Quantitation of beta-actin-specific mRNA transcripts using xeno-competitive PCR.

R M du Breuil, J M Patel and B V Mendelow

Genome Res. 1993 3: 57-59

Quantitation of β-Actin-specific mRNA Transcripts Using Xeno-competitive PCR

R.M. du Breuil, J.M. Patel, and B.V. Mendelow

Department of Haematology, School of Pathology of the University of the Witwatersrand, and the South African Institute for Medical Research, Johannesburg, South Africa

PCR is being used increasingly, not just to establish the presence of specific nucleotide sequences, but also to ascertain their quantity. The technique of competitive PCR involves the coamplification of a quantified competitive sequence with the target mRNA. Relative abundance after amplification of the target and competitor is then analyzed using a predefined and exploitable difference between the target sequence and that of the competitor.[1,2] Differences that have been reported previously involve restriction site variations induced by site-directed mutagenesis and incorporation of an intron within the competitor.[3]

In this paper we report a simplified competitive PCR system based on interspecies sequences differences and similarities. The technique has been applied to study quantitative variations in β-actin mRNA within serum-deprived human hepatocellular carcinoma cells (Hep 3B) in response to serum addition. By comparing the β-actin gene nucleotide sequence of the rat with that of the human[4,5] and obtaining a range of consensus sequences, an appropriate set of nucleotide sequences that best fit the criteria for primers and were identical in both species was selected (Fig. 1). By exploiting existing nucleotide differences between the two species, unique restriction sites present in only one of the sequences could be identified (Fig. 1). Two β-actin primers were designed that produced a 289-bp product after reverse transcriptase–PCR (RT–PCR), which upon complete restriction with *Pvu*II yielded two fragments of 132 and 157 bp in rat only (Fig. 1).

RNA was extracted by the acid–guanidium thiocyanate method.[6] Human or rat RNA was submitted to a RT reaction using random hexamers or the downstream primer XAHR17. Upon the addition of the remaining primer, the cDNA was subjected to standard PCR.

Commercial reagents (GeneAmp RNA PCR kit, Perkin-Elmer) and the manufacturer's suggested reaction conditions were employed for the reverse transcription of the mRNA into cDNA. A master mix consisting of 5 mM $MgCl_2$, 1 × PCR buffer II, 0.35 μM dNTPs, 1 U/μl of RNase inhibitor, 2.5 U/μl of RT, and 0.75 μM of the downstream primer (XAHR17) was used. Prior to PCR, ~0.5 μCi per sample of [α-^{32}P]dATP or [α-^{32}P]dCTP was added to enable sample analysis.[1,2,7] PCR was carried out in 50- to 100-μl volume of a master mix containing 2 mM $MgCl_2$, 1× PCR buffer II, 0.15 μM upstream primer (XAHR20), and 0.025 U/μl of AmpliTaq DNA polymerase at a cycle program for

