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Reverse Transcription and Polymerase Chain Reaction Technique for Quantifications of mRNA in Primary Astrocyte Cultures

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The reverse transcription and polymerase chain reaction technique (RT-PCR) was assessed for the quantification of changes in mRNA levels from primary astrocyte cultures. The effects of dibutyryl cyclic AMP (dBcAMP) on glial fibrillary acidic protein (GFAP) mRNA and the effects of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and lipopolysaccharide (LPS) on interleukin-6 (IL-6) mRNA were examined. Two quantitative PCR methods were used: one involved carrying out the reaction in the exponential phase and the other involved the coamplification of a competitive target sequence. Increased GFAP mRNA in response to chronic dBcAMP treatment and increased IL-6 mRNA in response to TNF-α/IL-1β were readily detected. Both RT-PCR techniques were found to be suitable for the detection of large as well as smaller (twofold) changes in mRNA levels. The advantages and limitations of RT-PCR for mRNA quantification are discussed.

Key words: mRNA expression, glial fibrillary acidic protein, interleukin-6, TNF-α, IL-1β

INTRODUCTION

Recently reverse transcription in combination with polymerase chain reaction (RT-PCR) has been used to detect and measure messenger RNA (mRNA). A number of approaches have been proposed (Wang et al., 1989; Gilliland et al., 1990; Murphy et al., 1990). In this paper we describe the use of RT-PCR to quantify changes in mRNA in primary astrocyte cultures in response to a variety of stimuli. The feasibility of this technique for both large and small changes in mRNA levels is examined.

Two strategies for quantitative RT-PCR were used in the present study. The first is a modification of a method developed by E. Murphy et al. (1993) for cytokine mRNA quantification. This approach is based on the fact that the amount of PCR product is proportional to the amount of starting material when amplification is performed within the exponential phase of the reaction. The second method employs coamplification of a competitive DNA standard with the experimental sample in a single reaction tube (Wang et al., 1989; Gilliland et al., 1990). By performing PCR reactions with varying amounts of standard, but a constant amount of the experimental sample, the starting amount of the experimental sample can be determined.

We examined levels of mRNA coding for interleukin-6 (IL-6) and glial fibrillary acidic protein (GFAP) in primary mouse astrocyte cultures. IL-6 belongs to a family of neuroactive cytokines, which also includes leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) (Patterson, 1992), and has been implicated in the pathology of Alzheimer's disease (Vandenabeele and Fiers, 1992). Constitutive IL-6 mRNA expression by unstimulated astrocytes has been reported by some investigators (Wesselingh et al., 1990), but not others (Benveniste et al., 1990; Sawada et al., 1992). However, prior studies are in agreement that stimulation of astrocytes with a combination of interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) or with lipopolysaccharide (LPS) causes an increase in IL-6 mRNA. In the present study we used TNF-α/IL-1β stimulation of astrocytes to assess the suitability of RT-PCR for quantification of large changes in astrocyte IL-6 mRNA expression, as well as detection of low levels of constitutive expression.

Morphologic differentiation can be induced in astrocyte cultures by stimulation with dibutyryl cyclic AMP (dBcAMP) (Hertz, 1990), which mimics activation of the cAMP second messenger system. There is also evidence that chronic treatment with dBcAMP increases GFAP

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mRNA in astrocyte cultures by about twofold, based on Northern blotting (Le Prince et al., 1991). GFAP is the key biochemical marker for astrocytic activation, and effective measurement of GFAP and its message is important in studying the regulation of astrocytic activation (Eng et al., 1992). In this study we used chronic dBcAMP stimulation to examine the utility of RT-PCR for measuring smaller changes in transcript levels.

MATERIALS AND METHODS

Cell Culture

Primary astrocyte cultures were established from neonatal mice neopallia according to the method of Yu et al. (1982). Cultures were grown to confluence for 10–14 days in 10% fetal calf serum (FCS; Hyclone, Ogden, UT) in Dulbecco's modified essential medium (GIBCO BRL, Gaithersburg, MD) with 2 mM L-glutamine and 0.2 mM penicillin and 0.05 mM streptomycin at 37°C in a humidified 95%/5% (vol/vol) mixture of air and CO_2. Culture media were changed twice weekly. At confluence, the cultures were greater than 95% positive for GFAP by immunostaining. Immunostaining with a Mac-1 antibody detected an average of 2–3 microglial cells per 100× field in a 35 mm dish.

For dBcAMP stimulation, cultures were treated with dBcAMP (Sigma, St. Louis, MO) at a concentration of 1 mM in FCS-containing medium, whereas the controls were continued on FCS-containing medium. Media for both groups were changed twice per week, with the addition of fresh dBcAMP to the treatment group. After 14 days both groups of cells were harvested and total RNA was extracted.

