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The Presence of Two Cytochrome *P*450 Aldosterone Synthase mRNAs in the Hamster Adrenal

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We isolated a cDNA from a hamster adrenal cDNA library which was similar in sequence to those of the mouse and rat $P450_{c18}$ cDNAs. The hamster $P450_{c18}$ cDNA, however, was shorter than the rat and mouse $P450_{c18}$ cDNAs at its 5′-end and the peptide leader sequence was absent. From a hamster genomic library we isolated and sequenced the first seven exons and a 5′-flanking region of the first $P450_{c18}$ gene exon. With this information we were able to generate a $P450_{c18}$ cDNA containing the peptide leader sequence using the polymerase chain reaction. Northern analyses were performed on adrenals from hamsters maintained on a low sodium diet for 0, 4, 7 and 10 days using a ^{32}P-labeled sequence specific to $P450_{c18}$; two mRNA bands were found at 2 and 3.4 kb. The intensity of both bands was increased about 3- to 5-fold under sodium restriction compared to controls. A distinct mRNA band of 2.3 kb hybridized with an oligonucleotide specific to $P450_{11\beta}$ and its intensity did not change following low sodium intake. Immunoblotting analyses were performed using an antibovine adrenal $P450_{11\beta}$ antibody that does not discriminate between $P450_{11\beta}$ and $P450_{c18}$ proteins. Three bands were detected at 52, 48 and 45 kDa in homogenate preparations of entire glands. Furthermore, the 45 kDa protein band was present in homogenates of the zona glomerulosa and absent in homogenates of the zone fasciculata-reticularis. In conclusion, these results show that the hamster adrenals express $P450_{c18}$ as do mouse, rat and human adrenal glands. Furthermore, two $P450_{c18}$ mRNAs, which are inducible by a low sodium intake, are present in the hamster adrenal *vs* one for the rat. The physiological role of these two hamster adrenal mRNA species remains to be elucidated.

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INTRODUCTION

The mineralocorticoid aldosterone, an attribute of terrestrial vertebrates [1], is involved in the homeostasis of sodium and potassium and in the maintenance of the volume of extracellular fluids. Consequently, the involvement of potassium and sodium in the regulation of aldosterone biosynthesis is not surprising. The adrenal cortex of most mammalian species, including human [2–4], mouse [5] and rat [6–10], expresses two distinct enzymes to catalyze the terminal steps of the biosynthesis of glucocorticoids and mineralocorticoids. These enzymes, 11β-hydroxylase ($P450_{11\beta}$) and aldosterone synthase ($P450_{c18}$) are the products of the *CYP11B1* and *CYP11B2* genes. In bovine [11, 12] adrenals, however, the product of a single *CYP11B* gene has been shown to transform both deoxycorticosterone to corticosterone and corticosterone to adlosterone.

We have reported that the rat *CYP11B1* and *CYP11B2* genes are expressed differently in the adrenal [13–17]. Indeed, a low sodium or a high potassium intake increases the levels of the rat adrenal *CYP11B2* mRNA but not that of *CYP11B1* [15, 16]. We further established that these increases in *CYP11B2* mRNA levels are due to a transcriptional activation of the gene [15, 17]. The participation of angiotensin-II in the induction of the *CYP11B2* gene in the rat adrenal by low sodium or high potassium intake was also demonstrated, since an increase in the levels of the *CYP11B2* mRNA was blocked by feeding an inhibitor of the angiotensin conversion enzyme [16, 18].

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We have established that hamster adrenals synthesize the mineralocorticoid aldosterone [19] and the glucocorticoid cortisol [20], and that the regulation of the secretion of these two steroids differs. The regulation of the last steps of aldosterone formation in hamster adrenal had not hitherto been studied.

In this article, we report the expression of $P450_{c18}$ gene in the hamster adrenal. Two $P450_{c18}$ mRNA bands were found by Northern analysis and one mRNA band for $P450_{11\beta}$. The intensity of the two $P450_{c18}$ mRNA bands was increased by a low sodium intake, whereas the $P450_{11\beta}$ band was unchanged.

