Targeted Ablation of Pituitary Pre-Proopiomelanocortin Cells by Herpes Simplex Virus-1 Thymidine Kinase Differentially Regulates mRNAs Encoding the Adrenocorticotropin Receptor and Aldosterone Synthase in the Mouse Adrenal Gland

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We have produced and characterized lines of transgenic mice expressing a fusion gene composed of the pituitary expression-specific promoter region of the POMC gene, driving the herpes simplex viral-1 thymidine kinase. Adult mice were treated with the antiherpes agent ganciclovir at 70 mg/kg body weight (ip, twice daily for 10–12 days). Approximately 98% of the pituitary intermediate lobe melanotropes and anterior lobe corticotropes were ablated as determined by immunocytochemistry and RIA specific for the POMC-derived peptides, ACTH, β-endorphin, and α-MSH. The number of lactotropes, somatotropes, thyrotropes, and gonadotropes was not altered compared with controls, indicating that in the adult pituitary, POMC products are not required to maintain the distribution of cell types. As expected, plasma corticosterone levels were substantially decreased after POMC cell ablation. *In situ* hybridization studies showed that the mouse ACTH receptor was expressed uniformly throughout the adrenal cortex, and RNase protection assays revealed that the ACTH receptor mRNA decreased after pituitary POMC cell ablation. Additionally, RNase protection assays showed that pituitary POMC cell ablation resulted in the decrease of adrenal p450c11β transcripts while p450c11AS (aldosterone synthase) mRNA levels remained constant. These data demonstrate differential regulation of steroid pathway-specific enzymes by POMC products. Our results also suggest that the thymidine kinase cell obliteration technique may not be dependent on cell division as a prerequisite for cytotoxicity, thus supporting the idea that targeted molecular ablation using cell- and tissue-specific promoter sequences to drive viral thymidine kinase expression can be refined further to study other nonmitotic cells. (Molecular Endocrinology 9: 1005–1016, 1995)

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INTRODUCTION

POMC cells are found in the brain, pituitary, and other tissues (1, 2); however, the source of virtually all peripherally circulating POMC peptides is the pituitary gland. There are three families of peptides derived from POMC: the adrenocorticotropins (ACTHs), the α-, β-, and γ-MSHs, and β-endorphin. These peptides have important roles in the molecular physiology of the adrenal cortices and of pigmentation and analgesia (3–6). Certain lines of evidence indicate that POMC peptides may mediate multiple functions through their receptors (7–9).

In response to stress, biochemical signals (such as CRH) emanating from the brain can stimulate the secretion of POMC peptides from the pituitary gland (10). These peptides can then signal distant target tissues and by signal transduction mediated through their receptors

produce and integrate additional responses (11). For example, it is well known that the major role of ACTH secreted by the anterior lobe (AL) corticotropes is to stimulate glucocorticoid (GCs) secretion from the adrenal cortex. In addition, ACTH has multiple effects on the fetal and adult adrenal gland such as growth, cytodifferentiation, and maintenance of adrenal architecture (12–15). GCs can further coordinate the hypothalamic-pituitary-adrenal (HPA) axis by regulating gene and cognate protein expression mediated through the intracellular GC receptor and (in the brain) the mineralocorticoid receptor acting as transcription factors (16).

To more thoroughly elucidate the complex and multiple roles of peripherally circulating POMC-derived peptides in the regulation of the adrenal cortex, a method is required to either specifically delete POMC peptides or ablate POMC cells. Techniques such as surgical excision of the pituitary gland have a major drawback in that other cell types in addition to POMC cells are removed. Further, it would be advantageous if the system were reversible. To accomplish the specific ablation of pituitary POMC cells we have used the thymidine kinase cell obliteration (TKO) technique (17, 18).

Transgenic mice were produced harboring a fusion gene consisting of nucleotides −706 to +64 of the rat (r) POMC 5′-flanking region that is pituitary tissue- and POMC cell type-specific ligated to the coding region of the herpes simplex viral type 1 thymidine kinase (hsv1-TK) enzyme (Fig. 1) (19). This enzyme can phosphorylate the modified nucleoside ganciclovir [9-[1, 3-dihydroxy-2-propoxy(methyl)guanine] causing cytotoxicity to only the targeted pituitary POMC cells by an unknown mechanism (20). Here we report multiple effects at key points in the HPA axis after pituitary POMC cell ablation. We also demonstrate the utility of this transgenic model for the study of POMC peptide regulation of gene expression in the adrenal gland.

RESULTS

General Characteristics of the POMC/TK Transgenic Mice

We obtained three founder lines of transgenic mice designated 401–21, 401–57, and 401–66. The 401–21 line of mice had no visible alterations in phenotype, was fertile, and has been used in the majority of studies described herein. The 401–57 line was infertile, and when an infrequent pregnancy occurred the fetuses were usually found resorbed *in utero*. The 401–66 line exhibited some male sterility. The Southern blot shown in Fig. 2A demonstrates that the 401–66 (lanes 5, 6, and 8) line has the highest copy number of transgene integration. Additionally, this line of transgenic mice consistently had a substantially smaller adult intermediate lobe (IL) in the absence of ganciclovir treatment in the adult IL; however the IL

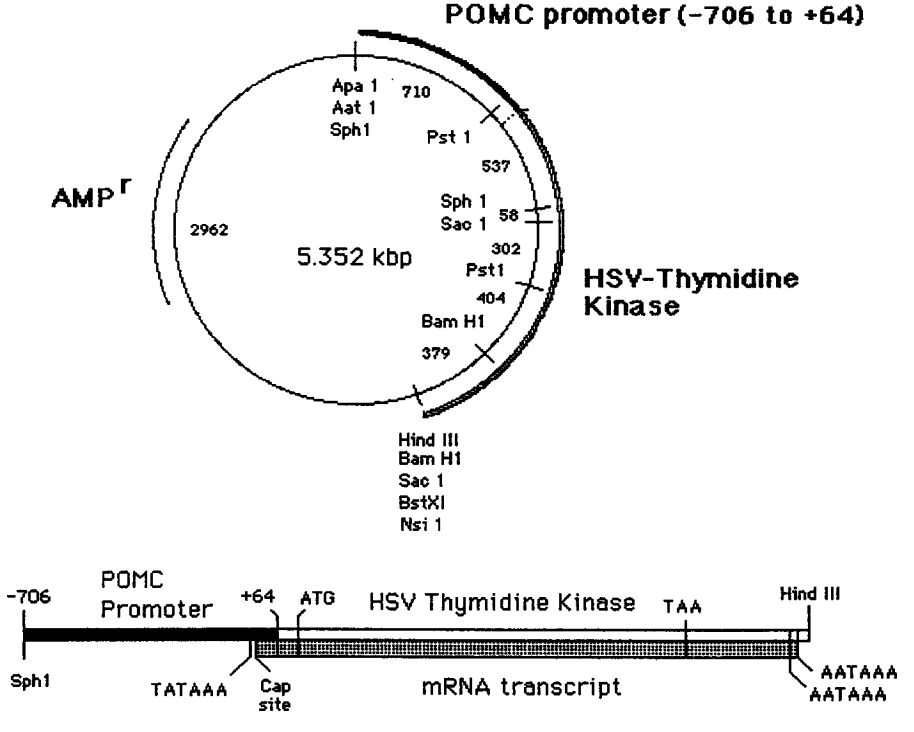

Fig. 1. Schematic Diagram of the Rat (r)POMC/hsv-1tk (POMC/TK) Construct Used to Produce Transgenic Mice

The vector was constructed as described in *Materials and Methods* and linearized, and approximately 500 copies were injected into fertilized mouse oocytes. The 379-bp *Bam*HI restriction fragment in the TK coding region was used to make random primer probe for the dot blots and the Southern analysis shown in Fig. 2.

