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Steroid 21-Hydroxylase in the Kidney: Demonstration of Levels of Messenger RNA Which Correlate with the Level of Activity

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Steroid 21-hydroxylase activity was assayed in low-speed supernatants prepared from whole cell homogenates of mouse and rat tissues. Kidney supernatants had an activity which was approximately 2–5% that of adrenal preparations while heart muscle was found to be without 21-hydroxylase activity. When the enzyme kinetics were characterized, both adrenal and kidney low-speed supernatants demonstrated saturation kinetics, but with very different V_{max} and K_m values. Using polymerase chain reaction amplification after reverse transcriptase synthesis of cDNA from isolated RNA (RT-PCR), we found low levels of mRNA for steroid 21-hydroxylase in mouse kidney, but none in heart muscle. Thus, extra-adrenal steroid 21-hydroxylase activity in the kidney may be mediated by the same enzyme as found in adrenals.

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INTRODUCTION

Several reports on steroid 21-hydroxylase activity in other organs than the adrenal gland have been published during the last few decades. One explanation for this can be found in the embryonic development of the adrenal glands, which sometimes results in the presence of adrenal cells in neighboring tissues. This phenomenon is of no clinical significance, except when such islets of adrenal cells become hyperactive or form malignant tumours [1]. Furthermore, it has been difficult to explain how high levels of progesterone are converted to deoxycorticosterone (DOC) during pregnancy without the involvement of extra-adrenal 21-hydroxylation. The rate of blood flow through the adrenals is not sufficient to transport all the progesterone required to attain the levels of DOC observed [2, 3]. Finally, a discrepancy between suspected genetic mutation/deletion and the clinical picture in certain cases of adrenogenital syndrome has indicated the existence of extra-adrenal activity. Such enzymes (other than 21-hydroxylase) would protect against severe salt-wasting, that could be predicted from a genetic analysis [4].

While undertaking different types of investigations, several authors have obtained the same results. 21-Hydroxylation activity is found in a variety of tissues, including the kidney, skin, bowel, liver and pancreas. Heart muscle cells are without such activity [5, 6]. Kidney cells are the most consistent in demonstrating this activity, with levels around 1% of those in adrenal cells. Since the total volume of the kidneys is much greater than that of the adrenals, several investigators have argued that the low rate of renal hydroxylation could be physiologically significant.

The present study characterizes the kinetics of steroid 21-hydroxylase activity in rat and mouse kidney cells and demonstrates the presence of mRNA for this enzyme in mouse kidney cells.

MATERIALS AND METHODS

Steroids

17α-OH-[1,2,6,7-^{3}H]-progesterone (65 Ci/mmol) was purchased from Amersham International (U.K.). Unlabelled 17α-OH-progesterone and 11-deoxycortisol were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Tissue preparations

Adrenal, kidney, pancreatic, liver and heart tissue samples were taken from adult NMRI mice and also from adult Sprague–Dawley rats. Skin, aorta, small intestine, colon and testis tissue samples were taken only from adult Sprague–Dawley rats. The animals were killed by CO_2-inhalation and the tissues removed and dissected free of extraneous tissue. Approximately 250 mg of each

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tissue was homogenized in 1 ml of buffer A (10 mM Hepes; 10 mM NaCl; 1.5 mM $MgCl_2$, pH 6.2) in a glass homogenizer. After centrifugation at 800*g* for 10 min, the supernatants were used as the enzyme source.

Assay of steroid 21-hydroxylase activity

This assay was performed essentially as described by Tusie-Luna *et al.* [7]. To 25–200 μl of each supernatant, 50 μl 2 mM NADPH (final concentration 0.2 mM) and buffer B (200 mM KH_2PO_4, pH 7.2) were added to give a total volume of 500 μl. Finally, radiolabelled 17α-OH-progesterone (0.2 μCi/sample) was added and the sample incubated at 37°C. The incubation was terminated by the addition of 100 μl methylene chloride (Sigma Chemical Co, St Louis, MO).

Each tube was mixed vigorously for 5 min and the methylene chloride phase containing the extracted steroids then pipetted into a new tube and evaporated to dryness. Markers dissolved in ethanol were added and the steroids resolved by thin-layer chromatography with chloroform:ethyl acetate (80:20) as eluant. Slices were taken from the TLC plate and their radioactivity determined by liquid scintillation spectrophotometry.

