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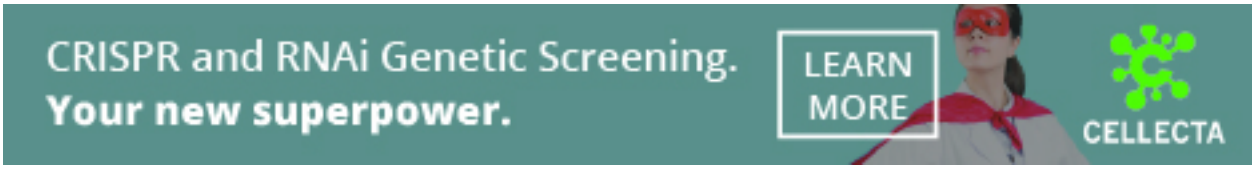

Generation of Competitor DNA Fragments for Quantitative PCR

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A convenient and generally applicable method for the generation of competitor DNA fragments for quantitative PCR is described. Using mouse-specific primers, fragments are amplified from DNA of an evolutionarily distantly related species under low-stringency annealing conditions. Because these artificially created fragments contain the mouse primer specific ends, they can be used for the quantification of the mouse DNA amplified by these primers. Competitor DNA fragments that differ in size from the corresponding mouse DNA are selected to distinguish both fragments visually by gel electrophoresis. Competitor DNA fragments were generated for mouse β-actin, interleukin-1, and tumor necrosis factor (TNF). Co-amplification of β-actin cDNA for adjustment of equal amounts of input cDNA and subsequently TNF cDNA from lipopolysaccharide (LPS)-activated and nonactivated spleen cells with serial dilutions of the respective competitor DNA fragments allowed a semiquantitative comparison of the ratio of TNF mRNA present in both cDNA samples. Under certain conditions, the competitor DNA fragments can be used to determine the approximate molar concentration of a mRNA.

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Due to its high sensitivity, the polymerase chain reaction (PCR) allows the detection of rare mRNA species. Because the amplification of reverse-transcribed mRNA usually does not allow the amount of a given mRNA species to be deduced, methods must be developed to quantify the level of transcription. The most reliable approach for quantifying specific mRNA species by PCR consists of co-amplification of the target cDNA and a control fragment, which is flanked by the primer-specific sequences of the target cDNA.[1] Co-amplification of an internal standard serves as a control for comparing the efficiency of the PCR in different reactions. Since the target cDNA and the control fragment compete for the same primers, the amount of amplification product of either fragment reflects the ratio of input DNA. During the amplification, the molar ratio of the target cDNA and the competitor DNA fragment of known concentration should remain constant. To determine the weight ratio and, therefore, to deduce the molar ratio of the amplified products on an agarose gel, the amplified cDNA and the competitor DNA fragment must differ in size. Such competitor DNA fragments can be obtained by mutagenesis of cloned cDNA fragments,[1] by isolation of intron-containing genomic fragments,[1] or by synthesis of appropriate DNA fragments.[2] This paper describes a convenient alternative approach for the generation of competitor DNA fragments and their application.

METHODS

DNA and RNA extraction

Genomic chicken liver DNA was isolated by standard procedures.[3] Cytoplasmic RNA was extracted from non-stimulated or lipopolysaccharide (LPS)-stimulated BALB/c spleen cells (1×10^6 cells/ml, 10 μg LPS/ml, for 4 hr in Click's RPMI-1640 containing 10% FCS, 2 mM glutamin, 100 units of penicillin/ml, and 100 μg streptomycin/ml). A total of 5×10^6 to 2×10^7 cells were lysed in a solution containing 10 mM Tris (pH 7.5), 10 mM NaCl, 1.5 mM $MgCl_2$ in the presence of 50 μl of ribosyl complexes (prepared as described in ref. 3), and 0.5% Triton X-100 in a volume of 1 ml. Nuclei were pelleted by centrifugation at 10,000*g* for 5 min at 4°C. SDS and EDTA were added to the supernatant at final concentrations of 0.5% and 5 mM, respectively. Proteins were removed by phenol, phenol/chloroform, and chloroform extraction. Subsequently, the RNA was ethanol-precipitated and resuspended in 500 μl H_20. The RNA was precipitated by LiCl at a final concentration of 2 M at −20°C for 12 hr. After centrifugation (10,000*g*, 5 min at 4°C), the RNA was resuspended in 500 μl of H_20, ethanol-precipitated, and resuspended in 20 μ1 of H_20, and an aliquot was analyzed on an agarose gel. RNA (2.5 μg) was reverse-transcribed in a volume of 50 μl using Superscript Reverse Transcriptase (GIBCO/BRL) and oligo(dT) as primer following the supplier's recommendations. Whenever possible, reagents were DEPC treated.

