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Quantitation of mRNA Species by RT–PCR on Total mRNA Population

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PCR is commonly used for mRNA quantitation. Previously described procedures are applied to one or a few specific mRNA sequences. We show here that methods used for amplifying heterogeneous cDNA populations can be applied to the quantitation of many mRNA species. This quantitation is achieved by dot blotting and hybridization with the corresponding probes after amplifying a bulk mRNA population. Only a single, two-round-amplification assay is required for quantitation of a whole set of mRNA species. The proportionality of input molecules to output signal was shown by performing a series of control experiments. We applied this technique to measure the relative variations of the MBP, Po, and MAG mRNA sequences in the normal trembler mouse model. The results were consistent with previously described Northern blot data. This quantitative PCR method provides a rapid and reliable way to quantify relative amounts of mRNA species in small amounts of total RNA by using internal controls.

Estimation of the amount of one or several mRNA species is a common way to monitor responses of biological systems to environmental stimuli or changes in develomental programs. The classic RNA hybridization technique has the disadvantage of requiring a considerable amount of mRNA (at least 10^5–10^6 specific molecules per sample). With reverse transcription polymerase chain reaction (RT–PCR) the detection threshold has been lowered at least 1000-fold. Various protocols using PCR for quantitating specific RNA species have been developed in the last few years.(1–13) Reasonably good results have been obtained when an RNA molecule of known concentration is added to the sample and used as a standard and when the reaction is monitored during its exponential phase.(1,2,12) Alternative quantitative procedures involving reactions that have been driven to the plateau have been also developed.(9,10,13) However, these methods have some drawbacks. First, preliminary calibration analysis has to be carried out for each individual template. Second, such procedures allow one analysis and therefore give only one answer per PCR assay for the sample under study. Alternatively, amplification of complex DNA or cDNA mixtures have already been described(14–17) for the constitution of DNA and cDNA libraries. Here, we show that such amplification techniques can be adapted to a quantitation end. Our strategy is outlined in Figure 1. First, a double-stranded cDNA population is synthesized from the RNA population to be studied. Second, a specially designed linker–adapter is blunt-end ligated to this cDNA; third, the population is amplified in two successive rounds of 20-cycle PCR; fourth, aliquots of the amplification products are taken during the exponential phase of the second PCR round, dot-blotted, and hybridized with different specific probes. Finally, hybridization signals are plotted on a semi-log scale against the number of cycles, and quantitation of a given mRNA species is obtained by extrapolating to zero cycle. This provides an easy, accurate, and reproducible relative quantitation. This was shown explicitly by measuring expression levels of several mRNAs in the Trembler mouse model.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 350±25 grams were used. Brains were removed rapidly on ice and cortices were isolated for RNA extraction. Normal and heterozygous 8-day old trembler mice (strain B6-CBA) were bred locally and used in the experiments. After cervical rupture, sciatic nerves were isolated for RNA extraction.

RNA and cDNA Preparation

RNAs were purified by the method of Chirgwin et al.(18) Total RNA from brain (2 μg) or the whole preparation from a single mouse sciatic nerve, (~1 μg) was reverse transcribed using a cDNA synthesis kit (Amersham). Reverse transcription was initiated by a 17-nucleotide oligo(dT), and its efficiency was estimated by measuring [α-^{32}P]dCTP incorporation. The specific activity of [α-^{32}P]dCTP was kept low so as to not interfere with the subsequent measurements. Amounts of 8 to 10 ng of cDNA were usually obtained when starting with 1 μg of total RNA. The cDNA was then purified on a

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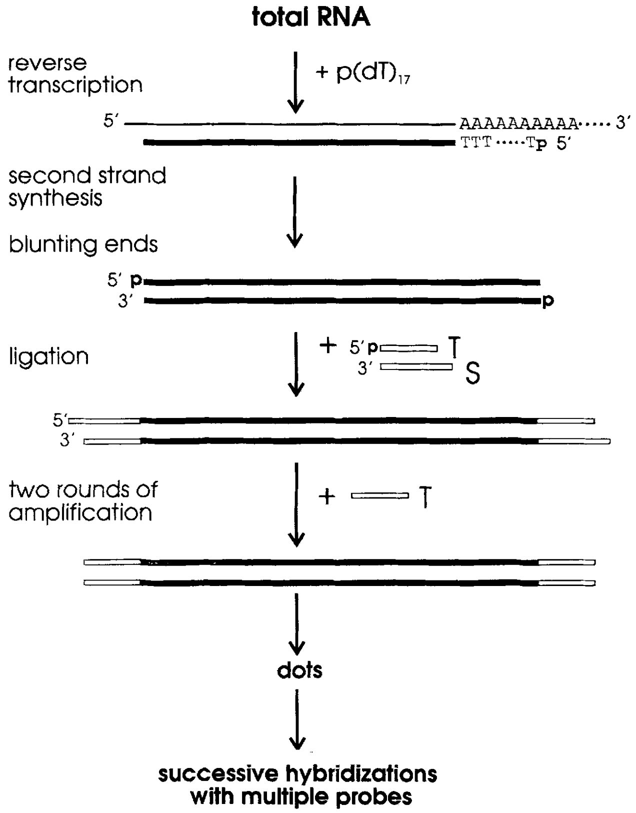

