European Journal of Endocrinology (2002) **147** 795–802 ISSN 0804-4643

CLINICAL STUDY

Quantitative assessment of *CYP11B1* and *CYP11B2* expression in aldosterone-producing adenomas

F Fallo, V Pezzi[1], L Barzon[2], P Mulatero[3], F Veglio[3], N Sonino and J M Mathis[4]

Department of Medical and Surgical Sciences, Division of Endocrinology, University of Padua, Via Ospedale 105, 35128 Padua, Italy, [1]Department of Pharmaco-Biology, University of Calabria, Arcavacata di Rende, Italy, [2]Department of Histology, Microbiology and Medical Biotechnologies, University of Padua, Padua, Italy, [3]Department of Medicine and Experimental Oncology, University of Turin, Turin, Italy and [4]Department of Cellular Biology and Anatomy, LSU Health Sciences Center, Shreveport, Louisiana, USA

(Correspondence should be addressed to F Fallo; Email: francesco.fallo@unipd.it)

Abstract

Background: The presence and pathophysiological role of *CYP11B1* (11β-hydroxylase) gene in the zona glomerulosa of human adrenal cortex is still controversial.
Methods: In order to specifically quantify *CYP11B1*, *CYP11B2* (aldosterone synthase) and *CYP17*(17α-hydroxylase) mRNA levels, we developed a real-time RT-PCR assay and examined the expression in a series of adrenal tissues, including six normal adrenals from patients adrenalectomized for renal cancer and twelve aldosterone-producing adenomas (APA) from patients with primary aldosteronism.
Results: *CYP11B1* mRNA levels were clearly detected in normal adrenals, which comprised both zona glomerulosa and fasciculata/reticularis cells, but were also measured at a lower range ($P < 0.05$) in APA. The levels of *CYP11B2* mRNA were lower ($P < 0.005$) in normal adrenals than in APA. *CYP17* mRNAlevels were similar in normal adrenals and in APA. In patients with APA, *CYP11B2* and *CYP11B1* mRNA levels were not correlated either with basal aldosterone or with the change from basal aldosterone in response to posture or to dexamethasone. No correlation between *CYP11B1* mRNA or *CYP11B2* mRNA and the percentage of zona fasciculata-like cells was observed in APA.
Conclusions: Real-time RT-PCR can be reliably used to quantify *CYP11B1* and *CYP11B2* mRNA levels in adrenal tissues. Expression of *CYP11B1* in hyperfunctioning zona glomerulosa suggests an additional formation of corticosterone via 11β-hydroxylase, providing further substrate for aldosterone biosynthesis. *CYP11B1* and *CYP11B2* mRNA levels in APA are not related to the *in vivo* secretory activity of glomerulosa cells, where post-transcriptional factors might ultimately regulate aldosterone production.

European Journal of Endocrinology **147** 795–802

Introduction

In the human adrenal cortex, cortisol and aldosterone are synthesized by the isozymes CYP11B1 (11β-hydroxylase) and CYP11B2 (aldosterone synthase) respectively. CYP11B1 catalyzes the 11β-hydroxylation of 11-deoxycortisol to cortisol and of 11-deoxycorticosterone (DOC) to corticosterone and is regulated by adrenocorticotropin (ACTH) (1, 2). CYP11B2 catalyzes the 11β-hydroxylation of DOC to corticosterone, the 18-hydroxylation of corticosterone to 18-hydroxycorticosterone (18-OHB), and the 18-oxidation of 18-OHB to aldosterone, predominantly under the control of angiotensin II and K^+ (1–3). The two enzymes are respectively encoded by the *CYP11B1* and *CYP11B2* genes, which are 95% identical in coding sequence (4) and are located in close proximity (5). It is well accepted that the *CYP11B2* gene is exclusively expressed in the zona glomerulosa and that the *CYP11B1* transcript is localized in the zona fasciculata/reticularis of human adrenal cortex (6). The presence and pathophysiological role of the *CYP11B1* gene in the zona glomerulosa are still controversial. Recent studies with conventional reverse transcription-polymerase chain reaction (RT-PCR) (3, 7) and *in situ* hybridization (8, 9) have shown the presence of *CYP11B1* mRNA in human aldosterone-producing adenomas (APA), which are commonly believed to originate from glomerulosa cells, while others have not (10). To further investigate this issue, we quantified by real-time RT-PCR the *CYP11B1* and *CYP11B2* mRNA levels in a series of adrenal tissues. In comparison with conventional RT-PCR, this method allows an absolute quantitation, reduces many sources of error, and is much less labour and reagent

intensive (11). In addition, levels of *CYP11B1* and *CYP11B2* transcripts in APA were correlated with both the cell histotype of tumors and aldosterone response to dynamic tests *in vivo*.