Isolation of mRNA and cDNA synthesis

For isolation of mRNA from tissue homogenates, the Micro Fast Track mRNA Isolation Kit (Invitrogen Corp, San Diego, CA) was used as recommended by the manufacturer. The final ethanol precipitate was vacuum-centrifuged to dryness and the pellet directly dissolved in reverse transcriptase buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$) to give a final volume of 20 μl containing dNTPs (0.5 mM of each dATP, dCTP, dGTP and dTTP), 10 mM DTT, 2.7 nM primer, 1.5 μl RNasin (40 U/μl) and 2 μl superscript RT (GIBCO BRL) and was subsequently incubated for 2 h at 45°C. The primer employed was 5'-GTGCCAGAG-GCTCTCCCAG (2445–2426) [8].

Polymerase chain reaction (RT-PCR)

The entire transcriptase reaction mixture was used for PCR in a final volume of 50 μl containing PCR buffer (5 μl 10-times concentrated PCR buffer, Perkin Elmer Cetus), 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs and 0.27 μM of each primer. After incubation at 96°C for 10 min, 2 units of amplitaq DNA polymerase (Perkin Elmer Cetus) was added (hot start). The primers employed were: 5'-CGTGCAACTAGGGCT-AGCAG (2085–2105), (upstream) and 5'-TTCCCAGGTTC-CAGGAAGCG (2382–2362), (downstream) [8]. The program was as follows: 96°C/1 min, 55°C/30 s and 72°C/2 min, 35 cycles were run. The PCR products were visualized and photographed under UV transillumination in a 2% agarose gel containing ethidium bromide. For isolation and restriction enzyme cleavage of PCR products, the amplified bands were cut out from low-gelling temperature agarose [9] and agarose removed using the Magic Prep Kit (Promega Corp, Madison, WI). Treatment with SrcF I (New England Biolabs, Berverly, MA) was performed in supplemented buffer at an approximate concentration of 5 U/μg DNA at 37°C for 1 h.

RESULTS

Low-speed supernatants from different tissues were analyzed for steroid 21-hydroxylation activity by incubation with radiolabelled 17-α-OH-progesterone. The conversion of radioactivity into 11-deoxycortisol was estimated by thin-layer chromatography (TLC) of methylene chloride extracts. Adrenal, kidney, heart and testis from both mice and rats were assayed, whereas low-speed supernatants from intestine, skin, liver and pancreas were only from rats.

In the case of adrenal, kidney (Fig. 1), colon, skin and aorta, radioactivity was recovered exclusively in spots demonstrating exactly the same mobility as the 17-α-OH-progesterone and 11-deoxycortisol standards. The specific activities (nmol product formed/mg protein/h incubation) expressed as percentages of adrenal activity were as follows: kidney, 2–5%; colon, 3–8%; skin, 2–3% and aorta, 2–4%. Low-speed supernatants from heart muscle and small intestine showed no activity, even when the incubation time was extended to 2 h (data not shown). In liver, pancreas and testis samples, peaks of radioactivity also appeared in other areas of the chromatograms. We concluded that our method utilizing crude low-speed supernatants was unable to distinguish between 21-hydroxylation and other metabolic events in these latter tissues.

Kidney preparations were found to demonstrate extra-adrenal steroid 21-hydroxylase activity most consistently and were therefore selected for kinetic analysis. First, linearity of product formation as function of incubation time was investigated (Fig. 2). The adrenal and kidney samples showed linearity for at least 10 min and this incubation time was thus selected for all subsequent experiments.

A crucial question was whether the dependence of steroid 21-hydroxylation on substrate concentration was the same in adrenals and kidneys. Increasing amounts of 17-OH-progesterone were added and the specific activities (nmol/mg protein/h) measured. In the experiment presented in Fig. 3, this activity in the kidney samples was approx. 3% of that in adrenal cells. In repeated saturation experiments with tissues from both mice and rats, the renal hydroxylation activity was found to be 2–5%, of its adrenal counterpart. Lineweaver–Burk plots of the data from Fig. 3 (Fig. 4) yielded apparent K_m values in rat adrenal and kidney samples of 6 and 25 μM and V_{max} values of 360 and 12 nmol/mg/h, respectively. Repeated experiments gave K_m values of 2–7 μM in adrenal preparations and 3–25 μM in kidney samples. The calculated maximal velocities were 200–400 and 10–20 nmol/mg/h, respectively.

