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A polymerase chain reaction assay for simultaneous detection and quantitation of proto-oncogene and GAPD mRNAs in different cell growth rates

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Abstract. A reverse transcriptase followed by a polymerase chain-reaction (RT-PCR) assay was developed for the simultaneous detection and quantitation of proto-oncogene (c-fos and c-myc) mRNAs using an internal standard mRNA glyceraldehyde-6-phosphate dehydrogenase (GAPD). Total cellular RNA was reverse transcribed and PCR amplified with oligonucleotide primers specific to GAPD and either c-fos or c-myc genes. In contrast to Northern blot analysis, the RT-PCR assay is rapid and sensitive enough to quantitate specific proto-oncogene levels from as little as 12–25 ng of total cellular RNA. The reliability of the assay was tested by measuring c-fos and c-myc expression in C3H 10T1/2 mouse embryo fibroblast cells under two different growth states: (a) quiescent cell entry into the proliferative cycle, and (b) plateau phase. Furthermore, the assay was used in measuring variations in c-fos or c-myc expression in HA-1 hamster cells following exposure to the cellular stressing agent, nitric oxide. In serum-stimulated cells, the RT-PCR measurements of transient increase in c-fos (16-fold at 30 min) and c-myc (10-fold at 1 h) mRNA levels were comparable to previously reported results in the literature using a Northern blotting assay. In addition, a two- to fivefold increase in c-fos mRNA levels was observed in plateau phase cells when compared to log phase growth. Furthermore, a transient increase in c-fos mRNA levels (threefold at 2 h) was also observed following cells' exposure to the stressing agent nitric oxide. These results suggest that the multiplex RT-PCR assay represents a significant improvement over current methods to quantitate specific cellular mRNAs under different growth conditions or following environmental insults.

Proto-oncogenes are evolutionarily conserved genes encoding proteins that are known to play a vital role in the growth and differentiation of mammalian cells (Muller *et al*. 1982, Bishop 1983). Continuous expression of c-fos or c-jun can result in transformation of fibroblasts *in vitro* and the induction of tumour formation *in vivo* (Lee, Lin & Curran 1988, Wang *et al*. 1991). Increased expression of c-myc has been observed in different human and murine malignancies (Croce 1987) and conditional expression of c-myc accelerates cells' transit through the G_1 phase of the cell cycle (Karn *et al*. 1989). Furthermore, increased cellular

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expression of c-fos, c-jun, or c-myc genes occurs in response to a variety of environmental insults including ultraviolet light (Devary *et al.* 1991), X-irradiation (Hallahan *et al.* 1991), changes in redox potential (Yao *et al.* 1994), hypoxia (Fornace *et al.* 1989), single electric pulse (Pazmany *et al.* 1995), low-level electromagnetic fields (Goodman, Basset & Henderson 1989), etc. Because altered expression of c-fos, c-jun, or c-myc gene could be used as an early prognostic indicator for longer term changes in cell growth and/or transformation, a rapid, sensitive and accurate assay is necessary for the determination of the steady-stage levels of specific proto-oncogene mRNAs.

Methods such as Northern blotting and RNAase protection assays are routinely used for determination of the steady-state level of mRNAs. In general, quantitation of results is often time consuming and the assays require a large number of cells for the detection of low abundance messages. Furthermore, stripping of probes and re-hybridization during Northern blotting of multiple mRNAs also decrease the sensitivity of the assay. As an alternative to these methods of detection, several studies have applied the reverse transcription followed by a polymerase chain reaction (RT-PCR) assay for determination of the steady-state levels of mRNAs (Gilliand *et al.* 1990, Murphy *et al.* 1990, Sugimoto *et al.* 1993). However, the high efficiency of the PCR method may also cause amplification of small differences due to input cDNA, primer concentration, etc., that may lead to inconsistent results from experiment to experiment. Although co-amplification of competitive genomic DNA as an internal control has been suggested (Gilliand *et al.* 1990), the use of an exogenous internal standard often becomes cumbersome if the levels of different mRNAs in several samples have to be measured. An alternative approach is to co-amplify the target mRNA in different samples simultaneously with a structurally unrelated endogenous mRNA as an internal standard (Sugimoto *et al.* 1993). However, utilizing such an approach often amplifies disproportionately the highly abundant internal standard mRNA as opposed to the low abundant target mRNA.

In this report, we describe the application of a RT-PCR assay for simultaneous detection and quantitation of c-fos or c-myc mRNA using a highly abundant glyceraldehyde-6-phosphate dehydrogenase (GAPD) mRNA as an internal control. This was accomplished by a series of standardization reactions which determined that a 1:5 ratio of GAPD to c-fos or c-myc primers resulted in a linear response of the PCR amplified products. The reliability and sensitivity of the assay was tested by determining the changes in the steady-state level of either c-fos or c-myc and GAPD mRNAs in quiescent cells as they enter into the proliferative cycle, plateau phase cells and following cellular exposure to the stressing agent, nitric oxide.

