Cyclic AMP-Dependent Protein Kinase Controls Basal Gene Activity and Steroidogenesis in Y1 Adrenal Tumor Cells†

Christopher H. Clegg,‡,§ Mitchell S. Abrahamsen,∥,⊥ Jay L. Degen,▽ David R. Morris,∥ and G. Stanley McKnight*,‡

Departments of Pharmacology, SJ-30, and Biochemistry, SJ-70, University of Washington, Seattle, Washington 98195, and Children's Hospital Research Foundation and Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45229

Received October 10, 1991; Revised Manuscript Received January 28, 1992

ABSTRACT: Transfection of mouse Y1 adrenal tumor cells with DNA encoding mutant type I regulatory subunit generated stable transformants in which the basal activity of cAMP-dependent protein kinase was repressed. As expected, steroidogenesis in these kinase-deficient cells was no longer stimulated by corticotropin or cAMP analogues, and the expression of three cAMP-regulated genes (ornithine decarboxylase, urokinase-type plasminogen activator, and P450 side-chain cleavage) could no longer be induced. However, in addition to the loss of hormone responsiveness, the basal level of steroidogenesis and the constitutive expression of these cAMP-inducible genes was also repressed in kinase-defective mutant clones. To verify that functional cA-PK would revert this repressed phenotype, we transfected a cA-PK defective subclone of Y1 cells, Kin 8, with DNA encoding the Cα and Cβ subunits of cAMP-dependent protein kinase. Basal levels of steroid production were restored to normal in stable transformants, and the elevation of kinase activity following induction of the C-subunit expression vectors elicited a steroidogenic response. Gene transcription was also shown to be regulated by either Cα or Cβ as measured by the induction of plasminogen activator and ornithine decarboxylase mRNA levels and transcription rates. The dominant role played by cAMP-dependent protein kinase in these adrenal cells was demonstrated by experiments showing the regulation of ornithine decarboxylase gene expression by protein kinase C requires basal cAMP-dependent protein kinase activity.

Maintenance of the differentiated phenotype displayed by many tissues requires the presence of specific hormones. The adrenal cortex provides an example in which the pituitary peptide hormone, corticotropin (ACTH),[1] regulates steroidogenesis, gene expression, and cell division. Remarkably, it appears that this spectrum of cellular activities controlled by ACTH is dependent on a single signal transduction pathway involving cAMP. This second messenger, which accumulates in cells following ACTH receptor occupancy, associates with cAMP-dependent protein kinase (cA-PK), a tetrameric enzyme containing two regulatory (R) and two catalytic (C) subunits. The binding of four cAMP molecules to the R-subunit dimer leads to holoenzyme dissociation and C-subunit release, causing the phosphorylation of numerous protein substrates.

The prominence of cA-PK in signal transduction is evident by the number of hormones and neurotransmitters that elevate cAMP and the phenotypes that cA-PK controls. Among the rapid hormonal responses mediated by this enzyme are changes in ion channel activity (Rogers et al., 1990), cytoskeleton conformation (Lamb et al., 1988), intermediary metabolism (Krebs & Beavo, 1979), and peptide secretion (Scheckterson & McKnight, 1991). Long-term changes in tissue differentiation depend on the regulation of gene expression by cA-PK (Mellon et al., 1989; Gonzalez & Montminy, 1989). It is now recognized that transcription involves protein recognition of specific DNA binding sites in gene promoters and enhancers and that protein phosphorylation modifies the participation of these transcription factors in the initiation complex. One class of DNA binding proteins identified as substrates for cA-PK belong to the CREB/ATF family (Hai et al., 1989; Montminy et al., 1990), but additional DNA consensus sequences that mediate cAMP action suggest that other transcription factor substrates may exist (Imagawa et al., 1987; Roesler et al., 1988).

The requirement for the cAMP signaling pathway in regulating ACTH activity is based on experiments in which adrenal functions were induced with agents that bypassed the ACTH receptor and raised intracellular cAMP levels directly (Kramer et al., 1984; Schimmer, 1980). In addition, mutations that prevent cA-PK activation in cells have been shown to correlate with the loss of ACTH responsiveness and adrenal cell function (Schimmer, 1980). Questions still remain, however, as to whether cAMP is the sole signal pathway involved in ACTH activity. These are based in part on the kinetics of ACTH versus cAMP analogue induction of steroidogenic enzymes in primary adrenal cells and their relative strengths of induction (Hanukoglu, 1990). It has been suggested that ACTH binding might also lead to the production

† This work was supported by National Institutes of Health Research Grants GM 32875 to G.S.M., CA44611 to J.L.D., and CA39053 and DEO8229 to D.R.M. M.S.A. was supported by a training grant from the same agency (GM07270).

‡ Department of Pharmacology, University of Washington.

§ Present address: Bristol-Myers Squibb Pharmaceutical Research Institute-Seattle, 3005 1st Ave., Seattle, WA 98122.

∥ Department of Biochemistry, University of Washington.

⊥ Present address: Veterinary Molecular Biology Laboratory, Montana State University, Bozeman, MT 59717.

▽ University of Cincinnati College of Medicine.

[1] Abbreviations: ACTH, corticotropin; cA-PK, cAMP-dependent protein kinase; R, regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; PKC, protein kinase C; ODC, ornithine decarboxylase; uPA, urokinase type plasminogen activator; SCC, P450 side-chain cleavage; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

0006-2960/92/0431-3720$03.00/0

of diacylglycerol and protein kinase C (PKC) activation (Kimura & Armelin, 1990).

