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Differentiation of the Expression of Aldosterone Synthase and 11β-Hydroxylase mRNA in the Rat Adrenal Cortex by Reverse Transcriptase–Polymerase Chain Reaction

Martin K. Oaks[1] and Hershel Raff [1,2*]

[1]*Immunology and Endocrine Research Laboratories, St Luke's Medical Center, Milwaukee, WI 53215 and*
[2]*Department of Medicine, Medical College of Wisconsin, Milwaukee, WI 53226, U.S.A.*

The adrenocortical enzymes of the steroidogenic late pathway in the rat are aldosterone synthase (*P*450aldo), which catalyzes the production of aldosterone, and 11β-hydroxylase (*P*45011β), which catalyzes the production of corticosterone throughout the cortex. These two enzymes are highly homologous and are encoded by the genes CYP11B2 and CYP11B1, respectively. The purpose of the present study is to describe the development of two sets of primers and the reverse transcription-polymerase chain reaction (RT–PCR) conditions that are capable of discriminating between rat *P*450aldo and *P*45011β mRNAs. The *P*450aldo primer set did not amplify full length cDNA *P*45011β plasmid and the *P*45011β primer set did not amplify full length cDNA *P*450aldo plasmid indicating minimal crosstalk. The fidelity of the PCR primers and method was further established by sequencing the PCR products and demonstration of virtual identity with the published sequences of *P*450aldo and *P*45011β. RT–PCR of mRNA from adrenal capsules (zona glomerulosa) and subcapsules (zona reticularis/fasciculata) from rats demonstrated no effect of sodium diet on the expression of *P*45011β mRNA but an ~8-fold greater expression in *P*450aldo mRNA on low vs high sodium intake. Similar results were found when single hemicapsules were subjected to RT–PCR, demonstrating the sensitivity of the method. We conclude that the two sets of PCR primers and the RT–PCR method described are capable of evaluating the expression of the highly homologous mRNAs for *P*450aldo and *P*45011β with great precision and sensitivity.

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INTRODUCTION

The synthesis of steroid hormones by the adrenal cortex is catalyzed by specific cytochrome *P*450 enzymes. The nomenclature of the genes which encode the late pathway enzymes in the rat, and the nature of the enzymes which synthesize aldosterone in the zona glomerulosa and corticosterone in the zona reticularis-fasciculata has been the subject of considerable confusion and controversy [1–4]. It is now accepted that aldosterone synthase (*P*450aldo) which catalyzes the production of aldosterone in the rat is expressed primarily in the zona glomerulosa and is encoded by the gene CYP11B2. 11β-hydroxylase (*P*45011β), which catalyzes the production of the majority of corticosterone released in the rat, is an isoenzyme of *P*450aldo with >88% homology, expressed in the inner zones and, perhaps, the zona glomerulosa of the adrenal cortex, and encoded by the gene CYP11B1.

Several methods have been used to measure the expression of *P*450aldo and *P*45011β mRNA produced by changes in sodium intake in rats. Using Northern analysis, several studies demonstrated that a low sodium diet increased *P*450aldo mRNA in the capsule (zona glomerulosa) without an effect on the expression of *P*45011β mRNA [5–8]. This effect was attenuated by captopril, indicating that the effect of a low sodium diet on *P*450aldo mRNA expression was mediated by angiotensin II [7]. A high sodium diet was shown to decrease expression of *P*450aldo mRNA without an effect on *P*45011β [5]. Using the RNase protection (liquid hybridization) assay, no effect of

*Correspondence to H. Raff at: St Luke's Health Science Center, 2901 W. KK River Parkway Suite 503, Milwaukee, WI 53215, U.S.A.
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high salt but an increase in *P*450aldo mRNA expression by a low salt diet was described [9].

A recent study used a reverse transcriptase–polymerase chain reaction (RT–PCR) with a generic set of primers to create a cDNA library of 11β-hydroxylase isoenzymes. The relative abundance of mRNAs for *P*45011β and *P*450aldo was assessed by counting the frequency of subcloned colony hybridizations; a low sodium/high potassium diet resulted in an increase in the colony frequency of mRNA for *P*450aldo only [10]. This study did not use specific primers for each enzyme and, therefore, is not a practical approach for the routine measurement of the differential expression of *P*450aldo and *P*45011β mRNA in small samples.

