Journal of Cellular Biochemistry 39:1–11 (1989)
Growth Factors and Their Receptors: Genetic Control and Rational Application 71–81

Novel Method for Studying mRNA Phenotypes in Single or Small Numbers of Cells

Daniel A. Rappolee, Alice Wang, David Mark, and Zena Werb

Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, California 94143 (D.A.R., Z.W.); Department of Molecular Biology, Cetus Corporation, Emeryville, California 94608 (A.W., D.M.).

Biological processes, such as growth control, are often governed by biochemical steps involving mRNA transcripts that are short-lived and have a low copy number. Furthermore, the cells involved in these processes are often available in low numbers from in vivo sources. We now report a method that is superior to in situ hybridization, RNA blot analysis, and the nuclease protection assay for the study of short-lived, low-copy-number mRNA transcripts. The method consists of a microprocedure for isolating RNA from one to a few thousand cells and two coupled enzymatic steps: reverse transcription of whole cellular RNA, followed by amplification of the cDNA by a specifically primed polymerase chain reaction to give specific cDNA fragments that can be visualized on agarose gels by ethidium bromide staining. With this method we have detected actin mRNA from a single cell, or < 100 cRNA molecules, and have quantified differences in RNA concentrations of less than threefold. The reverse transcription reaction products can be divided for the polymerase chain reaction, and several mRNA species can be assayed simultaneously. Therefore, we call the method single-cell mRNA phenotyping. This technique is applicable to the analysis of low-copy-number growth factor transcripts in cells in culture and in vivo.

Key words: cDNA, mRNA, reverse transcription, polymerase chain reaction, single-cell mRNA detection, actin, growth-factor transcripts

The study of growth factor transcription has been slowed by a number of obstacles. Growth factors are generally transcribed in low copy numbers and have a short half-life [1]. In addition, often low numbers of primary cells can be obtained from in vivo sources. In situ hybridization analysis for mRNA in cells in tissue is

Abbreviations used: GuSCN, guanidine thiocyanate; IL-1α, interleukin-1α; MMLV-RT, Moloney murine leukemia virus reverse transcriptase; NGF, nerve growth factor; PCR, polymerase chain reaction; RT, reverse transcription; Taq, *Thermus aquaticus*.

Received March 22, 1988; accepted July 6, 1988.

difficult and insensitive [2]. One approach has been to use continuous cell lines to obtain sufficient material for growth factor studies, but immortalization of cell lines is often produced by ectopic expression of the growth factors in question [3].

Two developments have now permitted development of a novel method for RNA analysis. First, a method for cell-free DNA replication was reported [4]. This method, the polymerase chain reaction (PCR), involves two oligonucleotide primers that flank the DNA sequence to be amplified, repeated cycles of thermal denaturation of the DNA, annealing of primers to their complementary sequences, and primer extension to give an exponential accumulation of the target fragments. PCR has been used for selective enrichment of DNA sequences and sequence variations and for high-efficiency cloning of genomic sequences and has been improved by the use of a newly isolated thermostable DNA polymerase from *Thermus aquaticus* (Taq) [5]. In addition, the reverse transcriptase from Moloney murine leukemia virus (MMLV—RT), which produces cDNA from whole cellular RNA mixtures, was recently isolated [6].

We now report that a microadaptation of the guanidine thiocyanate/cesium chloride (GuSCN/CsCl) gradient ultracentrifugation technique [7], followed by MMLV-RT production of cDNA and PCR amplification of specific cDNA subfragments, allows the quantitative assay of multiple transcript types from a low number of total transcripts. We have found that a) a single cell yields a signal for β-actin mRNA; b) as few as 100 cRNA transcripts can be detected; c) each cDNA molecule can generate 10^9 to 10^{10} PCR fragments; and d) $<$ threefold differences in mRNA can be resolved after PCR amplification of cDNA.

