BIOCHEMICAL LETTERS

Competitive reverse-transcriptase PCR: a useful alternative to Northern blotting for quantitative estimation of relative abundances of specific mRNAs in precious samples

We have read with interest the report by Zhang et al. [1] describing two variants of competitive reverse-transcriptase PCR (RT-PCR) in which an internal competitor was utilized to quantify, as absolute number of mRNA copies, the levels of α -, β - and γ -fibrinogen transcripts in rat liver lobes. As pointed out by the authors, competitive RT-PCR, in addition to representing the most sensitive method so far available for studying genes expressed at a very low level, must be currently considered the technique of choice for accurate quantification of mRNA copy number [1]. However, a competitive RT-PCR approach may also be exploited for estimating the relative abundances of given transcripts, especially when the availability of very limited RNA amounts does not allow the application of conventional

techniques, such as Northern blotting or ribonuclease protection assays. In this regard, competitive RT-PCR procedures could gain a more general impact by a wider application in those laboratories which frequently deal with fresh biological samples directly obtained from patients through invasive diagnostic procedures (e.g. bone-marrow aspirates, small-needle biopsies) usually leading to small RNA yields.

We have previously demonstrated the constitutive expression of CD30 ligand (CD30L), a membrane glycoprotein with pleiotropic cytokine-like activities, in human haematopoietic malignancies of myeloid and lymphoid origin [2]. The presence of CD30L transcripts and protein was investigated in a wide series of fresh samples from leukaemia/lymphoma patients by standard RT-PCR and immunostaining with specific anti-CD30L monoclonal antibodies [2]. The use of conventional methods for evaluating relative levels of specific transcripts, such as Northern blotting, was possible only in a fraction of cases, being hampered by the lack of adequate quantities of total RNA in other samples.

Here we report the development of a competitive RT-PCR method based on an internal DNA competitor through which

Figure 1 Set-up of competitive RT-PCR strategy by a non-homologous DNA fragment (CD30L_{Comp}) containing specific CD30L primer templates

(A) Kinetics of amplification of CD30L cDNA and CD30L_{Comp} fragments. Constant amounts (0.1 amol) of CD30L cDNA and CD30L_{Comp} fragments were amplified in a single reaction tube with specific CD30L primers. After 23 amplification cycles and after each of seven additional cycles, a 10 μ l aliquot of the reaction mixture was removed and the products resolved on agarose gel (upper panel). The relative intensities of the bands corresponding to CD30L cDNA (689 bp) and CD30L_{Comp} (475 bp) amplified products were quantified by computer imaging. The amount of specific amplified products for CD30L cDNA ($\circ$) and CD30L_{Comp} ($\bullet$), expressed in arbitrary units (A.U.), was plotted as a function of the number of cycles (lower panel). Equations of regression lines were $y = 1224x - 26095$ ($r = 0.98$, CD30L) and $y = 1113x - 21466$ ($r = 0.97$, CD30L_{Comp}). (B) Determination of relative levels of CD30L mRNA in DG-75 cells by competitive RT-PCR. Tenfold serial dilutions ($100-1 \times 10^{-4}$ amol) of CD30L_{Comp} were amplified with CD30L primers together with constant aliquots (2 μ l) of cDNA from the DG-75 cell line. After 35 cycles, amplified products (10 μ l/lane) were resolved on agarose gel (upper panel). Relative intensities of the bands were densitometrically determined and the logarithm of their ratios was plotted as a function of the logarithm of the amount of CD30L_{Comp} added (lower panel). The equivalence point (arrows) was inferred to be at about 10^{-1} amol. Sequences of CD30L primers and amplification conditions have been reported previously [2].