For TNF-α/IL-1β and LPS stimulation, astrocytes were grown to confluence on FCS-containing medium with twice weekly changes, then changed to either chemically defined medium (CDM) (Morrison and De Vellis, 1981), or medium containing horse serum (HS). Recombinant basic fibroblast growth factor (bFGF) used in the CDM was a gift from Dr. Barbara Cordell, Scios/Nova (Mountain View, CA). The cells were maintained on CDM for 11 days, with medium changes twice per week. The cultures were then divided into three groups. The first group received fresh CDM, the second group received CDM containing murine recombinant TNF-α, 100 ng/ml of medium, and murine recombinant IL-1β, 1 ng/ml (both from R & D, Minneapolis, MN), and the third group received CDM containing LPS (10 μg/ml) plus calcium ionophore A23187 (5 μM) plus cycloheximide (5 μg/ml) (all from Sigma). After 15 hr the cells were harvested for total RNA extraction. The cells grown in HS-containing medium were maintained with twice weekly medium changes and harvested at the same time as the other cultures.

RNA Extraction

Total cellular RNA was prepared according to the method of Chomczynski and Sacchi (1987), with the addition of a second phenol-chloroform-isoamyl alcohol extraction to increase RNA purity. RNA integrity was verified on agarose gels with ethidium bromide staining.

RNA was quantified using a spectrophotometer at a dilution of 1:100. In addition to determining the ratio of absorbance at 260 and 280 nm (ratios were 1.8–2.0), we also assessed each sample for absorbance at 230 nm, which reflects residual contamination with guanidinium thiocyanate, used in the extraction process. RNA samples employed in this study had 260/230 ratios of at least 1.8.

RT

RT was performed from 1 μg of total RNA. The 20 μl reaction mixture consisted of 5 mM $MgCl_2$, 1:10 dilution of PCR buffer II (Perkin-Elmer, Norwalk, CT), 2 mM dNTPs (Pharmacia, Piscataway, NJ), 20 U RNasin (Promega, Madison, WI), 1 μg random hexamers (Pharmacia), 1 μl of AMV reverse transcriptase diluted to 1 U/μl (IBI, New Haven, CT), and 6.5 μl of nuclease-free water (Promega). The reaction mixture was incubated in a Microamp tube (Perkin-Elmer) at 42°C for 60 min, then 95°C for 5 min in a Perkin-Elmer 9600 Thermal Cycler. RT products (cDNA) were used immediately, or stored at −20°C. For comparisons of mRNA levels among different RNA samples, RT was performed simultaneously using reagents from a single master mix.

PCR

Four sets of PCR primers were used: GFAP and IL-6, the genes of interest, and hypoxanthine phosphoribosyl transferase (HPRT) and β-actin, which were used to assure equal starting amounts of cDNA for each sample. For comparisons among samples, PCR was performed simultaneously from a single master mix (but in different tubes) with the primers for the cDNA of interest (GFAP or IL-6) and the control primers (HPRT or β-actin). PCR was carried out in a reaction volume of 50 μl using a master mix containing 5 μl of 10× PCR buffer (5 U/μl, Perkin-Elmer), 0.2 mM dNTP (Pharmacia), 1 μl each of the forward and reverse primers (10 O.D. stock concentration), 0.2 μl of Taq polymerase (Perkin-Elmer), 37 μl of nuclease-free water (Promega), and 5 μl of RT product (cDNA). The reaction was performed in a Microamp tube (Perkin-Elmer) in a Perkin-Elmer 9600 Thermal Cycler. After 2 min at 92°C, 30 cycles of amplification were performed consisting of 91°C for 10 sec, followed by 59°C for 25 sec, followed by 72°C for 25 sec. This PCR cycle was determined by E. Murphy et al. (1993) to be optimal for the HPRT and IL-6 primers. We found it also effective for the β-actin primers. For

GFAP amplification, after 2 min at 94°C, 30 cycles were performed consisting of 94°C for 20 sec, 60°C for 60 sec, and 72°C for 140 sec.

For GFAP quantification, the forward primer was located at nucleotides 423–437 of the mouse GFAP genomic sequence, whereas the reverse primer was located at nucleotides 3806–3825 (Balcarek and Cowan, 1985), spanning 4 introns. The size of the fragment amplified from cDNA was 656 bp. For HPRT amplification, the forward primer was located at nucleotides 318–339 (based on sequence of clone pHPT5 given in Melton, 1987), whereas the reverse primer was located at nucleotides 473–495, yielding a product of 178 bp. The intervening genomic sequence included a large intron. Further, the primer sites each contained a splicing point for large introns. For IL-6 amplification, the forward primer was located at nucleotides 1420–1540, whereas the reverse primer was located at nucleotides 6076–6093, yielding a product of 341 bp. The primer sites spanned 2 large introns. The β-actin forward primer was located at nucleotides 25–45 (Alonso et al., 1986), whereas the reverse primer was located at nucleotides 531–564), producing a product of 540 bp. According to the manufacturer (Clonetech, Inc., Palo Alto, CA), amplification of genomic DNA does not result in a 540 bp band, suggesting the presence of introns in the region amplified. The GFAP primers were synthesized by Operon, Inc. (Alameda, CA) and the HPRT and IL-6 primers were gifts from Dr. Anne O'Garra of DNAX Research Institute (Palo Alto, CA). The β-actin primers were purchased from Clonetech, Inc. PCR products were examined on agarose gels with ethidium bromide staining to verify the expected size of the amplified fragment using a 123 bp DNA ladder (GIBCO BRL, Gaithersburg, MD) as a standard and to rule out amplification of high molecular weight products derived from genomic DNA (see Fig. 1).