Subsequently, we investigated expression of the

steroid 21-hydroxylase gene in the kidney. Previous reports have indicated the presence of transcripts of this gene in human liver [10], but RNase protection analysis [11] showed that only low levels of such transcripts could be expected. We decided to employ PCR amplification after reverse transcription (RT-PCR), since this is a very sensitive method.

Sequence information for mouse steroid 21-hydroxylase cDNA was available and the mouse gene was therefore selected for study. After preparation of polyA-RNA, RT-PCR demonstrated the presence of mRNA for steroid 21-hydroxylase in the kidney, while preparations from heart muscle were negative (Fig. 5). In the kidney and heart, DNA contaminants led to amplification of a longer fragment as well. The cDNA, as well as the DNA fragments, were of the expected sizes, i.e. 146 and 297 bp, respectively.

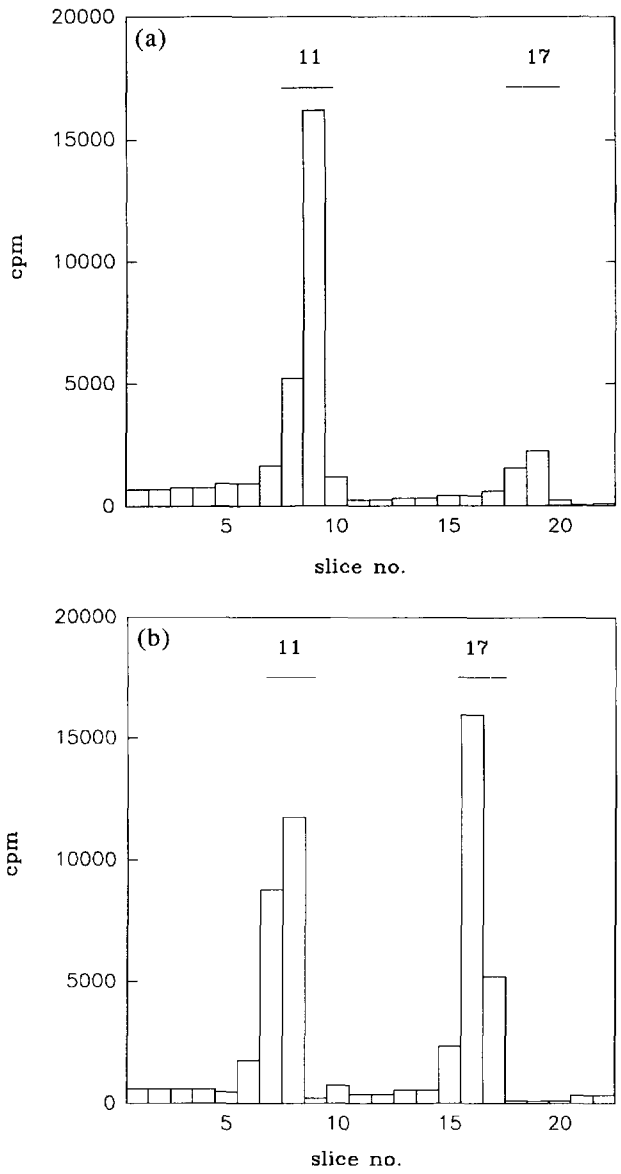

Fig. 1. Thin layer chromatography of steroids. Low-speed supernatants prepared from the adrenals (a) and kidneys (b) of rat were incubated with 0.5 μM [^{3}H]17α-OH-progesterone for 10 min. After incubation, steroids were extracted with methylene chloride and separated by TLC. The horizontal bars indicate the positions of the standards (11, 11-deoxycortisol; 17, 17α-OH-progesterone).

