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Quantitative Reverse Transcriptase Polymerase Chain Reaction Assay for Mouse Androgen Receptor mRNA

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A quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay for mouse androgen receptor (AR) mRNA was developed to study relative changes in AR gene expression. Serial dilutions of a standard comprising a fragment of the ampicillin resistance gene flanked by the primer sequences of the AR mRNA were added to a constant amount of total RNA for RT-PCR. Primers were designed to generate a 541-bp fragment of mouse AR mRNA (target [T]) and a 460-bp fragment of the standard (S). PCR products were resolved by gel electrophoresis and quantitated by densitometry. A standard curve was generated for each sample by plotting the logarithm of T/S products vs the logarithm of the amount of S added. The amount of T was determined from the standard curve where intensities of PCR products of T and S were equal. The assay was validated by measuring the relative abundance of AR mRNA in 10 mouse tissues, and results were consistent with studies of AR expression in rat tissues. Assay reproducibility, tested by repeating assays on four different tissues on different days from the RT step, had a coefficient of variation of 6–16%. The current assay is thus both reproducible and valid in quantitation of mouse AR mRNA.

Key Words: Reverse transcriptase polymerase chain reaction; androgen receptor; mouse.

Introduction

The androgen receptor (AR) functions as a ligand-activated transcription factor, regulating the expression of genes involved in various biologic functions. In the male reproductive system, it mediates the effects of androgens at induction and maintenance of spermatogenesis *(1)*. It is unknown, however, how androgens affect in vivo AR mRNA levels in testicular cells during these processes. The aim of the current study was to develop a method for measuring relative changes in AR mRNA (target [T]) at induction of mouse spermatogenesis.

Three methods used for studying steady-state AR mRNA levels are Northern analysis *(2)*, RNase protection assay *(3)*, and reverse transcriptase polymerase chain reaction (RT-PCR) *(4)*. Northern analysis can provide information on mRNA size and alternate splicing in addition to quantitation. RNase protection assay is more sensitive than Northern analysis since target molecules remain mobile in solution during hybridization. Both these methods, however, require microgram quantities of RNA, prompting us to use RT-PCR for quantitation since tissue from undeveloped mouse testis is limited and AR expression in testis is low *(5,6)*.

Quantitative RT-PCR can be performed by measuring signal intensities at the end of amplification (end-point systems) or while products accumulate from cycle to cycle (real-time systems) *(7)*. Fully automated real-time systems provide a high degree of precision and reproducibility, assay parameters that are essential when monitoring clinical intervention over time *(8)*. End-point systems, however, are infinitely cheaper and offer levels of reproducibility that are adequate for most biologic research applications such as the current one, in which the primary aim of quantitating RNA was to compare relative changes in gene expression.

By far the most common end-point method is to use an internal standard (S) to control for variable efficiencies during RT-PCR. RNA standards are more useful than DNA standards because they control for variable efficiencies during the entire RT-PCR process. It remains common practice to use one or more housekeeping genes in the same sample as RNA standards *(9,10)*. Some problems with this method, however, are differences in primer-annealing efficiencies during PCR, differences in the abundances of target and housekeeping genes, possible treatment effects on housekeeping genes, and the potential for coamplification of pseudogenes if samples are contaminated with traces of genomic DNA. To overcome these problems, a synthetic standard is added to each sample for RT-PCR. We describe here an RT-PCR assay for relative quantitation of mouse AR mRNA using a synthetic standard of bacterial origin.