Fig. 1. Two percent agarose gel stained with ethidium bromide showing PCR products amplified from cDNA derived from astrocyte total RNA. Lane 1: DNA 123 bp molecular size ladder; lane 2: GFAP; lane 3: IL-6 (RNA from cells stimulated with TNF-α/IL-1β); lane 4: HPRT; lane 5: β-actin. The intensities of the signals are not necessarily indicative of differences in expression among the various mRNAs, as amplification efficiencies differ. Note that there is no signal from genomic DNA detected in the starting wells in lanes 2–5.

Slot Blot

One quarter (12.5 μl) of the product from each PCR reaction was denatured at room temperature with a solution of 0.4 N NaOH and 25 mM EDTA. The denaturing reaction was stopped after 10 min with 1 M Tris, pH 8.0. The product was then transferred to Nytran, 0.2 μm (Schleicher and Schuell, Keene, NH), which had been wet with 5× SSC, in a slot blot apparatus (Bio-Rad, Richmond, CA). The Nytran containing the PCR product was dried at 80°C for 1 hr, or exposed to ultraviolet (UV) light with a total energy of 120 mJ/cm^2 (Spectronics UV linker, Westbury, NY), and stored desiccated at 4°C until ready for use.

Probe Labeling

Oligonucleotide probes complementary to sites internal to the upstream and downstream primers were used for detection of PCR products transferred to nylon membranes. The probe for IL-6 [27-mer, bases 1594–1620 (Tanabe et al., 1988), sense orientation] was a gift from Dr. Anne O'Garra, as was the probe for HPRT [28-mer, bases 440–467 (Melton, 1987), sense orientation]. The β-actin probe [30-mer, bases 281–310 (Alonso et al., 1986), antisense orientation] was purchased from Clonetech, Inc. The GFAP probe [21-mer, bases 1806–1826 (Balcarek and Cowan, 1985), sense orientation] was synthesized by Operon, Inc. 5′-End labeling of probes with γ-^{32}P-ATP (NEN, Boston, MA) was accomplished using T4 polynucleotide kinase (Promega) following the protocol of Sambrook et al. (1989). After labeling, probes were Sephadex column (Boehringer Mannheim, Indianapolis, IN) purified to remove unincorporated nucleotides.

Hybridization

Nylon membranes were met with 2 ml prehybridization solution (6× SSC, 0.5% SDS, 5× Denhardt's solution, 100 μg/ml denatured salmon sperm DNA) at 42°C for 1 hr in a 50 ml centrifuge tube rotating in a hybridization oven (Robbins Scientific, Sunnyvale, CA). The prehybridization solution was removed and 2 ml of hybridization solution (6× SSC, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA) and 50 μl of purified probe (approximately 25 × 10^6 cpm) were added and incubated with rotation at 50–60°C for 2–3 hr, depending on the particular probe. After hybridization Nytran membranes were washed twice for 15 min in 2× SSC/0.1% SDS at 50–60°C and then once for 15 min in 1× SSC/0.1% SDS at 37°C. X-ray film was exposed to the washed membranes with an intensifying screen for varying times depending on the activity of the particular probe.

Quantification

Hybridization signals on developed autoradiographs were quantified using a Pharmacia LKB Ultroscan XL Laser densitometer, and results expressed in densitometer units, which reflect signal intensity. All samples were initially assessed for HPRT and β-actin expression. Expression of these mRNAs were generally equal among samples, although small variations existed. Where variation existed, the amount of cDNA used for PCR was adjusted and the reaction was performed again. This was repeated until the results showed equal HPRT and/or β-actin levels among samples. Then, the levels of the mRNAs of interest (GFAP, IL-6) were determined using the adjusted cDNA amounts. In general, levels of HPRT and β-actin were equal in each sample relative to others.

Negative controls for contamination were performed routinely in our laboratory, by doing RT without RNA, or PCR amplification without cDNA. No products were detected from these negative control experiments. Likewise, the entire slot blot and hybridization protocols were performed without including the PCR product, or with a PCR product not complementary to the probe used. Neither of these negative controls produced a signal on autoradiographs.

Competitive PCR

To construct the competitive PCR standard for quantification of GFAP, a 633 bp DNA fragment of known sequence was used (PCR MIMIC kit, Clonetech, Inc.). Composite primers consisting at the 5′-end of the GFAP primers and at the 3′-end sites on the DNA fragment were synthesized by Operon, Inc. Amplification of the DNA fragment with the composite primers according to the manufacturer's directions resulted in a new fragment (competitive standard) of 470 bp with the GFAP primer sites at the 5′-ends of the sense and antisense strands of the fragment. This competitive standard differed by 186 bp from the 656 bp GFAP fragment amplified from cDNA derived from astrocyte total RNA. The competitive standard was purified through a Sephadex column (Boehringer Mannheim) and visualized on an ethidium bromide-stained agarose gel, which demonstrated the predicted size. The yield of the 470 bp competitive standard was determined by comparison on an ethidium bromide-stained gel with dilutions of a øX174/HaeIII-digest DNA of known concentration provided by the manufacturer (data not shown).