Materials and methods

Patients

Twelve patients (eight women and four men, aged 31–64 years) with APA were studied at our centres (Table 1). All were hypertensive and had hypokalemia of varying degree, compared with the normal patients. Plasma renin activity (PRA) was suppressed and unresponsive to stimuli such as upright posture and captopril administration, and the plasma aldosterone (pmol/l)/PRA ratio (ng/ml/h) was greater than 1385 (i.e. 50 expressing aldosterone as ng/dl). The differential diagnosis between APA and hyperaldosteronism due to bilateral idiopathic adrenal hyperplasia was made by computerized axial tomography, adrenal scintiscan with ^{75}Se-methyl-nor-cholesterol after dexamethasone suppression, and/or aldosterone/cortisol ratio measurements in adrenal venous blood (12). Glucocorticoid-remediable aldosteronism associated with adrenal tumors was excluded by either negative long PCR or Southern blotting for the chimeric gene in leukocyte DNA, as previously described (13). The subjects were on a diet containing 120–150 mmol sodium and 60 mmol potassium daily for 2 weeks, and all medications, including spironolactone, were withdrawn for at least 2 weeks before the diagnostic tests. None of the patients had any other disease. Specifically, the patients with APA underwent: (a) postural test for PRA and plasma aldosterone defined by the hormone response, after overnight recumbency, to 2 h upright position from 0800 to 1000 h; and (b) supine plasma aldosterone change at 0800 h after short-term dexamethasone administration trial (0.5 mg 6-hourly for 4 days), with plasma cortisol suppression, i.e. <138 mmol/l, as index of the dexamethasone effect. All patients underwent unilateral adrenalectomy, and the diagnosis of adenoma was surgically confirmed. After surgery, all APA patients showed normalization of serum potassium and hormone levels, with restoration of a normal aldosterone response to upright posture. Blood pressure normalization or satisfactory control by low-dose conventional anti-hypertensive drugs paralleled restoration of electrolyte and hormonal pattern. Six adrenal glands were obtained from patients undergoing unilateral expanded nephrectomy for kidney cancer and used as controls (Table 1); none of these patients (four men and two women, aged 47–66 years) had clinical symptoms of adrenal dysfunction. All patients gave informed consent before participating in this study, which was approved by the local Ethics Committees.

Tissues

After removal of adrenal tissue from patients who underwent unilateral adrenalectomy for APA or nephrectomy for renal cancer, a portion was frozen

Table 1 Details of patients with APA and normal adrenals.

Patient no.	Age/sex	BP (mmHg)	K (mmol/l)	Sup/Upr PRA (ng/ml per h)	Sup/Upr ALDO (pmol/l)	Post-dex ALDO (pmol/l)	Tumor size (cm)	ZF-like cells (%)
APA								
1	43/M	205/110	2.8	0.1/0.1	1120/1310	980	1.6	90
2	57/F	190/110	3.0	0.2/0.1	1240/1080	1280	1.1	95
3	31/M	200/100	3.1	0.1/0.1	1688/2120	1330	2.4	80
4	64/F	210/110	2.7	0.1/0.1	920/1110	910	1	35
5	44/M	190/100	3.3	0.2/0.2	1775/2040	1460	2.2	50
6	53/F	200/120	2.5	0.1/0.2	1390/1270	1810	1.5	95
7	51/F	170/100	3.0	0.1/0.1	620/1250	410	1.5	70
8	42/F	205/115	3.4	0.2/0.1	760/1190	610	1.8	85
9	39/M	190/110	2.8	0.1/0.1	1800/1560	1740	2.4	90
10	58/F	210/120	2.6	0.1/0.3	2240/2010	2560	1.2	70
11	41/F	200/100	2.8	0.2/0.2	720/1270	940	2	30
12	37/F	190/110	3.2	0.1/0.1	1380/1060	730	1.7	80
Means±S.E.M.	48±3	197±3/108±2	2.9±0.1	0.1±0.01/0.1±0.02	1304±146/1439±114	1230±158	1.7±0.1	72±7
Normal								
1	61/M	140/90	4.4	0.8/2.8	128/610			
2	66/M	160/100	4.8	2.1/4.9	180/580			
3	54/M	135/80	4.0	3.0/5.2	244/760			
4	58/M	150/80	4.4	1.6/3.8	240/690			
5	63/F	130/90	4.1	1.4/3.6	310/520			
6	47/F	140/80	4.5	1.0/2.2	110/410			
Means±S.E.M.	58±3	143±3/86±3	4.4±0.1	1.7±0.8/3.7±0.5	202±31/595±51			

BP, blood-pressure; Sup/Upr PRA, supine/upright plasma renin activity; Sup/Upr ALDO, supine/upright plasma aldosterone; Post-dex ALDO, aldosterone levels after dexamethasone; ZF, zona fasciculata.

immediately in liquid nitrogen and stored at −80 °C until processing. The adrenal cortex was separated visually from adrenal medulla, while it was not possible to obtain an adequate separation of the outer zone, i.e. the glomerulosa part, from the remaining tissue of normal adrenal specimen. The central portion of each APA was studied. The adenomas weighed between 2.8 and 15.7 g.

Hormone assays

Plasma aldosterone and PRA were determined by radioimmunoassay (RIA), with kits purchased from Sorin Biomedical Diagnostics (Vercelli, Italy). The intra- and interassay coefficients of variation (CV) for aldosterone were 7.9% and 9.6% respectively; the normal range is 55–330 pmol/l supine and 140–830 pmol/l upright. The intra- and interassay CV values for PRA were 5.4% and 9.1% respectively; the normal range is 0.4–3.0 ng/ml per h supine and 1.5–6 ng/ml per h upright. Plasma cortisol was measured using an RIA kit from Diagnostic Products (Los Angeles, CA, USA). Intra- and interassay CV values were 4.1 and 5.0% respectively; the normal range is 138–550 nmol/l.

