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Post-transcriptional Action of ACTH in the Control of P450c17 Expression in Rabbit Adrenal Glands

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Stimulation of transcription of the *CYP17* gene by ACTH has been demonstrated previously by others using cultures of adrenal tissue from several species. In the present investigations we have demonstrated from measurements in pooled rabbit adrenal glands that after 4 or 6 days of ACTH injections no difference in amounts of CYP17 mRNA per μg of total adrenal RNA was observed between ACTH and control animals. While the total amount of CYP17 mRNA per adrenal increased 1.4- to 1.7-fold over the injected and non-injected controls due to an increase in total RNA in the ACTH-stimulated adrenals, Western analysis of adrenal microsomal protein demonstrated that the amount of adrenal *P*450c17 protein, the product of the *CYP17* gene, increased over 50-fold. The data show that ACTH is acting at both transcriptional and post-transcriptional loci to increase the amount of rabbit adrenal *P*450c17 and that the greater effect is post-transcriptional.

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INTRODUCTION

Transcription of the genes coding for the *P*450 family of enzymes is subject to complex regulation (reviewed in Ref. [1]). Steroidogenic *P*450s are no exception and the control of their expression is predominantly transcriptional (reviewed in Refs [2,3]). Post-transcriptional control of steroidogenic *P*450s has not been demonstrated conclusively.

Specific regulation of adrenal 17α-hydroxylase activity is effected by ACTH via a cAMP-dependent pathway and was originally suggested by the work of Kass *et al.* [4] after demonstrating an increase in cortisol and a decrease in corticosterone secretion by rabbit adrenals following ACTH injections. This effect of steroid synthesis was shown subsequently to be due to an increase in 17α-hydroxylase activity, rather then to an inhibition of a competing enzyme [5, 6]. Maximum stimulation was demonstrated after 3 days of injections, and elevated levels could be maintained for at least 28 days [7]. It was also demonstrated that the increase in enzyme activity correlates with an increase in the *P*450c17 protein [8]. In bovine and human adrenal cells in culture, the control of 17α-hydroxylase activity and *P*450c17 protein has been shown to involve stimulation of transcription and increased *CYP17* mRNA accumulation [9, 10]; however, ACTH-stimulated 17α-hydroxylase activity and *CYP17* gene expression in these cell cultures do not generally exceed levels found in normal tissue. For example, ACTH stimulation of bovine cells in culture brings the 17α-hydroxylase level back to about the level found in the original tissue prior to culture [11]. In experiments using rabbit adrenal cell cultures or intact animals, ACTH stimulation results in levels of 17α-hydroxylase activity greater than that found in normal, non-stimulated tissue [12, 13]. This difference, the fact that rabbit adrenal tissue can be stimulated to express much higher than normal levels of *P*450c17, whereas adrenal tissue from some other species cannot, led to the investigations reported here. We wished to determine whether ACTH-stimulated increases of rabbit adrenal 17α-hydroxylase activity and *P*450c17 protein are due primarily to increases in transcription of the *CYP17* gene and the amount of CYP17 mRNA available for translation, as is the case in humans and cows [9, 10]. We report that the primary locus of ACTH stimulation of rabbit *P*450c17 expression is

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post-transcriptional and that this defines a new locus of ACTH action.

MATERIALS AND METHODS

Male New Zealand White rabbits weighing 2–3 kg were injected intramuscularly twice daily with 28–40 IU ACTH in an oil/beeswax suspension as described previously [12], or as a commercial preparation, Acthar gel (Armour). Injection periods were either 4 or 6 days as indicated in the results. Control animals were either injected with oil vehicle or not injected. Animals were anesthetized with pentobarbital and exsanguinated on the morning following the last day of injections. Adrenal glands, testes, and a portion of the liver were removed and immediately frozen in liquid nitrogen. Each group contained 4 or 6 animals depending on the experiment, and tissues were pooled within a group for RNA and microsomal protein preparations.