FIGURE 1 Strategy for mRNA quantitation. (Details are provided in the text.)

spin G50 column (Boehringer Mannheim).

Oligonucleotides and Linker

Oligonucleotides S (20-nucleotide) and T (17-nucleotide) were synthesized on an Applied Biosystems 381A DNA automated synthesizer (IBGC/CNRS-Bordeaux) and purified on a Cytolab column. The oligonucleotide T was phosphorylated prior to its annealing to oligonucleotide S. The linker S/T was used for ligation and the oligonucleotide S for amplification. Oligonucleotide and linker are the following: S, 5'-GAGATATTAGAATTCTACTC-3'; T, 5'-pGAGTAGAATTCTAATAT-3'; and S/T, 5'-pGAGTAGAATTCTAATAT-3' and 3'-CTCATATTAAGATTATAGAG-5'.

cDNA Ligation to S/T Linker

cDNA ligation was performed in a 10-μl mixture containing 2.5 units of T4 DNA ligase (Pharmacia), 10 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM ATP, 1 mM S/T linker, and 4 ng of cDNA.

PCR Amplification

The standard amplification mixture contained 10 U/ml of *Taq* polymerase (Perkin-Elmer Cetus), 50 mM KCl, 10 mM Tris-HCl, (pH 8.3), 3 mM $MgCl_2$, 200 μM dNTP, 0.5 μM of the S primer, 2 μCi/ml of [γ-^{32}P]ATP (included for pipetting and aliquoting normalization), and 4 ng of cDNA. The amplification mixture was overlaid with 50 μl of mineral oil (Pharmacia). Amplification was usually achieved by two consecutive rounds. The first round was carried out in a 50-μl reaction for 20 cycles, and the second round was performed after adding 1 μl of the first-round amplification reaction to a fresh 100-μl reaction mixture for an additional 4–20 cycles. The two rounds of amplification started with a 5-min incubation at 95°C and consisted of sequential cycles (usually 20) of denaturation for 1 min at 95°C, annealing for 30 sec at 42°C, and extension for 1 min at 72°C on a Biometra Trio-thermoblock PCR automated apparatus.

For the model system, known amounts of a 620-bp fragment of the *Podospora anserina het-s* gene were added to the ligated cDNA at the first round.

Preparation of the *P. anserina* DNA

A double digestion by *Bam*HI and *Kpn*I of Bluescript II containing 2601 bp of the *P. anserina het-s* gene(19) generated a 620-bp fragment. After electrophoresis on low-melting Seaplaque GTG agarose (FMC), this 620-bp fragment was purified and then ligated to the S/T linker and PCR amplified for 20 cycles. The amount of this fragment was estimated by running a last PCR cycle in the presence of 2 μCi of [α-^{32}P]dCTP.

Amplification Analysis of the Amplified cDNA

After 5 min of denaturation at 95°C, the amplified products were dot blotted, and hybridized with different specific probes. Probes were labeled with [α-^{32}P]dCTP by random primed synthesis (Megaprime kit, Amersham). Final washing of the filters was performed at 65°C in 0.1% (mass/volume) SDS; 0.1× SSPE (0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA at pH 7.4) for 1 hr.

