starting points for more detailed studies on the scope of ACTH action in the adrenal cortex and on the signaling pathways that mediate the hormone's effects. Our observation that ACTH affects the accumulation of a large number of transcripts suggests that the hormone causes a significant remodeling as the cells switch from the basal to hormone-stimulated state.

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Annotations for gene symbols, including gene names, can be retrieved from the Stanford Online Universal Resource for Clones and ESTs (S.O.U.R.C.E.) database (http://genome-www5.stanford.edu/cgi-bin/SMD/source/sourceSearch).

The supplemental information provided with this article is also available on our web site at http://www.utoronto.ca/schimmer/supplements/supplements.html.

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Brunton L, Lazo J, Parker K, eds. Goodman and Gilman's the pharmacological basis of therapeutics. 11th ed. New York: McGraw-Hill, Inc.; 1587–1612.

References

1. **Sewer MB, Waterman MR** 2001 Insights into the transcriptional regulation of steroidogenic enzymes and StAR. Rev Endocr Metab Disord 2:269–274
2. **Rainey WE, Saner K, Schimmer BP** 2004 Adrenocortical cell lines. Mol Cell Endocrinol 228:23–28
3. **Cooke BA** 1999 Signal transduction involving cyclic AMP-dependent and cyclic AMP-independent mechanisms in the control of steroidogenesis. Mol Cell Endocrinol 151:25–35
4. **Gallo-Payet N, Payet MD** 2003 Mechanism of action of ACTH: beyond cAMP. Microsc Res Tech 61:275–287
5. **Enyeart JJ** 2005 Biochemical and ionic signaling mechanisms for ACTH-stimulated cortisol production. Vitam Horm 70:265–279
6. **Lotfi CFP, Todorovic Z, Armelin HA, Schimmer BP** 1997 Unmasking a growth-promoting effect of the adrenocorticotropic hormone in Y1 mouse adrenocortical tumor cells. J Biol Chem 272:29886–29891
7. **Kargul GJ, Dudekula DB, Qian Y, Lim MK, Jaradat SA, Tanaka TS, Carter MG, Ko MS** 2001 Verification and initial annotation of the NIA mouse 15K cDNA clone set. Nat Genet 28:17–18
8. **Rae PA, Gutmann NS, Tsao J, Schimmer BP** 1979 Mutations in cyclic AMP-dependent protein kinase and corticotropin (ACTH)-sensitive adenylate cyclase affect adrenal steroidogenesis. Proc Natl Acad Sci USA 76:1896–1900
9. **Olson MF, Krolczyk AJ, Gorman KB, Steinberg RA, Schimmer BP** 1993 Molecular basis for the 3′,5′-cyclic adenosine monophosphate resistance of kin mutant Y1 adrenocortical tumor cells. Mol Endocrinol 7:477–487
10. **Wong M, Krolczyk AJ, Schimmer BP** 1992 The causal relationship between mutations in cAMP-dependent protein kinase and the loss of adrenocorticotropin-regulated adrenocortical functions. Mol Endocrinol 6:1614–1624
11. **Schimmer BP** 1985 Isolation of ACTH-resistant Y1 adrenal tumor cells. Methods Enzymol 109:350–356
12. **Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ** 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299
13. **Schena M, Shalon D, Davis RW, Brown PO** 1995 Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270:467–470
14. **Tseng GC, Oh MK, Rohlin L, Liao JC, Wong WH** 2001 Issues in cDNA microarray analysis: quality filtering, channel normalization, models of variations and assessment of gene effects. Nucleic Acids Res 29:2549–2557
15. **Tusher VG, Tibshirani R, Chu G** 2001 Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 98:5116–5121
16. **Fisher RA** 1932 Statistical methods for research workers. 4th ed. London: Oliver and Boyd
17. **Benjamini Y, Hochbert Y** 1995 Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Royal Stat Soc B 57:289–300