EXPERIMENTAL

Animals

Male Syrian golden hamsters were purchased from Charles River Canada Inc. (St-Constant, QC, Canada). The animals were fed Purina rat chow and tap water *ad libitum* or were maintained on a sodium-deficient diet (<0.01 mEq Na^+/g; ICN Biochemicals, Cleveland, OH) with demineralized water to drink. After various times on the diet, hamsters were killed by decapitation and blood was collected for hormone analysis. The adrenal glands were removed, freed of fat and used as whole gland, or separated into zona glomerulosa and zona fasciculata-reticularis (with medulla).

RNA isolation, electrophoresis and blotting

Adrenal tissues were homogenized in 15 vol of 7 M guanidine–HCl solution containing 20 mM iodoacetic acid, 1% lauroyl sarcosine and 1 mM EDTA, pH 5, and the RNA was isolated as described previously [21]. Total RNA was denatured with glyoxal [22], electrophoresed in 1% agarose in 0.01 M phosphate buffer pH 6.5 and transferred to GeneScreenPlus™ (Dupont, Canada, Inc., Mississauga, ONT, Canada). The membrane was hybridized for 4 h at 42°C and then hybridized for 16 h at 42°C in the presence of specific [^{32}P]oligonucleotides derived from hamster adrenal $P450_{11\beta}$ and $P450_{c18}$ cDNA sequences. When total RNA was analyzed the blots were also treated with a [^{32}P]ribosomal probe for the quantitation of mRNA. PolyA$^+$ was also prepared by chromatography of total RNA on a small oligo(dT)-cellulose column

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ATG GCA CTC AGG GCA AAG GCA GAT GTG TGG CTG GCA AGA CCC TGG   45
CAG TGC CTG CCC AGG ACG AGG GCA CTG GGC ACC ACA GCA GCA CTG   90
GCC CCC AAC ACA CTG CGG CCC TTT GAA GCC ATA CCG CAG TAC TCC  135
AGA AAC AGG TGG CTG AAG ATG CTA CAG ATC CTG AGG GAG GAG GGC  180
CAA GAG GGC CTG CAC CTG GAG ATG CAT GAG GCC TTC CGG GAG CTG  225
GGG CCC ATT TTC AGG TAC AGC ATG GGA AGA ACA CAG GTT GTG TCT  270
GTG ATG TTG CCA GAG GAT GCC GAG AAG CTG CAC CAG GTG GAG AGT  315
ATG CAC CCT CGT CGG ATG CAC CTG GAA CCT TGG GTA GCC CAC AGA  360
GAA CAC CGT GGC CTG AGT CGT GGA GTG TTC TTG CTA AAT GGG CCT  405
GAA TGG CGC TTC AAC CGA CTG AGG CTC AAC CCA CAC GTG CTG TCC  450
CCA AAG GCC GTT CAG AAG TTT GTC CCC ATG GTG GAC ATG GTA GCA  495
CGG GAC TTC TTG GAG TCC CTG AAG AAG AAG GTG TTT CAG AAT GCT  540
CGT GGG AGC CTC ACC ATG GAT GTG CAG CAA AGC CTT TTT AAC TAC  585
AGT ATA GAA GCC AGC AAC TTT GTT CTT TTT GGG GAG CGG CTG GGA  630
CTC CTT GGC CAT GAC CTG AGC CCT GCC AGC CTG ACG TTC ATC CAT  675
GCC TTG CAT TCC GTG TTC AAG ACG ACC CCA CAG CTC ATG TTC TTG  720
CCC AGG AGC CTG ACT CGC TGG ACA AGC ACC CGG GTG TGG AAA GAG  765
CAT TTT GAG GCC TGG GAT GTC ATC TCT GAG TAT GTC AAC AGA TGT  810
ATC CGG AAG GTG CAC CAG GAG CTC AGA CTT GGC AGC CCT CAC ACC  855
TAC AGT GGC ATC GTG GCA GAA CTA ATG TCC CAG GGA GCT TTG CCT  900
CTC GAC GCC ATC AGA GCC AAC TCA ATT GAG CTC ACC GCT GGG AGT  945
GTA GAC ACG ACA ACC TTC CCC CTG GTC ATG GCT CTC TTT GAG CTG  990
GCT CGG AAC CCA GAT GTT CAG CAG GCT GTG CGG CAG GAG AGC CTG 1035
GCA GCT GAG GCC AGC GTG GCT GCA AAT CCC CAG AGG GCT ATG TCG 1080
GAT CTG CCC CTG CTG CGG GCT GTC CTT AAA GAG ACC TTG AGG CTC 1125
TAT CCT GTT GGT GGC TTT TTG GAG AGA ATT CTA AGC TCG GAC TTG 1170
GTG CTT CAG AAC TAC CAC GTC CCT GCT GGG ACA TTG GTC CTA CTT 1215
TAT CTC TAC TCC ATG GGC CGA AAC CCT GCA GTA TTT CCG AGG CCC 1260
GAG CAC TAC TTG CCC CAG CGC TGG CTG GAG AGG AAT GGG AGT TTC 1305
CAG CAC CTG ACC TTC GGC TTT GGG GTG CGC CAG TGC CTG GGG AAG 1350
CGC CTG GCT CAG GTG GAG ATG CTC CTC CTG CTG CAC CAT GTG CTG 1395
AAA TCC TTC AGG GTG GAG ACG CAG GAG CGA GAG GAT GTG CGG ATG 1440
GTG TAC CGC TTT GTT CTG GCG CCC AGC TCC AGC CCC CTG CTC ACT 1485
TTC CGG CCT GTC AGC TAG  1503
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Fig. 1. Primary structure of the hamster adrenal $P450_{c18}$ cDNA. Base number starts at the initiating methionine.

