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Ubiquitous Expression of the Androgen Receptor and Testis-specific Expression of the FSH Receptor in the Cynomolgus Monkey (*Macaca fascicularis*) Revealed by a Ribonuclease Protection Assay

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Androgens are known to exert a variety of effects on an organism while follicle-stimulating hormone (FSH) seems to act specifically on the gonads. To investigate whether these effects are reflected by the expression pattern of the androgen receptor (AR) or the FSH receptor (FSHR) we screened 38 different tissues and organs of one intact and one castrated male non-human primate (*Macaca fascicularis*). By means of a highly sensitive ribonuclease protection assay (RPA) we demonstrated AR mRNA expression in all tissues of the intact monkey investigated. Immunohistochemistry of selected organs from this monkey revealed a good correlation between AR mRNA and protein expression. In the castrated monkey, the overall AR mRNA expression was markedly lower compared with the intact monkey, although higher expression was present in the pituitary, thyroid and prostate glands. FSHR mRNA was only detected in testicular tissue. This study has revealed, for the first time, ubiquitious expression of the AR mRNA in a non-human primate. The testis-specific expression of the FSHR highlights the importance of FSH for spermatogenesis with the testis being apparently the only target organ.

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INTRODUCTION

Androgens are essential for the development and physiological function of male reproductive organs. In addition, they exert a variety of effects on many other non-reproductive organs and tissues. These effects are mediated by the androgen receptor [1] which belongs to the superfamily of steroid receptors. Activation of the receptor by either testosterone or dihydrotestosterone leads to the expression of a network of specific androgen-dependent genes [2]. Androgens and follicle-stimulating hormone (FSH) together are the key hormones for the regulation of mammalian spermatogenesis [3]. The action of FSH is mediated by a specific, cell-surface receptor expressed by Sertoli cells [4]. The FSH receptor (FSHR) belongs to the superfamily of G-protein coupled receptors [5], which are characterized by seven transmembrane domains. Binding of FSH to its receptor leads to the activation of the cAMP pathway and to a sequential elevation of cytosolic calcium levels [6].

Previous studies have investigated, in different tissues of human and rat, the expression of androgen receptor (AR) and FSHR mRNA [7,8] and AR protein [9,10], but a comprehensive study of the level and specificity of occurrence of their mRNAs throughout a whole organism is lacking. The detection and determination of AR mRNA and protein levels would be an important step towards the understanding of the great variety of androgen actions in different organs and tissues. Furthermore, it should clarify whether FSHR mRNA expression is restricted to the testis or is also present in other organs, possibly indicating a non-reproductive function of FSH, as has been recently suggested by Garde *et al.* [11]. For these purposes we have established a ribonuclease protection assay (RPA) which combines high sensitivity and quantitation with ease of performance and

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applicability. The study was performed using two non-human primates in an animal model, which is considered a representative model for the human. In order to obtain information about regulatory aspects of the two receptors, one castrated and one intact monkey were used.

MATERIALS AND METHODS

Animals

Two adult cynomolgus monkeys (*Macaca fascicularis*) were investigated. An intact animal was sacrificed because of an acute obstruction of the urethra. Body weight at death was 6.5 kg and serum testosterone levels were 14.6 nmol/l. In addition, a castrated monkey (orchidectomized 10 years ago) with a body weight of 3.3 kg and a serum testosterone value of <2 nmol/l was sacrificed. Animals were sedated with ketamine hydrochloride and exsanguinated by an incision into the inguinal veins. The tissues were removed as quickly as possible and frozen in liquid nitrogen for subsequent analysis of AR and FSHR expression (see Fig. 1).

Detection and quantitation of mRNA

The RPA was performed according to previously published protocols [12]. Total RNA was prepared from frozen monkey tissues using RNAzol [13]. The following cDNA clones were used as template for synthesis of both antisense and sense cRNAs: the monkey FSHR cDNA of 353 bp (covering 169 bp of exon 9 and 184 bp of exon 10 [14]), linearized by BamH I for synthesis of the antisense cRNA using T7 RNA polymerase and by Kpn I for synthesis of the reference cRNA with T3 RNA polymerase; and the human AR cDNA of 269 bp (covering bases 1094 to 1363 of the AR cDNA sequence [1]) linearized by Hind III was used for generating the antisense probe by T3-RNA polymerase transcription and was linearized by Sac I and transcribed by T7-RNA polymerase for generating the sense probe. Radioactive cRNA synthesis using [α-^{32}P]CTP was performed using a commercial transcription kit (Promega).