As a general approach for studying the role of cA-PK in complex biological processes, we have developed DNA transfection methods that control enzyme activity directly in cells. Elimination of cAMP-induced phosphorylation, for instance, can be achieved by the expression of mutant RIα subunit cDNAs (Clegg et al., 1987). Similar to normal RIα subunits, these variant proteins associate with the C subunit to form inactive holoenzyme. As a result of mutations preventing cAMP binding, however, these holoenzymes fail to release C subunit in response to cAMP. As an alternative approach, constitutive phosphorylation can be achieved by overexpression of the C subunit beyond the level of endogenous R subunit. Free C subunit is thus generated in an active form without elevation of cAMP (Clegg et al., 1989; Uhler & McKnight, 1987).

The following report describes the stable transfection of Y1 adrenocortical tumor cells with these expression vectors. Our results indicate that cA-PK mediates hormone and cAMP induction of gene expression and steroidogenesis and that either of two isoforms of the mouse C subunit, Cα and Cβ, can regulate these processes. We show that the constitutive expression of these functions, ongoing when cells are cultured in the absence of hormone, are regulated by the kinase even when 10% or less of the enzyme is activated. Finally, the dominant role of this enzyme in maintaining the differentiated state is illustrated by experiments that show that the regulation of gene expression by an alternate signaling pathway, involving PKC, requires basal cA-PK activity.

Experimental Procedures

Plasmid Construction. MT-REV(AB)^{neo} is a 7.6-kb expression vector that contains a mutant cDNA of the mouse RIα subunit inserted between the mouse metallothionein-1 promoter and the polyadenylation signal sequence of the human growth hormone gene. MT-REV(AB)^{neo} differs from the original MT-REV (Clegg et al., 1987) in that the RIα cDNA contains two mutations in the site B cAMP-binding domain (Asp 324 and His 332) in addition to the site A mutation (Glu 200). This was accomplished by substituting a 284-bp *Eco*RI/*Apa*I fragment with a derivative containing two amino acid substitutions in the site B region. We have also introduced into this plasmid the transcription unit for the neomycin resistance gene isolated from pKOneo (van Doren et al., 1984). This 2.2-kb *Eco*RI/*Bam*HI fragment begins with the *lacUV5* bacterial promoter, followed by the SV40 early promoter and the Tn5 neomycin resistance gene, and ends with the SV40 small t intron and polyadenylation signal sequence. The ends of this fragment were filled in with Klenow enzyme and ligated into the *Bam*HI site of the multiple cloning site of MT-REV that lies 5′ of the MT-1 promoter. Plasmids were chosen in which transcription from the SV40 and MT-1 promoters initiated in the opposite direction. Construction of CEVα and CEVβ have previously been described (Uhler & McKnight, 1987). The neomycin resistance gene from pKO^{neo} was inserted into these plasmids in a manner similar to MT-REV-(AB)^{neo} (see above).

Cell Cultures and Transfections. Mouse Y1 and Kin 8 adrenocortical cells (Doherty et al., 1982) were maintained in Ham's F10 plus 15% horse serum and 2.5% fetal calf serum. DNA transfections were performed by calcium phosphate precipitation as previously described (Clegg et al., 1987). Stable transformants were selected in 500 μg/mL Geneticin (Gibco) and isolated using cloning cylinders. Clones expressing either mutant RIα subunit, Cα subunit, or Cβ subunit were identified by measuring mRNA levels for these genes and by testing cell extracts for repressed or elevated cA-PK activity.

cA-PK Activity. Cells were washed twice with PBS and harvested in homogenization buffer as previously described (Clegg et al., 1987). Cell extracts were assayed for cA-PK by measuring the phosphorylation of the peptide substrate Kemptide in the presence and absence of 5 μM cAMP (Clegg et al., 1987). Units of activity are expressed as picomoles of P_i transferred per minute per milligram of protein. Data points are the average of triplicate measurements.

Steroidogenesis. Steroid levels were measured in culture medium as previously described (Clegg et al., 1987). One hundred microliters of medium was extracted with 0.7 mL of methylene chloride. The organic phase was extracted with 0.7 mL of 65% sulfuric acid in ethanol. Five hundred microliters of the acid phase was then transferred to a glass tube containing 1.5 mL of 65% sulfuric acid in ethanol. After 1.5 h, steroids were quantitated using a fluorometer with an excitation wavelength of 470 nm and an emission wavelength of 536 nm. Total steroids measured per culture were normalized to cell number by measuring protein with the Bradford assay. Each data point is the average of two experiments using duplicate cultures.

Northern Blot Analysis and mRNA Quantitation. Total RNA was extracted from cell cultures (Uhler & McKnight, 1987) and 30 μg of RNA/lane was electrophoresed under denaturing conditions through 1% agarose gels and blotted onto nitrocellulose. The same filter was successively probed using ^{32}P-labeled cDNA fragments and standard conditions of hybridization for ornithine decarboxylase (ODC) mRNA [386-bp *Sal*I/*Pst*I fragment (Gupta & Coffino, 1985)], urokinase-type plasminogen activator (uPA) mRNA [398-bp *Pst*I/*Sma*I fragment (Degen et al., 1987)], and P450 side-chain cleavage (SCC) mRNA [1700-bp *Pvu*II fragment (Fujii-Kuriyama et al., 1984)]. The sizes for the respective mRNAs were verified by comparison with denatured ^{32}P-labeled DNA markers. Application of equivalent amounts of RNA to the gel was verified by ethidium bromide staining. As an added control, the level of Cα mRNA was also measured in each sample and no significant changes were observed in the expression of this constitutively active gene.