(Pharmacia Biotechnology Inc. Baie d'Urfé, QC, Canada) according to the published protocol. A ^{35}S-labeled kb ladder was used to determine the mass of the hybridized mRNA.

Immunoblotting

Adrenal tissues were homogenized and solubilized in Laemmli buffer [23]. They were passed through a 26-gauge needle and then boiled for 5 min. Proteins were electrophoresed on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate, as described by Laemmli [23], and subsequently transferred onto nitrocellulose according to the method of Towbin *et al.* [24]. Cytochrome $P450_{11\beta}$ was detected with an antibovine adrenal cytochrome $P450_{11\beta}$ antibody (kindly provided by Dr M. R. Waterman, School of Medicine, Department of Biochemistry, Nashville, TN, U.S.A.). [^{125}I]Protein-A (Dupont Canada, Inc.) and autoradiography was used to analyze antibody–antigen complexes.

Plasma aldosterone, corticosterone and cortisol determination

Specific antibodies, obtained from ICN Immunobiological (Lisle, IL, U.S.A.), ICN Biochemical, and Kallestad Labs, Inc. (Austin, TX, U.S.A.), were used to determine plasma aldosterone, corticosterone, and cortisol.

RESULTS

In preliminary experiments, using a mouse adrenal $P450_{11\beta}$ cDNA probe, we screened and isolated two cDNAs from a hamster adrenal cDNA library, which were similar in sequence to the mouse and rat $P450_{11\beta}$ (unpublished data) and rat $P450_{c18}$. Compared with these species, however, the hamster $P450_{c18}$ cDNA lacked 380 bp in its 5′-end. Subsequently, from a hamster genomic library we isolated and sequenced a DNA similar to the mouse and rat $P450_{c18}$ genes. With this information we were able to generate a $P450_{c18}$ cDNA containing the peptide leader sequence by the polymerase chain reaction. Figure 1 shows the sequence of this $P450_{c18}$ cDNA.