To determine the sensitivity of the assay, known amounts of reference cRNAs (sense) were diluted serially and hybridized against their respective radioactive cRNA probes. Approximately 125 fg of the monkey FSHR reference RNA and at least 250 fg of the human AR reference RNA could be detected with this assay. On the other hand a minimum of 1–2 μg of total RNA isolated from a monkey testis was necessary to obtain a quantifiable signal of both receptors. 20 μg of total RNA were also hybridized to achieve a quantifiable signal in organs with presumably low FSHR and AR mRNA expression. For determination of the intra-assay variations of FSHR and AR mRNA expression, identical amounts of total RNA (10 μg) isolated from the same monkey testis were probed 20 times for FSHR and AR mRNA expression using GAPDH as an internal standard. After normalizing the values the coefficient of variance for FSHR mRNA levels ranged from 10.2 to 13.8% and for AR mRNA levels from 10.8 to 13.0%. In comparison with classical methods like Northern blot analysis, a significantly better reproducibility in quantification of mRNA transcripts could be achieved by this RPA. Hybridization of total RNA samples isolated from the human prostate cancer cell line LNCaP with the human AR probe showed a very high expression of AR mRNA whereas AR mRNA in samples isolated from the AR-negative human prostate cancer cell line DU 145 could not be detected, demonstrating the high specificity of AR mRNA detection [12].

Taking particular care to use equal amounts of total RNA from each tissue in the assay, 20 μg of total RNA of each tissue, except hypothalamus (8 μg) and thyroid gland (10 μg) of the intact monkey, was dissolved in hybridization buffer (80 % formamide; 0.5 M NaCl; 40 mM Pipes (pH 6.4); 0.2 mM EDTA (pH 8.0)) and hybridized at 53°C with the radioactive probes. In order to generate the standard curves, known amounts of serially diluted AR and FSHR reference cRNAs were assayed along with the samples to estimate the absolute amounts of the protected target RNAs. Following hybridization, single stranded RNA was eliminated by RNase treatment (300 mM NaCl; 10 mM Tris–Cl (pH 7.5); 5 mM EDTA (pH 8.0); 5 μg/ml RNase A–100 U/ml RNase T1-mixture) for 35 min at 37°C. After denaturing the RNase using proteinase K and SDS for 45 min at 37°C, the remaining protected double-stranded cRNA–mRNA hybrids were electrophoresed through a 7% denaturating polyacrylamide sequencing gel. Following electrophoresis the gels were dried by a Vacuum Slap Gel-Dryer (Renner) and exposed at least overnight at −80°C to an X-ray film using intensifying screens (Fig. 1). Quantitation was performed by densitometric scanning using a GelScan XL (Pharmacia). The amounts of protected mRNA fragments in each sample were calculated directly from the standard curves generated from serially diluted reference RNAs (FSHR: 0–10 pg; AR: 0–20 pg).

Immunohistochemical analysis

Frozen tissue samples (see Table 3) were cut (7μm) using a Jung Frigocut 2800 E (Leica Instruments, Nußloch, Germany) and placed onto poly-L-lysine coated slides, dried (>15 min), fixed for 5 min in 3% paraformaldehyde–5% sucrose in PBS (pH 7.4) and washed in running tap water for 15 min. For immunohistochemical localization of the AR, the peroxidase–anti-peroxidase (PAP) method was performed. After washing in TBS-buffer (50mM Tris, 150 mM NaCl, pH 7.6) the tissue specimens were incubated overnight at 4°C with a monoclonal mouse antibody against the AR (MAB 394.1, diluted 1:2000 in TBS-

buffer containing 0.5% BSA to suppress non-specific background staining). After washing in TBS (3 × 5 min) the sections were incubated for 60 min with rabbit anti-mouse antiserum (Dako Diagnostica, Hamburg, Germany, code No. Z 456, diluted 1:25 in TBS-buffer containing 0.1% BSA). After removal of unbound antibody by washing in TBS-buffer (3 × 5 min), the incubation of the PAP-complex (Sigma Chemie GmbH, Deisenhofen, Germany, code No. 3039, diluted 1:25 in TBS-buffer containing 0.1% BSA) was performed for 60 min. After the next wash in TBS-buffer (3 × 5 min), the slides were incubated with the substrate diaminobenzidine (0.05%, H_2O_2 0.03% in TBS) for 5–10 min. This substrate is converted by the action of the peroxidase into an insoluble brown reaction product readily observed under the light microscope. After brief washing in deionized water, the sections were dehydrated through an ethanol series and finally mounted in Merckoglas (Merck, Darmstadt, Germany).