Histology

Adrenal tissues were fixed in neutral formalin and paraffin embedded: 3–5 μm tissue sections were stained with hematoxylin–eosin, periodic acid–Schiff (PAS) and PAS–diastase reactions. Histological examination revealed the absence of tumor in the adrenal tissue specimen collected from nephrectomized subjects. The adrenal cells from confirmed APA were classified microscopically into different types, following the morphological criteria of Neville & O'Hare (14). Two main populations were considered. A first population included zona fasciculata-like cells, which are clear cells having large vacuolated lipid-laden cytoplasm and central round nuclei very similar to the normal counterpart cells of the fasciculata zone. A second population comprised non-zona fasciculata-like cells, including: (a) compact eosinophilic cells similar to those normally seen in the reticularis zone; (b) cells showing variable contents of clear lipid micro-vacuoles and granular eosinophilic cytoplasms, referred to as intermediate-type (hybrid) cells because of sharing some features of glomerulosa and some of fasciculata cells; and (c) lipid-poor glomerulosa-type cells. Cells were examined by the same observer from at least ten high-power fields (×250) randomly, and the percentage of zona fasciculata-like cells vs non-zona fasciculata-like cells were calculated.

Molecular analysis

Total RNA was isolated from tumor portions of APA specimens, and from whole adrenal glands of nephrectomized patients using TRIZOL reagent according to the protocol provided by the manufacturer (Molecular Research Center, Cincinnati, OH, USA). Single-strand cDNA was prepared from 250 ng cell or tissue total RNA using a TaqMan reverse transcription kit (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA, USA). The reverse transcription reaction was initiated using random hexamer primers and the reaction were carried out at 48 °C for 30 min, followed by heat inactivation at 95 °C for 5 min.

Human CYP11B1, CYP11B2 and CYP17 cDNA templates The human *CYP11B1* cDNA (GenBank ID NM 000498) and *CYP11B2* cDNA (GenBank ID NM 000498), sequences from +1208 to +1522, and the human *CYP17* cDNA (GenBank ID NM 000102) sequence from +341 to +403 were isolated by PCR and subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). The identity of each clone was confirmed by sequence analysis. Serial dilution of these plasmids marked as p*CYP11B1*, p*CYP11B2* and p*CYP17* were used as template to generate standard curves for absolute quantification of the expression of *CYP11B1*, *CYP11B2* and *CYP11B17* genes respectively, by real-time RT-PCR.

Primers, probes, and real-time PCR The primers and probes for the *CYP11B1*, *CYP11B2*, and *CYP17* genes were determined with the assistance of the computer program Primer Express (Perkin Elmer Applied Biosystems), which selected the theoretically optimized sequences for this system. Primer pairs were selected so that they were located on different exons to prevent amplification from any contaminating genomic DNA. The same forward amplification primer 5′-GGCAGAGGCAGAGATGCTG-3′ for both the *CYP11B1* and *CYP11B2* genes and different reverse primers 5′-TCTTGGGTTAGTGTCTCCACCTG-3′ for *CYP11B1* and 5′-CTTGAGTTAGTGTCTCCACCAGGA-3′ for *CYP11B2* were respectively used. The sequence of probes for *CYP11B1* and *CYP11B2* were 5′-TGCTGCACCATGTGCTGAAACACCT-3′ and 5′-CTGCACCACGTGCTGAAGCACT-3′ respectively. The primers and probes for *CYP17* analysis were: forward 5′-TCTCTGGGCGGCCTCAA-3′; reverse 5′-AGGCGATACCCTTACGGTTGT-3′; probe 5′-TGGCAACTCTAGACATCGCGTCC-3′. Probes were labeled with a reporter fluorescent dye FAM (6-carboxyfluorescein) at the 5′ end and a quencher-fluorescent dye TAMRA (6-carboxy-tetramethylrhodamine) at the 3′ end. PCR reactions were performed using the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems) in a total volume of 50 μl reaction mixture containing 5 μl cDNA template, 25 μl TaqMan Universal PCR Master Mix (Perkin Elmer Applied Biosystems), 0.1 μmol/l probe and 0.1 μmol/l of each primer. Negative controls contained water instead of first-strand cDNA. The PCR conditions were established as follows: after incubation at

