Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 2725–2729, April 1990
Genetics

Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction

(gene expression/gene dosage/hematopoietic growth factor(s)/granulocyte–macrophage colony-stimulating factor/interleukin 3)

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Communicated by Arthur Pardee, January 26, 1990 (received for review November 14, 1989)

ABSTRACT **The expression of two cytokines, granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3), has been investigated in MLA-144 cells before and after induction with phorbol 12-myristate 13-acetate. We describe an adaptation of the polymerase chain reaction (PCR) for highly accurate quantitation of mRNA or DNA from a small number of cells. Aliquots of the PCR mixture containing cDNA copies of the RNA to be assayed were added to serial dilutions of a competitor DNA fragment that differed from the cDNA of interest by having either a small intron or a mutated internal restriction enzyme site. Therefore, the same primers were used to coamplify the unknown and the competitor. The ratio of products remains constant through the amplification and can be readily quantitated. In unstimulated cells, no GM-CSF or IL-3 mRNA could be detected. However, with appropriate induction, mRNA for both cytokines was detected and quantitated in as few as 200 cells. Competitive PCR was also used to accurately quantitate the copy number of the human GM-CSF gene in normal human cells, in a clonal population of cells from a patient with 5q$^-$ syndrome, and in a human–hamster cell line known to have only one copy of the human GM-CSF gene.**

Understanding the regulation of gene expression depends in part on the ability to accurately measure mRNA species in defined cell populations. Recent advances in fluorescent cell sorting as well as cell culture technology provide access to homogeneous cell samples with well characterized maturational and/or developmental features. However, conventional methods of mRNA analysis such as Northern and "dot blot" hybridization and even nuclease protection mapping are not sensitive enough to detect mRNA in samples limited by either low cell number or low copy number per cell. Moreover, these methods permit only crude quantitation of mRNA. *In situ* hybridization allows detection of mRNA in single cells but is insensitive and nonquantitative. Because of its extraordinarily high sensitivity, the polymerase chain reaction (PCR) is being widely used for amplifying cDNA copies of low abundance mRNA (1–6). However, quantitation is unreliable because the amount of PCR product increases exponentially with each cycle of amplification; therefore, minute differences in any of the variables that affect the efficiency of amplification can dramatically alter product yield.

This report describes analyses of mRNA levels of two cytokines, interleukin 3 (IL-3) and granulocyte–macrophage colony-stimulating factor (GM-CSF) in MLA-144 cells after induction with phorbol 12-myristate 13-acetate (7). We have devised a simple and inexpensive method in which competitive PCR is used for highly accurate quantitation of mRNA and DNA from a small number of cells. Rather than analyze a different reporter gene product (2, 5), we add a competitor DNA fragment that differs from the cDNA of interest by having either a small intron or a mutated restriction enzyme site. Therefore, the same primers are used to coamplify the unknown and the competitor. The ratio of products remains precisely constant through the amplification. The relative amount of each product can be readily determined either by densitometric scanning of ethidium bromide-stained gels or by excising and counting bands radiolabeled with [α-^{32}P]-dNTP. This strategy can be applied to a variety of studies of gene expression requiring accurate measurement of mRNA species in low abundance or from small numbers of cells. Furthermore, as presented in this report, competitive PCR can provide accurate quantitation of gene copy number. A portion of this work has been described (8).

METHODS

Materials. HPLC purified deoxyribonucleotides (United States Biochemical) were diluted to a stock solution of 2.5 mM each nucleotide. Other materials included avian myeloblastosis virus reverse transcriptase (Life Sciences, Saint Petersburg, FL), recombinant *Thermus aquaticus* DNA polymerase (*Taq* polymerase) (Cetus), and RNasin (Promega). All of the primers used in the PCRs were 30-mers, had 50–60% G+C content, and lacked 3′ complementarity between primer pairs. Primers were prepared on an Applied Biosystems 380B automated synthesizer.

Plasmids. HTB194B contains human GM-CSF cDNA in the expression plasmid PXM; pIB124 contains the human *Hin*dIII/*Eco*RI fragment of the GM-CSF gene; gibbon IL-3 cDNA is subcloned in PXM and the human IL-3 gene in pUC8. These plasmids were the generous gift of Steven Clark (Genetics Institute, Cambridge, MA).

