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Adrenocorticotropin-Dependent Changes in SF-1/DAX-1 Ratio Influence Steroidogenic Genes Expression in a Novel Model of Glucocorticoid-Producing Adrenocortical Cell Lines Derived from Targeted Tumorigenesis

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We established the first adrenocortical tumor cell lines with complete zona fasciculata (ZF) cell phenotype from tumors induced in transgenic mice by large T-antigen of simian virus 40 under the control of the aldose reductase-like *akr1b7* gene promoter. Adrenocortical tumor cell lines produced high amounts of corticosterone and were responsive to ACTH. All genes that are supportive for glucocorticoid synthesis including *cyp21a1* and *cyp11b1* were expressed, and most of them were transiently up-regulated by ACTH at transcriptional level: stimulation culminated after 3–6 h and returned to basal levels after 24 h. Taking advantage of these cells, we have examined the effect of ACTH on DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on X-chromosome, gene 1) and SF-1 (steroidogenic factor 1), two transcription factors known to respectively repress and activate adrenocortical steroidogenesis by acting on common target genes. According to their antagonistic activities, DAX-1 mRNA and protein levels were transiently down-regulated by ACTH, whereas those of SF-1 were stimulated, with kinetics paralleling those of steroidogenic genes expression, notably of two known SF-1 target genes, *star* and *akr1b7*. This suggests an essential role of SF-1/DAX-1 proteins ratio to achieve proper ACTH control of steroidogenic gene expression in cells derived from ZF. This was confirmed in mice adrenals, where repression of *dax-1* gene and concomitant up-regulation of *sf-1*, *star*, and *akr1b7* genes were observed in response to ACTH stimulation. In conclusion, using both unique differentiated cell lines and *in vivo* approaches, we provide the first evidence that hormonally induced changes in SF-1/DAX-1 ratio are part of the molecular arsenal of ZF cells to fine tune ACTH responsiveness. (*Endocrinology* 147: 1805–1818, 2006)

THE MAMMALIAN adrenal cortex is an endocrine gland composed of cells that segregate into separate zones with distinct functions: the zona glomerulosa (ZG) produces aldosterone, the zona fasciculata (ZF) synthesizes corticosterone in rodents and cortisol in humans, and the zona reticularis (ZR) secretes androgen precursors in humans but not in rodents (1, 2). Differential production of adrenal steroid hormones results from both the proper sensitivity of each zone to respond to specific secretagogues (angiotensin II, potassium, ACTH) that stimulate steroidogenic genes transcription and the restricted localization of the hydroxylases that catalyze the terminal steps of steroidogenesis. Indeed, aldosterone production in the ZG is essentially under the control of angiotensin II and potassium acting via the calcium messenger system, whereas ACTH is the main regulator of corticosterone production in the ZF and exerts its effect at least by generating cAMP as an intracellular messenger. On the other hand, zonal differences in the steroid products are attributable to the zone-specific expression of two structurally related cytochrome P450 hydroxylases, aldosterone synthase (CYP11B2) and 11β-hydroxylase (CYP11B1) that convert deoxycorticosterone into aldosterone in the ZG and into corticosterone in the ZF, respectively (3). By contrast, all the initial reactions leading to the conversion of cholesterol to deoxycorticosterone are catalyzed by the sequential action of three enzymes expressed throughout the adrenal cortex, cytochrome P450 cholesterol side-chain cleavage (CYP11A1), 3β-hydroxysteroid dehydrogenase (3β-HSD), and cytochrome P450 21-hydroxylase (CYP21A) (4). In rodents adrenals, intracellular uptake and intramitochondrial transfer of cholesterol are facilitated by the scavenger type B class I receptor (SR-BI), the high-density lipoprotein receptor and by the steroidogenic acute regulatory protein (StAR), respec-

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Abbreviations: ATC, Adrenocortical tumor cell lines; CYP11A1, cytochrome P450 cholesterol side-chain cleavage; CYP11B1, cytochrome P450 hydroxylase; 11β-hydroxylase; CYP11B2, cytochrome P450 hydroxylase, aldosterone synthase; DAX-1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on X-chromosome, gene 1; E, embryonic day; FACS, fluorescence-activated cell sorting; gapdh, glyceraldehyde-3-phosphate dehydrogenase; HPA, hypothalamo-pituitary-adrenal; 3β-HSD, 3β-hydroxysteroid dehydrogenase; SF-1, steroidogenic factor-1; StAR, steroidogenic acute regulatory protein; SV40, simian virus 40; ZF, zona fasciculata; ZG, zona glomerulosa; ZR, zona reticularis.

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tively (5–7). Thus, ACTH triggers glucocorticoid biosynthesis in the ZF by inducing a coordinated up-regulation of all the genes involved in the cholesterol delivery and in its conversion into steroid hormones (8). In addition, maintaining full steroidogenic activity of ZF cells may require the coordinated control by ACTH of a third group of genes encoding detoxifying enzymes, the aldose reductases AKR1B7 and AKR1B1, and the manganese-dependent superoxide dismutase that scavenge deleterious byproducts of steroidogenesis, *e.g.* aldehydes (isocaproaldehyde and 4-hydroxynonenal) and superoxide radicals, respectively (9–11).

Genetic ablation of the steroidogenic factor-1 (SF-1) showed that it is essential for both adrenal and gonadal differentiation and development, but the complete lack of these organs in SF-1 knockout mice impedes evaluation of its role in the maintenance of differentiated functions (12, 13). Many studies, essentially based on cell transfections, have focused on the identification of transcription factors that could explain hormonal and cell-specific control of genes encoding steroid hydroxylases, StAR protein, high-density lipoprotein receptor or the detoxifying enzyme AKR1B7 (14–19). These studies have prominently shown that 1) based on mutational analyses of promoter sequences, SF-1 behaves as a common mediator of the hormonal/cAMP response of many steroidogenic genes in both adrenals and gonads, and 2) SF-1 is not sufficient for this process, which requires functional interactions between SF-1 and other transcription factors *e.g.* Sp1, CCAAT/enhancer binding protein (C/EBPβ), GATA-binding factor 4 (GATA-4), cAMP response element binding protein (CREB), AP-1 (reviewed in Ref. 13), as shown by the inability of a pure SF-1 binding site to confer hormonal/cAMP responsiveness to a minimal promoter (19). Therefore, the molecular mechanisms involved in SF-1-mediated hormonal/cAMP response remain poorly understood, although variations in its accumulation and phosphorylation upon cAMP stimulation have been reported (20–23). In both human and mouse, mutations in either SF-1 or DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on X-chromosome, gene 1) lead to impaired adrenal function, and because their expression colocalizes in many endocrine cells (24, 25), it was proposed that these two factors may interact to control a common genetic cascade (26, 27). Cell transfection experiments suggested that DAX-1 functions antagonistically with SF-1 and acts as a powerful repressor of basal and cAMP-stimulated steroid production (28–31). Introduction of the DAX-1 mutation (DAX-1$^{-/Y}$) in SF-1 heterozygous mice rescues the adrenal phenotype and reverses the blunted stress response observed in SF-1+/− animals, indicating that the two factors antagonistically interact in the adrenal *in vivo* to regulate ACTH sensitivity (32). Hence, endocrine cells may modulate their response to pituitary hormones by controlling the balance between SF-1 and DAX-1. Indeed, DAX-1 expression was shown to be repressed by FSH, LH, and angiotensin II in Sertoli, Leydig, and adrenal glomerulosa cells, respectively (33–36). However, because adrenocortical cell lines exhibiting a complete ZF phenotype were not available, a potential effect of ACTH on the endogenous balance between SF-1 and DAX-1 levels in the ZF could not be evaluated. Indeed, most of our knowledge of the mechanisms regulating adrenocortical steroidogenesis stems from studies in either mouse Y-1 or human NCI-H295 tumoral cell lines, both of which are incompletely differentiated. Although Y-1 cells are devoid of DAX-1 and cytochrome P450 21-hydroxylase, H295 cells simultaneously exhibit phenotypes of ZG and ZF. Furthermore, both lines are poorly responsive to ACTH (30, 37–41).

Taking advantage of the promoter for the *akr1b7* gene, which is able to target adrenal expression in the ZF *in vivo*, we developed adrenocortical cell lines derived from simian virus 40 (SV40) large T antigen-induced tumors obtained by targeted tumorigenesis in transgenic mice (42–44). These cell lines showed a complete and stable ZF phenotype over more than 25 passages. By analyzing expression of all the major actors of steroidogenesis in response to ACTH in these newly developed cell lines as well as in mouse adrenal cortex, we provide evidence for the essential role of the balance between SF-1 and DAX-1 levels in ACTH-mediated stimulation of steroidogenic genes expression and steroidogenesis in the ZF. Our data also point out the detoxifying enzyme gene *akr1b7* as a new target for the antagonistic partners DAX-1 and SF-1.