The purpose of the present report is to describe the development of specific PCR primers and methodology for the differentiation of the expression of mRNA for *P*450aldo (CYP11B2) and *P*45011β (CYP11B1) in the adrenal cortex of the rat. Specificity of these primers was confirmed by determining cross-reactivity with full length cloned cDNAs for *P*45011β and *P*450aldo. To further ensure the fidelity of the reaction, the RT–PCR product from each set of primers was sequenced. Finally, to compare this new method with previous studies using Northern analysis and RNase protection assay and to demonstrate its sensitivity, we used RT–PCR to determine the effect of high and low salt diet on the expression of mRNA for *P*450aldo and *P*45011β in pooled adrenal capsules and subcapsules and from single adrenal hemicapsules.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley (CD) rats (200–250 g) were obtained from Harlan Sprague–Dawley (Indianapolis, IN). The rats were fed a diet with either high sodium (4.0%) or low sodium (0.4%) content (Dyets, Bethlehem, PA) and had access to tap water *ad libitum*.

Plasmids

Plasmids pc*P*45011β, aldo-46 and pc*P*45011β-62 were a generous gift from Dr M. Okamoto (Osaka, Japan) and are full length cDNA clones of the *P*450aldo and *P*45011β genes, respectively, in pUC119 vectors [11]. The plasmids were transformed into *E. coli* strain INV1αF′ by standard methods and miniprep DNAs were prepared by Wizard chromatography (Promega, Madison, WI).

RNA isolation

Total cellular RNA from adrenal tissue was extracted by the guanidine thiocyanate method using kit-supplied reagents with minor modifications (RNAgents, Promega Biotec, Madison, WI). Portions of adrenal tissues were placed in 750 μl of guanidine thiocyanate and homogenized with a few strokes of a glass Dounce homogenizer. The RNA was extracted with phenol–chloroform, and the aqueous phase was precipitated with 1/10 vol. of 2 M sodium acetate and an equal volume of isopropanol. The RNA was pelleted by centrifugation, washed twice with 75% ethanol in diethylpyrocarbonate-treated water (DEPC-water), and dissolved in 500 μl of 10 mM Tris–HCl (pH 7.4). Contaminating DNA was digested in the presence of 80 U/ml RNAse inhibitor (Promega) and the addition of 1 U RQ-1 DNase (Promega). DNAse digestion was performed for 30 min at 37°C, and RQ-1 DNAse was heat-killed at 95°C for 15 min. The RNA was then reprecipitated overnight at −20°C as described above. The resultant pellet was then washed in 75% ethanol and air dried. The dried pellet was dissolved in 25 μl DEPC-water, and the RNA content quantified by spectrophotometry and adjusted to 200 ng/μl in DEPC-water. The RNA was then reverse transcribed immediately or stored at −80°C until analysis by PCR.

Reverse transcription

Single-strand cDNA was generated from total cellular RNA with the use of Superscript preamplification reagents (Life Technologies, Bethesda, MD) and our optimized protocol. To this end, 12.5 ng random hexamers were added to 500 ng total cellular RNA in a final volume of 3.5 μl and incubated for 10 min at 70°C to allow annealing of the hexameric primers. The reaction was chilled to 5°C, and to a final volume of 5 μl the following components were added to the reaction tube: 0.5 μl 10 synthesis buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl, 25 mM $MgCl_2$, 1 μg/ml bovine serum albumin)], 0.25 μl dNTP mixture (10 mM each dNTP), 0.5 μl of 0.1 M dithiothreitol, and 0.25 μl (50 U) Superscript reverse transcriptase. The reaction was incubated for 10 min at 22°C and then for 1 h at 45°C. The RT was inactivated by heating to 70°C for 15 min, and then 0.25 μl (0.5 U) of RNase H was added. RNA was digested for 20 min at 37°C and the reaction was chilled to 5°C.

Primers

Primers were designed with the aid of commercially available software (Primer Designer; S&E Software, State Line, PA) from previously published sequences of the *P*450aldo (CYP11B2) and *P*45011β (CYP11B1) genes [11]. The primers were devised to provide maximum 3′ mismatches while still providing similarity in GC content and melting temperature. The following are the 5′→3′ sequences of the sense (s) and antisense (as) primers used in these studies: Aldo(s)=ACCATGGATGTCCAGCAA, Aldo(as)=GAGAGCTGCCGAGTCTGA, 11β(s)=GCTGGAGAATGTTCATGG, 11β(as)=CTCTGCCAGTTCGCGATA, rat β-actin (RBA)(s)=GCCATGTACG-

TAGCCATCCA, RBA(as)=GAACCGCTCATTGCCGATAG. The *P*450aldo primers were designed to amplify a 297 bp fragment corresponding to positions 657–954 of the *P*450aldo (CYP11B2) gene, and the *P*45011β primers were designed to amplify a 312 bp segment corresponding to positions 528–840 of the *P*45011β (CYP11B1) gene [11]. The RBA primers were designed from Genbank sequence J00691 [12] and amplified a 374 bp fragment of the rat β-actin gene. Primers were synthesized by Operon Technologies (Alameda, CA).