MATERIALS AND METHODS

Materials

Cells were thioglycollate-elicited peritoneal macrophages [8] harvested on day 4 after stimulation from CF1 mice (Charles River Laboratories). Hep-SK cells were obtained from the American Type Culture Collection. Restriction endonucleases were obtained from BRL and Promega Biotech and used according to the manufacturer's instructions. Taq DNA polymerase and DNA Thermocycler programmable heating block were obtained from Perkin Elmer-Cetus Corporation. MMLV-RT was purchased from BRL. cDNA probe for chick actin was a gift of M. Kirschner [9]. Primers were purchased from UCSF Biomolecular Resource Center or were provided by Cetus Corporation. [^{32}P]dCTP (3,000 Ci/mmol) was purchased from Amersham. SeaKem ME and NuSieve GTG agaroses were purchased from FMC Corporation. DNA 1-kb molecular weight standards were purchased from BRL, and Zeta-bind nylon membranes were purchased from Bio-Rad. Apparatus for agarose gel electrophoresis was purchased from American Bionetics. CsCl was purchased from Pharmacia, and GuSCN was purchased from Fluka. Oligo-dT primers and dNTPs (dATP, dCTP, TTP, dGTP) were purchased from Pharmacia. RNAsin and T7 polymerase were purchased from Promega Biotech. Acetylated bovine serum albumin was purchased from Sigma. Nerve growth factor (NGF) cRNA, produced from an SP6 clone, was a gift of Dr. Dennis Clegg. The poly A$^+$-pBR322-IL-1α cRNA was synthesized from a plasmid template (D. Mark, unpublished results) by T7 polymerase, purified by oligo-dT chromatography, and quantified by absorbance at 260 nm.

The mouse β-actin [10] primers were (5′-primer) 5′-GTGGGCCGCTCTAGGCACCA-3′, 5′ = residue 125, and (3′-primer) 5′-TGGCCTTAGGGTGCAGGGGG-3′, 3′ = residue 264. The mouse NGF [11] primers were (5′ primer) 5′-CCAAGGACGCAGCTTTCTAT-3′, 5′ = residue 246, and (3′ primer) 5′-CTCCGGTGAGTCCTGTTGAA-3′, 3′ = residue 649.

RNA Preparation

RNA was prepared by a microadaptation of the GuSCN/CsCl gradient ultracentrifugation method [7]. Briefly, macrophages (as few as one cell per microtiter well, as determined by microscopic examination) were cultured, washed with 0.9% NaCl, lysed in 100 μl of 4 M GuSCN containing 10–20 μg *Escherichia coli* ribosomal RNA as carrier, layered over 100 μl 5.7 M CsCl, and centrifuged for approximately 20×10^6 *g*-min/cm of gradient length, in a Beckman TL-100 tabletop ultracentrifuge or in a Beckman Airfuge. RNA was ethanol precipitated, centrifuged, and then washed in 80% ethanol. The dried RNA pellet was redissolved directly in reverse transcription (RT) buffer (described below) or in diethyl pyrocarbonate-treated H_2O containing RNAsin.

Reverse Transcription

RNA was reverse transcribed into cDNA essentially as previously described [6]. RNA (from one cell equivalent to 1 μg) was incubated at 37°C for 60 min with a mixture of 100 U of MMLV-RT and the following reagents: 0.2 μg oligo-dT primer, 3 mM $MgCl_2$, 10 mM Tris-HCl buffer, pH 8.3, 75 mM KCl, 1 μg acetylated bovine serum albumin, 0.5 mM dNTP, and 5 U of RNAsin in 10 μl volume. The RT can be repeated by addition of 50 U of fresh MMLV-RT after a 93°C, 5-min denaturing step, followed by flash cooling to 4°C.

Polymerase Chain Reaction

PCR was performed essentially as previously described [5]. A small portion of RT products (1 μl) was mixed with 1 U of Taq DNA polymerase, 50 pmol of 20–25 base-long oligonucleotide 5′ and 3′ sequence-specific primers, in a buffer containing 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 50 mM KCl, 5 μg acetylated bovine serum albumin, pH 8.3, in 50 μl volume. The mixture was overlaid with mineral oil to prevent evaporation and then amplified by PCR in a repeated 3-temperature cycle on the Thermocycler programmable heating block. The temperature used in the annealing cycle was varied because groups of primers had different melting temperatures; temperatures were also lowered when primers were used in other species for which the DNA sequence was not known. For samples to be amplified for >60 cycles of PCR, 1 U of Taq polymerase was added at 60 cycles.