Materials and Methods

Establishment and culture of adrenocortical tumor cell (ATC) cell lines

Two cell lines were established from adrenal tumors of two transgenic mice harboring the large T-antigen of SV40 under the control of the adrenocortical-specific promoter of *akr1b7*. These cell lines were designated ATC1 and ATC7-L for ATC of founder transgenic mice 1 and 7-Left adrenal, respectively (44). ATC cells were cultured on polylysin-coated 10-cm tissue culture dishes in a 1:1 mixture of DMEM and Ham's F-12 medium (DMEM-F12) at 37 C in a 5% CO_2-95% air atmosphere. The medium was supplemented with insulin (10 mg/ml), transferrin (5.5 mg/ml), and selenium (5 ng/ml) (ITS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), 2.5% horse serum, and 2.5% fetal bovine serum. The culture medium was changed every 2 d. Cells were starved 24 h in minimum medium (without serum) before the addition of ACTH (fragment 1–24), forskolin, actinomycin D, or cycloheximide (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France) at times and concentrations indicated in the figure legends (Figs. 1–6).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 10 min, and permeabilized in the presence of PBS and 0.2% Triton for 8 min. Cells were incubated overnight at 4 C with anti-SV40 Large T antigen (kindly provided by Dr. D. Hanahan, University of California, San Francisco, CA) or anti-AKR1B7 antiserum L3 (10) polyclonal antibodies used at a dilution of 1:2000 and 1:500, respectively. After washing in PBS, nonspecific protein-binding sites were blocked by incubation with PBS and 5% goat serum. Incubation with the fluorescin isothiocyanate antirabbit IgG (1:200) was carried out for 1 h at room temperature. Cells were washed in PBS and were observed under UV irradiation with a photonic microscope (Carl Zeiss, Le Pecq, France).

Flow cytometry

Cell cycle analysis for the determination of the cell DNA content was undertaken after propidium iodide labeling of cells. ATC1 ant ATC7-L cells were harvested and washed in PBS. Pellets were resuspended in ribonuclease A (500 μg/ml), propidium iodide (50 μg/ml) solution and kept 1 h at 4 C in the dark. Cell suspensions were analyzed using Beckman Coulter FACS (fluorescence-activated cell sorting) apparatus (Beckman Coulter, Paris, France). For control samples, total blood from mouse was collected from the inferior vena cava, and a 200-μl aliquot was used in FACS analyses.

Analysis of steroid production

Cells were starved 24 h in serum-free medium and then cultured in the same medium in the presence or absence of ACTH. Gas-liquid chromatography-mass spectrometry analysis of steroids secreted in serum-free media of cells cultured for 30 h in presence or absence of ACTH was done as previously described (45). Corticosterone concentrations in culture media were determined by RIA using a commercially available kit (ICN Biomedicals, Orsay, France).

Analysis of RNA

Total RNA was isolated from cells and tissues with TRIzol (Invitrogen Life Technologies, Cergy Pontoise, France) according to the manufacturer's instructions. Total RNA (25 μg) was separated in denaturing formaldehyde/formamide agarose gel and transferred onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech, Orsay, France). cDNA fragments of $p57^{kip2}$, *igf2*, *mc2r*, *sr-b1*, *star*, *cyp11a1*, and *cyp21a1* were isolated by RT-PCR, starting from 2 μg mouse adrenal total RNAs, using Moloney murine leukemia virus reverse transcriptase and *Taq* polymerase (Promega Corp., Charbonnière, France) with the following primer pairs: $p57^{kip2}$ (forward, 5′-TGAACGCCGAGGACCAGAA-3′; reverse, 5′-TCTCTTGCGCTTGGCGAAGA-3′), *igf2* (forward, 5′-CCAGTGGGGAAGTCGATGTT-3′; reverse, 5′-GATGGTTGCTGGACATCTCC-3′), *mc2r* (forward, 5′-CTGACATGTTGGGCAGTCTG-3′; reverse, 5′-TCTGGGCTCCGAAAGGCATA-3′), *sr-b1* (forward, 5′-TCCCTCATCAAGCAGCACGT-3′; reverse, 5′-ATTCTGGACGCCCGTGAAGA-3′), *star* (forward, 5′-CAGATGTGGGCAAGGTGTT-3′; reverse, 5′-GATAGGACCTGGTTGATGAT-3′), *cyp11a1* (forward, 5′-ACCCCAAGGATGCGTCGATA-3′; reverse, 5′-CTGAAGTCTCGCTTCTGCCT-3′), *cyp21a1* (forward, 5′-ACCCTTCACGACTGTGTCCA-3′; reverse, 5′-TGCTAGCCCTAGTTGCACGA-3′), rat *cyp11b1* (forward, 5′-AGGGAGCCTTACCATGGATG-3′; reverse, 5′-CCTGAGTTATTAGTGCCGCCA-3′). The ligation of RT-PCR products was performed in pGEMT-easy vector (Promega Corp.) and the fragments for the cDNA probes were generally excised by *Eco*RI digestion with the exception of *cyp11b1* cDNA probe that was excised by *Sph*I/*Nde*I digestion. The probe used to detect *akr1b7* mRNA was obtained by pGEM-akr1b7 digestion with *Eco*R1 and *Bam*H1 (18). Mouse *gata4* and *gata6* probes were excised from pBS-m-*gata4* by *Not*I/*Kpn*I digestion and from pCRII-m-*gata6* by *Eco*RV/*Hin*dIII digestion (kindly provided by Dr. D. B. Wilson, Washington University School of Medicine, St. Louis, MO). Mouse *sf-1* probe was extracted from pCMV5-*sf-1* by *Eco*RI digestion (a kind gift of Dr. K. L. Parker, University of Texas Southwestern Medical Center, Dallas, TX). Mouse *dax-1* probe was extracted from pSG.mDAX-1 by *Eco*RI/*Bam*HI digestion (kindly provided by Dr. E Lalli, Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne Sophia-Antipolis, France). To normalize the loading of RNAs, Northern blots were stripped and rehybridized with a mouse *β-actin* or *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) probes excised from pGEM-7ZF-*β-actin* by *Eco*RI/*Bam*HI digestion and from pGEMT-easy-*gapdh* by *Eco*RI digestion, respectively. All cDNA fragments used as probes were labeled with the α^{32}P-dCTP NEBlot kit (New England Biolabs, Ozyme, Saint-Quentin en Yvelines, France). Prehybridization was carried out for 2 h at 65 C in a solution containing 3× SSC (standard sodium citrate buffer), polyvinylpyrrolidone (0.2%), Ficoll (0.2%), polyethyleneglycol (5%), glycine (1%), sodium dodecyl sulfate (1%), and 100 μg/ml sonicated salmon sperm DNA. Hybridization was performed overnight in the same solution in the presence of labeled probes. After washing, blots were exposed to an imaging screen and visualized using a Bio-Rad molecular imager FX phosphor-imager (Bio-Rad, Marnes-la-Coquette, France). Hybridization signals were analyzed using Quantity One quantification software (Bio-Rad). All Northern blot analyses using total RNA of ATC1 or ATC7-L lines were performed in at least three independent experiments.

RT-PCR

The isolated total RNAs were treated with amplification grade deoxyribonuclease I (Invitrogen Life Technologies) to remove trace amounts of genomic DNA. The deoxyribonuclease-treated total RNAs (1 μg) were reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Promega Corp.) according to the manufacturer's instructions and amplified using *Taq* DNA polymerase (Promega Corp.) by PCR. Nucleotide sequences of the specific primers used were: *dax-1* (forward, 5′-AAGCCAGGTCCCTCTTGTAC-3′; reverse, 5′-TACAGTAGGACAGCTGCCAC-3′); *β-actin* (forward, 5′-CGTGGGCCGCCCTAGGCACCA-3′; reverse, 5′-TTGGCCTTAGGGTTCAGGGGGG-3′). These primer pairs yielded PCR products of 365 and 242 bp for *dax-1* and *β-actin*, respectively. PCR consisted of 20 cycles of the following steps: denaturation for 1 min at 94 C, annealing for 30 sec at 59 C, extension for 50 sec at 72 C. Twenty percent of the PCR product was resolved on a 1.5% agarose gel and transferred onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech). The specificity of the RT-PCR products was determined by hybridizing the membranes with cDNA fragment of *dax1* and *β-actin* labeled with the α^{32}P-dCTP NEBlot kit (New England Biolabs, Ozyme, Saint-Quentin en Yvelines, France). Blots were visualized and analyzed with Bio-Rad molecular imager FX phosphor-imager (Bio-Rad).

Western blot analysis

Cell samples and frozen adrenals samples were homogenized in buffer C: 20 mM HEPES (pH 7.6)/0.2 mM EDTA/1.5 mM $MgCl_2$/0.42 M NaCl/0.5 mM dithiothreitol/25% glycerol/ plus 0.2% Nonidet P-40 and a cocktail of protease inhibitors (Complete protease inhibitor cocktail tablets; Roche Diagnostics, Meylan, France). The concentration of soluble proteins was determined by the Bradford method (Bio-Rad). Proteins were subjected to SDS-PAGE on 10% acrylamide gels and transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech). Nonspecific protein-binding sites were blocked by incubation for 1 h at room temperature in 50 mM Tris-HCl (pH 8), 150 mM NaCl and 0,1% Tween 20 (TBS-T) containing 10% nonfat dry milk. Incubation with primary polyclonal antibodies [anti-AKR1B7 L3 antiserum at 1:3000 (10); anti-StAR at 1:2000 (46); anti-SF-1 at 1:2000 (Upstate Biotechnology, Euromedex, Mundolsheim, France); anti-β-ACTIN at 1:2000 (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France) or monoclonal anti-DAX-1 2F4 at 1:1000 (33) was carried in the same buffer overnight at 4 C. After washing in TBS-T, membranes were incubated for 1 h at room temperature with the peroxidase-conjugated antirabbit IgG at 1:10,000 (P.A.R.I.S., Compiègne, France) or peroxidase-conjugated antimouse κ at 1:5000 (Southern Biotechnology, Clinisciences, Montrouge, France). The specific complexes were detected using the enhanced chemiluminescence (ECL) system from Amersham Pharmacia Biotech.