18. **Blake JA, Richardson JE, Davisson MT, Eppig JT** 1997 The Mouse Genome Database (MGD). A comprehensive public resource of genetic, phenotypic and genomic data. The Mouse Genome Informatics Group. Nucleic Acids Res 25:85–91
19. **Zhang W, Morris QD, Chang R, Shai O, Bakowski MA, Mitsakakis N, Mohammad N, Robinson MD, Zirngibl R, Somogyi E, Laurin N, Eftekharpour E, Sat E, Grigull J, Pan Q, Peng WT, Krogan N, Greenblatt J, Fehlings M, van der Kooy D, Aubin J, Bruneau BG, Rossant J, Blencowe BJ, Frey BJ, Hughes TR** 2004 The functional landscape of mouse gene expression. J Biol 3:21.21–21.22
20. **Harris MA, Clark J, Ireland A, Lomax J, Ashburner M, Foulger R, Eilbeck K, Lewis S, Marshall B, Mungall C, Richter J, Rubin GM, Blake JA, Bult C, Dolan M, Drabkin H, Eppig JT, Hill DP, Ni L, Ringwald M, Balakrishnan R, Cherry JM, Christie KR, Costanzo MC, Dwight SS, Engel S, Fisk DG, Hirschman JE, Hong EL, Nash RS, Sethuraman A, Theesfeld CL, Botstein D, Dolinski K, Feierbach B, Berardini T, Mundodi S, Rhee SY, Apweiler R, Barrell D, Camon E, Dimmer E, Lee V, Chisholm R, Gaudet P, Kibbe W, Kishore R, Schwarz EM, Sternberg P, Gwinn M, Hannick L, Wortman J, Berriman M, Wood V, de la Cruz N, Tonellato P, Jaiswal P, Seigfried T, White R** 2004 The Gene Ontology (GO) database and informatics resource. Nucleic Acids Res 32:D258–D261
21. **Kent WJ** 2002 BLAT—the BLAST-like alignment tool. Genome Res 12:656–664
22. **Harper J** 1984 Peritz's F test: basic program of a robust multiple comparison test for statistical analysis of all differences among group means. Comput Biol Med 14:437–445
23. **Tushinski RJ, Warner JR** 1982 Ribosomal proteins are synthesized preferentially in cells commencing growth. J Cell Physiol 112:128–135
24. **Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F** 1991 The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 266:15771–15781
25. **Simpson ER, Waterman MR** 1988 Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annu Rev Physiol 50:427–440
26. **Karpac J, Ostwald D, Bui S, Hunnewell P, Shankar M, Hochgeschwender U** 2005 Development, maintenance, and function of the adrenal gland in early postnatal proopiomelanocortin-null mutant mice. Endocrinology 146:2555–2562
27. **Le T, Schimmer BP** 2001 The regulation of mitogen-activated protein kinases in Y1 mouse adrenocortical tumor cells. Endocrinology 142:4282–4287
28. **Schimmer BP, Zimmerman AE** 1976 Steroidogenesis and extracellular cAMP accumulation in adrenal tumor cell cultures. Mol Cell Endocrinol 4:263–270
29. **Schimmer BP, Schulz P** 1985 The roles of cAMP and cAMP-dependent protein kinase in forskolin's actions on Y1 adrenocortical tumor cells. Endocr Res 11:199–209
30. **Kudlow JE, Rae PA, Gutmann NS, Schimmer BP, Burrow GN** 1980 Regulation of ornithine decarboxylase activity by corticotropin in adrenocortical tumor cell clones: Roles of cyclic AMP and cyclic AMP-dependent protein kinase. Proc Natl Acad Sci USA 77:2767–2771
31. **Yao B, Rakhade SN, Li Q, Ahmed S, Krauss R, Draghici S, Loeb JA** 2004 Accuracy of cDNA microarray methods to detect small gene expression changes induced by neuregulin on breast epithelial cells. BMC Bioinformatics 5:99–114
32. **Yuen T, Wurmbach E, Pfeffer RL, Ebersole BJ, Sealfon SC** 2002 Accuracy and calibration of commercial oligonucleotide and custom cDNA microarrays. Nucleic Acids Res 30:e48–e56