PCR. The components of the PCR were tested for contaminants by 60 cycle reactions without added DNA template. The 10-fold PCR buffer contained 500 mM Tris·HCl (pH 8.2), 15 mM $MgCl_2$, 500 mM KCl, and 0.01% (wt/vol) gelatin. The gelatin was made soluble by autoclaving the stock solution. Unless otherwise specified, each reaction mixture contained dNTPs (200 μM final concentration in each, including [α-^{32}P]dCTP at 50 μCi/ml; 1 Ci = 37 GBq), 0.2 μM each primer, 5 unit of *Taq* polymerase per ml, 1× PCR buffer, and H_2O to a final vol of 100 μl. In all experiments, the presence of possible contaminants was checked by control reactions in which amplification was carried out on samples in which (*i*) reverse transcriptase was omitted from the reverse transcription reaction mixture; (*ii*) lysis buffer alone was added to the reverse transcription reaction mixture. Samples were amplified by repeated cycles at 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min. An aliquot of each

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Abbreviations: GM-CSF, granulocyte–macrophage colony stimulating factor; IL-3, interleukin 3; PCR, polymerase chain reaction.
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reaction mixture was subjected to electrophoresis on 1% agarose/2% NuSieve gels.

Competitive Templates. Templates were subcloned fragments of genomic DNA containing an intron. Stock competitive templates from 0.25 amol/liter to 2.5×10^6 amol/liter were prepared by serial dilutions with calibrated microcapillary pipettes. Competitive templates that differ from the target sequence by a single base pair (bp) were prepared by site-directed mutagenesis using PCR (9).

MLA-144 Cell Growth and RNA Preparation. This primate T-cell line, gift of Charlotte Niemeyer (Harvard Medical School), was maintained in RPMI 1640 medium/10% fetal calf serum. Cells were resuspended at a density of 2×10^6 cells per ml in RPMI 1640 medium/10% fetal calf serum with or without phorbol 12-myristate 13-acetate (10 ng/ml). After 18 hr of incubation, admixtures of stimulated and unstimulated cells were prepared. Total cellular RNA (10) was analyzed by competitive PCR as well as by standard Northern blot hybridization, using as probes the *Xho* I/*Xho* I fragment of human GM-CSF cDNA and human β-actin cDNA.