Using a specific ^{32}P-labeled oligonucleotide derived from the hamster $P450_{c18}$ cDNA (5′-ACAGTGGCATCGTGGCAGAACTAATGTCCCAGGGA-3′), two mRNA bands were detected at 2.0 and 3.4 kb by Northern blotting analysis in the hamster adrenal zone glomerulosa (Fig. 2). On the same membrane, the oligonucelotide (5′-TGCAAAGTGGTGGCCCACAGTCCTGGAGTGTCATATCACAGCTGGT-3′) specific for $P450_{11\beta}$ hybridized to a single mRNA band at 2.3 kb (Fig. 2), which clearly differed from those revealed by the $P450_{c18}$ probe.

We previously reported that two protein bands of 52 and 48 kDa reacted with a bovine adrenal cytochrome $P450_{11\beta}$ antibody [20] when hamster adrenal mitochondrial proteins were analyzed by Western blotting. Figure 3 shows that the same antibovine cytochrome $P450_{11\beta}$ antibody also coupled to an additional band at 45 kDa when a whole gland homogenate was analyzed. The 45 kDa protein was present in homogenates of the zona glomerulosa but absent from the zona fasciculata-reticularis. In contrast the 52 kDa band was only present in homogenates of zona fasciculata-reticularis, showing a differential adrenal zone distribution for the 45 and 52 kDa protein.

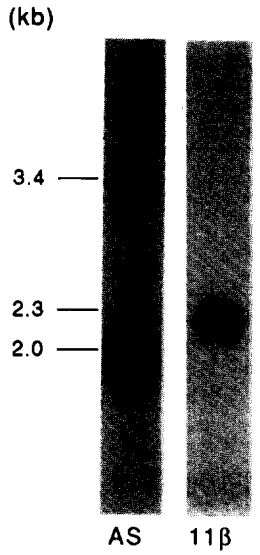

Fig. 2. Northern analysis of hamster adrenal zona glomerulosa cytochrome $P450_{c18}$, and cytochrome $P450_{11\beta}$. Total RNA was electrophoresed, transferred onto GeneScreenPlus membrane and hybridized with ^{32}P-labeled oligonucleotides specific to $P450_{c18}$ (AS) and $P450_{11\beta}$ (11β).

We then evaluated the effects of a low sodium diet on hamster adrenal function. Hamster plasma aldosterone levels were elevated after 2, 4, 7 and 10 days of low sodium intake, whereas cortisol and corticosterone levels were similar to controls, indicating a different regulation for these corticosteroids (Table 1). Northern analyses were performed on separated adrenal zones of hamsters which had been sodium restricted for 0, 4, 7 and 10 days. A cDNA specific for the 3′-end of

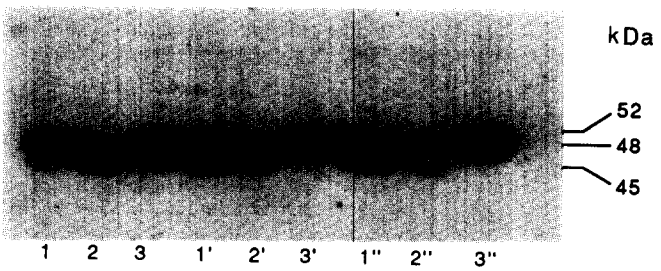

Fig. 3. Immunoblotting analysis of hamster adrenal cytochrome $P450_{11\beta}$. Adrenal tissues were homogenized and solubilized in sodium dodecyl sulfate and then electrophoresed in a 10% polyacrylamide gel. Cytochrome $P450_{11\beta}$ was detected with an antibovine adrenal cytochrome $P450_{11\beta}$ that recognizes both $P450_{11\beta}$ and $P450_{c18}$. 1,1′,1″: whole gland; 2,2′,2″: zona glomerulosa; 3,3′,3″: zona fasciculata-reticularis.