50 °C for 2 min and denaturing at 95 °C for 10 min, 45 cycles were performed at 95 °C for 15 s, at 60 °C for 60 s. To quantify transcripts of the genes precisely, we monitored 18S ribosomal RNA (18S) levels as the quantitative control and each sample was normalized on the basis of its 18S content. The 18S quantification was performed using a TaqMan Ribosomal RNA Control Reagent kit (Perkin Elmer Applied Biosystems) and the method of PCR was followed using the manufacturer's protocol. Briefly, 50 μl reaction mixture containing 5 μl cDNA template, 25 μl TaqMan Universal PCR Master Mix, 50 nmol/l specific primer and 200 nmol/l probe (VIC™) was amplified by the program as follows: after incubation at 50 °C for 2 min and denaturing at 95 °C for 10 min, 40 cycles were performed at 95 °C for 15 s and at 60 °C for 1 min. The standard curves for *CYP11B1* and *CYP11B2* are shown in Fig. 1 and were generated using serially diluted solutions (21.75–0.002 175 attomoles) of plasmid clones containing either the *CYP11B1* cDNA (Fig. 1A) or the *CYP11B2* cDNA (Fig. 1C) as templates. The standard curve for *CYP17* was generated using serially diluted solutions (19.38–0.001 938 attomoles) of p*CYP17*. The standard curve for 18S was generated using serially diluted solutions of standard cDNA derived from human mRNA included in the TaqMan Ribosomal RNA Control Reagent kit (Perkin Elmer Applied Biosystems). ABI Prism 7700 measured the ratio (defined as the normalized reporter signal Rn) between the fluorescence signal of reporter dye and the fluorescence of the passive reference dye of each sample in every cycle and calculated the ΔRn defined as Rn minus the baseline signals established in the first 15 cycles. The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point at which the fluorescent signal is first recorded as statistically significant above background. This point is defined as Ct, and occurs during the exponential phase of amplification. The amount of target gene expression was calculated from the standard curves

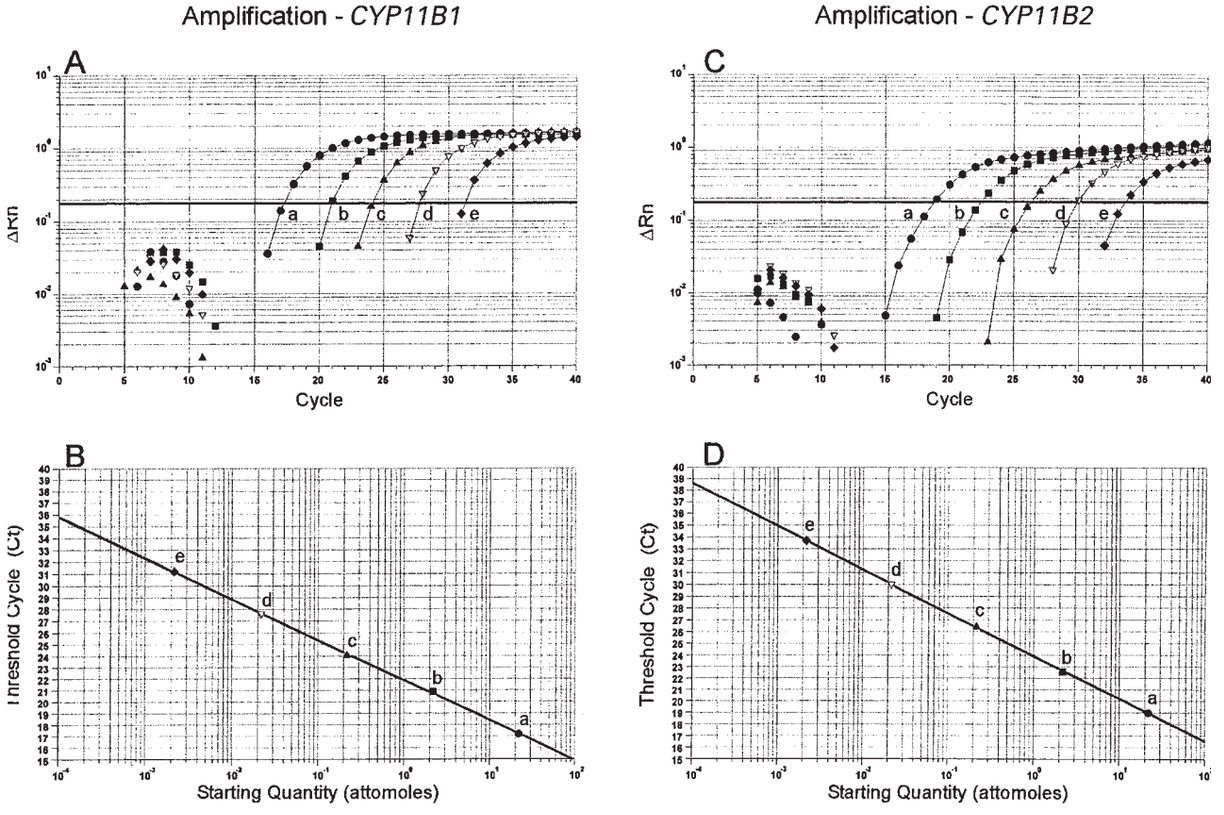

Figure 1 *CYP11B1* and *CYP11B2* standard curves by real-time RT-PCR. Samples containing five different concentrations (a, 21.75 attomoles; b, 2.175 attomoles; c, 0.2175 attomoles; d, 0.02175 attomoles; e, 0.002 175 attomoles) of plasmid clones containing either *CYP11B1* cDNA (A) or *CYP11B2* cDNA (C) were subjected to real-time PCR. Cycle number is plotted vs change in normalized reporter signal (ΔRn). ΔRn increases during PCR as the *CYP11B1* or the *CYP11B2* PCR product copy number increases until the reaction reaches a plateau. Standard curves plotting log starting copy number of *CYP11B1* cDNA (B) and *CYP11B2* cDNA (D) versus threshold cycle (Ct). The parameter of Ct represents the fractional cycle number at which a significant increase in the fluorescence signal above a baseline signal can be first detected. Dots represent data obtained from standard curve point samples. Two replicates for each standard curve point sample were performed, but the data for only one are shown here.