RNA was isolated by the acid guanidinium thiocyanate–phenol–chloroform extraction method of Chomczynski and Sacchi [14], and the amount of RNA isolated determined spectrophotometrically. Northern analyses were performed [15] using Zeta Probe membranes (Biorad). The same type of membrane was used for dot hybridization analyses [16]. Probes used for hybridization were derived from a rat CYP17 cDNA [17] or a bovine CYP21 clone [18] and labeled using either a random primed DNA labeling kit from Boehringer Mannheim and α-[^{32}P]dCTP (3000 Ci mmol^{-1}, NEN) or PCR [19]. Labeled probes were purified by filtration through Sephadex G-50 (Pharmacia) [20], and hybridized bands were detected by autoradiography. When used for hybridizing with a second probe, membranes were stripped of the original probe by washing at 80°C for 30 min in 0.1 × SSC containing 0.1% sodium dodecyl sulfate (SDS). Efficiency of probe removal was confirmed by autoradiography prior to hybridizing the membrane with the second probe. An EC densitometer was used to scan the autoradiograms and obtain an estimate of the relative amounts of probe-specific RNA in different lanes of a given gel.

Microsomes were prepared as described previously [8]. The final microsomal pellet was suspended in 0.25 M sucrose in 0.1 M sodium phosphate buffer, pH 7.0, 1.0 mM dithiothreitol (DTT), made 20% in glycerol and frozen at −20°C. Protein concentration was determined by the Lowry method [21]. Microsomal proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes for Western analysis as described previously [8]. *P*450c17 and *P*450c21 were detected using ^{125}I-labeled protein A and antibodies raised against pig and bovine proteins, respectively. In one experiment a hybridized membrane was stripped of its first antibody and ^{125}I-label by incubation of the membrane at 70°C for 30 min in