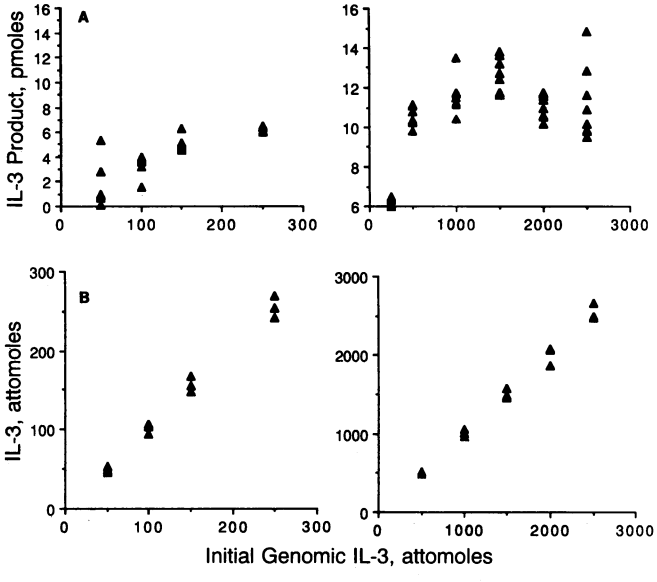

FIG. 1. Comparison of noncompetitive (*A*) and competitive (*B*) PCR. (*A*) Generation of amplified product by standard PCR. Nine separate master mixtures contained IL-3A and IL-3B primers, [α-^{32}P]dCTP, the designated amounts of IL-3 genomic template, and standard PCR components in a final vol of 1.0 ml. For each amount of genomic IL-3 (shown on the *x*-axis), a master mixture was prepared and then split into eight separate tubes in vol of 100 μl each. After 25 cycles of amplification, triplicate 15-μl samples from each tube were electrophoresed and bands corresponding to amplified genomic IL-3 (301 bp) were excised and counted. Triplicate values were averaged. There was <5% variance among triplicate samples. IL-3 product yield in pmol was calculated assuming a 30% ratio of cpm/dpm. Yield is plotted as pmol of IL-3 product per 100-μl reaction volume vs. amol of input IL-3 template per 100-μl reaction volume. (*B*) Quantitation of genomic IL-3 by competitive PCR. Nine separate 1.0-ml master mixtures contained [α-^{32}P]dCTP and genomic IL-3 ranging from 50 to 2500 amol per 90 μl. The concentration of genomic IL-3 in each master mixture was then determined by adding 90 μl to each of 10 tubes which contained 10-μl solutions of IL-3 cDNA of concentrations ranging from 1 to 100 times that of the genomic DNA in the master mixture. Thus, tubes containing 50 amol of IL-3 genomic DNA also contained 5–500 amol of IL-3 cDNA. The following primers were used: IL-3A, 5′-ATGAGCCGCCTGCCCG-TCCTGCTCCTGCTC-3′; IL-3B, 5′-CATCAGAATGTCTTGGTC-TTCCCCATTGAG-3′. These primers span a small intron so that genomic DNA may be readily distinguished from cDNA based on size. After amplification and electrophoresis of triplicate samples, the amount of genomic DNA was calculated as described in the legend to Fig. 2.

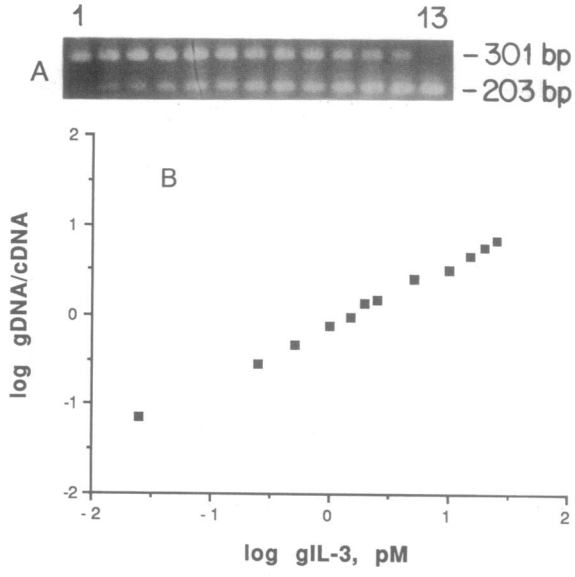

FIG. 2. (*A*) A PCR master mixture was prepared containing IL-3A and IL-3B primers, radiolabeled dCTP, and IL-3 cDNA plasmid at a final concentration of 1.4 pM. Ninety microliters of master mixture was aliquoted into each of 13 tubes containing 10 μl of genomic IL-3 template from a dilution series. The final concentration of genomic IL-3 in each tube (left to right) was 250, 25, 20, 15, 10, 5, 2.5, 2.0, 1.5, 1.0, 0.5, 0.25, and 0.025 pM, respectively. Twenty-five cycles of PCR and electrophoresis of samples were performed. Bands corresponding to genomic IL-3 and IL-3 cDNA were excised and a ratio of genomic IL-3 to IL-3 cDNA was calculated. To correct for differences in molecular weight, cpm obtained from genomic IL-3 bands were multiplied by 203/301. (*B*) Data are plotted as log ratio of IL-3 genomic DNA (gDNA)/cDNA vs. log input genomic IL-3 (gIL-3).

RESULTS

Noncompetitive Amplification of IL-3. To determine the effectiveness of PCR for quantitation of low levels of DNA, we amplified replicate samples of plasmid containing a genomic copy of the IL-3 gene. Despite the use of master mixtures, as described in the legend to Fig. 1*A*, the yield of amplified IL-3 product varied as much as 6-fold within the same dilution, and concentrations over a logarithmic range of dilutions could not be reliably distinguished. This variability was independent of cycle number over the range 15–30. With higher initial concentrations of plasmid, the amount of product appeared to reach a plateau, probably owing to rate-limiting reaction components (e.g., primers, dNTP, enzyme) or by inhibitory reaction by-products such as pyrophosphate. Thus, even under carefully controlled conditions, variables that affect efficiency of amplification preclude accurate quantitation of starting DNA in a given sample.