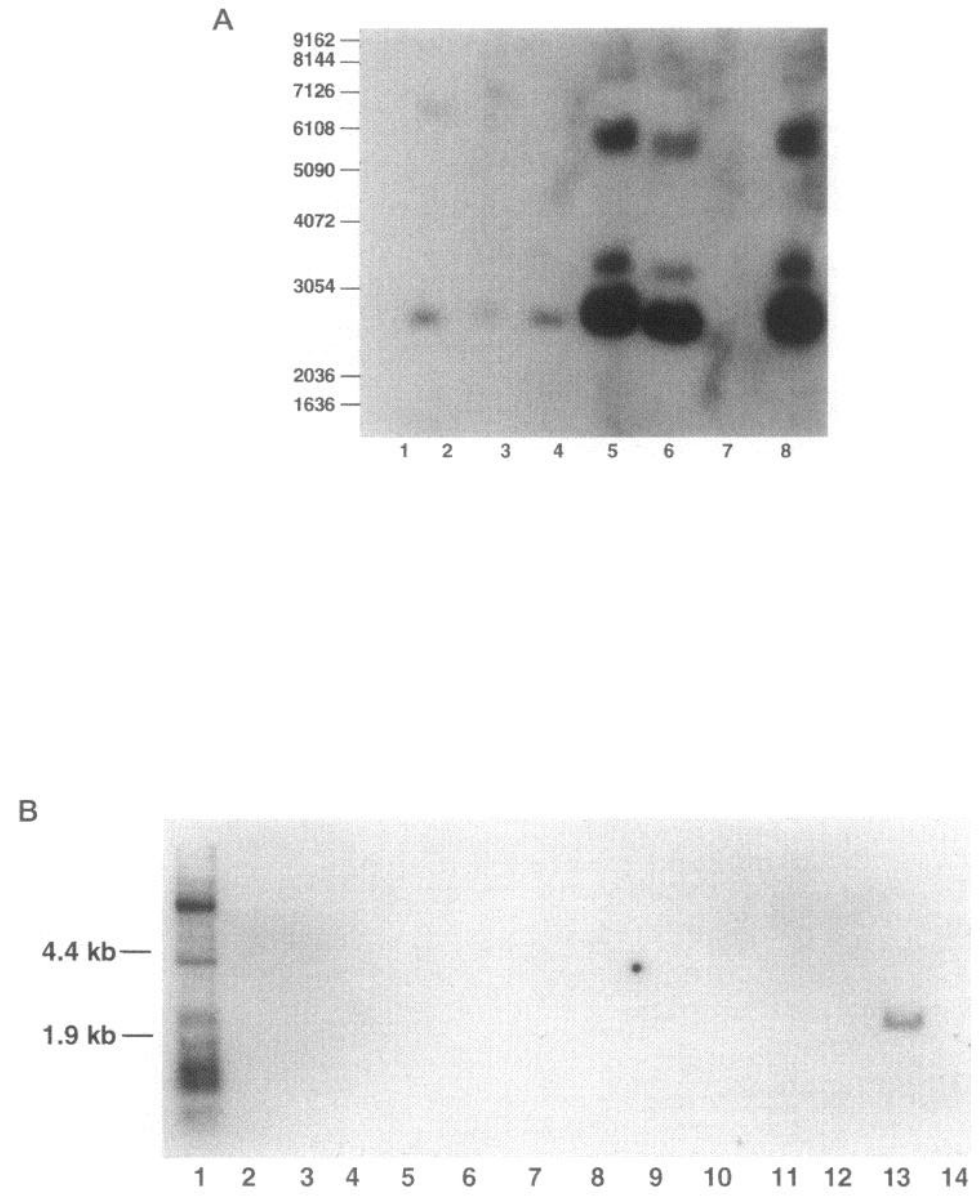

Fig. 2. Expression of POMC/hsv1-TK in Transgenic Mice

A, Southern analysis of tail DNA from progeny of founder transgenic mice harboring the POMC/TK construct. Southern analysis was performed as described in *Materials and Methods*. Lanes 1 and 7, Tail DNA from TK^- mice; lane 2, tail DNA from a 401–57 line TK^+ mouse; lanes 3 and 4, tail DNA from 401–21 line TK^+ mice; lanes 5, 6, and 8, tail DNA from 401–66 line TK^+ mice. B, Northern analysis of hsv1-TK expression in tissues of the 401–21 line of transgenic mice. Northern analysis was performed using a random primer 790-bp probe generated from the hsv1-TK-coding region as described in *Materials and Methods*. Five to 10 μg total RNA were loaded in each lane. The blots were stained with methylene blue to localize 4.4 kb and 1.9 kb rRNA, and their migration positions are indicated on the blot. The autoradiogram was exposed for 48 h. 1) RNA ladder; 2) blank; 3) hypothalamus; 4) whole brain; 5) cerebellum; 6) heart; 7) spleen; 8) testis; 9) lung; 10) adrenal; 11) liver; 12) kidney; 13) pituitary; and 14) blank.

was normal in size at birth. The 401–21 line has at least one copy of the transgene (lanes 3 and 4).

Expression of hsv1-TK is Pituitary-Specific in the 401–21 Line of Transgenic Mice

To determine whether the expression of the transgene was pituitary-specific we isolated total RNA from several tissues and performed Northern blot analysis. The approximately 2 kilobase (Kb) hsv1-TK transcript was expressed exclusively in the pituitary gland (lane 13) and not in hypothalamus, whole brain, cerebellum, heart, spleen, testis, lung, adrenal, liver, and kidney (Fig. 2B). In contrast, the relatively infertile 401–66 line of mice also had TK expression in the testis (data not shown).

We also performed double indirect immunofluorescence on cryostat sections of pituitary tissue using an-

tiserum directed at hsv1-TK and ACTH and analyzed them by confocal photomicroscopy. We found that the epitopes colocalized to the melanotropes and corticotropes in the pituitary (data not shown). Other studies (performed by M.J.L.) have used the same POMC promoter region (−706 to +64) ligated to other reporter genes to show that expression is pituitary and POMC cell-specific (21, 22).

