Exp Clin Endocrinol Diabetes 106 (1998) 506–513

Experimental and Clinical
Endocrinology
&Diabetes

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Current Methods

Quantification of insulin-like growth factor-1 (IGF-1) mRNA: Development and validation of an internally standardised competitive reverse transcription-polymerase chain reaction

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Key words: IGF-1 mRNA, quantitative RT-PCR, castration, androgen status, skeletal muscle, liver, bovine

Summary: To investigate the role of local IGF-1 mRNA expression in various tissues, we developed and validated a method which allows for a specific, sensitive and reliable quantification of IGF-1 mRNA: an internally standardised Reverse Transcription-Polymerase Chain Reaction (RT-PCR). A synthetic competitive template IGF-1 standard cRNA (IGF-1 cRNA) was designed, which contains the same flanking primer sequences used to amplify the wild type IGF-1 mRNA, but differs by 56 bp in length. To obtain the IGF-1 mRNA concentration present in tissue RNA samples, series of 250 ng total-RNA were spiked with three known quantities of the standard IGF-1 cRNA, incubated for competitive RT-PCR reactions and the two amplificates obtained (184 bp from IGF-1 cRNA and 240 bp from the wild type IGF-1 mRNA) were subsequently separated and quantified by HPLC-UV. For every individual tissue RNA sample, the ratio R (R = competitor PCR product / wild type PCR product) was plotted against the number of starting molecules of the competitor IGF-1 cRNA. The initial amount of IGF-1 mRNA present in the sample can then be read off where R = 1. The validated assay had a detection limit of 1600 IGF-1 cRNA molecules/reaction, the intra-assay variation was 7.4% ($n = 5$) and linearity ($r = 0.997$) was given between 140 ng to 840 ng total-RNA input. The present method was first applied to study the effect of long term castration on the IGF-1 expression rates in bovine tissues. The hepatic IGF-1 mRNA concentrations were well correlated ($r = 0.81$) with the plasma concentrations as quantified by RIA and were higher in intact than in castrated animals. In two skeletal muscles (m. splenius and m. gastrocnemius) IGF-1 mRNA concentrations were 20- and 35- times lower than in liver, respectively, without any differences between steers and bulls. In bulls, the IGF-1 mRNA expression was higher in m. splenius ($p < 0.01$) than m. gastrocnemius, indicating that locally produced IGF-1 might be important for sexually dimorphic muscle growth patterns.

Introduction

IGF-1 mediates the anabolic growth hormone actions in skeletal tissues. Above that locally expressed IGF-1 is an important growth regulator acting in auto- and paracrine way (Thissen et al., 1994). To investigate the tissue specific expression in low abundant tissues a method is required which allows for a reliable quantification of IGF-1 mRNA. Considering these limitations, RT-PCR offers the most potent instrument to detect low-abundance mRNAs and the detection limit can been increased up to 1000-fold in comparison to other methods, e.g. Northern hybridisation (Saiki et al., 1988). The relationship between the initial amount A of target mRNA present in the tissues and the amount Y_n of DNA produced after n PCR cycles can be expressed as $Y_n = A \cdot (1 + E)^n$, where E is the amplification efficiency of one reaction step (Chelly et al., 1988). Small variations in the reaction efficiency, therefore, translate into large differences in the amount of RT-PCR product generated after n cycles. These limitations in quantitative analyses can be compensated by parallel co-amplification of the native mRNA together with known amounts of an internal standard cRNA. The

Support: This study was supported by a grant (Sa 432/2-2) from the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany.

amplification efficiency should affect both templates similarly. Several designs have been used in quantitative RT-PCR to obtain an internal standard cRNA that suits the characteristics of having an identical amplification efficiency as the wild-type mRNA template and of being easy distinguishable from it (Nedelman et al., 1992). Hereby the construction of an internal standard by inserting (Martini et al., 1995) or deleting (Becker-André and Hahlbrock, 1989; Piatak et al., 1993; Malucelli et al., 1996) a relatively small sequence within the wild type template are common practice. Due to the negative relationship between the efficiency of amplification and the length of the amplified sequences, the both templates should be as short as possible (Rolfs et al., 1992). Analysis and quantification of competitive PCR products can be done either by electrophoretic separation with densitometric quantification or by HPLC and following UV detection at 260 nm. HPLC-UV is the most exact quantification method for PCR products in terms of accuracy, precision and linearity (Katz et al., 1990). In consequence we designed, developed and validated an internally standardised IGF-1 mRNA RT-PCR assay with subsequent HPLC-UV quantification for quantitative comparisons in tissues of low IGF-1 mRNA abundance. The method was first applied to investigate the effect of castration on IGF-1 mRNA expression in bovine liver and two different skeletal muscles.