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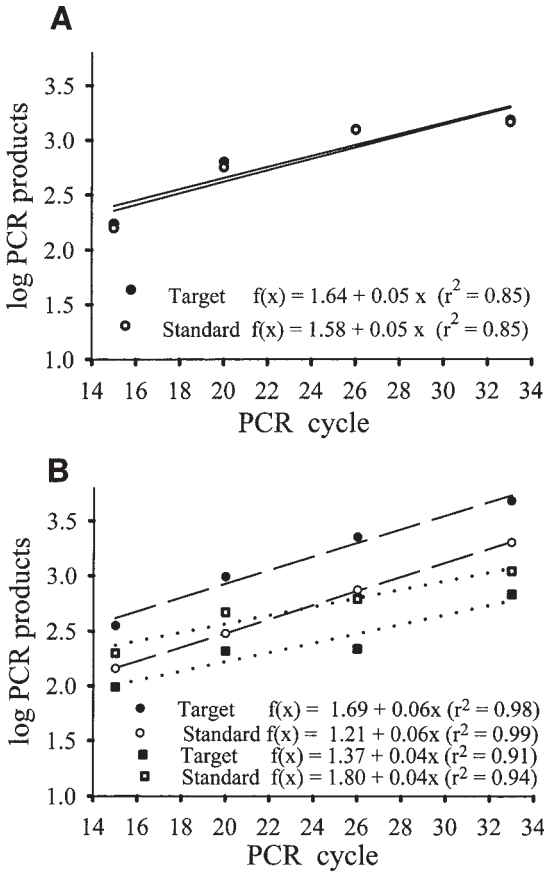

Fig. 1. Regression lines of target and standard PCR products. Accumulation of T and S using a sample with (**A**) similar starting T and S (T/S = 1.11), or (**B**) more starting S (T/S = 0.47) (dotted line) or more starting T (T/S = 2.47) (dashed line) when the log of products was plotted against the number of PCR cycles. The slopes of each T and S pair, determined by least-squared regression, were not significantly different ($p > 0.05$) when compared using student's *t*-test for two slopes *(17)*.

Results

Amplification Efficiency

To test whether T and S amplified with similar efficiencies, a previous PCR product that was shown by densitometry to contain similar amounts of T and S (T/S = 1.11) was diluted 500-fold and amplified with the same primers in a second round of PCR. Aliquots of PCR products were removed at different intervals after products first became visible and plotted on a log scale against the cycle number (Fig. 1A). T/S was 1.11 at the start of PCR and 1.04 after 33 PCR cycles. A similar test was conducted on samples in which starting S was either double or half that of starting T (Fig. 1B). T/S was 0.47 before PCR and 0.49 after 33 PCR cycles for the sample with more S. T/S was 2.47 before PCR and 2.43 after PCR for the sample with more T. These results indicated that differences in amplification efficiencies between T and S did not affect T/S in the linear phase of amplification when starting S was either double or half that of starting T.

Slopes of standard curves of –1 (or +1 for log S/T) can be used to indicate whether amplification efficiencies were similar at assay. The slopes from 30 standard curves, obtained from assaying 10 tissues from 3 mice, ranged from –0.8 to –1.4, suggesting that there was a difference in the amplification efficiency of T and S and that this difference was not identical in all the tubes in the dilution series *(11)*. One explanation is early plateauing in reactions containing more S and a consequent tilting of slopes of standard curves. This was demonstrated by a noticeable improvement in slopes when one or more reactions at the high-S end of each dilution series were omitted from the standard curve. Other explanations for nonideal slopes include under- or overestimation of faint gel bands at the extreme ends of the dilution series *(11)* and the use of agarose gels for quantitation. For reasons that remain unclear, the same amplification products that produce a slope of –1 on polyacrylamide gels produce inferior slopes when quantitated on agarose gels with no effect on the point of equivalence *(12)*.

Validation of Assay

The assay was validated by determining the relative abundance of AR mRNA (relative amount per 50 ng of total RNA) in 10 mouse tissues. As a first step, samples were ranked in order of AR abundance by performing RT-PCR without S. This step was repeated at least once to overcome some of the variability in efficiency of RT-PCR among tubes. Products were quantitated by densitometry and ranked in decreasing order of AR abundance. Total RNA (50 ng) from epididymis (highest-T sample), testis (intermediate-T sample), and spleen (lowest-T sample) was then amplified with three fourfold dilutions of S to obtain an approximate determination of equivalence point (E) for each sample. Quantitative RT-PCR was then performed using six twofold dilutions of S and 50 ng of total RNA from 10 tissues from three mice. The descending order of AR mRNA level was epididymis, prostate, kidney, adrenal, seminal vesicle, testis, heart, lung, liver, and spleen (Fig. 2). The abundance of AR mRNA (molecules/50 ng of total RNA) was only a crude estimate based on the approximate number of S molecules that produced the same amount of T product. The abundance of AR mRNA in mouse tissues relative to epididymis was 4-fold lower in testis ($p < 0.05$); 5-fold lower in heart, lung, and liver ($p < 0.05$); and more than 100-fold lower in spleen ($p < 0.01$). Levels of AR mRNA in epididymis, prostate, kidney, adrenal, and seminal vesicle were not significantly different.