Competitive PCR was performed by amplifying in the same tube 2 μl of RT product (cDNA; equivalent to about 0.1 μg of total RNA) and 2 μl of serial diluted competitive standard. The competitive standard 1:2 dilution series began at 1 × 10^{-1} attomol and proceeded to 3 × 10^{-3} attomol. Total reaction volume, including the master mix, was 52 μl. Amplification was performed by incubating the reaction mixture at 94°C for 120 sec, followed by 30 cycles of 94°C for 20 sec, 60°C for 60 sec, and 72°C for 140 sec. One fifth of the product from each reaction was examined on ethidium bromide-stained agarose gels.

RESULTS

Treatment with 1 mM dBcAMP resulted in differentiation of polygonal astrocytes into process-bearing cells within 4 days. They remained in this state for the duration of the experiment without morphologic evidence of toxicity. Cells treated with CDM also underwent differentiation (Morrison and De Vellis, 1983), although at a slower rate, and the morphologic change was less dramatic.

Figure 2 shows the effect of chronic dBcAMP treatment on GFAP mRNA expression. Quantification of a standard dilution curve shown in lane 1 (Fig. 2) demonstrates that the reaction occurred largely within the exponential range (Fig. 3). Three dilutions were performed for the dBcAMP and for the control cells (Fig. 2, lane 2), and these are also shown to be within the exponential range (Fig. 4). When the data were averaged across the three dilutions and compared, the GFAP mRNA level in the dBcAMP cells was found to be increased 2.55 times compared to that in cells maintained on FCS (Fig. 4). Note that each signal in Figure 2, lane 2 is from a separate PCR amplification and that the difference between the dBcAMP and the FCS signals remains nearly constant at each of the three dilutions. Also note that Figure 2, lane 3 represents a replication (separate PCR amplifications) of the data in lane 2. An additional separate set of PCR amplifications performed on a different day from the same cDNA showed a 2.57 times

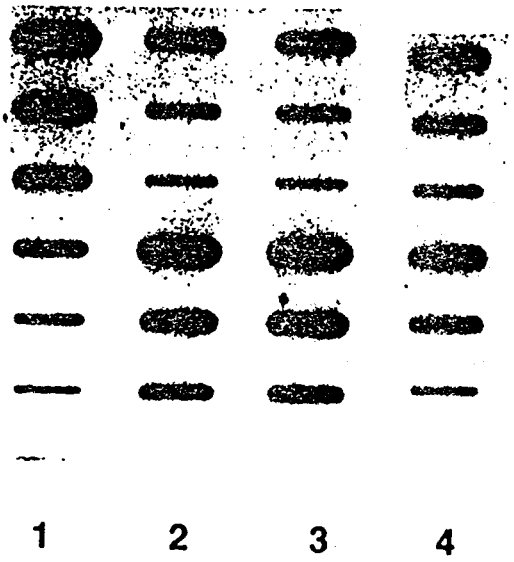

Fig. 2. Autoradiograph showing the effects of chronic dBcAMP treatment on GFAP mRNA. **Lane 1:** PCR products derived from serial 1:2 dilutions of cDNA from unstimulated confluent astrocytes [standard curve; 0.5–7.8 × 10^{-3} μl RT product (cDNA) after dilution]. Each signal represents a separate PCR reaction. **Lane 2:** The upper three signals represent serial dilutions of the cDNA from cells maintained on FCS, whereas the lower three represent serial dilutions of the cDNA from dBcAMP-treated cells. Stronger signals are seen with the dBcAMP cells. **Lane 3:** A replication of the amplifications performed in lane 2. **Lane 4:** HPRT signal after PCR amplification from the same cDNA samples, demonstrating equal loading of RNA for the two conditions.

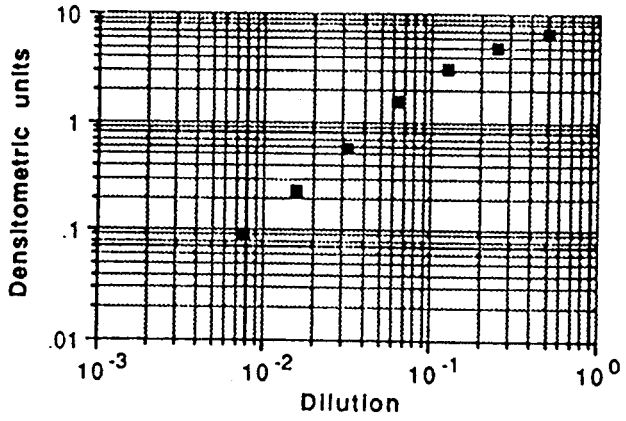

Fig. 3. Densitometric analysis of standard curve shown in Figure 2, lane 1. It is evident that the first 5 points approximate the theoretical slope for exponential amplification of 1.0, whereas the last 2 points show a plateau effect.

increase in GFAP mRNA with dBcAMP treatment. A separate RT performed on still another day followed by another separate set of PCR amplifications showed an increase in GFAP mRNA of 2.54 times with dBcAMP.