For evaluation, the intensity of positive staining was expressed in terms of relative intensities: high (++), moderate (+), low (+/−) and no detectable staining (−) (see Table 1).

Table 1. Distribution of androgen receptor protein in different organs/tissues of the intact monkey

Tissues	Relative intensity
Epid. (caput)	++
Epid. (corpus)	++
Epid. (cauda)	++
Seminal vesicle	++
Testis	+
Prostate	+/−
Liver	+
Adrenal gland	+/−
Pituitary	+
Gall bladder	−
Esophagus	−
Muscle	
Spleen	
Ischiadic nerve	−
Aorta	
Jejunum	−
Ileum	
Caecum	
Colon	−
Rectum	
Lung	
Heart	
Kidney	
Scrotal skin	
Tongue	−
Salivary gland	−
Larynx	−
Trachea	−
Lymph nodes	

The intensity of positive staining is expressed in terms of relative intensities: high (++), moderate (+), low (+/−) and no detectable staining (−).

RESULTS

Distribution of AR mRNA

In the intact monkey AR mRNA expression was detectable in all tissues/organs investigated [Fig. 1(A)]. In general the castrated monkey showed a decreased expression of AR mRNA, which was no longer detectable in most tissues [Fig. 1(B)].

Quantitation of the AR mRNA is shown in Fig. 2, giving a ranked list of organs and tissues expressing AR mRNA in the intact monkey compared to AR mRNA levels in similar tissues of the castrated monkey. Organs and tissues of the intact monkey that are not illustrated in this figure showed AR mRNA expression below 0.1 pg protected AR mRNA/20 μg total RNA and were detectable but not quantifiable.

Reproductive organs. In the intact monkey expression of AR mRNA was the highest in the seminal vesicles and the three regions (caput, corpus, cauda) of the epididymis, which did not differ widely from each other, whereas the testis displayed a clearly lower expression level. Expression in the prostate and penis of the intact monkey was markedly lower than in the other reproductive organs, being detectable but not quantifiable in the castrated monkey. In the seminal vesicle of the castrated monkey, only a weak expression could be observed, whereas the AR mRNA levels in the prostate were elevated in comparison with the intact monkey.

Digestive, respiratory and urinary tracts. AR mRNA expression in the intact monkey was variable among these organs: low in stomach, caecum, colon and bladder and higher in the gall bladder, jejunum and esophagus. Expression in the salivary gland, lung, kidney, pancreas, ileum and rectum could only be detected but not quantified. In the castrated monkey, lung, gall bladder, bladder, caecum, colon and rectum AR mRNA was also detectable but not quantifiable, while the esophagus, stomach, pancreas, kidney, salivary gland, ileum and jejunum lacked any detectable expression.

Brain tissues. Expression of AR mRNA in the hypothalamus and pituitary of both the intact and castrated monkey was similar, whereas expression in the cortex, cerebellum and medulla oblongata that was seen in the intact monkey was absent in the castrated monkey.

Other tissues and organs. Liver and adrenal gland of the intact monkey displayed moderate AR mRNA expression that was markedly weaker in the liver and not quantifiable in the adrenal gland of the castrated monkey. In contrast to the other non-reproductive organs, AR mRNA was more highly expressed in the thyroid gland of the castrated monkey compared with the intact monkey.