Interassay Reproducibility

Interassay reproducibility was tested by repeating assays on four different tissues (epididymis, prostate, kidney, and testis) on different days from the RT step and comparing the molar quantity of S at equivalence point for each tissue. Inter-assay coefficient of variation (CV) was 9.8, 6.5,

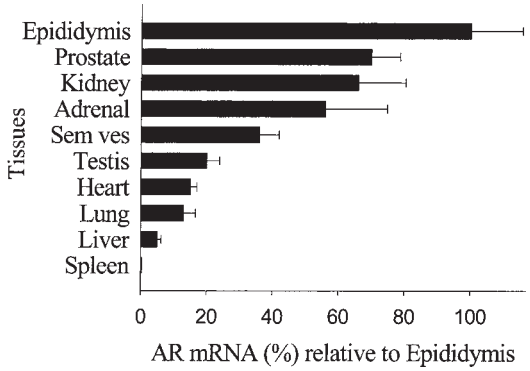

Fig. 2. AR mRNA expression in 10 adult mouse tissues as determined by quantitative RT-PCR. Horizontal bars represent the mean abundance of AR mRNA ± SEMs. Abundances of AR mRNA per 50 ng of total RNA were relative to S added at assay and were determined from standard curves comprising an average of five data points. See text for details of assay conditions.

16.0, and 6.3% for epididymis, prostate, kidney, and testis, respectively.

Discussion

The aim of the current study was to develop a quantitative RT-PCR assay to measure relative differences in the abundance of mouse AR mRNA. A synthetic standard was constructed and added to each RT-PCR reaction to control for variability in tube-to-tube efficiencies. The use of a synthetic standard circumvented the potential problem of treatment effects on housekeeping genes serving as internal standards. The design of the current standard for AR mRNA conferred two additional advantages. First, the use of common primers for the AR and standard ensured that these primers annealed to both AR and standard with identical efficiencies. Second, the use of a heterologous intervening sequence between primer sites prevented heteroduplex formation between AR and the standard strands since they did not share homology.

A potential problem with heterologous standards, however, is that amplification efficiencies of both templates are likely to be different despite a similarity in overall G/C content, template sizes, and the use of identical primers. Because differences in efficiencies are likely to become more apparent at plateau, it was very important to restrict quantitation to the linear phase of PCR amplification. To obtain a conservative estimate of the duration of the linear phase for all reactions in a dilution series, this preliminary test should be conducted on high-AR tissues using the lowest working dilution of the standard. It is worth pointing out that while it is necessary to show identical amplification efficiencies for absolute quantitation, small differences in amplification efficiencies are insignificant when comparing relative abundances of the target gene transcript *(13)*.

Merits of the current assay include the simplicity of the method chosen to construct the standard; it involved two rounds of PCR and a short in vitro transcription step. This method can be contrasted to traditional cloning involving restriction endonuclease digestion of plasmid and religation after insertion of a template, which can be inconsistent. There is also more flexibility in choosing the size of standard, without the inhibitions imposed by the fixed position of restriction enzymes in commercially prepared plasmids. Some preliminary work was necessary to determine the linear range of each sample for a given amount of starting template, but once this was established, quantitation was rapid. There was minimal post-PCR manipulation of samples compared with assays that distinguish target from standard by enzyme digestion, reducing the chance for error. The use of a nonradioactive assay circumvents problems associated with the use of radioisotopes, such as safety, limited half-life, and disposal of radioisotopes. From an economic perspective, the current assay does not require sophisticated instrumentation or expensive fluorescent probes.