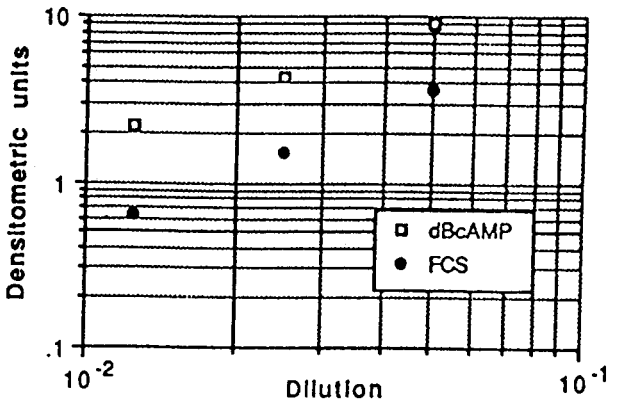

Fig. 4. Densitometric analysis of signals shown in Figure 2, lane 2. The upper points represent data from the dBcAMP-treated cells, whereas the lower points represent the FCS (control) cells. The increase in GFAP mRNA is consistent across the three serial dilutions, although a mild plateau effect is seen with the dBcAMP-treated cells. The HPRT signals shown in Figure 2, lane 4, also increase exponentially with increasing cDNA concentration (data not shown).

Figure 2, lane 4 shows equal expression of HPRT mRNA in the dBcAMP and control samples, demonstrating equal RNA loading.

Figure 5 shows the results of coamplification of astrocyte-derived cDNA and the competitive GFAP standard. As explained in the figure legend, the increase in GFAP mRNA after dBcAMP treatment was shown to be about twofold with this method, confirming the results shown in Figure 2, which were obtained by carrying out serial dilutions in the exponential range.

Figure 6 shows the effects of TNF-α/IL-1β and LPS/Ca^{2+} ionophore/cycloheximide on IL-6 mRNA. For the cells maintained on HS or CDM, there were no signals detectable on an ethidium bromide-stained agarose gel, but there were strong signals from cells treated with TNF-α/IL-1β or LPS/Ca^{2+} ionophore/cycloheximide. Figure 7 shows autoradiograph signals from serial dilutions of cDNA at 1:800 through 1:6,400 from the TNF-α/IL-1β cells, along with a 1:2 through 1:16 dilution series from the CDM-maintained cells. As shown in Figure 8, amplifications for both conditions occurred at the same rate (equal slopes) and in the exponential range (points fall approximately on straight line). All of these points were derived from individual PCR amplifications. Note that the difference between the treated and untreated cells remains approximately the same at different cDNA dilutions.

To determine the magnitude of the increase in IL-6 mRNA, we compared the intensity of the CDM signals with those from the TNF-α/IL-1β cells (Fig. 7) and adjusted for the differences in the starting cDNA dilutions. This yielded an average (across dilutions) increase in IL-6 mRNA of 646 times. Two separate RT and PCR

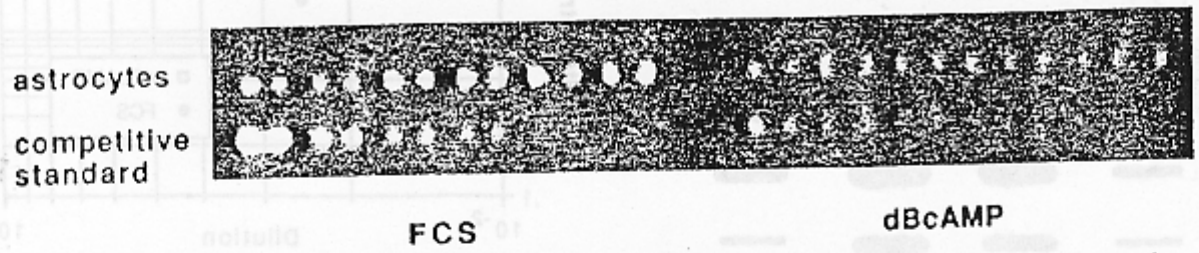

Fig. 5. Effect of dBcAMP on GFAP mRNA determined by RT/competitive PCR (1.4% agarose gel, ethidium bromide staining). Lanes 1–12: The lower band represents the competitive standard (470 bp) and the upper band represents amplified astrocyte-derived cDNA (656 bp). The first 6 bands represent cells maintained on FCS, whereas the second 6 bands represent cells stimulated with 1 mM dBcAMP for 14 days. Within each group, the lower band shows serial 1:2 dilutions of the competitive standard (starting at 1×10^{-1} attomol), whereas the upper band represents a constant amount of astrocyte-derived cDNA. The point at which the upper and lower bands are of equal intensity represents equal starting amounts. For FCS, lane 3 shows equal starting amounts, whereas for dBcAMP, lane 2 shows equal starting amounts. This demonstrates that the dBcAMP-treated cells have about twice as much GFAP mRNA as those maintained on FCS.