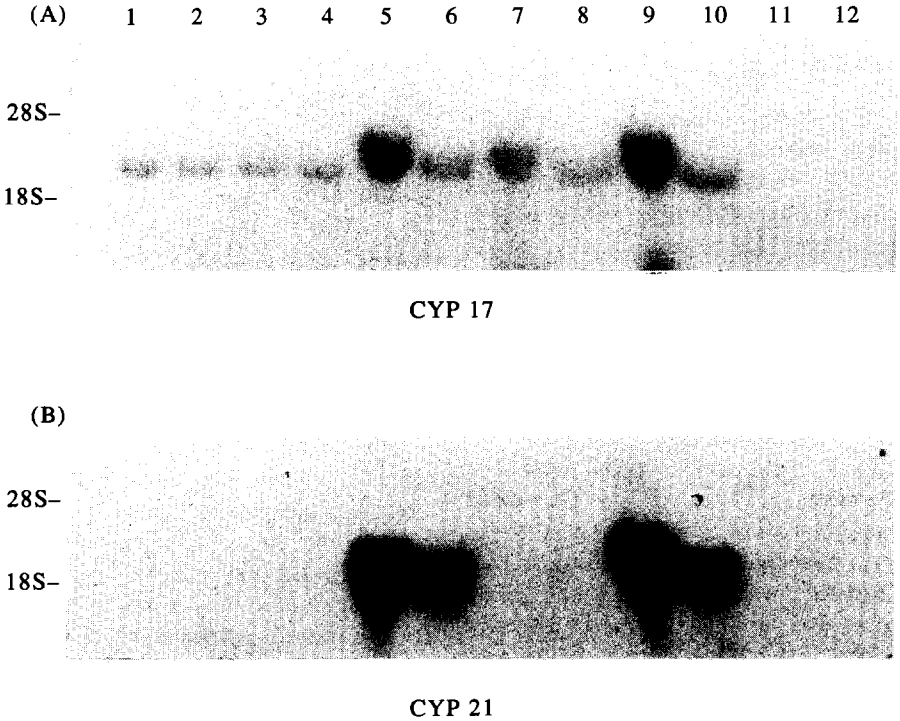


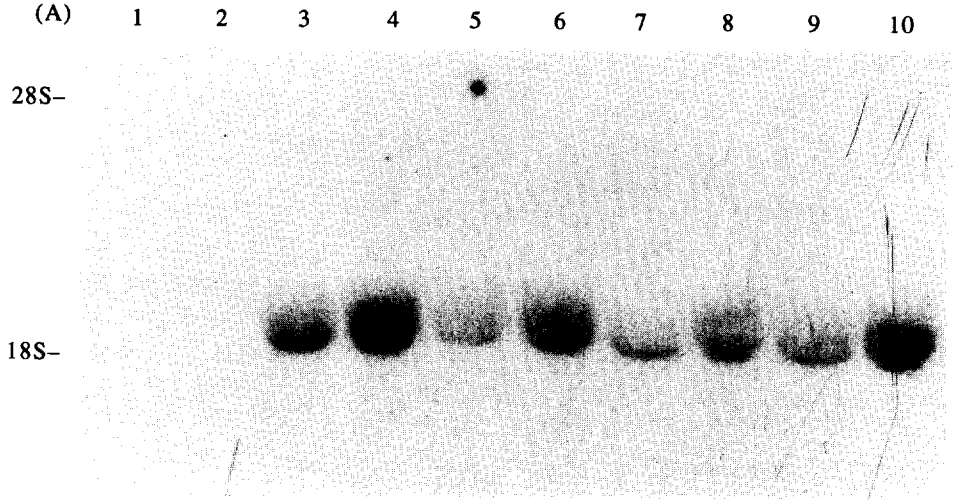
Fig. 1. Northern analysis of rat and rabbit CYP17 and CYP21 specific RNA. 4 Day ACTH injection period. Lanes 1–4: rat testis RNA, 20 μg each; 5 and 6: ACTH-stimulated rabbit adrenal RNA, 40 and 20 μg; 7 and 8: rabbit testis RNA, 40 and 20 μg; 9 and 10: control rabbit adrenal RNA, 40 and 20 μg; 11 and 12: rabbit liver RNA, 40 and 20 μg. (A) Rat CYP17 cDNA probe; (B) bovine CYP21 cDNA probe hybridized after stripping the blot in A.

62.5 mM Tris buffer, pH 6.8, containing 2% SDS and 100 mM β-mercaptoethanol.

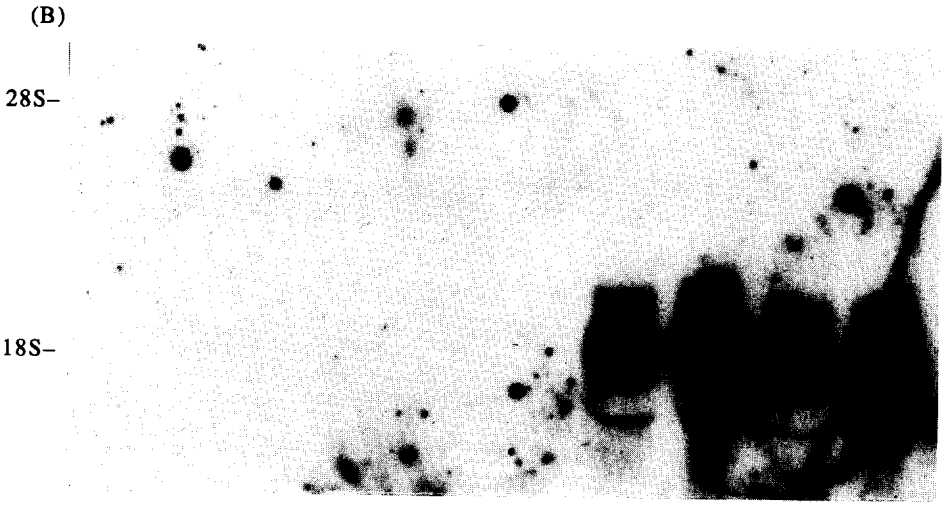
RESULTS

After 4 days of ACTH injection, successful adrenal stimulation was evidenced by a 57% increase in adrenal weight and a 19% increase in total adrenal RNA. There was no apparent difference, however, between CYP17 mRNA levels in rabbits that had been injected with ACTH and those that had been injected with the oil vehicle [Fig. 1(A), lanes 5, 6, 9 and 10]; reprobing the same blot for CYP21 mRNA, which codes for an enzyme activity minimally affected by ACTH stimulation [22, 23], demonstrated equivalent amounts of RNA on the blots [Fig. 1(B)]. Testis and liver RNA were included in Northern analyses as positive and negative controls. Densitometric scanning and the appropriate calculation showed that the amount of CYP17 and CYP21 mRNA per adrenal was the same in both groups: 3.15 ± 1.20 (average deviation of duplicate electrophoretic patterns) vs 3.10 ± 1.56 for CYP17 and 21.8 ± 3.0 vs 19.2 ± 2.3 for CYP21, ACTH-stimulated and control values, respectively, in arbitrary units.