Material and methods

RNA extraction

0.5 g frozen tissue sample was homogenised in 4 M guanidinium thiocyanate (GU-SCN) buffer according to Chirgwin et al. (1979) to destroy RNase activity. In the following steps, the RNA-Clean™ protocol (AGS, Heidelberg, Germany) with phenol/chloroform extraction for total RNA was used. In order to quantify the amount of RNA extracted, the optical density was determined in 3 different dilutions of the final RNA preparations at 260 nm and the concentration was then calculated. RNA integrity was electrophoretically verified by ethidium bromide stain.

Construction of the competitive standard template IGF-1 cRNA

The three primers used for the construction of IGF-1 cRNA (internal standard IGF-1 cRNA) and for quantitative RT-PCR were derived from the bovine IGF-1 sequence (Fotsis et al., 1990). They were designed to produce an amplification product spanning two RNA-splicing sites in the highly conserved region (exon 3 and 4) of the IGF-1 sequence coding for the mature 70 amino acid IGF-1 protein (Gilmour et al., 1992).

The competitive standard IGF-1 cRNA should have the same properties like the native IGF-1 mRNA in RT-PCR, but the products should be distinguishable for their individual quantification. We therefore used RT-PCR to generate an internal deletion of 56 bases within the IGF-1 DNA target for length differentiation (Fig. 1). Bovine liver total RNA was reverse transcribed into cDNA and amplified using reverse primer *P1b* (5′-GCA GTA CAT CTC CAG CCT CCT CAG A-3′) and forward primer *P1a* (5′-TCG CAT CTC TTC TAT CTG GCC CTG T-3′). All details of the RT-PCR are given below. From the 240 bp DNA fragment obtained, which includes a native *Xho I* restriction site, a second PCR was performed using a different forward primer *P2* (5′-CAT CTC GAG AGC ATC CAC CAA CTC A-3′) which introduces an artificial restriction site for *Xho I* into the resulting 105 bp PCR product. 240 bp and 105 bp fragment were gel purified using the QUIAEX DNA gel extraction kit (Qiagen, Chatsworth, CA, USA) and were then digested with *Xho I* restriction enzyme (Pharmacia, Uppsala, Sweden). The derived two DNA fragments, each with one sticky *Xho I* end, were subsequently ligated at the *Xho I* recognition site with Ready-To-Go T4 DNA Ligase™ (Pharmacia) to form a 184 bp fragment. The ligated product was cloned into pCRII™ (Invitrogen BV, Leek, The Netherlands), and transformed into competent *E. Coli* INVF' cells (Invitrogen). Compared to the native sequence, the ligated IGF-1 cDNA construct had a 56 bp sequence deletion, but is flanked by the same primers *P1a* and *P1b* used to amplify the wild type IGF-1 mRNA as confirmed by sequence analysis.

SP6 Polymerase transcription of IGF-1 cRNA

After linearisation of the plasmid, a cRNA was transcribed, containing the 184 bp IGF-1 cRNA standard fragment, using SP6 polymerase (MBI Fermentas, Vilnius, Lithuania) as described by Sambrook et al. (1989). RNase-free DNase™ (Pharmacia) was added to a concentration of 1 U/µg DNA for 60 min at 37°C. The preparation was then extracted and precipitated in the presence of ammonium acetate and ethanol overnight at −20°C. This procedure was repeated to ensure a complete elimination of the original plasmid pCRII and IGF-1 standard DNA. After two washing, pelleting and solving steps, the IGF-1 cRNA standard was finally stored in diethyl pyrocarbonate (DEPC, Aldrich-Chemie, Steinheim, Germany) treated H_2O. The purity of the cRNA preparation was confirmed by PCR amplification without prior reverse transcription, thus demonstrating the absence of residual pCRII IGF-1 DNA. IGF-1 cRNA standard was quantified in a series of 5 dilutions at 260 nm. Aliquots were diluted from factor 10^1 to 10^{10} in DEPC water, representing $1.6 \cdot 10^{10}$ to 16 IGF-1 cRNA molecules/µL. After addition of