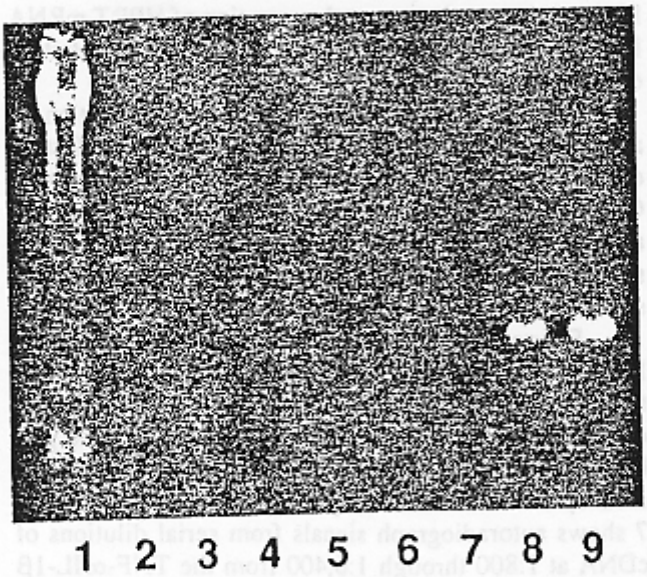

Fig. 6. Effects of TNF-α/IL-1β on IL-6 mRNA, also demonstrating constant levels of HPRT mRNA. **Lane 1:** DNA 123 bp molecular size ladder. **Lanes 2–9:** PCR products from cDNA derived from astrocyte total RNA. **Lane 2:** HPRT for HS cells; **lane 3:** HPRT for CDM cells; **lane 4:** HPRT for TNF-α/IL-1β cells; **lane 5:** HPRT for LPS/cycloheximide/Ca^{2+} ionophore cells; **lane 6:** IL-6 for HS cells; **lane 7:** IL-6 for CDM cells; **lane 8:** IL-6 for TNF-α/IL-1β cells; **lane 9:** IL-6 for LPS/cycloheximide/Ca^{2+} ionophore cells. Note that PCR product from IL-6 mRNA in unstimulated astrocytes is not detectable on the ethidium bromide-stained gel.

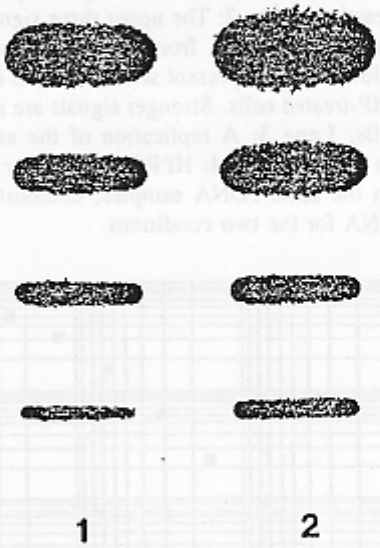

Fig. 7. Autoradiograph demonstrating relative increase in IL-6 mRNA after TNF-α/IL-1β treatment compared to CDM-treated cells. **Lane 1:** PCR product from CDM cells, with serial cDNA dilutions from 1:2 through 1:16. **Lane 2:** TNF-α/IL-1β-treated cells with 4 serial cDNA dilutions from 1:800 through 1:6,400.

amplifications were performed on these RNA samples. They showed increases in IL-6 mRNA after TNF-α/IL-1β stimulation of 556 and 554 times, respectively. Cells maintained on HS also showed a low but detectable signal for IL-6 on autoradiographs of approximately the same intensity as that for CDM-maintained cells (data not shown). Figures 9 and 10 illustrate that all conditions showed equal HPRT expression and that HPRT amplifications were exponential.

DISCUSSION

These results demonstrate the utility of RT-PCR for the relative quantification of mRNA from primary astrocyte cultures. Chronic stimulation of cultures with dBcAMP was shown to result in a 2.5 times increase in GFAP mRNA. This is in agreement with a previous report using Northern blotting to quantify GFAP mRNA (Le Prince et al., 1991). Both strategies employed in the

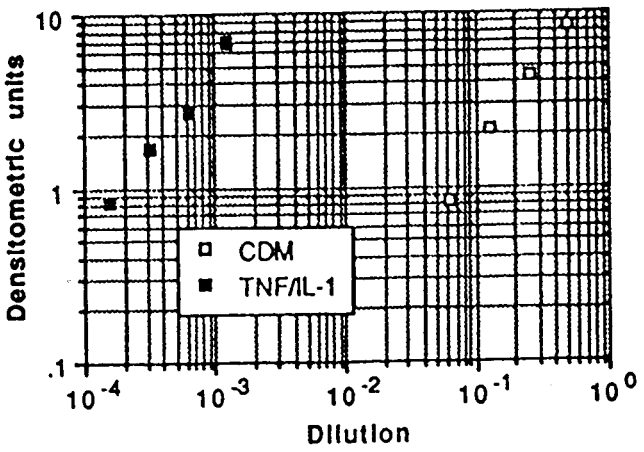

Fig. 8. Densitometric analysis of data in Figure 7. The data fall on lines which are parallel and have slopes of approximately 1.0, demonstrating that all PCR reactions occurred in the exponential range. However, the level of IL-6 mRNA expression is approximately 600 times greater after TNF-α/IL-1β treatment than with CDM.