The presence of the same amount of CYP17 mRNA in contol and ACTH-stimulated tissues was unexpected. To address the possibility that the level of stimulation attained was insufficient, an experiment using a 6-day injection period was performed. Figure 2 shows the results of Northern analyses with the CYP17 probe (A) and, after stripping the membrane, with the



CYP 17



CYP 21

Fig. 2. Northern analysis of rabbit CYP17 and CYP21 specific RNA. 6 day ACTH injection period. Lanes 1 and 2: rabbit liver RNA, 20 and 40 μg; 3 and 5, 4 and 6: rabbit testis RNA, 20 and 40 μg, respectively; 7 and 8: control rabbit adrenal RNA, 20 and 40 μg; 9 and 10: ACTH-stimulated rabbit adrenal RNA. (A) Rat CYP17 cDNA probe; (B) bovine CYP21 cDNA probe, as in Fig. 1.

CYP21 probe (B). Comparing lanes 9 and 10, ACTH-stimulated adrenal RNA, with lanes 7 and 8, control adrenal RNA, it is apparent that there is little difference in the amount of CYP17 mRNA [Fig. 2(A)]; this was also true of the amount of CYP21 mRNA [Fig. 2(B)]—the comparable CYP21 mRNA band densities again demonstrating that equivalent amounts of RNA had been applied to the gel. Adrenal stimulation was confirmed by demonstration of a marked increase in *P*450c17 protein by Western analysis (Fig. 3). For comparison, the amount of *P*450c21 protein was determined after stripping and reprobing the membrane, and appeared actually to decrease with ACTH stimulation when expressed on a microgram of microsomal protein basis. This is consistent with earlier reports [22, 23], and reflects the fact that the trophic action of ACTH results in a general increase in the total amount of RNA and

