Expression of the progesterone receptor and progesterone-metabolising enzymes in the female and male human kidney

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Abstract

Due to high binding affinity of progesterone to the human mineralocorticoid receptor (hMR), progesterone competes with the natural ligand aldosterone. In order to analyse how homeostasis can be maintained by mineralocorticoid function of aldosterone at the MR, especially in the presence of elevated progesterone concentrations during the luteal phase and pregnancy, we investigated protective mechanisms such as the decrease of free progesterone by additional binding sites and progesterone metabolism in renal cells. As a prerequisite for sequestration of progesterone by binding to the human progesterone receptor (hPR) we demonstrated the existence of hPR expression in female and male kidney cortex and medulla at the level of transcription and translation. We identified hPR RNA by sequencing the RT-PCR product and characterised the receptor by ligand binding and Scatchard plot analysis. The localisation of renal hPR was shown predominantly in individual epithelial cells of distal tubules by immunohistology, and the isoform hPR-B was detected by Western blot analysis. As a precondition for renal progesterone metabolism, we investigated the expression of steroid-metabolising enzymes for conversion of progesterone to metabolites with lower affinity to the hMR. We identified the enzyme 17α-hydroxylase for renal 17α-hydroxylation of progesterone. For 20α-reduction, different hydroxysteroid dehydrogenases (HSDs) such as 20α-HSD, 17β-HSD type 5 (3α-HSD type 2) and 3α-HSD type 3 were found. Further, we detected the expression of 3β-HSD type 2 for 3β-reduction, 5α-reductase (Red) type 1 for 5α-reduction, and 5β-Red for 5β-reduction of progesterone in the human kidney. Therefore metabolism of progesterone and/or binding to hPR could reduce competition with aldosterone at the MR and enable the mineralocorticoid function.

Journal of Endocrinology (2002) **175,** 349–364

Introduction

Progesterone is one of the main steroid hormones involved in the regulation of female reproductive function (Graham & Clarke 1997). Its effects are mediated by the progesterone receptor (PR), a member of the nuclear receptor family of ligand-activated transcription factors (Tsai & O'Malley 1994). Upon binding of progesterone to the ligand-binding domain (Williams & Sigler 1998), the whole receptor protein undergoes conformational changes leading to dissociation from heat shock protein (hsp) such as hsp90 (Pratt & Toft 1997). This enables dimerisation of receptor monomers and binding to hormone-responsive elements of DNA within the regulatory region of target genes (Beato *et al.* 1987). Transcription or suppression of the target gene depends on the context of promoter and the distribution of PR isoforms in target cells. Two isoforms of the PR have been described (Horwitz & Alexander 1983): PR-B (933 amino acids) and PR-A (769 amino acids), the latter lacking 164 N-terminal amino acids of PR-B. Both isoforms are expressed from the same gene by transcription from two alternative promoters and translation from two different start codons located in the transcript of the first exon (Kastner *et al.* 1990*a*). PR expression has been described in classical target organs like the uterus (Bergeron *et al.* 1988), ovary (Duffy & Stouffer 1995), vagina (Batra & Iosif 1985), breast (Horwitz & McGuire 1975) and brain (pituitary gland and hypothalamus) (Kato *et al.* 1978). In endometrial stromal cells, progestins induce target genes encoding transforming growth factor-β and insulin-like growth factor-binding protein (IGFBP)-1. The IGFBP-1 promoter is more

strongly induced by PR-A than by PR-B (Gao *et al.* 2000). In uterine epithelia, the stimulating effect of progesterone on the expression of histidine decarboxylase is mediated by PR-B, as shown by experiments with knockout mice for PR-A, while PR-A seems to be essential for a progesterone activation of amphiregulin and calcitonin expression (Mulac-Jericevic *et al.* 2000). In the breast cancer cell line T-47D, progestins induce the expression of desmoplakin, CD59/protectin, FKPB51, and the Na^+/K^+-ATPase subunit α1. The latter is also found in normal breast tissue (Kester *et al.* 1997). In T-47D, expression of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 is increased by progestins (Arcuri *et al.* 2000). For 11β-HSD type 2, co-localisation with the mineralocorticoid receptor (MR) has been demonstrated in normal and malignant human breast tissue (Sasano *et al.* 1997), where it could facilitate selective binding of aldosterone to MR, as has been shown in the kidney (Edwards *et al.* 1988). McDonnell *et al.* (1994) described the possibility of a repression of MR transcriptional activity by ligand-activated PR-A via cross-talk of MR with this potent transdominant inhibitor competing for a common transcription factor or 'adaptor'.

The question whether the PR is also expressed in the human kidney is of special interest, since progesterone also binds with high affinity to the renal MR but confers only weak transcriptional activity. Hence progesterone is a strong MR antagonist (Rupprecht *et al.* 1993*a*, Myles & Funder 1996). Geller *et al.* (2000) have described an agonistic progesterone function at the mutated MR_{L810}, found in a family of patients with early onset of severe hypertension. Pregnant members developed preeclampsia caused by a normal increase of progesterone. To determine how homeostasis is maintained in late pregnancy, for example, when the plasma progesterone concentration can rise to 700 nM (Johansson & Jonasson 1971) with an aldosterone increase to 5·8 nM (Nolten *et al.* 1978), we examined two mechanisms for avoiding excessive progesterone binding at the MR.

One explanation for the discrepancy of high progesterone concentration in the presence of wild-type MR and a still functioning renin–aldosterone system (Oelkers 1996) could be a competition of PR with MR for binding of progesterone in the kidney. Therefore we examined the expression of the human (h)PR in renal cortex and medulla of female (pre- and postmenopausal) and male origin. The possibility of high-specificity binding of progesterone to hPR in mineralocorticoid target cells could prevent antagonism or agonism of progesterone at the MR in the kidney.

Another mechanism for reducing progesterone binding to MR is the metabolic conversion of progesterone to derivatives with lower affinity to hMR. In renal cell fractions an effective metabolism of progesterone has been described as: 17α-OH(hydroxy)-progesterone(P) (23–32%), 20α-DH(dehydro)-P (24–27%), 17α-OH,20α-DH-P (9–11%), 5α-DH-P (7–8%), 20α-DH,5α-DH-P (5%), 3β,5α-TH(tetrahydro)-P (2–3%), 20α-DH,3β,5α-TH-P (2%), 3α,5α-TH-P or 3β,5β-TH-P (1–2%), and 5β-DH-P (<1%) (total conversion of more than half of progesterone in the range of 1 nM–1 μM) (Quinkler *et al.* 1999, 2001). These metabolites exhibit reduced affinity to the MR in comparison with progesterone: 7% for 17α-OH-P, 11% for 20α-DH-P, 0·2% for 17α-OH,20α-DH-P, 9% for 5α-DH-P, 0·4% for 20α-DH,5α-DH-P, 0·5% for 3β,5α-TH-P,<0·1% for 20α-DH,3β,5α-TH-P,<0·1% for 3α,5α-TH-P,<0·1% for 3β,5β-TH-P, and 0·6% for 5β-DH-P (Quinkler *et al.* 2002). Thus the following steroid-metabolising enzymes were examined for renal expression in female (premenopausal) and male kidney cortex and medulla: for 17α-hydroxylation, 17α-hydroxylase/17,20-lyase (P450c17 or CYP17) (Chung *et al.* 1987), for 20α-reduction, 20α-HSD (aldo-keto reductase AKR1C1) (Nishizawa *et al.* 2000), 3α-HSD type 3 (AKR1C2) (Shiraishi *et al.* 1998), prostaglandin F synthase (PGFS, AKR1C3) (Suzuki-Yamamoto *et al.* 1999), and 17β-HSD type 5 (Dufort *et al.* 1999) with 20α-HSD activity, for 5α-reduction, both isoforms 5α-reductase (Red) type 1 and type 2 (Andersson & Russell 1990, Andersson *et al.* 1991), for 5β-reduction, 5β-Red (Kondo *et al.* 1994), and for 3β-reduction, both isoforms 3β-HSD type 1 and type 2 (Rheaume *et al.* 1991, Dumont *et al.* 1992).

Materials and Methods

Human tissues and cell lines

Kidney specimens were obtained from nephrectomies of female and male patients with renal carcinoma. Tissue not needed for histological diagnosis was separated into medulla and cortex specimens and immediately snap frozen in liquid nitrogen. Myometrium and endometrium were obtained from a hysterectomy of one postmenopausal patient with the indication of prolapsus uteri. The cell lines MCF7 (human breast adenocarcinoma cells), T-47D (human breast ductal carcinoma cells) and CV-1 (African green monkey kidney fibroblasts) were obtained from ATCC Cell Lines (Rockville, CA, USA).

Cell culture

CV-1 and MCF7 cells were cultured in Dulbecco's MEM (modified Eagle's medium) supplemented with 10% fetal calf serum (FCS) and 100 000 IU/l penicillin and 100 mg/l streptomycin. For culture of CV-1, 2·5 mg/l amphotericin B and for MCF7, 2 mM glutamine were added. T-47D were maintained in RPMI 1640 medium with 10% FCS, 100 000 U/l penicillin and 100 mg/l streptomycin, 1 mM sodium-pyruvate and

200 IU/l insulin. All materials used for cell culture were obtained from Seromed Biochrom, Berlin, Germany. Cells were grown in 70 cm^2 flasks at 37 °C under a 95% air–5% CO_2 humidified atmosphere.