The levels of ODC and uPA mRNA were also quantitated by solution hybridization (Uhler et al., 1986a). Antisense RNA probes synthesized in the presence of [^{32}P]UTP (ICN) were prepared from a fragment of the ODC cDNA cloned into pGEM (Promega) using SP6 or T3 polymerase. A single-stranded cDNA probe for measurement of uPA gene expression was prepared as described previously (Degen et al., 1985) using a 398-bp *Pst*I/*Sma*I fragment of the murine uPA cDNA (Degen et al., 1987). The respective mRNA molecules were quantitated using a standard curve constructed with known amounts of either single-stranded M13 DNA containing the sense strand of the appropriate cDNA or with sense strand RNA transcripts synthesized with T7 or SP6 polymerase. Picogram levels of mRNA hybridized per microgram of total RNA was converted to molecules per cell by assuming that RNA in Kin 8 cells represents 66% of the total nucleic acid and that these cell lines contain 10 pg of DNA/cell.

Nuclear Run-On Analysis. Kin 8 cells transfected with pKO^{neo}, CEVα, and CEVβ were incubated in the absence or presence of 100 μM $ZnCl_2$ for 5 h. Nuclei were isolated, nascent RNA transcripts were labeled with [^{32}P]UTP, and nuclear RNA was extracted using 4 M guanidine thiocyanate (Abrahamsen & Morris, 1990). Equal counts from each RNA preparation (1×10^7 cpm/mL) were hybridized to nitro-

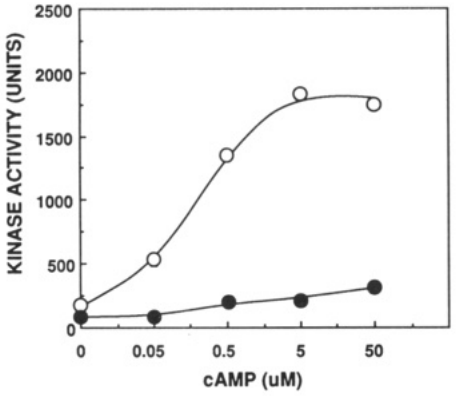

FIGURE 1: Inhibition of cA-PK in Y1 cells expressing mutant RIα subunit protein. A control Y1 subclone (○), transfected with pKOneo, and RAB2 cells (●), a subclone of Y1 cells transfected with MT-REV(AB)^{neo}, were incubated with 80 μM Zn^{2+} for 16 h to induce overexpression of mutant RIα protein. Cell extracts were then prepared, diluted to a concentration of 1 mg/mL protein with homogenization buffer, and assayed for kinase activity at various concentrations of cAMP by monitoring ^{32}P incorporation of Kemptide substrate.

cellulose filters containing 5 μg of single-stranded M13 DNA with a specific ODC cDNA fragment (386-bp *Sal*I/*Pst*I) in either orientation so as to detect sense (+) and antisense (−) transcription. Transcription of the β-actin gene was measured in a similar manner using a bovine β-actin cDNA (Degen et al., 1983).

RESULTS

Inhibition of cA-PK Activity in Y1 Cells Using Dominant Mutations of the RIα Subunit. On the basis of in vitro reconstitution of holoenzymes using purified subunits, Woodford et al. (1989) have demonstrated that the site A (Glu 200) and site B (Asp 324, His 332) mutations shift the apparent K_a for holoenzyme activation 4- and 100-fold, respectively. In order to maximize the repression capacity of the mutant RI, a new expression vector was constructed in which both site A and site B mutations were combined within the same RIα cDNA. This expression vector, MT-REV(AB)^{neo}, is regulated by the mouse metallothionein-1 (MT-1) promoter and contains the neomycin resistance gene. Y1 adrenocortical cells were transfected and stable transformants were isolated as previously described (Clegg et al., 1987).

The basal and cAMP inducible cA-PK activity in extracts from one expressing clone, RAB2, is compared to control Y1 cell extracts in Figure 1. In the absence of added cAMP, the basal phosphorylation of the synthetic substrate Kemptide was reduced by 50% in the RAB2 extract compared to control. It is not known whether all of this residual kinase activity is cA-PK because of the presence of other kinases that can phosphorylate Kemptide. The addition of up to 50 μM cAMP caused less than a 10% activation of cA-PK in mutant RAB2 extracts compared to full activation in the control.

Basal Kinase Activity Maintains Steroidogenesis and Gene Expression in Adrenal Cells. Many of the biological responses controlled by cA-PK are stimulated maximally when only a fraction of the kinase is activated (Honnor et al., 1985; Litvin, et al., 1984). This suggests that basal levels of kinase activity could be important for maintaining differentiated functions in the absence of cAMP elevation. On the basis of the results shown in Figure 1, less than 10% of the C subunit is active in normal untreated Y1 cells. To test whether the inhibition of basal cA-PK observed in RAB2 cells would have an effect, we examined two parameters of adrenal cell function, steroidogenesis and gene expression.