PCR amplification

PCR was carried out in 25 μl volumes of 1 × PCR buffer [10 mM Tris–HCl (pH 8.4), 50 mM KCl, 3.75 mM $MgCl_2$] containing the entire contents of the RT reaction, 0.2 mM each dNTP, 0.5 μM of each primer, 0.25 U perfect match polymerase enhancer (Stratagene, La Jolla, CA), and 0.025 U/μl Taq DNA polymerase (Promega, Madison, WI). The reaction mixtures were overlaid with mineral oil, centrifuged for 1 min at 2000 ***g***, and "hot started" with the addition of the Taq polymerase after a 5 min incubation at 95°C. The reactions were subjected to 35 amplification cycles on a Perkin-Elmer-Cetus thermal cycler. The amplification cycle profile was 95°C denaturation for 1 min, annealing of primers at 65°C for 1 min, and primer extension at 72°C for 1 min. Optimization of PCR conditions was first performed on plasmids pc*P*45011β, aldo-46 and pc*P*45011β-62 using 100 ng of plasmid DNAs and varying the annealing temperature, pH, and $MgCl_2$ concentration.

A number of precautions were applied to prevent contamination of reagents and samples used for PCR amplification. Working areas were separated for pre- and post-PCR experiments, and filtered pipet tips were used for all reagent manipulations. Separate groups of pipets were used for extraction, PCR set-up, and electrophoresis of PCR products. Pipets were cleaned daily with 10% household bleach. All reagents were aliquoted in a laminar flow hood and were systematically tested for possible contamination.

Detection of PCR products

Ten μl of the PCR product were separated by electrophoresis in a 2% agarose gel (Sea-Kem GTG agarose, FMC Marine Colloids). The gel was visualized by ethidium bromide staining and analyzed with the use of CCD camera/video gel documentation system (Foto/Analyst Archiver, Fotodyne Inc., Hartland, WI) and a Hoefer Model GS300 densitometry system equipped with GS365W electrophoresis data software (Hoefer Scientific Instruments, San Francisco, CA). In some experiments, PCR product detection was enhanced by incorporation of trace amounts (5 μM) of digoxigenin-dUTP during PCR. For this purpose, the DNA was denatured before blotting by incubating the gels for 30 min in 0.4 N NaOH. The gels were then "dry-blotted" overnight onto nylon membranes (BMB Biochemical, Indianapolis, IN), and baked for 1 h at 120°C. Chemiluminescent detection was performed with the use of anti-digoxigenin alkaline phosphatase conjugate system (Boehringer Mannheim Biochemicals, Indianapolis, IN). The lumigrams were exposed to Kodak X-AR film in cassettes equipped with DuPont Lightning-Plus intensifying screens for 1–10 min at room temperature, and were developed manually.

PCR product sequencing

Aliquots of *P*450aldo and *P*45011β RT–PCR products were sequenced by Sequetech Corp. (Mountain View, CA) using the PCR primers described above.

Statistical analysis

The expression of *P*450aldo mRNA in hemicapsules from high vs low salt diet rats were compared by Mann–Whitney rank sum test and by unpaired *t*-test.

RESULTS

Our first series of experiments was designed to determine the optimum PCR conditions for amplification of *P*450aldo and *P*45011β DNAs while minimizing potential "crosstalk" between primers designed from cDNAs with extensive sequence similarity. For this purpose, we first optimized the PCR conditions for amplification of *P*450aldo and *P*45011β cDNA-containing plasmids by varying anneal temperature, buffer pH, and $MgCl_2$ concentration. Once optimal PCR conditions were obtained, we then evaluated the specificity of PCR by attempting to amplify *P*450aldo plasmid DNA with *P*45011β primers and *P*45011β plasmid DNA with *P*45011β primers [Fig. 1(A)]. Primers designed to recognize *P*450aldo-specific sequences amplified a 297 bp fragment of the cloned *P*450aldo cDNA, but no signal was obtained using cloned *P*45011β cDNA. Likewise, the *P*45011β primer pair successfully amplified sequences of *P*45011β cDNA but not *P*450aldo cDNA. Attempts to enhance the sensitivity of detection of these PCR products by incorporating digoxigenin-dUTP during PCR followed by chemiluminescent detection of digoxigenin showed complete absence of crosstalk between the *P*450aldo and *P*45011β primer sets and their reciprocal cloned cDNAs [Fig. 1(B)].