MATERIALS AND METHODS

Cell culture

The C3H 10T1/2 mouse embryo fibroblast cell lines were obtained from ATCC (American Type Culture Collection, Rockville, MD) and were cultured in basal minimal essential medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone Labs, Logan, UT), 100 U/ml penicllin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2. Cells were plated at 2×10^5 cells per ml in 100-mm dishes and exponentially growing cells were serum starved by replacing the regular growth medium with medium containing 0.5% fetal bovine serum. After 48 h of serum starvation, quiescent cells were stimulated by adding fresh medium supplemented with 20% fetal bovine serum and cells were harvested for RNA isolation at representative time points. For plateau phase

experiments, RNA was isolated when monolayer cultures were 30–40%, 70–80%, and 100% confluent, and at 4 days following each of the preceding time points.

For the nitric oxide experiments, Chinese hamster fibroblast (HA1) cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 as described earlier (Spitz, Malcolm & Roberts 1990). Exponentially growing cells were treated for 30 min at 37°C with nitrogen or nitric oxide saturated medium. The medium without phenol red, glutamine and serum was first made anoxic with nitrogen gas followed by saturation with nitric oxide gas (3.45×10^4 Pa per 100 ml volume for 20 min). The concentration of nitric oxide in the medium was determined to be 1.87 mM using an extinction coefficient of 5725 M^{-1} cm^{-1} measured at 496 nm wavelength as described earlier (Nims *et al.* 1996).

Isolation of RNA

Total cellular RNA was isolated by using TRI reagent (Molecular Research, Cincinnati, OH) following the manufacturer's protocol. Briefly, cells were homogenized in 1 ml of TRI-reagent and the extracted total cellular RNA was precipitated with isopropanol. The RNA pellet was washed once with 70% ethanol and resuspended in diethylpyrocarbonate treated water. The purity and the yield of total RNA were determined spectrophotometrically and the integrity of the rRNA bands were checked by denaturing agarose gel electrophoresis.

First-strand cDNA synthesis

For the synthesis of cDNAs, 1 μg of total cellular RNA was reverse transcribed in buffer containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 2.5 μM Oligo $(dT)_{16}$, 1U RNase inhibitor, dNTPs 1 mM each, and murine leukaemia virus reverse transcriptase (MuLV RT) 2.5 U in a final volume of 20 μl. Appropriate dilutions of the cDNA pool representing 12–250 ng equivalent of total cellular RNA was used in the PCR assay. Reverse transcription reaction was carried out in polypropylene reaction tubes (Perkin-Elmer, Foster City, CA) and the tubes were kept at room temperature for 10 min before transferring to a Perkin-Elmer GeneAmp PCR system 9600. The reaction cycle consisted of 20 min at 42°C, 20 min at 37°C, 5 min at 99°C, then cooling to 5°C. To minimize errors in pipetting among samples, master mixes of cDNA synthesis buffer containing the dNTPs, reverse transcriptase, oligo dT were prepared and used for reverse transcription of all RNA samples in an experiment.

Oligonucleotides used for amplification

Primer sequences for c-fos, c-myc and GAPD were selected on separate exons flanking an intron to distinguish amplified products of mRNA from possible contaminating DNA or precursor RNA. The primer sequences and PCR product sizes are as follows: c-fos-5′-GAGCTGACAGATACACTCCAAGCG-3′ and 5′-CAGTCTGCTGCATAGAAGGAA-CCG-3′ (432 base pairs (bp), Meijlink *et al.* 1985); c-myc-5′-AGTGCATTGATCCC-TCAGTGGTCTTTCCCTA-3′ and 5′-CAGCTCGTTCCTCCTCTGACGTTCCAAGACG-TT-3′ (548 bp, Stanton *et al.* 1984); GAPD-5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (983 bp, Sabath, Broome & Prystowsky 1990). Optimal melting temperature (T_m) for each primer was estimated by using the PC-GENE software from Oxford Molecular Group (Campbell, CA).

Polymerase chain reaction

For PCR amplification, appropriate dilutions of cDNA samples representing 12–250 ng of total cellular RNA were mixed with PCR buffer containing 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 200 μM dNTPs, 0–1 μM each of the primers and 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) in a total volume of 50 μl. To ensure accuracy of this method, master mixes of PCR amplification buffer was prepared and used for amplification of all cDNA samples in an experiment. This approach minimized errors associated with pipetting different solutions among various samples in the PCR reaction. One microlitre of (1:50 dilution) α^{32}P-dATP (6000 Ci/mMol, NEN) was included in the PCR master mix for quantitation of incorporated radioactivity in each amplified product. The samples were denatured initially at 94°C for 2 min and amplification was performed on a DNA thermal cycler (Perkin-Elmer GeneAmp PCR system 9600) at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s for varying cycle numbers. The final cycle was followed by a 10 min extension step at 72°C. The absence of contaminants was routinely checked by omitting the reverse transcriptase enzyme from the cDNA synthesis step.