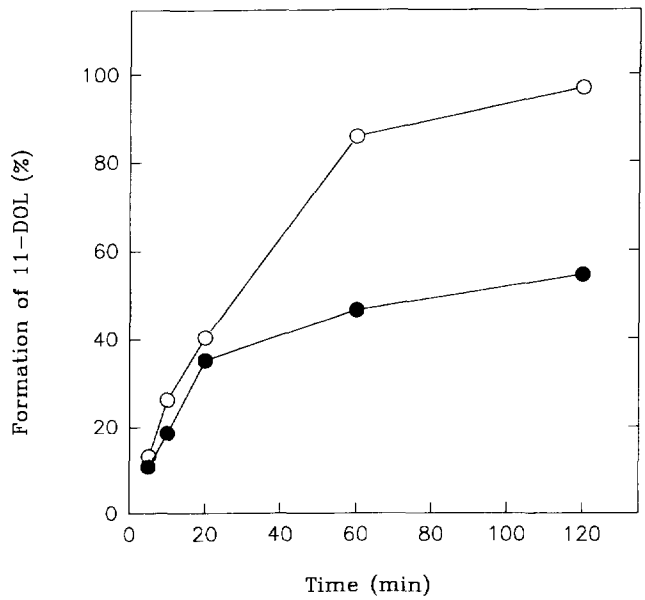

Fig. 2. Time curve for steroid 21-hydroxylation. Low-speed supernatants prepared from the adrenals (○) and kidneys (●) of rat were incubated with [^{3}H]17α-OH-progesterone for 5–120 min. The conversion of label from 17α-OH-progesterone to 11-deoxycortisol was analyzed by TLC. Radioactivity in 11-deoxycortisol is expressed as a percentage of the total radioactivity added.

The cDNA band (146 bp) was removed from the gel and subjected to restriction enzyme cleavage (Scr FI), which resulted in fragments of the expected sizes from both adrenal and kidney RT-PCR products (data not shown). RT-PCR resulted in the expected fragments in five of six experiments (one experiment failed to show any PCR products in the kidney sample). To exclude the possibility of contaminating fragments, three of these six experiments were performed in another laboratory with different equipment, including pipettes, and chemicals. Standard controls (lacking template or primers) were negative in all experiments.

DISCUSSION

In spite of the fact that several investigators have demonstrated steroid 21-hydroxylase activity in extra-adrenal tissues, the nature of this activity remains obscure. Some investigators have found variations in the K_m values for steroid 21-hydroxylation in different tissues, while others have reported enzyme kinetic data supporting the hypothesis that the same steroid 21-hydroxylase gene is expressed both in the adrenal and in other tissues [11, 12]. Using RNase protection assay analyses, Mellon and Miller [3] clearly demonstrated that only the adrenals produce high levels of mRNA for

steroid 21-hydroxylase in humans and rats. In contradiction, Sasano and Sasano [4] reported the presence of proteins which cross react immunochemically with antibodies towards the adrenal steroid 21-hydroxylase protein in human kidney, pancreas and salivary glands, whereas colon and liver tissues were negative.

In the present study we present data on steroid 21-hydroxylation in adrenals and kidneys. In repeated experiments the activity in kidney preparations was 2–5% that of adrenal samples while heart muscle had no capacity for 21-hydroxylation. Increasing substrate concentrations indicated saturation kinetics in both adrenals and kidneys, but these experiments could neither confirm nor exclude enzymatic identity in the two tissues. Substantial variation between experiments, especially in the kidney samples, was the main reason for this uncertainty.

