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Quantification of Androgen Receptor mRNA in Tissues by Competitive Co-amplification of a Template in Reverse Transcription–Polymerase Chain Reaction

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We describe a polymerase chain reaction (PCR)-based method for the quantification of androgen receptor (AR) mRNA in tissues. The amount of PCR products depends on the exponential amplification of the initial cDNA copy number; therefore minor differences in the efficiency of amplification may dramatically influence the final product yield. To overcome these tube-to-tube differences in reaction efficiency, an internal control AR cRNA was reverse transcribed along with the target mRNA using the same primers. This standard was obtained by deleting a 38 bp fragment from an amplified bovine AR sequence, which was then subcloned and transcribed into cRNA. Known dilutions of the competitor cRNA were spiked into a series of RT–PCR reaction tubes containing equal amounts of the target mRNA. Following RT–PCR, the co-amplified specimens obtained were separated by gel electrophoresis and quantified by densitometric analysis of ethidium bromide stain. We applied this method to quantify the AR-mRNA in skeletal muscle of castrated as well as from intact male cattle. The applicability of the quantification system for AR-mRNA described herein was demonstrated for other species, e.g. man. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

Androgens regulate the development, differentiation and growth of the male reproductive tract and of male secondary characteristics [1]. The molecular mechanism involves the binding to a specific receptor protein to form an androgen-receptor complex which then interacts with specific DNA sequences to activate the transcription of selected sets of genes [2]. Besides the physiological actions, androgens can play a role in tumorigenesis as demonstrated by qualitative and/or quantitative alterations in androgen receptor (AR) mRNA expression, e.g. in prostate or breast cancer [3–6]. To investigate the regulation of AR expression at the level of transcription both under physiological as well as under pathological circumstances, valid and sensitive methods for the quantification of AR-mRNA are required. Expression of AR-mRNA has traditionally been studied using blotting techniques [7–11]. However, these methods have several disadvantages such as limited sensitivity, requiring considerable amounts of RNA. Moreover, the procedure can take several days to obtain results. Using the polymerase chain reaction (PCR) after reverse transcribing the RNA, the detection threshold has been lowered up to 1000-fold [12, 13]. PCR therefore offers the most potent instrument to detect low-abundance mRNA. In spite of these advantages over RNA blotting methods, the quantification can often be compromised due to variations in the efficiency of the PCR reaction [14]. The relationship between the initial amount of target present (A) and the amount of

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DNA produced after n cycles of PCR (Y_n) can be expressed as $Y_n = A(1 + R)^n$, where R is the efficiency of the reaction [13]. Small variations in the reaction efficiency, therefore, translate into large differences in the amount of product generated at the end of the reaction. To compensate for these limitations in quantitative analyses, different standardization protocols have been proposed: the most simple approach i.e. the use of external standard reference curves, does not allow reliable quantitations, due to the unpredictable variability of individual amplification reactions, and is thus unsatisfactory. More recently, the co-amplification of internal reference templates, together with the target sequence, has been used to quantitate a number of different mRNAs e.g. [14–17]. The rationale supporting this technique is that alterations in the amplification efficiency should affect both templates similarly. In this case, the major problem is that the different nature of the template sequences might influence the amount of products unpredictably [18]. Considering these limitations we developed a reverse transcription–polymerase chain reaction (RT–PCR) that uses an internal control RNA template which has the same primer binding sites as the target sequence of interest. Designs for such control templates have been reported in the literature [16, 19–21]. The competitive RT–PCR presented herein for AR-mRNA allows for a reliable quantification in a number of species, and was first applied to investigate the effect of long-term castration on AR expression in bovine skeletal muscle.