Analysis of amplified products

Five microlitres of the PCR amplified products were analysed by electrophoresis on a 1.5% agarose gel. Gels were stained with ethidium bromide, photographed, dried and exposed to a phosphoimager screen for quantitation of the incorporated radioactivity in each individual band. Quantitation was obtained using a STORM 840 Phosphoimager (Molecular Dynamics, Sunnyvale, CA). Relative mRNA levels of each target gene were first normalized to the corresponding GAPD mRNA level in individual samples and the fold increase was determined relative to the 0 h time point.

Northern blotting

To determine the reliability and sensitivity of the RT-PCR assay, duplicate RNA samples were subjected to Northern blot analysis following standard procedures with only minor modifications (Goswami *et al.* 1996). Briefly, 20 μg of total RNA was solubilized and separated by formaldehyde-agarose gel electrophoresis. After transfer to a Gene Screen membrane (NEN), the blots were sequentially hybridized with ^{32}P-radiolabelled probes for c-fos, c-myc and GAPD. Each probe was stripped from the blot prior to the next hybridization. Hybridization probes were first generaated by PCR amplification of each individual cDNA fragment using the primer pairs as described above. Individual plasmid DNAs containing the c-fos, c-myc or GAPD template were obtained from Clontech Laboratories (Palto Alto, CA) and used in the PCR reactions. The PCR amplified products were purified by agarose gel electrophoresis and ^{32}P-radiolabelled by random priming. The blots were washed and autoradiographed for visualization of bands. Quantitation of results was performed using a Personal Densitometer from Molecular Dynamics. Relative levels of c-fos and c-myc mRNAs were determined after normalization for the amount of GAPD mRNA in individual samples.

RESULTS

In order to develop a method for the detection and quantitation of low abundance proto-oncogene mRNA simultaneously with the highly abundant GAPD mRNA, various conditions for the cDNA synthesis step were optimized. First strand cDNA was synthesized from total cellular RNA using MuLV RT with oligo $(dT)_{16}$ priming. The use of oligo $(dT)_{16}$ selectively

reverse transcribed poly $(A)^+$ RNA and minimized non-specific amplifications that are generally associated with reverse transcription using random priming (data not shown). This difference was probably due to the interference between the random primed reverse transcribed rRNAs, which represent the majority of total cellular RNA, and the mRNAs. A temperature combination of 42°C for 20 min and 37°C for a subsequent 20 min was found to be optimal for the synthesis of cDNA. The buffer compositions were standardized for maximal efficiency of the cDNA synthesis and optimal PCR amplification with the Taq DNA polymerase enzyme.

As reported earlier, linearity in the multiplex PCR assay is often difficult due to the different amounts of target mRNAs present initially during the cDNA synthesis step (Sugimoto *et al.* 1993). Therefore, serial dilutions of primer concentrations were tested such that a highly abundant GAPD mRNA did not affect the amplification efficiency of low abundant c-fos and c-myc mRNAs. In Figure 1A the primer concentrations for either c-fos or c-myc were kept constant at 0.5 μM and the primer concentrations for GAPD varied from 0–1 μM. The concentration range of GAPD primer used in these reactions did not have any significant effect in the amplification of c-fos or c-myc cDNAs. Next in a separate set of PCR