for *CYP11B1* (Fig. 1B) or the *CYP11B2* (Fig. 1D). The amount of target gene expression was calculated from the standard curve plotting log starting copy number versus Ct. Quantitative normalization of cDNA in each sample was performed using expression of the 18S as an internal control, *CYP11B1*, *CYP11B2* and *CYP17* mRNA levels are shown as attomoles/μg total RNA. Three different real-time PCR assays were conducted in duplicate for each sample and a mean value was used for calculation of the mRNA levels. The variability of Ct values between duplicates within the same run was no more than 3% while the CV values between different runs were no more than 5%. Tests of sensitivity revealed that our method can reliably detect samples as low as 0.001 attomoles mRNA/μg total RNA for each mRNA target investigated. Considering the high homology between *CYP11B1* and *CYP11B2*, we tested the specificity of our assays running a real-time PCR reaction with *CYP11B2* primers and probes at different concentrations of p*CYP11B1* as template and a second reaction in which the primers and probes used to detect *CYP11B1* were added as serial dilutions of p*CYP11B1* as template. In both experiments no fluorescence was detected, demonstrating that non-cross-reactivity was produced in both reactions.

Statistics

Data are expressed as means±S.E.M. Intergroup differences in means were tested using unpaired Student's *t*-test, and the least square method was used for correlations. When there was evidence of abnormal value distribution, non-parametric tests were used for testing differences in means (Wilcoxon signed rank) and for correlations (Spearman rank correlation). A $P < 0.05$ was considered significant.

Results

The mRNA levels of *CYP11B1*, *CYP11B2* and *CYP17* from APA and normal adrenals are shown in Table 2 and Fig. 2. Levels of *CYP11B1* mRNA were clearly detected in normal adrenals, which comprised both zona glomerulosa and fasciculata/reticularis cells, but were also detected at a lower range in APA tissues (37.82±4.17 vs 4.04±0.59 attomoles/μg total RNA, $P < 0.05$). The levels of *CYP11B2* mRNA were lower in normal adrenals than in APA (0.044±0.014 vs 0.344±0.056 attomoles/μg total RNA, $P < 0.005$). No correlation between *CYP11B2* mRNA level and APA size was found (Z, i.e., correlation coefficient $= -0.15$, $P = 0.88$). In patients with APA, the levels of *CYP11B1* mRNA ($Z = -0.55$, $P = 0.57$ supine; $Z = 1.07$, $P = 0.28$ upright) and *CYP11B2* mRNA ($Z = -0.16$, $P = 0.87$ supine, $Z = -0.89$, $P = 0.37$ upright) were not correlated with basal aldosterone. In addition, there was no correlation between *CYP11B1* mRNA ($Z = 1.70$, $P = 0.09$; $Z = -1.04$, $P = 0.29$) as well as *CYP11B2* mRNA ($Z = 1.58$, $P = 1.1$; $Z = 0.69$, $P = 0.48$) and the change from basal aldosterone in response to posture (mean change +127±99 pmol/l) or to dexamethasone administration (mean change −159±119 pmol/l) respectively. No correlation between *CYP11B1* mRNA ($Z = 2.21$,

Table 2 Individual *CYP11B1, CYP11B2* and *CYP17* mRNA levels of patients with APA and with normal adrenals.

Patient no.	***CYP11B1* mRNA** (attomoles/μg total RNA)	***CYP11B2* mRNA** (attomoles/μg total RNA)	***CYP17* mRNA** (attomoles/μg total RNA)
APA			
1	4.86	0.118	673.91
2	5.02	0.710	246.10
3	6.49	0.424	174.42
4	3.69	0.301	437.13
5	6.67	0.176	141.47
6	2.04	0.226	388.42
7	6.41	0.117	720.64
8	1.51	0.650	380.84
9	1.27	0.199	480.12
10	2.66	0.391	610.80
11	5.71	0.456	188.16
12	2.24	0.355	144.84
Means±S.E.M.	4.04±0.59	0.344±0.056	373.90±60.19
Normal			
1	25.38	0.084	225.77
2	44.35	0.010	814.49
3	46.95	0.013	319.83
4	39.99	0.045	708.21
5	45.70	0.018	222.92
6	24.59	0.079	664.80
Means±S.E.M.	37.82±4.17	0.044±0.014	492.67±108.56