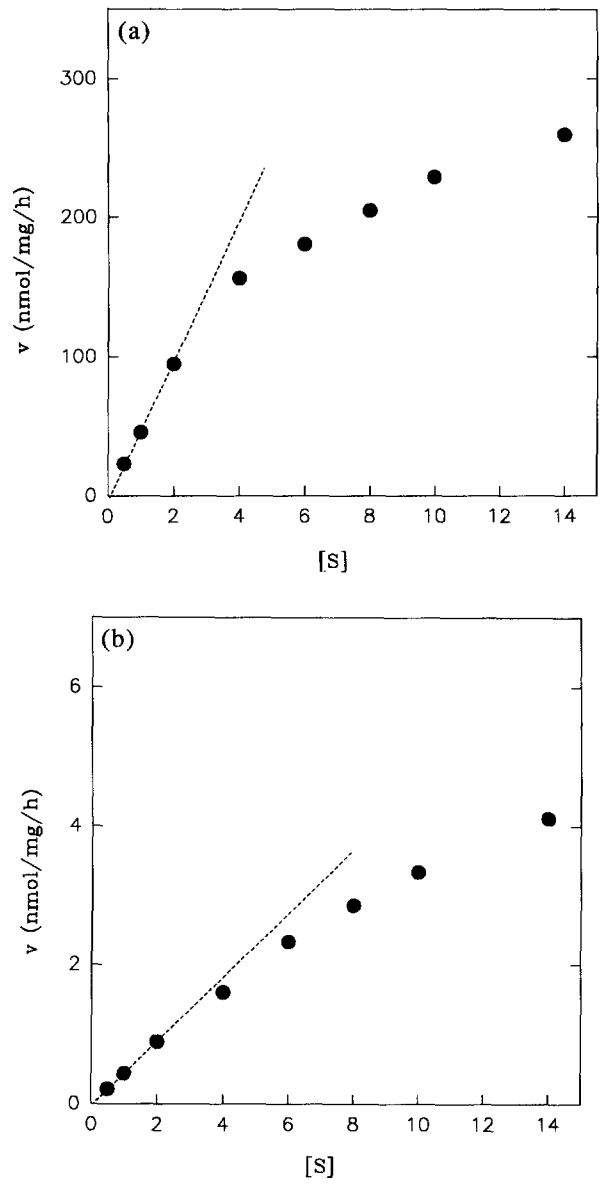

Fig. 3. Saturation kinetics of steroid 21-hydroxylation. Low-speed supernatants prepared from the adrenals (a) and kidneys (b) of rat were incubated with [^{3}H]17α-OH-progesterone at different concentrations for 10 min and the specific activities expressed as nmol product formed/mg protein/h. The dotted lines indicate the initial linear slope of the curves. [S], substrate concentration in μM.

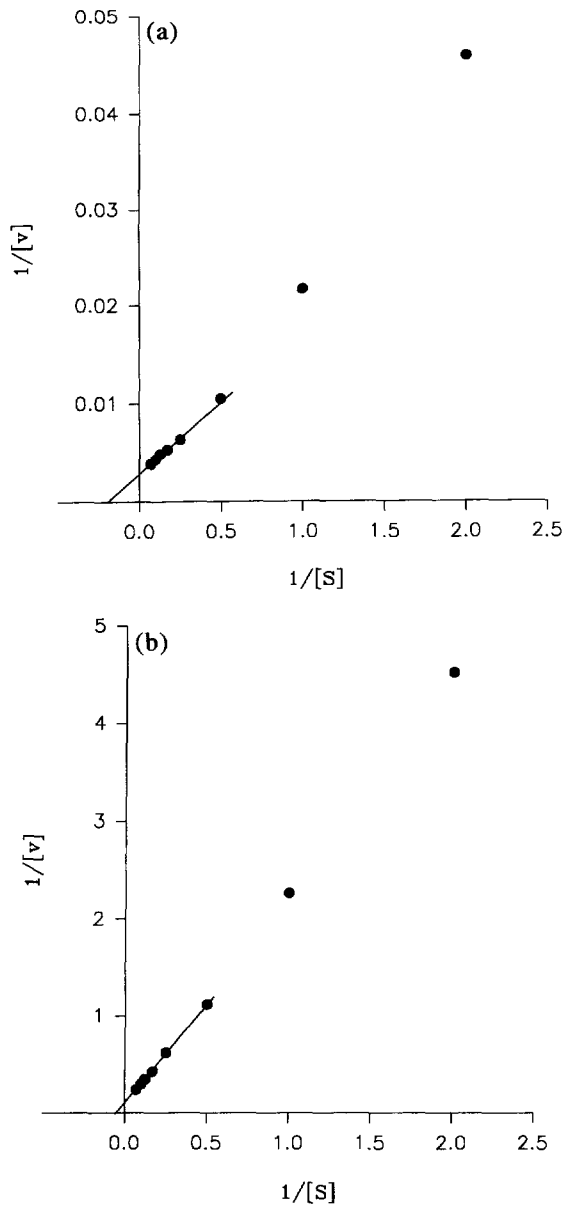

Fig. 4. Lineweaver–Burk plot of steroid 21-hydroxylation. The experimental data from Fig. 3 were used.

When our activities were compared with previously published results, it was found that several authors have reported similar levels of activity [7, 15]. In contrast other articles report steroid 21-hydroxylation activity in the pmol/mg/h range, i.e. 100–1000-fold lower [5, 6, 11]. It is, however, striking that the relationship between the activities in different tissues is consistent.

Therefore, we conclude that the wide variations in enzyme activities reported reflects methodological

differences. One major problem is the difficulty in obtaining comparable substrate concentrations, due to the hydrophobicity of the steroids. In addition, different homogenization and centrifugation procedures and purification steps result in widely different specific activities for this enzyme. In the present investigation no purification, apart from a brief centrifugation to remove intact cells and cell debris, was performed. The activities reported in this and many other studies can, therefore, be questioned and serve perhaps only for making comparisons between different tissues.

