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A rapid procedure for the quantitation of low abundance RNAs by competitive reverse transcription-polymerase chain reaction

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During the last years, the extensive utilization of the reverse transcription-polymerase chain reaction (RT-PCR) technique has allowed the specific detection of several RNA molecules, both of cellular and viral origin, in biological samples of almost any source. However, the precise quantitation of such molecules by conventional RT-PCR is quite difficult, since the yield of both the amplification (1–3) and the reverse-transcription (4) steps can be grossly variable in different reactions.

Among the several strategies proposed for RNA quantitation, the competitive RT-PCR procedure, which involves the simultaneous reverse transcription and amplification of a competitive RNA template, appears to be the most appropriate one (5–10). Recently, we have developed a method for the construction of competitive templates for the quantification of low amounts of DNA molecules (9, 11, 12). In this report, we describe a modification of this method for the construction of RNA templates to be used as competitors in RT-PCR experiments, and the utilization of these competitors for the quantitation of the human gp91-*phox* mRNA. This transcript encodes for the large subunit of a multicomponent NADPH oxidase which is absent or defective in patients with X-linked chronic granulomatous disease (X-CGD), a rare inherited disease resulting in the inability of phagocytic cells to generate O_2 with consequent recurrent severe infections (13,14). The gp91-*phox* mRNA is expressed at physiological levels exclusively in terminally differentiated myelomonocytic cells (phagocytes); even in these cells, its very low abundance renders its quantitation in peripheral blood samples very arduous by conventional techniques.

Competitor RNAs for each competitive RT-PCR experiment were obtained by *in vitro* transcription from DNA fragment templates having the same sequence as the final amplification products except for the addition of 20 bp extra in the middle and of a 5′ extension on the coding strand corresponding to the T7 RNA polymerase promoter sequence; these DNA templates were obtained by the recombinant PCR procedure (9) described in Figure 1. Three competitors were constructed, corresponding, respectively, to adjacent exons of the 5′ end (primer set CGD 1-2) and 3′ end (primer set CGD 3-8) of the gp91-*phox* mRNA coding region and of the β-actin mRNA; the first two were chosen to provide two independent quantitations of the same transcript in order to validate the method; the last was utilized as a measure of the amount of total mRNAs in each sample.

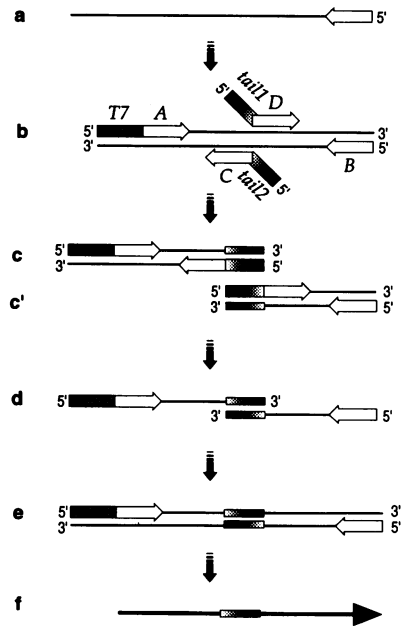

Figure 1. Construction of competitors for competitive RT-PCR. Each competitor was obtained starting from an RT-PCR product obtained by RT with primer B (step a) and amplification with primers A and B (step b), by a modification of an already published procedure (9). Using this product as template, two separate amplifications were carried out (steps c and c′), using, respectively, a modified primer A carrying at its 5′ end the recognition sequence for T7 RNA polymerase (T7 tail) plus primer C carrying at its 5′ end the 20 nt sequence indicated as tail2, and with primer B plus primer D carrying at its 5′ end the 20 nt sequence indicated as tail1. The sequences of tail1 and tail2 are unrelated to the template and complementary each other. The resulting PCR products were recovered, mixed, denatured, renatured (step d) and amplified by the addition of the external primers T7/A and B (step e). The final DNA product was transcribed with T7 RNA polymerase (step f).