Table 1. Effects of a low sodium intake on the levels of hamster plasma aldosterone, corticosterone and cortisol

Time (days)	Aldosterone (ng/dl)	Corticosterone (μg/dl)	Cortisol (μg/dl)
0	5.4	1.8	0.41
2	12.8	2.5	0.61
4	42.6	ND	0.04
7	69.8	ND	0.34
10	63.9	3.7	0.33

Groups of hamsters were fed a low sodium diet and killed at times 0, 2, 4, 7 and 10 days. Steriods were analyzed by radioimmunoassay. Groups 0, 2 and 10 days, $n = 3$; groups 4 and 7 days, $n = 1$; ND = not determined.

$P450_{c18}$ also demonstrated the two above mentioned mRNA bands. The upper 3.4 kb band was less abundant than the lower 2.0 kb band but the intensity of both bands increased with the duration of the treatments (Fig. 4), suggesting a functional role for these two mRNAs. In this series of experiments, a similar pattern was found in the zona fasciculata-reticularis which is not surprising since, in contrast to the rat, it is difficult to cleanly separate the hamster adrenal zona glomerulosa from the zona fasciculata-reticularis. In another series of experiments, however, we have been able to better separate hamster adrenal zona glomerulosa from zona fasciculata-reticularis. In this case, as shown in Fig. 5, $P450_{c18}$ was mainly located in the zona glomerulosa, whereas $P450_{11\beta}$ was nearly equally distributed in both zones. The lower panel of Fig. 5 shows the results of hybridization of the same membrane with a 28 S ribosomal probe to measure the relative quantity of RNA in each lane. After correction, with the 28 S values, we calculated that the low sodium intake for 7 days provoked 3.5- and 5-fold increases

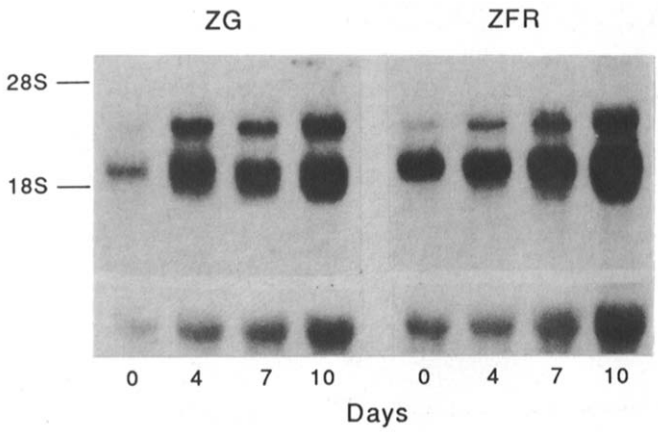

Fig. 4. Effects of a low sodium intake on $P450_{c18}$ mRNA in hamster adrenals. Groups of hamsters were fed on low sodium diet for 0, 4, 7, and 10 days. Total RNA was extracted from the adrenal zona glomerulosa (ZG) and the zona fasciculata-reticularis (ZFR). 20 μg of RNA was analyzed by Northern blotting. A ^{32}P-labeled cDNA probe specific to the 3′-end of hamster adrenal $P450_{c18}$ was used for hybridization (upper panels). The same membranes were also analyzed with a 28 S ribosomal probe (lower panels).