Other tissues and organs with low abundance of AR mRNA. Low amounts of AR mRNA could be seen in the spinal cord of the intact monkey, whereas in the

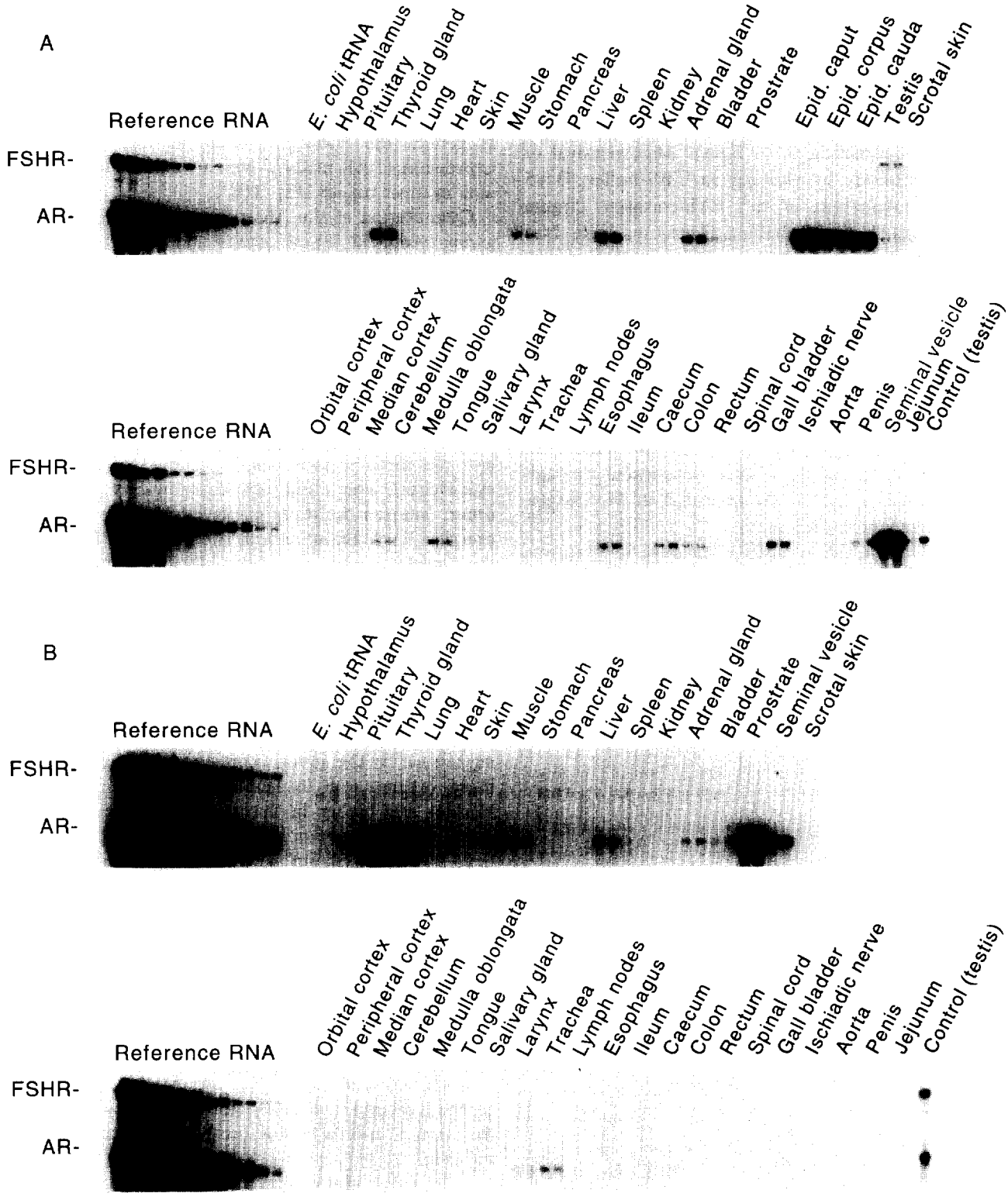

Fig. 1. Autoradiogram of protected FSHR and AR mRNA fragments: Total RNA of different tissues obtained from an intact monkey (A) and a castrated monkey (B) was hybridized in duplicate against a monkey FSHR probe and a human AR probe. The protected fragment sizes were 353 bp for the FSHR and 269 bp for the AR. Concurrently, each of the respective reference RNAs (0, 0.31, 0.62, 1.25, 2.5, 5 and 10 pg FSHR cRNA and 0, 0.62, 1.25, 2.5, 5, 10 and 20 pg AR cRNA) were hybridized in duplicate to the two radioactive receptor probes. The film exposure time for the intact monkey was 4 days, for the castrated monkey 12 days.