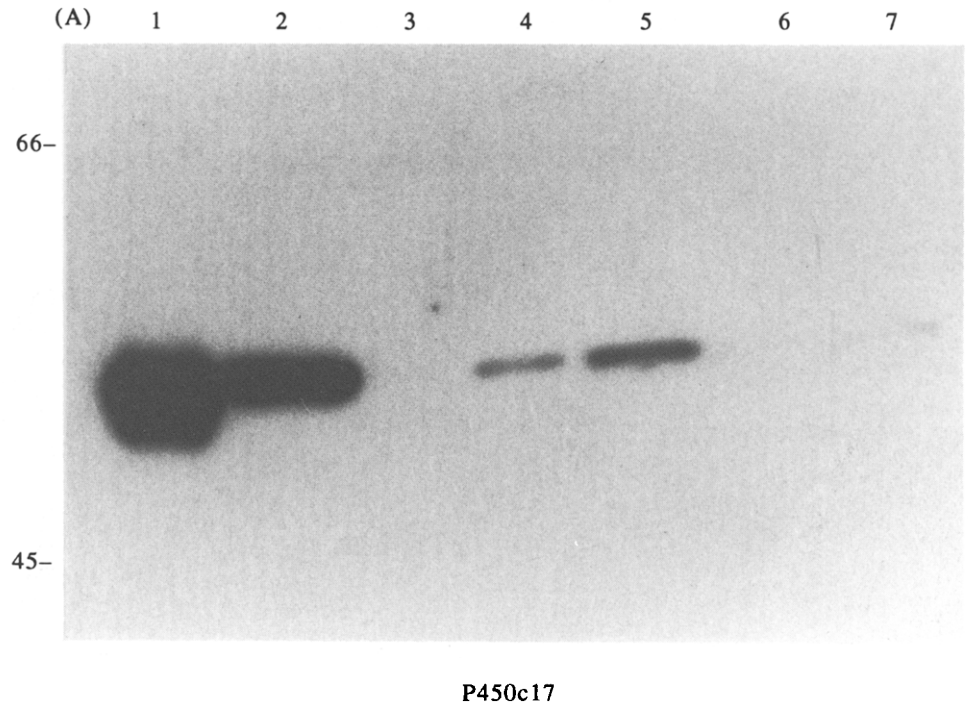


Fig. 3. Western analysis of *P*450c17 and *P*450c21 in guinea pig and rabbit microsomal protein. 6 Day ACTH injection period. Lane 1: guinea pig adrenal, 40 μg; 2: rabbit testis, 40 μg; 3: rabbit liver, 40 μg; 4 and 5: ACTH-stimulated rabbit adrenal, 40 and 80 μg; 6 and 7: vehicle-injected control rabbit adrenal, 40 and 80 μg. (A) Anti-porcine *P*450c17 used as the first antibody; (B) anti-bovine *P*450c21 used as the first antibody after stripping anti-*P*450c17 antibody from the membrane in A.

protein in adrenal cortical cells. Guinea pig adrenal, rabbit testis and rabbit liver microsomal preparations were included in the Western analyses as positive and negative controls.

Because no apparent increase in CYP17 mRNA resulted from 6 days of ACTH stimulation, we were concerned that the trauma from the control injections themselves might have been sufficient to elicit endogenous ACTH secretion, resulting in the presence of CYP17 mRNA in the injected control animals. Therefore, another 6-day stimulation experiment was performed in which a third group of animals, not injected or handled, was included. Armour's Acthar gel, a more potent ACTH preparation, was also used in this experiment. The results of the Northern analyses are shown in Fig. 4 and the quantitative data from densitometric scans in Table 1. On a microgram of RNA basis, the ACTH injected animals (lanes 7 and 8) had essentially the same amount of CYP17 mRNA as did the non-injected controls (lanes 11 and 12), whereas the value for the injected controls (lanes 9 and 10) was 50% higher. The total amount of CYP17 mRNA in the ACTH-stimulated tissue, calculated from the amount of RNA isolated, increased 1.4- and 1.7-fold compared to injected and non-injected controls, respectively. The value for total amount of CYP21 mRNA in ACTH-stimulated tissue is, for unknown reasons, less than for control values. Extreme care was taken to assure that equal amounts of RNA from the three groups of animals were applied to the gels, and ethidium bromide staining gave no contrary indication. Dot blot analyses performed with three different amounts of RNA (data not shown) confirmed the results from Northern analyses.