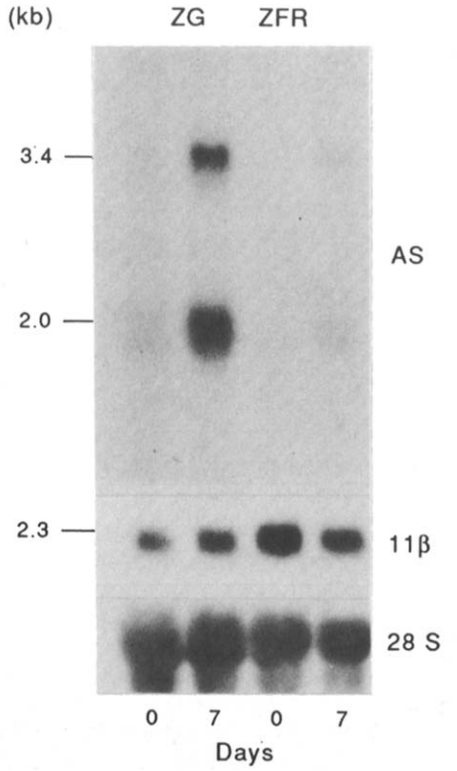

Fig. 5. Effects of low sodium intake on $P450_{c18}$ and $P450_{11\beta}$ mRNAs in hamster adrenals. Groups of hamsters were fed a low sodium diet for 0 and 7 days. Total RNA was extracted from the adrenal zona glomerulosa (ZG) and the zona fasciculata-reticularis (ZFR). 5 μg of RNA was analyzed by Northern blotting. Membranes were probed with ^{32}P-labeled oligonucleotides specific to $P450_{c18}$ (AS) and $P450_{11\beta}$ (11β). Membranes were also hybridized with a 28 S ribosomal probe (28 S).

in the level of the 2 and 3.5 kb $P450_{c18}$ mRNA, respectively in zona glomerulosa. In the zona glomerulosa the mRNA level of $P450_{11\beta}$ was 1.0 for control and 1.3-fold for the low sodium intake (Fig. 5, middle panel); also, in another series of experiments this level was 1.0 for control and 0.8 for the low sodium intake. In the zona fasciculata-reticularis the mRNA level of $P450_{11\beta}$ was 1.0 for control and 0.6 for the low sodium intake group; also in another series of experiments, this level remained near control value for the low sodium intake group (1.1-fold), indicating that sodium restriction was without effect on the expression of $P450_{11\beta}$ gene in hamster adrenal.

DISCUSSION

The hamster adrenal expresses a gene whose product is closely akin to the mouse [5], rat [6–10] and human [2–4] $P450_{c18}$. The homology of $P450_{c18}$ cDNA and $P450_{c18}$ amino acid sequences between the hamster, rat, human and bovine genes and proteins are shown in Fig. 6 and Table 2. Table 2 also shows the comparisons between $P450_{11\beta}$ and $P450_{c18}$. It can be seen that the hamster adrenal $P450$ cDNA and amino acid sequences