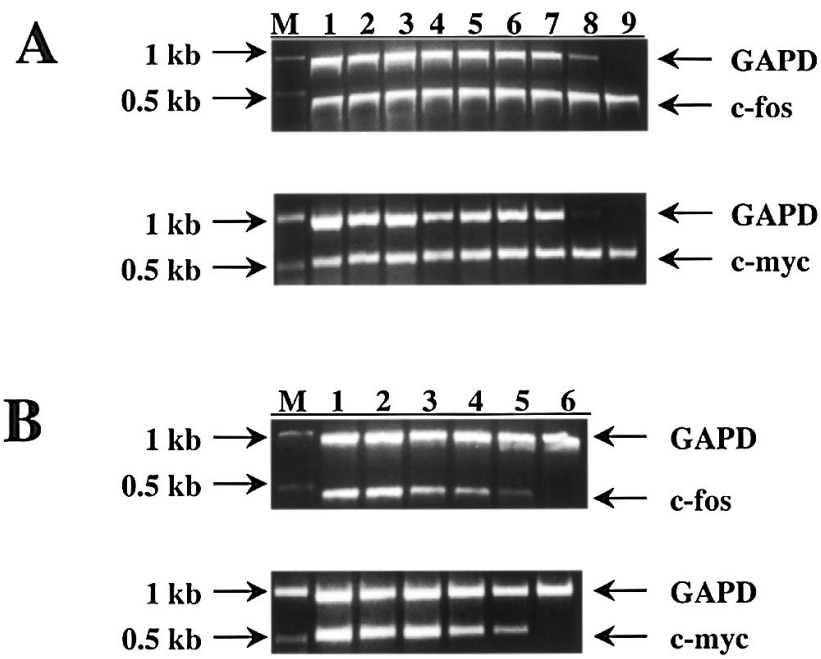

Figure 1. Optimization of primer concentrations in the RT-PCR assay. The first strand cDNA was synthesized by reverse transcription of total cellular RNA and PCR amplified with varying primer concentrations: (A) The primer concentrations of c-fos or c-myc were kept constant at 0.5 μM and GAPD primer concentrations were varied between 0–1 μM (lane 1, 1 μM; lane 2, 0.5 μM; lane 3, 0.1 μM; lane 4, 0.05 μM; lane 5, 0.03 μM; lane 6, 0.025 μM; lane 7, 0.02 μM; lane 8, 0.01 μM; and lane 9, 0 μM). (B) The primer concentrations of GAPD were kept constant at 0.1 μM and the primer concentrations for c-fos, or c-myc were varied between 0–1 μM (lane 1, 1 μM; lane 2, 0.75 μM; lane 3, 0.5 μM; lane 4, 0.25 μM; lane 5, 0.1 μM; and lane 6, 0 μM). Lane M, molecular size markers. The PCR products were separated on 1.5% agarose gels which were then stained with ethidium bromide and photographed under UV light. Gels were dried and exposed to a phosphoimager screen for quantitation of results.

reactions the primer concentrations for GAPD were kept constant at 0.1 μM and the concentrations of primer for the target mRNAs were varied between 0–1 μM (Figure 1B). A linear response in signal intensities for c-fos was obtained between 0.1–0.5 μM primer concentrations (lanes 5–3). For c-myc co-amplification the linear response was obtained between 0.1–0.75 μM primer concentrations (lanes 5–2). From these results an optimal ratio of 1:5 primer concentrations for GAPD to either c-fos or c-myc was selected for co-amplification. In addition, increased specificity was obtained by optimization of temperature and time of primer annealing which was found to be 60°C for 30 s.

The utilization of the multiplex RT-PCR assay as a relative measure of gene expression required optimization of the number of PCR cycles and the input RNA concentration. To determine the optimal cycle number, co-amplifications were carried out for 22–35 cycles. Results from such a study indicted that 25 PCR cycles for co-amplifications of either c-fos or c-myc and GAPD were sufficient to observe a quantifiable signal within the linear range of the amplification (data not shown).

The amount of cellular mRNA levels is known to be a relative measure of gene expression. Therefore, varying aliquots of input cDNAs representing 12.5–250 ng of total cellular RNA (Figure 2) were used in the assay to co-amplify either c-fos or c-myc and GAPD mRNAs. Co-amplification of cDNAs synthesized from 12 to 150 ng of total cellular RNA showed a linear relationship between the amounts of input RNA and PCR products. The co-amplification ratio under these conditions was found to be independent of the template concentration. These results showed that variations in c-fos and c-myc expressions can be accurately measured in as little as 12–25 ng of input RNA. The reproducibility of the assay was tested by performing separate cDNA syntheses from the same batch of RNA and PCR amplification on two different days. The results obtained from such experiments showed less than 2% variation between each PCR product (data not shown).

The reliability of the assay was tested by determining the variations in the steady-state level of c-fos, c-myc, and GAPD mRNAs in serum stimulated and plateau-phase C3H 10T1/2 mouse embryo fibroblast cells. Exponentially growing cells were serum starved for 48 h then stimulated to enter the proliferative cycle by the addition of medium containing 20% serum. Results from Figure 3A showed little variation in the steady-state level of GAPD mRNA following entry into the proliferative cycle suggesting that the GAPD mRNA level could provide a reliable cellular standard for the study of gene expression under these conditions. It can be used as an internal control for variability in RNA input in the cDNA synthesis step, reverse transcription, and subsequent PCR amplification. The relative level of c-fos was low at 0 h (lane 1), increased more than 16-fold by 30 min (lane 3) after serum stimulation, then decreased rapidly to basal levels at 2 h (lane 5) post-stimulation. In contrary to c-fos, the increase in the c-myc mRNA level was slightly delayed peaking at more than 10-fold by 1 h (lane 4) after serum stimulation. The increase in c-myc mRNA level followed a slower decay kinetics and remained elevated at 5 h (lane 7) after serum stimulation. Next we determined c-fos and c-myc mRNA levels in continuously growing C3H 10T1/2 cells when they reached 30–40% (Figure 3B, lane 1), 70–80% (Figure 3B, lane 3), 100% (Figure 3B, lane 5) confluent monolayer cultures and 4 days after each of these growth stages (Figure 3B, lanes 2, 4 and 6). Thus, lanes 2, 4 and 6 represent different times in plateau phase. Results from these experiments showed a two- to fivefold increase in c-fos mRNA levels in plateau phase (lanes 2, 4 and 6) cells when compared to exponential (lane 1), transit to plateau (lane 3) and early plateau (lane 5) phases. Since there were no changes detected for cell number, fraction of S-phase cells, or pH of the medium, the increase in c-fos mRNA levels was considered to be a cell growth stage related effect. In contrast to the increase in c-fos mRNA levels, the