Animals and treatments

Animal studies were conducted in agreement with standards described by the National Institutes of Health Guide for Care and Use of Laboratory Animals as well as with the local laws and regulations applicable to animal manipulations in France. Adult male CD1 mice were injected with vehicle (sesame oil), dexamethasone acetate for 5 d (75 μg twice daily; Sigma-Aldrich, L'Isle d'Abeau Chesnes, France), or dexamethasone acetate (5 d) plus long-acting ACTH (1.2 U, im, daily; Synacthene, Novartis Pharma S.A., Rueil-Malmaison, France) for the last 6 or 24 h. Adrenal glands were removed, and total RNAs or proteins were extracted. The Southern blot in Fig. 5A represents a typical experiment from one animal (two adrenals), and the corresponding quantification shown below is the mean of experiments performed with four animals for each condition; in Fig. 5B, each lane represents pooled samples from three animals (six adrenals).

Cell transfection

HeLa cells were maintained in DMEM with Glutamax supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 5% fetal bovine serum. HeLa cells were transfected in six-well plates 24 h after seeding at a density of 300.10^3 cells per well in complete medium. Cells were transfected for 29 h with Exgen500 reagent (Euromedex, Mundolsheim, France) according to manufacturer's instructions. In Fig. 6A, HeLa cells were transfected with 1 μg reporter gene, 5 ng pCMV5-SF1, encoding human SF-1 and increasing amounts of pCMV-DAX-1 expression vector, encoding human DAX-1 (a kind gift of Dr. J. L. Jameson, Chicago, IL) or corresponding pCMV empty vector (Stratagene, Amsterdam, The Netherlands). Total vector amount was kept constant to 1.1 μg by cotransfection of pBL-CAT3 empty vector as carrier DNA. In Fig. 6B, HeLa cells were transfected with 1 μg reporter gene, 2 ng pRLSV40, 5 ng pCMV5-SF-1, and 5 ng pCMV-DAX-1 or empty pCMV. The *luc* reporter constructs driven by the 0.5-kb *akr1b7* gene promoter (−510/+41) pGL3–0.5 wild-type and the corresponding mutants for the SF-1 sites were already described (19). Transfection of ATC1 cells was con-

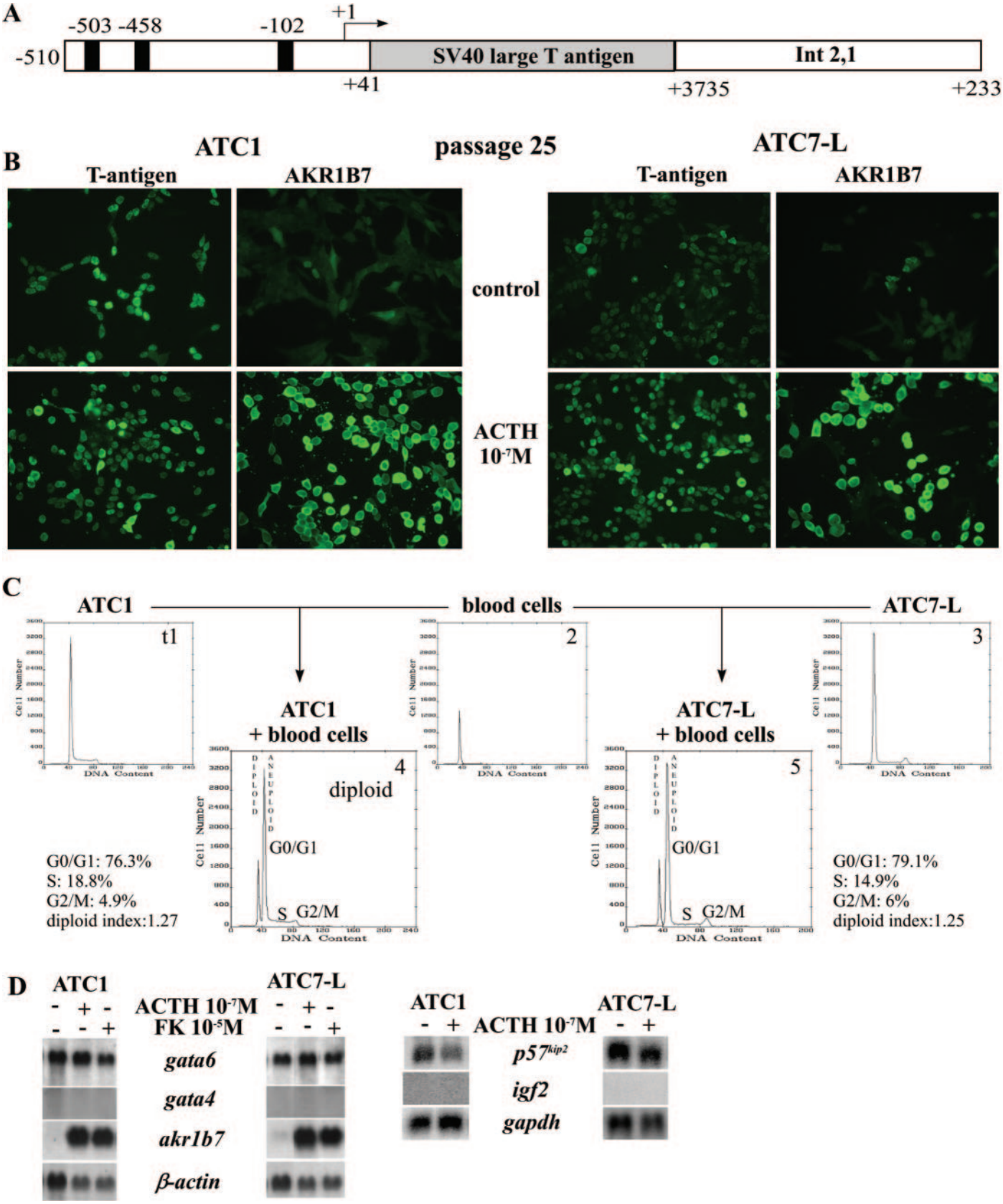

FIG. 1. A, Structure of the transgene used for production of transgenic mice carrying the SV40 large T antigen gene under control of the *akr1b7* promoter (−510/+41). The *arrow* points out the transcription start site (+1). Binding sites for SF-1 factor (−503, −458, −102) are indicated. Int 2, 1 (+3735/+233) sequence containing the *akr1b7* first intron, second exon, and second intron was inserted in an inverted position

ducted in six-well plates using Metafectene reagent (Biontex Laboratories GmbH, Münich, Germany) according to the manufacturer's instructions. ATC1 cells were seeded at a density of 400.10^3 cells per well and transfected with 2 μg of reporter constructs and 5 ng pRLSV40 in serum-free ATC medium for 19 h and then induced for 6 or 12 h with 10^{-7} M ACTH (fragment 1–24) or 1 mM 8Br-cAMP in absence or presence of 5 μM H89 PKA inhibitor (Sigma-Aldrich). The *luc* reporter constructs transfected in ATC1 cells were driven either by murine *dax-1, star* or *akr1b7* genes promoters: −2.5kDax was a gentle gift of Dr. K. I. Morohashi (National Institute of Basic Biology, Okazaki, Japan), −966Star was provided by Dr. D. J. Mangelsdorf (University of Southwestern Medical Center, Dallas, TX), and pGL3–1.8 was constructed by subcloning the −1804/+41 fragment of the *akr1b7* gene excised from the p1.8CAT reporter construct (47) into the Sma1 and *Bgl*II sites of pGL3 vector. Luciferase and *Renilla* assays were performed in an automated luminometer with GenofaxA and GenofaxB reagents (Yelen, Ensue la Redonne, France).

Statistical analysis

Statistical analyses were performed by a one-way ANOVA followed by Fisher's *t* test. Values of $P < 0.05$ were considered significant and are presented in *Results*.

Results

Establishment of ACTH-responsive adrenocortical cell lines from akr1b7-SV40 Tag transgenic mice

We had previously demonstrated that DNA fragments of the *akr1b7* gene enclosing the 0.5-kb promoter and intronic sequences (Fig. 1A) were able to confer ZF-specific and ACTH-regulated expression to reporter genes in transgenic mice, recapitulating the essential features of the endogenous gene expression in the adrenal gland (42, 43). These *cis*-acting sequences driving the SV40 large T antigen were shown to induce the formation of cortical tumors in two founder transgenic mice (44). The cells from two of these independent tumors, ATC1 and ATC7-L, were propagated for up to 40 passages without crisis, indicating immortalization. The analyses presented hereby were all conducted between 25 and 35 passages. The doubling times for both the ATC1 and ATC7-L cells were about 22–24 h in basal conditions. To determine the homogeneity and the responsiveness of these cells to ACTH, we assayed control and ACTH-treated cells for T-antigen and AKR1B7 by immunocytochemistry (Fig. 1B). In the control condition, T-antigen-positive nuclear staining was detected in ATC1 and ATC7-L cells, whereas AKR1B7 staining remained undetectable or very low. When 10^{-7} M ACTH was added to the medium for 24 h, we observed an increased intensity of T-antigen nuclear staining and a vigorous responsiveness of AKR1B7 cytoplasmic staining in both lines. Note that both lines responded to ACTH with a clear change in cell morphology from flat to round shape. The superposition of immunocytochemical stainings and Hoechst nuclear staining indicated that the whole cell population appeared sensitive to the hormone (not shown). Homogeneity of the cell population was further confirmed using flow cytometry as shown by the unique profile exemplified by the major peaks recorded with the ATC1 and ATC7-L cell samples (Fig. 1C). This analysis also indicated that ATC1 and ATC7-L tumoral cells had retained a near-diploid state (diploid index 1.27 and 1.25, respectively).