Preparation of cell and tissue homogenates and fractionation

Cells were harvested with trypsin/EDTA (0·05%/0·02%), washed with PBS and lysed in ice-cold buffer containing potassium phosphate (10 mM K_2HPO_4 and 10 mM KH_2PO_4, 1·5 mM EDTA, 3 mM NaN_3, 10% glycerol pH 7·5) (PENG buffer) (Pollow *et al.* 1989) supplemented with protease inhibitors obtained by dilution of Complete Mini protease inhibitors from Boehringer Mannheim, Mannheim, Germany. Three cycles of freezing in liquid nitrogen and thawing at 37 °C or homogenisation with ten strokes in a cooled Potter homogeniser were used for lysis, and centrifugation at 4 °C (30 min, 15 000 ***g***) for removal of debris and nuclei. Tissues of myometrium and endometrium were minced in 3 volumes (w/v) of ice-cold PENG buffer with an Ultra Turrax (3 × 2 s) and homogenised as described above. Centrifugation at 4 °C (10 min, 800 ***g***) sedimented the nuclear fraction. Cytosol was obtained by further centrifugation at 4 °C (30 min, 15 000 ***g***) as the supernatant fraction. Tissue homogenates of kidney cortex and medulla were prepared in 3 volumes of ice-cold lysis buffer containing 10 mM sodium phosphate pH 7·4 and 250 mM sucrose (Monder & Lakshmi 1989). Centrifugation at 4 °C (30 min, 750 ***g***) pelleted the nuclear fraction and (60 min, 100 000 ***g***) gave the cytosol fraction with soluble proteins in the supernatant. Proteins from the nuclear fraction were extracted with 0·4 M KCl in the presence of 1·5 mM EDTA, 1 mM dithiothreitol (DTT), 10 mM $Na_2MoO_4.2H_2O$, and 10% glycerol in lysis buffer. The protein contents of cytosols and nuclear fractions were analysed by using the Bio-Rad protein assay (Bio-Rad, Munich, Germany).

Ligand binding assays and Scatchard plot analysis

Promegestone (R5020) and [17α-methyl-^{3}H]R5020 (NEN, Zaventem, Belgium) were used as high-specificity ligands for the progesterone receptor. [17α-methyl-^{3}H]R5020 with a specific activity of 80 Ci/mmol was diluted in ethanol for concentrations of 0·05, 0·1, 0·2, 0·4, 0·8, 1·6, 3·2, 6·4 and 12·8 nM. Additionally a 200-fold excess of cortisol for saturation of the corticoid-binding globulins and the glucocorticoid receptor (GR), and aldosterone for saturation of the MR were added. Rupprecht *et al.* (1993*b*) published affinities for aldosterone to the MR (K_d=0·09 nM and K_i=0·08 nM measured by competition), and for cortisol to the MR (K_i=0·13 nM) and to the GR (K_i=15 nM). For each concentration a 100-fold excess of unlabelled R5020 was used to determine the extent of non-specific binding. Steroids were evaporated and each dissolved in 100 µl PENG buffer with 0·2% ethanol and kept on ice.

Cytosol fractions of cells (MCF7 and T-47D) and tissues (human renal cortex and medulla of postmenopausal patients) were pretreated with dextran-coated charcoal (DCC) (0·5% DCC in PENG buffer) for removal of endogenous steroids by incubation at 4 °C for 1 h. DCC with adsorbed steroids was sedimented by centrifugation at 4 °C (10 min, 15 000 ***g***) and each 100 µl aliquot of supernatant cytosol plus 100 µl of dissolved steroids was incubated at 4 °C for 16 h. Unbound steroids were removed following addition of 500 µl 1% DCC in PENG buffer by adsorption at 4 °C for 10 min and centrifugation at 4 °C (10 min, 15 000 ***g***). Aliquots (600 µl) of the supernatant were transferred into scintillation vials with 10 ml LSC Cocktail Ultima Gold (Packard, Dreieich, Germany) and counted for radioactivity. The binding parameters were calculated by linear regression analysis.

Immunohistology

Paraffin-embedded renal tissue specimens were retrieved from the files of the Department of Pathology at the Benjamin Franklin Clinic of the Free University of Berlin, Germany. They originated from the surroundings of renal cell carcinomas obtained in the course of nephrectomy. Myometrial tissue was obtained by hysterectomy. Sections of 4 µm were cut, deparaffinised and subjected to an antigen-retrieval protocol to optimally visualise antigens in paraffin-embedded tissue. Tissue sections were immersed in citrate buffer (10 mM, pH 6·0) and cooked under high pressure for 5 min. Sections were than incubated with the anti-PR IgG1 monoclonal antibody (clone PgR 636) in a dilution of 1:100. Bound antibody became visualised using the alkaline phosphatase–anti-alkaline phosphatase (APAAP) complex method and Fast Red as chromogen (Dako, Glostrup, Denmark).

Western blotting

Preparation of protein samples and immunoprecipitation of PR Cytosols or total protein preparations including nuclear extracts of cultured cells or tissues were used for immunoprecipitation of PR as described (Attia *et al.* 2000) with some modifications. Samples containing 500, 1000 and 5000 µg protein were incubated for 1 h at 4 °C with 2–4 µg rabbit polyclonal antibody PR(C20) sc539 (recognising a common region of PR-A and PR-B) obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Protein A-Sepharose (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) in a mixture of packed swollen particles 1:1 in buffer A (10 mM Tris–HCl pH 7·5 with 1·5 mM EDTA and 12 mM monothioglycerol) was added (100 µl per sample) and incubated for 1 h at 4 °C. Immunocomplexes were precipitated by centrifugation

(1 min, 10 000 ***g***) and each pellet washed twice with 500 µl buffer A. Following resuspension in 20 µl 2 × SDS loading buffer containing 215 mM Tris–HCl pH 6·7, 17·4% glycerol, 5·2% SDS, 8·7% β-mercaptoethanol and 0·27% bromphenol blue, samples were denatured at 97 °C for 5 min. Protein A-Sepharose was pelleted by centrifugation (1 min, 10 000 ***g***) and the supernatant used for protein analysis.

SDS-PAGE, Western transfer blotting and immunodetection Polyacrylamide mini-gels for protein electrophoresis were prepared as described in the manual (Hoefer, San Francisco, CA, USA) with two layers of 8 and 5% polyacrylamide gels containing 0·1% SDS respectively. Protein samples in SDS loading buffer and a prestained protein ladder (Bio-Rad) were separated during electrophoresis with 20 mA/gel for 1 h and proteins were blotted by electrotransfer with 400 mA for 2 h to PVDF membranes (Bio-Rad). Membranes were incubated overnight in a blocking solution containing 0·5% casein and 0·1% Tween-20 in PBS (Vector Laboratories, Burlingame, CA, USA). Blocked membranes were incubated for 1 h at room temperature (RT) with primary antibody NCL-PGR 312 (mouse monoclonal anti-hPR (PR-A and PR-B) IgG) (Novocastra, Newcastle, UK) diluted 1:1000 in blocking solution and, following extensive washing, for 1 h at RT with the secondary antibody goat anti-mouse IgG conjugated to peroxidase (Sigma, Deisenhofen, Germany), diluted 1:4000 in blocking solution. Chemiluminescence of the peroxidase reaction was detected by ECL or ECL Plus (Amersham Pharmacia Biotech). PVDF membranes were stripped for 30 min at 50 °C in 62·5 mM Tris–HCl pH 6·7, 100 mM β-mercaptoethanol, 2% SDS, blocked and reincubated with NCL-PGR 312, followed by a different secondary antibody (biotinylated anti-mouse IgG diluted 1:300) and horseradish peroxidase-streptavidin diluted 1:500 (Vector).

Preparation of RNA for gene expression analysis

RNAs from tissues (150–250 mg snap-frozen kidney cortex and medulla specimens) and from cultured cells (10^6–10^7 cells of MCF7 or CV-1) were isolated immediately after thawing of tissues or directly after harvesting of cells respectively with the RNeasy Kit (Qiagen, Hilden, Germany) including an on-column DNase digestion with the RNase-free DNase set also provided by Qiagen. The concentration and quality of RNA was determined photometrically (260/280 nm).

RT-PCR

RNA (2·5 µg) was annealed with random hexamer primers, 15 µg/45 µl incubation volume, for 10 min at 70 °C. First-strand reverse transcription was performed in 100 µl mixtures with 1000 U RT II Superscript polymerase in the presence of 40 U RNase inhibitor, 1 µmol DTT, 50 nmol dATP, 50 nmol dGTP, 50 nmol dCTP, 50 nmol dTTP in first-strand buffer for 1 h at 37 °C followed by denaturation for 5 min at 95 °C. All supplements were obtained from Gibco BRL (Karlsruhe, Germany). Specific PCRs with synthesised cDNA templates (transcribed from 40 ng RNA) were carried out in amplification mixtures of 25 µl containing 20 mM Tris–HCl pH 8·4, 50 mM KCl, 1·5 mM $MgCl_2$, and 0·2 mM each of dATP, dCTP, dGTP, dTTP using 1 U Taq DNA polymerase (Gibco BRL) and 12 pmol specific sense and antisense primers (Tib Molbiol, Berlin, Germany) listed in Table 1.