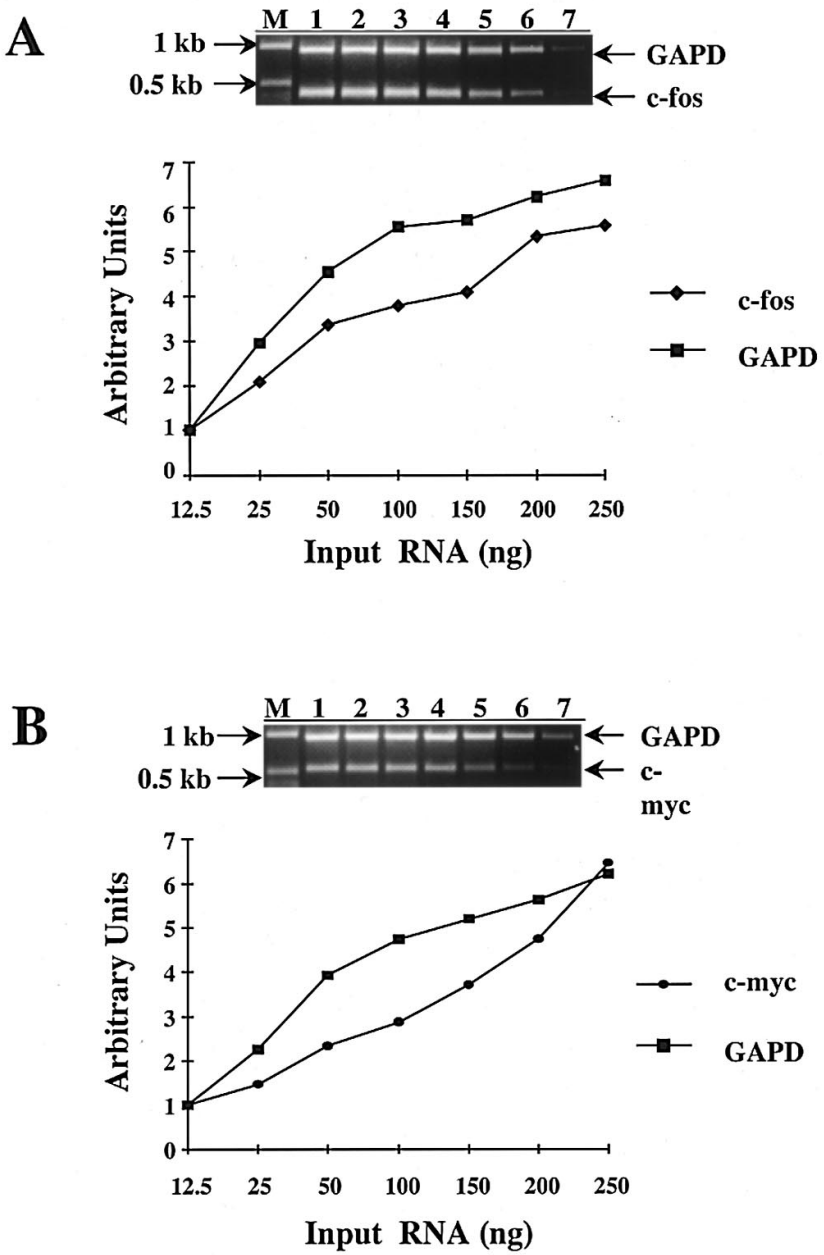

Figure 2. Simultaneous PCR amplifications starting from serial dilutions of input cDNAs: lane 1, 250 ng; lane 2, 200 ng; lane 3, 150 ng; lane 4, 100 ng; lane 5, 50 ng; lane 6, 25 ng; and lane 7, 12.5 ng equivalent of total cellular RNA. Twenty-five cycles of PCR amplifications were carried out using 0.1 μM of GAPD and 0.5 μM of either c-fos (A), or c-myc (B) primers. Relative levels of c-fos or c-myc was determined after normalization for the amount of GAPD mRNA levels in each sample and relative to 12.5 ng sample.

mRNA levels of c-myc did not change significantly as exponential cells transit to the plateau phase (Figure 3B).