33. **Hughes TR, Marton MJ, Jones AR, Roberts CJ, Stoughton R, Armour CD, Bennett HA, Coffey E, Dai H, He YD, Kidd MJ, King AM, Meyer MR, Slade D, Lum PY, Stepaniants SB, Shoemaker DD, Gachotte D, Chakraburtty K, Simon J, Bard M, Friend SH** 2000 Functional discovery via a compendium of expression profiles. Cell 102:109–126
34. **Tavazoie S, Hughes JD, Campbell MJ, Cho RJ, Church GM** 1999 Systematic determination of genetic network architecture. Nat Genet 22:281–285
35. **Martini CN, Vaena de Avalos SG, Romero DG, San Martin de Viale L, Vila Mde C** 1997 Heme availability affects corticosterone and aldosterone biosynthesis in rat adrenal. Steroids 62:767–770
36. **Dobrzyn A, Ntambi JM** 2005 The role of stearoyl-CoA desaturase in the control of metabolism. Prostaglandins Leukot Essent Fatty Acids 73:35–41
37. **Zhang YM, Rock CO, Jackowski S** 2005 Feedback regulation of murine pantothenate kinase 3 by coenzyme A and coenzyme A thioesters. J Biol Chem 280:32594–32601
38. **Lee CY, Lee SM, Lewis S, Johnson FM** 1980 Identification and biochemical analysis of mouse mutants deficient in cytoplasmic malic enzyme. Biochemistry 19:5098–5103
39. **Pan Q, Shai O, Misquitta C, Zhang W, Saltzman AL, Mohammad N, Babak T, Siu H, Hughes TR, Morris QD, Frey BJ, Blencowe BJ** 2004 Revealing global regulatory features of mammalian alternative splicing using a quantitative microarray platform. Mol Cell 16:929–941
40. **Wu W, Kamma H, Ffujiwara M, Yano Y, Satoh H, Hara H, Yashiro T, Ueno E, Aiyoshi Y** 2005 Altered expression patterns of heterogeneous nuclear ribonucleoproteins a2 and b1 in the adrenal cortex. J Histochem Cytochem 53:487–495
41. **Schimmer BP** 1980 Cyclic nucleotides in hormonal regulation of adrenocortical function. Adv Cyclic Nucleotide Res 13:181–214
42. **Widmaier EP, Hall PF** 1985 Protein kinase C in adrenal cells: possible role in regulation of steroid synthesis. Mol Cell Endocrinol 43:181–188
43. **Al-Hakim A, Rui X, Tsao J, Albert PR, Schimmer BP** 2004 Forskolin-resistant Y1 adrenal cell mutants are deficient in adenylyl cyclase type 4. Mol Cell Endocrinol 214:155–165
44. **Parissenti AM, Kirwan AF, Sim SA, Colantonio CM, Schimmer BP** 1996 Molecular strategies for the dominant inhibition of protein kinase C. Endocr Res 22:621–630
45. **Martin TF, Kowalchyk JA** 1981 Growth inhibition by adenosine 3′,5-monophosphate derivatives does not require 3′,5′ phosphodiester linkage. Science 213:1120–1122
46. **Laurenza A, Sutkowski EM, Seamon KB** 1989 Forskolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action? Trends Pharmacol Sci 10:442–447
47. **Silinsky EM, Searl TJ** 2003 Phorbol esters and neurotransmitter release: more than just protein kinase C? Br J Pharmacol 138:1191–1201
48. **Saez JM, Morera A-M, Haour F** 1979 Hormonal-induced refractoriness of steroidogenesis in testicular and adrenal cells. Horm Cell Regul 3:187–216
49. **Wong M, Rice DA, Parker KL, Schimmer BP** 1989 The roles of cAMP and cAMP-dependent protein kinase in the expression of cholesterol side chain cleavage and steroid 11β-hydroxylase genes in mouse adrenocortical tumor cells. J Biol Chem 264:12867–12871
50. **Clegg CH, Abrahamsen MS, Degen JL, Morris DR, McKnight GS** 1992 Cyclic AMP-dependent protein kinase controls basal gene activity and steroidogenesis in Y1 adrenal tumor cells. Biochemistry 31:3720–3726
51. **Parker KL, Chaplin DD, Wong M, Seidman JG, Smith JA, Schimmer BP** 1985 Expression of murine 21-hydroxylase in mouse adrenal glands and in transfected Y1 adrenocortical tumor cells. Proc Natl Acad Sci USA 82:7860–7864
52. **Dee A, Carlson G, Smith C, Masters BS, Waterman MR** 1985 Regulation of