Interassay reproducibility was assessed by repeating assays on epididymis, prostate, kidney, and testis on different days from the RT step and comparing absolute values of AR mRNA. The CV ranged between 6 and 16%. In assays repeated over an extended period of weeks, a consistent shift in the equi-valence points for each sample was noted. Consistently higher predetermined concentrations of the standard were required to produce equivalence points for each sample. This suggested that the changes were owing to degradation of the standard over time. Problems with maintaining similar concentrations of RNA standards for sufficient interassay reproducibility have been reported elsewhere *(14)*. To ensure that the level of AR mRNA was comparable across assays, all 10 tissues from three animals were assayed within a week of preparing dilutions.

Validity of the assay was assessed by measuring the abundance of AR mRNA in 10 mouse tissues. Differential AR expression in these tissues was consistent with AR mRNA and protein reported in rat tissues using radioactive quantitative RT-PCR *(4)* and immunohistochemistry *(15)*. This assay can be readily applied to mouse tissues and cultured cells and is currently being used to monitor changes in AR mRNA levels in mouse testis after induction of spermatogenesis.

Materials and Methods

Animals and Tissues

Mice (C3HeX101 strain) were bred at the Bosch Animal House, University of Sydney and handled in accordance with National Health and Medical Research Council guidelines for animal experimentation. Mice were killed by cervical dislocation while under anesthesia administered by ip injection (0.01 mL/g of body wt) of a solution of ketamine (0.4%) (Parke-Davis, Caringbah, Australia) and xylazine (0.4%) (Bayer, Botany, Australia). Small pieces of tissue

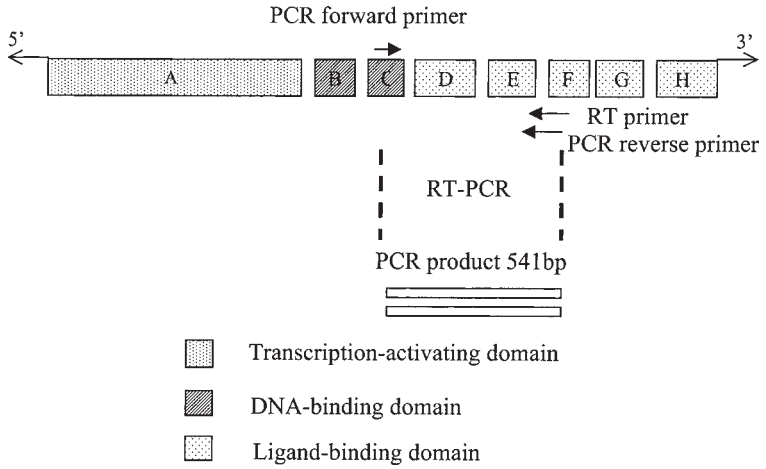

Fig. 3. Primers for amplification of mouse AR mRNA. Primers were designed to isolate and amplify a 541-bp fragment of mouse AR mRNA. Boxes labeled A–H represent the eight exons of the AR gene, and shaded regions represent the three functional domains of the AR. The PCR forward and reverse primers and the RT primer comprised nucleotide sequences 1753–1776, 2270–2293, and 2278–2301 of mouse AR cDNA, respectively *(16)*.

from 10 different organs (liver, lung, heart, kidney, spleen, adrenal, testis, epididymis, seminal vesicle, and prostate) were rapidly cleared of fat, excised, and snap-frozen in liquid nitrogen. Tissues were stored at −70ºC until RNA isolation.