Sequence analysis of PCR products confirmed the specificity of the primer sets for their appropriate cDNAs. A 259 base stretch of DNA was successfully sequenced from *P*450aldo PCR product and revealed identity at 258 bases with the published cDNA sequences (data not shown). For *P*45011β PCR products, a 278 base sequence was obtained and showed identity at 277 sites. Furthermore, comparison of the *P*450aldo and *P*45011β cDNA sequences predicts 51

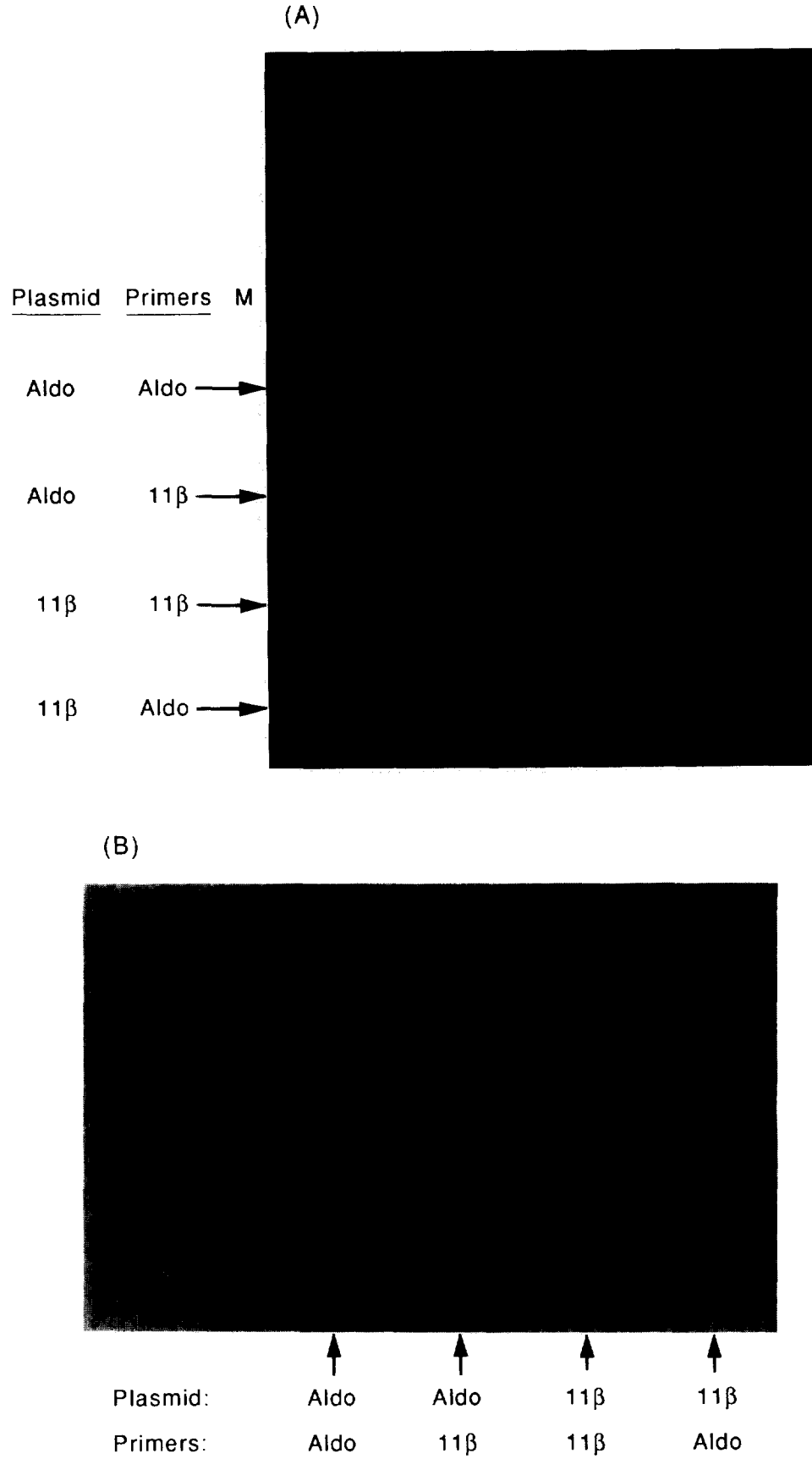

Fig. 1. (A) Gel electrophoresis of PCR products using plasmids containing cDNAs of *P*450aldo (Aldo) and *P*45011*β* (11*β*) as templates and Aldo and 11*β* primer sets. The Aldo primer set amplified a 297 bp fragment of the *P*450aldo gene; the 11*β* primer set amplified a 312 bp segment of the *P*45011*β* gene. There is no evidence of "cross-priming" on this ethidium bromide stained gel. (B) Southern lumigram of PCR products shown in (A). 5 *μ*M digoxigenin was incorporated in the PCR and detected by anti-digoxigenin alkaline phosphatase. The lumigram was intentionally overexposed in order to enhance possible detection of cross-primed PCR product.