PCRs were performed with an initial step of denaturation for 2 min at 94 °C and were run for 35 cycles with denaturation for 45 s at 94 °C, annealing for 1 min at the thermodynamic melting temperature of the specific primer pair (Table 1), and extension for 90 s at 72 °C followed by a final extension for 10 min at 68 °C. Amplicons were resolved by electrophoresis in 1% agarose gels stained with ethidium bromide. The size and quantity of PCR products were approximated by comparison with a 1 kb Plus-DNA ladder and DNA low-mass ladder (Gibco BRL). PCR products of expected size were re-extracted from agarose gels (QIAquic gel extraction kit; Qiagen) for direct sequencing. Each sample was sequenced twice with the specific sense and antisense primers (Table 1) respectively using an automated sequencing device (ABI Prism 377; Perkin-Elmer, Überlingen, Germany), as described previously by Herrmann *et al.* (2001). Sequences were identified by blast search GenBank analysis.

hPR-specific high-stringency PCR

The conditions of hPR-specific RT-PCR were similar to those described (Misao *et al.* 1998) with 35 instead of 25 cycles using an annealing temperature of 55 °C for the primer pair hPR sense and hPR antisense (Table 1). We investigated cDNAs from tissue specimens of kidney cortex and medulla of a premenopausal, a postmenopausal, and a male patient. The PR-expressing breast carcinoma cell line MCF7 was used as positive control. The vector pRShMR which contains the complete coding sequence (CDS) for the hMR (Arriza *et al.* 1987), kindly provided by R Evans, was used as negative control for the hPR-specific PCR.

High-stringency PCRs specific for progesterone-metabolising enzymes

Specific primers were designed according to published sequences of steroid-converting enzymes and PCRs were performed with 35 cycles using primer pair-specific annealing temperatures as summarised in Table 1.

Table 1 Gene-specific primers for high-stringency PCRs. Sets of gene-specific primer pairs (s=sense, as=antisense) for amplification of cDNA according to the published CDS (first reference) and genomic location according to the exons of the published gene sequences (second reference) of the hPR and progesterone-metabolising enzymes are shown. Calculated sizes of the expected amplicon and the primer pair specific annealing temperatures are indicated for PCRs (2 min 94 °C, 35 cycles: 45 s 94 °C, 1 min × °C, 90 s 72 °C, and 10 min 68 °C). Note that 17β-HSD type 5 specific primers were designed to the high homologous sequence of 3α-HSD (clone HAKRb) because no sequence for 17β-HSD type 5 was available in the GeneBank

	Primer (sense); primer (antisense)	Genomic location	Annealing temperature (°C)	Expected size of amplified cDNA (bp)	According to published sequences (references)
Specific primers for hPR and enzymes					
hPR s	5′-AGCCCTAAGCCAGAGATTCA-3′	Exon 4	55	303	Misrahi *et al.* (1987)
hPR as	5′-TAGGATCTCCATCCTAGACC-3′	Exon 5			Misrahi *et al.* (1993)
17α-hydroxylase s	5′-TCTCTTGCTGCTTACCCTAG-3′	Exon 1	55	527	Chung *et al.* (1987)
17α-hydroxylase as	5′-TCAAGGAGATGACATTGGTT-3′	Exon 3			Picado Leonard & Miller (1987)
20α-HSD s	5′-GGGATCCCACCGAGAAGAACC-3′	Exon 6	65	416	Stolz *et al.* (1993)
20α-HSD as	5′-TAACCAGAGGCGATGTGTCCAGTC-3′	Exon 9			Lou *et al.* (1994)
3α-HSD type 3 s	5′-TAAAAGTAAAGCTCTAGAGGCCGT-3′	Exon 2	55	191	Ciaccio & Tew (1994)
3α-HSD type 3 as	5′-ACTCTGGTCGATGGGAATTGCT-3′	Exon 3			Khanna *et al.* (1995)
PGFS s	5′-ATTCCGGGCAGCAGCAAACA-3′	Exon 1	55	562	Suzuki-Yamamoto *et al.* (1999)
17β-HSD type 5 as1*	5′-CTGCCTGCGGTTGAAGTTTGATA-3′	Exon 5			Khanna *et al.* (1995)
17β-HSD type 5 s1	5′-ACTTCATGCCTGTATTGGGATTTG-3′	Exon 1	60	476	Qin *et al.* (1993)
17β-HSD type 5 as1*	5′-CTGCCTGCGGTTGAAGTTTGATA-3′	Exon 5			Khanna *et al.* (1995)
17β-HSD type 5 s2	5′-GGAGGCCATGGAGGAGTGTAAGGA-3′	Exon 4	65	335	Qin *et al.* (1993)
17β-HSD type 5 as2	5′-GGTAGCGCAGGGCAATCAGG-3′	Exon 7			Khanna *et al.* (1995)
17β-HSD type 5 s3	5′-AACGAGACAAACGATGGGTGGAC-3′	Exon 6	60	469	Qin *et al.* (1993)
17β-HSD type 5 as3	5′-GTATTTCTGGCCTATGGAGTGAGC-3′	Exon 9			Khanna *et al.* (1995)
3α-HSD s	5′-GTGACAGGGAATG-3′	Exon 1	40	248	Qin *et al.* (1993)
3α-HSD as	5′-ATATGTCTTCTCTCTCA-3′	Exon 2			Khanna *et al.* (1995)
3β-HSD type 1 s	5′-GATCATCCGCCTCCTGGTG-3′	Exon 2	55	485	Dumont *et al.* (1992)
3β-HSD type 1 as	5′-GGGTGCCGCCGTTTTTCA-3′	Exon 4			Lachance *et al.* (1990)
3β-HSD type 2 s	5′-GATCGTCCGCCTGTTGGTG-3′	Exon 2	65	382	Rheaume *et al.* (1991)
3β-HSD type 2 as	5′-CTCTTCTTCGTGGCCGTTCTGGATGAT-3′	Exon 4			Lachance *et al.* (1991)
5α-Red type 2 s	5′-GCCCGGCAGCCCCTCTCC-3′	Exon 1	60	244	Andersson *et al.* (1991)
5α-Red type 2 as	5′-CACCCAAGCTAAACCGTATGTCTG-3′	Exon 2/3			Labrie *et al.* (1992)
5α-Red type 1 s	5′-CGGGCCGCCTGGGTGGTG-3′	Exon 1	65	168 (gene)	Andersson & Russell (1990)
5α-Red type 1 as	5′-TCCTCGCATCAGAAACGGGTAAAT-3′	Exon 2		168 (pseudo)	Jenkins *et al.* (1991)
5α-Red type 1 preATG s	5′-CTGGGGCATGGAGCACGC-3′	Exon 1	65	351 (gene)	Andersson & Russell (1990)
5α-Red type 1 as	5′-TCCTCGCATCAGAAACGGGTAAAT-3′	Exon 2		357 (pseudo)	Jenkins *et al.* (1991)
5α-Red type 1 pseudo s	5′-GGCCATGTTCCTCGTCCACTAC-3′	Exon 1	60	—	—
5α-Red type 1 pseudo as	5′-CCGCTCATGATGCTCTTTTGCTCT-3′	Exon 1		454 (pseudo)	Jenkins *et al.* (1991)
5α-Red type 1 gene s	5′-CAGATCCCCGTTTTCTAATAGG-3′	Exon 2	55	239 (gene)	Andersson & Russell (1990)
5α-Red type 1 gene as	5′-AAACGTGAAGAAAGCAAAAGC-3′	Exon 4			Jenkins *et al.* (1991)
5β-Red s	5′-AACAAGCCAGGACTCAAACACAAG-3′	Exon 5	60	451 (gene)	Kondo *et al.* (1994)
5β-Red as	5′-CTCCCGGCAGTCAGTATTCATCA-3′	Exon 9		432 (pseudo)	Charbonneau & The (2001)

*The antisense primer (17β-HSD type 5 as1) was also used for PGFS-specific cDNA amplification as there is only one mismatch in position 22. preATG, 5′ to start codon ATG; pseudo, pseudogene.

Table 2 Comparison of ligand binding characteristics. Scatchard plot analyses were performed with [^{3}H] R5020 using steroid-depleted cytosol fractions from tissue specimens of human kidney cortex and medulla (postmenopausal), myometrium (postmenopausal) as classical target tissue control and from hPR-expressing breast carcinoma cells MCF7 and T-47D

	K_d (nM)	B_{max} (fmol/mg protein)
Tissues and cells		
Kidney cortex	0·76	7
Kidney medulla	0·86	17
Myometrium	0·98	213
MCF7	0·57	43
T-47D	0·44	347

K_d=dissociation constant for specific binding of promegestone (R5020) to the receptor (hPR); B_{max}=maximum concentration of available binding sites (calculated using the linear regression method).

Templates for enzyme expression analyses were cDNAs from female (premenopausal) and male kidney samples and from CV-1 monkey kidney cells as control. Additionally cDNA templates from human adrenals and testes, kindly provided by W Arlt, were used as positive controls for steroid-metabolising enzyme expression.