Agarose Gel Electrophoresis

A portion of the PCR mixture (5 μl) was added to 4 μl of loading dye mix and electrophoresed in an 80-V constant-voltage field in 3% NuSieve GTG/1% SeaKem ME agaroses until the bromphenol blue dye front had migrated 6 cm. Gels were stained for 10 min in ethidium bromide and destained in H_2O for at least 30 min.

DNA Blot Analysis

DNA separated on agarose gels was transferred to Zeta-bind nylon membranes as previously described [12]. Gels were incubated for two 10-min periods at ambient

temperature with 0.25 M HCl, incubated overnight in 0.4 M NaOH, and rinsed in 2 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). cDNA probes were prepared by random priming [13]. Zeta-bind transfers were hybridized by standard techniques [13] and washed at 60°C for 60 min in 0.2 × SSC containing 0.1% SDS before autoradiography.

Restriction Enzyme Analysis

PCR products were ethanol precipitated and resuspended in the appropriate restriction enzyme buffer. Three-quarters of the resuspended products were digested, and 5 μl of this digest and 5 μl of the undigested products were then separated on a 4% agarose gel as described above.

Quantitative Analysis

Two procedures were used to obtain quantitative data: a) An RT mixture from thioglycollate-elicited macrophages was mixed with PCR reagents (those previously described plus 100 μCi of [^{32}P]dCTP, spec. act. 3,000 Ci/mmol) in 2-μl aliquots after 1/100, 1/10, or no dilution. Samples of this mixture (5 μl) were removed after 35, 40, 45, 50, and 55 cycles of the PCR, mixed with loading dye, and then separated on a 1.5-mm-thick 5% polyacrylamide/7 M urea gel [13] and run at 20 mA constant current until the bromphenol blue front had migrated 9 cm into the separating gel. The gel was autoradiographed, appropriate bands were cut from the gel, and radioactivity was determined by scintillation fluorography. Data were plotted by exponential curve fitting with an Apple Cricket Graph® program. b) Samples of pBR322-IL-1α cRNA were serially diluted threefold and mixed with 1 μg of Hep-SK RNA so that the RT mixture contained 3 × 10^2 to 1.3 × 10^7 copies of cRNA. After RT, the cDNA was amplified by PCR for 40 cycles in the presence of [^{32}P]dCTP to follow incorporation into the specific cDNA fragment band.

RESULTS AND DISCUSSION

A diagram outlining the steps in the RNA phenotyping analysis is shown in Figure 1. The first step was to obtain the mRNA, which can be done by any conventional procedure for obtaining total or cytoplasmic RNA from tissue or large numbers of cells [13] or by a micromethod for obtaining total RNA from 1 to 10^5 cells (Fig. 1a). The RNA was then reverse transcribed to prepare cDNA (Fig. 1b). This cDNA preparation was divided as desired for analyzing specific mRNA transcripts. For each mRNA species to be analyzed, a designated sequence (Fig. 1b, arrowheads) was selected, and 5′ and 3′ sequence oligonucleotides were prepared according to the criteria outlined in Table I. The primers were added to the cDNA preparation along with Taq polymerase and dNTPs. The cDNA was then thermally denatured and afterward cooled to allow annealing of primers, and the temperature was then increased to the optimum for primer extension by Taq polymerase (Fig. 2). In the first PCR cycle only the 5′ primers annealed to the cDNA from the RT (Fig. 1c); after primer extension a cDNA sequence extending from the 5′ primer for various lengths, up to and including the 3′ end of the cDNA, was obtained. In the second cycle, the 3′ primers annealed to the DNA synthesized in the first cycle, and the 5′ primers annealed to the original cDNA; after primer extension the first copies