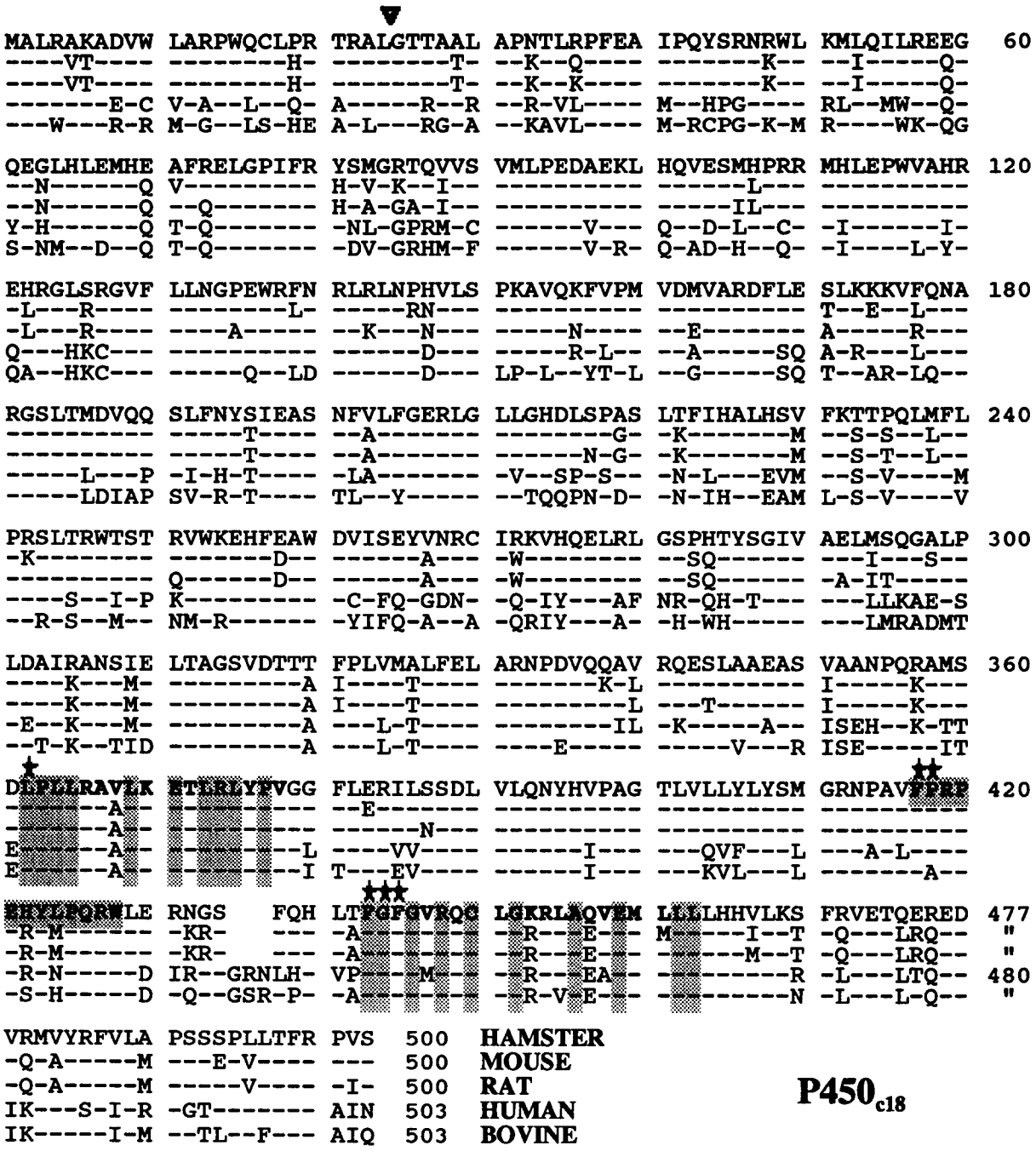

Fig. 6. Comparison of the amino acid sequence of hamster $P450_{c18}$ with that of mouse, rat, human and bovine enzymes. The peptides with one, two, and three asterisks are the putative heme/steroid binding site, the Ozol's tridecapeptide and the putative heme binding site, respectively. The arrow indicates the cleavage site for the processing enzyme.

are most closely related to those of mouse and rat, a little less so with human and still less with bovine adrenals, indicating that, as is to be expected, hamsters are more closely related to mice than to humans. The cDNA coding sequence and the deduced amino acid sequence of hamster $P450_{11\beta}$ and $P450_{c18}$ are highly homologous indicating that these two cytochromes originated from a common ancestral gene. When compared to the bovine $P450_{11\beta}$, the homology of hamster $P450_{c18}$ is 74% for cDNA and 65% for amino acid sequences. This homology is, however, higher than that between the hamster $P450_{11\beta}$ and bovine $P450_{11\beta}$.

Northern analyses of total hamster adrenal mRNA showed two mRNA bands that hybridized to an oligonucleotide specific to $P450_{c18}$. When polyA$^+$ mRNA was analyzed, the two mRNA species at 2.0 and 3.4 kb were also found, and the ratio between the two bands was the same as for total mRNA, indicating that both species were polyadenylated. In the

Table 2. Hamster adrenal $P450_{11\beta}$ and $P450_{c18}$ cDNA (coding region) and amino acid sequences: % homology within species