The Extent of Pituitary POMC Cell Ablation Is Dependent on the Dose of Ganciclovir

To demonstrate that our POMC cell ablation system was functional we performed dose-response studies injecting mice twice daily with ganciclovir at 20, 50, and 70 mg/kg body weight, for 11–12 days. Coronal sections of pituitary tissues were analyzed by indirect immunofluorescence in combination with a β-endorphin-specific antibody.

Figure 3A shows a representative coronal section from a 401–21 line control sibling pituitary (TK^-), injected with 70 mg/kg ganciclovir for 11–12 days. There is no effect of ganciclovir in the normal mouse. Injection of 20 mg/kg ganciclovir to a TK^+ mouse resulted in a 50% ablation of the POMC cells in the IL and a reduction of the corticotrope staining in the anterior lobe (AL) (Fig. 3B). Injection of 50 mg/kg produced an 80% reduction in β-endorphin-positive cells in the IL and substantial reduction in corticotropes populating the AL (Fig. 3C). A 70 mg/kg administration of ganciclovir for 11–12 days caused an almost complete ablation of pituitary POMC cells (Fig. 3D).

The Ablation of Adult Pituitary POMC Cells Does Not Alter the Distribution of Other Pituitary Cell Types

Because coexpression of POMC peptides has been reported in other pituitary cell types, it was important to assess whether the loss of POMC cells reduced populations of differentiated cells producing the other pituitary trophic hormones. After pituitary POMC-cell ablation, pituitary sections were analyzed by indirect immunofluorescence using antisera for FSH (Fig. 4A), GH (Fig. 4B), TSH (Fig. 4C), and PRL (Fig. 4D). Representative sections showed a normal distribution in the transgenic animals after POMC cell ablation when compared with sections of normal mouse pituitary (Fig. 4, E-H). These results demonstrate that the ablation of POMC cells and/or lack of POMC peptides in the adult pituitary does not significantly alter the distribution of the other pituitary cell types.

Peptide Analysis of Transgenic Mouse Pituitary Tissue after POMC Cell Ablation

Processing-specific POMC peptide RIAs were used to confirm biochemically that both corticotrope and melanotrope populations were ablated in 401–21 animals treated for 11–12 days with 70 mg/kg ganciclovir. These antisera can distinguish between the two pituitary POMC cell types by virtue of the absolute requirement of the ACTH antiserum for an intact KKRR sequence in the middle of the ACTH (ACTH 15–18) (specific to corticotropes), and the α-MSH antiserum preferentially binds to acetylated forms of mela-

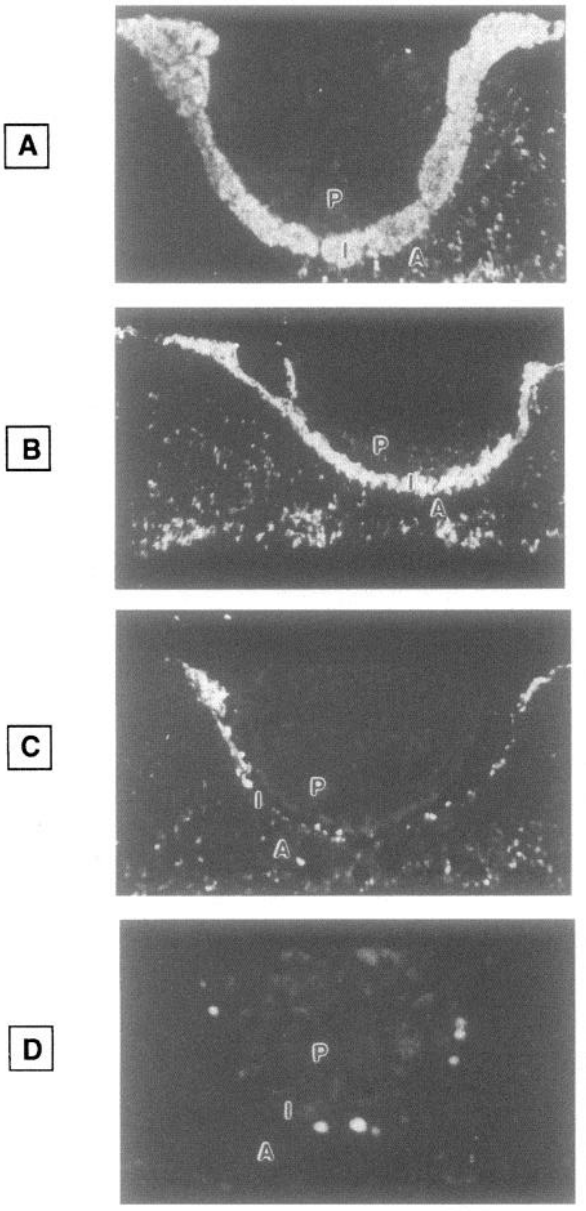

Fig. 3. Pituitary POMC Cell Ablation Is Dependent on the Dose of Ganciclovir

Adult transgenic mice were treated with drug or vehicle, pituitary tissues were processed, and indirect immunofluorescence using β-endorphin antiserum was used to locate pituitary POMC-producing cells as described in *Materials and Methods*. Transgenic (401–21) and nontransgenic control sibling mice were treated for 12 days with the following doses of ganciclovir injected ip. Representative coronal sections show the detection of β-endorphin-positive cells. A) Nontransgenic control, TK^-, 70 mg/kg; B) TK^+, 20 mg/kg; C) TK^+, 50 mg/kg; D) TK^+, 70 mg/kg. (Original 26×).