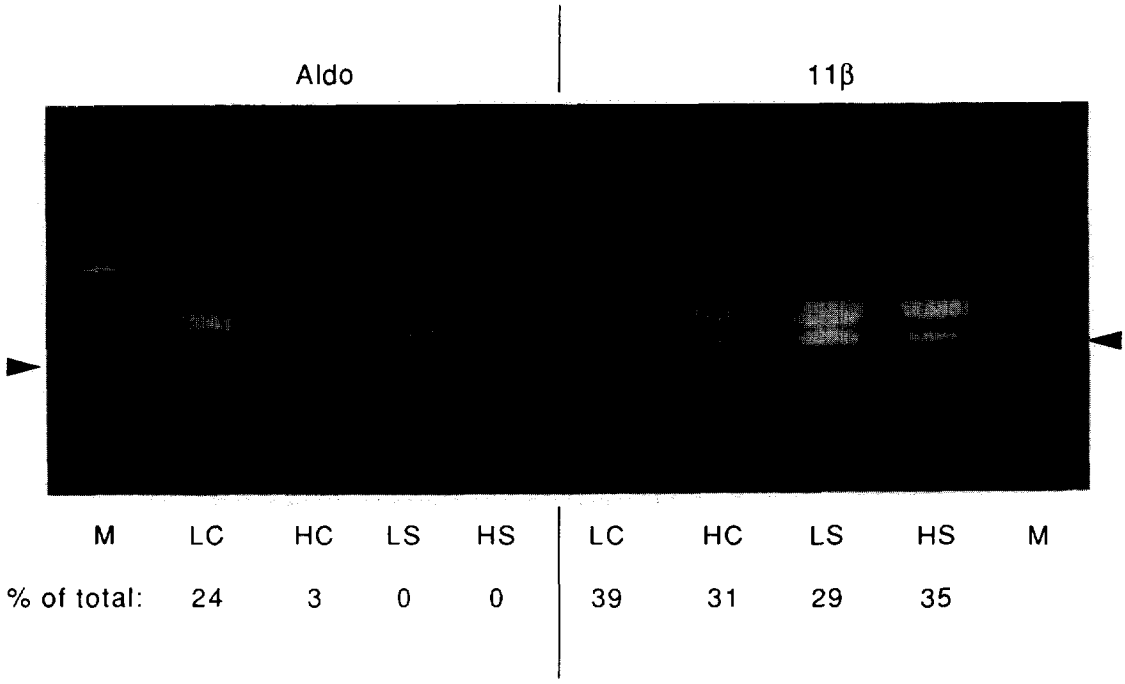

Fig. 2. Gel electrophoresis of PCR products of pooled adrenal capsules (C) and subcapsules (S) from rats fed low (L) and high (H) salt diets. M, molecular weight markers; left arrow indicates 297 bp product of aldo amplifications; right arrow indicates 312 bp product of 11β amplifications.

and 59 "diagnostic" nucleotides within the region of the P450aldo and P45011β primer sets, respectively. Of the 51 bases unique to the P450aldo gene, fifty were identical in the sequenced PCR product. The base assignment for the single discrepant site obtained from PCR product sequencing was ambiguous when sequenced from either direction. There was 100% concordance between the observed and predicted bases at the 59 diagnostic P45011β nucleotides.

Our next series of experiments was designed to determine whether we could distinguish P450aldo and P45011β mRNAs by RT–PCR. For this purpose, we chose to study the effects of high and low sodium diets on the relative abundance of P450aldo and P45011β mRNAs in pooled adrenal capsules and subcapsules. Figure 2 shows results of RT–PCR performed on pooled capsules and subcapsules of rats fed high and low sodium diets. Visual inspection of the gel shows that low salt diet induces P450aldo mRNA levels in the adrenal capsule but not the subcapsule. In contrast, P45011β mRNA levels do not appear to be significantly different with respect to adrenal capsule vs subcapsule nor changed by salt diet.