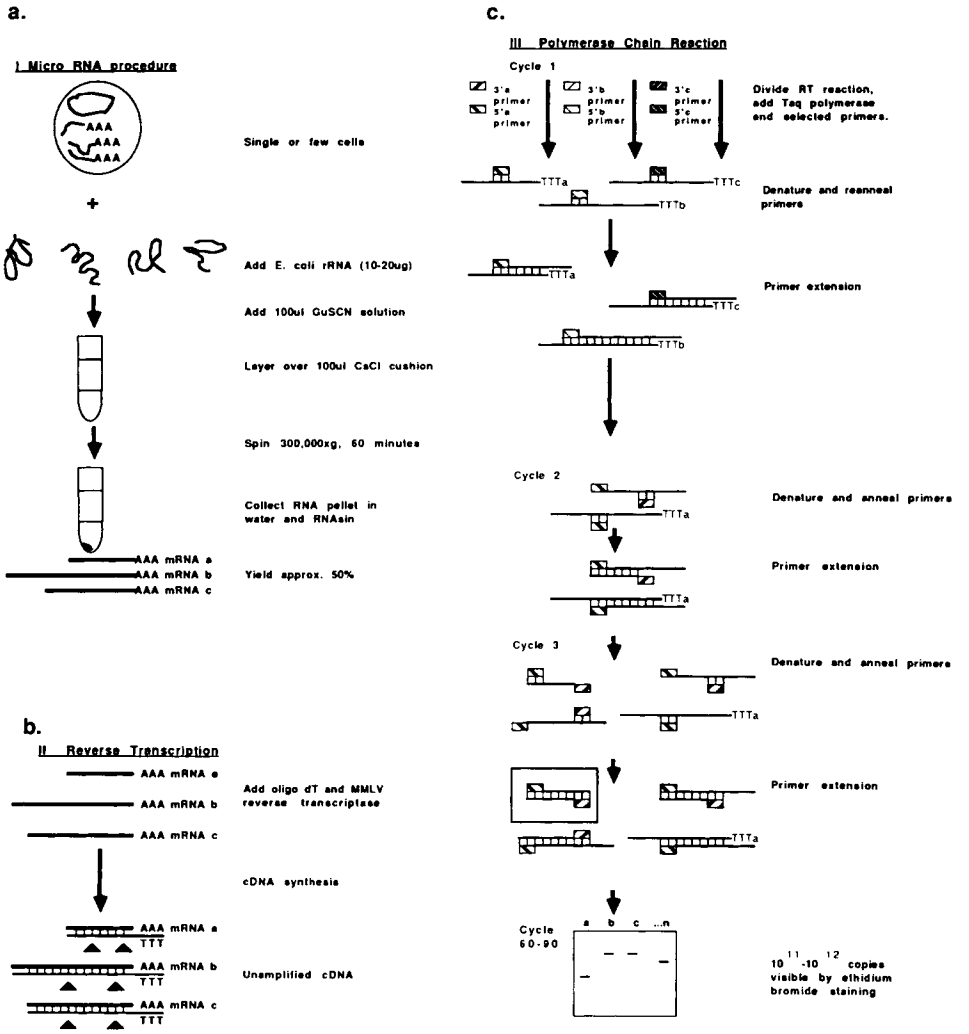

Fig. 1. Steps involved in mRNA phenotyping in low numbers of cells. **a:** Purification of whole cellular RNA from small numbers of cells. **b:** Reverse transcription using MMLV-RT. The arrowheads indicate the designated sequences for amplification by PCR. **c:** Early PCR cycles and the visualization of PCR products in an agarose gel. Note that cycles 2 and 3 focus on amplification of cDNA a only, but the schematic of the agarose gel at bottom represents cDNA a, b, and c.

of DNA of the exact size of the designated sequence flanked by the primers were produced. In subsequent cycles, this exact-size DNA became dominant, so that by 30 to 90 cycles, the 10^{11} to 10^{12} copies of cDNA, visible by ethidium bromide staining, contained only this specific sequence (Fig. 1c). The intensity of the ethidium bromide signal was related to the number of fragments in each well over a ~20-fold dilution range (data not shown). The ideal equation describing the generation of fragments in

TABLE I. Criteria for Choosing Oligonucleotide Primer Pairs for RNA Phenotyping Analysis

1. Target mRNA sequences should be ~0.2 to 0.5 kb in length.
2. For most efficient reverse transcription, target sequences should be toward the 3′ end of the coding region of the mRNA sequence; in general the 3′ untranslated region is not used because of the lack of conservation of these between species.
3. Target sequences should contain an intron/exon border so that genomic DNA and mRNA can be distinguished.
4. Target sequences should contain a diagnostic restriction endonuclease cleavage site to permit analytical validation of fragments.
5. Melting temperature of the two oligonucleotide sequences should be similar and close to the extension temperature in the assay.
6. Target sequences should be within available cDNA probes for validation by DNA blot analysis.