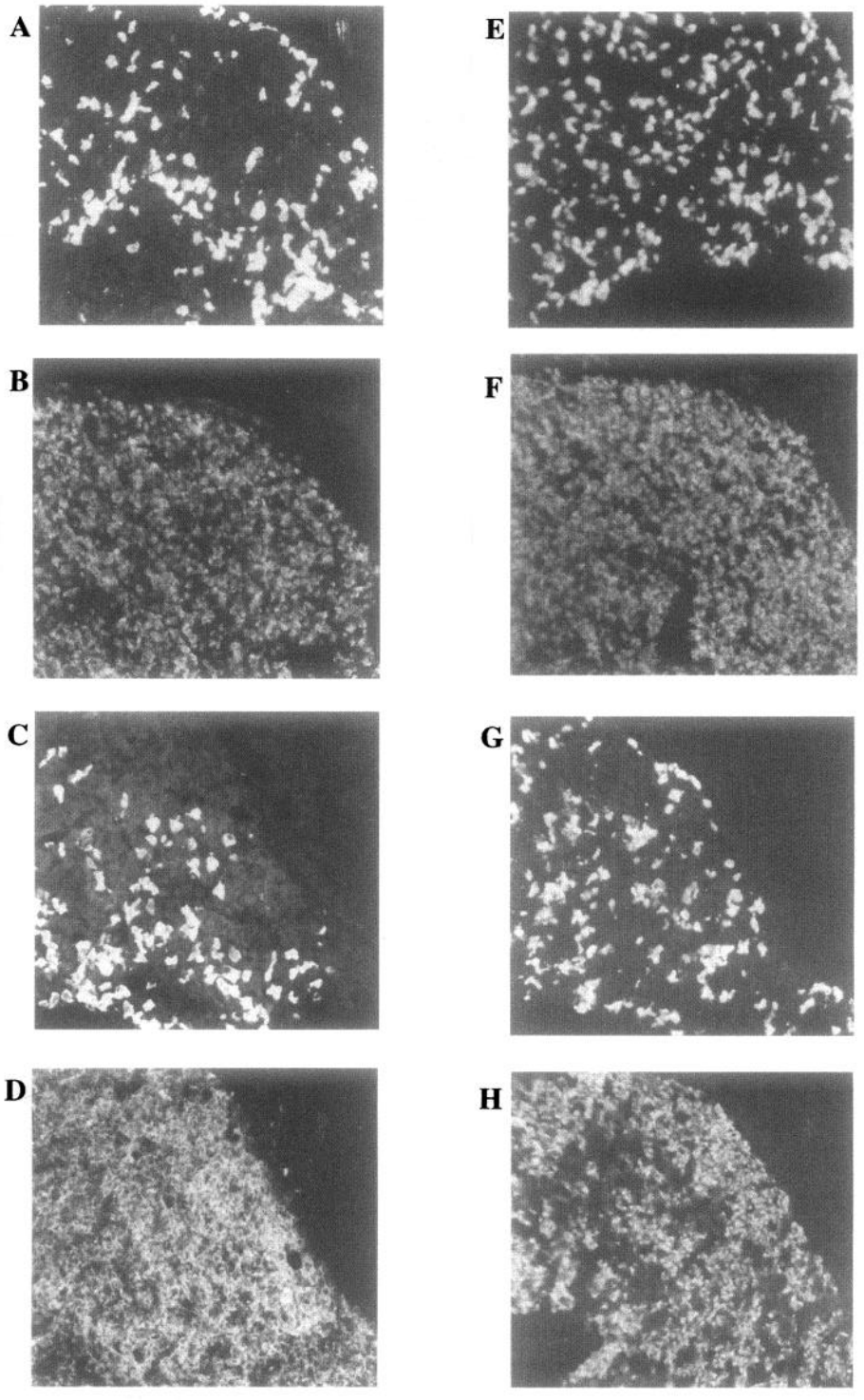

Fig. 4. Populations of Other Pituitary Cell Types Remain Unchanged after POMC Cell Ablation

Indirect immunofluorescence on coronal pituitary sections was used to identify hormone-producing pituitary cells after POMC cell ablation in a TK-positive mouse using 70 mg/kg ganciclovir treatment for 12 days. A) FSH; B) GH; C) TSH; D) PRL. Panels E-H are sections of control mouse pituitary tissue: E) FSH; F) GH; G) TSH; H) PRL. Animal treatment, tissue preparation, and immunohistochemistry were performed as described in *Materials and Methods*. (Original 26×)

notropins produced exclusively in melanotropes (23). Single pituitary glands were dissected, POMC peptides extracted, and the POMC peptide content measured by ACTH and α-MSH RIA. There was a greater than 98% reduction of the POMC peptide content in both the melanotropes and corticotropes of the pitu-

itary gland, confirming the immunohistochemical results (Fig. 5). TK-positive transgenic mice injected with vehicle had normal ACTH content. Circulating ACTH and β-endorphin levels were undetectable in unextracted plasma RIAs (data not shown).

Gene Expression Changes in the Mouse Adrenal Cortex after Pituitary POMC Cell Ablation

***In Situ* Hybridization** To determine whether ablation of POMC pituitary cells altered the distribution or levels of mRNA encoding the mouse adrenal ACTH receptor, we performed *in situ* hybridization studies. We found an apparent reduction in ACTH-receptor mRNA levels after a 12-day treatment with ganciclovir (Fig. 6). Of further interest was the observation that mouse ACTH-receptor transcripts were expressed uniformly throughout the adrenal cortex, before and after pituitary POMC cell ablation.

RNase Protection Assays To further analyze potential influences on mouse adrenocortical gene expression, total RNA was extracted from adrenal tissue, and the levels of the p450c11β receptor, p450c11AS, and ACTH mRNAs were measured by RNase protection assays (Fig. 7, A-C). These mRNA transcripts are expressed zone-specifically in the adrenal cortex, with the first gene product encoding the critical enzyme involved in corticosterone synthesis. We found that pituitary POMC cell ablation reduces the levels of mRNA expression for the ACTH receptor and p450c11β (11β). In contrast, the mRNA levels of p450c11AS (aldosterone synthase; AS) remained unchanged. The gels were scanned by densitometry to quantitate the differential expression of these adrenal cortical gene products (Fig. 7, D-F). These analyses confirmed the differences seen in the autoradiograms. The reduction in 11β and ACTH receptor mRNA expression probably reflects a combination of the disappearance of adrenocortical cells and the down-regulation of the specific mRNAs. POMC cell ablation was virtually complete as determined by measuring the β-endorphin content in the pituitary glands of treated animals (see legend to Fig. 7). Irrelevant lanes have been removed from these scans for clarity. However, it is important to note that incomplete ablation of POMC peptides and saline-injected controls were indistinguishable from control mRNA levels (data not shown).

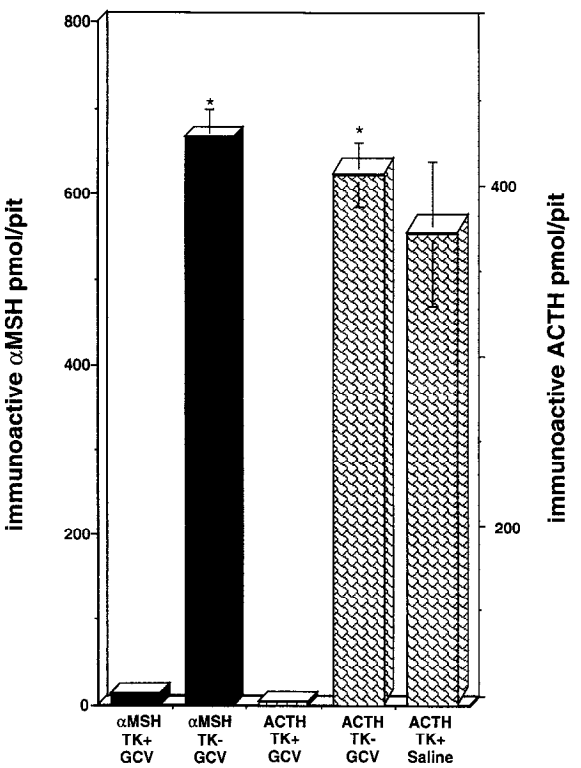

Fig. 5. Tissue-Specific POMC Peptide RIA Analysis of Mouse Pituitary Extracts after Ganciclovir (GCV) Treatment

Whole pituitary tissues from transgenic TK^+ 401–21 line (ganciclovir 12 days) and control TK^- negative mice were extracted, and the extracts were assayed for ACTH (AL) and α-MSH (IL) immunoactivity. α-MSH TK^+, GCV (n = 6); α-MSH TK^-, GCV (n = 5); ACTH TK^+, GCV (n = 6); ACTH TK^-, GCV (n = 5); ACTH TK^+ saline (n = 3). Errors are ± SEM. *, $P < 0.005$ as determined by Student's *t* test compared with TK^+, GCV-treated mice (StatView, Abacus Concepts, Inc., Berkeley, CA)

Plasma Corticosterone Levels Decrease Dramatically after Ganciclovir Treatment It was important to determine the influences of pituitary POMC cell ablation on the adrenocortical secretion of GCs. TK-positive transgenic mice were treated for 2 weeks with ganciclovir and plasma levels of corticosterone measured by RIA. Figure 8 shows that circulating corticosterone levels were reduced substantially in the TK^+ transgenic mice. TK^+ animals injected with saline vehicle had the same levels of corticosterone as control TK animals injected with ganciclovir.