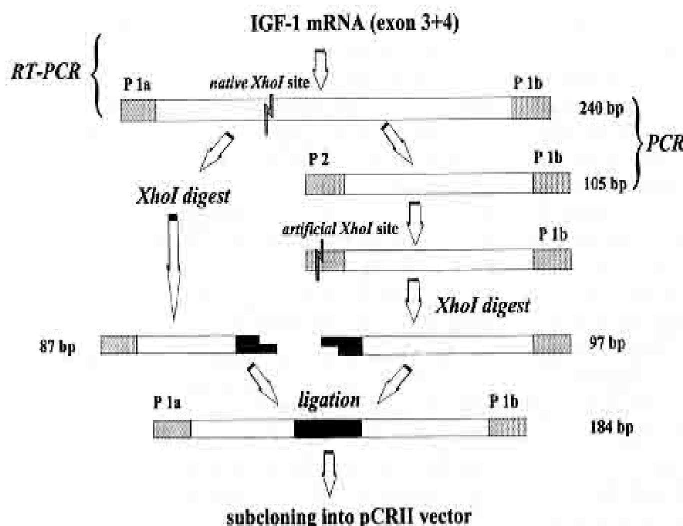

Fig. 1 Construction of the competitive standard IGF-1 cRNA: RT-PCR of bovine liver RNA resulting in a 240 bp fragment, bearing a native *Xho I* site. With this initial fragment, a second PCR was performed using the forward primer *P2* which introduces an additional artificial restriction site for *Xho I* into the resulting 105 bp PCR product. The 240 bp as well as the 105 bp fragment were gel purified and digested with *Xho I*. The two fragments were then ligated to a 184 bp fragment. This product was cloned into pCRII and transformed into competent *E. Coli* INVF' cells. Compared to the native sequence, the ligated IGF-1 cDNA construct has a 56 bp sequence deletion, but is flanked by the same primers *P1a* and *P1b*

20 U of Rnasin™ RNase inhibitor (MBI Fermentas) to prevent cRNA degradation, the aliquots were stored at −80 °C until needed.

Competitive IGF-1 mRNA and IGF-1 cRNA RT-PCR

For each tissue RNA preparation three reactions were performed, using 250 ng of total-RNA and fixed concentrations of the competitor IGF-1 cRNA ($8.0 \cdot 10^8$; $1.6 \cdot 10^9$ and $8.0 \cdot 10^9$ molecules for liver; $8.0 \cdot 10^7$; $1.6 \cdot 10^8$ and $8.0 \cdot 10^8$ molecules for muscles). RNA, RT buffer (50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT and 150 µM dNTPs (each of dATP, dGTP, dCTP, and dTTP) with three drops of Chill-Out 14™ Liquid Wax (MJ Research, Biozym Diagnostik GmbH, Oldendorf, Germany) were denaturated for 5 min at 65 °C. The subsequent RT was done at 42 °C for 60 min by adding 10 pmol of reverse primer *P1b*, 50 U of Super script II™ Plus RNase H⁻ Reverse Trancriptase (BRL, Gaithersburg, MD, USA), 10 U of Rnasin™ RNase inhibitor. The samples were then heated for 1 min at 95 °C to terminate RT. The following PCR was performed as a hot start PCR with 2.5 U of Prime Zyme™ thermostable DNA polymerase (Biometra Inc., Tampa, FL, USA) in an automatic DNA Thermal Cycler (Perkin-Elmer/Cetus) by adding 30 µL of a PCR master mixture containing 1× PCR puffer, $MgCl_2$ (to a final concentration of 1.45 mM) and 10 pmol of forward primer *P1a* to the cDNA samples. 30 cycles (1 min 94 °C, 30 sec 62 °C, 30 sec 72 °C) followed by additional 5 min at 72 °C for complete amplification of all PCR products were used. In each experiment water was used as a negative control.