To examine further the reliability of the PCR assay, RNA samples were also analysed by Northern blotting (Figure 4). The blot was sequentially hybridized with ^{32}P-radiolabelled probes for c-fos, c-myc and GAPD. The blot was stripped of each probe prior to the subsequent re-hybridization. The probes detected 2.2, 2.3 and 1.8 kb bands that corresponded to the transcript sizes of c-fos, c-myc and GAPD mRNAs, respectively. The GAPD mRNA levels showed no significant variation after serum stimulation, confirming the results obtained from the RT-PCR assay. Furthermore, the kinetics of c-fos and c-myc expression analysed by the Northern blotting method were identical to the results determined from the RT-PCR assay. The steady state level of c-fos mRNA began to increase at 0.25 h (lane 2), peaked at 0.5 h (lane 3) followed by a rapid decrease to basal level by 2 h (lane 5). The kinetics of c-fos expression determined by the Northern blotting technique were similar to results obtained from the RT-PCR assay (Figure 3A). However, in the Northern blotting assay (Figure 4), because c-fos mRNA levels at 0 h (lane 1) remained undetected, an accurate determination for the fold increase at 0.25 h (lane 2) or 0.5 h (lane 3) post-stimulation was not feasible. In contrast, the RT-PCR assay could detect c-fos mRNA levels at 0 h post-stimulation (Figure 3A, lane 1) and, therefore, an accurate quantitation for the fold increase was possible. The

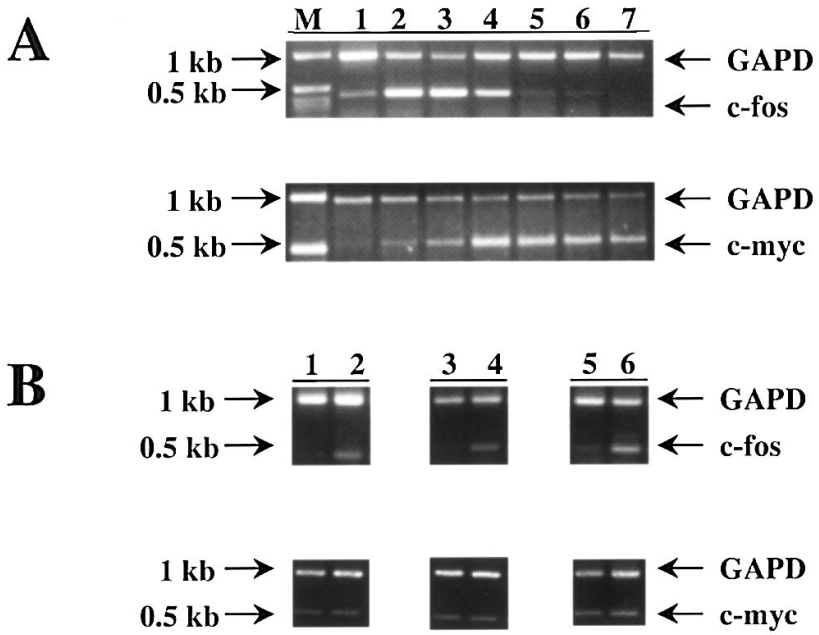

Figure 3. RT-PCR assay for changes in mRNA levels in serum stimulated and plateau phase C3H 10T1/2 mouse embryo fibroblast cells. (A) Total cellular RNA isolated at 0 (lane 1), 0.25 (lane 2), 0.5 (lane 3), 1 (lane 4), 2 (lane 5), 4 (lane 6) and 5 (lane 7) h after serum stimulation of quiescent cells were reverse transcribed and co-amplified for either c-fos, or c-myc and GAPD cDNAs. Lane M represents molecular size markers. (B) mRNA levels of c-fos or c-myc and GAPD representing cells from 30–40% (lane 1), 70–80% (lane 3), 100% (lane 5) confluent monolayer cultures and 4 days subsequent to each time point (lanes 2, 4 and 6). Quantitation of results was obtained by normalizing to the individual GAPD levels and relative to each growth states.