POMC Peptide Levels Increase Slowly after Discontinuing Ganciclovir Treatment Since repopulation of specific tissues was found after ablation of lymphocytes and GH-producing cells by TKO followed by antiviral withdrawal, we wanted to determine whether POMC cells would repopulate the pituitary after removal of ganciclovir. 401–21 line mice were treated with ganciclovir at 70 mg/kg for 11 days. One group of mice was analyzed for pituitary POMC peptide content at this point, and the remainder were withdrawn from ganciclovir administration; 35 days after the cessation of drug treatment the pituitary tissues were analyzed for POMC peptide content by RIA.

POMC cell ablation was virtually complete at the termination of treatment with ganciclovir (Table 1). After removal of ganciclovir and recovery, the levels of immunoactive POMC peptides found in the pituitary extracts were substantially higher than those of the animals in the initial set. Thirty five days after cessation of ganciclovir administration, anterior lobe POMC peptide levels (ACTH), reported as percent of control, increased to about 13% of normal, recovering from about 1% of normal after cell ablation. This difference was statistically significant at $P < 0.007$ using the analysis of variance (ANOVA) plus Fisher protected least significant differ-

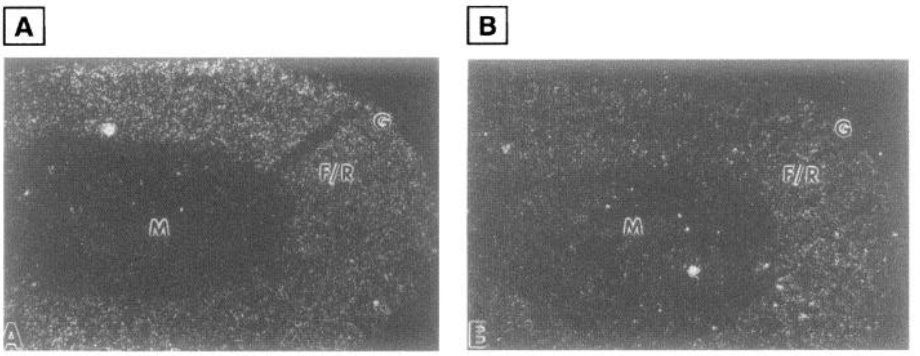

Fig. 6. *In situ* Hybridization of Mouse Adrenal Gland after Treatment with Ganciclovir

Ten-micron sections were hybridized with ^{35}S-labeled cRNA probes for the mouse ACTH receptor (MAD-26, ~200 bp). A, Saline-injected control. B, Ganciclovir treatment for 12 days. The mouse adrenal glomerulosa layer is indicated by "G," the fasiculata/reticularis by "F/R," and the adrenal medulla by "M." Sense probes showed no hybridization signal (data not shown).

ence. α-MSH levels (IL) only increased from 1.4-to 3.8% of control in the same time period. The difference in the α-MSH content was not statistically significant.

DISCUSSION

The coordination of the HPA axis is a complex process (10, 24). A molecule that is essential to the integration of this system is POMC. Tissue-specific posttranslational processing of the nonbioactive precursor POMC produces peptide hormones with a wide variety of biological activities (25). This processing requires proteolytic enzymes, such as the prohormone convertases, in concert with α-amidating, carboxy-shortening, and acetylating enzymes to produce mature peptides (26). The roles of all POMC peptides in adrenocortical regulation are not completely understood (27).

The adrenal cortex consists of three distinct zones. GCs are produced in the two inner zones (fasiculata/reticularis), while mineralocorticoids are synthesized in the outer zone (glomerulosa). Recent progress has been made in the identification and cloning of genes encoding enzymes critical for adrenocortical function. Different isozymes in the family of cytochrome p450 enzymes have been cloned from a mouse genomic library and have been shown to produce GCs (Cyp11β-1 or p450c11β) and mineralocorticoids [Cyp11β-2, aldosterone synthase (AS)] (28). Additionally, it has been determined that the mRNA transcripts encoding these enzymes are differentially expressed in cell types composing the mouse adrenal cortex, with AS transcripts confined to the glomerulosa layer while p450c11β is expressed predominantly in the inner two zones (28).

Production of aldosterone by the zona glomerulosa is largely controlled by the renin-angiotensin system (29), while ACTH regulates GC production by the fasiculata/reticularis zone. It had been thought that the 11β-OH was able to catalyze the final steps of both GC and aldosterone synthesis. Recent studies in the rat have shown that two enzymes were required for these final steps and led to the cloning of the 11β-OH homologs and the demonstration that only one of these enzymes was capable of producing mineralocorticoids while the other could produce GCs (30). The prevailing view is that there is a functional separation of these enzyme activities in most species, including humans (31). However, the bovine adrenal cortex does not exhibit this functional segregation (32).

ACTH binds to specific receptors expressed by adrenocortical cells and regulates steroid secretion by activating adenylyl cyclase (33). Prolonged exposure to ACTH causes an increase in GC production and the expression of steroid-metabolizing enzymes in adrenal fasiculata cells. We determined that ablation of pituitary POMC cells, whereby circulating ACTH and other peptides derived from POMC are removed, caused a substantial reduction of p450c11β and ACTH receptor mRNA levels. We also observed abnormal adrenal morphology (shrunken inner zone) after ganciclovir treatment (data not shown). In contrast, AS mRNA levels remained unchanged. We also have determined that mRNA transcripts encoding the ACTH receptor are expressed robustly throughout the normal mouse adrenal cortex (Fig. 6).