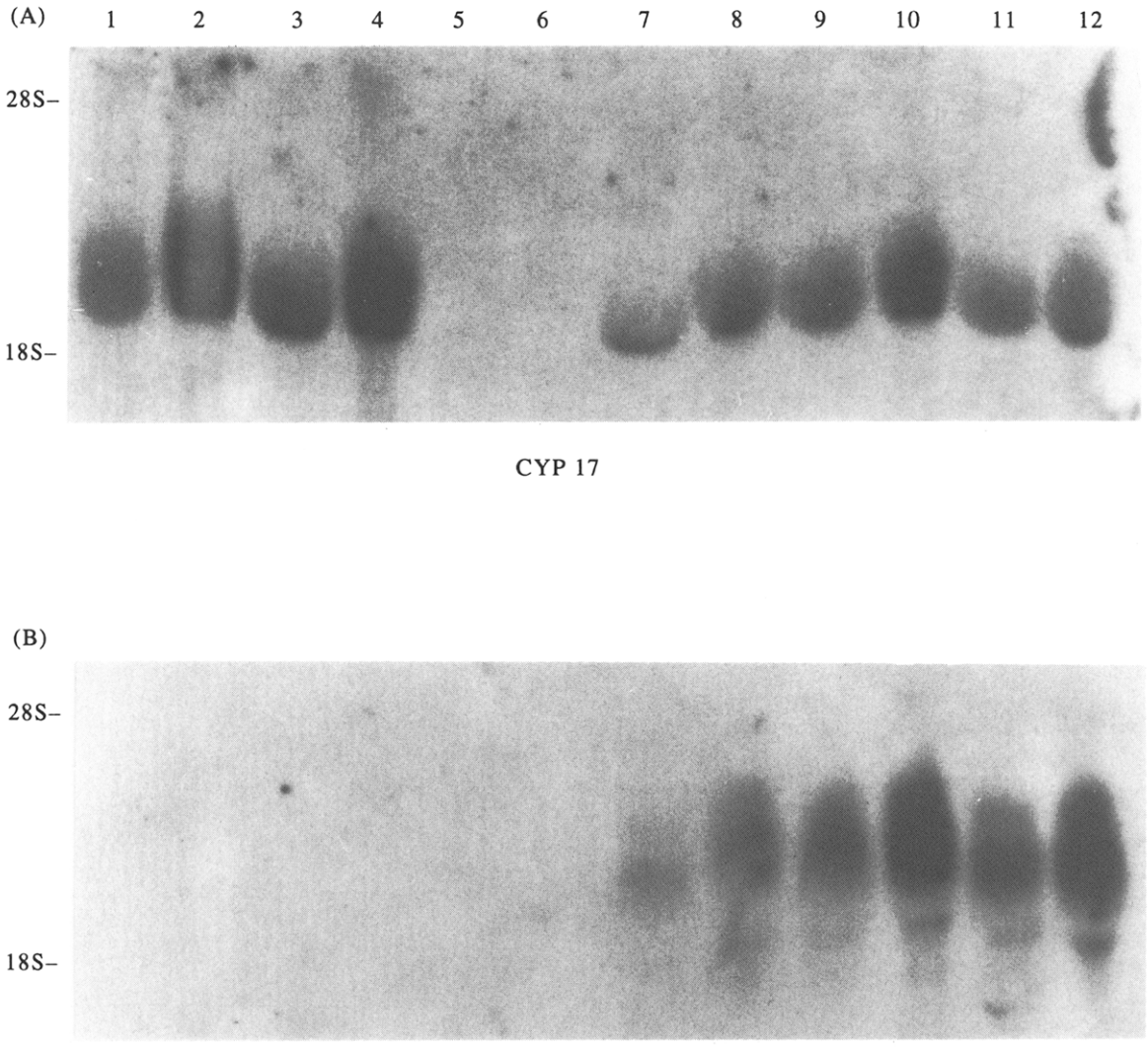


Fig. 4. Northern analysis of rabbit CYP17 and CYP21 specific RNA. 6 Day Acthar injection period. 40 and 80 μg of testis RNA from ACTH-stimulated rabbits (lanes 1 and 2), testis RNA from non-injected control rabbits (3 and 4), rabbit liver RNA (5 and 6), ACTH-stimulated adrenal (7 and 8), vehicle-injected control adrenal RNA (9 and 10), and non-injected control adrenal RNA (11 and 12). (A) Rat CYP17 cDNA probe; (B) bovine CYP21 cDNA probe, as in Fig. 1.