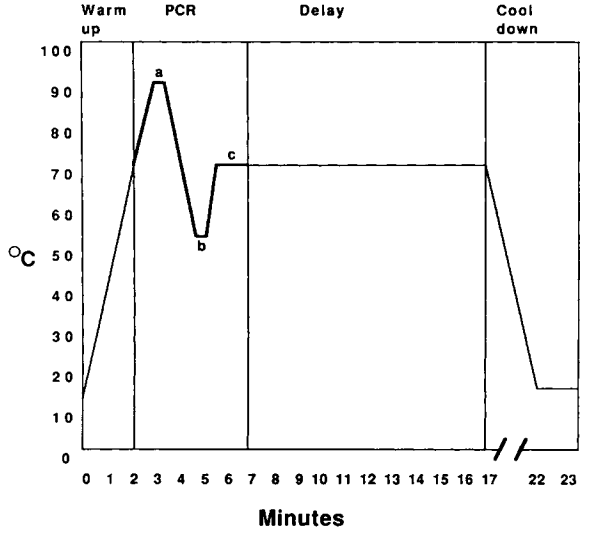

Fig. 2. The four stages in the operation of the programmable heating block for PCR. The three-temperature PCR cycle is repeated 30 to 90 times. PCR kinetics: **a** = dimer denaturing (95°C, 30 s); **b** = primer-template reannealing (50–70°C, 30 s); **c** = primer extension (72°C, 90 s).

the PCR is exponential, similar to equations for describing bacterial growth and compound interest:

$$N = N_0(\text{eff.})^n$$

where N is number of amplified fragments, N_0 is number of input cDNA molecules, eff. is efficiency (1–2 is equivalent to 0–100%), and n is cycle number. However, empirically we have found that cDNA generates fragments efficiently and exponentially only during the first 10–20 cycles of the PCR. PCR cycles 30–60 are often only 10% efficient (Fig. 3). These data also show that the theoretical equation does not describe the generation of the fragments. Moreover, extrapolation of the data by curve fitting does not work and shows that small changes in the efficiency of reaction make it impossible to predict N_0 from late cycles, except with the use of a standard, as shown below.

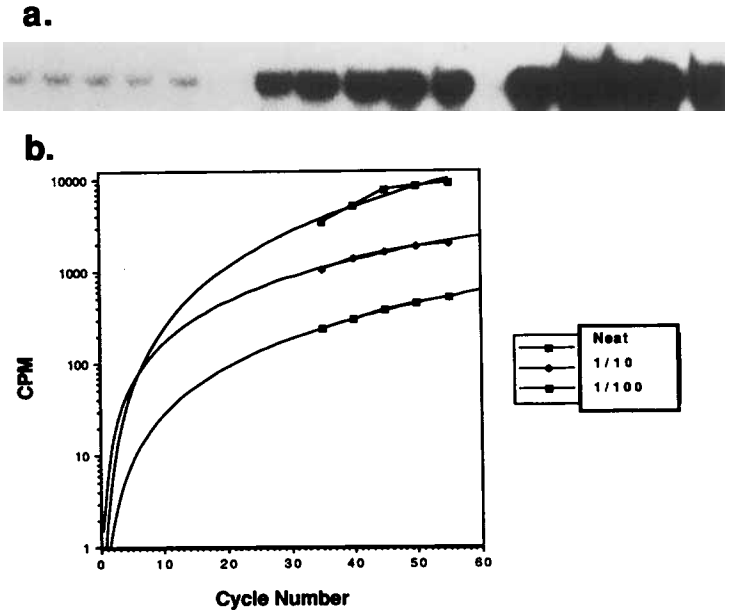

Fig. 3. Quantification of differences in input actin mRNA by sampling at various cycles. The efficiency of the generation of PCR products declines after the first 10 to 20 cycles. Note that tenfold differences in input cDNA result in resolvable differences in output specific signal at all time points measured. These data were obtained by polyacrylamide gel electrophoresis of ^{32}P-labeled cDNA products of PCR as described in Materials and Methods. **a:** Autoradiogram of gel. The three sections are 1/100, 1/10, and undiluted mRNA preparations, respectively, sampled at 35, 40, 45, 50, and 55 cycles; **b:** cpm of each band of the gel shown in **a**.