Even though the ACTH receptor is expressed throughout the adrenal cortex, the observation that there was no reduction in AS mRNA implies that removal of ACTH does not positively influence AS mRNA levels. In contrast, other studies demonstrate that injection of ACTH causes AS mRNA to fall to undetectable levels in the rat adrenal gland after 8 days of treatment (34), thus implying that factors other than ACTH maintain AS mRNA levels. Additionally, it should be noted that ACTH influences steroidogenic enzyme gene transcription, and these alterations differ widely *in vivo* and *in vitro*. For instance, in cell culture and gene transfer experiments, ACTH has been shown to influence the regulation of several gene products, including negative modulation of AS transcription (34, 35). Our studies demonstrate ACTH receptor mRNA regulation by its peptide ligand, although this result was probably due to both changes in transcrip-

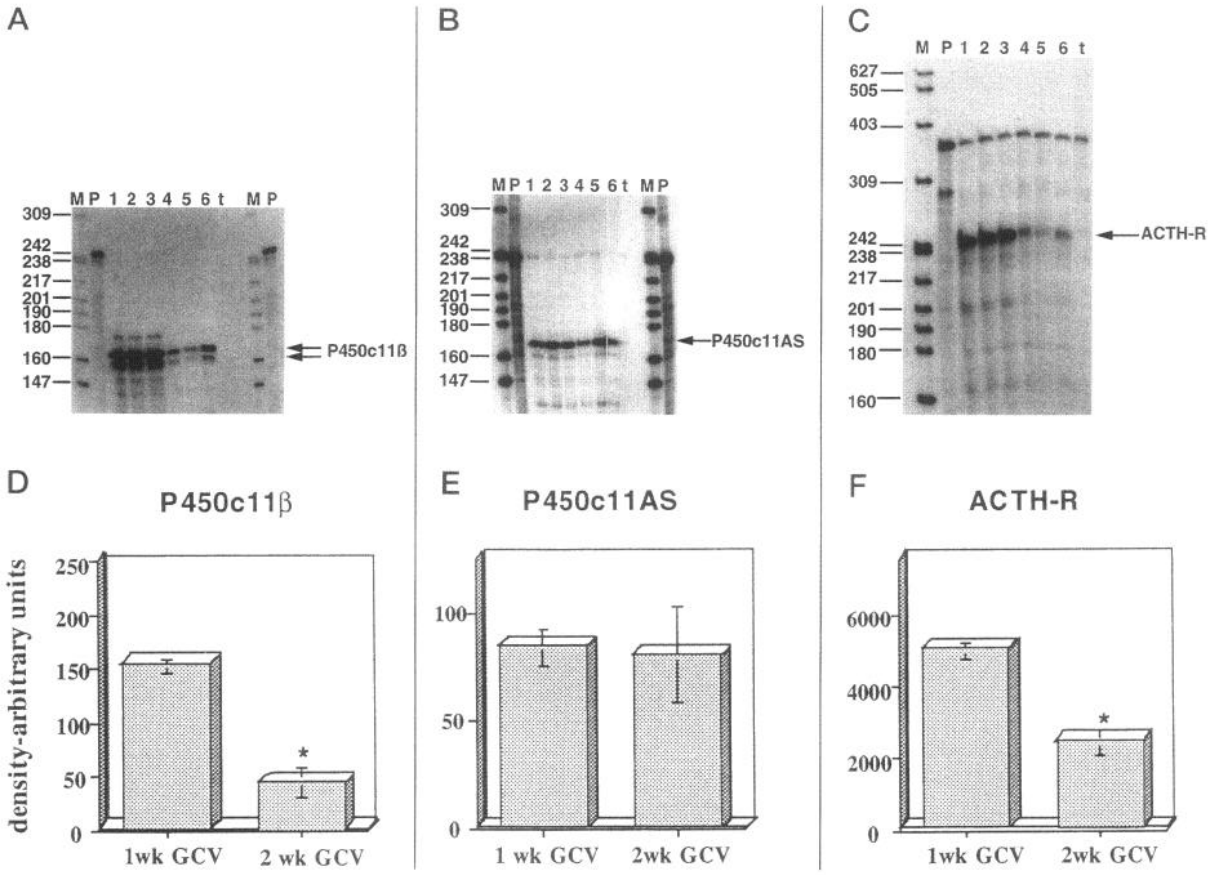

Fig. 7. RNase Protection Assay of Mouse Adrenal RNA from Transgenic TK$^+$ Mice Treated with Ganciclovir

Two micrograms of total RNA from single adrenal glands were combined with 5–6 $\times$ 10^5 cpm of ^{32}P-labeled mouse p450c11β (A), mouse p450c11AS (B), or mouse ACTH receptor (C) probes. Fifty micrograms of tRNA treated the same as the adrenal RNA is in the lane marked "t." Molecular weight markers are in the lanes marked M, and the individual unprotected probes are shown in the lanes marked "P." Lanes 1–3 were transgenic mice treated for 1 week with ganciclovir, and lanes 4, 5, and 6 were mice treated for 2 weeks. The values (picomoles of β-endorphin/pituitary) for each mouse represented in lanes 1–6 respectively were: 1) 100; 2) 100; 3) 30; 4) 1.1; 5) 1.3; and 6) 13.6. Saline- injected control mice had between 300–400 pmol β-endorphin/pituitary. The gels were scanned by densitometry and the results are shown in panels D-F, *, $P < 0.05$ by t test compared with 1 wk GCV treatment, errors = ± SEM.

tion and adrenocortical degeneration. There are studies that show that the ACTH receptor mRNA levels can be up-regulated by ACTH in cell culture (36). This is also consistent with the notion that ACTH can modulate mRNA levels of its own receptor.

The TKO technique has been used successfully to ablate cells in several organ systems including the pituitary gland using FIAU [(1-(2-deoxy-fluoro-β-d-arabinofuranosyl)-5-iodouracil] as the antiviral (17, 18). For instance, experiments were conducted to remove the somatotropes and lactotropes that populate the AL by TKO using GH and PRL promoter sequences to drive the expression of hsv1-TK (17). These studies showed that somatotropes could repopulate the pituitary gland after drug withdrawal, while lactotropes showed a much weaker and later recovery. The observations led to the conclusions that both somatotropes and lactotropes derive from a common GH-expressing stem somatotrope, and differentiation was a postmitotic event because pharmacological sensitivity to antiherpes agents is dependent on cell division. The interpretation of these study results was confounded because no experiments were performed using adult animals, where mitotic rates occur more slowly. Additionally, even though pregnant mothers were implanted with minipumps delivering FIAU, no data were shown regarding cell ablation during embryogenesis.

In contrast to the results obtained in the GH/PRL system were studies of the ablation of thyroid follicular cells by the TKO technique using ganciclovir as the nucleoside. After ablation, the remaining thyroid cells could not repopulate the tissue (37). It was concluded that cell ablation did not depend on cell division because the rate of adult follicle cell division in the adult has essentially ceased, whereas 50% of the cells were killed in 2 days. Our studies would appear to support these findings because the mitotic rate of adult pituitary POMC cells is in the range of 10^{-3}-10^{-4}/day, and thus could not account for 98% cell death in 12 days