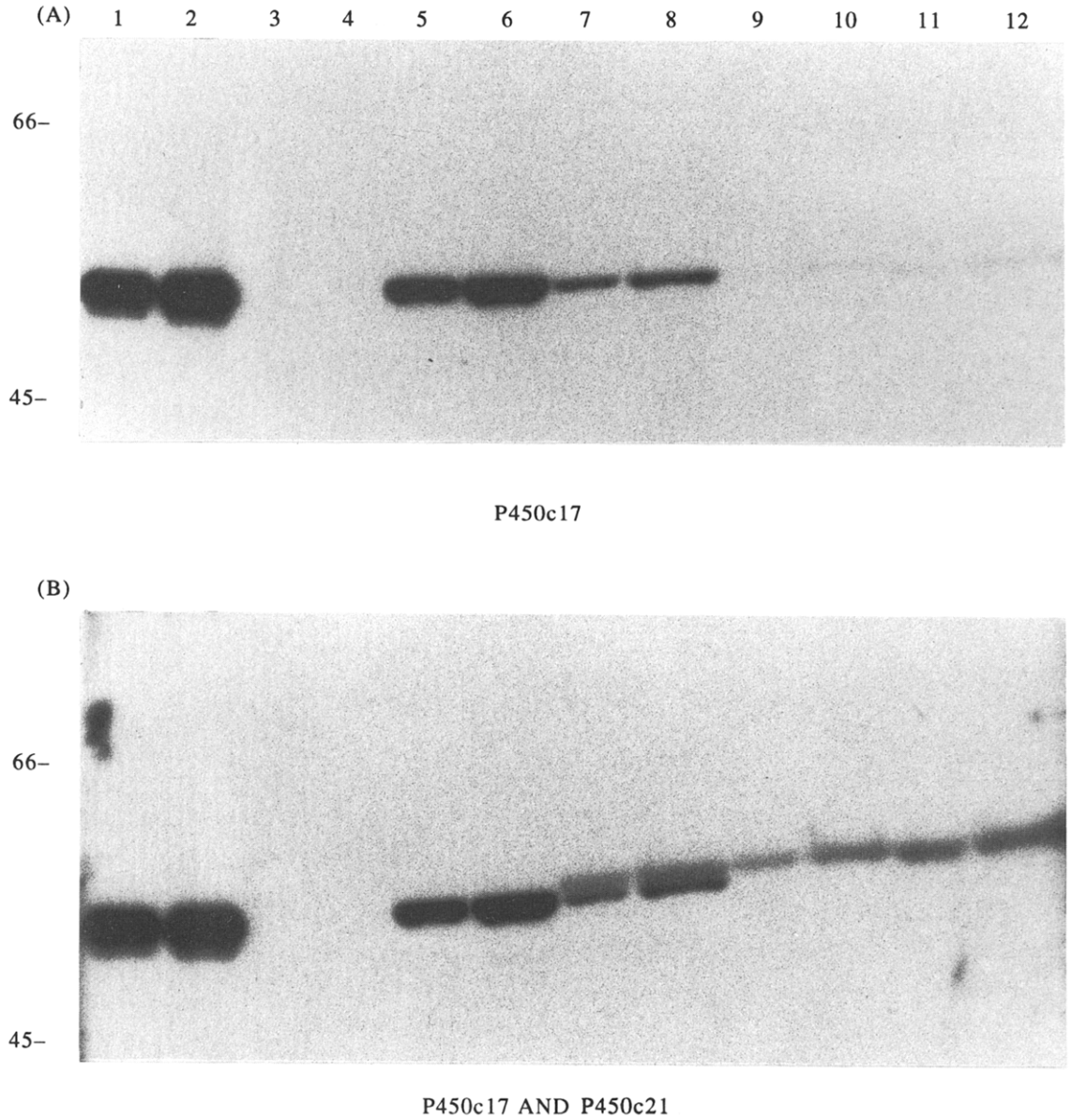


Fig. 5. Western analysis of *P*450c17 and *P*450c21 in guinea pig and rabbit microsomal protein. 6 Day Acthar injection period. 40 and 80 μg of microsomal protein from guinea pig adrenals (lanes 1 and 2), rabbit liver (3 and 4), rabbit testis (5 and 6), ACTH-stimulated rabbit adrenal (7 and 8), vehicle-injected control rabbit adrenal (9 and 10), and non-injected control rabbit adrenal (11 and 12). (A) Anti-porcine *P*450c17 used as first antibody; (B) reprobe with anti-bovine *P*450c21 antibody without prior stripping of the *P*450c17 antibody.

Western analysis of microsomal proteins using a *P*450c17 antibody (Fig. 5) demonstrated a marked increase in the amount of this protein in the tissue from ACTH-stimulated animals (lanes 7 and 8 vs 9–12). Reprobing of the membrane with antibody to *P*450c21 without prior stripping of the *P*450c17 antibody demonstrated that comparable amounts of protein had been applied in all rabbit adrenal samples (lanes 7–12).

Table 1. *Effect of ACTH stimulation on rabbit adrenal CYP17 and CYP21 mRNA*

Animal group	RNA per Adrenal, μg	CYP17[a] per μg RNA	CYP17[a] per Adrenal × 10^{-3}	CYP21 per μg RNA	CYP21 per Adrenal × 10^{-3}
ACTH-injected	293	23.2 ± 0.8	6.8 ± 0.3	17.7 ± 4.7	5.2 ± 1.4
Injected control	127	38.0 ± 1.8	4.8 ± 0.2	60.8 ± 4.4	7.7 ± 0.6
Non-injected control	154	26.6 ± 0.2	4.1 ± 0.0	73.2 ± 1.6	11.2 ± 0.3

[a]Relative intensity from densitometric scans or Northern transfer hybridizations.
[b]Average deviation of determinations on 40 and 80 μg RNA.