In order to make more objective determinations of the relative amounts of P450aldo and P45011β products, we determined the percentage of P450aldo and P45011β signals relative to co-amplified β-actin by video densitometric analysis. When expressed as a percentage of total product signal, P450aldo represented 24% of the total from pooled capsules of animals fed a low salt diet compared to 3% of the signal from capsules of animals fed a high salt diet. This represents an ~8-fold greater expression of P450aldo mRNA in rats fed a low salt diet as compared to a high salt diet. In contrast, the P45011β signal represented 39 and 31% of the signal obtained from capsules from animals fed low and high salt diets, respectively. The P450aldo signal was not detected in adrenal subcapsules regardless of salt intake (lanes HS and LS in Fig. 2), whereas P45011β levels were similar (29 vs 35%) in subcapsules of rats fed low and high salt diets.

To establish the sensitivity of the method and to estimate the variation of P450aldo and P45011β levels among individual rats, we performed RT–PCR on RNAs derived from single adrenal hemicapsules from rats fed high or low salt diets. For this purpose, RNA from one-half of the individual adrenal capsules from six rats (three per diet group) was extracted and quantified as outlined in Methods. RNA yields ranged from 8.4 to 16.1 μg total cellular RNA per sample. The results of a representative experiment are shown in Fig. 3. There was remarkably little variation in the ratio of P45011β–β-actin signals obtained from individual animals. The mean P45011β signal from animals fed a low salt diet was $40 \pm 1\%$ of the total signal (P45011β + β-actin). The P45011β signal represented $42 \pm 1\%$ of the total signal (P45011β + β-actin) in animals fed a high salt diet. We observed little variation in P450aldo mRNA levels ($34 \pm 2\%$) from animals fed a low salt diet, whereas individual hemicapsules from animals on a high salt diet showed a lower and more variable expression of P450aldo mRNA. Because of

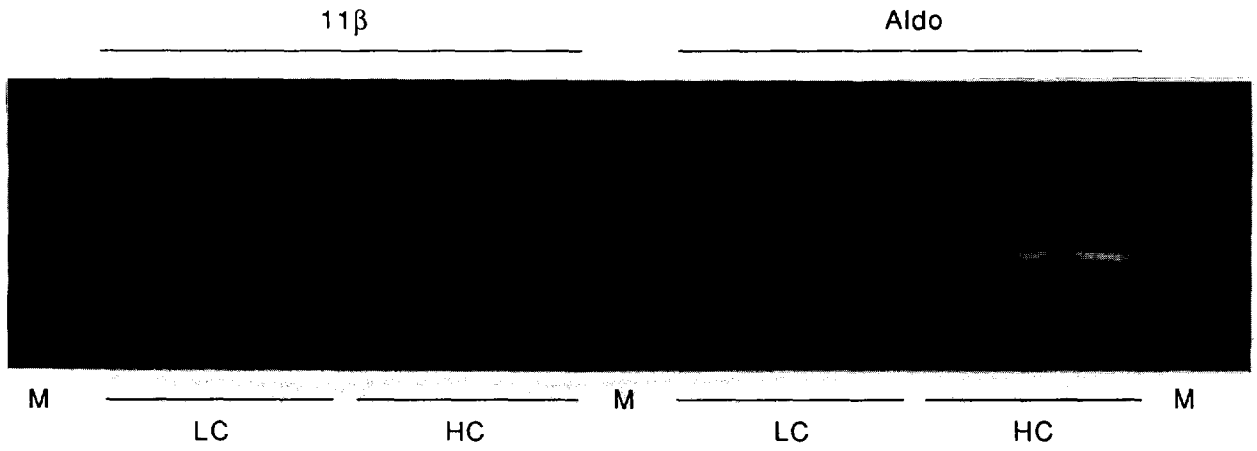

Fig. 3. Gel electrophoresis of PCR products of individual adrenal capsules from three rats fed high or low salt diets. M, molecular weight marker. Mean ± SEM of signal expressed as percent of the total signal (aldo or 11β plus β-actin). Due to relatively large variability in *P*450aldo expression under high salt diet (HC), an additional 3 hemicapsules in low salt (LC) vs high salt (HC) were analyzed. Combined with the results in Fig. 3, *P*450aldo mRNA expression was significantly less on a low salt (8 ± 4%) vs a high salt (34 ± 1%) diet ($P < 0.001$).

this greater variability, we analyzed *P*450aldo mRNA expression in additional individual hemicapsules from rats on high vs low salt diets such that the total was 6 hemicapsules per group. There was a significant difference in *P*450aldo mRNA expression in hemicapsules from rats fed low salt (34 ± 1%; n=6) vs high salt (8 ± 4%; n=6) diets (P<0.001). Taken together, these data show that it is feasible to differentiate between *P*450aldo and *P*45011β mRNA from individual adrenal capsules by the use of RT–PCR. In general, there is moderate variation of *P*450aldo and *P*45011β levels among individual animals within high or low salt diet groups.