Results

Characterisation of the hPR by binding of promegestone (R5020)

Scatchard plot analysis was performed using steroid depleted cytosol fractions from renal cortex and medulla as well as from myometrium (target tissue control). Additional positive controls were MCF7 and T-47D breast carcinoma cells, the latter overexpressing hPR-A. Specific binding of promegestone (R5020) as a highly specific ligand for the PR was found in kidney cortex and medulla specimens as well as in all controls. The binding affinities, characterised by their dissociation constant (K_d values) calculated by linear regression analysis, were in the same range (0·44–0·98 nM) for all samples (Table 2). Therefore the existence of the same kind of receptor in both kidney specimens and the progesterone target tissue myometrium as well as in hPR-expressing cells could be assumed. The amount of R5020-specific binding protein (B_{max}) in the kidney (7–17 fmol/mg protein) was less than 10% compared with the target tissue myometrium (213 fmol/mg protein). B_{max} in MCF7 cells was 43 fmol/mg protein and in T-47D cells 347 fmol/mg protein (Table 2).

Localisation of hPR in human kidneys by immunohistology

In tissue sections of female postmenopausal (Fig. 1A and B), premenopausal (Fig. 1C and D) and male (Fig. 1F) human kidneys and of myometrium (Fig. 1E) used as positive control, hPR was detected as nuclear staining following treatment with the antibody PgR 636, APAAP and Fast Red chromogen. A high percentage of myometrial smooth muscle cells stained positively for hPR (Fig. 1E). Labelling for hPR was also detected in kidney specimens. Positive immunodetections of hPR were found predominantly in some individual epithelial cells of distal tubules in female (Fig. 1A–C) and male (Fig. 1F), in single podocytes of glomeruli in postmenopausal (Fig. 1B), premenopausal (Fig. 1D), and male (Fig. 1F), in parietal cells in premenopausal (Fig. 1D) and in few interstitial cells of investigated renal tissues. The cytoplasmic staining (Fig. 1D and F) is probably not PR-specific but artefactual (reaction of anti-alkaline phosphatase, used in APAAP, with the renal alkaline phosphatase).

hPR-B is synthesised in female and male kidneys

Western blots of protein fractions prepared by immunoprecipitation of hPR gave chemiluminescent signals in human male and female kidney samples as a single band, corresponding to the middle band of the triplet for PR-B in cytoplasm of T-47D used as control (114, 117, 120 kDa) (Sheridan *et al.* 1989). This 117 kDa protein band was hardly detectable in a sample of cytosol from male kidney cortex with 200 μg protein content without immunoprecipitation of hPR, but the immunoprecipitates from cytosol aliquots with 5000, 1000 and 500 μg protein gave clear concentration-dependent chemiluminescent signals (Fig. 2A). This dependence on protein concentration used for immunoprecipitation was also found in total protein extracts from female kidney cortex and medulla (Fig. 2B). Smears of chemiluminescent signals in the range of slightly higher to lower size of PR-A (detectable in all samples) could not be identified as PR-A as in T-47D. After membrane stripping and reincubation with the identical first antibody, NCL-PGR 312, and a different second antibody (biotinylated anti-mouse IgG followed by horseradish peroxidase-streptavidin detection), chemiluminescence did not appear directly at the position of PR-A (compared with T-47D), but there were strong signals at the position of PR-B, besides non-specific bands smaller than PR-B and PR-A (Fig. 2A and B, lower panel). Therefore, only the synthesis of PR-B could be clearly shown in male and female kidney specimens.

hPR-gene expression in the kidney

The transcription of the hPR-gene analysed by RT-PCR was found in all of our kidney samples (Fig. 3). From cDNAs of renal cortex and medulla from premenopausal, postmenopausal and male patients, PCR products of 303 bp were amplified with the hPR-specific primer pair (Table 1), similar to the positive control MCF7 (PR-expressing cell line). The negative control did not give any DNA staining with ethidium bromide. In

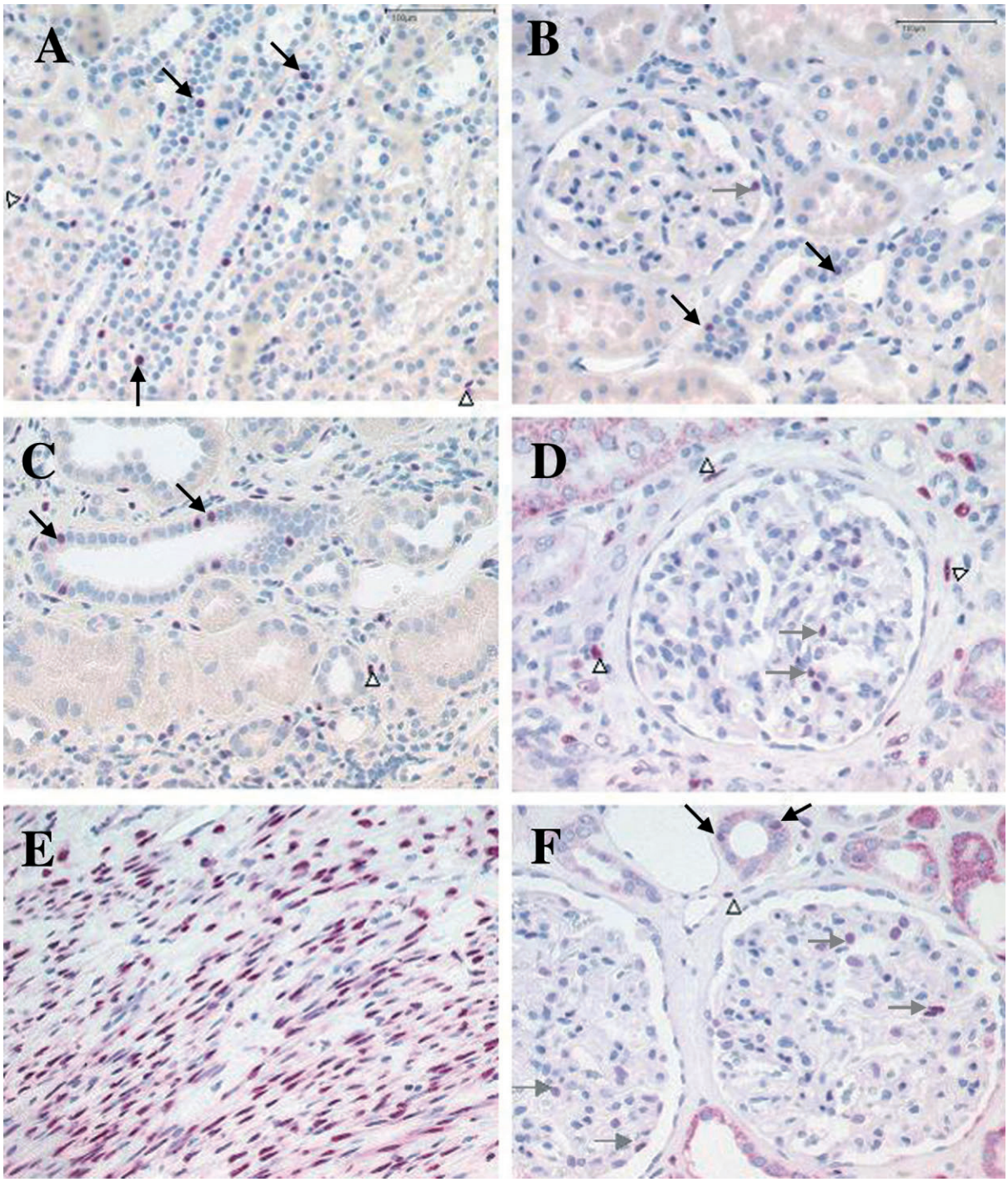

Figure 1 Immunohistological demonstration of PR expression in human kidney tissues. Immunohistological visualisation of hPR was performed on paraffin-embedded renal sections (4 µm) using PgR 636 (1:100) and APAAP complex formation with Fast Red chromogen. Demonstration of PR-positive cells in the epithelia of distal tubules (black arrows), glomeruli (grey arrows), and interstitium (white arrow-heads) in kidneys of different origin: postmenopausal (A, B), premenopausal (C, D), male (F) human kidney. Control staining for hPR was performed on myometrium (E).