Because immunocytochemistry is an approximate index to evaluate the hormonal responsiveness of the cells, we compared their responsiveness to ACTH and to forskolin, a potent inducer of cAMP-dependent protein kinase signaling, using Northern blot analyses. As seen in Fig. 1D (*left panel*), rather undetectable in the control conditions, the *akr1b7* mRNA was strongly induced to similar levels in both tumor lines treated with either 10^{-7} M ACTH or 10^{-5} M forskolin for 6 h. By contrast, angiotensin II or calcium ionophore A23187 failed to induce *akr1b7* gene expression, and CYP11B2 (aldosterone synthase) was neither detected in basal condition nor in presence of these inducers (not shown). Finally, because T-antigen is known to induce a broad spectrum of tumor phenotypes, we looked for the expression of markers of malignancy of adrenal tumors (*gata-4* and *igf2*) and of genes whose expression is associated to a more differentiated phenotype (*gata-6* and $p57^{kip2}$). As revealed by Northern blot analyses (Fig. 1D, *left* and *right panels*), ATC lines contain easily detectable levels of *gata-6* and $p57^{kip2}$ mRNAs, whereas neither *gata-4* nor *igf2* mRNAs are detected. Note that the abundance of *gata-4* mRNA was unchanged upon hormonal treatment, whereas $p57^{kip2}$ appeared slightly repressed.

Altogether, these data established that ATC1 and ATC7-L lines were composed of very homogenous adrenocortical cell populations that were derived from rather differentiated tumors and had conserved functional ACTH receptors.

Steroidogenic activity of the cell lines

Gas chromatography-mass spectrometry analysis of the serum-free media of the ATC1 line cultured for 30 h in presence of 10^{-7} M ACTH showed that the only steroid produced was corticosterone (Fig. 2A). A similar profile was observed in the absence of ACTH, indicating that corticosterone was the main if not the only product of basal steroidogenesis in these cells (not shown). Corticosterone secretion was then assayed in the serum-free media of each cell line (Fig. 2, B and C). The basal level of corticosterone secretion varied among the cells from 12 ng ± 4 to 85 ng ± 9 $/10^6$ cells/6 h for ATC1 and ATC7-L, respectively (Fig. 2B). Importantly for both lines, a significant stimulation of corticosterone secretion was observed starting from 10^{-11} M ACTH and then increased in a dose-dependent manner, reaching the greatest values at 10^{-7} M ACTH. As a consequence of the differences in the corticosterone basal production, the greatest amount of stimulation (observed at 10^{-7} M

downstream the polyadenylation signal. B, Hormonal responsiveness of *akr1b7* gene and transgene in ATC1 and ATC7-L cell lines. To test the hormonal responsiveness, cells (passage 25) were starved for 24 h in minimum medium and were cultured 24 h in the presence or absence of ACTH (10^{-7} M). Then, T antigen and AKR1B7 immunocytochemistry was performed as described in *Materials and Methods*. C, Flow cytometry analysis of ATC1 and ATC7-L cells (passage 27). Cells were stained with propidium iodide for FACS analysis. DNA content was analyzed by flow cytometry. Total diploid blood cells were used to standardize the apparatus. In addition to the major peak of diploid blood cells (graph 2), the DNA histograms of ATC1 and ATC7-L cells mixed with blood cells (graphs 4 and 5) exhibit two supernumerary peaks corresponding to the G0/G1 and G2/M peaks of ATC1 and ATC7-L cells. Graphs 1 and 3 show histograms of ATC1 and ATC7-L cells alone, respectively. D, Expression of markers of malignancy in ATC cells. The Northern blot was prepared with 25 μg of total RNA in each lane and transferred onto a nylon membrane, and the filter was successively hybridized with the indicated ^{32}P-labeled probes.

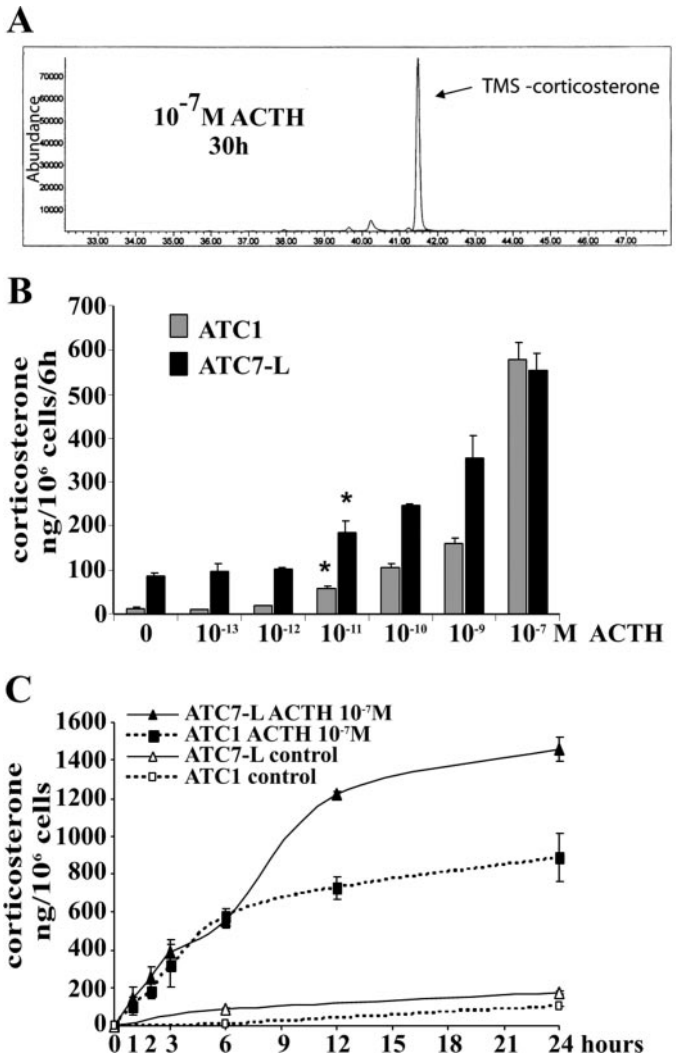

FIG. 2. Analysis of steroid production of ATC lines. A, Gas-liquid chromatography-mass spectrometry analysis of culture medium from ATC1 cells. B, Response of ATC1 and ATC7-L cells with increasing doses of ACTH. Cells were incubated for 6 h with increasing doses of ACTH and corticosterone productions in the culture medium were assayed by RIA. *, Significantly different from corticosterone productions recorded in presence of 10^{-12} M ACTH ($P < 0.01$). C, Time course of corticosterone production by ATC lines stimulated with 10^{-7} M ACTH (assayed by RIA). Results represent the mean of three independent experiments ± SD.

ACTH) was 49-fold in ATC1 line and only 6.5-fold for the ATC7-L line. However, the two cell lines produced equivalent accumulated amounts of corticosterone in response to 10^{-7} M ACTH, 578 ng ± 38 and 554 ng ± 38 /10^6 cells/6 h for ATC1 and ATC7-L, respectively. It is noteworthy that corticosterone production in ATC lines in the presence of 10^{-7} M ACTH (ATC1, 183 ± 43 ng/10^6 cells/2 h; ATC7L, 255 ± 56 ng/10^6 cells/2 h) was very similar to cortisol production induced by 10^{-8} M ACTH in primary culture of bovine adrenocortical cells, *i.e.* 127 ng/10^6 cells/2 h (48). As shown in Fig. 2C, the two lines responded to 10^{-7} M ACTH with similar kinetics up to 6 h, doubling corticosterone secretion in 2–3 h but behaved differently thereafter: steroid production of ATC1 cells slowed down, whereas that of ATC7-L went on rising until 12 h.

Effects of ACTH on steroidogenic genes expression in cell lines

To understand the patterns of steroid hormone synthesis in the ATC lines, we examined in detail the expression and hormonal responsiveness of the mRNAs for the genes involved in the different steps of the glucocorticoid synthesis, *i.e.* ACTH response (*mc2r*), cholesterol transport (*sr-b1*, *star*), steroid conversion (*cyp11a1*, *cyp21a1*, *cyp11b1*), and detoxification of harmful by-products (*akr1b7*). Because gene expression and hormonal sensitivity were equivalent between the two cell lines (data not shown), following experiments only focused on ATC1 cells. As shown in Fig. 3, all the mRNAs mentioned above were detected but differed in their response to ACTH. Indeed, *mc2r* and *cyp21a1* mRNAs were detected but were unresponsive to ACTH (Fig. 3A). By contrast, mRNAs levels for *sr-b1*, *star*, *cyp11a1*, *cyp11b1*, and *akr1b7* were strongly induced in a time-dependent manner by ACTH treatment and these inductions were not abolished when the protein synthesis inhibitor, cycloheximide was added together with the hormone. In addition, incubation