synthesis and activity of bovine adrenocortical NADPH-cytochrome P-450 reductase by ACTH. Biochem Biophys Res Commun 128:650–666
53. **Mountjoy KG, Bird IM, Rainey WE, Cone RD** 1994 ACTH induces up-regulation of ACTH receptor mRNA in mouse and human adrenocortical cell lines. Mol Cell Endocrinol 99:R17–R20
54. **Liu J, Heikkila P, Kahri AI, Voutilainen R** 1996 Expression of the steroidogenic acute regulatory protein mRNA in adrenal tumors and cultured adrenal cells. J Endocrinol 150:43–50
55. **Castilla R, Maloberti P, Castillo F, Duarte A, Cano F, Maciel FC, Neuman I, Mendez CF, Paz C, Podesta EJ** 2004 Arachidonic acid regulation of steroid synthesis: new partners in the signaling pathway of steroidogenic hormones. Endocr Res 30:599–606
56. **Enyeart JJ, Boyd RT, Enyeart JA** 1996 ACTH and AII differentially stimulate steroid hormone orphan receptor mRNAs in adrenal cortical cells. Mol Cell Endocrinol 124:97–110
57. **Mankowitz L, Staffas L, Bakke M, Lund J** 1995 Adrenocorticotrophic-hormone-dependent regulation of a mu-class glutathione transferase in mouse adrenocortical cells. Biochem J 305(Pt 1):111–118
58. **Cao G, Zhao L, Stangl H, Hasegawa T, Richardson JA, Parker KL, Hobbs HH** 1999 Developmental and hormonal regulation of murine scavenger receptor, class B, type 1. Mol Endocrinol 13:1460–1473
59. **Cavallaro S, Pani L, Guidotti A, Costa E** 1993 ACTH-induced mitochondrial DBI receptor (MDR) and diazepam binding inhibitor (DBI) expression in adrenals of hypophysectomized rats is not cause-effect related to its immediate steroidogenic action. Life Sci 53:1137–1147
60. **Bassett MH, Suzuki T, Sasano H, De Vries CJ, Jimenez PT, Carr BR, Rainey WE** 2004 The orphan nuclear receptor NGFIB regulates transcription of 3β-hydroxysteroid dehydrogenase. Implications for the control of adrenal functional zonation. J Biol Chem 279:37622–37630
61. **Altuvia S, Stein WD, Goldenberg S, Kane SE, Pastan I, Gottesman MM** 1993 Targeted disruption of the mouse *mdr*1b gene reveals that steroid hormones enhance *mdr* gene expression. J Biol Chem 268:27127–27132
62. **Hanukoglu I, Feuchtwanger R, Hanukoglu A** 1990 Mechanism of corticotropin and cAMP induction of mitochondrial cytochrome P450 system enzymes in adrenal cortex cells. J Biol Chem 265:20602–20608
63. **Liu J, Heikkila P, Meng QH, Kahri AI, Tikkanen MJ, Voutilainen R** 2000 Expression of low and high density lipoprotein receptor genes in human adrenals. Eur J Endocrinol 142:677–682
64. **Lehoux JG, Lefebvre A, Belisle S, Bellabarba D** 1989 Hormonal regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA in the rat adrenal gland. J Steroid Biochem 34:379–384
65. **Pomeraniec Y, Grion N, Gadda L, Pannunzio V, Podesta EJ, Cymeryng CB** 2004 Adrenocorticotropin induces heme oxygenase-1 expression in adrenal cells. J Endocrinol 180:113–124
66. **Estensen RD, Zustiak K, Chuang A, Schultheiss P, Ditmanson J** 1983 Action of 12-O-tetradecanoylphorbol-13-acetate on Y1 adrenal cells apparently requires the regulatory subunit of type 1 cyclic AMP dependent protein kinase. J Exp Pathol 1:49–60
67. **Voutilainen R, Eramaa M, Ritvos O** 1991 Hormonally regulated inhibin gene expression in human fetal and adult adrenals. J Clin Endocrinol Metab 73: 1026–1030
68. **John ME, John MC, Boggaram V, Simpson ER, Waterman MR** 1986 Transcriptional regulation of steroid hydroxylase genes by ACTH. Proc Natl Acad Sci USA 83:4715–4719
69. **Miller WL** 2005 Minireview: regulation of steroidogenesis by electron transfer. Endocrinology 146:2544–2550
70. **Schimmer BP, Kwan WK, Tsao J, Qiu R** 1995 Adrenocorticotropin-resistant mutants of the Y1 adrenal cell line fail to express the adrenocorticotropin receptor. J Cell Physiol 163:164–171
71. **Parker KL, Schimmer BP** 1997 Steroidogenic factor 1: A key determinant of endocrine development and function. Endocr Rev 18:361–377
72. **Papadopoulos V, Amri H, Boujrad N, Cascio C, Culty M, Garnier M, Hardwick M, Li H, Vidic B, Brown AS, Reversa JL, Bernassau JM, Drieu K** 1997 Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. Steroids 62:21–28
73. **Casey OM, Fitzpatrick R, McInerney JO, Morris DG, Powell R, Sreenan JM** 2004 Analysis of gene expression in the bovine corpus luteum through generation and characterisation of 960 ESTs. Biochim Biophys Acta 1679:10–17
74. **Azhar S, Reaven E** 2002 Scavenger receptor class BI and selective cholesteryl ester uptake: partners in the regulation of steroidogenesis. Mol Cell Endocrinol 195:1–26
75. **van Kalken CK, Broxterman HJ, Pinedo HM, Feller N, Dekker H, Lankelma J, Giaccone G** 1993 Cortisol is transported by the multidrug resistance gene product P-glycoprotein. Br J Cancer 67:284–289
76. **Laue L, Hoeg JM, Barnes K, Loriaux DL, Chrousos GP** 1987 The effect of mevinolin on steroidogenesis in patients with defects in the low density lipoprotein receptor pathway. J Clin Endocrinol Metab 64:531–535

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