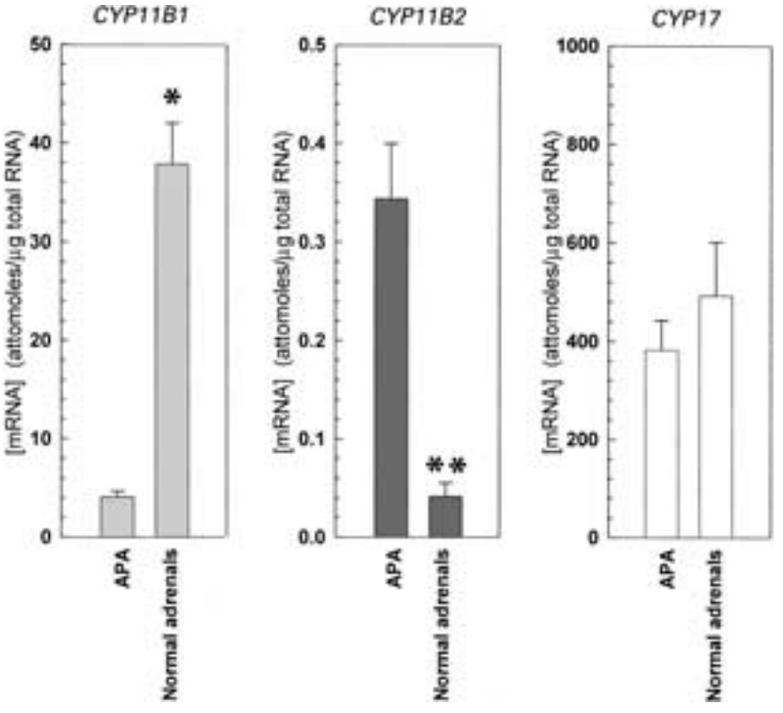

Figure 2 Mean±S.E.M. *CYP11B1*, *CYP11B2* and *CYP17* mRNA levels of patients with APA and with normal adrenals. $*P < 0.05$; $**P < 0.005$.

$P = 0.12$) or *CYP11B2* mRNA ($Z = 0.23$, $P = 0.81$) and the percentage of zona fasciculata-like cells was observed in APA.

CYP17 mRNA levels were similar in normal adrenals and in APA (492.67±108.56 vs 373.90±60.19 attomoles/µg total RNA, P = not significant).

Discussion

Functional zonation of the human adrenal cortex, i.e. the ability of each zone to differentially produce aldosterone and cortisol, relies on the zone-specific expression of CYP11B1 and CYP11B2 isozymes. Zonal expression of the isozymes results from transcriptional regulation of their coding genes (6, 15). The high similarity of the two isozymes has hampered the production of specific antibodies for their immunohistochemical detection in adrenal tissues (16), and measurement of enzyme activities in different types of adrenal cells (17–19) does not distinguish between 11β-hydroxylation due to *CYP11B1* or *CYP11B2* genes. In our study, we have shown a large amount of *CYP11B1* transcript in the whole normal adrenal, which included both zona glomerulosa and the much larger zona fasciculata/reticularis.

In agreement with previous reports using conventional RT-PCR (3) and *in situ* hybridization (8, 9), our technique has shown the presence of *CYP11B1* transcript in the zona glomerulosa-derived cells of APA, and in addition allowed its absolute quantification. The competitive RT-PCR analysis employed in a previous study (7) could not provide accurate measurements of *CYP11B1* mRNA; e.g. stable differences in amplification efficiency between target and competitor would remain undetected (20). Furthermore, in our method, the Ct value used for quantitation is measured during a period when the PCR amplification is still in the log phase of amplicon accumulation. This circumvents many of the problems associated with quantitation in the plateau stage of a PCR amplification. Expression of *CYP11B1* in hyperfunctioning zona glomerulosa suggests a formation of corticosterone via 11β-hydroxylase, additional to that driven by aldosterone synthase, which may be used as substrate for the increased aldosterone biosynthesis. *In vitro* studies on CYP11B2 transfected Chinese hamster COS-7 cells (21) and rat Leydig cells (22) show a better efficiency to metabolize DOC rather than corticosterone as exogenous substrate. However, in human APA, where high amounts of the corticosterone derivative 18-OHB are also secreted (23), the combination of abundant substrates available (i.e. DOC, corticosterone, 18-OHB) would reasonably contribute to the overproduction of aldosterone. The demonstration in APA of *CYP17*, encoding the 17α-hydroxylase enzyme required for cortisol but not for aldosterone biosynthesis, further supports the concept that cortisol can be produced by aldosteronoma cells, as shown in previous studies (9, 24–27).

At variance with *CYP11B1*, *CYP11B2* mRNA levels were very low in normal adrenals. This does not appear