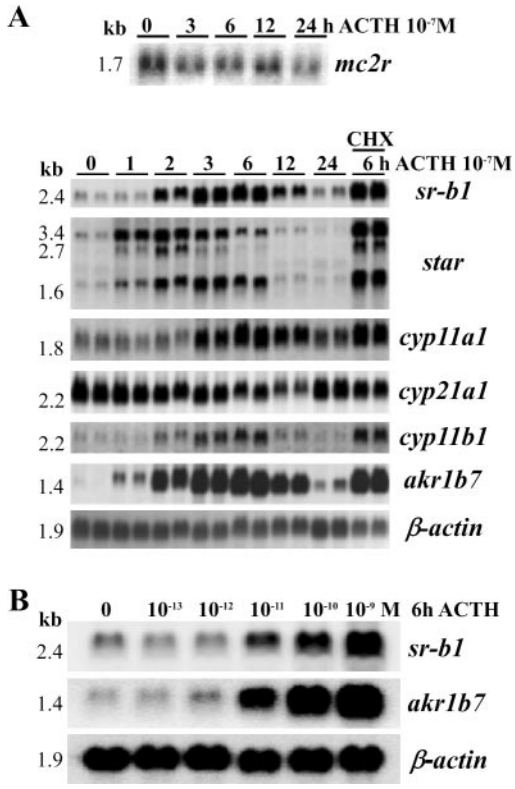

FIG. 3. Effects of ACTH on steroidogenic genes expression in ATC1 line. A, Time course effects of ACTH on the expression of genes involved in ACTH response (*mc2r*), cholesterol transport (*sr-b1*, *star*), steroid conversion (*cyp11a1*, *cyp21a1*, *cyp11b1*), and detoxification (*akr1b7*). Cells were starved 24 h in minimum medium (without serum) before the addition of ACTH (10^{-7} M) alone or in combination with cycloheximide (CHX, 10^{-5} M) for times indicated in figure. B, To determine the sensitivity of the response of steroidogenic genes, cells were incubated for 6 h with increasing doses of ACTH. The Northern blots were prepared with 25 μg of total RNA in each lane and transferred onto a nylon membrane, and the filters were successively hybridized with the indicated ^{32}P-labeled probes. The Northern blots were repeated at least three times in independent experiments.

with actinomycin D prevented ACTH mediated induction of all these mRNAs (not shown). Thus, the effect of ACTH on accumulation of these mRNAs is essentially transcriptional. Note that hormonal stimulation was transient because all responsive mRNAs returned to near control values after 24 h. Maximal mRNAs accumulation occurred after 3–6 h of ACTH treatment for most of the responsive genes and earlier, after 2–3 h, for *star*. Although *star* was clearly the earliest inducible gene, quantitative analyses of the Northern blots signals pointed *akr1b7* and *sr-b1* genes as being the most responsive genes to ACTH (Table 1). Indeed, a 6-h incubation of the cells with the hormone displayed a dose-dependent increase of *akr1b7* and *sr-b1* mRNAs levels, which was detectable from 10^{-11} M ACTH (Fig. 3B).

Effect of ACTH on DAX-1 and SF-1 expression in cell lines

Because the transcription factors DAX-1 and SF-1 are known to respectively repress and activate adrenal steroidogenesis, we next examined whether they were expressed in ATC lines and looked at their behavior upon hormonal stimulation. ATC1 cells were treated with 10^{-7} M ACTH for increasing periods of time and the accumulation of the mRNAs and proteins for DAX-1 and SF-1 were correlated to expression of *star* and *akr1b7*, two known SF-1 target genes. To prevent ACTH decay in long-time incubation (24–48 h), media were replaced every 12 h by fresh media containing 10^{-7} M ACTH. As shown in Fig. 4A, ATC1 cells were positive for *dax-1* gene expression. Interestingly, ACTH induced a dramatic time-dependent decrease in *dax-1* mRNA levels, which was clearly observed after 3 h reaching a maximum at 6 h (Table 1). By contrast, *sf-1* mRNA levels were significantly induced in the same conditions and maximal induction was reached in cells stimulated for 3 h with ACTH (Table 1). Corresponding kinetics of *star* and *akr1b7* mRNAs in response to ACTH are illustrated (Fig. 4A). To further understand the mechanisms involved in the ACTH-dependent changes in gene expression, we followed *sf-1* and *dax-1* mRNA levels under conditions of transcription (actinomycin D) or protein synthesis (cycloheximide) blockade (Fig. 4, A and B). As for *star* and *akr1b7* genes, the ACTH-dependent stimulation of *sf-1* mRNA was completely prevented by actinomycin D and was resistant to cycloheximide (Fig. 4, A and B), suggesting a direct action of ACTH mainly at the transcriptional level. The ACTH-dependent decrease in *dax-1* mRNA observed after 3 or 6 h treatment was almost completely prevented by coincubation with cycloheximide, indicating that at least one labile protein factor was required to repress the mRNA accumulation. When actinomycin D was applied alone, levels of *dax-1* mRNA decreased to 52% of the control value after 3 h, indicating the approximate half-life of the mRNA. Addition of ACTH did not accelerate this decay rate but rather prevented it, maintaining near-control mRNA levels after 3 h (85.5 ± 9.8%) and even after 6 h of coincubation (83.9 ± 18%). Altogether, these data suggested that two possible and not mutually exclusive mechanisms could lead to the observed down-regulation of *dax-1* mRNA: 1) ACTH induction of the expression of a labile repressor protein blocks the transcription of the *dax-1* gene and/or 2) ACTH induction of a labile mRNA-destabilizing protein is responsible for *dax-1* mRNA decay. To elucidate these mechanisms, we conducted transient transfection assays in ATC1 cells to test the ability of murine *dax-1* promoter to mediate the repression of *luc* reporter construct upon ACTH treatment (Fig. 4C). Control reporter constructs driven by the *akr1b7* gene 1.8-kb promoter (pGL3–1.8) or the *star* gene 0.9-kb promoter (−966Star) were both efficiently stimulated by either ACTH or 8Br-cAMP treatments and as expected, these stimulations were counteracted by H89, a PKA inhibitor. As illustrated in Fig. 4C, the murine 2.5-kb *dax-1* promoter *luc* reporter construct (−2.5kDax) showed a detectable basal activity in ATC1 cells but was not repressed upon 6 h or 12 h of ACTH stimulation. Thus, ACTH-mediated *dax-1* repression is unlikely to be at transcriptional level.

We next examined whether 10^{-7} M ACTH applied for 0–9 h (at a single dose) or for a prolonged time, 24–48 h (with hormone replacement every 12 h), induced similar changes at DAX-1 and SF-1 protein levels. As shown in Fig. 4D, DAX-1 protein levels were significantly decreased in a time-dependent manner from 3 h and up to 9 h, whereas SF-1 expression was increased by ACTH treatment and reached a plateau after 3 h. These kinetics were similar to those observed at the mRNA levels (Fig. 4A) and showed that during the 0- to 9-h period of hormonal treatment, ACTH-mediated changes in DAX-1 and SF-1 proteins occurred mainly by controlling their mRNA accumulation. Interestingly, a long-term hormonal exposure of the cells (24–48 h) induced a transient desensitization of the ACTH-mediated repression of DAX-1 expression and ACTH-mediated stimulation of StAR expression (Fig. 4D). Indeed, DAX-1 protein expression returned to initial levels by 24 h of ACTH treatment, began to decrease again by 36 h, and was fully repressed by 48 h. StAR protein levels followed an exact mirror pattern. The high accumulation of DAX-1 protein observed at 24 h without a concomitant rise in mRNA levels have also been observed in primary cultures of granulosa cells (49). This suggested that translational mechanisms might also participate in the regulation of DAX-1 expression upon sustained exposure to the hormone. Accumulation of both AKR1B7 and StAR proteins was correlated with low DAX-1 and high SF-1 contents found in ATC1 cells stimulated for 3–9 h with ACTH (Fig. 4D). However, there were clear differences between the AKR1B7 and StAR proteins kinetic responses to hormone stimulation: the former accumulated slowly probably because of delayed translation of its

TABLE 1. ACTH-dependent changes in mRNA levels in ATC1 line after 3 h and 6 h induction with 10^{-7} M ACTH

	3 h	6 h
sr-b1	↑ ×22 ± 3	↑ **×39 ± 9**
star (3.4 kb)	↑ **×23 ± 8**	↑ ×7.2 ± 3
star (1.6 kb)	↑ ×18 ± 8.5	↑ **×20 ± 8**
cyp11a1	↑ ×6.8 ± 1.2	↑ **×9.8 ± 2.1**
cyp11b1	↑ ×2 ± 0.5	↑ **×2.4 ± 0.6**
akr1b7	↑ ×78 ± 14	↑ **×196 ± 28**
sf-1	↑ **×2.9 ± 0.3**	↑ ×2.2 ± 0.3
dax-1	↓ ÷1.8 ± 0.15	↓ **÷11.7 ± 3**

Results were quantified from three independent Northern blot experiments and expressed as fold-induction (↑ ×) or fold inhibition (↓ ÷). Maximal effect is shown in *boldface*.