A single cell with approximately 1,000 β-actin transcripts [14] generated a signal after RT-PCR that was detectable by ethidium bromide staining after 65 PCR cycles (Fig. 4). With increasing actin mRNA input from 10^2 and 10^4 macrophages, the signal appeared at earlier cycles. At large cycle numbers, there was some variability in the strength of the ethidium bromide signal for individual samples; therefore the incorporation of [^{32}P]dCTP into the PCR-generated fragments was the preferred method for quantitative analysis. Signals were validated by four means: a) the size of the fragment; b) the presence of diagnostic restriction sites (Fig. 4a); c) detection by cDNA probes; and d) direct or indirect sequencing. The results from analysis of a single cell suggested that PCR is less efficient when cDNA mixtures, compared with genomic DNA mixtures, are used. Because the input number of cDNA molecules must be in the range of 1–1,000, we estimated (using the equation given earlier and a 10^{12}-fragment detection limit) that the PCR is 35–55% efficient when cDNA mixtures are used as templates. The PCR has previously been reported to be 67–90% efficient when genomic mixtures are used [4,5]. This discrepancy in efficiency is currently under investigation.

To test the sensitivity of the RT-PCR procedure, we used synthetic NGF cRNA produced from a plasmid containing the SP6 polymerase promoter. We have found that 100 copies of input cRNA can be detected by this method (Fig. 5). This finding agrees with the result from the single-cell experiment shown in Figure 4. In fact, because the input cRNA was not polyadenylated, the 3′ primer was used for priming

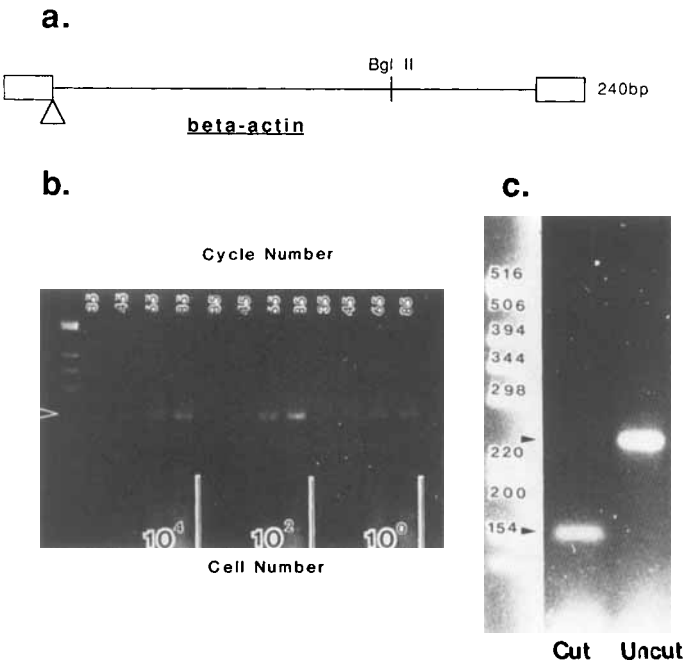

Fig. 4. Generation of a signal detectable by ethidium bromide staining from a single cell. RNA from 1, 10^2, and 10^4 macrophages was reverse transcribed, and the PCR analysis was performed with actin oligonucleotide primers. PCR products were separated by agarose gel electrophoresis, and the gel was stained with ethidium bromide. **a:** Diagram of target β-actin sequence between primers (**boxes**). The position of an intron (**triangle**) and the position of the *BglII* restriction cleavage site are shown. **b:** Initial signal validation by size (**arrowhead**). Numbers at the top are PCR cycles. **c:** Further signal validation by restriction enzyme cleavage of β-actin fragment with *BglII* (**Cut**). DNA molecular weight standards (in bp) are shown at left. **Arrowheads** indicate uncut (**top**) and cut (**bottom**) β-actin fragments.

the RT. Because the latter reaction is known to have a lower efficiency than oligo-dT priming, the sensitivity of the procedure may approach 1 copy of input mRNA with oligo-dT primer. Under ideal conditions the MMLV-RT is 10–50% efficient [13].