castrated monkey this tissue yielded only detectable but not quantifiable amounts. In addition, AR mRNA was only present in detectable amounts in spleen, heart, skin, tongue, larynx, trachea, scrotal skin and lymph nodes of both monkeys. By contrast with the intact monkey, the aorta and Nervus ischiadicus of

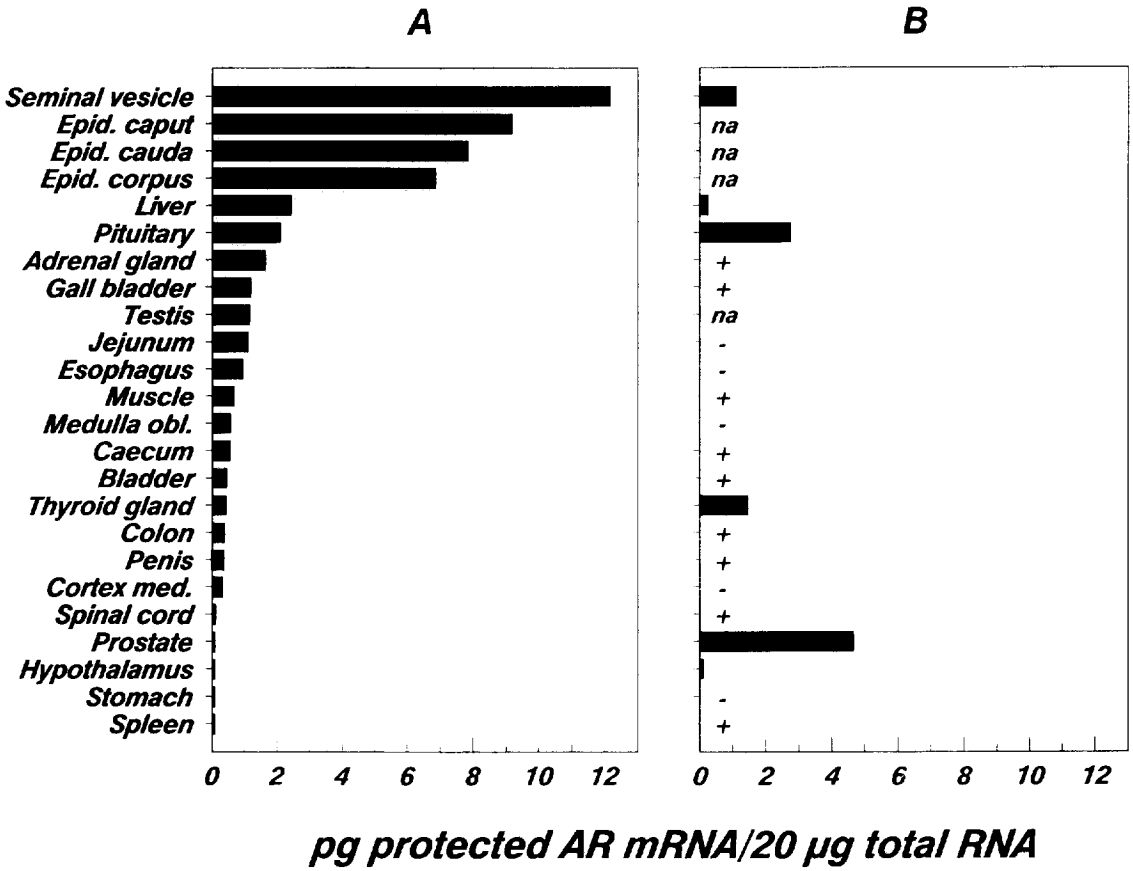

Fig. 2. Quantitation and comparison of AR mRNA expression in different organs of an intact and castrated non-human primate. Organs of the intact monkey not illustrated showed protected AR mRNA below 0.1 pg/20 µg total RNA. Terms: (na) not available; (+) expressed but not quantifiable; (−) not detectable.

the castrated monkey lacked detectable expression of AR mRNA.

Distribution of FSHR mRNA

FSHR mRNA was present in the testis of the intact monkey, but lacked detectable expression in all other organs. The expression level was approx. 2.5 pg/20 µg total RNA.