Quantification of PCR products

Aliquots of 20 µL PCR product were injected on a TSK-gel anion exchange column (DEAE-NPR, 2.5 µm particle size, 35 mm length, 1.6 mL/min maximal flow rate: 200 kg/cm² maximal pressure; Toso Haas, Montgomeryville, PA, USA) in a High Performance Liquid Chromatography (HPLC) system (Beckmann Instruments, Maryland, Columbia, USA). Separation and elution of the two successfully co-amplified PCR products (184 bp and 240 bp) was achieved in a NaCl concentration gradient (flow rate: 1 mL/min), starting at 450 mM NaCl, 25 mM Tris HCl pH 9.0 being elevated to 550 mM NaCl, 25 mM Tris HCl pH 9.0 within 20 min. UV detection was done at 260 nm (UV/VIS Detector LC 295 and Personal Integrator software Model 1020, both Perkin-Elmer/Cetus). Integrating the optical density ($OD_{260\,nm}$) versus the elution time (abscissa), an injection peak, where all the remaining dNTPs and primers were detected (after 0−1 min), a standard IGF-1 peak (~6 min) and a native IGF-1 peak (~8 min) were observed. Both integrals of interest, the IGF-1 cRNA product peak (184 bp) and the native IGF-1 product peak (240 bp) were recorded. Figs. 2a to 2c demonstrate the resolution of the two RT-PCR products derived from liver total-RNA and 3 different cRNA concentrations on the HPLC-UV system. The amount of competitor cRNA yielding equal molar amounts of PCR products gives the initial amount of target mRNA (Siebert and Larrick, 1992). Therefore the yields of the two products were compared by plotting their ratio R (R = competitor PCR product/ wild type PCR product) against the molecule numbers of internal standard template (abscissa) spiked to the RT-PCR reaction. To obtain the IGF-1 mRNA

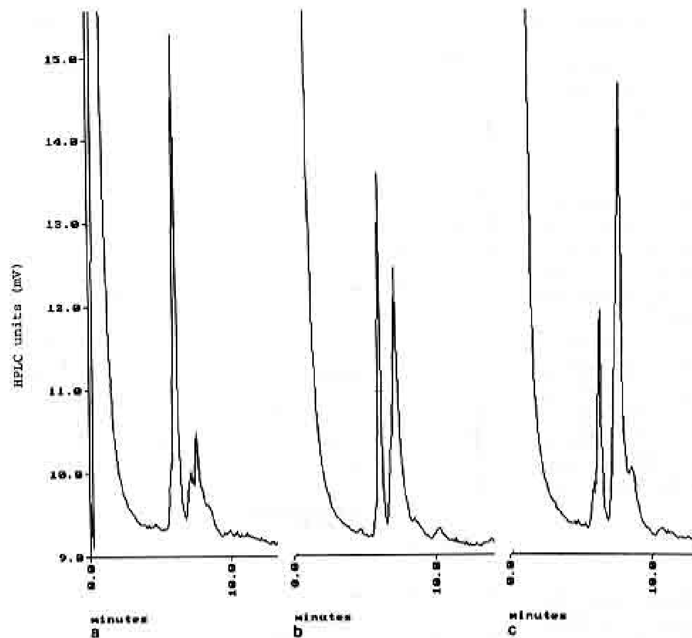

Fig. 2 Resolution of both RT-PCR products of total bovine liver mRNA and at 3 different IGF-1 cRNA concentration on the HPLC-UV system: a 250 ng RNA and $8.0 \cdot 10^9$ IGF-1 cRNA molecules; b 250 ng RNA and $1.6 \cdot 10^9$ IGF-1 cRNA molecules; c 250 ng RNA and $8.0 \cdot 10^8$ IGF-1 cRNA molecules

concentration present in the tissue sample, the ratio plot was extrapolated to where the amounts of target and competitor are equal ($R = 1$) to the x-axis as described by Siebert and Larrick (1992) and Zachar et al. (1993).

Reference quantities

The amount of extractable total RNA per g tissue was recorded as described above. DNA was quantified in 500 mg tissue samples according to the protocol of Bester et al. (1994) using the fluorescent dye 33258 Hoechst (Sigma). Fluorescence was measured in the Titertek Fluoroscan II (Labsystems, Helsinki, Finland) with simultaneous excitation (λ_{ex} = 355 nm) and emission (λ_{em} = 460 nm). DNA sample concentrations were calculated according to a DNA standard curve prepared from calf thymus DNA (Sigma) on each microtitration plate (Nunc) using the Sero Calc® 4.0 windows software (Merlin, Bornheim-Hersel, Germany). To compare the IGF-1 mRNA expression rates in different tissues of potentially varying RNA/DNA ratios, the number of IGF-1 mRNA molecules quantified in 250 ng total tissue RNA was finally related to a diploid genome basis: considering the amount of extractable RNA per g tissue, the number of IGF-1 mRNA molecules was first calculated per g tissue and was then related to the DNA content taking 6.2 pg DNA as a basis for the DNA content of the diploid genome (Cheek and Hill, 1970).