Quantitation by Competitive PCR. To circumvent the problem of variability in efficiency of amplification, plasmid containing GM-CSF and IL-3 cDNA was coamplified with a standard dilution series of plasmid containing genomic copies of GM-CSF and IL-3. Primers, specified in the legend to Fig. 1, were chosen that flank small (≈100 bp) introns in these genes. GM-CSF cDNA amplified with these primers is 196 bp, whereas genomic GM-CSF amplified with the same set of primers is 289 bp. Similarly, IL-3 cDNA amplified with the designated primers is 203 bp, while genomic IL-3 is 301 bp. Because the primers are identical, the cDNA and genomic DNA copies of these genes should be amplified with the same efficiency, provided that the amplification of the genomic DNA copy is unaffected by its small intron. In effect, the cDNA and genomic DNA templates compete for available PCR substrates. By titrating an unknown amount of a cDNA

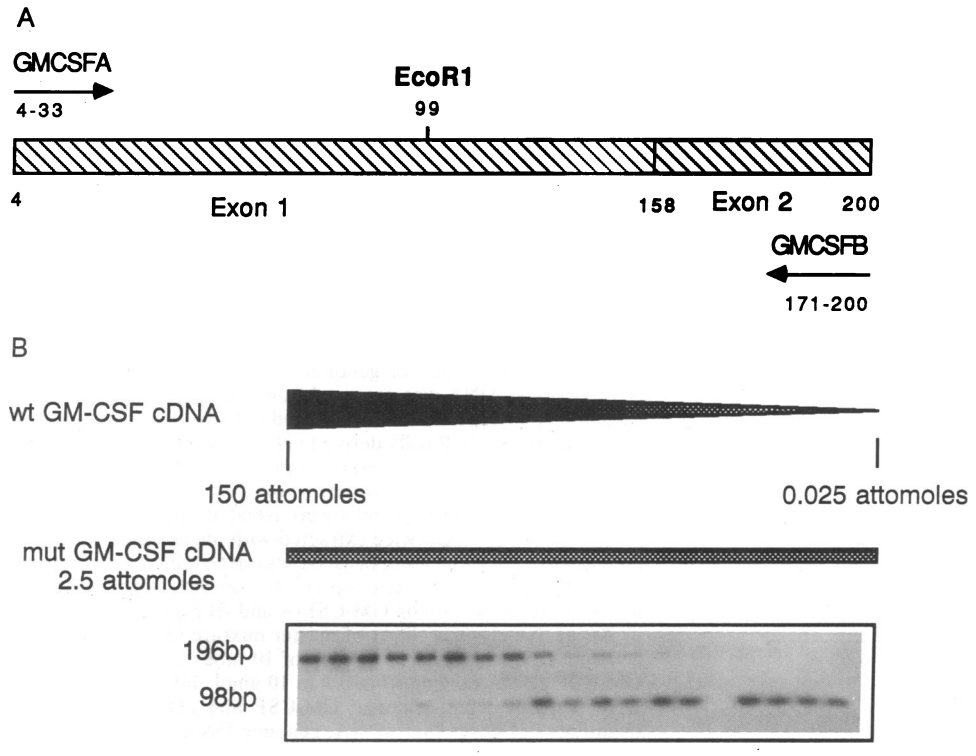

FIG. 3. Use of mutant competitor for quantitating GM-CSF. (*A*) Map of competitor. The flanking primers were as follows: GM-CSF-A, 5′-GGCTGCAGAGCCTGCTGCTCT-TGGGCACTG-3′; GM-CSF-B, 5′-CTGGAGGTCAAACATTTCTGA-GATGACTTC-3′. Using a mutant primer, 5′-AGCATGTGAATTCCA-TCCAGGAGGCCCGGC-3′, a single change, G → T, at base pair 103 creates a unique *Eco*RI site, which cleaves at base pair 99, generating two fragments of 99 and 103 bp. These two fragments comigrate on 1% agarose/2% NuSieve agarose gels. (*B*) A titration curve of wild-type (wt) GM-CSF cDNA from 15 amol/μl to 0.0025 amol/μl was constructed as described. The final concentration of mutant (mut) cDNA per tube was 2.5 amol. The reactions were amplified for 45 cycles and subjected to electrophoreses as described above.