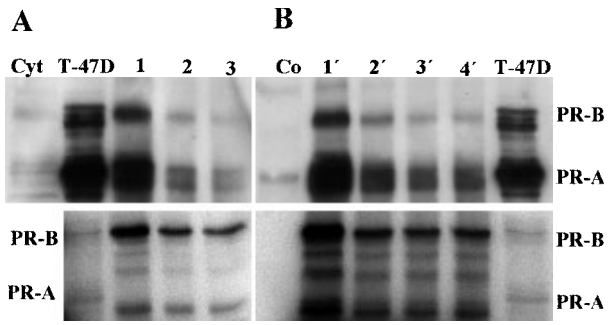

Figure 2 Western blot and immunodetection with NCL-PGR 312. (A) SDS-PAGE of 200 µg total protein from cytosol (Cyt) of male kidney cortex without immunoprecipitation, of 10 µg total protein from T-47D cells overexpressing PR-A as positive control, and immunoprecipitates obtained with PR(C20) sc539 rabbit polyclonal IgG from 5000 µg (lane 1), 1000 µg (lane 2), 500 µg (lane 3) protein in cytosol. (B) SDS-PAGE of PR(C20) sc539 under conditions of immunoprecipitation without renal proteins as negative control (Co), immunoprecipitates from 5000 µg (lane 1′), 1000 µg (lane 2′), 500 µg (lane 3′) total protein extract (cytosolic and nuclear proteins) of female kidney cortex and from 500 µg (lane 4′) of female kidney medulla, and 10 µg T-47D protein containing PR-A and PR-B as positive control. (A, B) Proteins were transferred to PVDF membranes and hPR immunodetected with NCL-PGR 312 mouse monoclonal anti-hPR IgG (1:1000) and goat anti-mouse IgG conjugated to peroxidase (1:4000) and visualised by chemiluminescence in the upper panel. There are concentration-dependent specific chemiluminescent signals as a single band at the position of PR-B in all kidney samples corresponding to the middle band in the triplet of PR-B (117 kDa) visible in T-47D, which was not detected in the negative control. A smear of chemiluminescence around the expected size of PR-A was found in all samples, but without concentration at the position of the PR-A-specific band as in the track of T-47D. There was a faint signal in the negative control evoked by the immunoprecipitating antibody itself as well. In the lower panel only PR-B could be detected after stripping of the membranes (A) and (B) and immunodetection with NCL-PGR 312 as well followed by biotinylated anti-mouse IgG and horseradish peroxidase-streptavidin. Chemiluminescent signals at positions smaller than PR-B and PR-A seem to be non-specific for PR.

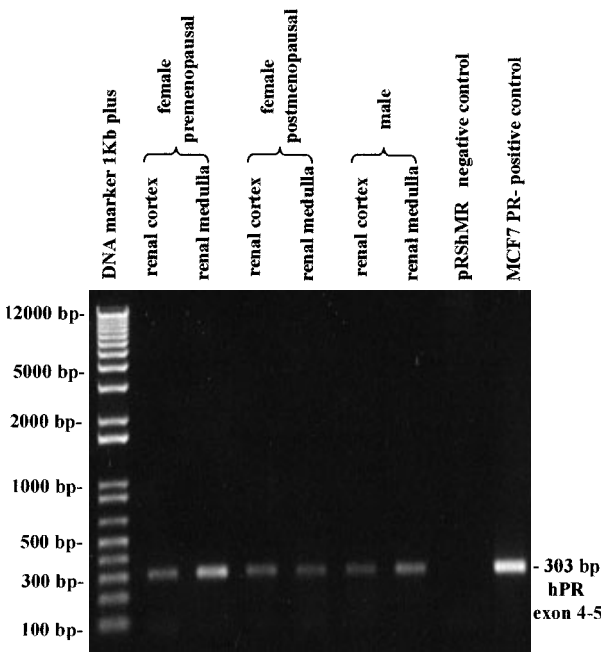

Figure 3 RT-PCR analysis for the expression of hPR. cDNAs obtained by reverse transcription of RNA from kidney cortex and medulla specimens of female (pre- and postmenopausal) and male origin and from MCF7 cells expressing the hPR as positive control were amplified by high-stringency hPR-specific PCR. pRShMR was used as template for the negative control. PCR products were separated on a 1% agarose gel with a 1 kb-Plus DNA ladder for sizing. Staining with ethidium bromide visualised the DNA marker (100–12 000 bp) and single bands of 303 bp amplicons specific for hPR (exon 4–5) from all kidney specimens and MCF7. pRShMR was not amplified by hPR-specific PCR.

addition to 35 cycles of PCR, lower cycle numbers were performed. Staining of specific PCR products was obtained from MCF7 cDNA after 25 cycles and from kidney samples after 30 cycles of PCR (data not shown). Direct sequencing (forward with the hPR sense primer and reverse with the hPR antisense primer) of the PCR product from the premenopausal kidney cortex revealed 100% sequence homology (Table 3) according to the published sequence (Misrahi *et al.* 1987). This confirmed the identity of the hPR-specific RT-PCR product in the human kidney.

Expression of different enzymes for progesterone metabolism

PCRs for steroid-metabolising enzymes were performed with different specific primer pairs (Table 1) tested with cDNA templates from human adrenals and testes as positive controls (Fig. 4). Amplicons of primer-specific calculated sizes (Table 1) were obtained for 17α-hydroxylase (527 bp), 20α-HSD (416 bp), 3α-HSD type 3 (191 bp), 3α-HSD type 2 (562 bp), 17β-HSD type 5 (335 bp), 3β-HSD type 2 (382 bp) and 5α-Red type 1 (239 bp) from both adrenals and testes. PCR products for 5α-Red type 2 (244 bp) and 5β-Red (451 bp) were synthesised only from testes but not from adrenals, and for 3β-HSD type 1 amplification could not be detected from adrenals (Fig. 4) or from testes (data not shown). PCRs with cDNA templates from human kidney cortex and medulla (premenopausal and male) and from the monkey kidney cell line CV-1 gave the following results.

17α-hydroxylase (P450c17=CYP17) The renal expression of 17α-hydroxylase (capable of hydroxylation at the angular C17 of the pregnane body) was analysed by RT-PCR slightly modified from the method described by Jose *et al.* (1999). Amplification of the expected PCR product of 527 bp was detected from cDNAs of all kidney samples as well as from CV-1 (Fig. 5A). Sequencing of the re-extracted and reamplified 527 bp PCR product

Table 3 Renal expression of PR and progesterone-metabolising enzymes. Expression analyses were performed from human kidney specimens (cortex and medulla) by RT-PCR and direct sequencing of specific amplicons from samples of one premenopausal patient. Detection is indicated by + and the homologies of the PCR product to the published sequences are given as percentage. − is used if expression (e.g. of isoenzymes) was not detected in the human kidney specimens used for analysis

Analysed receptor and enzymes	Amplification of cDNA (bp)	Found in the human kidney	Homology to published sequences (%)	References
hPR	303	+	100	Misrahi *et al.* (1987)
17α-hydroxylase (=CYP17)	527	+	100 99·8	Chung *et al.* (1987) Picado Leonard & Miller (1987)
20α-HSD (=DD1, AKR1C1)	416	+	100	Nishizawa *et al.* (2000)
3α-HSD type 3 (=DD2, AKR1C2)	191	+	100	Shiraishi *et al.* (1998)
PGFS (=3α-HSD type 2, AKR1C3)	562	+	99·8	Suzuki-Yamamoto *et al.* (1999)
17β-HSD type 5 amplicon 1	476	+	99·6	Qin *et al.* (1993)*
17β-HSD type 5 amplicon 2	335	+	99·1	Qin *et al.* (1993)*
17β-HSD type 5 amplicon 3	469	+	99·5 for complete CDS	Qin *et al.* (1993)*
17β-HSD/3α-HSD type 2			99·8 for complete CDS	Lin *et al.* (1997)
3α-HSD (=HAKRb)	—	−	—	Qin *et al.* (1993)
3β-HSD type 1	—	−	—	Dumont *et al.* (1992)
3β-HSD type 2	382	+	100	Rheaume *et al.* (1991)
5α-Red type 2		−	—	Andersson *et al.* (1991)
5α-Red type 1	168 (gene)	+	95·0 female and 99·0 male	Andersson & Russell (1990)
	168 (pseudo)	+	99·4	Jenkins *et al.* (1991)
5α-Red type 1	351 (gene)	+	99·0	Andersson & Russell (1990)
	357 (pseudo)	+	100	Jenkins *et al.* (1991)
5α-Red type 1 pseudogene	454 (pseudo)	+	100	Jenkins *et al.* (1991)
5α-Red type 1 gene	239 (gene)	+	100	Andersson & Russell (1990)
5β-Red	451 (gene)	+	89·0	Kondo *et al.* (1994)
	432 (pseudo)	+	99·0	Charbonneau & The (2001)

*Note that the sequence published for HAKRb was supposed to be 3α-HSD type 2, highly homologous to 17β-HSD type 5 (an enzyme with only two amino acid exchanges). pseudo, pseudogene.

from the premenopausal kidney cortex revealed 100% homology to the corresponding part of the CYP17 sequence (Chung *et al.* 1987) (Table 3).

For all investigated HSDs and reductases only those PCR products from premenopausal kidney of the same patient are shown in Fig. 5B and C. The same amplicons were used for sequencing.

AKR1C1 (=20α-HSD, dihydrodiol dehydrogenase 1 (DD1)) The expression of 20α-HSD (aldo-keto reductase AKR1C1) (Nishizawa *et al.* 2000) was analysed by sequencing a specific PCR product amplified with highly specific primers (Table 1) for the AKR1C1 sequence containing mismatches to sequences of other members of the AKR1C family. Amplification of the expected 416 bp product appeared in all human kidney samples as well as in CV-1 monkey kidney cells. One hundred per cent homology to the published sequence of 20α-HSD was found (Table 3).