IGF-1 radioimmuno-assay (RIA)

IGF-1 in blood plasma was measured by a binding protein blocked RIA which has been first described by Blum and Breier (1994). In brief, plasma was diluted (1:150) in acidic buffer (20 mM $NaPO_4$ pH 2.8, 0.1 mM NaCl, 0.1% (w/w) BSA, 0.02 Na_3N, 0.1% (v/v) Triton X-100) in order to dissociate IGFs from IGF-binding proteins. The first antibody (878/4, kindly provided by B. Breier, Auckland, New Zealand) together with excess IGF-2 was diluted in a buffer that re-neutralised the pH (100 mM $NaPO_4$ pH 7.8, 0.04 mM NaCl, 0.02% Na_3N, 0.2% (w/w) bovine serum albumin, 0.1% (v/v) Triton X-100) to 1:50000 (working dilution). 0.1 mL of diluted sample or standard (1 to 0.008 ng/0.1 mL; recombinant human IGF-1, Lilly, Indianapolis, Indiana, USA) were incubated together with 0.1 mL of the antibody-IGF-2 solution and 0.1 mL of ^{125}I-IGF-1 (15000–20000 cpm diluted in the re-neutralising buffer) at 4 °C. After 48 h 0.5 mL of the second antibody (sheep-anti-rabbit-serum), diluted 1:75 in 4% (v/v) polyethylenglycol 6000, were added and incubated for 1 h at 4 °C. After centrifugation (3800 rpm, 30 min, 4 °C) the supernatant was discarded and the pellet was washed once in 1 mL cold water and then counted. The

iodination of IGF-1 was done by the Chloramine-T method (Hunter and Greenwood, 1962). The recovery of rhIGF-1 added to the samples at four different concentrations (2, 20, 200 and 400 ng) was 100 ± 7.0% in blood plasma and assay-variation was 9.8%.

Animals and tissue collection

10 male Brown-Swiss calves were randomly allocated to two groups: Group 1 was left intact, whereas the calves in group 2 were castrated within their first week of life. All animals were slaughtered at 12–13 months of age at a final live weight of 356 ± 2 kg (steers) and 401 ± 25 kg (bulls), respectively. Bulls gained more weight per day (1040 ± 69 g/d) than steers did (816 ± 21 g/d; $p < 0.05$). Tissue sampling (liver, m. splenius and m. gastrocnemius) and storage was done as described earlier (Sauerwein et al., 1995). Blood samples for the determination of IGF-1 plasma concentrations were obtained at three different days during the week before slaughter via punction of the *vena jugularis*.

Statistics

All statistical comparisons were done using the Sigma-Stat® statistical software (version 2.0 for Windows 95, Jandel Scientific Software, San Rafael, CA, USA). Initially all group data were tested for normality with the Kolmogorow-Smirnov test. Parametric unpaired t-tests were used for comparisons of two independent groups. For non-parametric testing we used the Mann-Whitney Rank Sum Test. All data are presented as mean ± S.E.M. To test for relationships between parameters, the Pearson correlation procedure was used.

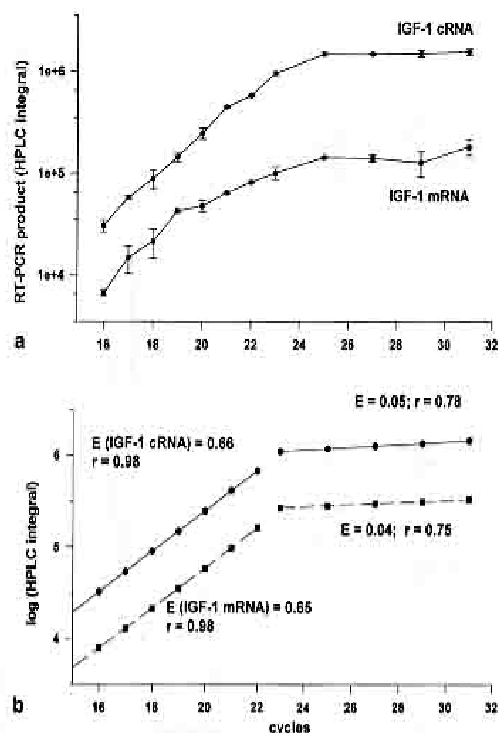

Fig. 3 a Competitive co-amplification of 36 individual RT-PCR reactions, containing 125 ng total bovine liver RNA and $4.5 \cdot 10^9$ molecules IGF-1 cRNA. After the indicated cycle numbers 3 RT-PCR reaction tubes were analysed and plotted versus of the respective HPLC-UV integrals (mean ± SEM); b Regressions were computed for the exponential phase and the plateau phase after transforming the equation $Y_n = A \cdot (1 + E)^n$ (Chelly et al., 1988). Reactions efficiencies were equal in exponential phase of competitive PCR: $E_{(IGF\text{-}1\ cRNA)} = 0.66$; r = 0.98 and $E_{(IGF\text{-}1\ mRNA)} = 0.65$; r = 0.98 and at plateau: $E_{(IGF\text{-}1\ cRNA)} = 0.05$; r = 0.78 and $E_{(IGF\text{-}1\ mRNA)} = 0.04$; r = 0.75