MATERIALS AND METHODS

RNA extraction and RT–PCR

Total cellular RNA was isolated by a combined guanidinium isothiocyanate and phenol/chloroform extraction method (AGS RNA-Clean™, Heidelberg, Germany). Reverse transcription of 0.2 μg total RNA using 20 pmol of reverse primer (S1) was performed for 30 min at 47°C with 50 units of Super script™ II (BRL, Gaithersburg, MD, U.S.A.) in 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 15 units of RNasin (MBI Fermentas Vilnius, Lithuania), and 0.44 mM each of dATP, dGTP, dCTP and dTTP. The samples were then heated for 1 min at 99°C to terminate the reverse transcription reaction. PCR was performed with 0.5 units of Gold Star polymerase (Eurogentec, Seraing, Belgium) in an automatic DNA thermal cycler (Perkin–Elmer/Cetus, Norwalk, CT, U.S.A.) by adding 30 μl of a PCR master mixture containing PCR buffer, $MgCl_2$ (to a final concentration of 1.45 mM) and 20 pmol of forward primer (S2) to the cDNA samples. Thirty cycles (1 min at 94°C, 45 s at 60°C, 45 s at 72°C) followed by an additional 5 min at 72°C were used. In each experiment water was used as a negative control for contamination.

Table 1. Oligonucleotides used to synthesize ARSt and to quantitate AR mRNA

Primer	Sequence
P1	5′–AGATGGGCTTGACTTTCCCAGAAAG–3′
P2	5′–ATGGCTGTCATTCAGTACTCCTGGA–3′
P3	5′–GGGGTGATTTGGAGCCATCCAAAT–3′
P4	5′–GCATTATTCCAGTGGATGGGCTGAA–3′
S1	5′–TTGATTTTTCAGCCCATCCACTGGA–3′
S2	5′–CCTGGTTTTCAATGAGTACCGCATG–3′

Oligonucleotides used for amplifications

The various primers for the construction of ARSt (androgen receptor internal standard) and for quantitative RT–PCR were derived from the human AR sequence [22]. They were designed to produce an amplification product spanning two RNA-splicing sites in the highly conserved region of the AR sequence coding for the hormone binding domain [23, 24]. A list of all sequences of the primers used herein is given in Table 1.

Construction of the competitive template ARSt-cRNA

We used PCR to generate an internal deletion within the target AR DNA sequence selected for quantification. A total RNA preparation from bovine liver was reverse transcribed into cDNA and amplified using primer P1 and P2 (Fig. 1) to obtain a DNA fragment of 545 bp. This fragment was then cloned into pCRII™ and transformed into *Escherichia coli* INVF′α cells (Invitrogen BV, Leek, The Netherlands). Starting from this construct (pCRII AR-A), a sequence deletion was done by amplifying two DNA-fragments with the two primer pairs P1–P3 and P2–P4 (fragments B and C in Fig. 1). These two fragments were ligated, cloned into pCRII, and transformed in *E. coli* INVF′α cells. Compared to the native sequence, the ligated construct had a 38 bp sequence deletion including the loss of a restriction site for EcoRI.

T7 polymerase transcription

After plasmid linearization, *in vitro* transcription was performed using T7 polymerase (MBI Fermentas) according to Sambrook *et al.* [25]. RNase-free DNase (Pharmacia Biotech, Uppsala, Sweden) was added to a concentration of 1 U/μg DNA and the sample was incubated for 30 min at 37°C. The preparation was then extracted and precipitated in the presence of 20 μg glycogen overnight at −20°C. RNA was pelleted and dissolved in RNase-free water. This procedure was repeated to ensure a complete elimination of the original DNA. The ARSt-cRNA preparation was submitted to a PCR amplification without previous reverse transcription to confirm