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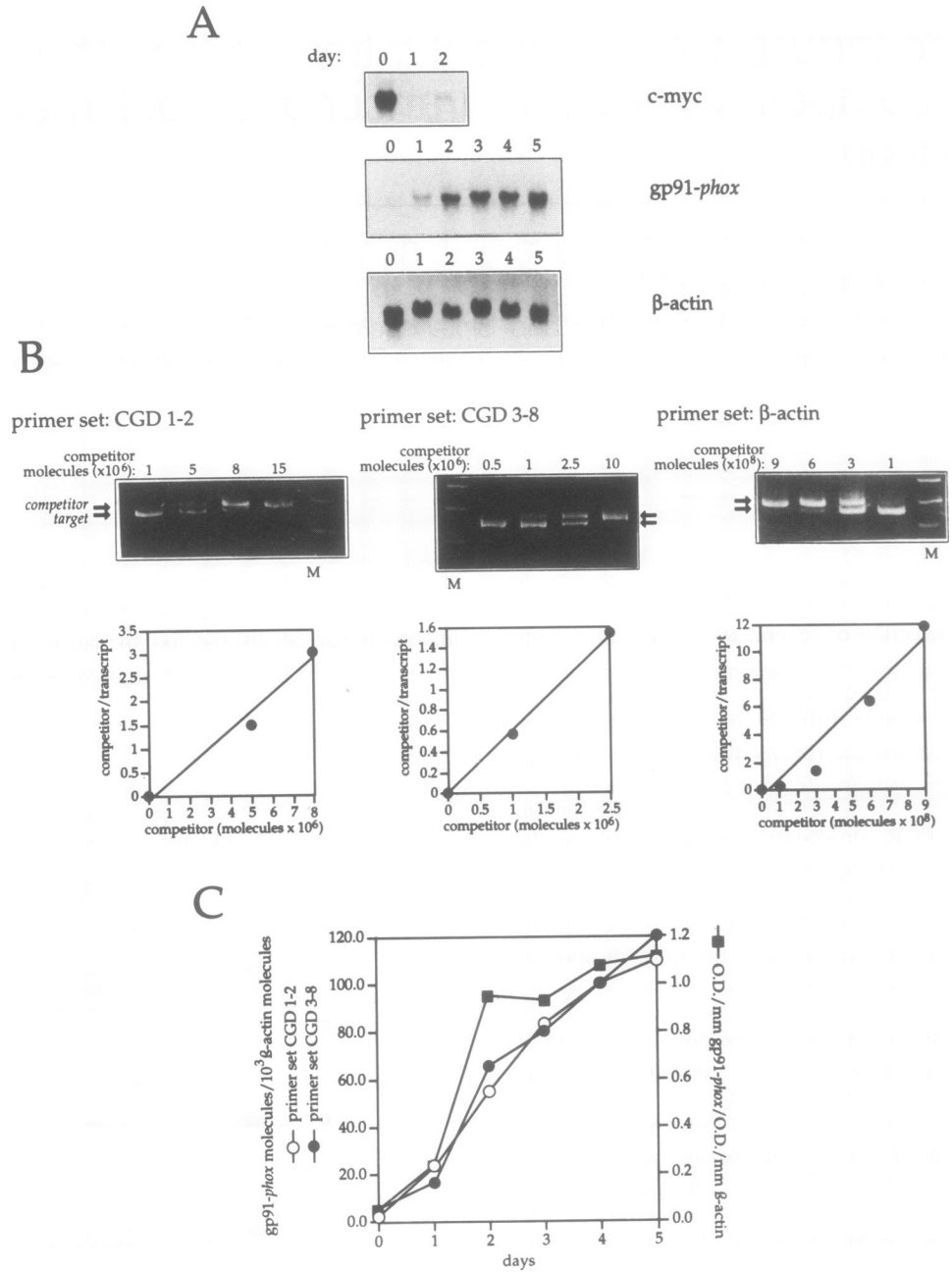

Figure 2. Quantitation of gp91-*phox* in differentiating HL-60 cells. **A.** Northern blotting. Ten μg of total cellular RNA of HL-60 cells extracted at days 0–5 after induction of differentiation was hybridized to a gp91-*phox* cDNA probe (a *PstI–SacI*, fragment from plasmid PKS-CGD, a kind gift of M.C.Dinauer), to a β-actin probe (the RT-PCR product obtained with primers BA1 and BA4), and to a probe for the c-*myc* gene (a 1500 bp *Eco*RI DNA fragment, including the first and second exons). **B.** Competitive RT-PCR. A fixed amount of the RNA sample from HL-60 cells at day one after induction of differentiation was mixed with increasing amounts of competitors (as indicated on top of the gels) and submitted to RT-PCR; the amplification products were resolved by polyacrylamide gel electrophoresis and stained with ethidium bromide (indicated by arrows). For each amplification, the ratio between the intensities of the bands (evaluated by densitometric scanning) was plotted against the amount of competitor added (lower part). As expected, the points are fitted by a straight line emanating from the origin. M indicates the molecular weight marker lane. **C.** Results of quantitation. The amount of molecules of gp91-*phox* mRNA detected by two independent quantifications (primer sets and competitors CGD 1-2 and CGD 3–8) in RNA samples from HL-60 cells at different days of differentiation are expressed per 10^3 molecules of β-actin mRNA (scale on the left side). For comparison, the results corresponding to the ratio between the intensity of the bands of gp91-*phox* and β-actin of the blot of panel A are shown (scale on the right side).