AKR1C2 (=3α-HSD type 3, DD2, bile acid binding protein BABP) Analysing the expression of the enzyme 3α-HSD type 3 (=AKR1C2, DD2, BABP), the sequence of a PCR product amplified with primers containing unique nucleotides for 3α-HSD type 3 at their 3′-ends (Table 1), revealed 100% homology to the sequence of 3α-HSD type 3 (Shiraishi *et al.* 1998) (Table 3).

AKR1C3 (=PGFS, 3α-HSD type 2) The expression of the enzyme PGFS (=AKR1C3) with a sequence (Suzuki-Yamamoto *et al.* 1999) identical with AKR1C3, which was reported to be 3α-HSD type 2 (Khanna *et al.* 1995), was investigated by sequencing a PCR product obtained with a PGFS-specific sense primer (Table 1) and the antisense primer (17β-HSD type 5 as1★), used because of high homology to the corresponding sequence of PGFS. For the 562 bp product, 99·8% homology was found to the PGFS sequence (Table 3) with G instead of A (312th nucleotide (nt) of the CDS), which does not alter the amino acid sequence.

17β-HSD type 5 similar to 3α-HSD (clone of human aldo-keto reductase b (HAKRb)) As the human enzyme 17β-HSD type 5 possesses a high 20α-HSD activity that inactivates progesterone to 20α-DH-P (Dufort *et al.* 1999), we analysed the expression of 17α-HSD type 5 in

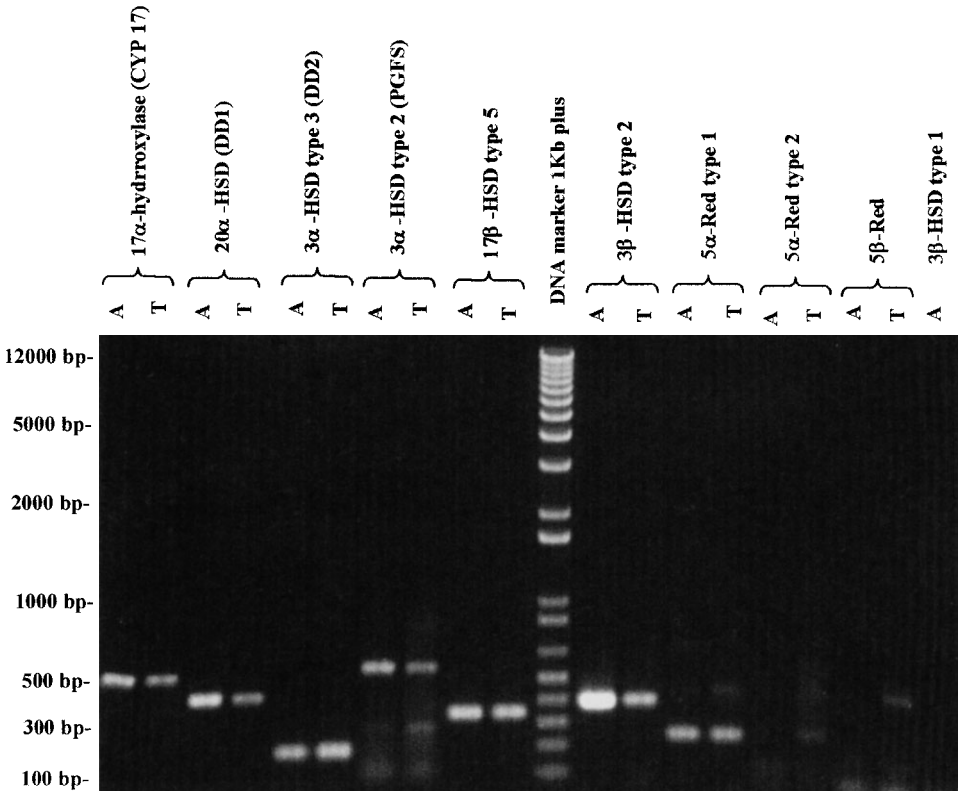

Figure 4 High-stringency PCR controls for steroid-metabolising enzymes. cDNAs from adrenals (A) and testes (T) as positive controls were amplified with specific primer pairs for different steroid-metabolising enzymes. PCR products of expected size of 527 bp for 17α-hydroxylase, 416 bp for 20α-HSD, 191 bp for 3α-HSD type 3, 562 bp for 3α-HSD type 2, 335 bp for 17β-HSD type 5, 382 bp for 3β-HSD type 2, and 239 bp for 5α-Red type 1 were obtained from both adrenals and testes. Amplifications of 244 bp for 5α-Red type 2 and 451 bp for 5β-Red were achieved only from testes, and a specific amplicon for 3β-HSD type 1 could not be shown.

the human kidney. Amplifications of three overlapping parts originating from exon 1–5, 4–7 and 6–9 covering nearly the complete CDS (Table 1) were performed with primers according to the homologous sequence of 3α-HSD clone HAKRb (Qin *et al.* 1993) encoding an enzyme differing only in the 75th and 175th amino acid from 17β-HSD type 5. All three expected PCR products of 476, 335 and 469 bp (Table 3) were amplified from cDNAs of all human kidney samples and CV-1 cells as well, the latter missing only the PCR product of the last segment. This suggests differences in human and monkey sequences located 3′ to the stop codon TAA for translation. Three 17β-HSD type 5-specific PCR products were used for complete sequencing. We found differences with respect to the sequence of HAKRb (Qin *et al.* 1993): G instead of T (222nd nt), G instead of A (495th nt), G instead of C (702nd nt), all of which do not alter the amino acid sequence. However, two additionally identified nucleotide substitutions resulted in amino acid changes; A instead of G (223rd nt) changing glutamic acid (75th amino acid) to lysine, and G instead of C (525th nt) changing isoleucine (175th amino acid) to methionine (such as in all other members of the AKR1C family). Our sequencing data show 99·5% homology to the CDS of HAKRb (Qin *et al.* 1993); 99·8% homology was found to the sequence of a recombinant 3α-HSD type 2 with 17β-HSD activity (Lin *et al.* 1997), identified as 17β-HSD type 5 (Penning 1999). Our sequence differed only in the 312th nt with G (such as in the sequence of HAKRb) instead of A, and in the 855th nt with G (such as in the sequences of PGFS and HAKRb) instead of A, both conserving the amino acid sequence of 17β-HSD type 5.

HAKRb (=3α-HSD) The expression of an enzyme structurally related to 3α-HSD with a sequence according to clone HAKRb (Qin *et al.* 1993) with nearly the same genomic sequence found for 3α-HSD type 2 (Khanna *et al.* 1995) was investigated by using a highly specific antisense primer (Table 1) ending 3′ with two unique nucleotides for HAKRb. The expected 248 bp PCR product was not amplified in any of our kidney samples even at low-stringency annealing temperature for this

specific primer. Therefore, an expression of an enzyme encoded by the sequence described by Qin *et al.* (1993) for 3α-HSD clone HAKRb could be excluded.

3β-HSD type 1 and 3β-HSD type 2 For 3β-reduction of the keto group in position 3 of steroids, the expressions of two different isoforms of the enzyme 3β-HSD were analysed with 3β-HSD type 1- and 3β-HSD type 2-specific primer pairs (Table 1). Only PCR products with the expected size of 382 bp for 3β-HSD type 2-specific amplification were obtained (Table 3). Sequencing of the re-extracted and reamplified PCR product (Fig. 5C) revealed 100% homology to the sequence of 3β-HSD/delta-4-delta-5 isomerase (Rheaume *et al.* 1991). Therefore, we conclude that only the isoform 3β-HSD type 2 is expressed in the human kidney, while the expression of 3β-HSD type 1 could not be detected in any of our kidney samples (Table 3).

5α-Red type 2, 5α-Red type 1 gene and pseudogene For ring A reduction of steroids, producing 5α-DH metabolites, the expression of the reductases 5α-Red type 1 and 5α-Red type 2 was examined. 5α-Red type 2-specific primers (Table 1) did not amplify the expected 244 bp product from any renal sample (Table 3), but PCR with the specific primer pair for 5α-Red type 1 (located in exon 1 and 2) resulted in amplification of the expected 168 bp PCR product with 99·4% homology (Table 3) to the published sequence of the 5α-Red-pseudogene (located on chromosome X) (Jenkins *et al.* 1991). There was only one nucleotide substitution: G (gene-specific at this position) instead of A. According to the cDNA sequence (Andersson & Russell 1990) from the gene of 5α-Red type 1 (located on chromosome 5), our main