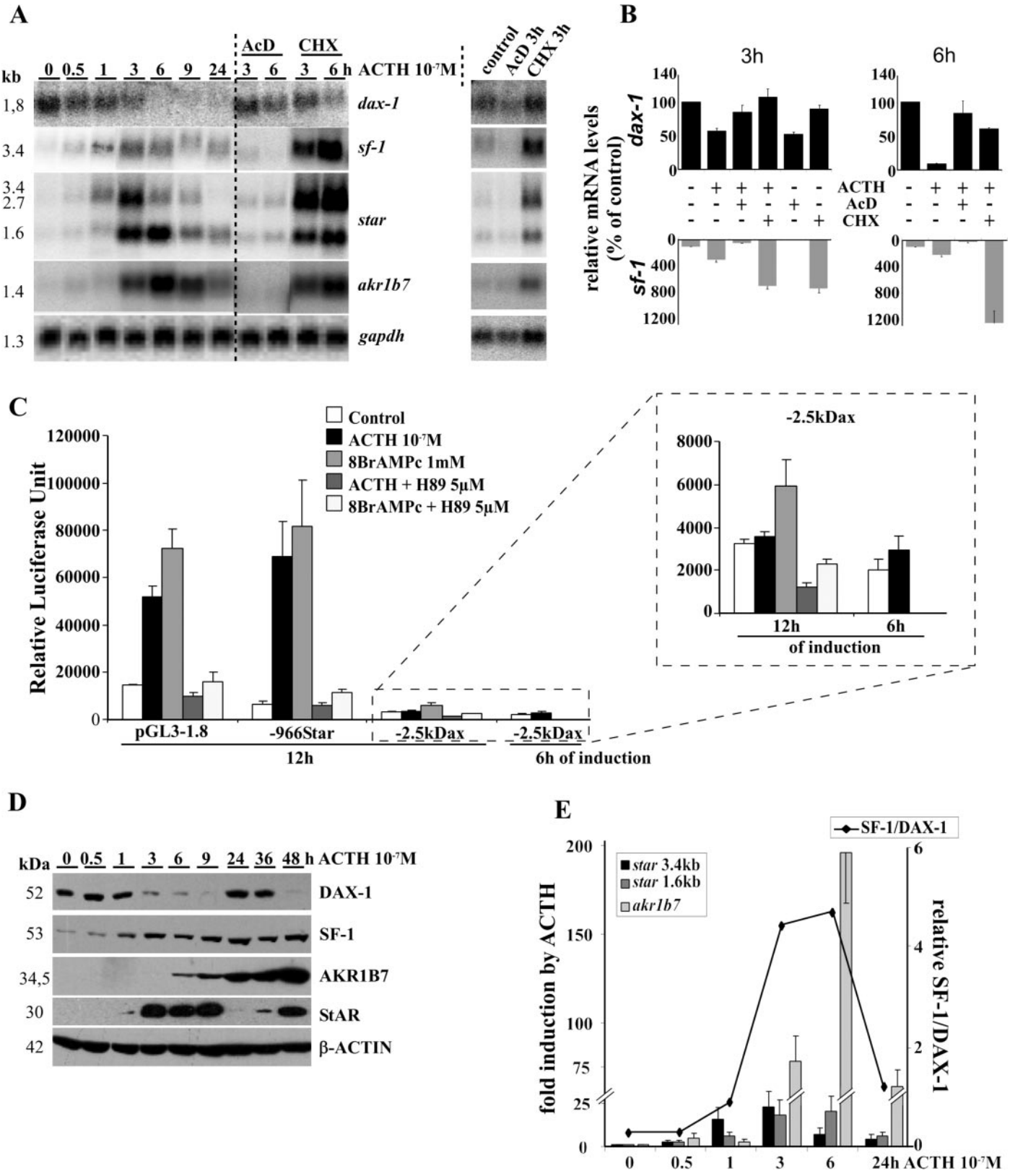

FIG. 4. Regulation by ACTH of DAX-1 and SF-1 in ATC1 line. A, Comparison of *dax-1*, *sf-1, star*, and *akr1b7* genes mRNA expression in ATC1 cells in response to increasing time induction with ACTH alone or in combination with actinomycin D or cycloheximide (*left panel*) or actinomycin D and cycloheximide alone (*right panel*) at 10^{-7} M, 1 μg/ml and 10^{-5} M, respectively. The Northern blots were prepared with 25 μg of total RNA in each lane and transferred onto a nylon membrane, and the filter was sequentially hybridized with the indicated ^{32}P-labeled probes. B, Histograms representing the quantification of *dax-1* (*black bar*) and *sf-1* (*gray bar*) mRNA Northern blot signals from three independent

mRNAs, whereas the latter reached immediately a plateau reflecting a concomitant increase in both transcription and translation. Thereafter (24–48 h), AKR1B7 went on accumulating regardless of DAX-1 levels, whereas StAR protein expression remained inversely correlated to low DAX-1 expression. These observations are likely to reflect the differences in protein turnover, AKR1B7 having a very long half-life (over 96 h; Manin, M., unpublished results) and StAR a short one [around 5 h, (50)]. Because DAX-1 represses SF-1 target genes by regulating at least SF-1 transactivation (31), one might expect that the SF-1/DAX-1 protein ratio in each cell would be determinant for the fine tuning of gene transcription. As shown in Fig. 4E, the time-dependent changes in *star* and *akr1b7* mRNAs levels after ACTH treatment reflected a parallel change in the SF-1/DAX-1 protein ratio.

Altogether, these results demonstrate that ATC1 cells (and also ATC7-L, not shown) have conserved both DAX-1 and SF-1 expression. Importantly, in these cells ACTH was shown to control the balance between repressor and inducer activities, by negatively regulating DAX-1 accumulation and stimulating SF-1 expression.

Effect of ACTH on DAX-1 and SF-1 expression in vivo

We next asked whether the ACTH-dependent regulation of DAX-1 and SF-1 expression observed in the cell lines might also occur *in vivo*. Therefore, we followed the changes in the expression of these two regulators in the adrenal glands of mice injected for 5 d with either dexamethasone alone to maintain a negative feedback on the hypothalamo-pituitary-adrenal (HPA) axis or in combination with long-acting ACTH (synacthene) for increasing periods of time. As shown in Fig. 5A, semiquantitative RT-PCR analyses showed that although HPA blockade caused no significant stimulation of *dax-1* mRNA levels, a drastic decrease was observed after ACTH treatment (over 70% ± 2.3 of the control value after 6 h). Importantly, similar results were obtained at the level of DAX-1 protein (Fig. 5, B and C). As expected, SF-1, AKR1B7 and StAR proteins levels exhibited an opposite behavior: they decreased upon dexamethasone treatment and were strongly reinduced after ACTH injections (Fig. 5, B and C). We conclude that the ACTH-mediated opposite regulation of DAX-1 and SF-1 expression observed in the cultured cell lines was consistent with the *in vivo* data. Thus, it appears that *ex vivo* as well as *in vivo*, ACTH imposes changes in SF-1/DAX-1 ratio that are likely to participate to hormonal regulation of at least *akr1b7* and *star* genes expression.

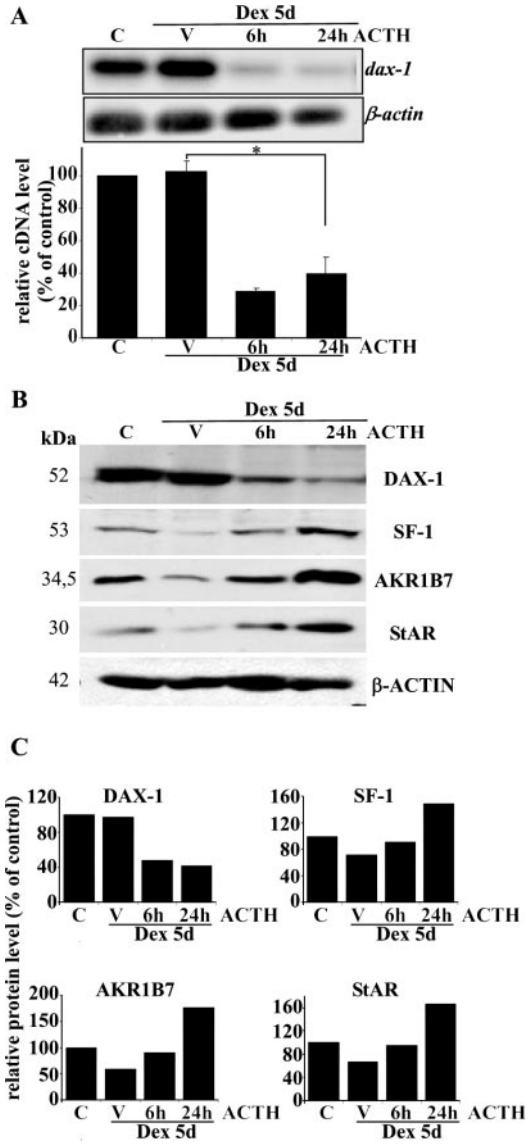

FIG. 5. DAX-1 and SF-1 expression in adrenal is regulated by ACTH. To determine the effect of ACTH on *dax-1* and *sf-1* expression *in vivo*, mice were treated by dexamethasone (Dex) alone or combination with ACTH (see *Materials and Methods*). C, Control; V, vehicle. A, RT-PCR analysis of relative *dax-1* mRNA levels *in vivo* in response to ACTH. Histograms represent the mean of three independent experiments ± SD. *, $P < 0.01$. cDNA level of control was set as 100%. B, Comparison of SF-1, DAX-1, AKR1B7, and StAR proteins levels in mice treated with dexamathesone alone or combination with ACTH. C, Histograms represent the quantification of DAX-1, SF-1, AKR1B7, and StAR proteins from pooled adrenal glands of three animals per condition. The control condition protein levels were set as 100%.