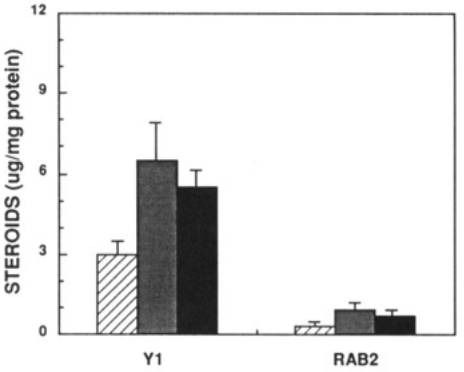

FIGURE 2: Inhibition of steroidogenesis in RAB2. Cultures of Y1 and RAB2 cells were pretreated for 16 h with 80 μM Zn^{2+}, rinsed, and incubated for an additional 5 h in fresh medium alone (no treatment, hatched bars) or fresh medium containing either 8-bromo-cAMP (1 mM, stippled bars) or ACTH (20 nM, solid bars). The culture medium and cells were collected separately, assayed for steroids, and then normalized to cellular protein. Each data point is the average of two experiments using duplicate cultures.

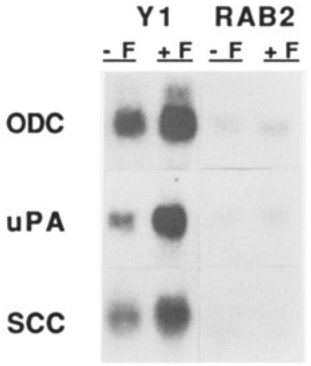

FIGURE 3: Inhibition of cA-PK causes the downregulation of basal as well as cAMP-induced gene expression. Y1 and RAB2 cells were pretreated with 80 μM Zn^{2+} for 16 h, rinsed, and incubated for an additional 5 h in the absence or presence of forskolin (10 μM). RNA was isolated and then assayed by Northern analysis for ODC, uPA, and SCC mRNA.

Cultures of RAB2 cells and a Y1 subclone transfected with a control plasmid, pKOneo (van Doren et al., 1984), were rinsed and incubated for 5 h in fresh medium or in fresh medium supplemented with either 1 mM 8-bromo-cAMP or 10^{-8} M ACTH. Cells and culture media were then collected and the concentration of secreted steroids was measured. As indicated in Figure 2, both the basal and cAMP-inducible levels of steroid production were reduced significantly in RAB2. Responsiveness to ACTH was also lost. These results confirm that cA-PK mediates ACTH and cAMP induction of steroid synthesis and that the basal rate of cA-PK phosphorylation controls steroid production in the absence of a hormonal signal.

The transcription of several genes in Y1 adrenal cells is induced by ACTH or agents such as forskolin that directly elevate intracellular cAMP. The genes affected include ODC and uPA as well as genes important for steroidogenesis such as SCC and 11β-hydroxylase (Wong et al., 1989). A Northern analysis of mRNA levels for three of these genes is shown in Figure 3. Not suprisingly, gene induction by forskolin is repressed in kinase-deficient RAB2 cells. This agrees with a number of studies showing that cAMP regulation of gene expression is mediated by cA-PK (Clegg et al., 1989; Gonzalez & Montiminy, 1989; Grove et al., 1989; Maurer, 1989; Mellon et al., 1989). However, the significant reduction in mRNA levels in untreated RAB2 shows that cA-PK is also required for the basal level of endogenous gene expression. This

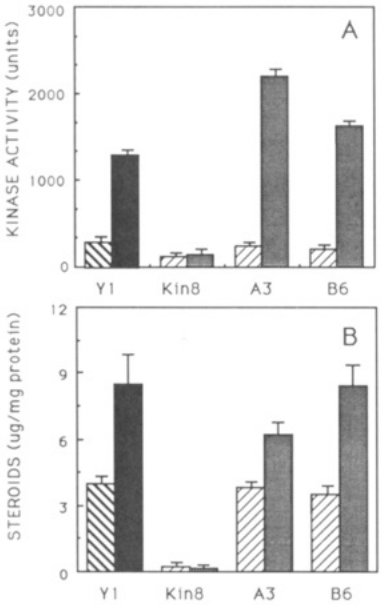

FIGURE 4: Regulation of steroidogenesis by the Cα and Cβ subunits of cA-PK. (A) Clones were incubated for 12 h in the absence (lightly hatched bars) and presence (stippled bars) of 80 μM Zn^{2+} to stimulate transcription of the respective catalytic subunit cDNAs. Cell extracts were then prepared and assayed for cA-PK. Kin 8 cells (transfected with pKOneo) and clones expressing either Cα (A3) or Cβ (B6) were assayed in the absence of exogenous cAMP to determine the amount of free C subunit. Y1 cell extracts were assayed in the absence (heavily hatched bar) and presence (solid bar) of 5 μM cAMP to determine the amount of cAMP-independent and -dependent protein kinase activity present in normal cells. (B) Following the 12-h incubation period, cultures were assayed for steroid synthesis. To compare the restoration of steroid production in clones A3 and B6 to normal Y1 cells, we incubated Y1 cells in the absence and presence of 500 μM 8-chlorophenylthio-cAMP for 12 h. Each data point is the average of two experiments using duplicate cultures.