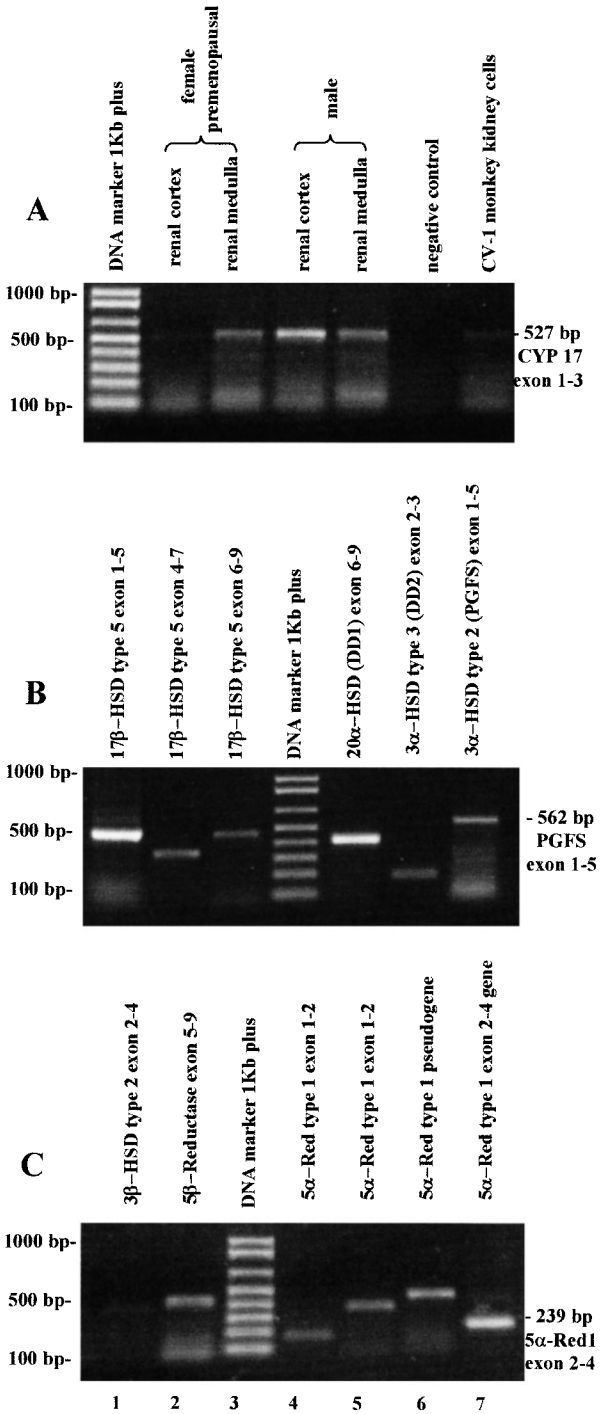

Figure 5 RT-PCR analysis for the expression of progesterone-metabolising enzymes. (A) 17α-hydroxylase (CYP17). cDNAs from premenopausal and male renal cortex and medulla and from CV-1 monkey kidney cells (control) were amplified in the first step with specific primers 17α-hydroxylase sense and antisense. The negative control without cDNA did not give any staining while all kidney samples stained positively for the expected 527 bp amplicon with a smear of smaller fragments. Therefore the DNA corresponding to the band of the correct size was re-extracted from the gel and reamplified for sequencing. (B) HSDs capable of 20α-reduction. Three sets of primers were used for overlapping amplicons of 17β-HSD type 5 covering nearly the complete CDS and each one single primer pair for 20α-HSD, 3α-HSD type 3, 3α-HSD type 2. Specific PCRs were performed with cDNAs from female and male kidney cortex and medulla and CV-1 as well. Here premenopausal samples used for sequencing are shown. Three specific PCR products for 17α-HDS type 5: amplicon 1 (exon 1–5) 476 bp, amplicon 2 (exon 4–7) 335 bp, and amplicon 3 (exon 6–9) 469 bp, each one single specific PCR products for 20α-HSD (exon 6–9) 416 bp, 3α-HSD type 3 (exon 2–3) 191 bp and 3α-HSD type 2 (exon 1–5) 562 bp. (C) 3β-HSD type 2 for 3β-reduction and reductases for 5β- and 5α-reduction. Specific amplicons of cDNA samples from premenopausal kidney specimens used for sequencing are shown for 3β-HSD type 2 (exon 2–4) 382 bp (lane 1), 5β-Red (exon 5–9) 451 bp (lane 2), DNA marker (100–1000 bp shown) (lane 3), 5α-Red type 1 (exon 1–2) 168 bp (lane 4), 5α-Red type 1 (exon 1–2 amplified with a sense primer located preATG) 351 bp and/or 357 bp for the gene and/or pseudogene transcript (lane 5), 5α-Red type 1 pseudogene-specific 454 bp (lane 6), and 5α-Red type 1 gene-specific (exon 2–4) 239 bp (lane 7).

sequence revealed only 95·0% homology (Table 3). In the chromatogram obtained by direct sequencing of the amplicon, additional underlying peaks of gene-specific nucleotides with lower concentration were also detectable at the positions of pseudogene-specific nucleotide substitutions. Therefore, in the sample of the premenopausal kidney cortex, mainly transcription of the pseudogene from chromosome X besides lower expression of the gene for 5α-Red type 1 could be found. Because of this phenomenon, the PCR product from a male kidney medulla cDNA was sequenced additionally. In the direct sequencing data, 99·0% homology to the sequence of 5α-Red type 1 gene (Table 3) was found with an underlying sequence of the pseudogene at lower levels. For analysing the region with six duplicated nucleotides in the pseudogene directly following the start codon ATG, the sense primer (5α-Red type 1 preATG sense) located 5′ of the CDS matching both sequences and the antisense primer (5α-Red type 1 antisense) were used for amplification. Sequencing of the PCR product from the premenopausal renal medulla (Fig. 5C, lane 5) again revealed high homologies (Table 3) to both the expected 351 bp for gene-specific and 357 bp for pseudogene-specific sequences (including the six additional nucleotides). Combination of the common sense primer (5α-Red type 1 preATG sense) with a pseudogene-specific antisense primer (5α-Red type 1 pseudo antisense) and further amplification of the PCR product in combining the same pseudogene-specific antisense primer with a pseudogene-specific sense primer (5α-Red type 1 pseudo sense) by a second (semi-nested) PCR resulted in the expected 454 bp product (Fig. 5C, lane 6). Sequencing of the amplified cDNA from the premenopausal renal medulla revealed a uniform sequence with 100% homology for the pseudogene (Table 3) including the internal stop codon TGA unique to the pseudogene. Therefore, transcription of the pseudogene in the human kidney can be assumed, but translation would not result in a full-length protein because of premature termination. For analysing the 5α-Red type 1 gene expression, a gene-specific primer pair ending 3′ with nucleotides unique only to the gene (5α-Red type 1 gene sense and antisense) (Table 1) was used for amplification of a uniform PCR product. The amplified expected 239 bp product (Fig. 5C, lane 7) was 100% homologous to the gene-specific sequence of 5α-Red type 1 (Andersson & Russell 1990) (Table 3). Therefore, the expression of the isoform 5α-Red type 1 was found in the human kidney, besides transcription of the pseudogene, while 5α-Red type 2 expression could not be detected.

5β-Red (=AKR1D1) gene and pseudogene For the synthesis of 5β-reduced metabolites, expression analyses were performed for the enzyme 5β-Red (AKR1D1). PCR products of about 430–450 bp using the primer pair 5β-Red sense and antisense (Table 1) were obtained (Fig. 5C). According to the sequence for the 5β-Red cDNA (Kondo *et al.* 1994), an amplicon of 451 bp was expected. By direct sequencing we found 99·0% homology (Table 3) to the sequence of the 5β-Red pseudogene (Charbonneau & The 2001) (reverse complement sequence located on chromosome 1), missing 19 nucleotides in the amplified cDNA of 432 bp compared with 451 bp from the 5β-Red gene transcript. To the 5β-Red gene cDNA sequence (Kondo *et al.* 1994) only 89·0% homology was found (Table 3). Another direct sequencing from a sample of another female patient revealed highly homologous sequences specific for the pseudogene and the gene at the same time. Therefore, the transcription of the pseudogene and the expression of the enzyme 5β-Red was confirmed in the human kidney.

Discussion

This study examined mechanisms by which the MR may be protected against binding and antagonism of progesterone, which binds to the MR with the same or even higher affinity, but confers only little transcriptional activity (Rupprecht *et al.* 1993*a*, Myles & Funder 1996). Therefore progesterone is assumed to be an MR antagonist. We found that progesterone has a higher antagonistic than agonistic function at the MR (Quinkler *et al.* 2002). It is still unclear how the mineralocorticoid function of aldosterone can be maintained especially at higher progesterone than aldosterone levels, e.g. during the luteal phase and pregnancy, when progesterone exceeds aldosterone concentrations 100-fold in the third trimester (Rosenthal *et al.* 1969).