In order to investigate the expression of the steroid 21-hydroxylase gene in kidney tissue, we employed the polymerase chain reaction after cDNA synthesis from polyA-RNA extracts (RT-PCR). We found low levels of mRNA for steroid 21-hydroxylase in kidney preparations, while heart muscle was negative (Fig. 5). Since PCR is an extremely sensitive method, we feared that positive results (in spite of standard controls) could reflect contamination by amplified DNA-fragments in the laboratory. Therefore, we repeated the experiments in another laboratory, with new stocks of all chemicals and pipettes etc., that had never been used in this project. The same results were obtained.

We conclude that kidney cells from rats and mice express low levels of the same steroid 21-hydroxylase expressed in adrenals. The RT-PCR procedure used in this investigation is not quantitative. It is, however, striking that all five positive experiments demonstrated adrenal:kidney ratio of PCR product intensity of approximately, 10:1. We have not yet analyzed human tissues and cannot exclude species differences in expression of the steroid 21-hydroxylase gene. As mentioned in the Introduction, one reason for the present interest in extra-adrenal steroid 21-hydroxylation is the finding that gene alterations thought to be responsible for severe deficiency in this activity are found in patients with only minor symptoms [4]. This can be interpreted as evidence for the presence of other enzymes catalyzing steroid 21-hydroxylation, perhaps in a salvage pathway. With the help of sequence-specific PCR and sequencing of mutated genes, the number of known mutations responsible for steroid 21-hydroxylase deficiency is increasing rapidly [16, 17]. Characterization of renal steroid 21-hydroxylation in an individual with severe steroid 21-hydroxylase deficiency remains to be performed. Until then, the biological significance of extra-adrenal steroid 21-hydroxylation will remain unexplained.

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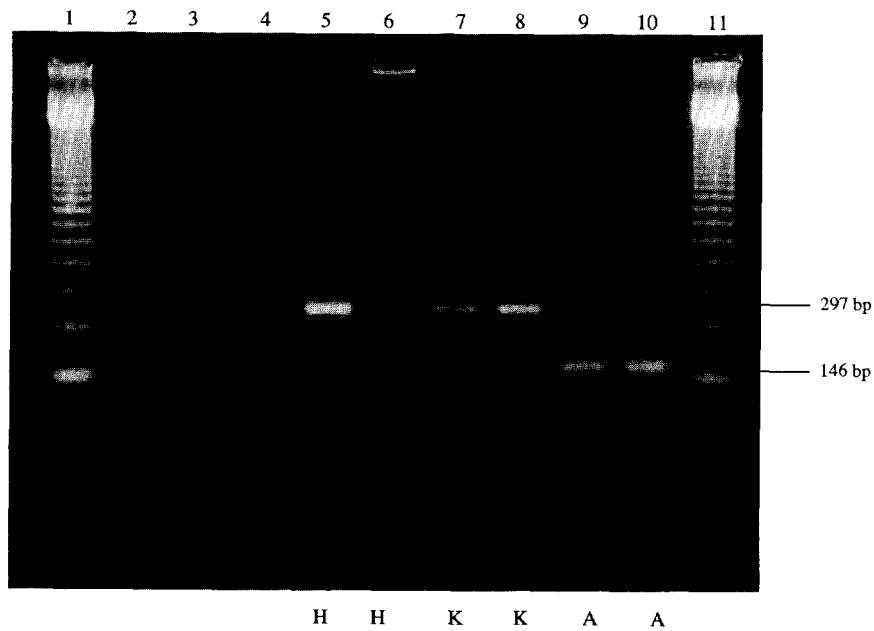

Fig. 5. PCR products obtained after reverse transcriptase synthesis of cDNA from mRNA extracts. Tissues from adult mice were homogenized and mRNA was prepared from heart (lanes 5 and 6), kidney (lanes 7 and 8) and adrenals (lanes 9 and 10). The cDNA products were subjected to PCR and the PCR products separated on a 2% agarose gel. Lanes 2–4 are negative controls (lacking template or primers or with a non-specific template, respectively). Lanes 1 and 11 contain DNA size markers (123 bp ladder).

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