Primers for Mouse AR mRNA

Primers were synthesized on an Oligo 1000 DNA synthesizer (Beckman, Fullerton, CA). Primers were designed to isolate a 541-bp fragment of the coding region of the AR gene (Fig. 3). This fragment included part of the DNA-binding domain (3' of exon C), the complete hinge region (5' half of exon D), and part of the ligand-binding domain (3' of exon D, exon E, and 5' of exon F). To prevent reverse-transcribing genomic DNA, the RT primer spanned the junction of two exons. The full primer sequences for amplification of AR mRNA were as follows: PCR forward primer (5'-TATGTGCCAGCAGAAACGATTGTA-3'), PCR reverse primer (5'-CGGTACTCATTGAAAACCAAGTCA-3'), and RT primer (5'-TGTGCATGCGGTACTCATTGAAAA-3'). The 541-bp PCR product obtained using these primers was sequenced and found to match the published sequence of mouse AR *(16)*. The assay can be used for quantitation of human AR mRNA by changing six nucleotides in the primers and incorporating this change into the standard.

Isolation of Total RNA

Total RNA was isolated from mouse tissues using Tri-reagent (Sigma, Castle Hill, NSW, Australia) according to the manufacturer's protocol. To minimize genomic DNA contamination, not more than 30 mg of tissue/mL of Tri-reagent was used. The RNA pellet was resuspended in RNase-free H_2O, quantitated by measuring absorbance at 260 nm, and stored at −70°C until assay. Tubes used to store RNA were treated with 0.1% diethylpyrocarbonate, incubated overnight at 42°C, and autoclaved. Since spectrophotometric quantitation of RNA does not differentiate RNA from DNA, all RNA samples were tested for genomic DNA contamination by performing PCR directly on total RNA (100 ng) using GAPDH primers (5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3') (Clontech, Palo Alto, CA). These primers are suitable for detecting genomic DNA because they amplify GAPDH-related pseudogenes of about 450-bp. Any contaminating genomic DNA was efficiently removed by repurifying samples with Tri-reagent.

Construction of S

S comprised a 400-bp fragment of the ampicillin resistance gene (with similar overall dG/dC content to T) flanked by the primer sequences of T. This ensures identical primer-annealing efficiencies for both T and S templates during assay. Two rounds of PCR were performed to isolate and modify the ampicillin resistance gene from Bluescript II SK plasmid (Stratagene, La Jolla, CA). The forward composite primer in the first round of PCR (5'-TATGTGCCAGCAGAAACGATTGTA/GAATTC/ATCCGCCTCCATCC-3') contained sequences of T, *Eco*RI, and the ampicillin resistance gene. The reverse composite primer in the first round of PCR (5'-ACTCATTGAAAAC CAAGTCA/AAGCTT/ATCCCGTATTGACG-3') contained sequences of T, *Hind*III, and the ampicillin resistance gene. The restriction enzyme sequences were included to facilitate endonuclease digestion if required. The forward composite primer in the second round of PCR (5'-GGATCC TAATACGACTCACTATAGGG/TATGTGCCAGCAG-3') contained sequences of the T7 promoter to facilitate in vitro transcription of the PCR product into complementary