A difficulty in quantification is suggested by the equation we have been using ($N = N_0(\text{eff.})^n$). A small difference in efficiency could lead to large differences in N. Ideally, small input (N_0) mRNA could be quantified most accurately by using an exogenous cRNA with the same sequence (from the 5′ primer to the poly A tail) as the endogenous mRNA in question. The exogenous cRNA would be diluted serially as an internal standard, and then the amount of endogenous mRNA could be extrapolated from the equation of the line. For this endogenous standard to succeed, we first needed to analyze the resolving power of the RT-PCR procedure. In the experiment shown in Figure 6, 6.5×10^3 to 1.3×10^7 copies of specific cRNA were used in the RT-PCR in threefold dilutions. The results demonstrate that the RT-PCR can resolve < threefold differences in N_0 cRNA, with the greatest sensitivity at low N_0. These data will allow us to construct standard curves in an attempt to extrapolate a copy number for the low-copy N_0 endogenous mRNA. Input RNA present in samples of tissue in which input cell number cannot be determined accurately can be measured by RNA blot analysis of the cellular ribosomal RNA with cDNA probes

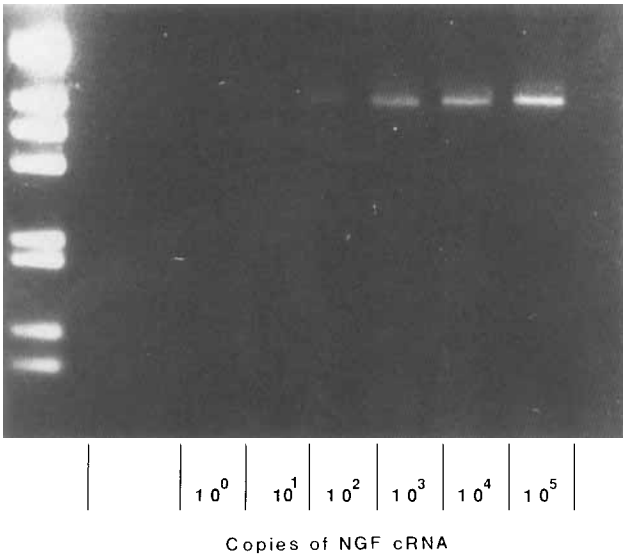

Fig. 5. Detection of a threshold of 100 molecules of NGF cRNA after RT-PCR, followed by agarose gel electrophoresis and ethidium bromide staining after 60 cycles of PCR.

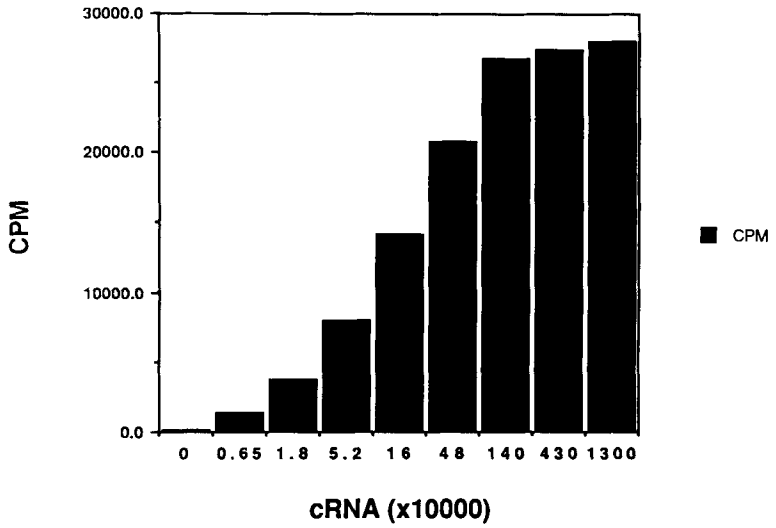

Fig. 6. Resolution of threefold differences in input (N_0) pBR322-IL-1α cRNA after PCR amplification of cDNA. Samples were serially diluted, then subjected to RT-PCR. The ^{32}P-labeled cDNA products were separated by gel electrophoresis and the radioactivity was determined.

for vertebrate 28S and/or 18S rRNA. This method can easily detect the rRNA in 2–10 cells (unpublished observation).