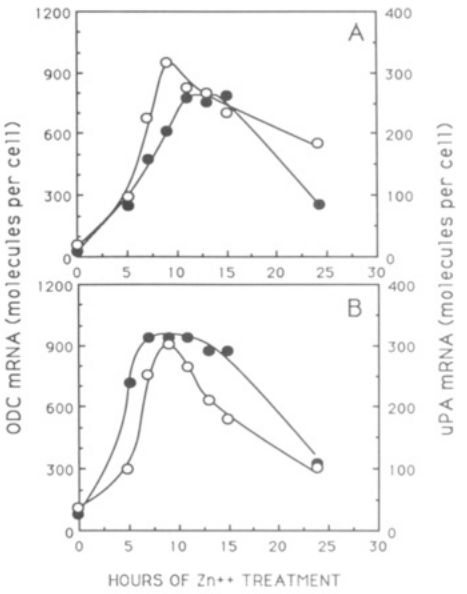

FIGURE 5: Regulation of gene expression by Cα and Cβ subunits of cA-PK. The A3 subclone of Kin 8 cells transfected with Cα (A) and the B6 subclone transfected with Cβ (B) were fed medium containing 80 μM Zn^{2+} and then assayed at the indicated times for the accumulation of ODC mRNA (○) and uPA mRNA (●) by solution hybridization. The data were expressed as mRNA (molecules per cell) by comparing the hybridization of samples to known amounts of complementary RNA or DNA. Each data point was assayed in triplicate.

downregulation is restricted to cAMP-inducible genes and is not a general effect on transcription because the mRNA level of genes unresponsive to cAMP such as Cα subunit and β-actin (not shown) remained normal in kinase-deficient cells.

Expression of Two Isoforms of the Catalytic Subunit Can Regulate Steroidogenesis and Gene Transcription in Y1 Cells. Two catalytic subunit genes (Cα and Cβ) have been identified in mouse and they encode proteins that are 91% identical (Uhler et al., 1986b). Y1 adrenal cells express Cα and Cβ in a 5:1 ratio, but it is not known whether they each play a distinct role in cellular regulation. To test whether one or both of these isoforms can control steroid biosynthesis and gene expression, we transfected Cα and Cβ expression vectors regulated by the zinc-inducible MT-1 promoter into a variant subclone of Y1 cells, Kin 8. These cells are a useful recipient for C-subunit transfection because, similar to RAB2 cells, their cA-PK activity is functionally inhibited as a result of a dominant mutation in one of the endogenous RIα subunit genes (Doherty et al., 1982). Overexpression of C subunit can restore cA-PK activity once the level of C exceeds the ability of the cell to produce the mutant RIα subunit (Clegg et al., 1989; Wong & Schimmer, 1989).

Figure 4 shows a comparison of cA-PK activity and steroidogenesis in normal Y1 (wt), Kin 8, and two Kin 8 subclones expressing the Cα (clone A3) and Cβ (clone B6) isoforms of the catalytic subunit. In this experiment the Kin 8, A3, and B6 clones were treated with 90 μM Zn^{2+} for 12 h to increase transcription from the MT-1 promoter. Control Y1 cells were incubated in the presence of 1 mM 8-bromo-cAMP for the same period to elicit kinase activation and steroid production. As indicated, the constitutive activity of the MT-1 promoter in clones A3 and B6 was sufficient to effect an approximate 2.5-fold increase in basal kinase activity. Corresponding to this increase, steroid production was restored to the basal Y1 level. Zinc treatment specifically raised kinase activity in clones A3 and B6, and steroid production increased to a level comparable to that in cAMP-treated Y1 cells. The effects of elevated kinase have been examined in five Cα- and six Cβ-expressing clones and in all cases steroid production was restored. No significant difference in steroidogenesis was observed between Cα- and Cβ-expressing clones.

Cα and Cβ regulation of gene expression is shown in Figure 5. In this experiment cultures of A3 and B6 cells were exposed to Zn^{2+} and harvested at various intervals. ODC and uPA mRNA was then quantitated by solution hybridization using radiolabeled nucleic acid probes. At $t = 0$, the levels of both ODC and uPA mRNA were 2–3-fold higher in A3 and B6 than in control Kin 8 cells. This demonstrates that the basal kinase in these cells (see above) had a positive effect on gene expression. During the next 10 h of Zn^{2+} treatment, ODC and uPA mRNA accumulated dramatically and reached comparable levels in both Cα- and Cβ-expressing cells. In control Kin 8 cells, however, the mRNA levels remained very low and were unaffected by Zn^{2+} treatment (Clegg et al., 1989).

To demonstrate that these changes in mRNA levels resulted from increased rates of transcription, a nuclear run-on assay was performed (Figure 6). Cultures were incubated in Zn^{2+} for 5 h to induce C-subunit gene expression. Nuclei were prepared and incubated with [^{32}P]UTP, and the radioactive RNA was then hybridized with immobilized DNAs. As indicated, A3 and B6 cells were stimulated upon Zn^{2+} treatment to synthesize RNA complementary to the sense strand of the ODC gene. Incubating control Kin 8 cells with Zn^{2+} had no effect. Transcription of the actin gene, which is not regulated by cAMP, was also examined and found to be unaffected by Zn^{2+} or C-subunit expression. These results demonstrate that both isoforms of the C subunit can stimulate transcription of

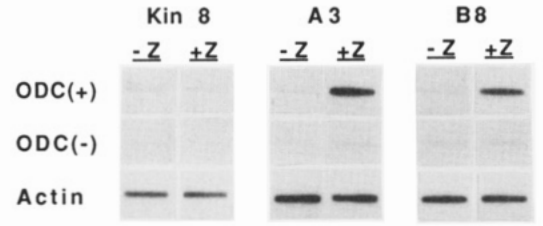

FIGURE 6: ODC gene transcription is regulated by Cα and Cβ subunits of cA-PK. Kin 8, A3, and B6 cells were incubated in the absence or presence of 80 μM Zn^{2+} for 5 h. Nuclei were then prepared and used to measure transcription levels of the ODC and β-actin genes as described (Experimental Procedures).

the ODC gene. They also indicate that the loss of basal mRNA levels shown in Figure 3 in kinase-deficient clones is likely due to decreased transcription.