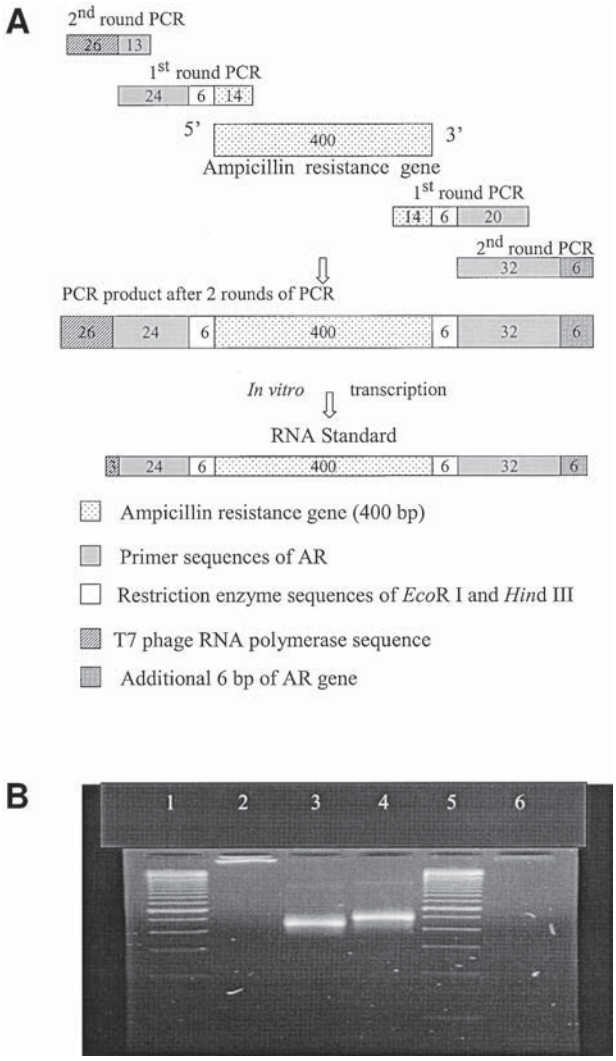

Fig. 4. Construction of S for quantitative RT-PCR. (**A**) Composition of two pairs of composite primers used to isolate and modify a fragment of the ampicillin resistance gene. (**B**) PCR products after first (456-bp, lane 3) and second (500-bp, lane 4) round of PCR. As a negative control, plasmid was used as template in the second round of PCR (lane 6). Also present are plasmid only (lane 2) and 100-bp DNA ladder (lanes 1 and 5).

RNA and some sequences of T to facilitate annealing of this primer to the first-round product. The reverse composite primer in the second round of PCR (5'-GAGACT/TGTGCATGCGGTACTCATTGAAAACCAAGTCA-3') contained an additional six bases of the AR gene to promote annealing of the RT primer to S during assay and some sequences of T to facilitate annealing of this primer to the first-round product. A schematic diagram showing construction of S is given in Fig. 4A.

PCR reactions were conducted in a final volume of 25 µL containing 1X PCR buffer (Applied Biosystems, Foster City, CA), 0.2 m*M* dNTPs (Roche, Castle Hill, NSW, Australia), 0.6 µ*M* of the first pair of composite primers, 0.02 U of *Taq* DNA polymerase (Applied Biosystems), and 50 ng of plasmid as template. Amplification was conducted with an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 45 s, 58°C for 15 s, and 72°C for 30 s. First-round product (456-bp) was diluted 1000-fold and used as template in a second round of PCR using the second pair of composite primers (Fig. 4B). Second-round products (500-bp) were gel purified and in vitro transcribed using MAXIscript™ (Ambion, Austin, TX) to generate complementary RNA according to the manufacturer's protocol. The RNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1) and precipitated with ethanol. The pellet was washed with 80% ethanol, suspended in RNase-free H_2O, and quantitated by ultraviolet absorbance at 260 nm. To show that no traces of DNA product remained, a small aliquot of S was amplified directly by PCR (35 cycles) using AR forward and reverse primers. An absence of a band indicated a DNA-free RNA preparation. The solution containing S was aliquoted into "single-use" twofold dilutions and stored at –70°C until assay.

Quantitative RT-PCR Protocol

An average of six PCR tubes containing a constant amount of total RNA (50 ng) were spiked with twofold dilutions of S. Templates were denatured at 70°C for 10 min. A master mixture containing 0.5 m*M* dNTPs, 0.1 µ*M* gene-specific RT primer, 1X RT buffer, 0.01*M* dithiothreitol, and 200 U of Superscript II (last three reagents from Life Technologies, Melbourne, Victoria, Australia) was added to each tube to make up a final volume of 10 µL, and cDNA synthesis was performed at 42°C for 55 min. The reaction was stopped by denaturing the enzyme at 70°C for 15 min. For PCR amplification, 10% of the cDNA product was transferred to a PCR mixture comprising 1X PCR buffer, 0.2 m*M* dNTPs, 0.5 µ*M* each of forward and reverse primers, and 0.06 U of *Taq* DNA polymerase in a final volume of 25 µL. Amplification was performed with an initial denaturation at 94°C for 2 min followed by 25–35 cycles of 94°C for 45 s, 65°C for 15 s, 72°C for 30 s, and a final extension step at 72°C for 5 min. As a negative control, cDNA template was substituted with DNase-free H_2O to confirm that all PCR reagents were free from contaminating cDNA and products from previous amplifications.