Results

Establishment and validation of the quantitative IGF-1 mRNA RT-PCR

Assay conditions

Considering the described criteria we designed a short internal standard IGF-1 cRNA, for which the same flanking primers are used as for the wild-type IGF-1 mRNA. The conditions for the RT-PCR as described in Materials and Methods were optimised with regard to PCR buffer pH, primer and $MgCl_2$ concentration in the PCR reaction, dNTPs concentration and annealing temperatures. To ensure a parallel start in all individual reaction tubes and to increase specificity, yield and precision of the PCR, a "hot-start" amplification with a melting wax barrier between RT reagents and PCR master-mix was applied. The quantification of wild-type IGF-1 mRNA in different tissues required a preliminary estimation of the IGF-1 cRNA start-molecule concentration range to be used for individual tissues. This was performed by 7 titration steps from $1.6 \cdot 10^8$ to $1.6 \cdot 10^{11}$ cRNA start-molecules versus 250 ng total tissue RNA. For routine comparisons, three standard concentrations covering the range in which equal amounts of the two amplification products are to be expected for a certain tissue were selected.

Amplification efficiencies

The amplification efficiencies for the wild-type and the standard template were recorded during the exponential and the plateau phase of the PCR. Fig. 3a shows the results of the competitive co-amplification for the two amplificates. Until cycle numbers 23–25 there was an exponential increase in the amount of both products, followed by the plateau phase. In order to compare the amplification efficiencies of target IGF-1 mRNA and standard IGF-1 cRNA, the $^{10}\log$ of the HPLC integrals ($^{10}\log Y_n$) was plotted versus

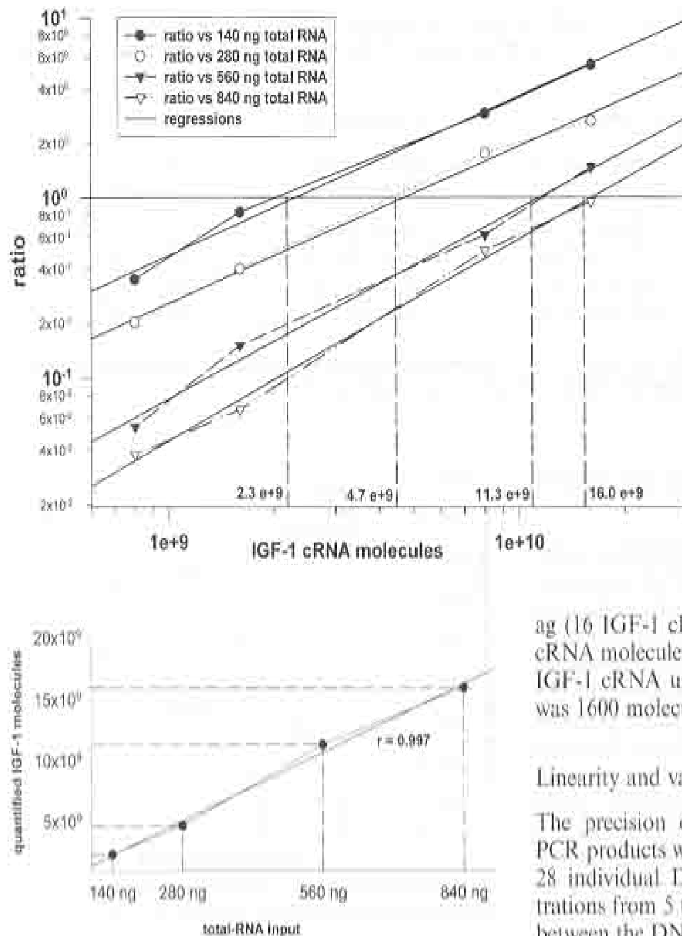

Fig. 4 Quantitative analysis of IGF-1 mRNA concentrations in a dilution series of total bovine liver RNA (140, 280, 560 and 840 ng). The log ratio R between standard PCR product and wild-type PCR product was plotted against the amount of standard cRNA molecules spiked into the four RT-PCR reactions. For each individual RNA input the respective regression lines are included. The given numbers of IGF-1 mRNA molecules present in the different RNA dilutions were read off from the intersection of the individual regression lines with the $R = 10^0 = 1$ line