Human myeloid HL-60 cells were treated for five days with retinoic acid and dimethylformamide (15); under these conditions, cells stop dividing, switch off transcription of the c-*myc* gene and start expressing high levels of the gp91-*phox* mRNA (Northern blots in Figure 2 panel A). The amount of the gp91-*phox* mRNA in the same RNA samples utilized for the Northern blots was determined by competitive RT-PCR. Increasing amounts of competitor RNA were added to the RNA sample to be quantified, and submitted to RT-PCR. The reaction products were resolved by gel electrophoresis, stained with

ethidium bromide, and quantified by densitometric scanning. An example of the procedure adopted is shown in Figure 2 panel B for the RNA at day 1. According to the principles of competitive PCR (9, 10), when the ratio between the amounts of amplification products of competitor and template is plotted against the amount of competitor added, the experimental points are fitted by a straight line; as a consequence, the number of molecules of competitor corresponding to a 1:1 ratio is equivalent to the number of molecules of input RNA in the sample. The results of the quantitation of the gp91-*phox* mRNA, are shown in Figure 2 panel C. The amounts of this transcript, which is several hundred fold less represented than the β-actin mRNA, increase linearly of more than 50 fold up to day 5 after induction of differentiation. The independent quantitation of the same transcript with the two different competitors gives equivalent results.

Competitive RT-PCR has several well established advantages over other methods for RNA quantitation utilizing RT-PCR (8–10), in particular, being the quantification procedure based on the calculation of the ratio between the amounts of competitor and target products, the technique is unaffected by the overall yield of either the RT or PCR steps, allows the experimenter to reach the plateau phase of amplification, and is insensitive to the formation of aspecific amplification products.

The limiting step in the development of a competitive RT-PCR assay, however, is represented by the construction of competitive RNA templates. To this purpose, the method we have developed is easy, does not require the utilization of complex mutagenesis protocols, does not require cloning of template DNA, and is extremely reproducible (according to this method, we have so far constructed over 30 different competitors for DNA and RNA quantitation). Finally, since the competitor RNAs obtained differ in size from the targets, the resulting amplification products can be simply resolved by gel electrophoresis and detected by ethidium bromide staining, avoiding the need for complex separation or internal hybridization procedures, in contrast to other competitive methods (16,17).

METHODS

Five oligonucleotides were synthesized for each amplification set (generically named A, T7-A, B, tail1-D, and tail1-C in Figure 1). For amplification set β-actin, oligo A corresponds to nt 1201–1220 of the sequence in the GenBank file *humaccyba*, oligo B to nt 1560–1541, oligo C to nt 1279–1260, and oligo D to nt 1414–1433. For set CGD 1-2, oligo A corresponds to nt 32–51 of the sequence in file *humxcgd*, oligo B to nt 311–292, oligo C to nt 120–101, and oligo D to nt 121–140. For set CGD 3-8, oligo A corresponds to nt 1588–1607 of the sequence in file *humxcgd*, oligo B to nt 1932–1913, oligo C to nt 1660–1641, and oligo D to nt 1680–1661. The sequences of the 5′ extensions are: 5′-CGGGATCCGGATCC-TAATACGACTCACTATAGGGAGA-3′ for T7, 5′-ACCTGC-AGGGATCCGTCGAC-3′ for tail1, and 5′-GTCGACGGAT-CCCTGCAGGT-3′ for tail2. Primers A and B of each set were chosen in different exons in order to avoid amplification from contaminating DNA template.

Competitor RNA fragments were obtained as shown in Figure 1 by run-off transcription of 40 ng of DNA template using a T7-RNA polymerase-based *in vitro* transcription kit (Promega, Madison, WI); two μl of [^{32}P]UTP (Amersham, UK; 3000 Ci/mmol; 10 mCi/ml) were included in the reaction. After completion of the reaction, the template DNA was removed either by RNase-free DNase I (Pharmacia, Uppsala, Sweden) digestion or by elution of the RNA fragment after resolution by denaturing gel electrophoresis. The amount of competitor was calculated according to the final specific activity of the incorporated UTP. The absence of contaminating DNA from the competitor preparation was estimated by assembling a control reaction with omission of the RT step.

The competitive RT-PCR procedure was as follows: a fixed amount of each sample was added to increasing amounts of each competitor species, and the mixture was reverse transcribed by extension of the antisense primer B and amplified with primers A and B. PCR cycle profiles were the following: 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, 40 repetitions. The amplification products were resolved on a 8% polyacrylamide gel, stained with ethidium bromide, and quantified by densitometric scanning.

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