Optimizing Amount of Starting Template and Number of PCR Cycles

To determine the range over which T and S amplified exponentially for each sample, total RNA was mixed with a relatively low dilution of S and amplified over 40 cycles under standard assay conditions. The accumulation of products was determined by removing an aliquot of the PCR reaction every three cycles from cycle 15. A cycle in the lower end of the linear range was selected for assays. The use of 500 ng of total RNA as starting template was reduced to

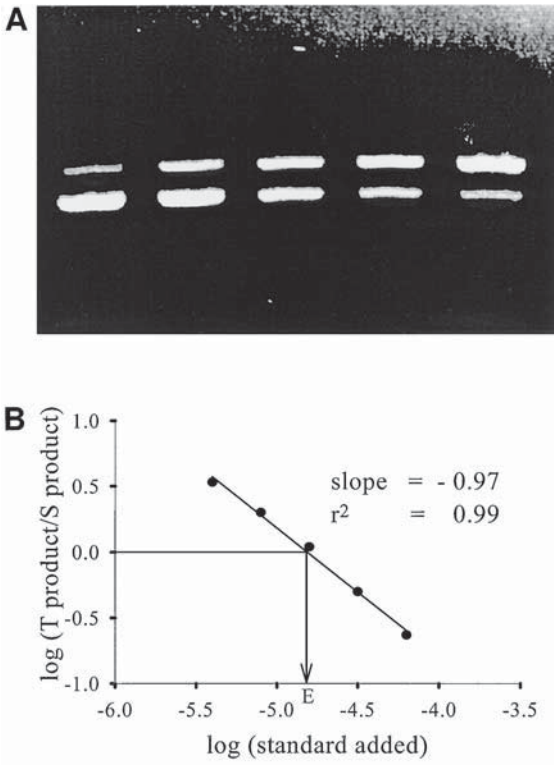

Fig. 5. Quantitative RT-PCR. Serial dilutions of S were mixed with 50 ng of total RNA from mouse testis for RT-PCR. (**A**) Ethidium bromide staining of T products (upper band, 541 bp) and S products (lower band, 460 bp) separated in 2% agarose. (**B**) Standard curve generated by plotting the log of T product/S product against the log of the amount of S added. The amount of T was determined by interpolation from the point where T and S were equal (E) and is relative to the amount of S added at assay.

50 ng for all tissues to prevent high AR-expressing tissues from reaching plateau soon after bands became visible.

Gel Electrophoresis

The RT-PCR products were resolved in a 2% (w/v) agarose gel in Tris-borate/EDTA buffer (Fig. 5A). Bands were visualized by ethidium bromide staining (0.5 µg/mL) and gel images were photographed (Grab-It™; UVP, Cambridge, England, UK). Care was taken to avoid saturation of band intensities during photography of gels. Band intensities of T and S were quantitated by densitometry (NIH Image, Bethesda, MD).

Computation of T

A standard curve was generated by plotting the log of T products/S products vs the log of the amount of S added. Only reactions in the linear range of PCR were used to generate standard curves. The amount of T was determined by interpolation on the standard curve from the point where the band intensities of T and S were equal. This was represented by 0 on the *y*-axis and E on the *x*-axis (Fig. 5B). Values of T were relative to the number of S molecules added at E. The latter was calculated from the molarity of S after in vitro transcription and purification of S. Values at E were used for relative comparison of T in different samples.

Statistical Analyses

Data were expressed as mean ± SEM unless otherwise stated. The data were analyzed by analysis of variance (Graph PAD/Instat), and differences were regarded as significant at $p < 0.05$. The slopes of the lines showing accumulation of PCR products were determined by least-squared regression, and the slopes of the regression lines for each sample were compared using student's *t*-test for two slopes *(17)*.

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