Fig. 5 Linearity between RNA input into the RT-PCR reaction and the amount of IGF-1 mRNA molecules measured

the number of PCR cycles (abscissa) and the linear regressions were then calculated for the exponential and the plateau phase (Fig. 3b). The relationship $Y_n = A \cdot (1 + E)^n$, in which E is the amplification efficiency of interest, can be transformed to ${}^{10}\log Y_n = n \cdot {}^{10}\log(1 + E) + {}^{10}\log A$, yielding a linear equation: $y = x \cdot a + t$. The resulting efficiencies of competitive IGF-1 RT-PCR during the exponential phase were nearly identical (Fig. 3b; E (IGF-1 cRNA) = 0.66 ; r = 0.98 and E (IGF-1 mRNA) = 0.65 ; r = 0.98). Similarly, during the plateau phase the amplification efficiencies were parallel with E (IGF-1 cRNA) = 0.05 (r = 0.78) and E (IGF-1 mRNA) = 0.04 (r = 0.75). The initial ratio R of the both products remained constant throughout the amplification cycles.

Sensitivity

The sensitivity of the RT-PCR was evaluated using different starting amounts of IGF-1 cRNA from 2.8 ag (16 IGF-1 cRNA molecules) to 28 ng ($1.6 \cdot 10^{11}$ cRNA molecules). The minimal detectable amount of IGF-1 cRNA using the HPLC-UV detection modus was 1600 molecules/tube.

Linearity and variability

The precision of the HPLC-UV quantification of PCR products was initially established by quantifying 28 individual DNA samples at 7 different concentrations from 5 to 325 ng DNA. A linear relationship between the DNA concentration injected (d) onto the DEAE column and the respective peak integral (i) could be demonstrated ($i = 1.13 \cdot d + 3.62$; r = 0.99). The linearity of the RT-PCR was determined by quantifying the IGF-1 mRNA in serial dilutions of a liver RNA preparation (140, 280, 560 and 840 ng). Each RNA dilution was assayed together with four different IGF-1 cRNA standard concentrations. Fig. 4 shows the resulting ratio plots for the four individual RNA input concentrations. The IGF-1 mRNA molecule numbers initially present were read off at R = 1. In Fig. 5 the amount of IGF-1 mRNA molecules (a) measured in the different RNA dilutions is plotted versus the total-RNA input (t) into the RT-PCR assay. A linear relationship between the amount of analyte and the measured IGF-1 mRNA concentration could thus be demonstrated ($a = 2.0 \cdot 10^7 \cdot t + 5.4 \cdot 10^6$; r = 0.997).

To confirm the reproducibility of the competitive IGF-1 RT-PCR, the assay variation was determined: five identical RT-PCR experiments were set up; each with three different standard dilutions and 250 ng liver RNA. Quantification resulted in $1.069 \pm 0.079 \cdot 10^9$ IGF-1 mRNA molecules (n = 5) and thus in an assay variation of 7.4%.

Table 1 DNA concentration per gram tissue (means ± SEM), total-RNA concentration per gram tissue (means ± SEM) and IGF-1 mRNA concentrations (molecules per diploid genome (i.e. 6.2 pg DNA; means ± SEM) in liver and in two skeletal muscles of steers and bulls

		m. Splenius	m. gastrocnemius	liver
DNA	steers	1428.6 ± 94.5 a A	1027.7 ± 37.8 b A	6274.7 ± 333.7 B
(mg DNA/g tissue)	bulls	1344.5 ± 78.2 a A	1093.8 ± 34.7 b A	6976.4 ± 257.7 B
total-RNA	steers	98.8 ± 21.1 a A	30.5 ± 6.0 b A	1067.7 ± 130.9 B
(µg RNA/g tissue)	bulls	124.2 ± 29.0 a A	32.9 ± 4.2 b A	1268.3 ± 186.7 B
IGF-1 mRNA	steers	361 ± 153 a A	214 ± 144 a A	7498 ± 719 B*
(molecules/genome)	bulls	1048 ± 288 b A	272 ± 107 a A	14261 ± 1351 B

The asterisk designates differences between steers and bulls ($p < 0.05$); different small letters designate differences between the two muscles ($p < 0.05$); different capital letters designate differences between liver and the two muscles ($p < 0.001$)