Role of DAX-1 on SF-1-dependent akr1b7 promoter activity

It is well established that SF-1 or cAMP-induced transactivation of the *star* gene is inhibited by DAX-1 (28, 30). The *akr1b7* gene 0.5-kb promoter (−510/+41) was previously shown to be stimulated by SF-1 in cell transfections studies, through the recruitment of three binding sites located at −503, −458 and −102 (19). In addition, we had shown that constitutive DAX-1 ectopic expression in Y-1 adrenocortical

experiments using ATC1 cells treated for 3 h (*left part*) and 6 h (*right part*). C, To test the hormonal responsiveness of the murine promoters of *dax1*, *star*, and *akr1b7* genes in ATC1 line, cells were transfected with the corresponding Luc reporter constructs and submitted to either ACTH or cAMP inductions in absence or presence of the PKA inhibitor H89. Results represent the mean of two or three independent experiments performed in triplicate. D, Western blot analysis of DAX-1, SF-1, StAR, and AKR1B7 proteins accumulation in ATC1 cells in response to increasing time induction with ACTH. E, Comparison between *star* and *akr1b7* relative mRNA levels (histograms) and the relative ratio of DAX1/SF1 protein accumulation (*line*). Quantifications represent the mean of at least three independent experiments ± SD.

cells was able to abolish cAMP responsiveness of the *akr1b7* endogenous gene (18). Because our *ex vivo* and *in vivo* data show a clear correlation between SF-1/DAX-1 ratio and *akr1b7* expression, we thus asked whether the *akr1b7* gene transcription could also be regulated by SF-1/DAX-1 balance in transfected HeLa cells (Fig. 6A). DAX-1 overexpression led to a marked dose-dependent decrease in SF-1 stimulated promoter activity. This decrease was not observed when the cells were solely transfected with pCMV-DAX-1 (data not shown). This suggests that DAX-1 effect on *akr1b7* promoter activity was strictly dependent on SF-1 activity. As expected, all the individual mutations previously shown to affect SF-1 responsiveness of the 0.5-kb promoter also affected DAX-1 repressive activity, although to a different degree (Fig. 6B). Indeed, the individual mutation of either the −503 or −458 sites abrogates DAX-1 repressive capacity, whereas mutation of the −102 site only modestly alters it, a result consistent with its poor responsiveness to SF-1 in HeLa cells (19). Interestingly, combined mutations of the −458 or −503 sites were not more efficient than their individual mutations. This observation is reminiscent of early results from Hanley and colleagues, who suggested that, to be efficient, DAX-1-mediated repression of SF-1 target genes requires multiple SF-1 sites within the promoter region (27).

Discussion

We undertook this study in an attempt to develop novel cell lines maintaining differentiated endocrine functions of ZF adrenocytes to investigate the mechanisms of steroidogenic genes regulation in response to the physiological activator of glucocorticoid synthesis, ACTH. Using genetically targeted oncogenesis, we have obtained the first immortalized cell lines, ATC1 and ATC7-L, that have retained a complete ZF phenotype. This phenotype was manifest at multiple levels: steroidogenic capacity, ACTH responsiveness, and expression profile of specific genes. Indeed, cultured ATC lines only produced corticosterone either in basal condition or under ACTH stimulation, a feature characteristic of the normal mouse ZF cells. Both lines appeared equally sensitive to ACTH because significant stimulation of corticosterone biosynthesis could be recorded from 10^{-11} M, a dose similar to ACTH concentration in mouse plasma. At maximally effective concentrations, ACTH causes a 6.5- to 49-fold increase in corticosterone production over the basal levels in ATC7-L and ATC1 lines, respectively. As expected, both lines expressed the genes that support the initial steps of steroid biosynthesis (*mc2r*, *sr-b1*, *star*, *cyp11a1*, and *akr1b7*) as well as the genes required for the final steps of corticosterone synthesis, *i.e.* the 21-hydroxylase (*cyp21a1*) and the ZF-specific

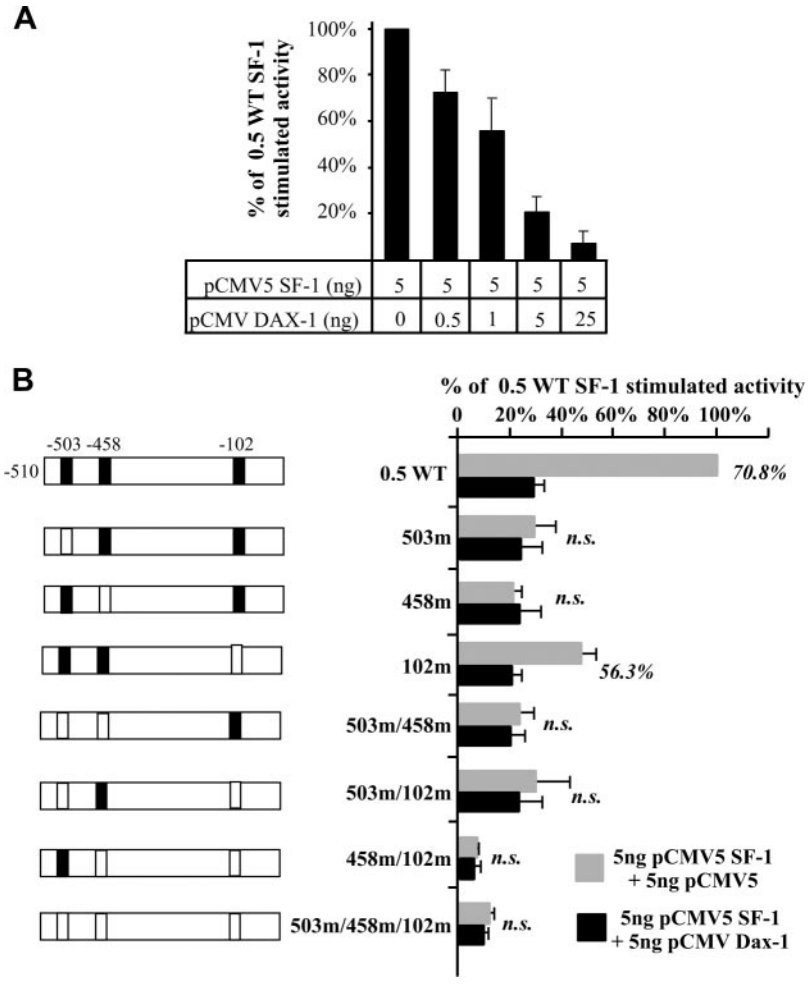

FIG. 6. Role of DAX-1 on *akr1b7* gene promoter activity. A, DAX-1 repress *akr1b7* promoter activity. To test the ability of DAX-1 to repress SF-1-mediated *akr1b7* activity, HeLa cells were transfected with 1 μg of the 0.5-kb wild-type promoter together with 5 ng of pCMV5-SF-1 expression vector and increasing doses of pCMV-DAX-1 expression vector that were normalized to the corresponding amounts of empty vector. Results represent the mean of two or three independent experiments performed in triplicate. SF-1 activated 0.5-kb wild-type construct was set as 100%. B, Single SF-1 response element (SFRE) mutation impair DAX-1 repression. To assess the effect of mutations of the SFREs on DAX-1 repression in the context of 0.5-kb promoter, HeLa cells were transfected with 1 μg of the mutant constructs, cotransfected with 5 ng pCMV5-SF-1 and 5 ng pCMV-DAX-1 (*black bar*) or the same amount of the corresponding empty vector (*gray bar*). Results represent the mean of the least three independent experiments performed in triplicate ± SD. The relative luciferase activity was normalized to the *Renilla* activity of a cotransfected expression vector. SF-1 activated 0.5-kb wild-type promoter activity was set as 100%. DAX-1 repressive activity displayed on the *right* of the *bars*. n.s., No significant repression by DAX-1 ($P > 0.05$).

enzyme 11β-hydroxylase (*cyp11b1*). In agreement with the high hormonal responsiveness of corticosterone production, most of these genes were strongly induced by ACTH. However, for unknown reasons and in contrast to the normal gland, *mc2r* (51, 52) and *cyp21a1* (53) genes were found unresponsive to ACTH but constitutively expressed. Currently available models of adrenocortical cell lines are almost exclusively represented by mouse Y-1 cells (54) and human NCI-H295 cells (40). Although both models have been extensively used and have proved their considerable value in studying regulatory mechanisms of adrenocortical steroidogenesis, these cells do not present most of the physiological features of the adrenal cortex (for review, see Ref. 55). Indeed, although Y-1 line exhibits features of ZF cells they are defective in 21-hydroxylase activity because of *cis*-inactivation of the *cyp21a1* gene (37) and therefore does not synthesize corticosterone. In addition, propagation of this line over years in different laboratories has led to spurious accumulation of unwanted variants, some of which became completely or partially resistant to ACTH. On the other hand, NCI-H295 cells exhibit simultaneous characteristics of both ZF and ZG cells: they produce cortisol and aldosterone but respond very poorly to ACTH, reflecting the low expression level of MC2R. Therefore, most if not all transfection experiments using Y-1 or NCI-H295 cells that were designed to examine the role of the cAMP-dependent pathway on transcriptional regulation were conducted with either forskolin (an activator of adenylate cyclase) or cAMP analogs instead of the physiological inducer, ACTH. It is noteworthy that we establish here, that ATC1 cells transfection is efficient and allows ACTH responsiveness of both *akr1b7* and *star* genes promoters. Up to now, very few alternative models of adrenocortical cell culture have been described. Two of them were derived from tumors induced in transgenic mice by SV-40 large T-antigen targeted in the adrenal glands using either the *CYP11A1* (56, 57) or the α-*inhibin* (58, 59) promoter regions. More recently, a third model was established from transgenic mice adrenals harboring a temperature-sensitive T-antigen under the control of an ubiquitously active promoter (60). However, none of these lines were reported to show ACTH responsiveness or efficient corticosterone secretion. Although it is unclear why previous targeted oncogenesis attempts failed to produce differentiated immortalized cells, we can hypothesize that the choice of the *akr1b7* gene promoter to target the expression of the large T antigen of SV40 is of paramount importance (because this is the only major difference with previously reported experiments). Indeed, to generate differentiated tumors the T-ag has to provide a growth advantage to a cell population that is already committed to produce the desired steroids (*i.e.* glucocorticoids in our case). *Akr1b7* mRNA expression is first detected at embryonic d 13.5 (E13.5) (44), a stage occurring soon after the onset of corticosteroid biosynthesis at E12.5 (reviewed in Ref. 61). By contrast, the *cyp11a1* gene, the earliest steroidogenic gene expressed in the adrenocortical anlage, is turned on from E11 (62, 63), before cells have acquired full steroidogenic capacity. This could explain why the cell lines derived from targeted adrenal tumors using the *CYP11A1* promoter, remain poorly differentiated and lose ACTH responsiveness by the fourth passage (56). On the other hand, the cell lines isolated from targeted tumors using the α-*inhibin* promoter were supposed to originate from the X zone of adrenal cortex that contains a cell population with a poorly differentiated steroidogenic phenotype (58). Thus, by comparison with the available cell lines above cited and according to their steroidogenic activity, ATC lines appeared to be the best differentiated adrenocortical cell lines (64). Finally, reminiscent of their differentiated endocrine phenotype, several lines of evidence indicate that ATC lines are derived from differentiated tumors that have not achieved malignant transformation yet. Indeed, as in normal adult tissue the mRNAs for cyclin-dependent kinase inhibitor $p57^{KIP2}$ (65) and transcription factor GATA-6 (66) were found highly expressed, whereas markers associated with malignancy, *i.e.* IGF-II (67, 68) and GATA-4 (69, 70) remained down-regulated in ATC lines.