template against a dilution series containing known amounts of the corresponding genomic template, it should be possible to reliably and reproducibly quantitate the amount of cDNA present. As shown in Fig. 2, a single concentration of IL-3 cDNA was coamplified with a dilution series of a known concentration of genomic IL-3 plasmid. After electrophoresis, the amount of IL-3 cDNA and genomic IL-3 in each sample was quantitated in triplicate by using radiolabeled dCTP (Fig. 2*A*). As would be predicted of competitive templates, a plot of the ratio of genomic IL-3 to IL-3 cDNA versus the known concentration of input genomic IL-3 is linear when plotted on an arithmetic scale (not shown) or on a log-log scale (Fig. 2*B*). At the point where genomic GM-CSF and GM-CSF cDNA product are in equivalence (i.e., ratio = 1.0), the starting concentration of GM-CSF cDNA prior to PCR is equal to the known starting concentration of the competing genomic GM-CSF plasmid. In this case, concentration of IL-3 cDNA calculated from competitive PCR was 1.5 pM, in good agreement with the spectrophotometric determination of IL-3 cDNA concentration. In three separate experiments (not shown), identical results were obtained.

Competitive PCR was used to quantitate IL-3 genomic DNA using the same dilutions depicted in Fig. 1*A*. In contrast to results obtained in Fig. 1*A* with noncompetitive PCR, triplicate results of competitive amplification of IL-3 cDNA, as shown in Fig. 1*B*, show a precise linear relationship (R = 1.00) between input genomic IL-3 and calculated genomic IL-3 concentrations from competitive PCR. As predicted, results were not dependent on cycle number or on concentrations of primers or dNTPs (data not shown).

Use of a Site-Specific Mutant GM-CSF as a Competitive Template. A potential drawback to the use of competitive templates that differ from the unknown DNA by a small intron is that the presence of the intron may alter amplification efficiency. Furthermore, intron structure is not known for many genes, and not all genes will have conveniently small introns to allow amplification of the type described above. For these reasons, we constructed competitive templates that differ from the template of interest by a single base pair generating or obliterating a unique restriction site. This minor modification would not be expected to affect amplification efficiency, but it allows one to distinguish the amplified product of the competitor (mutant) template from the unknown (wild type) template after restriction enzyme digestion. As shown in Fig. 3*A*, mutant GM-CSF cDNA template was prepared by a single G → T change at base pair 103, creating a unique *Eco*RI site. The mutant GM-CSF template can be used to accurately quantitate wild-type cDNA (Fig. 3*B*). Results were identical to titrations in which the intron-containing template was used.†

Quantitation of GM-CSF mRNA from MLA-144 Cells. Mixtures of unstimulated and phorbol 12-myristate 13-acetate stimulated MLA-144 cells were analyzed by standard Northern blots. Each sample contained a total of 10^7 cells that included a range from 10^1 to 10^7 stimulated cells. Unstimulated MLA-144 cells produced no detectable GM-CSF mRNA. Equal loading of each lane in this Northern blot was confirmed by means of a β-actin probe. Stimulated cells produce GM-CSF mRNA, which was easily detected as shown in Fig. 4*A* (lane 1). However, as the fraction of stimulated cells was decreased, the signal for GM-CSF mRNA decreased and was virtually undetectable in lane 6, which contained RNA from 10^6 stimulated cells. The same preparations were analyzed by competitive PCR. GM-CSF mRNA could not be detected in unstimulated MLA-144 cells, while 0.5 amol of GM-CSF mRNA could be detected and quantitated in RNA from 200 stimulated cells (Fig. 4*B*). In three separate experiments (data not shown), values ranged from 0.47 to 0.55 amol per 200 stimulated cells. In additional experiments not shown, GM-CSF mRNA was quantitated from 2000 and 20,000 cells and was found to be 4.8 and 57

†One caveat in the use of mutant templates as competitors is that under conditions in which primer is rate limiting, annealing may occur between heterologous strands of mutant and wild-type templates. Heteroduplexes would not be expected to be cleaved with *Eco*RI. To standardize conditions, heteroduplex formation is maximized by heating samples to 94°C followed by cooling. Under these conditions, when mutant and wild-type templates are present in a 1:1 molar ratio prior to PCR, the apparent ratio of products following PCR will be 1:3 because of heteroduplex formation, which follows a binomial distribution.