to be due to sample damage during processing, since *CYP11B1* and *CYP17* mRNA were measurable in the same specimens, but probably reflects anatomical zone differences. As we measured *CYP11B2* expression in the mixed extract of zona glomerulosa and fasciculata/reticularis, we cannot discuss its presence outside the zona glomerulosa. However, studies reporting lack of *in vitro* release of aldosterone from human zona fasciculata cells (9), as well as barely distinguishable expression of *CYP11B2* by *in situ* hybridization in the same zone (8), do not support this possibility. As expected (3, 7, 28), the levels of *CYP11B2* mRNA were consistently higher in APA. The aldosterone-producing tumors are commonly believed to originate from zona glomerulosa; however, most adenoma cells have the histological appearance of fasciculata cells and only a minority are small and compact, similar to normal glomerulosa cells. Several studies have shown distinct biochemical characteristics of patients with primary aldosteronism due to APA, based on different aldosterone responses to upright posture (7, 29–31) and dexamethasone administration (12, 13, 32–34). Responsiveness to endogenous angiotensin II and ACTH inhibition may reflect a different prevalence of zona fasciculata-like cells within the tumor (30, 35). No relationship was found in our patients with APA between *CYP11B1* and *CYP11B2* transcript levels and aldosterone secretion, either basal or in response to dynamic testing. In this regard, lack of data on related protein concentrations in our study did not allow a thorough evaluation. Although a limitation could also be the small number of tumors examined, post-transcriptional factors might ultimately regulate aldosterone production, determining the various functional features of APA. The lack of correlation between the percentage of fasciculata-like cells and the mRNA levels of either *CYP11B1* or *CYP11B2* seems to exclude the possibility that expression of these genes in the tumors defines cell phenotype in APA.

In conclusion, real-time RT-PCR can be reliably used to quantify *CYP11B1* and *CYP11B2* mRNA levels in adrenal tissues. Expression of *CYP11B1* in APA suggests an additional formation of corticosterone via 11β-hydroxylase. The mRNA levels of *CYP11B1* and *CYP11B2* in APA are not related to the *in vivo* secretory activity of glomerulosa cells, where post-transcriptional factors might ultimately regulate aldosterone production.

References

1 White PC, Curnow KM & Pascoe L. Disorders of steroid 11β-hydroxylase isozymes. *Endocrine Reviews* 1994 **15** 421–438.

2 Kawamoto T, Mitsuuchi Y, Toda K, Yokoyama Y, Miyahara K, Miura S *et al.* Role of steroid 11β-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans. *PNAS* 1992 **89** 1458–1462.

3 Curnow KM, Tusie-Luna MT, Pascoe L, Natarajan R, Gu JL, Nadler JL *et al.* The product of *CYP11B2* gene is required for aldosterone biosynthesis in the human adrenal cortex. *Molecular Endocrinology* 1991 **5** 1513–1522.

4 Mornet E, Dupont J, Vitek A & White PC. Characterization of two genes encoding human steroid 11beta-hydroxylase (P-450(11)β). *Journal of Biological Chemistry* 1989 **264** 20961–20967.

5 Taymans SE, Pack S, Pak E, Torpy DJ, Zhuang Z & Stratakis CA. Human CYP11B2 (aldosterone synthase) maps to chromosome 8q24.3. *Journal of Clinical Endocrinology and Metabolism* 1998 **83** 1033–1036.

6 Rainey WE. Adrenal zonation: clues from 11β-hydroxylase and aldosterone synthase. *Molecular and Cellular Endocrinology* 1999 **151** 151–160.

7 Wu KD, Chen YM, Chu JS, Hung KY, Hsieh TS & Hsieh BS. Zona fasciculata-like cells determine the response of plasma aldosterone to metoclopramide and aldosterone synthase messenger ribonucleic acid level in aldosterone-producing adenoma. *Journal of Clinical Endocrinology and Metabolism* 1995 **80** 783–789.

8 Pascoe L, Jeunemaitre X, Lebrethon MC, Curnow KM, Gomez-Sanchez CE, Gasc JM *et al.* Glucocorticoid-suppressible hyperaldosteronism and adrenal tumors occurring in a single French pedigree. *Journal of Clinical Investigation* 1995 **96** 2236–2246.

9 Enberg U, Farnebo LO, Wedell A, Grondal S, Thoren M, Grimelius L *et al. In vitro* release of aldosterone and cortisol in human adrenal adenomas correlates to mRNA expression of steroidogenetic enzymes for genes CYP11B2 and CYP17. *World Journal of Surgery* 2001 **25** 957–966.

10 Erdmann B, Gerst H, Bulow H, Lenz D, Bahr V & Bernhardt R. Zone-specific localization of cytochrome P45011B1 in human adrenal tissue by PCR-derived riboprobes. *Histochemistry and Cell Biology* 1995 **104** 301–307.

11 Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* 2000 **25** 169–193.

12 Pilon C, Mulatero P, Barzon L, Veglio F, Garrone C, Boscaro M *et al.* Mutations in CYP11B1 gene converting 11β-hydroxylase into an aldosterone-producing enzyme are not present in aldosterone-producing adenomas. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 4228–4231.

13 Mulatero P, Veglio F, Pilon C, Rabbia F, Zocchi C, Limone P *et al.* Diagnosis of glucocorticoid-remediable aldosteronism in primary aldosteronism: aldosterone response to dexamethasone and long polymerase chain reaction for chimeric gene. *Journal of Clinical Endocrinology and Metabolism* 1998 **83** 2573–2575.

14 Neville AM & O'Hare MJ. Histopathology of the human adrenal cortex. *Clinics in Endocrinology and Metabolism* 1985 **14** 791–820.

15 Denner K, Rainey WE, Pezzi V, Bird IM, Bernhardt R & Mathis JM. Differential regulation of 11β-hydroxylase and aldosterone synthase in human adrenocortical H295R cells. *Molecular and Cellular Endocrinology* 1996 **121** 87–91.