The combination of techniques for reverse transcribing mRNA into cDNA by using MMLV-RT and amplifying specific cDNA segments > 10^9-fold (using Taq DNA polymerase in the PCR) has allowed us to develop a method for phenotyping mRNA in low numbers of cells or with low amounts of mRNA. Some of the advantages and disadvantages of the method are listed in Tables II and III. The major

TABLE II. Advantages of RNA Phenotyping Method

1. Rapid: results in < 1 day
2. Sensitive: < 100 mRNA molecules detected
3. Low numbers of cells: RNA from 1 to a few thousand cells can be analyzed
4. mRNA phenotyping: > 10 different mRNA species within a single sample can be analyzed
5. Rare mRNA: specific detection is possible
6. Resolution: < threefold differences can be determined
7. Alternative splicing: easy analysis
8. Multiple species: primers often work across species
9. Rapid cDNA cloning: preparation of species-specific probes is possible
10. Rapid sequencing: amplified sequences can be sequenced directly
11. Safe: analysis does not require ^{32}P
12. Probes: rapid preparation of high specific activity probes
13. Easy: many samples can be analyzed simultaneously
14. Related molecules: easy discrimination between molecules

TABLE III. Disadvantages of RNA Phenotyping Method

1. Does not give mRNA sizes directly
2. Sensitive to mRNA secondary structure
3. Does not give three-dimensional information about transcript distribution in cells and tissues
4. Quantification takes effort
5. Less resolution (3:1) than other methods
6. Some sequence information needed
7. Error rate is high (1/6000) for Taq polymerase

advantages of this novel RNA phenotyping procedure are the ease and rapidity with which mRNA transcripts from limited material can be unambiguously demonstrated. The simultaneous analysis of up to ten different mRNA species in a single small sample of RNA is possible [15]. Specific detection of rare mRNA species, such as those for growth factors, is possible [15,16]. By specific primer design, alternatively spliced mRNAs can be determined. With careful design (e.g., use of conserved amino acids with low degeneracy of codons), primers often work for several species (e.g., human PDGF-A in mouse) [15], even when the species-specific cDNA sequences have not been determined. The PCR fragments so generated can be sequenced and also used as homologous cDNA probes for other types of RNA analysis [17]. Specific primer design or specific restriction endonuclease analysis allows rapid analysis and discrimination of closely related molecules in very small samples [17]. Simultaneous analysis of several dozen samples differing in either input RNA and/or primer pairs is possible.

The major disadvantage of this method is that the size of mRNA species is not a direct result of the analysis. Knowledge about alternative splicing patterns is needed to circumvent this problem. As is true for any reaction involving reverse transcription, the technique is sensitive to secondary structure in mRNA. Of >30 primer pairs designed, a few have been unsuccessful even for abundant mRNA species. Not all primer pairs work with the same efficiency or reliability. Quantitative analysis is cumbersome and difficult, although semiquantitative data can be obtained readily

with dilution curves. Although this RT-PCR procedure can be more sensitive than in situ hybridization and has shown the presence of mRNA transcripts not found previously by the latter procedure, it does not give three-dimensional information about transcript distribution in cells and tissues. Good sequence homology is needed to cross species. Although the error rate for Taq polymerase has been estimated at about 1/6,000, only one base-pair error has been detected in sequencing about 5,000 bp of known sequence [17]. However, this could present a problem in analysis of point mutations in transcripts.

The potential exists for obtaining these data quantitatively. The RNA phenotyping procedure is superior in ease, speed, sensitivity, and resolution to mRNA analysis by in situ hybridization, RNA (Northern) blots, and nuclease protection assays. In other work, we have used this procedure to demonstrate growth-factor transcripts in macrophages stimulated by inflammatory agents in culture and in macrophages obtained directly from healing wounds in vivo [15]. We have also used this method to analyze the regulation of maternal and embryonic transcripts for growth factors [16] and the regulation of proteolytic enzymes [17] in single eggs and preimplantation embryos.

ACKNOWLEDGMENTS

This work was supported by the Office of Health and Environmental Research, U.S. Department of Energy (contract DE-AC03-76-SF01012). D.A.R. was the recipient of a U.C. Regents Fellowship and a U.C. Patent Fund Award. We thank Mike Doyle for providing the Hep-SK cells, Mary McKenney for editing the manuscript, and Renee Horiuchi for typing it.

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