From densitometric scans the ratio of the amounts of these *P*450 proteins in adrenals from ACTH-stimulated and non-injected control animals showed a 50.9-fold increase in *P*450c17 protein per adrenal compared to the smaller 1.7-fold increase in mRNA (Table 1). It is apparent that the increase in *P*450c17 protein after ACTH stimulation exceeds the increase in the CYP17 mRNA. For comparison *P*450c21 protein increase 1.8-fold per adrenal over non-injected controls.

DISCUSSION

Our data clearly demonstrate a post-transcriptional stimulation by ACTH of rabbit adrenal cytochrome *P*450c17 expression, since there is no more than a 1.7-fold increase in CYP17 mRNA resulting from ACTH stimulation while the level of *P*450c17 protein increases 50-fold. Whether this is due to an increase in the rate of translation or to a decrease in the rate of *P*450c17 degradation is not known. The information available on regulation of the expression of steroidogenic cytochrome *P*450 enzymes documents transcription as the primary site [2, 3] in tissue culture. However, some evidence exists to suggest alternative sites of control *in vivo*. Provencher *et al.* [24] reported that ACTH caused an increase in both 17-hydroxylase activity and CYP17 mRNA when added to guinea pig adrenal cell cultures. In contrast, when guinea pigs were injected with ACTH for 7 days a 2- to 3-fold increase in 17-hydroxylase activity was accompanied by a decrease in CYP17 mRNA on a microgram of total RNA basis [25]. However, increases in total RNA resulting from the ACTH stimulation can mask an increase in the amount of a specific mRNA when results are reported per microgram of total RNA. Nevertheless, if the increase in total guinea pig adrenal CYP17 mRNA was less than the observed 2- to 3-fold increase of 17-hydroxylase activity, post-transcriptional control would be indicated as suggested by the authors; these reports also demonstrate a difference between *in vitro* (tissue culture) and *in vivo* results. On the other hand, after *in vivo* stimulation of hamster adrenal tissue by ACTH both *P*450c17 protein and mRNA levels increase, suggesting that the rate of protein synthesis is dependent on the mRNA concentration in this species [26].

In vivo stimulation with ACTH has usually produced smaller effects on adrenal 21-hydroxylase and *P*450c21 levels than on 17α-hydroxylase and *P*450c17 in rabbits [8, 22, 23], guinea pigs [25, 27] and hamsters [26]. In the present experiments the effects on *P*450c21 and CYP21 mRNA were minimal (Table 1). The reason for the 1.5- to 2-fold decrease in mRNA per adrenal accompanied by a 1.8-fold increase in *P*450c21 protein is unknown (Fig. 6), but could also indicate post-transcriptional control.

Clearly the validity of the results of our experiments depend on the ability of cDNA probes and antibodies to monospecifically hybridize or bind with homologous nucleotide sequences or proteins from other species. The presence of single bands in both Northern and Western analyses suggests that this is the case and, for the bovine and porcine antibodies, confirms our previous results [8]. The results from the present experiments also confirm our earlier results [8] with regard to the relative apparent molecular weights of the rabbit enzymes compared with those of guinea pig enzymes; the *P*450c17 proteins have equivalent M_r values (52 kDa), whereas the rabbit *P*450c21 has a somewhat greater M_r value (54 kDa) than that of guinea pig *P*450c21 (49 kDa). CYP17 cDNAs from different species also have sufficient sequence identity to allow use of probes from one species to detect the homologous DNA or RNA from another [17], even at relatively high stringency as evidenced in the present studies by the single bands on the Northern analyses.

The results reported here show that the major effect of ACTH stimulation on rabbit adrenal *P*450c17 is post-transcriptional and differs from the primarily transcriptional action reported for several other species. It is possible that this difference reflects both species differences, in the case of the hamster [26], and/or different experimental systems—*in vivo* stimulation in the rabbit versus *in vitro* stimulation of human and bovine cells in culture [9, 10]. In hepatic tissue the induction of *P*450s is largely transcriptional (reviewed in Ref. [1]). However, the expression of the acetone/alcohol inducible hepatic *P*450IIE1 is increased by post-transcriptional mechanisms [28] including increased rate of translation [29, 30], and both mRNA [31] and protein [32] stabilization, the latter involving a hormonally regulated cAMP-dependent phosphorylation mechanism [33]. The possible analogy with a cAMP mechanism resulting from ACTH stimulation is apparent.

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