Although the activator SF-1 and the repressor DAX-1 are known to regulate common genetic cascades that ensure normal adrenal organogenesis and adult adrenocortical function (25), murine Y-1 cells, by far the most studied adrenocortical cell model, are devoid of DAX-1 (30). We focused our attention on the ability of the ATC lines to express these essential transcriptional regulators. ATC lines were found positive for the expression of both SF-1 and DAX-1. Moreover, careful analysis of the impact of ACTH on gene expression allowed us to reveal mechanisms of hormonal regulation of steroidogenic activity that were, until now, uncharacterized in ZF cells. First, ACTH influenced the cellular balance between SF-1 and DAX-1 expression levels by stimulating the former and repressing the latter at both the mRNA and protein levels. Second, ACTH-dependent changes in SF-1/DAX-1 proteins ratio are likely to fine tune the glucocorticoid response because they are accurately correlated to changes in mRNA levels of steroidogenic genes. In addition, these observations allowed the identification of a novel DAX-1 target gene participating in detoxification of steroidogenesis byproducts, *i.e.* aldose reductase-like *akr1b7*. Finally, it is essential to stress that these observations were confirmed *in vivo* in the adrenals of mice subjected to HPA blockade and ACTH injections, demonstrating the physiological relevance of the ATC lines model.

Until now, DAX-1 down-regulation has only been described in primary cultures of adrenal ZG, in ovarian granulosa cells and in MA-10 Leydig cell line after hormonal stimulation by angiotensin II or forskolin, FSH and LH, respectively (34, 36, 49). Although the precise mechanism underlying the down-regulation of DAX-1 expression by ACTH is not clear, our data support the idea of a labile protein that would act at posttranscriptional level to accelerate *dax-1* mRNA decay. First, the similar kinetics of DAX-1 mRNA and protein down-regulation patterns indicates that ACTH mainly controls mRNA concentrations. Second, the cycloheximide sensitivity of ACTH-mediated down-regulation of *dax-1* mRNA levels, points out the involvement of at least one labile protein factor acting to prevent or to decrease accumulation of mRNA. Third, ACTH does not accelerate the decay rate of *dax-1* mRNA upon transcription blockade (actinomycin D treatment) but rather maintains mRNA levels to control values. This rules out the possibility that ACTH may enhance the translation of preexisting messengers for a labile

mRNA-destabilizing factor. However, these experiments cannot distinguish between the possibilities that the hormone-induced labile protein either represses *dax-1* gene transcription or accelerates its mRNA degradation, because both mechanisms would require *de novo* synthesis of the mRNA encoding this protein and hence would be sensitive to actinomycin D blockade. Finally, cell transfection studies conducted in ATC1 line showed that the transcriptional activity of the 2.5-kb upstream region of *dax-1* gene promoter was not repressed by ACTH. Interestingly, an ACTH-regulated protein involved in VEGF mRNA-destabilization was recently described in bovine adrenocortical cells (11, 71). The idea that a similar protein could be involved in *dax-1* gene down-regulation in ZF cells should be investigated. Although our data are very similar to what was previously reported in bovine ZG cells (36), our interpretation diverges from Osman and colleagues' hypothesis favoring down-regulation of *dax-1* gene transcription that was not directly demonstrated in their experiments. However, it is still possible that transcriptional repression was not observed in our transfection assays because key *cis*-acting sequences lying more upstream from the 2.5-kb *dax-1* promoter are essential to ACTH-mediated repression. Interestingly, an 11-kb 5′ regulatory region of the murine *dax-1* gene was shown not to be sufficient to direct adrenal expression in transgenic mice. This suggests that adrenal expression of *dax-1* requires yet uncharacterized far upstream (or downstream) sequences (72).

Up-regulation of SF-1 in response to secretagogues has been observed in primary cultures of bovine adrenocortical cells (36, 73) and in human H295 cells (74), whereas its expression was unaffected by hormonal induction in gonadal cells (34, 49). Hormonal regulation of SF-1 remains a conflicting subject because SF-1 was initially considered as insensitive to hormones and/or to the cAMP pathway in Y-1 cells (20, 75) as well as in the rat adrenal cortex (76). Although reasons for SF-1 unresponsiveness in previous experiments conducted in Y-1 cells remain unclear, it could be reasonably accounted for by the high variability of Y-1 cells, as discussed above. In agreement with this proposal, recently, SF-1 expression was shown to be stimulated by ACTH/cAMP in Y-1 adrenocortical cells at both the mRNA and protein levels (77), and the same results were also obtained in our laboratory (Aigueperse, C., unpublished data). Although the persistent expression of SF-1 in adrenal cortex sections of hypophysectomized rats (76) pointed out the dispensable role of ACTH for SF-1 expression, it is reasonable to hypothesize that an immunohistochemical approach was not sensitive enough to detect moderate quantitative variations. Indeed, the present study conducted in ATC lines and in mice subjected to hormonal manipulations allowed us to record a 2- to 3-fold increase in SF-1 mRNA or protein levels over basal levels. Importantly, the results reported by Lehmann *et al.* (77) in Y-1 cells were in the same range. Thus, by comparison with steroidogenic genes, the moderate ACTH sensitiveness of SF-1 was probably unnoticed in previous *in vivo* experiments performed by Nomura *et al.* (76). Anyway, converging data obtained either *in vivo* in mice (present results), in bovine primary cultures (36) or in highly differentiated murine cell lines (present results) argues undoubtedly for a hormonal modulation of SF-1 and DAX-1 cellular contents in the adrenal cortex. This could have important physiological consequences considering the critical nature of both *sf-1*and *dax-1* gene dosage in development and adaptative response-like stress (78). In ATC lines, ACTH treatment gave rise to a transient induction of mRNA levels of most steroidogenic genes (culminating at 2–6 h and returning to initial values after 24 h) and notably of *star* and *akr1b7*, both of which are targets for SF-1 and DAX-1. This temporal pattern mirrors that of DAX-1 and suggests that the ratio of SF-1 and DAX-1 intracellular levels determines whether their target genes are activated or repressed. When SF-1/DAX-1 ratio in ZF cells is low (0–1 h or 12–24 h after ACTH induction), SF-1-DAX-1 complexes are supposed to outnumber SF-1 molecules. Steroidogenic genes are then repressed and corticosterone production remains basal or decreases. Conversely, when SF-1/DAX-1 ratio is high (3–9 h after ACTH induction), SF-1 molecules are supposed to outnumber SF-1-DAX-1 complexes. Steroidogenic genes are activated and corticosterone production increases. However, this mechanism is unlikely to be the only one ensuring that steroidogenic activity is responsive to ACTH. Indeed, although Y-1 cells are devoid of DAX-1, they are still responsive to hormonal or cAMP induction. In fact, other transcriptional factors such as cAMP response element binding protein (CREB), CCAAT/enhancer binding protein (C/EBPβ), or Sp1 are known to participate to the cAMP/ACTH regulation of SF-1 target genes in adrenocortical cells (13). The control of SF-1 and DAX-1 levels might therefore be part of the molecular arsenal of ZF cells to fine tune ACTH responsiveness. Consistent with a direct role of these two regulators in hormonal responsiveness is the recent demonstration of a cAMP-dependent dissociation of DAX-1-SF-1 nuclear complexes that promotes SF-1 interactions with coactivators and subsequent activation of SF-1 target genes (79). Our data show that, at least in adrenocortical cells, DAX-1 does not only carry out a tonic inhibition of SF-1 (25), but rather that both *sf-1* and *dax-1* gene dosage is a dynamic process under the control of ACTH. Thus, one can speculate that returning to high DAX-1 protein levels after prolonged ACTH stimulation may be part of an intracellular feedback, limiting steroidogenic response over time. Interestingly, Y-1 cells that lack DAX-1 showed no transient hormonal stimulation of steroidogenic genes but exhibited an uninterrupted accumulation of *akr1b7* mRNA levels up to 24 h (19). Considering that mice lacking DAX-1 showed an increased adrenal responsiveness to ACTH (32), whereas haploinsufficient SF-$1^{+/-}$ mice were less responsive and displayed a lower response to stress (80), one can assume that the ability of ZF cells to induce a rebound of DAX-1 levels may provide a mechanism limiting the adrenal response to stress.

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