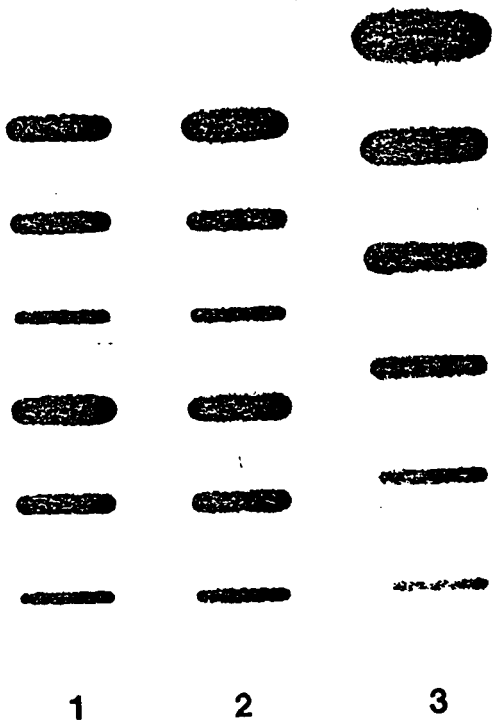

Fig. 9. Autoradiograph showing HPRT signal derived from the same cDNA as that used for Figures 6 and 7. **Lane 1:** Upper three signals, cells maintained on HS, lower three signals, CDM cells. **Lane 2:** Upper three signals, TNF-α/IL-1β-treated cells; lower three signals, LPS/cycloheximide/Ca^{2+} ionophore-treated cells. **Lane 3:** Standard curve for HPRT using serial 1:2 dilutions beginning with 0.5 μl cDNA from unstimulated cells.

present paper, measurement in the exponential phase of the reaction and competitive PCR, proved sufficiently sensitive to detect a twofold change. This result was reliable across multiple RT and PCR amplifications.

Treatment with TNF-α/IL-1β resulted in a large increase in IL-6 mRNA which was readily detectable after RT-PCR on an ethidium bromide-stained agarose gel. In a previous study this had been demonstrated by Northern blotting (Benveniste et al., 1990). Also in that study, IL-6 mRNA was not detectable in unstimulated astrocytes. Sawada et al. (1992) used a non-quantitative RT-PCR technique and ethidium bromide detection to demonstrate TNF-α induction of IL-6 mRNA in astrocytes, but not microglia. They did not observe IL-6 message in unstimulated cells. Using quantitative RT-PCR and radioisotope detection, we were able to demonstrate IL-6 mRNA in unstimulated cells at a level of about 600 times less than that seen in the TNF-α/IL-1β-stimulated cells. This sensitivity constitutes a distinct advantage of RT-PCR over Northern blotting. Wesselingh et al. (1990) found low levels of IL-6 mRNA in unstimulated astrocytes, but noted variation among cultures, which they attributed to factors in serum-containing medium. In our study, astrocytes raised in CDM (serum-free) showed small amounts of IL-6 mRNA. The intensity of the signal from these cultures showed some variation among RT-PCR replications, probably due to errors related to working with the very small amount of starting material, although a signal was always detectable.

As noted by Benveniste et al. (1990), a possible source of IL-6 in unstimulated cultures may be contributing microglia. However, we have also detected low levels of IL-6 mRNA in unstimulated secondary murine astrocyte cultures which have been purified by shaking, trypsinization, and replating (data not shown). Nevertheless, a few microglia remain even after this procedure. Benveniste et al. (1990) attempted to solve this problem by subjecting their cells to four rounds of shaking and replating. However, it is possible that cells may lose some of the properties of primary astrocytes after multiple passaging, including the ability to express IL-6 mRNA constitutively at a low level.

In the present study we were able to induce IL-6 mRNA in cells which had undergone morphological differentiation after treatment with CDM. This demonstrates that differentiated astrocytes, like polygonal resting astrocytes, can respond to TNF-α/IL-1β with increased IL-6 mRNA. This is important because differentiated cultured astrocytes may be more similar than polygonal astrocytes to the reactive astrocytes seen in multiple sclerosis, Alzheimer's disease, and other neuropsychiatric conditions (Federoff et al., 1984).