PCR

For the generation of the competitor DNA fragments, the PCR was performed in a volume of 100 μl containing 1x reaction buffer (Perkin-Elmer, Norwalk,

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CT), 1 μg of genomic chicken DNA, 0.5 μM primers, 200 μM of deoxynucleotides (dATP, dCTP, dGTP, dTTP), and 2 units of *Taq* polymerase (Perkin-Elmer). The mouse interleukin-1 (IL-1), tumor necrosis factor (TNF), and β-actin primer sequences were taken from ref. 4. Amplification occurred at the following conditions in a Perkin-Elmer Thermal Cycler: (1 min, 94°C; 2 min, 37°C; 2 min, 72°C) for three cycles followed by 35 cycles (1 min, 94°C; 2 min, 60°C; 2 min, 72°C). After agarose gel electrophoresis, fragments of appropriate size were excised and the gel slices were dissolved in NaJ in a final concentration of 4.5 M. A 1-μl amount of a 1:10,000 dilution was amplified again, size-separated by gel electrophoresis, and purified by Gene Clean (Bio 101 Inc., La Jolla, CA). DNA concentrations were determined by ethidium bromide staining in comparison to dilutions of DNA of known concentration. The IL-1 cDNA fragment was generated by amplification of cDNA de-rived from LPS-stimulated spleen cells with the IL-1 primers and purified as described above.

Competitive PCR

The competitive PCR was performed in a volume of 50 μl containing 1x reaction buffer, 5–10 ng of reverse-transcribed RNA, different amounts of competitor DNA fragment, 0.2 μM of primers, 200 μM deoxynucleotides (dATP, dCTP, dGTP, dTTP), and 1 unit of *Taq* polymerase. Amplification occurred during 35 cycles (1 min, 94°C; 2 min, 60°C; 2 min, 72°C). One-fifth of the PCR products were analyzed on 1.5% agarose gels containing 0.1 μg/ml ethidium bromide. Size markers were the 1-kb ladder or the 123-bp ladder from GIBCO BRL.

RESULTS

Generation of Competitor DNA Fragments

Amplification of mouse IL-1 cDNA with the IL-1–specific primers results in a 308-bp PCR product.[4] As a competitive template, we wished to obtain a fragment differing slightly in size from that of the cDNA amplification product. Therefore, the genomic DNA of a distantly related species (in our case chicken) was amplified by the mouse IL-1 primers. During the first three cycles of the PCR, the low annealing temperature (37°C) allowed multiple, presumably unspecific, priming, which became visible after a further 35 cycles (annealing temperature 60°C) (Fig. 1, lane 2). A fragment of about 240 bp was excised from the gel and amplified further, yielding a fragment of the same size. The fragment was purified (Fig. 1, lane 3) and, subsequently, it could be used as a competitive template, since the amplified chicken DNA fragment is flanked by the mouse IL-1 primers. To exclude the possibility that the competitor DNA fragment is flanked by one of the primers at both ends, the competitive template was either amplified by both primers (Fig. 1, lane 4), or each primer alone (lanes 5 and 6). Similarly, competitor DNA fragments were prepared for mouse β-actin and mouse TNF cDNA.