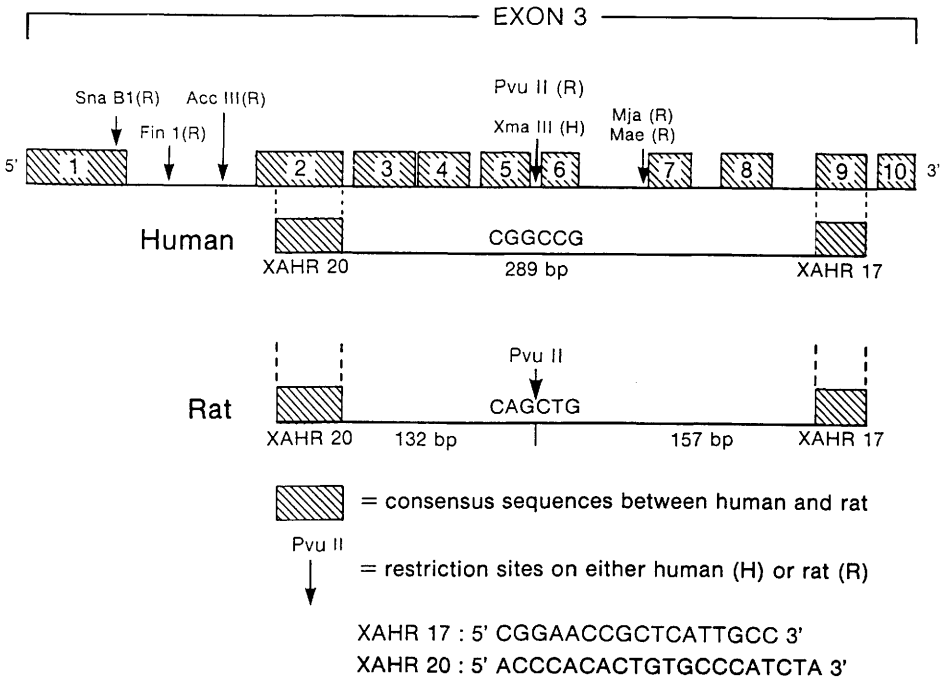

FIGURE 1 Exon 3 of the human and rat β-actin gene showing consensus regions and restriction sites. Consensus sequences 2 and 9 were chosen for primers. A *Pvu*II restriction site is present in the rat sequence.

3:57–59©1993 by Cold Spring Harbor Laboratory Press ISSN 1054-9803/93 $5.00

15–30 cycles of 92°C for 1 min, 54–56°C for 1 min, and 72°C for 2 min, followed by soaking at 15°C. Blank controls were set up with no RNA, rat RNA only, and human RNA only.

The 289-bp PCR product was then restricted with *Pvu*II to give, as predicted, restriction of the parent fragment into the two smaller fragments in the case of the rat sample (Fig. 2, lanes iii and iv), and no restriction occurred in the case of the human sample (Fig. 2, lanes ii and v). *Pvu*II restriction was carried out directly on an aliquot of amplified DNA with 30 units of enzyme at 37°C for ~12 hr.

The resulting reaction mixtures were then run on a 2% agarose gel with ethidium bromide, and the specific bands were excised and the radioactive counts in each determined.[2] Background counts representing the unrestricted rat fragment were subtracted, and the ratio of rat/human determined and plotted.

To demonstrate "linearity of quantitation," a xeno-competitive PCR standard curve was carried out by modification of the approaches described by Gilliland et al.[1,2] In brief, varying volumes (0.25–1 μl) of a standardized stock solution of rat competitor were dispensed into master mixes for reverse transcription and aliquoted into varying but known volumes of human mRNA stock, prepared from a human leukemic cell line (HL-60). The amplification step was subsequently carried out by aliquoting a PCR master mix into the human/rat

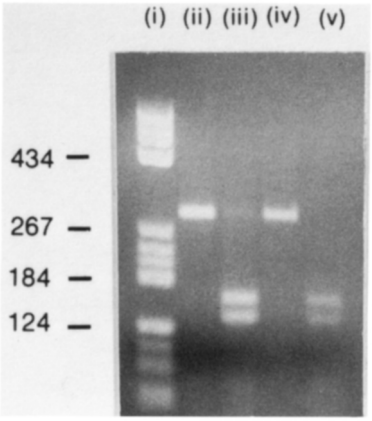

FIGURE 2 A 2% agarose gel showing PCR products after restriction with *Pvu*II. Amplified β-actin fragments of human (lanes *ii* and *iv*) and rat (lanes *iii* and *v*) showing no restriction and restriction, respectively. It is preferable that restriction is carried to completion, as seen in lane *v*, as it would facilitate analysis of the results.