Regarding RT-PCR as a method of quantifying

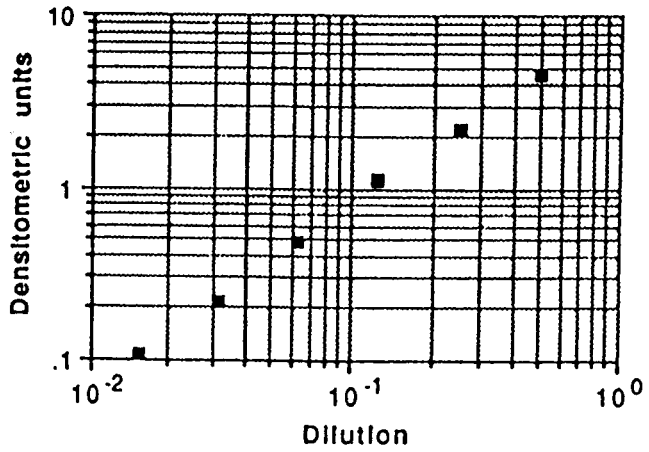

Fig. 10. Graph showing densitometric analysis of standard curve for HPRT from Figure 9, lane 3. The data closely approximate the theoretical curve for exponential amplification of 1.0. Comparison of lanes 1 and 2 with lane 3 in Figure 9 shows that the reactions in lanes 1 and 2 are within the exponential range.

mRNA, several limitations must be taken into consideration in interpretation of results. First, unless the difference among two samples in starting RNA is very large, it must be demonstrated that the reaction is occurring in the exponential phase. Otherwise, one or the other sample may have reached the plateau phase and the results will not be indicative of the true starting amounts. Thus, a single signal derived by RT-PCR from mRNA may not accurately represent the amount of starting material.

A second problem infrequently addressed in RT-PCR studies is the efficiency of RT among samples. It is possible that differences in PCR products could be due to varying efficiencies in RT rather than starting mRNA amounts. One might actually assess the efficiency of RT for the various samples each time the procedure is performed. We have instead repeated RT reactions on multiple occasions to check for consistency. However, if a particular RNA sample contained materials inhibitory to RT, this sample might still produce an inaccurate, albeit reliable, result. To minimize this possibility, we have performed simultaneous processing of RNAs and have been careful to remove residual guanidinium thiocyanate, a powerful inhibitor of reverse transcriptase. RNA samples were not used if they differed significantly from others in purity.

A key assumption in any attempt to quantify mRNA is that equal starting amounts of RNA are used for each sample. Spectrophotometric readings are dependent on accurate and reproducible pipetting. A time-honored approach to assessing RNA loading in Northern blotting studies has been comparison of the expression of a control gene such as β-actin among samples. Such genes are assumed to be independent of the treatment, an assumption which may not be valid in all cases. Other control genes have been proposed, including HPRT, G3PDH, dihydrofolate reductase, and cyclophilin. Each has advantages and disadvantages (Mohler and Butler, 1991). We addressed this problem by using spectrophotometric readings for initial determination of RNA concentration, and then making further adjustments based on two control genes (HPRT and β-actin). An optimal strategy might be to assess a battery of constitutively expressed "housekeeping" genes in pilot studies and to chose several that appear to be unaffected by the treatment under study.

The competitive PCR strategy provides a useful confirmation of the results obtained using the exponential phase technique. In this method the internal standard and the competitive template compete for primers and other reagents in the reaction (Wang et al., 1989). When the standard and the cDNA of interest are present in equal quantities, they result in equal amounts of products. By performing amplifications containing serial dilutions of the competitive standard, but equal amounts of the cDNA of interest, the point of equal starting material can be determined. By repeating this procedure for different samples, differences in the amount of the cDNA of interest can be determined. In the present study we were able to demonstrate with competitive PCR an approximately twofold increase in GFAP mRNA with chronic dBcAMP treatment.

Competitive PCR uses an internal standard known concentration, which is amplified in the same tube, under identical conditions as the cDNA of interest. If the standard and the cDNA of interest are amplified with the same efficiency and are present in equal amounts, they will reach the plateau phase after the same number of cycles and produce equal amounts of PCR products. The amount of standard required to achieve this result can be compared among samples. A potential problem with competitive PCR is that amplification efficiencies may vary with the size of the target (Chelly et al., 1990) and this may affect the final ratio of the amplified products. For absolute quantification of the amount of starting material, this error can be corrected by determining the actual difference in amplification efficiency between the cDNA of interest and the standard (Wang et al., 1989). However, for relative quantification, as in the present study, the same differences in efficiency (due to varying size) between the standard and the target cDNA are present in both the treated and control samples. The only other difference between the amplifications performed on the treated and control material is the amount of cDNA required to achieve equal signal intensities. This difference provides an indication of how the samples vary in starting mRNA. Competitive RT-PCR for GFAP mRNA yielded similar results to those obtained using the

method of E. Murphy et al. (1993) in which reactions are performed in the exponential range.

In summary, RT-PCR is suitable for quantification of large and small differences in mRNA expression in astrocytes. It is highly sensitive, allowing detection of low levels of mRNA. Reliability is enhanced by performing multiple replications and verifying results using competitive and non-competitive strategies. Using RT-PCR, increased expression of GFAP can be detected after chronic stimulation with dBcAMP. This technique provides a useful assay which may be used to study changes in GFAP mRNA in response to other stimuli. RT-PCR can also be used to demonstrate that IL-6 mRNA is induced about 600-fold by TNF-α and IL-1β in astrocytes and that a low level constitutive processing is also detectable. This increase occurred in morphologically differentiated astrocytes, supplementing previous observations based on resting, polygonal astrocytes.

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