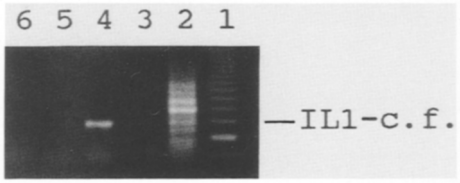

FIGURE 1 Analysis of amplification products obtained by cross-species PCR with mouse IL-1 primers and genomic chicken DNA. (Lane *1*) 1 μg 123-bp ladder size marker; (lane *2*) equivalent of 0.2 μg of genomic chicken DNA amplified under low-stringency annealing conditions; (lane *3*) purified IL-1 competitor DNA fragment (IL-1-c.f.); (lanes *4–6*) PCR in the presence of 1 fg of competitor DNA fragment and either both primers (lane *4*), or sense (lane *5*) and anti-sense (lane *6*) primers alone.

Semiquantitative Analysis

To demonstrate the usefulness of the competitor DNA fragments for determining the relative amount of a mRNA within two cell populations, we wanted to compare the TNF mRNA levels in nonstimulated versus activated spleen cells. Therefore, RNA was extracted from untreated spleen cells and from spleen cells stimulated for 4 hr with lipopolysaccharide (LPS). Similar amounts of RNA were reverse-transcribed. To compare identical amounts of cDNA, the cDNAs were adjusted to equal concentrations using the β-actin cDNA for calibration. For this purpose, the amount of cDNA required to obtain equal amounts of PCR products for the β-actin control fragment and β-actin cDNA in the presence of the same amount of input control fragment was determined in a competitive PCR for both cell populations. Tenfold serial dilutions of the competitor β-actin fragment were co-amplified with equal amounts of cDNA. After identifying the order of magnitude of the amount of β-actin competitor DNA fragment needed to obtain roughly equal amounts of the amplified fragments, a second PCR with 2- to 2.5-fold dilutions of the cDNA in the presence of identical amounts of the competitor DNA fragment was carried out to determine carefully the amount of cDNA required to obtain equal amounts of PCR products. A twofold higher concentration of the cDNA from LPS-induced cells as compared to the control cDNA is needed to achieve equal band intensities of the PCR products (Fig. 2A, arrows). Thus, this analysis allows comparison in subsequent experiments of precisely the same amounts of cDNA. For quantification of the TNF mRNA, serial 10-fold dilutions of the TNF competitor DNA fragment were co-amplified with equal amounts of the adjusted cDNAs. The TNF-cDNA of the LPS-stimulated cells competes for amplification with the control fragment at a 10-fold higher concentration of TNF control fragment as compared to the TNF-cDNA of nonstimulated cells. Therefore, LPS induces approximately a 10-fold increase of TNF-mRNA in spleen cells.

Quantitative Analysis

Although in most experimental situations the knowledge of the relative amount of a mRNA compared between two cDNAs may be sufficient, sometimes the number of mRNA molecules within a defined cell population may be of interest. The control fragment may be used for this purpose, once it has been shown that during amplification the molar ratio of competitor DNA fragment and target DNA remains constant.