DISCUSSION

The purpose of the present study was to develop and validate RT–PCR methods to differentiate the two, highly homologous "late pathway" enzymes of the adrenal cortex of the rat. These two enzymes, *P*450aldo and *P*45011β, are encoded by genes CYP11B2 and CYP11B1, respectively. Specificity of PCR was established since the *P*450aldo primers did not produce significant product from cloned *P*45011β plasmids and the *P*45011β primers did not produce significant product from cloned *P*450aldo plasmids. In addition, the fidelity of the reaction was confirmed as the sequenced PCR products were virtually identical to the published sequences [11].

Estimates of the change in *P*450aldo and *P*45011β mRNA levels with high and low salt diet were made by comparison to levels of the "housekeeping" gene β-actin and agreed remarkably well with previously published results using Northern analysis and RNase protection assays [5–9]. We found that expression of *P*450aldo mRNA in the capsule (zona glomerulosa) was induced approx. 8-fold by a low sodium diet and not detected in the subcapsule on either diet. In contrast, expression of *P*45011β mRNA was similar between capsule and subcapsule and not affected by a change in sodium in the diet. These results are consistent with previously published data using other methods and with the known regulation of the renin–angiotensin–aldosterone system [5–9, 13]. That is, a low salt diet, by increases in renin and angiotensin II, augments the production of aldosterone from the zona glomerulosa but does not increase ACTH or corticosterone production. It is logical that the expression of the "late pathway" enzyme of the zona glomerulosa (*P*450aldo) would be increased to augment the synthesis of aldosterone from its precursors [8].

The zonation of CYP11B1 (*P*45011β) has recently been questioned. Several recent studies have failed to find *P*45011β in the zona glomerulosa by *in situ* histochemistry [14–16]. Interestingly, two recent studies using *in situ* hybridization histochemistry are conflicting, with one demonstrating *P*45011β mRNA [17] and the other failing to find *P*45011β mRNA [16] in the zona glomerulosa. This suggests either a post-translational dissociation and/or that the capsular cells were contaminated with subcapsular cells in our experiments.

We were able to demonstrate the sensitivity of the method by measuring the effect of a low sodium diet which increased the expression of *P*450aldo detected in single adrenal hemicapsules. In agreement with the findings in pooled capsules and the work of others [5–9], changes in sodium diet did not alter *P*45011β expression in single hemicapsules.

RT–PCR has been used previously to detect *P*45011β (CYP11B1) and *P*450aldo (CYP11B2) mRNA expression. However, these studies used generic primers to simultaneously amplify both enzyme mRNAs and then attempted to differentiate their expression by counting the frequency of subcloned colony hybridizations [10] or by Southern blotting using oligonucleotide probes specific for each enzyme sequence [18,19]. The method described in this communication avoids these complicating and tedious steps by the use of primers which are highly specific for *P*450aldo vs *P*45011β cDNAs.

There are more quantitative approaches to RT–PCR than using the expression of β-actin as a reference housekeeping gene. The need for more quantitative approaches is highlighted by the finding that dietary manipulation in the rat may alter expression of β-actin mRNA in the adrenal cortex [20]. We are currently developing competitive PCR mimics [21] for these two sets of primers which will provide a highly quantitive analysis of the differential expression of *P*450aldo and *P*45011β without the need for using β-actin mRNA as a reference.

We conclude that PCR amplification obtained with the use of the described *P*450aldo and *P*45011β primers is specific and not complicated by cross-priming of highly homologous cDNAs, will prime only their respective cDNA and shows no crossreactivity with non-homologous sequences, and that RT and subsequent PCR can accurately detect changes in expression in very small tissue samples. The advantage of this approach over previous studies with Northern analysis and RNase protection assays is its relative simplicity and the ability to measure expression in very small samples from individual animals.