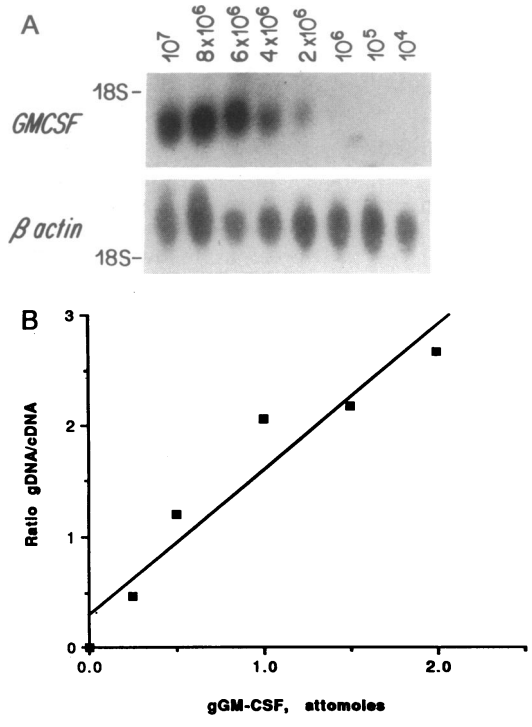

FIG. 4. Demonstration of GM-CSF expression in phorbol 12-myristate 13-acetate-stimulated MLA-144 cells. (*A*) Northern blot hybridization. Total RNA from admixtures of stimulated and unstimulated cells was analyzed with GM-CSF and β-actin probes. Each sample contained a total of 10^7 cells. The number of stimulated cells in each sample is shown above the lanes. (*B*) Quantitation of GM-CSF mRNA using competitive PCR. RNA from a mixture of 2000 stimulated MLA-144 cells and 2×10^5 unstimulated cells (≈100 ng of total RNA) was heated to 65°C for 10 min and reverse transcribed in a reaction mixture containing 10 nmol each dNTP, GM-CSF-B primer (20 pmol), 5 units of RNasin, and 20 units of avian myeloblastosis virus reverse transcriptase in 20 μl total vol of 1-fold PCR buffer plus 1 mM dithiothreitol for 1 hr at 37°C. The reaction mixture was heated to 100°C for 3 min and added to a PCR master mixture in a total vol of 900 μl as described above, except that master mixture concentrations were corrected for input GM-CSF-B and dNTP from the reverse transcription reaction. Separate experiments showed that efficiency of reverse transcription was the same using PCR buffer or the manufacturer's buffer. For each experiment, control experiments in which reverse transcriptase or RNA was excluded gave no signal after amplification, documenting the absence of contamination. Ninety microliters of PCR master mixture, containing RNA from the equivalent of 200 stimulated cells, was added to each of 10 tubes containing GM-CSF genomic DNA plasmid ranging from 0.1 to 10 amol per tube. Forty cycles of PCR were performed and genomic GM-CSF and cDNA signals were quantitated as described. Equivalence [genomic DNA (gDNA)/cDNA = 1] was obtained at ≈0.5 amol of input genomic GM-CSF (gGM-CSF).

amol, respectively. Thus, our measurement was linear throughout a 100-fold range. It can be calculated from these data that each stimulated cell contained ≈1500 GM-CSF mRNA molecules. Separate control experiments in which reverse transcriptase was excluded and in which mRNA was excluded showed no detectable signal.

Quantitation of Genomic Copy Number. We analyzed several DNA preparations that contain either one or two copies of the GM-CSF gene. Genomic DNA from normal individuals would be expected to contain two copies of GM-CSF per genome. In contrast, in patients with the $5q^-$ syndrome, hematopoietic cells contain one normal chromosome 5 and a homolog in which the long arm (5q) has a large interstitial deletion. This deleted chromosomal fragment includes a number of hematopoietically relevant genes such as GM-CSF and IL-3. Therefore, isolated neutrophils from a $5q^-$ patient