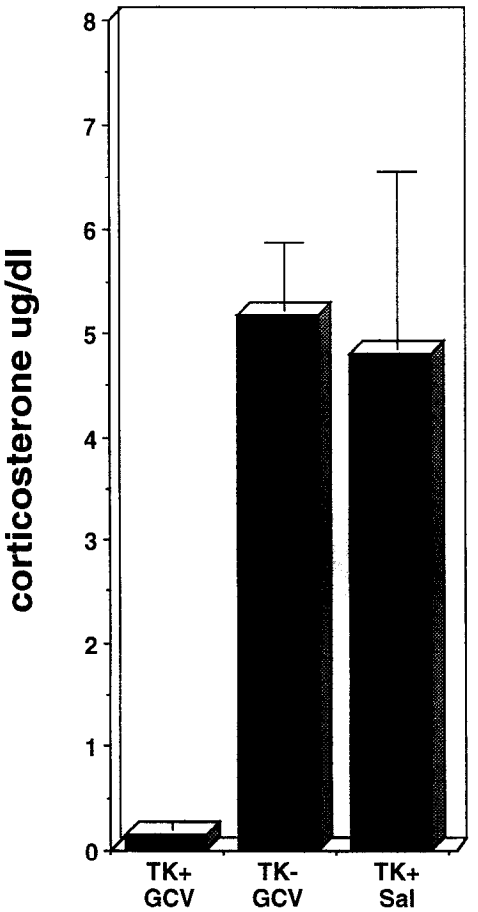

Fig. 8. Measurement of Plasma Corticosterone after Ganciclovir Treatment

Plasma corticosterone levels were measured in the 401–21 line of animals given ganciclovir or saline injections ip as described in *Materials and Methods*. Transgenic, TK^+ (ganciclovir 12 days, n = 3); control sibling, TK^- (ganciclovir 12 days, n = 3); transgenic, TK^+ (saline 12 days, n = 3); error bars are ± SEM. $P < 0.005$ as determined by one-way analysis of variance *t* test (StatView, Abacus Concepts, Inc., Berkeley, CA).

Table 1. Recovery of Pituitary POMC Cells after Treatment with Ganciclovir[a]

	Immunoactive POMC peptides after 11 days ganciclovir		
	pmol/pituitary	% of control	*N*
Before recovery			
α-MSH TK^-	667 ± 33	100	6
α-MSH TK^+	13 ± 1.1	1.9	5
ACTH TK^-	360.3 ± 22	100	6
ACTH TK^+	5.0 ± 2.2[b]	1.4	5
After recovery			
α-MSH TK^+	25.6 ± 3.4	3.8	3
ACTH TK^+	45.9 ± 2.1[b]	12.7	3

[a] A group of mice from the experiment described in Fig. 5 were treated with ganciclovir for 12 days and then allowed to recover for 35 days. Whole pituitary tissues were extracted and the individual extracts assayed for ACTH and αMSH immunoactivity as described in *Materials and Methods*. Ganciclovir-treated mice are from the data shown in Fig. 5, changed to tabular form.

[b] Statistically significant at $P < 0.007$ using the ANOVA. Errors are ± SEM.

(38, 39). However, lack of cell division must be determined directly to resolve this issue.

We found differential rates of increase in POMC peptide levels after recovery from ganciclovir. This result implies that POMC cells are repopulating the pituitary. It is also possible that after drug removal POMC synthesis increases in a population of cells resistant to ganciclovir. Thus, additional studies will be required to show POMC cell repopulation directly. Taken together, the results of Borelli *et al.* (thymus ablation, and ablation of GH/PRL cells), Wallace *et al.* (thyroid follicular cells), and the studies presented here seem to indicate that the regeneration of the ablated cell-type is related to its general mitotic rate. However, this may not be true for cells of any given type ablated at different pre- and postnatal periods.

The mechanism(s) of action of the antivirals remains largely unknown (40, 41). Several groups have predicated their conclusions on the fact that this process can only kill dividing cells. There are major differences in the structure/function of antiherpetic agents. ganciclovir is a DNA chain terminator, while FIAU can also be inserted into DNA chains (42). Thus, a comparison of the two compounds, and other antiherpes agents, is certainly warranted, considering the recent deaths of transplant patients receiving one of these drugs (42).

The observation that nondividing POMC cells might be susceptible to ablation by the TKO method is an important finding. If this technique is indeed independent of cell division, it may enable the use of neuronal-specific promoter regions ligated to hsv-1 TK to accomplish ablation of specific brain systems. The process by which this cell death occurs needs to be understood to further develop and refine targeted molecular cell ablation. Furthermore, future studies may shed light on mechanism(s) of antiherpes drug-induced cytotoxicity that are, at present, poorly understood (42).

The results shown here support the idea that the ablation of POMC cells does not affect the distribution of other pituitary cell types and may preclude any high level coexpression of POMC in other adult pituitary cells. However, if POMC was expressed in other cell types, its expression would probably be low, and correspondingly low levels of hsv-1 TK would be produced, which might be insufficient for drug-induced cytotoxicity. Other studies have suggested the existence of a subpopulation of common corticotroph/gonadotroph cells that peaks in the neonatal rat pituitary during the stress hyporesponsive period (43, 44). It may be possible to characterize further the developmental fate of this transient cell population in our transgenic mouse model by treatment of neonatal mice with ganciclovir and subsequent analysis of gonadotropes in adult animals. This result is consistent

with the observation that the ACTH receptor is not expressed in adult pituitary tissue (3) but does not preclude possible paracrine influences of POMC peptides during prenatal development utilizing transiently expressed POMC peptide receptors. Additionally, receptors for POMC-derived peptides other than ACTH may be expressed in other pituitary cell types and possibly at different times during embryogenesis.

The mouse model system we have produced will be extremely valuable in understanding the regulation of mRNAs encoding POMC peptide receptors. For instance, after pituitary POMC cell ablation, each individual peptide and/or GC can be replaced systematically, thus allowing the function of a single POMC-derived peptide hormone to be examined. This system will be amenable to molecular dissection of the roles of all POMC peptides in the maintenance of adrenal function at the molecular level, the regulation of POMC peptide receptor populations in peripheral tissues, and the establishment of specific roles of POMC peptides in the molecular mechanisms of adrenal homeostasis. Studies characterizing the interaction of GC production and brain development with a reversible system will also now be possible.

MATERIALS AND METHODS

Transgenic Mice

A plasmid (D1), including the rat (r) POMC pituitary expression-specific (21) 5′-flanking sequence from −706 to +64, was constructed using pGem7Zf(+) (Fig. 1). The hsv1-TK coding region was ligated into the plasmid by standard cloning techniques (45). The pronuclei of $(B6D2F_1 \times B6D2F_1)F_2$ hybrid fertilized mouse oocytes were microinjected with approximately 500 copies of the linearized fusion gene as described previously (46). Transgenic mice were identified by dot-blot hybridization of tail DNA using a radiolabeled random primer probe generated from the *Bam*HI 379 base pair (bp) fragment of the construct (47). Animals (both males and females) were outbred to Swiss Webster (SW) and B6CB F_1 mice. All animal studies were conducted in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals.

Southern Analysis

Mouse tail DNA was extracted from progeny of the three transgenic lines designated 401–21, 401–57, and 401–66. Southern blotting was performed using standard methods and *Eco*RI to digest the genomic DNA (45). A ^{32}P-labeled random primer probe used for tail-blot analysis was generated using the *Bam*HI fragment, at 5×10^7 cpm/reaction; 10^6 cpm/ml was hybridized to the Nytran membrane. Washes were as follows: 2 × sodium, sodium citrate (SSC) (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), twice for 15 min at 25 C; 1 × SSC, 0.5% SDS, twice for 15 min at 37 C; 0.1 × SSC, 1% SDS, twice for 15 min at 65 C. Autoradiography was performed as described previously (47).