Protein Kinase C Mediated Induction of ODC Gene Expression Is Dependent on Basal cA-PK Activity. The ODC gene is regulated transcriptionally by both the cAMP/cA-PK and phorbol ester/PKC pathways, and a strong synergistic induction is observed when both kinases are activated (Abrahamsen & Morris, 1990). Our demonstration that the uninduced level of ODC mRNA was dependent on the basal activity of cA-PK (Figure 3) suggested that ODC gene induction by PKC might depend on a synergistic interaction between PKC and basal cA-PK activity.

In order to test this possibility, the induction of ODC mRNA by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was measured in Y1 subclones possessing normal or inhibited cA-PK. Parallel experiments were done in proliferating Y1 cells and cells exposed to low serum (0.5%) for 48 h, since the TPA response has been shown to be somewhat greater in serum-deprived, growth-arrested cells. The control Y1 cells showed a rapid induction of ODC mRNA in both growth-arrested (Figure 7A) and proliferating cells (Figure 7B), and by 6 h, ODC mRNA had increased 8.5- and 5-fold, respectively. Cells expressing the RIα mutant (RAB2) had depressed levels of ODC mRNA prior to TPA addition, as expected, and the TPA response was nearly eliminated. Kin 8 cells were also unable to support a normal TPA induction, but subclones of Kin 8 transfected with a Cα expression vector (A3) regained their ability to respond to TPA.

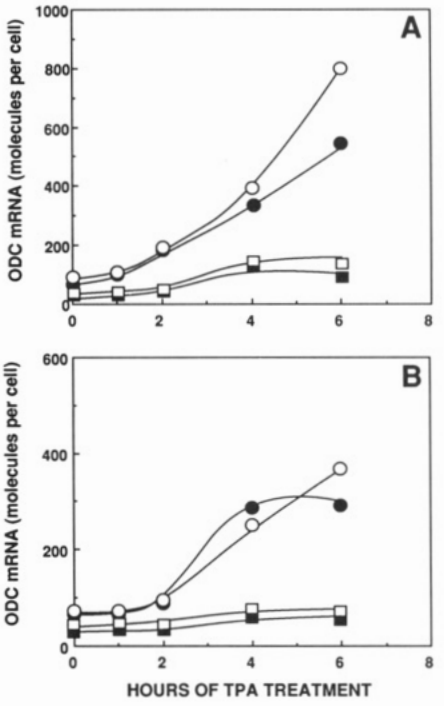

FIGURE 7: cA-PK is required for TPA induction of ODC gene expression. Quiescent (A) and proliferating (B) cultures of cA-PK-deficient cells [RAB2 (■) and Kin 8 (□) and cA-PK-containing cells [Y1 (○) and A3 (●)] were exposed to the phorbol ester TPA (100 ng/mL). At the indicated times, total RNA was prepared from the various cell types and assayed for ODC mRNA by dot-blot analysis. The relative levels of ODC mRNA were determined by densitometric scanning of autoradiograms. These values were then converted to mRNA molecules per cell using predetermined amounts of mRNA from each cell type.

Discussion

The signaling pathways that control the differentiated state of an adrenal cortical cell were dissected using expression vectors that manipulate cA-PK activity. Our results emphasize the importance of cA-PK in regulating responses to ACTH including steroid synthesis and gene induction. A major question addressed in these studies concerns the role of basal cA-PK activity in maintaining the functional integrity of the adrenal cell and the activity of endogenous genes known to be inducible by ACTH through the cAMP pathway. By developing a dominant cA-PK inhibitor construct, MT-REV-$(AB)^{neo}$, which was capable of decreasing the basal activity of cA-PK in stable transformants, we have been able to establish the role of this basal activity in steroid biosynthesis, gene expression, and the responses of adrenal cells to activation of the PKC second messenger pathway.

Repression of cA-PK phosphorylation in cells, caused by the expression of the mutant regulatory subunit encoded in MT-$REV(AB)^{neo}$, eliminated both the basal and ACTH-induced levels of steroid biosynthesis. The manner in which cA-PK mediates steroidogenesis is not fully understood but appears to involve multiple control points. Acute responses to ACTH involve an increase of free cytosolic cholesterol, cholesterol transport to mitochondria, and the conversion of cholesterol to pregnenolone by the SCC enzyme. cA-PK has been implicated in regulating all three steps by altering, respectively, the activities of cholesterol ester hydrolase (Beckett & Boyd, 1977), cytoskeleton conformation (Lamb et al., 1988), and a labile phosphoprotein colocalized with SCC (Besman et al., 1989).

The levels of mRNA for a number of steroidogenic genes are controlled by cAMP (Wong et al., 1989). In this report we have confirmed that the cAMP regulation of gene expression in Y1 cells is mediated by cA-PK. Repression of kinase with mutant RIα subunit significantly reduced ODC, uPA, and SCC mRNA levels in cells and prevented cAMP induction of these genes. The importance of C-subunit activity was further demonstrated when overexpression of either isoform of C subunit in a kinase-deficient subclone of Y1 cells (Kin 8) produced a 15- and 28-fold increase in uPA and ODC mRNA levels. Nuclear run-on assays confirmed that the regulation of the ODC gene by Cα and Cβ was transcriptional. These results are consistent with transient transfection assays that have utilized various promoters with cAMP response elements (CREs). Expression vectors for mutant regulatory subunit or the protein kinase inhibitor peptide prevented cAMP induction of gene expression, whereas Cα and Cβ subunit expression stimulated transcription (Day et al., 1989; Grove et al., 1989; Maurer, 1989; Mellon et al., 1989).