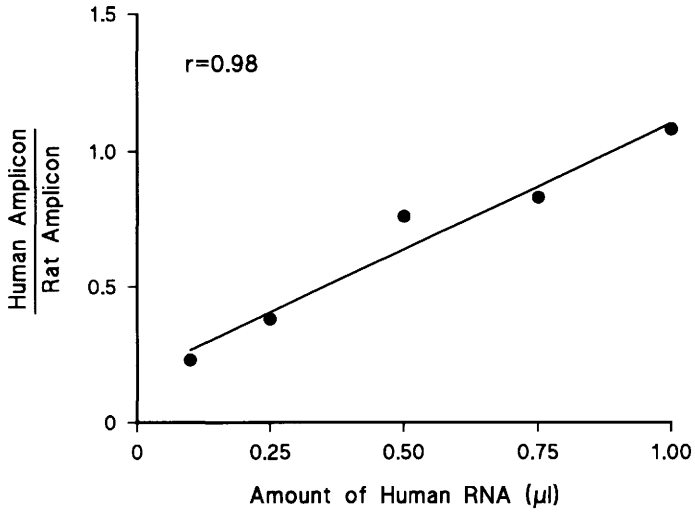

FIGURE 3 Graphic representation of varying volumes of human stock mRNA (μl) reverse transcribed and amplified with 0.25 μl of competitor rat mRNA. Radioactive counts representing the human and rat bands are depicted as human/rat ratios. The ratios which were comparable within, but not among, batches, showed a linear relationship to actual quantities of human RNA. Different batches contained varied volumes of rat competitive stock (r for 0.5 μl of rat stock = 0.95: r for 1 μl of rat stock = 0.99).

cDNA mixture. The ratios of human/rat counts plotted against the increase of human RNA are depicted in Figure 3. Each experiment represented samples run within a single batch, with identical aliquots of rat competitor RNA. For each of the batches analyzed, a linear response of ratios, was obtained for the varying amounts of human mRNA. However, it was not possible to compare ratios among batches. The results suggest that samples within a single batch may be compared with one another with respect to their relative quantities but that the values obtained are not comparable with those obtained from different batches.

To test further the within-batch precision of the technique, tube-to-tube variation was deliberately induced by varying the number PCR cycles for otherwise identical tubes. Four tubes were set up for RT–PCR, each with 0.25 μl of human and 0.25 μl of rat RNA stock solutions, and subjected to 15, 20, 25, and 30 cycles, respectively. A duplicate experiment was carried out using one-tenth the volume of human RNA. At 15 cycles, no PCR product was visible, and it was not possible to obtain accurate results. For 20, 25, and 30 cycles it was found that the coefficient of variation was <30% with high amounts of human RNA (0.25 μl) and <20% with lower amounts of human RNA (0.025 μl).

In experiments designed to apply the system to a real-life example, the postulate that β-actin mRNA is constitutive and unregulated was tested and found to be flawed. The Hep 3B cell line was cultured under serum-deprived conditions (no fetal calf serum for 24 hr), followed by the addition of fresh serum to a concentration of 10%. β-Actin mRNA was then quantified as a function of time after addition of serum, using xeno-competitive PCR (Fig. 4A). All analyses were carried out within a single batch. The results showed a time-related fluctuation in β-actin mRNA, with a rapid initial decline, followed by a gradual increase, presumably reflecting disturbances in the relative rates of transcription and catabolism, after addition of serum. The same result was reproduced in a similar but separate experiment, where Hep 3B cells were cultured in partially serum-deprived conditions (1% fetal calf serum for 60 hr; Fig. 4B). These results were also analyzed within a single batch, in which the volume of human mRNA was deliberately varied by 500-fold with respect to the experiment depicted in Figure 4A, to exclude the possibility of high- and low-dose hook effects. In both cases, within 15 min after serum addition, the β-actin mRNA fell to undetectable or virtually undetectable levels, possibly reflecting translation-related mRNA consumption, and then rose again, presumably reflecting enhanced serum-induced transcription. The conclusion the β-actin mRNA