Northern Analysis

Total RNA was isolated by the guanidinium isothiocyanate method as described in Ref. 48. For Northern blot analysis, total RNA was fractionated on a formaldehyde (6%)/agarose (1.2%) gel using HEPES electrophoresis buffer. After capillary transfer in 10× SSC to Nytran (Schleicher & Schuell, Keene, NH), the RNA was fixed by UV light to the membrane. The prehybridization conditions were: 2 h in 5% SDS in 400 mM NaPO4, 1 mM EDTA, 1 mg/ml BSA, and 50% formamide, at 42 C. The hybridization buffer was changed to fresh buffer containing 3×10^6 cpm of a 790 bp random primer probe excised from the plasmid using *Bam*HI and *Sac*I. The blot was hybridized overnight at 42 C, then washed 2 times at 55 C for 15 min in 0.1× SSC (0.1% SDS), and then washed once at 58 C for 10 min. The autoradiograph was exposed for 48 h and developed as described by Low *et al.* (47).

Ganciclovir Administration

TK^+ and TK^- (wild type siblings) 5- to 7-week-old adult mice were injected ip twice daily for 11–12 days with 20, 50, and 70 mg ganciclovir (Syntex, Palo Alto, CA)/kg body weight in physiological saline. As additional controls, TK^+ animals were subjected to the same injection schedule using the vehicle alone. Longer term injections were administered every 1–3 days up to 60 days. The efficacy of drug delivery by osmotic minipumps (Alzet, Palo Alto, CA) was tested with daily delivery rates of 50–200 mg ganciclovir/kg body weight using 7 and 14 day pumps. Minipumps were primed according to manufacturer's instruction and implanted sc in animals anesthetized with 0.15 ml 2% tribromoethanol/10 g body weight ip. For the recovery experiments, the injections were terminated and the animals killed mid-afternoon at the recovery times indicated in each experiment.

Histology and Immunohistochemistry

Mice were anesthetized with 2% tribromoethanol and perfused transcardially with 4% paraformaldehyde in borate buffer at 4 C. Tissues were postfixed at 4 C overnight in perfusion buffer with 10% wt/vol sucrose added or stored at 4 C in 10% buffered formalin with 10% sucrose. Sections (10–20 μm) of pituitary, adrenal glands, testis, and brain embedded in O.C.T. (TissueTek, Miles Inc., Elkhart, IN) were cut on a cryostat, and free floating sections were stored in sodium phosphate buffer (pH 7.3)-ethylene glycol/glycerol until used for hematoxylin-eosin staining or immunohistochemistry. Primary antisera directed against the following antigens were detected by indirect immunofluorescence as described previously (47) and used at the following dilutions: rat β-LH, 1:5000; rat β-FSH, 1:2000; rat β-TSH, 1:5000; human GH S2, 1:1000; rat PRL S9 (rPRL), 1:25 (donated by the National Hormone and Pituitary Program, NIDDK, Bethesda, MD); ACTH, 1:200; β-endorphin, 1:200. The hsv1-TK antiserum was a generous gift of Dr. Bill Summers and was used at a dilution of 1:50. Secondary antibodies were purchased from Tago, Inc. (Burlingame, CA) and used at a 1:200 dilution. These were goat anti-rabbit fluorescein isothiocyanate and goat anti-mouse rhodamine isothiocyanate.

In Situ Hybridization

In situ hybridization of adrenal glands was performed as previously described (49, 50). After linearization with *Sal*I, sense and antisense ^{35}S-labeled cRNA probes were generated using T3 and T7 polymerase from a mouse adrenal ACTH receptor partial clone (MAD-26), kindly provided by Dr. Roger Cone. This probe is 200 bp in length and is contained in the coding region of the ACTH receptor subcloned into Bluescript II SK±. [^{35}S]cRNA probe (10^6 cpm/ml) was hybridized to the sections. The slide-mounted sections were first exposed to Kodak X-omat film, then dipped in emulsion and exposed for 10 days.

RIAs

Pituitary tissues were homogenized in 0.5 ml 10% acetic acid containing 0.3 mg/ml phenylmethyl sufonylfluoride (PMSF)-0.5 mg/ml BSA, frozen and thawed three times, and centrifuged, and an aliquot (100 μl) diluted 1-fold with water was frozen at −20 C for RIA as previously described (51). The remaining supernatant was divided into aliquots, lyophilized, and stored at −20 C.

The β-endorphin antiserum is midportion specific and recognizes POMC and all molecules containing β-endorphin on an equimolar basis (51). The α-MSH, and the ACTH immunoassays have also been described in detail (23, 51). The ACTH RIA recognizes the midportion of ACTH (1–39) and has an absolute requirement for an intact KKRR sequence at residues 15–18 of ACTH (1–39), and the α-MSH assay is 85% acetyl-specific (23). The sensitivities of the RIAs ranged between 2–25 pg/tube. Synthetic peptides were obtained from Peninsula Laboratories (Belmont, CA) and BaChem (Torrance, CA).

Corticosterone RIA

Trunk blood was collected from nonperfused animals after decapitation. Blood (150–400 μl) was collected on ice with 15 μl (10 U/μl) heparin and centrifuged for 5 min at 10K rpm, and the plasma was stored at −80 C until RIA. Corticosterone was measured in unextracted plasma using a RIA kit (Ventrex, Portland, ME) and the manufacturer's instructions.

RNase Protection Assays

Adrenal glands were dissected and frozen immediately on dry ice. Total RNA was extracted by the acid guanidinium thiocyante-phenol-chloroform method (52). The basic RNase protection assay procedures have been described previously (53). To generate the adrenal-specific probes used in the present studies, cDNA was generated from total RNA obtained from the mouse Y-1 adrenal cell line and was amplified by polymerase chain reaction (PCR) using oligonucleotide primers specific for murine p450c11β and p450c11AS (28). The oligonucleotides for c11β were directed against bases 766–928, and the oligonucleotides c11AS were directed at bases 766–931. The PCR fragments were cloned into Bluescript KS +/- and sequenced. Adrenal-specific RNA probes: The p450c11β-containing plasmid was linearized with *Bam*HI and T3 RNA polymerase used to generate a 163-bp fragment. The p450c11AS-containing plasmid was linearized with *Eco*RI, and the probe was synthesized using T7 RNA polymerase to generate a 166-bp fragment. The ACTH receptor-containing plasmid (MAD-26; a generous gift of Dr. Roger Cone) was linearized with *Sal*I and T7 polymerase used to synthesize a 200-bp antisense RNA probe. For protection assays 5–6 $\times$ 10^5 cpm per sample were used. The RNAse-resistant fragments were separated by electrophoresis in 5 or 6% polyacrylamide-7.5 M urea gels followed by autoradiography as previously described (34).

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