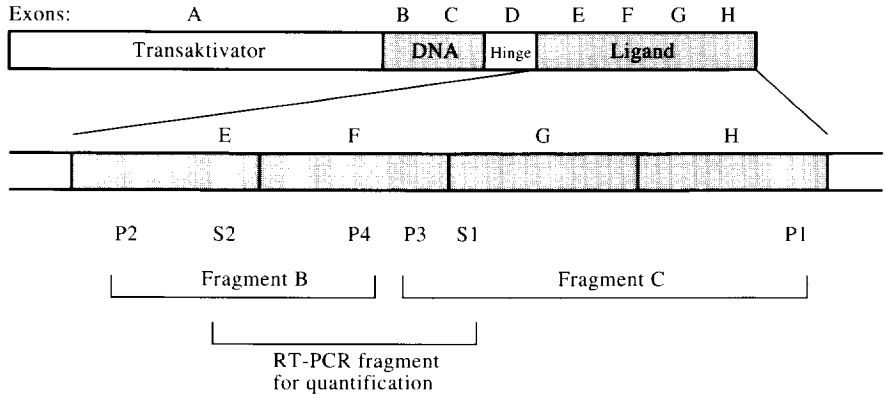

Fig. 1. Structure of the androgen receptor gene and primer design.

the absence of residual pCRII-ARSt DNA molecules. Aliquots were stored at −80°C until needed.

Quantification of PCR products by densitometry

Aliquots (8 µl) of the PCR products were electrophoresed on a VISIGEL™ Separation matrix (Stratagene, Heidelberg, Germany) and visualized by ethidium bromide staining. Polaroid pictures of the gels were scanned with an Agfa Arcus Scanner (Agfa PhotoScan Software, Agfa-Gevaert, Germany) and the intensity of the bands was analysed densitometrically by the NIH Image 1.41 programme. To obtain the AR-mRNA concentration present in the tissue sample, the intensities of the bands of the native AR amplificate (172 bp) and of the ARSt (134 bp) were compared. Briefly, known dilutions of the competitor cRNA were spiked into a series of four RT–PCR reaction tubes containing equal amounts of total RNA. The yield of products generated after co-amplification of AR-mRNA and ARSt-cRNA was compared by plotting their ratio against the log of the internal standard template (abscissa). The amount of competitor cRNA yielding equal molar amounts of PCR products was then calculated by extrapolating from the intersection of the curves, where the amounts of target and competitor are equal ($\log_{10} = 0$) to the x-axis (Fig. 2) as described by Siebert and Larrick [26].

Biological comparisons

AR-mRNA was quantified in bovine skeletal muscle (m. splenius) from five intact and five castrated males of the same age (10–11 months; castration within the first week of life). Tissue sampling and storage was done as described earlier [27].