Our results confirm the model that adrenal functions are induced by the increase in cAMP and cA-PK activity that follows ACTH binding. Most importantly, these results establish that basal cA-PK activity, estimated to amount to 10% or less of total cAMP-inducible kinase activity, maintained significant levels of steroid production and gene expression in

the absence of hormone. A similar situation, in which the kinase regulates gene expression at basal cAMP levels, has been reported following the transient transfection of a number of fusion gene constructs (Day et al., 1989; Grove et al., 1989). The physiological relevance of kinase activation in response to basal cAMP levels in adrenal tissue becomes apparent when one considers that ACTH is secreted in circadian pulses lasting only a few hours (Avgerinos et al., 1986). Thus, basal kinase activity preserves steroid synthetic capacity during periods of low ACTH concentration, whereas hormone-induced phosphorylation drives steroid production when ACTH levels are high. A critical aspect of this regulation is the mechanism controlling basal phosphorylation, below which cells would stop functioning. This must involve ambient cAMP concentrations as determined by the activities of adenylate cyclase and phosphodiesterase, both of which can be influenced by other hormones and signaling pathways in addition to ACTH.

Adrenal cells can also respond to second messengers other than cAMP, and our ability to produce a substantial block in the cA-PK pathway allowed us to test whether responses to PKC stimulation would occur independent of cA-PK activity. In Swiss 3T3 cells and T lymphocytes, the ODC gene can be induced by either cAMP or phorbol esters, and in combination the two inducers give a synergistic response (Abrahamsen & Morris, 1990). The decrease in ODC expression when basal cA-PK activity is inhibited suggests that the observed induction of ODC when cells are treated with phorbol esters might involve a synergistic interaction between transcription factors activated by basal cA-PK activity and induced PKC. The demonstration (Figure 7) that ODC induction in response to phorbol esters is nearly eliminated in cells expressing the dominant mutant R subunit supports this hypothesis.

Definition of the exact mechanism of interaction between the cA-PK and PKC pathways awaits identification of the regulated transcription factors interacting with the ODC promoter. The region of the ODC promoter contains many potential binding sites for known factors that respond to these signals (Abrahamsen & Morris, 1991), but protein binding and mutagenesis studies to define their functional significance have not yet been reported. There are potential binding sites upstream of the ODC gene (Abrahamsen & Morris, 1991) for the members of the CREB/ATF family of factors (Hai et al., 1989; Montminy et al., 1990; Roesler et al., 1988). These sites are of interest not only because these factors are cAMP-regulated but also because in many contexts these sites contribute strongly to basal-level transcription activity (Comb et al., 1988; Deutsch et al., 1988; Metcalf et al., 1990; Rice et al., 1989; Runkel et al., 1991). In addition to the CREB/ATF sites, there are also DNA sequences that could interact with the phorbol ester-regulated factor AP-1 and there are other potential binding sites for factors AP-2 and NF-kB, which have been reported to respond to both cAMP and TPA in the appropriate contexts (Abrahamsen & Morris, 1991). One could envision a model where one or more of the other sites serves to augment transcription in response to phorbol esters. This would be reminiscent of the complex promoters associated with the proenkephalin and c-*fos* genes (Comb et al., 1988; Runkel et al., 1991). More complicated possibilities are tenable on the basis of the recent observations that some members of the CREB/ATF family can form heterodimers with members of the phorbol ester-regulated *jun* family of transcription factors (Benbrook & Jones, 1990; MacGregor et al., 1990).

The conclusions of this study emphasize the role of cA-PK in both basal and hormone-induced activities of the adrenal cell. It is likely that these observations are equally relevant to other cell types where cA-PK has been shown to modulate gene expression (Mellon et al., 1989), ion channel activation (Rogers et al., 1990), receptor downregulation (Scherer & Nathanson, 1989), and peptide secretion (Scheckterson & McKnight, 1991). The importance of the basal activity of the cA-PK in setting the threshold level in each of these responses is open to scrutiny using the dominant inhibitor described in this study.

Registry No. ACTH, 9002-60-2; cAMP, 60-92-4; ODC, 9024-60-6; PA, 105913-11-9; protein kinase, 9026-43-1; cytochrome P450, 9035-51-2.

References

Abrahamsen, M. S., & Morris, D. R. (1990) *Mol. Cel. Biol. 10*, 5525–5528.

Abrahamsen, M. S., & Morris, D. R. (1991) in *Perspectives on Cell Regulation: From Bacteria to Cancer* (Inouye, M., Campisi, J., Cunningham, D. D., & Riley, M., eds.), Wiley-Liss Inc., New York, pp 107–119.

Avgerinos, P. C., Schurmeyer, T. H., Gold, P. W., Tomai, T. P., Loriaux, D. L., Sherins, R. J., Cutler, G. B., & Chrousos, G. P. (1986) *J. Clin. Endocrinol. Metab. 62*, 816–821.

Beckett, G. J., & Boyd, G. S. (1977) *Eur. J. Biochem. 72*, 223–233.

Benbrook, D. M., & Jones, N. C. (1990) *Oncogene 5*, 295–302.

Besman, M. J., Yanagibashi, K., Lee, T. D., Kawamura, M., Hall, P. F., & Shively, J. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4897–4901.

Clegg, C. H., Correll, L. A., Cadd, G. G., & McKnight, G. S. (1987) *J. Biol. Chem. 262*, 13111–13119.