16 Sasano H, Okamoto M & Sasano N. Immunohistochemical study of human adrenal cortex with mineralo- and glucocorticoid excess. *Virchows Archiv (A)* 1988 **413** 313–318.

17 Takasaki H, Miyamori I, Nahgai K, Takeda R, Mochizuki H & Katagiri M. Mitochondrial P-450 activities in aldosteronoma tissues. *Journal of Steroid Biochemistry and Molecular Biology* 1991 **38** 533–535.

18 Ogishima T, Shibata H, Shimada H, Mitani F, Suzuki H, Saruta T *et al.* Aldosterone synthase cytochrome P-450 in the adrenals of patients with primary aldosteronism. *Journal of Biological Chemistry* 1991 **266** 10731–10734.

19 Suzuki H, Shibata H, Maruyama T, Ishimura Y & Saruta T. Significance of steroidogenic enzymes in the pathogenesis of hyperfunctioning and non-hyperfunctioning adrenal tumors. *Steroids* 1995 **60** 42–47.

20 Raeymaeckers L. Quantitative PCR: theoretical considerations with practical implications. *Analytical Biochemistry* 1993 **214** 582–585.

21 Denner K, Doehmer J & Bernhardt R. Cloning of CYP11B1 and CYP11B2 from normal human adrenal and their functional

expression in COS-7 and V79 Chinese hamster cells. *Endocrine Research* 1995 **21** 443–448.
22 Zhou M, Xue D, Foecking MF & Gomez-Sanchez C. Stable expression of rat cytochrome P450 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) in MA-10 cells. *Journal of Steroid Biochemistry and Molecular Biology* 1995 **52** 523–528.
23 Biglieri EG, Schambelan M, Hirai J, Chang B & Brust N. The significance of elevated levels of plasma 18-hydroxycorticosterone in patients with primary aldosteronism. *Journal of Clinical Endocrinology and Metabolism* 1979 **49** 87–91.
24 Nomura K, Naruse M, Demaura H & Shizume K. *In vivo* evidence of cortisol secretion by aldosterone-producing adenomas. *Acta Endocrinologica* 1984 **106** 516–520.
25 Pham-Huu-Trung MT, Duclos JM, Pagny JY, Bogyo A, Leneuve P & Girard F. *In vitro* studies in primary aldosteronism: baseline steroid production and aldosterone response to ACTH and angiotensin II. *Acta Endocrinologica* 1988 **117** 135–144.
26 Carbaillera A, Fishman LM, Brown JW & Trujillo D. Content and biosynthesis of cortisol in aldosterone-producing adenomas. *Journal of Laboratory and Clinical Medicine* 1989 **114** 120–128.
27 Racz K, Pinet F, Marton T, Szende B, Glaz E & Corvol P. Expression of steroidogenic enzyme messenger ribonucleic acids and corticosteroid production in aldosterone-producing and 'nonfunctioning' adrenal adenomas. *Journal of Clinical Endocrinology and Metabolism* 1993 **77** 677–682.
28 Takeda Y, Furukawa K, Inaba S, Miyamori I & Mabuchi H. Genetic analysis of aldosterone synthase in patients with idiopathic hyperaldosteronism. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 1633–1637.
29 Fontes RG, Kater EG, Biglieri EG & Irony I. Reassessment of the predictive value of the postural stimulation test in primary aldosteronism. *American Journal of Hypertension* 1991 **4** 786–791.
30 Tunny TJ, Gordon RD, Klemm SA & Cohn D. Histological and biochemical distinctiveness of atypical aldosterone-producing adenomas responsive to upright posture and angiotensin. *Clinical Endocrinology* 1991 **34** 363–369.
31 Nomura K, Toraya S, Horiba N, Ujihara M, Alba M & Demura H. Plasma aldosterone response to upright posture and angiotensin II infusion in aldosterone-producing adenoma. *Journal of Clinical Endocrinology and Metabolism* 1992 **75** 323–327.
32 Ganguly A, Chevarri M, Luetscher JA & Dowdy AJ. Transient fall and subsequent return of high aldosterone secretion during continued dexamethasone administration in primary aldosteronism. *Journal of Clinical Endocrinology and Metabolism* 1977 **44** 775–779.
33 Wenting GJ, Man In't Veld AJ, Dercks FH, Brummelen FV & Schalekamp MADH. ACTH-dependent aldosterone excess due to adrenocortical adenoma: a variant of primary aldosteronism. *Journal of Clinical Endocrinology and Metabolism* 1978 **46** 326–335.
34 Fardella C, Mosso L, Gomez-Sanchez C, Cortes P, Soto J, Gomez L *et al.* Primary hyperaldosteronism in essential hypertensives: prevalence, biochemical profile, and molecular biology. *Journal of Clinical Endocrinology and Metabolism* 2000 **85** 1863–1867.
35 Fallo F, Barzon L, Biasi F, Altavilla G, Boscaro M & Sonino N. Zona fasciculata-like histotype and aldosterone response to upright posture are not related in aldosterone-producing adenomas. *Experimental and Clinical Endocrinology and Diabetes* 1998 **106** 74–78.

Received 19 March 2002
Accepted 29 August 2002