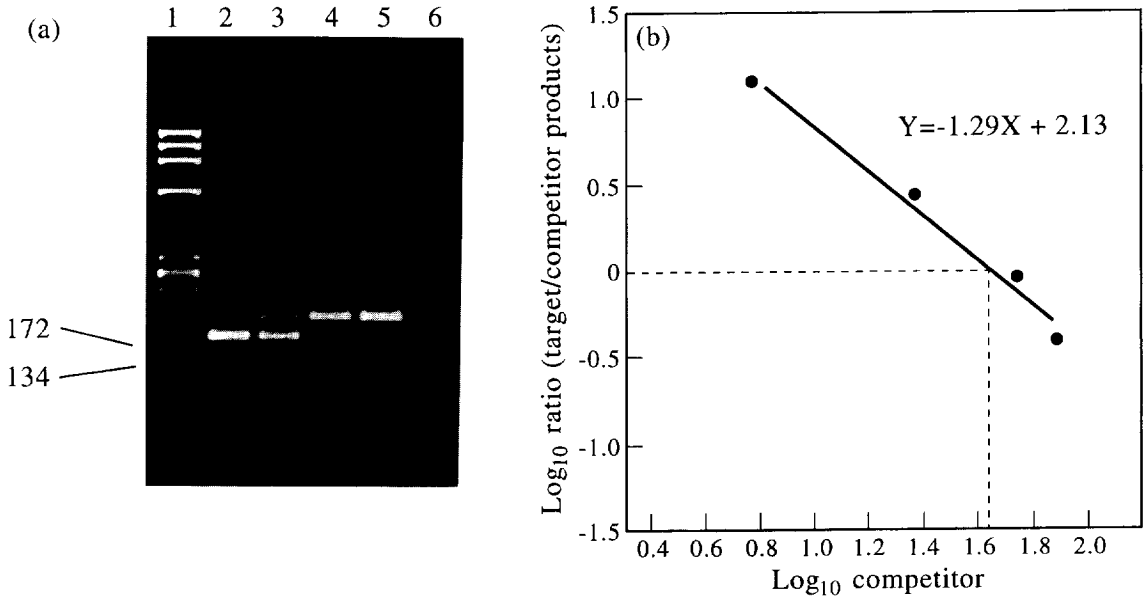

Fig. 2. Representative results of quantitative RT–PCR for AR mRNA. (a) Ethidium bromide staining of RT–PCR co-amplificates separated on a Visigel separation matrix. RT–PCR was carried out using 300 ng of total RNA from bovine skeletal muscle and four different dilutions of AR standard-cRNA (lanes 2–5; lane 1: DNA molecular weight standard). (b) Regression curve constructed from the data obtained by quantitative analysis of the electrophoretic pattern in (a). The logarithm of the ratios of the amplificates from the wild type and the standard (ordinate) was plotted against the log of the known input of AR cRNA standard (abscissa).

RESULTS AND DISCUSSION

Several designs have been used in quantitative RT–PCR to obtain an internal standard that suits the characteristics of having identical amplification efficiency with the wild-type template and of being easily distinguishable from it, allowing the detection and quantification of the two specimens. The insertion of a new endonuclease restriction site by mutation of a single base pair [16, 28] produces an internal standard which is nearly homologous to the wild-type template, and thus comes closest to the theoretical assumption of identical amplification efficiencies for both the standard and the wild-type template [29]. Edelstein *et al.* [30] used a similar approach for the quantification of human AR-mRNA; however, the necessity for two rounds of amplifications and the additional enzyme digestion step prior to quantification increases the expenditure and most probably decreases the assay reliability [18]. Instead, the construction of an internal standard by inserting [17, 31, 32] or deleting [33, 34] a relatively small sequence within the wild-type template is gaining increasing attention. Due to the negative relationship between the efficiency of amplification and the length of the sequences selected to be amplified, the templates should be as short as possible [35]. Moreover, degraded mRNA thus eventually has equal chances of being amplified [15]. Considering these criteria, we designed an internal AR standard which can easily be generated, and in which a small deletion allows for a simple one-step distinction and quantification of the amplication products from standard and from wild-type AR templates. In contrast to the protocol for AR-mRNA quantification proposed by Prins and Woodham [36], our amplificates are considerably shorter (134 and 172 vs. 321 and 486 bp) and they are more closely related.

Fig. 3a shows that the efficiency of amplification is identical for ARSt-cRNA and the native AR-mRNA. The sensitivity of the RT–PCR was evaluated using different amounts of total RNA prepared from bovine prostate tissue (5–360 ng; Fig. 3b). Even at the lowest initial RNA concentration (5.5 ng) amplificates were obtained. Although the sensitivity of this method allows for the detection of products from even smaller starting amounts of total RNA, we noticed alterations in the constancy of the products ratio, probably due to random variations when starting templates are present in very low copy numbers. The precision of the co-amplification was validated in 10 individual samples at four different dilutions. The measurements of the AR-mRNA concentration yielded an average coefficient of correlation (r^2) of 0.96 with a coefficient of variation of 3%. With the assay system described herein we obtained amplificates from various bovine tissues, from human prostate and from dog, pig and rat uterus. The identity of the amplificates of human and from dog tissue was additionally confirmed by sequence analysis.