Clegg, C. H., Ran, W., Uhler, M. D., & McKnight, G. S. (1989) *J. Biol. Chem. 264*, 20140–20146.

Comb, M., Mermod, N., Hyman, S. E., Pearlberg, J., Ross, M. E., & Goodman, H. M. (1988) *EMBO J. 7*, 3793-3805.

Day, R. N., Walder, J. A., & Maurer, R. A. (1989) *J. Biol. Chem. 264*, 431–436.

Degen, J. L., Neubauer, M. G., Degen, S. J. F., Seyfried, C. E., & Morris, D. R. (1983) *J. Biol. Chem. 258*, 12153–12162.

Degen, J. L., Estensen, R. D., Nagamine, Y., & Reich, E. (1985) *J. Biol. Chem. 260*, 12426–12433.

Degen, S. J. F., Heckel, J. L., Reich, E., & Degen, J. L. (1987) *Biochemistry 26*, 8270–8279.

Deutsch, P. J., Hoeffler, J. P., Jameson, J. L., Lin, J. C., & Habener, J. F. (1988) *J. Biol. Chem. 263*, 18466–18472.

Doherty, P. J., Tsao, J., Schimmer, B. P., Mumby, M. C., & Beavo, J. A. (1982) *J. Biol. Chem. 257*, 5877–5883.

Fujii-Kuriyama (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4647–4651.

Gonzalez, G. A., & Montminy, M. R. (1989) *Cell 59*, 675–680.

Grove, J. R., Deutsch, P. J., Price, D. J., Habener, J. F., & Avruch, J. (1989) *J. Biol. Chem. 264*, 19506–19513.

Gupta, M., & Coffino, P. (1985) *J. Biol. Chem. 260*, 2941–2944.

Hai, T., Liu, F., Coukos, W. J., & Green, M. R. (1989) *Genes Dev. 3*, 2083–2090.

Hanukoglu, I., Feuchtwanger, R., & Hanukoglu, A. (1990) *J. Biol. Chem. 265*, 20602–20608.

Honnor, R. C., Dhillon, G. S., & Londos, C. (1985) *J. Biol. Chem. 260*, 15130–15138.

Imagawa, M., Chiu, R., & Karin, M. (1987) *Cell 51*, 251–260.

Kimura, E., & Armelin, H. A. (1990) *J. Biol. Chem. 265*, 3518–3521.

Kramer, R. E., Rainey, W. E., Funkenstein, B., Dee, A., Simpson, E. R., & Waterman, M. R. (1984) *J. Biol. Chem. 259*, 707–713.
Krebs, E. G., & Beavo, J. A. (1979) *Annu. Rev. Biochem. 48*, 923–959.
Lamb, N. J. C., Fernandez, A., Conti, M. A., Adelstein, R., Glass, D. B., Welch, W. J., & Feramisco, J. R. (1988) *J. Cell Biol. 106*, 1955–1971.
Litvin, Y., PasMantier, R., Fleischer, N., & Erlichman, J. (1984) *J. Biol. Chem. 259*, 10296–10302.
Macgregor, P. F., Abate, C., & Curran, T. (1990) *Oncogene 5*, 451–457.
Maurer, R. A. (1989) *J. Biol. Chem. 264*, 6870–6873.
Mellon, P. L., Clegg, C. H., Correll, L. A., & McKnight, G. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4887–4891.
Metcalf, R. L., Ruegg, M., & Schleuning, W. (1990) *J. Biol. Chem. 265*, 14618–14626.
Montminy, M. R., Gonzalez, G. A., & Yamamoto, K. K. (1990) *Trends Neurosci. 13*, 184–188.
Rice, D. A., Aitken, L. D., Vandenbark, G. R., Mouw, A. R., Franklin, A., Schimmer, B. P., & Parker, K. L. (1989) *J. Biol. Chem. 264*, 14011–14015.
Roesler, W. J., Vandenbard, G. R., & Hanson, R. W. (1988) *J. Biol. Chem. 263*, 9063–9066.
Rogers, K. V., Goldman, P. S., Frizzell, R. A., & McKnight, G. S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8975–8979.
Runkel, L., Shaw, P. E., Herrera, R. E., Hipskind, R. A., & Nordeim, A. (1991) *Mol. Cell. Biol. 11*, 1270–1280.
Scherer, N. M., & Nathanson, N. M. (1989) *Biochemistry 29*, 8475–8483.
Schimmer, B. P. (1980) *Adv. Cyclic Nucleotide Res. 13*, 181–214.
Sheckterson, L. A. C., & McKnight, G. S. (1991) *J. Mol. Endocrinol. 5*, 170–178.
Uhler, M. D., & McKnight, G. S. (1987) *J. Biol. Chem. 262*, 15202–15207.
Uhler, M. D., Carmichael, D. F., Lee, D. C., Chrivia, J. C., Krebs, E. G., & McKnight, G. S. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1300–1304.
Uhler, M. D., Chrivia, J. C., & McKnight, G. S. (1986b) *J. Biol. Chem. 261*, 15360–15363.
van Doren, K., Hanahan, D., & Gluzman, Y. J. (1984) *J. Virol. 50*, 606–614.
Wong, M., & Schimmer, B. P. (1989) *Endocr. Res. 15*, 49–55.
Wong, M., Rice, D. A., Parker, K. L., & Schimmer, B. P. (1989) *J. Biol. Chem. 264*, 12867–12871.
Woodford, T., Correll, L., Landiss, L., Corbin, J., & McKnight, G. S. (1989) *J. Biol. Chem. 264*, 13321–13328.