To analyze whether IL-1 cDNA and control fragment are stoichiometrically

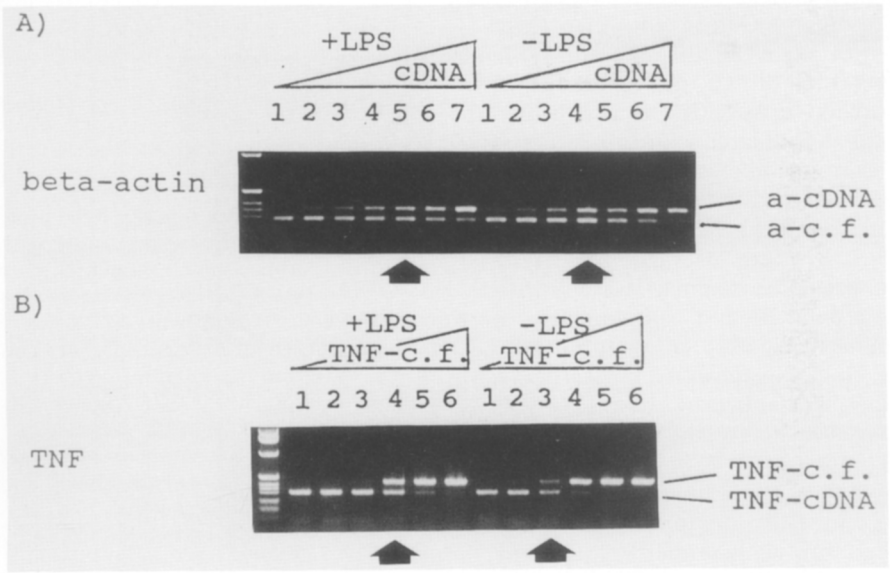

FIGURE 2 Determination of TNF mRNA levels in LPS-stimulated versus nonstimulated spleen cells by semiquantitative PCR. (*A*) Adjustment of cDNA concentrations of LPS-stimulated and nonstimulated cells by co-amplification of β-actin cDNA and β-actin control fragment. The equivalent of 50, (lanes *1*), 100 (lanes *2*), 250 (lanes *3*), 500 (lanes *4*), 1000 (lanes *5*), 2500 (lane *6*), and 5000 (lanes *7*) pg of reverse-transcribed mRNA were co-amplified with 0.5 pg of β-actin competitor DNA fragment. The dilutions resulting in equal band intensities of amplified β-actin cDNA (a-cDNA) and amplified β-actin competitor DNA fragment (a-c.f.) are indicated by arrows. (*B*) TNF competitor DNA fragment in the amounts of 0.02 ag (lane *1*), 0.2 ag (lane *2*), 2 ag (lane *3*), 20 ag (lane *4*), 200 ag (lane *5*), and 2 fg (lane *6*) was co-amplified with the equivalent of 10 ng of reverse-transcribed RNA of the LPS-stimulated cells (+LPS) or with the equivalent of 5 ng of reverse-transcribed RNA of nonstimulated cells (–LPS). The dilutions resulting in equal band intensities of amplified TNF cDNA (TNF-cDNA) and amplified TNF competitor DNA fragment (TNF-c.f.) are indicated by arrows. The size marker is 1 μg of DNA of the 1-kb ladder.

amplified, 0.5 fg of competitor DNA fragment was co-amplified with 0.05, 0.5, and 5 fg of IL-1 cDNA amplification product (Fig. 3A). Equal amounts of input competitor DNA fragment and IL-1 cDNA resulted in the same amount of PCR products (Fig. 3A, arrow), whereas a change in the amount of the IL-1 cDNA resulted in a bias of the two PCR products. Therefore, we conclude that during amplification the weight ratio and consequently the molar ratio of both fragments remains constant. To determine the absolute amount of IL-1 cDNA molecules present in the cDNA of LPS-stimulated cells, 0.005, 0.05, and 0.5 fg of competitor DNA fragment were co-amplified with equal amounts of cDNA. Equal band intensities of the amplified fragments were obtained at an input of 0.05 fg of competitor DNA fragment (Fig. 3B, arrow). Therefore, the cDNA used for PCR was determined to contain approximately 0.05 fg of the single-stranded 308-bp IL-1 cDNA, corresponding to 270 molecules (4.5×10^{-22} mole).

DISCUSSION

We have described a convenient method for the generation of PCR competitor DNA fragments by cross-species PCR. As demonstrated in three cases with mouse-specific primers and genomic chicken DNA (β-actin, IL-1, TNF), it is easily possible to isolate fragments of defined size with mouse primer-specific ends allowing competitive conditions during co-amplification experiments. Since appropriate annealing conditions during the initial PCR cycles usually yielded multiple priming, fragments that differ only slightly in size from the target sequence can be selected. Therefore, elongation times for cDNA and competitor fragment should be similar and, additionally, both fragments should stain approximately proportionally to their molar amount.

Several approaches for generating control fragments for competitive PCR have been described. These include the use of genomic regions containing short introns,[1] the engineering of restriction sites into the control fragment[1] to distinguish cDNA and control fragment by restriction enzyme digestion, or to mutagenize the control fragment site-specifically, allowing the separation of both PCR products by temperature gradient gel electrophoresis.[5] Since these methods are either rather laborious or may not be suitable for all genes, the generation of control fragments by cross-species PCR might be advantageous because it does not require any further genetic manipulations such as subcloning or the use of additional primers.