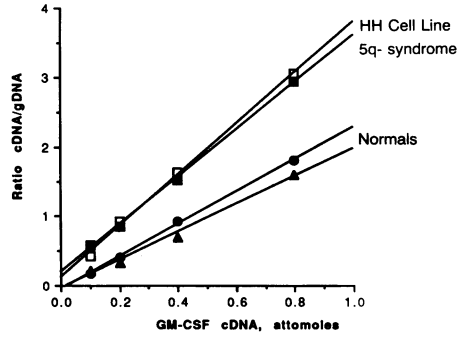

FIG. 5. Quantitation of genomic copy number by competitive PCR. Genomic DNA was prepared from isolated (13) polymorphonuclear leukocytes from two normal individuals, from a patient with $5q^-$ syndrome with clonally derived polymorphonuclear leukocytes, and from a human–hamster (HH) hybrid cell line (CQ4) containing a single copy of human chromosome 5 (14). Nuclei from polymorphonuclear leukocytes or CQ4 cells were isolated, digested with SDS/proteinase K, and then twice extracted with phenol/chloroform and precipitated. Concentration of DNA was determined spectrophotometrically. DNA from each source (10 μg) was added to a PCR master mixture containing GM-CSF-A and -B primers in a total vol of 900 μl. Ninety microliters of master mixture (containing 1 μg of genomic DNA) was added to each of 10 tubes containing 10 μl of GM-CSF cDNA ranging from 0.1 to 10 amol. Forty cycles of PCR were performed and genomic GM-CSF and cDNA signals were quantitated as described. gDNA, genomic DNA.

would be expected to have a single copy of the GM-CSF gene per genome. In separate experiments, we have shown that this DNA was derived from a single, mutated, hematopoietic clone (11). In addition, we analyzed DNA from a stable human–hamster cell line (CQ4), which contains a single copy of normal human chromosome 5 (12) and, therefore, a single copy of human GM-CSF per genome. DNA was isolated from each of these sources and purified as described in the legend to Fig. 5. Equivalent amounts (1 μg) of genomic DNA competed with GM-CSF cDNA in a titration series.

As shown in Fig. 5, genomic DNA isolated from two normal individuals (KLS and LMA) contained twice the number of genomic GM-CSF copies per μg of DNA as did genomic DNA from an individual with $5q^-$ syndrome (LY) or from the CHO cell line containing one copy of human 5q. Importantly, no amplified signal could be detected in a partner human–hamster cell line (CQ5) that contained only the abnormal $5q^-$ chromosome. The titrations in Fig. 5 give estimates of 0.45 and 0.50 amol (or 270,000–300,000 molecules) of GM-CSF gene per μg of normal diploid genomic DNA. Since the complexity of the diploid human genome is $\approx 4 \times 10^9$ bp, one cell contains ≈4 pg of DNA. Therefore, 1 μg of DNA would represent 250,000 cells. Considering the potential error in spectrophotometric estimations of DNA concentrations and potential for loss of DNA during purification, our titration measurements are in reasonable agreement with the expected value of two gene copies per cell or 500,000 gene copies per sample.

DISCUSSION

These experiments, as well as a number of recent studies (2–4), demonstrate that very small amounts of specific mRNAs can be amplified by the PCR. The major problem has been to obtain accurate quantitation. The amount of amplified product produced by PCR amplification (X) can be estimated from the formula $X = I(1 + E)^n$, where I is the initial amount of target DNA, E is the average efficiency of a cycle of amplification, and n is the number of cycles. E has a theoretical maximum value of 1, but it declines during multiple cycles of amplification as a result of consumption of primer and dNTPs, production of inhibitory pyrophosphates,

and other factors. Furthermore, as shown in Fig. 1*A*, efficiency can vary substantially among identical samples prepared with master mixes. Thus, significant error may result from extrapolating the initial amount of DNA from yield following PCR.