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REFERENCES

1. Hanukoglu I.: Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. *J. Steroid. Biochem. Molec. Biol.* **43** (1992) 779–804.
2. LeHoux J.G. and Tremblay A.: *In vivo* regulation of gene expression of enzymes controlling aldosterone synthesis in rat adrenal. *J. Steroid Biochem. Molec. Biol.* **43** (1992) 837–846.
3. Muller J.: Final steps of aldosterone biosynthesis: molecular solution of a physiological problem. *J. Steroid Biochem. Molec. Biol.* **45** (1993) 153–159.
4. Ogishima T., Suzuki H., Hata J., Mitani F. and Ishimura Y.: Zone-specific expression of aldosterone synthase cytochrome *P*-450 and cytochrome *P*-45011β in rat adrenal cortex: histochemical basis for functional zonation. *Endocrinology* **130** (1992) 2971–2977.
5. Holland O.B. and Carr B.: Modulation of aldosterone synthase messenger ribonucleic acid levels by dietary sodium and potassium and by adrenocorticotropin. *Endocrinology* **132** (1993) 2666–2673.
6. Imai M., Ogishima T., Shimada H. and Ishimura Y.: Effect of dietary sodium restriction on mRNA for aldosterone synthase cytochrome *P*-450 in rat adrenals. *J. Biochem.* **111** (1992) 440–443.
7. Tremblay A., Parker K.L. and LeHoux J.G.: Dietary potassium supplementation and sodium restriction stimulate aldosterone synthase but not 11β-hydroxylase *P*-450 messenger ribonucleic acid accumulation in rat adrenals and require angiotensin production. *Endocrinology* **110** (1992) 3152–3158.
8. Adler G.K., Chen R., Menachery A.I., Braley L.M. and Williams G.H.: Sodium restriction increases aldosterone biosynthesis by increasing late pathway, but not early pathway, messenger ribonucleic acid levels and enzyme activity in normotensive rats. *Endocrinology* **133** (1993) 2235–2250.
9. Malee M.P. and Mellon S.H.: Zone-specific regulation of two messenger RNAs for *P*450c11 in the adrenals of pregnant and nonpregnant rats. *Proc. Natn. Acad. Sci.* **88** (1991) 4731–4735.
10. Nomura M., Morohashi K., Kirita S., Nonata Y., Okamoto M., Nawata H. and Omura T.: Three forms of rat CYP11B genes: 11β-hydroxylase gene, aldosterone synthase gene, and a novel gene. *J. Biochem.* **113** (1993) 144–152.
11. Matsukawa N.M., Nonaka Y., Ying Z., Higaki J., Ogihara T. and Okamoto M.: Molecular cloning and expression of cDNAs encoding rat aldosterone synthase: variants of cytochrome *P*-45011β. *Biochem. Biophys. Res. Commun.* 169 (1990) 245–252.
12. Nudel Y., Zakut R., Shani M., Neuman S., Levy Z. and Yaffe D.: The nucleotide sequence of the rat cytoplasmic beta-actin gene. *Nucl. Acids Res.* **1** (1983) 1759–1771.
13. Reid I.A.: The renin–angiotensin system and body function. *Arch. Intern. Med.* **145** (1985) 1475–1479.
14. Mitani F., Suzuki H., Hata J-I., Ogishima T., Shimada H. and Ishimura Y.: A novel cell layer without corticosteroid-synthesizing enzymes in rat adrenal cortex: histochemical detection and possible physiological role. *Endocrinology* **135** (1994) 431–438.
15. Ogishima T., Suzuki H., Hata J-I., Mitani F. and Ishimura Y.: Zone-specific expression of aldosterone synthase cytochrome *P*-450 and cytochrome *P*-45011β in rat adrenal cortex: histochemical basis for the functional zonation. *Endocrinology* **130** (1992) 2971–2977.
16. Ho M.M. and Vinson G.P.: 11β-hydroxylase gene expression in the rat adrenal cortex. *J. Endocr.* 139 (1993) 301–306.
17. Yabu M., Senda T., Nonaka Y., Matsukawa N., Okamoto M. and Fujita H.: Localization of the gene transcripts of 11β-hydroxylase and aldosterone synthase in the rat adrenal cortex by *in situ* hybridization. *Histochemistry* **96** (1991) 391–394.
18. Mellon S.H. and Deschepper C.F.: Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. *Brain Res.* **629** (1993) 283–292.
19. Takeda Y., Miyamori I., Yoneda T., Iki K., Ama H.H., Blair I.A., Hsieh F-Y. and Takeda R.: Synthesis of corticosterone in the vascular wall. *Endocrinology* **135** (1994) 2283–2286.
20. Lauber M., Boni-Schentzler M. and Muller J.: Potassium raises cytochrome *P*45011β level in zona glomerulosa of rat adrenals. *Molec. Cell. Endocr.* **72** (1990) 159–166.
21. Wang A.M., Doyle M.V. and Mark D.F.: Quantitation of mRNA by the polymerase chain reaction. *Proc. Natn. Acad. Sci. U.S.A.* 86 (1989) 9717–9721