The competitor DNA fragments generated by cross-species PCR most likely have no internal homology to the target sequence. This may be useful because heteroduplex formation between the two PCR product molecules is prevented. On the other hand, the two fragments may have different reannealing behavior and/or different amplification rates. This can be disregarded if the competitor DNA fragment is used for semiquantitative PCR, that is, the determination of the *ratio* of a given mRNA between two different cell populations. If, in contrast, determination of the precise molar amount of a mRNA within a defined cell population is desired, then the competitor DNA fragment must be characterized further. We provide one example each for the two applications of the control fragments.

For semiquantitative PCR, two cDNAs first are adjusted to equal amounts of input cDNA by co-amplifying constant amounts of cDNA with 10-fold serially diluted competitor DNA fragment and, subsequently, vice versa, by co-amplifying constant amounts of the control fragment at the appropriate concentration and twofold dilutions of the cDNA. Equilibration of cDNA amounts may be done by amplifying β-actin cDNA or any other gene that may be considered as noninducible and being transcribed at a similar rate in different cells. The fact

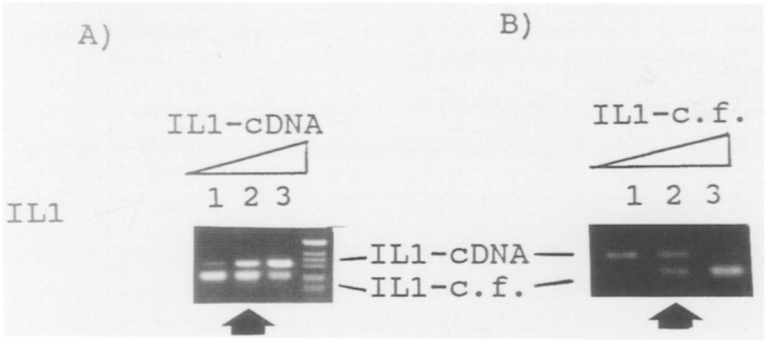

FIGURE 3 Quantitative PCR analysis. (*A*) Demonstration of stoichiometrical amplification of IL-1 cDNA and IL-1 competitor DNA fragment. A 0.5-fg amount of IL-1 competitor DNA fragment was co-amplified with 0.05 (lane *1*), 0.5 (lane *2*), and 5 fg (lane *3*) amplified IL-1 cDNA fragment (IL1-cDNA). (*B*) Determination of IL-1 cDNA derived from LPS-activated spleen cells. The equivalent of 5 ng of reverse-transcribed RNA of LPS-stimulated cells was co-amplified with 0.5 (lane *1*), 0.05 (lane *2*), and 0.005 (lane *3*) fg of IL-1 cDNA competitor DNA fragment (IL1-c.f.) The dilution resulting in equal band intensities of amplified IL-1 cDNA and amplified IL-1 competitor DNA fragment is marked by an arrow. The size marker is 1 μg of the 1-kb ladder DNA.

that a small change in the amount of input cDNA results in a bias of the two PCR products shows that the results obtained by competitive PCR are not falsified by the "saturating" nature of PCR.

Thus, the determination of the amount of cDNA required to obtain equal band intensities in the presence of the same amount of competitor DNA fragment allows comparison in later experiments of equal amounts of "amplifiable" cDNA for the abundance of other mRNA species. Other efforts to determine the relative amount of mRNA, e.g., by Northern blot or dot-blot analysis are far less sensitive. The careful equilibration of cDNAs enabled us to measure, again by co-amplifying cDNA and competitor fragment, the approximately 10-fold induction of TNF mRNA in LPS-stimulated versus nonstimulated spleen cells.

To quantify IL-1 mRNA in LPS-stimulated spleen cells, the artificially generated IL-1 competitor fragment first was shown to be amplified stoichiometrically with the IL-1 cDNA, although this may not hold true for all competitor DNA fragments generated by cross-species PCR. Appropriate PCR conditions (e.g., prolonged extension times or changes in the reannealing temperature) may lead to equal amplification rates. This can be controlled by co-amplification of known amounts of both cDNA and competitor DNA, as demonstrated in Figure 3A.

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