Recently, this problem has been addressed by coamplification of the mRNA of interest with a "reporter" DNA template as an internal standard (2, 5). Although this approach offers a means for quantitation, it depends on the unknown and reporter cDNAs having the same efficiency of amplification. When performed in a single tube, the two amplification processes share the following variables: concentrations of Mg^{2+} and deoxynucleotides, cycle number, temperature, and length of cycle steps. However, the two amplification reactions differ in the melting temperatures of DNA templates and primer/templates and in concentration and length of DNA template. Because of differences in this latter set of variables, the efficiencies of amplification of the two unrelated cDNAs are likely to be significantly different, owing to (*i*) different initial reaction rates; (*ii*) differential decline in reaction rates, which depend largely on initial concentration of DNA template; (*iii*) different efficiencies of amplification during each cycle, which depends on melting temperature of the primer/template duplex among other variables; and (*iv*) potential confounding effects arising from the formation of primer dimers. This strategy often necessitates blotting PCR products with labeled probe and it is difficult to apply to low abundance mRNA from small numbers of cells.

We have circumvented this problem by using a competitor DNA which is identical to the cDNA of interest, save for the presence of a mutated restriction site or a small intron. The two amplifications share all of the variables mentioned above. Accordingly, the ratio of unknown product to competitor product should remain constant throughout the amplification process, irrespective of cycle number or changes in any of the variables mentioned above. In contrast to noncompetitive amplification (Fig. 1*A*) or to the use of unrelated internal standards, the ratio of these amplified products should precisely reflect the initial concentration of unknown cDNA versus that of the added competitor. Because competitive PCR is not cycle dependent, amplification can be performed over many cycles so that PCR products can be visualized and quantitated by ethidium bromide staining. Thus, this strategy offers both a high degree of specificity as well as remarkable accuracy and sensitivity. Moreover, the assay is rapid, modestly labor intensive, and relatively inexpensive. We have observed no difference between competitive templates containing a small intron versus one having a single base substitution. In contrast, a competitor containing a large intron or unrelated sequence may have significantly different efficiency of amplification.

In applying competitive PCR to the quantitation of low abundance mRNA, several pitfalls should be considered. Variability in the efficiency of the reverse transcriptase can be evaluated by using as the competitor mutated RNA transcribed from an RNA expression vector. However, this control would not correct for losses of mRNA during processing. Moreover, the amplified PCR fragments may not recognize heterogeneity in mRNA, owing to differential splicing or to transcripts from duplicated genes. Therefore, the use of competitive PCR should be restricted to analyses of mRNA species that have been well characterized by Northern blotting and/or nuclease protection mapping.

Competitive PCR lends itself to a broad array of useful applications. Because it can accurately measure mRNA species in a small number of cells, this strategy can be used to analyze gene expression in cells isolated by fluorescent sorting or plucked from colonies grown in semisolid medium. Because competitive PCR enables quantitative determination of low abundance mRNAs, low level (constitutive) expression of genes can be characterized. Recently, several investigators (3, 4) have suggested that because of its extraordinarily high sensitivity PCR could possibly detect virtually any species of mRNA in any cell, thereby defining the inherent leakiness of transcription initiation. As reported above, we were not able to detect GM-CSF and IL-3 mRNA in uninduced MLA-144 cells. The regulation of these two cytokines is both transcriptional (15–17) and at the level of mRNA stability (13, 14). It seems plausible that species of mRNA, such as GM-CSF and IL-3, that are unstable in nonproducing cells may escape detection even by PCR, whereas more stable mRNA species such as dystrophin (2) and β-globin (3) can be detected. In any event, meaningful interpretation of low abundance mRNAs as revealed by PCR should be enhanced by quantitation with a competitor template, as described above.

The sensitivity and specificity of this method also lends itself to the quantitation of mRNAs expressed by reporter genes such as human growth hormone, bypassing the need for translation, secretion, and detection of a protein product from transfected cells. By means of an appropriate mutation within the region that is amplified, the gene under study can serve as its own reporter. As described above, the exogenous mRNA can be directly compared to the endogenous mRNA after digestion of the amplified products with the appropriate restriction enzyme. This approach should permit study of regulatory elements of a gene in the most "native" possible context.

Competitive PCR should also be useful in the detection and quantitation of low abundance species of DNA such as somatic cell mutations and integration of viral DNA, both of which may involve only a small minority of a given cell population. Efficiency of transfection of plasmids can also be accurately monitored. Finally, as illustrated in Fig. 5, this approach can be applied to measurements of gene dosage, both deletion and amplification.

We thank Drs. Arthur Nienhuis and Janet Rowley for providing the CQ4 and CQ5 human–hamster cell lines. This work was supported in part by National Institutes of Health Grant RO1-DK41234.

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