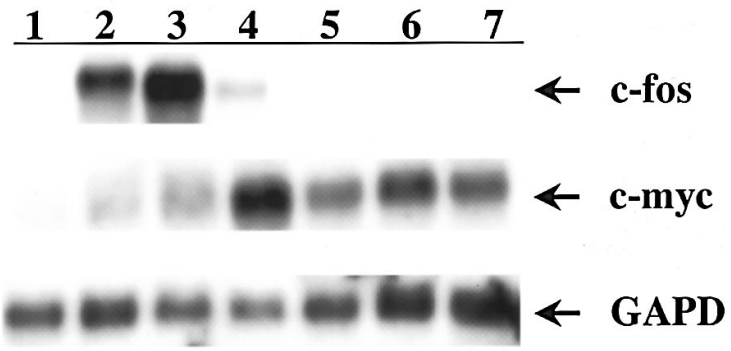

Figure 4. Northern blot analysis. Twenty micrograms of total cellular RNA isolated at 0 (lane 1), 0.25 (lane 2), 0.5 (lane 3), 1 (lane 4), 2 (lane 5), 4 (lane 6) and 5 (lane 7) h after serum stimulation of quiescent C3H 10T1/2 mouse embryo fibroblast cells were separated by agarose-formaldehyde gel electrophoresis. After separation, RNA was transferred to Gene Screen Plus hybridization membrane and the blot was sequentially hybridized with ^{32}P-radiolabelled probes of c-fos, c-myc and GAPD. Each probe was stripped from the blot prior to the next hybridization. Quantitation of results was performed by densitometric scanning of the autoradiographs. Results were first normalized to GAPD mRNA levels in each sample and the fold increase was determined relative to the 0 h time point.

kinetics of c-myc expression as determined from the Northern blotting method were also identical to the results obtained from the RT-PCR assay, suggesting that the RT-PCR assay could provide a suitable alternative to the Northern blotting technique.

The applicability of the RT-PCR assay was further tested by determining whether c-fos or c-myc expression changed in proliferating cells following exposure to a stress agent nitric oxide. Continuously proliferating Chinese hamster (HA1) cells were exposed to either nitric oxide (Figure 5A) or nitrogen (control, Figure 5B) and total cellular RNA isolated at 0, 0.5, 1, 2 and 4 h after the treatment. Results from Figure 5A showed a transient nitric oxide-induced increase in c-fos mRNA levels peaking threefold at 2 h (lane 5) followed by a rapid decrease at 4 h (lane 6) post-treatment. Since in proliferating cells the steady-state level of c-fos mRNA was relatively low (lane 1), the increase in c-fos mRNA levels at 2 h after the nitric oxide treatment was believed to be a stress response. This is particularly relevant since in the nitrogen treated control (Figure 5B), no changes were detected in the relative level of c-fos mRNA (lanes 1–5). In contrast to the transient increase in c-fos mRNA levels, the relative levels of c-myc mRNA did not change following the nitric oxide or nitrogen treatments.

These results showed that the RT-PCR assay is sensitive enough to determine changes in specific proto-oncogene mRNA levels following cells' exposure to environmental stress agents.

DISCUSSION

In this study we describe the application of a RT-PCR assay for simultaneous detection and quantitation of specific proto-oncogene mRNA levels in small quantities of total cellular RNA. The major procedural modification employed was the adjustment of relative primer concentrations to simultaneously amplify a highly abundant housekeeping gene mRNA GAPD, and a relatively low abundance of proto-oncogene c-fos or c-myc. The oligonucleo-

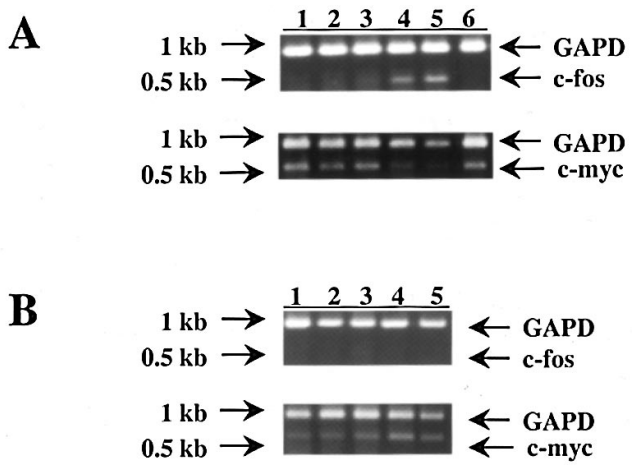

Figure 5. RT-PCR assay for changes in mRNA levels in Chinese Hamster (HA1) cells exposed to nitric oxide. (A) Total cellular RNA isolated at 0 (lane 2), 0.5 (lane 3), 1 (lane 4), 2 (lane 5) and 4 (lane 6) h after a 30 min nitric oxide treatment was reverse transcribed and co-amplified with either c-fos and GAPD, or c-myc and GAPD primers. Lane 1 represents RNA from exponentially growing cells prior to the nitric oxide treatment. (B) Cells in duplicate dishes were treated with nitrogen gas for 30 min and total cellular RNA isolated for RT-PCR analysis at 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 2 (lane 4) and 4 (lane 5) h after the treatment.

tide primers were chosen in separate exons flanking an intron allowing the amplified target cDNA product to be readily distinguishable from any genomic DNA contamination or unspliced mRNA products. The RT-PCR assay is rapid, accurate and more sensitive than the Northern blotting assay. This is particularly significant in situations where sample size may limit the use of the Northern blotting assay.