One protective mechanism could be achieved by an additional binding site for progesterone in renal cells, e.g. the PR for high-specific progesterone binding , resulting in a sequestration of progesterone. This could enable binding of aldosterone to the MR. Therefore, we investigated the renal expression of the hPR. We found the expression of the hPR in kidney specimens from female (pre- and postmenopausal) and male patients in cortical as well as in medullary tissue preparations. The function of the receptor protein, tested in binding assays with the specific ligand R5020 and Scatchard plot analysis, revealed K_d values of about 0·8 nM (mean of cortex and medulla) in kidney specimens compared with 0·98 nM in the target tissue myometrium, consistent with the published K_d of 0·89 nM for the myometrial hPR (Keightley 1979). These K_d values for binding of R5020 characterise the same kind of receptor (hPR) in the myometrium and in the human kidney, supporting our hypothesis of renal hPR expression. The identity of the hPR was further tested by immunodetection. In tissue sections, a nuclear localisation of hPR was detected with the IgG monoclonal PgR 636 predominantly in epithelial cells of distal tubules, the mineralocorticoid target cells of the kidney. Western blots

(differentiating PR-A and PR-B) were performed for isoform analysis of hPR by immunodetection with the hPR-specific IgG1 monoclonal antibody NCL-PGR-312. A protein of about 117 kDa corresponding to hPR-B (Sheridan *et al.* 1989, Kumar *et al.* 1998) was found in protein extracts from male and female kidney specimens, but a specific immunoreaction with a protein of the size corresponding to the isoform hPR-A (Kastner *et al.* 1990*b*) could not be shown. This is of special interest because hPR-A is discussed as a co-repressor of hMR expression and function (McDonnell *et al.* 1994). Therefore a renal downregulation of hMR function by PR-A is not likely. In addition, our results of RT-PCRs from RNA of kidney cortex and medulla specimens from male and female patients and sequencing data with 100% homology to hPR cDNA sequence (Misrahi *et al.* 1987), confirmed the identity of hPR and the renal transcription of the hPR gene. Therefore, the expression of hPR could be shown for the first time in the human kidney.

Renal progesterone-specific functions via hPR remain to be investigated. To date it is not known whether there is an additional influence on the mineralocorticoid function by a possibility of heterodimerisation, e.g. of hPR-B with the hMR co-localised in epithelial cells of distal tubules in the human kidney. Rather low levels of hPR in the kidney of postmenopausal origin were detected by Scatchard plot analyses, reflecting the mean of all cells in renal cortex or medulla. As only in a small number of cells was PR detected by immunohistology, especially in epithelia of distal tubules, local PR levels may be able to compete with MR for progesterone binding. Whether the expression of hPR would be raised in kidneys of premenopausal women by oestrogen for compensation of elevated progesterone could not be analysed because tissues from females during different cycle stages and/or pregnancy were not available. To discuss the role of the renal hPR for the hMR specificity, experiments investigating the number of both receptors and the co-localisation in renal cells would be helpful.

Additional mechanisms for protecting the hMR in the kidney against binding of progesterone appear to be essential. Another way of reducing progesterone binding to MR is the metabolism of progesterone to derivatives with lower affinity to the MR. More than 50% of progesterone is converted to 17α-OH-P, 20α-DH-P, 17α-OH,20α-DH-P, 5α-DH-P, 5β-DH-P, 3β,5α-TH-P, 3α,5α-TH-P, 3β,5β-TH-P, 20α-DH,5α-DH-P and 20α-DH,3β,5α-TH-P in the human kidney (Quinkler *et al.* 1999, 2001). These metabolites exhibit reduced affinity to the MR depending on hydroxylated and/or reduced groups (Quinkler *et al.* 2002). For different pathways of progesterone metabolism (Fig. 6), we examined the expression of corresponding steroid-converting enzymes in the human kidney by RT-PCR.

Metabolism of progesterone to 17α-OH-P is achieved by 17α-hydroxylase (CYP17). The expression of this

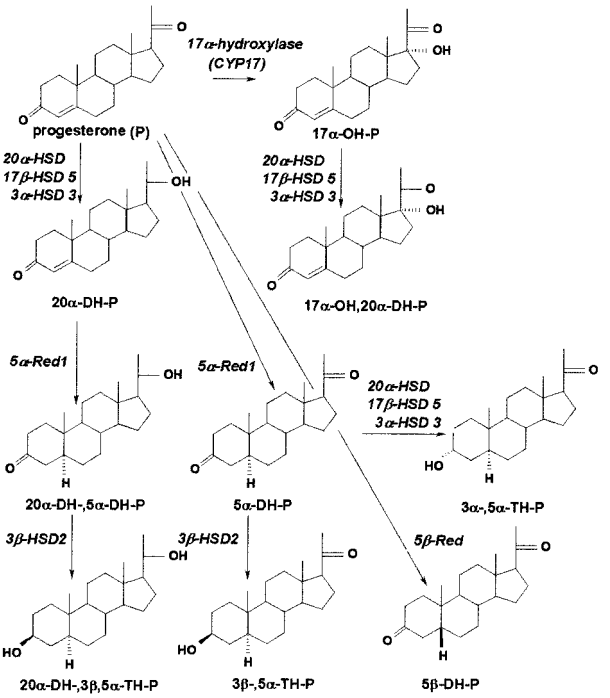

Figure 6 Scheme for progesterone metabolism with enzymes identified in the human kidney. Steroid-metabolising enzymes, confirmed by sequencing of RT-PCR products from human kidney specimens, are shown in different pathways of progesterone.

enzyme was found in renal cortex and medulla. One of 527 analysed nucleotides differed from the published sequence of exon 1 (Picado-Leonard & Miller 1987) of the gene of CYP17, but this difference had been previously described by Chung *et al.* (1987). Therefore, in the kidney, hydroxylation by CYP17 of progesterone to 17α-OH-P with lower affinity to the hMR is likely. For the reduction of progesterone to 20α-DH-P we found the expression of different enzymes in our kidney specimens. Direct sequencing of a 20α-HSD-specific RT-PCR product from renal RNA preparations revealed 100% homology to the sequence for 20α-HSD (AKR1C1) (Nishizawa *et al.* 2000). By sequencing of three overlapping 17β-HSD type 5-specific amplicons covering nearly the complete CDS, we identified RNAs specific for the enzyme 17β-HSD type 5 with a strong 20α-Red activity (Dufort *et al.* 1999). Amplification with a PGFS-specific sense primer resulted in a PCR product with 99·8% homology to the published sequence (Suzuki-Yamamoto *et al.* 1999) for PGFS (AKR1C3). This amplicon contained identical sequences as obtained by the first 17β-HSD type 5-specific PCR. Comparison of the complete CDS of PGFS (AKR1C3) and the sequence of 3α-HSD type 2 (AKR1C3) transcript from chromosome 10p15–p14 (deduced from six published sequences in GeneBank accession No. 003739·2) exhibits about

99·7% homology. Our complete sequencing data of the CDS for 17β-HSD type 5 reveal also 99·7% homology to both AKR1C3 sequences described for PGFS and 3α-HSD type 2. Therefore we think that the sequence of the renal cDNA we found encodes 17β-HSD type 5. It is probably the same enzyme as AKR1C3, as recently described (Penning *et al.* 2001). 3α-HSD type 3 (AKR1C2, DD2, BABP)-specific RNA with 100% homology (Shiraishi *et al.* 1998) was also identified in our kidney specimens, but this enzyme shows only weak activity of 20α-reduction. Therefore the enzymes 20α-HSD and 17β-HSD type 5 are the main candidates for the 20α-reduction of progesterone and 17α-OH-P, while 3α-HSD type 3 may contribute to the 3α-reduction of 5α-DH-P to 3α,5α-TH-P in the human kidney. 3β-Reduced progesterone metabolites are synthesised in the kidney by 3β-HSD type 2. The expression was found in renal cortex and medulla by RT-PCR with 100% homology to the published sequence (Rheaume *et al.* 1991). As 3β-HSD type 1 was not detected, the isoform 3β-HSD type 2 must be responsible for renal formation of 3β,5α-TH-P. For synthesis of this metabolite and direct 5α-reduction of progesterone, the expression of the isoform 5α-Red type 1 was found in the kidney. The amplicon of gene-specific RT-PCR exhibits 100% sequence homology to the 5α-Red type 1 gene-specific CDS located on chromosome 5 (Andersson & Russell 1990). Additionally a pseudogene-specific RT-PCR product reveals 100% homology to the pseudogene with X-chromosomal location (Jenkins *et al.* 1991) as well. The role of the transcription of the intronless pseudogene (Mighell *et al.* 2000) with a stop codon in the region corresponding to the CDS, in the context of simultaneous gene expression, remains to be investigated. For the renal 5β-reduction of progesterone, we found the expression of the enzyme 5β-Red (AKR1D1) from the gene located on chromosome 7 q32–q33 and also the transcription of the pseudogene located on chromosome 1 q23–q25 (reverse complement sequence) (Charbonneau & The 2001) in the human kidney. There may be a regulatory role by transcription of the reverse complement sequence (antisense RNA) of 5β-Red gene on expression of the enzyme 5β-Red.

In conclusion, we produced evidence for two mechanisms for the mineralocorticoid function of aldosterone at the renal hMR in the presence of elevated progesterone. Binding of progesterone to the renal MR may be partly prevented by competitive binding to the PR, and the concentration of free progesterone should be lowered by steroid-metabolising enzymes producing progesterone metabolites with reduced affinity to the MR in renal cells (Quinkler *et al.* 2002).

Acknowledgements

This work was supported by the grants DI 741/1–3 and Graduierten-Kolleg 426/2–00 (Molekularbiologische Grundlagen der Therapie) as well as 754 (Myokardiale Genexpression und Funktion, Myokardhypertrophie) from the Deutsche Forschungsgemeinschaft (DFG). We thank Katrin Kossatz-Eskandani for sequencing, Uschi Gruber for cell culture, Dr Alexandra Perez-Canto for histological inspection, Dr Ronald Evans for providing the plasmid pRShMR, and Dr Wiebke Arlt for putting at our disposal cDNAs from adrenals and testes.

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Received 24 April 2002
Accepted 29 July 2002