Figure 2 Expression of CD30L in lymphoid malignancies

Expression of CD30L in malignant cells from lymphoid malignancies [1, B lineage acute lymphoblastic leukaemia (ALL); 2, T-ALL; 3, B chronic lymphocytic leukaemia; 4, hairy-cell leukaemia; 5, low-grade non-Hodgkin's lymphoma NHL; 6, high-grade NHL; 7, the MN-60 NHL cell line] as assessed by Northern-blot analysis (upper panels) and competitive RT-PCR (lower panels). Upper panels: the Northern blot was carried out as previously reported [2]. Briefly, 10 µg/lane of each RNA sample was size-fractionated on 1% agarose gels containing 6.7% formaldehyde and subsequently blotted on to nylon membranes. Filters were hybridized in 1.0 M NaCl and 1% (w/v) SDS at 68 °C with 1.0×10^6 c.p.m./ml of random primed-labelled CD30L probe [2], washed to a final stringency of $0.2 \times$ standard sodium citrate and 0.1% (w/v) SDS at 65 °C and exposed to XAR-5 films at -80 °C. Bands corresponding to CD30L-specific transcripts were quantified by computer imaging of gel, expressed in arbitrary units (A.U.) and plotted as a histogram (black bars). Hybridization with a housekeeping gene (that of glyceraldehyde-3-phosphate dehydrogenase) and quantification of bands with computer imaging demonstrated less than 10% of differences in RNA loading (not shown). Lower panels: for competitive RT-PCR, 2 µl of cDNA for each sample was amplified with primers specific for CD30L [2] in the presence of a constant amount (10^{-2} amol) of CD30L_{comp} fragment. Following amplification, 10 µl of PCR products was resolved on 1.5%-agarose gel. For evaluation of relative abundances of CD30L transcripts, ratios of the relative intensities of bands corresponding to CD30L cDNA (689 bp) and CD30L_{comp} (475 bp) amplified products were quantified by computer imaging of the gel, expressed in arbitrary units (A.U.) and plotted as a histogram (hatched bars) after correction for the difference in size. cDNAs were always tested with β -actin-specific primers [2] as a check for first-strand synthesis (results not shown).

estimates of the relative abundances of CD30L transcripts in fresh samples from leukaemia/lymphoma patients were obtained utilizing a total RNA amount at least 100-fold lower than that required for other more conventional procedures.

For this purpose, an internal competitor (CD30L_{comp}), of a size different from that of the CD30L-specific amplicons (689 bp) [2], was prepared from an unrelated DNA fragment engineered to contain specific CD30L primer templates [3–5]. In particular, a *Bam*HI/*Eco*RI *v-erb B* DNA fragment (Clontech Laboratories Inc., Palo Alto, CA, U.S.A.) was first amplified with composite

primers containing both CD30L and *v-erb B*-specific sequences, and then with CD30L-gene-specific primers alone. This procedure gave rise to a 475 bp non-homologous DNA fragment (CD30L_{comp}) containing at its ends the appropriate templates for CD30L primers. CD30L_{comp} fragments, when amplified with CD30L-specific primers, yielded a band (475 bp) which was easily discriminated from the 689 bp CD30L-related amplicon on 1.5%-agarose/ethidium bromide-stained gels.

The next step was to verify whether CD30L_{comp} could actually act as an optimal competitor for CD30L amplicons. To do this, a comparison between the amplification kinetics of purified CD30L amplicons (obtained from the DG-75 cell line expressing high amounts of CD30L mRNA [2,6]) and CD30L_{comp} was performed by amplifying, in the same tube, 0.1 amol of each fragment, previously purified and quantified, removing 10 µl aliquots of reaction mix after 23–30 cycles, and analysing them separately on agarose gels (Figure 1A). A stepwise increase of specific amplified products corresponding to CD30L (689 bp) and CD30L_{comp} (475 bp) was observed between 23 and 30 amplification rounds (Figure 1A, upper panel). Quantification of CD30L- and CD30L_{comp}-specific bands with a gel analyser (Gel Doc 1000; Bio-Rad Laboratories, Hercules, CA, U.S.A.) resulted in two regression lines with comparable slopes (Figure 1A, lower panel), indicating a similar amplification efficiency for both fragments.