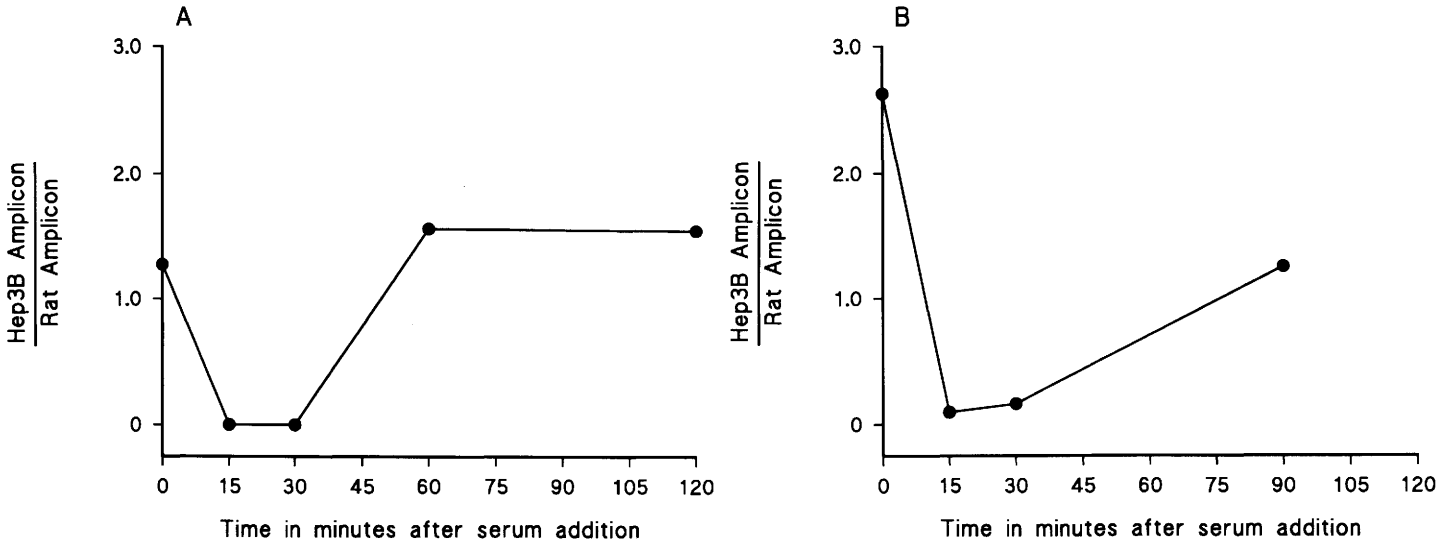

FIGURE 4 (*A*) Quantitative analysis of the effect of 10% fetal calf serum on β-actin mRNA levels in Hep 3B cells precultured under totally deprived serum conditions for 24 hr before serum addition. (*B*) Similar results were obtained using Hep 3B cells partially deprived or serum for 60 hr before serum addition.

is regulated by unidentified factors within serum is in accordance with the work of Jamal and Ziff.[8]

In summary, we have designed a simplified RNA quantitation approach, using xeno-competitive PCR analysis (X-PCR). The similarities between species provided us with the primers, whereas the differences provided us with a restriction site applicable to one species only, enabling us to distinguish one product from the other. We have used this method to quantify β-actin mRNA as an example. The same approach can be exploited with other mRNAs. In this connection we are using X-PCR to quantify variations in erythropoietin mRNA in response to various stimuli.

Variations of this method could use known polymorphic differences among individuals within a species to generate the restriction sites needed for allo-competitive PCR (A-PCR). X-PCR and A-PCR, therefore, alleviate the need for site-directed mutagenesis in a setting where mutations are readily available. Obviously, the same approach could be applicable to specific DNA quantitation.

ACKNOWLEDGMENTS

This work was supported by funding from the South African Institute for Medical Research, the Medical Research Council, and the National Cancer Association.

REFERENCES

1. Gilliland, G., S. Perrin, and H.F. Bunn. 1990. Competitive PCR for Quantitation of mRNA. In *PCR protocols: A guide to methods and applications* (ed. M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White), pp. 60–69. Academic Press, San Diego, CA.
2. Gilliland, G., S. Perrin, K. Blanchard, and H.F. Bunn. 1990. Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction. *Proc. Natl. Acad. Sci.* **87:** 2725–2729.
3. Higuchi, R. 1990. Recombinant PCR. In *PCR protocols: A guide to methods and applications* (ed. M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White), pp. 171–183. Academic Press, San Diego, CA.
4. Nudel, U., R. Zakut, M. Shani, S. Neuman, Z. Levy, and D. Yaffe. 1983. The nucleotide sequence of the rat cytoplasmic β-actin gene. *Nucleic Acids Res.* **11:** 1759–1771.
5. Nakajima-Iijima, S., H. Hamada, P. Reddy, and T. Kakunage. 1985. Molecular structure of the human cytoplasmic β-actin gene: Interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci.* **82:** 6127–6133.
6. Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
7. Dean, M. and B. Gerrard. 1991. Helpful hints for the detection of single-stranded conformation polymorphisms. *BioTechniques* **10:** 331–332.
8. Jamal, S. and E. Ziff. 1990. Transactivation of c-fos and β-actin genes by raf as a step in early response to transmembrane signals. *Nature* **344:** 463–466.

Received January 20, 1993; accepted in revised form June 22, 1993.