Distribution of AR protein

Immunohistochemistry of the different organs/tissues of the intact monkey revealed high expression of AR protein in the three regions of the epididymis and in the seminal vesicles. Moderate expression was found in testis, liver and pituitary, whereas the prostate and adrenal glands expressed only low amounts of AR protein. In all other organs/tissues examined by immunohistochemistry no AR staining could be detected (Table 1).

DISCUSSION

The use of the RPA not only offers the possibility of accurate detection and quantitation of transcripts, but also keeps the risk of contamination, known to be a problem in reverse-transcription–polymerase chain reaction (RT–PCR), to a minimum. Furthermore, there is no detection of illegitimate transcripts [15], as has been shown to occur in RT–PCR.

Although the AR mRNA expression levels varied markedly between the different tissues/organs of the intact monkey, they can be roughly classified into three categories (see also Fig. 2): (1) tissues/organs with high AR mRNA expression (e.g., seminal vesicle, epididymis); (2) those with medium AR mRNA expression (e.g., testis, adrenal gland); and (3) those with low AR mRNA expression (e.g., hypothalamus, spleen). These different expression patterns indicate that androgen dependency is not necessarily reflected by the AR mRNA expression status. For instance, the anabolic effects of androgens on muscle are well known [16] but the AR mRNA expression is low in this tissue. In contrast, the testis, the main source of androgens, has extremely high intratesticular androgen levels but only medium AR mRNA levels. The AR mRNA expression pattern of the adrenal, the other source of androgens, might point to a paracrine regulation of the AR by adrenal androgens. The surprisingly low AR mRNA levels in the prostate of the intact monkey may result from the indifferent morphological structure of the prostate as estimated by histological evaluation (data not shown) or differential

regulation of the AR mRNA in the different lobes of the prostate [17]. The detection of AR transcripts in the spleen is in contrast to earlier studies that used the spleen as a negative control for AR mRNA expression. This finding could be due to the superior sensitivity of the RPA compared with the Northern blot techniques used by others [8,18].

A comparison of selected tissues from the intact monkey with those from the castrated monkey (Fig. 2) provides some interesting clues to possible different AR mRNA regulatory mechanisms, although based on only two animals. In general, in most of the tissues of the castrated monkey, AR mRNA expression was down-regulated in the absence of androgens. However, the similar pituitary levels of the AR mRNA in both monkeys indicate that androgens might not always be directly involved in up- or down-regulation of AR mRNA in all organs. This hypothesis is supported by a recent report of differential regulation of the AR mRNA in certain regions of the rat brain [19].

To investigate whether the mRNA expression patterns also reflect the AR protein content, immunohistochemical analysis was performed in some of the tissues. In general good agreement with observed mRNA levels was obtained, which confirms the high correlation between mRNA and protein expression found in earlier studies [20]. In the gall bladder, which expressed moderate levels of AR mRNA, AR staining could not be detected, indicating that these transcripts may not have been translated into receptor proteins. AR protein was not detected in tissues with low mRNA expression, which might be due to the lower sensitivity of the immunocytochemical techniques applied.

Expression of the FSHR mRNA was located solely in the testis of the monkey. This finding is in contrast to the finding by Harvey *et al.* [21] who alleged the presence of FSHR transcripts in the hypothalamus, pituitary and prostate of rats by RT–PCR. To exclude the possibility of very low expression in these organs we increased the amount of RNA used for the RPA to as much as 80 μg, but again did not detect any specific signal (data not shown).

The testis-specific expression of the FSHR is perhaps surprising because recent data from the LHR and TSHR [22,23], receptors closely related to the FSHR, indicated expression of full length or truncated forms of the receptor mRNA in different tissues. Since the receptor protein is present, at least for the LHR, this might indicate hithherto unknown functions of LH and TSH on other non-classical target tissues. The cRNA used in the RPA of this study only detects transcripts covering exon 9 and exon 10 of the FSHR gene. Therefore, we can not exclude the possibility that there might be expression of truncated forms of the FSHR mRNA, excluding these two exons, in other tissues. However, functional studies with a truncated isoform of the FSHR lacking exon 9 [24], indicated that cAMP could not be accumulated after FSH stimulation (data not shown). For this reason we consider this isoform as non-functional. Alternative isoforms non-functional as receptors could have roles as binding proteins or autoantigens, as has been suggested for the LHR and TSHR [22].

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