The simultaneous amplification of a structurally unrelated and highly abundant GAPD mRNA provides rapid and accurate quantitation of the steady state level of low abundance proto-oncogene mRNAs. Additionally, the assay also minimizes signal intensity losses that generally occurs during the Northern blotting analysis by sequential hybridization for multiple genes. Furthermore, in contrast to the Northern blot assay where detection of c-fos or c-myc bands required at least 4 days of exposure of the autoradiographs, the RT-PCR assay was rapid enough to obtain results in 1 day. In addition, the assay is sensitive enough to detect c-fos and c-myc mRNA from as little as 12–25 ng of total cellular RNA (Figure 2) whereas 20 μg of total cellular RNA was required for a standard Northern blot assay (Figure 4). The use of total cellular RNA in contrast to poly A^+ RNA in the RT-PCR assay minimized the risk of losing weakly expressed proto-oncogene mRNAs. Moreover, the use of oligo $(dT)_{16}$ instead of random priming during the reverse transcription step also increased the specificity of the reaction by transcribing only the poly A^+ RNA. In general, random priming during the cDNA synthesis step resulted in non-specific amplification of PCR products (data not shown). This difference was probably due to interference between the random primed reverse transcribed rRNAs, which represent the majority of total cellular RNA, and the mRNAs. Previous reports in the literature described the use of genomic DNA as a competitive target in the RT-PCR assay for quantitation of gene expression (Gilliand *et al.* 1990, Sugimoto *et al.* 1993). One potential limitation of such a competitive RT-PCR assay is the dependence upon the efficiency of reverse transcription. If this is less than 100% or

varies from sample to sample then the assay will underestimate the mRNA level among samples. The inclusion of GAPD mRNA as an internal control in our assay eliminates such artefacts during the cDNA synthesis and subsequent PCR amplification steps.

In this report we have also demonstrated the utility of the RT-PCR assay for determining variations in c-fos or c-myc mRNA levels in three different cell growth conditions: (a) quiescent cells' entry into the proliferative cycle (Figure 3A); (b) plateau phase (Figure 3B); and (c) following proliferating cells' exposure to stress agent nitric oxide (Figure 5). The kinetics of c-fos and c-myc expression in serum stimulated C3H 10T1/2 cells as determined by the RT-PCR assay (Figure 3A) were identical to the Northern blot analysis (Figure 4) and were similar to previously reported results in the literature using Balb\c-3T3 cells (Kruijert *et al*. 1984, Muller *et al*. 1984). In addition, significant differences in c-fos mRNA levels were observed in different cell growth states by both methods of analysis. The expression of c-fos was more pronounced at different times in plateau phase growth as compared to exponential, transit to plateau or early plateau phase growth (Figure 3B). The increase in c-fos mRNA levels in plateau phase growth remained elevated in contrast to a transient increase in serum stimulated cells (Figure 3A). Since there were no changes detected for cell number, fraction of S-phase cells, or pH of the medium, the increase in c-fos mRNA levels was considered to be a cell growth state related effect. Furthermore, using the RT-PCR assay (Figure 5) we have extended and repeated our earlier results of Northern blot analysis showing a nitric oxide-induced increase in c-fos mRNA levels in HA1 cells (Ridnour, unpublished results). The increase in c-fos mRNA is considered to be a nitric oxide induced stress response in this cell line as such a response was absent in nitrogen treated cells (Figure 5B) and in exponentially growing cells (lane 1, Figure 5A). The kinetics of c-fos expression were, however, different from serum stimulation in which the amximal increase was at 30 min (lane 3, Figure 3A) instead of 2 h for the nitric oxide treatment (lane 5, Figure 5A). In addition reuslts from this study showed no significant variations in the steady state level of c-myc mRNA when HA1 cells were exposed to similar nitric oxide treatments.

In summary, we have described a rapid, accurate and sensitive RT-PCR assay for simultaneous detection and quantitation of cellular mRNA levels for c-fos and c-myc proto-oncogene. Using this assay, increased levels of c-fos mRNA were detected in plateau phase growth and in cells exposed to the stressing agent nitric oxide. Because variations in the expression of c-fos or c-myc genes are commonly associated with cell proliferation and/or transformation, the RT-PCR assay may represent a significant improvement over current methods to quantitate specific cellular mRNAs under different growth conditions or following environment insults.

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