To determine the optimal amount of CD30L_{comp} for further studies, 10-fold serial dilutions of CD30L_{comp} (100 – 1.0×10^{-4} amol) were amplified together with constant aliquots (2 µl) of cDNA from the DG-75 cell line used as a positive control for CD30L transcripts [2,6] and resolved on agarose gels (Figure 1B, upper panel). Relative intensities of the bands were densitometrically quantified by computer imaging, expressed as arbitrary units (A.U.) after correction for the size difference between CD30L and CD30L_{comp}, and the base-10 logarithms of their ratios were plotted as a function of the logarithm of the amount of CD30L_{comp} added (Figure 1B, lower panel). This plot was used to determine the equivalence point, that is, the point where the logarithm of the ratio of CD30L to CD30L_{comp} is equal to 0, i.e. the amount of CD30L is equal to the amount of CD30L_{comp}. In Figure 1B, the equivalence point was inferred to be close to 10^{-1} amol (arrows). Owing to the abundance of CD30L-specific RNA in the DG-75 cell line [2,6], the use of a CD30L_{comp} amount about an order of magnitude lower than the equivalence point (i.e. 10^{-2} amol) was also judged to be optimal for detecting CD30L transcripts in samples with a low expression rate.

For studies of competitive RT-PCR we have selected six patient samples (lymphoid leukaemias and lymphomas) and the lymphoma cell line MN-60, on the basis of their different levels of CD30L mRNA expression, as shown by Northern-blot analysis (Figure 2, upper panels). Total RNA (1.0 µg) was reverse-transcribed by avian-myeloblastosis-virus RT (Promega Co., Madison, WI, U.S.A.) in a 20 µl reaction mix containing 0.4 µg of hexadeoxyribonucleotide random primers (Promega Co.) for 1.0 h at 42 °C. Constant amounts of CD30L_{comp} (10^{-2} amol) were amplified by CD30L specific primers in the same tube together with 2 µl of reverse-transcribed cDNA from the experimental cell samples, in a final 50 µl volume of reaction mixture. After resolution on agarose gels, band intensities were quantified by computer imaging and the relative A.U. ratios were calculated (Figure 2, lower panels). Differences in these ratios indicated the relative differences in mRNA levels among the different samples (Figure 2, lower panels). As indicated in Figure 2, totally overlapping results were obtained by analysing the expression levels of CD30L, as detected by our competitive RT-PCR approach and a conventional Northern-blot analysis. The

highest amount of CD30L transcripts was detected in B-lineage acute lymphoblastic leukaemias and high-grade non-Hodgkin's lymphoma, as well as in the MN-60 cell line (Figure 2). Accordingly a correlation curve obtained by comparing densitometric quantifications of Northern-blot and competitive RT-PCR results yielded an r value of greater than 0.98.

Our results demonstrate that a competitive RT-PCR method, in addition to being usefully employed to precisely determine mRNA copy numbers, may be also successfully utilized for estimating relative abundances of specified transcripts in different samples, yielding results as reliable as a conventional Northern blot. More importantly, however, whereas Northern-blot results were obtained by loading 10 μ g of total RNA/lane, in the case of competitive RT-PCR each experimental point was carried out by amplifying 2 out of 20 μ l of cDNA, roughly corresponding to at least a 100-fold lower (0.1 μ g) amount of total RNA. This advantage makes competitive RT-PCR an ideal technique for investigating relative levels of specific transcripts in 'precious' samples consisting of a limited cellular amount. These may include biopsy material, highly purified bone-marrow progenitors and *in vitro*-grown haematopoietic colony-forming cells. In particular, competitive RT-PCR may turn of great value in detecting gene overexpression in small biopsy fragments for diagnostic procedures, e.g. cyclin D1 in mantle-cell lymphoma [7], *c-erb B-2* or cyclin D1 in breast cancer [8] and multidrug-resistance genes in different tumours [9]. In addition, a competitive RT-PCR approach has been successfully employed for monitoring minimal residual disease after bone-marrow transplantation in chronic-myeloid-leukaemia patients [5].

Several RT-PCR approaches have been so far reported, some involving a titration of cDNA or amplification kinetics in the absence of internal standard, others utilizing a co-amplification of target and control genes to gain some form of quantification of the amplified products [10,11]. However, problems of poor reproducibility, as well as concern about their value as truly quantitative methods, have hampered a wider application of this technique for screening analysis in a clinical setting. In contrast, in the case of competitive RT-PCR, as described here and by others [3–5], a precise set-up of validation procedures, along with a careful choice of the optimal dilution of competitor, may

definitely eradicate these problems, eventually producing an assay, straightforward to apply and alternative to Northern blotting, for gene-expression studies when 'precious' samples are involved [2,7–9,12].

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