We first applied the method described herein to quantify AR-mRNA expression in bovine skeletal muscle. In this particular tissue the concentration of AR is exceedingly low compared to other 'classical' androgen target tissues, e.g. in prostate the AR concentrations are about 70 times higher [37]. Previous studies of the regulation of AR in skeletal muscle, which were based on radio ligand assays have made important contributions to AR regulation. However, when the role of endogenous ligand had to be considered, this approach was compromised because in the presence of ligand, AR are tightly bound to the nucleus [38]. Therefore, they are not quantitatively extractable into the cytosolic fraction during homogenization with low ionic strength buffers. In consequence, the AR binding characteristics measured in

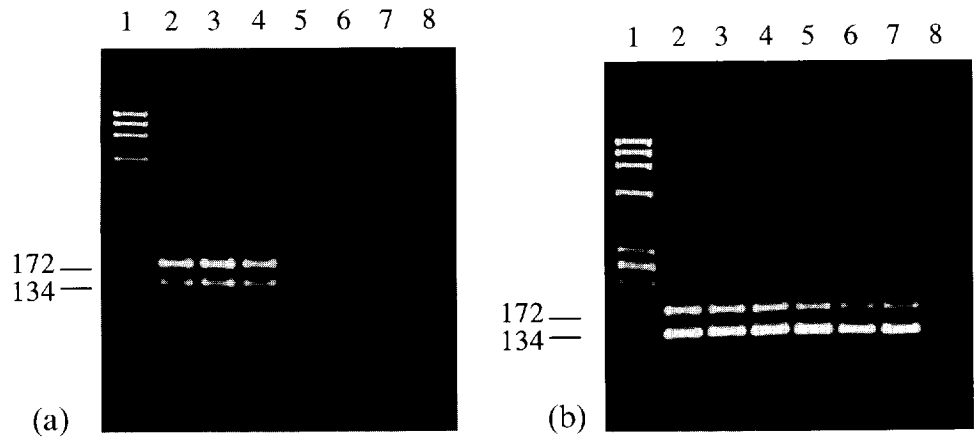

Fig. 3. Efficiencies of the amplification reactions and sensitivity. (a) Ethidium bromide staining of PCR amplificates from wild-type (174 bp) and standard DNA (134 bp) templates separated on a Visigel separation matrix; lane 1, DNA molecular weight standard; lanes 2–7, amplificates from 1:10 serial dilutions of both templates; lane 8, negative control. (b) The sensitivity of the RT–PCR was evaluated using different starting amounts of RNA from bovine prostate in the presence of a constant standard cRNA concentration. Lane 1, DNA molecular weight standard; lanes 2–7, amplificates from 1:2 serial dilutions of RNA (350–5.5 ng) and a constant amount of cRNA standard template; lane 8, negative control.

tissues from animals with different concentrations of the endogenous ligand do not reflect the actual androgen sensitivity [39]. In this case, measurements of AR-mRNA are of particular interest to elucidate the role of endogenous ligands for AR expression rates. To investigate the effect of castration on AR-mRNA concentration in muscular tissue, we selected the m. splenius from the neck because this particular muscle has been demonstrated to react over-proportionally to castration with regard to muscle growth [40] and, moreover, it contains relatively higher AR concentrations than muscles from other parts of the body, such as the hind leg or abdomen [39]. Comparing the AR-mRNA quantities in the m. splenius from intact and from long-term castrated males, we observed 1.3-fold higher AR-mRNA concentrations in castrates ($P < 0.05$). Indeed, this finding is different to earlier investigations on the effect of castration on androgen binding capacity in murine and rat muscle. Dahlberg *et al.* [41] reported that in rats AR binding capacity is increased for about 50 days after castration but decreases in long-term (190 days) castrates to levels lower than in intact males. Although the quoted study in rodents might not be strictly comparable to our investigations in cattle, our results demonstrate that AR expression is not decreased but is actually increased in long-term castrates. However, the effect of castration observed in our study is relatively small and thus indicates that androgens might only modulate rather than actually determine AR expression rates in skeletal muscle.

CONCLUSIONS

We presented herein a fast and reliable assay to quantify AR-mRNA by an internally standardized RT–PCR. The procedure is very sensitive and precise within the RNA concentration range tested. It was demonstrated to be applicable for the quantification of AR-mRNA in human, bovine, porcine, canine and rodent tissues. The described method thus provides an important tool for the study of the regulation of AR expression in physiological as well as in malignant conditions.

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