Species	Hamster $P450_{11\beta}$ cDNA (%)	Hamster $P450_{11\beta}$ Amino acids (%)	Hamster $P450_{c18}$ cDNA (%)	Hamster $P450_{c18}$ Amino acids (%)	Refs
Hamster $P450_{11\beta}$	100	100	90	84	
Mouse $P450_{11\beta}$	84	77	82	76	[5]
Rat $P450_{11\beta}$	82	74	81	75	[6, 28]
Human $P450_{11\beta}$	73	63	76	68	[2, 25]
Bovine $P450_{11\beta}$	72	60	74	65	[26, 29]
Hamster $P450_{c18}$	90	84	100	100	
Mouse $P450_{c18}$	81	75	89	86	[5]
Rat $P450_{c18}$	81	75	88	85	[7, 28]
Human $P450_{c18}$	73	63	76	69	[2, 27]

Homology was determined using DNASIS and PROSIS programs.

rat, using specific oligonucleotides, [16] only a single $P450_{c18}$ mRNA species was demonstrated, indicating a difference between these two quite closely related species.

We isolated and sequenced a short $P450_{c18}$ cDNA from a hamster adrenal cDNA library. This cDNA was shorter at its 5′-end by 380 bp compared to the cDNA generated by PCR. The short cDNA, however, possesses an ATG at the beginning of the NH_2-terminal, in frame with the full length coding sequence. It is tempting to speculate that the short cDNA originates from one of the two $P450_{c18}$ detected by Northern analysis. As we have not yet expressed this short cDNA, we consequently cannot say whether it will be translated into an active protein. However, as truncated cDNA are present in many libraries and are artefacts of library construction, we will screen another hamster adrenal cDNA library and if the short $P450_{c18}$ cDNA is still present we will study the product of its expression in COS cells. The Western analysis indicates that the zona glomerulosa protein reacting with the antibovine $P450_{11\beta}$ antibody might be extramitochondrial. An intracellular distribution study of this component will be done to clarify this point. We will also develop an antibody against a specific amino acid sequence deduced from the hamster $P450_{c18}$ cDNA sequence, which could discriminate between $P450_{11\beta}$ and $P450_{c18}$. The use of this tool will help us to determine the exact intracellular distribution of $P450_{c18}$ in hamster adrenal and to discriminate between $P450_{11\beta}$ and $P450_{c18}$ protein bands, when analyzed by Western.

Hamster adrenals responded to a low sodium diet by increasing the levels of plasma aldosterone, but not that of plasma corticosterone and cortisol, indicating that this deficient intake affected the mineralocorticoid pathway but not that of glucocorticoid formation. Moreover, the low sodium intake selectively affected the mRNA of $P450_{c18}$ but not that of $P450_{11\beta}$, showing specific regulation at the final step of aldosterone synthesis which is mediated by sodium restriction. These results are in agreement with previous findings demonstrating that low sodium intake affected the expression of $P450_{c18}$ but not that of $P450_{11\beta}$ in the rat adrenal [16].

In the rat adrenal the effects of sodium restriction on $P450_{c18}$ are mediated by angiotensin-II, since they are blocked by feeding an inhibitor of its convertion enzyme [16]. In the hamster, angiotensin-II is also involved in the control of aldosterone. Indeed, angiotensin-II produced dose-dependent increases in aldosterone output in hamster adrenal cell suspensions [19]. In contrast, the same system ACTH induced no changes in aldosterone output whereas it produced dose-dependent increases in corticosteroid output. Moreover, *in vivo*, a sustained ACTH stimulus resulted in a 48% decrease in the capacity of hamster mitochondrial preparations to transform corticosterone to aldosterone [20]. These results clearly demonstrate the differential effects of angiotensin-II and ACTH on the last step of aldosterone formation.

Collectively these results show that the hamster adrenal expresses $P450_{c18}$. This P450 is more closely related to murine than to human and bovine species. The hamster also possesses an additional adrenal $P450_{c18}$ mRNA species, inducible by a low sodium intake, the physiological relevance of which is yet to be established.

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