Quantification of DNA, total-RNA and IGF-1 mRNA in bovine tissues

Table 1 summarises the DNA concentration, total-RNA concentration and IGF-1 mRNA expression rates in liver and in two different muscles from steers and bulls. No significant changes in total transcriptional activity (total-RNA/DNA) within different tissue types could be observed. Hepatic IGF-1 mRNA concentrations were higher in intact than in castrated males ($p < 0.05$) and were correlated with the mean IGF-1 plasma concentratioms recorded one week before slaughter (Fig. 6). In bulls higher ($p < 0.01$) IGF-1 mRNA expression was observed in m. splenius than in m. gastrocnemius. In both muscles the difference between bulls and steers did not reach the level of significance ($p = 0.07$ for m. splenius and $p = 0.22$ for m. gastrocnemius, respectively).

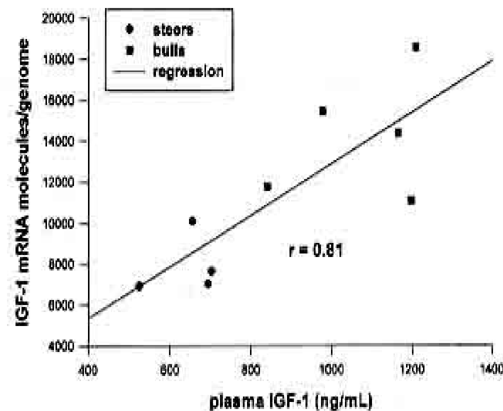

Fig. 6 Correlation of IGF-1 plasma concentrations and hepatic IGF-1 mRNA expression in bulls and steers (n = 9)

Discussion

RT-PCR is a potent and sensitive methodology to detect low amounts of mRNA molecules and offers important insights into the local expression system in low abundant tissues. Using competitive systems with an internal standard, the limitations of quantitative power can be circumvented (Becker-André and Hahlbrock, 1989; Piatak et al., 1993; Martini et al., 1995). The reliability of this approach depends on the condition of identical amplification efficiencies for both, the wild-type and the standard RNA. Wang et al. (1989) postulated that a reliable quantification of PCR products should remain within the exponential phase for both. In the IGF-1 mRNA quantification system described herein a parallel co-amplification of the two fragments could be substantiated, similarly as reported by Bouaboula et al. (1992) and Zimmermann and Mannhalter (1996) for other mRNA species. As demonstrated herein, the ratio between the two products remained constant throughout the amplification, i.e. during the exponential and the plateau phase. Thus the present IGF-1 mRNA quantification is not necessarily limited to the exponential phase of the reaction.

In view of the data provided for sensitivity, linearity and reproducibility, the competitive RT-PCR assay developed herein allows for the absolute and accurate quantification of IGF-1 mRNA molecules with a sufficiently high sensitivity even for tissues or cells with low abundancies or for very small amounts of RNA available.

We have first applied this IGF-1 mRNA quantification system to compare the IGF-1 expression rates in bovine tissues, however, the method can not only be applied in ruminant tissues but also for comparisons in other species with sufficiently high homologies of the amplified IGF-1 fragment. Besides bovine tissues, we have successfully applied this method in porcine tissues (Pfaffl et al., 1998). Considering the close relationship between the hepatic IGF-1 mRNA concentrations and the IGF-1 plasma concentrations, a biological parallelism of IGF-1

mRNA transcription rates and IGF-1 protein translation might be postulated. The two muscles investigated were selected according to their overproportional (m. splenius) or underproportional (m. gastrocnemius) growth response to testicular steroids (Brännäng, 1971). Castration divergently influenced the IGF-1 mRNA expression rates in liver and in skeletal muscles. Comparing the IGF-1 mRNA quantities in the two investigated skeletal muscle, we observed higher concentrations in m. splenius than in m. gastrocnemius in bulls. Total transcriptional activity (RNA/DNA ratio) remained constant within all tissues and the differences in IGF-1 mRNA were specific up- or down-regulations of the IGF-1 gene expression. These findings are in accordance with earlier investigations on the higher growth impetus of m. splenius compared to m. gastrocnemius in bulls (Berg and Butterfield, 1976). The molecular basis for this sexually dimorphic muscle growth pattern might be attributed to relativly higher sensitivities to testicular steroids in neck muscle (Sauerwein and Meyer, 1989). Above that, the present study implies that local differences in IGF-1 expression might be one of the mediators of the allometric growth of these individual muscles in intact males.

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