Quantitation

Quantitation of the hybridization signals was performed by autoradiograph densitometry or by Cerenkov counting. Autoradiographic exposure of filters to Amersham Hyperfilm MP was done for variable durations at −70°C, with a CGR micron R/S intensifying screen. Appropriate exposures (in the linear range of the film) were scanned using image analysis software (Samba 2005, Alcatel, TITN, or NIH Image). For the Cerenkov counting, the dots were cut out and counted by liquid scintillation.

cDNA Probes

Hamster β-actin, *P. anserina het-s*, mouse MBP (myelin basic protein), rat Po (major protein of peripheral nervous system), and MAG (myelin-associated glycoprotein) clones were gifts, respectively, from P. Chambon (Strasbourg, France), J. Bégueret (Bordeaux, France) Dr. A.R. Lazzarini (National Institutes of Health, Bethesda), Dr. G. Lemke (Columbia University, NY) and Dr. J.L. Salzer(20). Human glyceraldehyde-3 phosphate dehydrogenase (G3PDH)-purified cDNA probe was purchased by Clontech.

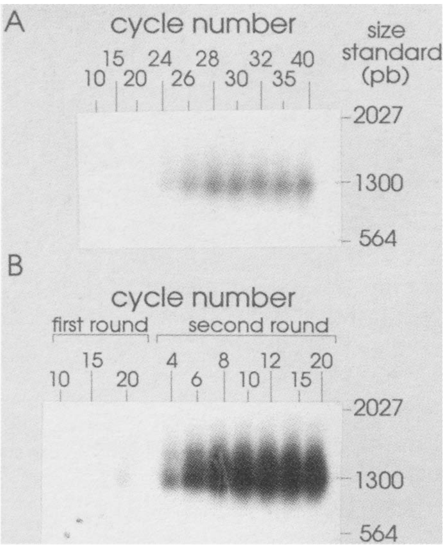

FIGURE 2 Amplification yields from one or two rounds PCR. Double-stranded cDNA obtained from 2 μg of total cerebral cortex rat RNA was ligated to the S/T linker. Amplification was performed on 4 ng of cDNA. (*A*) Amplification was performed for one round of 40 cycles. Aliquots were removed as indicated, analyzed by gel electrophoresis on 1% agarose, transferred to a Hybond N membrane (Amersham), and hybridized with a G3PDH probe labeled with [α-^{32}P]dCTP. (*B*) Amplification was performed for two rounds of PCR (20 cycles each). Aliquots were removed as indicated and were processed as for *A*.

RESULTS

Amplification Design

Our objective was to amplify the total mRNA population of given samples and measure the relative quantity of one or several mRNA species by hybridization with specific probes. We chose the amplification protocol described previously.(14,15) After oligo(dT)-primed reverse transcription, a common end sequence was added to the heterogeneous cDNA molecules through blunt-end ligation of the specially designed linker (S/T) (see Materials and Methods; Oligonucleotides and Linker). The asymmetric ends of this linker ensure directional ligation to the target cDNA population. This permits one strand of the (S/T) linker (oligonucleotide S) to be used as a primer in the subsequent enzymatic amplification (see Fig. 1).

To obtain high amounts of PCR products, the cDNA amplification procedure was performed by two consecutive rounds, usually 20 cycles for the first round and 4–25 cycles for the second. Figure 2 shows PCR amplification kinetics obtained with 4 ng of rat normal cerebral cortex cDNA. In Figure 2A, PCR was achieved wih a single round of 40 cycles. In B, PCR was performed, with two rounds of PCR. After electrophoresis on a 1% agarose gel, the amplified products were transferred and hybridized with a G3PDH probe. Figure 2 shows that PCR performed with two rounds of PCR gives a much better yield of amplified products.

Coamplification and Quantitation With the *P. anserina* Fragment

To test the usefulness of such an approach for mRNA quantitation, we first used our RT–PCR assay on a model system. We prepared a series of samples by adding known amounts of a 620-bp fragment of the *P. anserina het-s* gene to the same amount of brain cDNA (the *P. anserina* gene does not cross-hybridize with rat cDNA extracted from the cortex; data not shown). Blunt-ending and S/T linker ligation of the rat cDNA and of the *P. anserina* sequence were done under the same conditions as those for cDNA.

Amounts of 10, 100, and 1000 pg (Fig. 3A) and 1, 3, 10, and 30 pg (Fig. 3C) of the *P. anserina* fragment were added to the rat cDNA and coamplified as described in Figure 3. Kinetics were monitored in the second round in the presence of [γ-^{32}P]ATP. (This radionucleotide cannot be incorporated to the PCR product and is so used to normalize for DNA pipetting and dotting between the different stop points of the kinetics.) Aliquots of the amplified products were removed every two cycles and spotted onto membrane for hybridization. After hybridization of the 620-bp *P. anserina* probe, the dots were cut out and counted by liquid scintillation for their radioac-

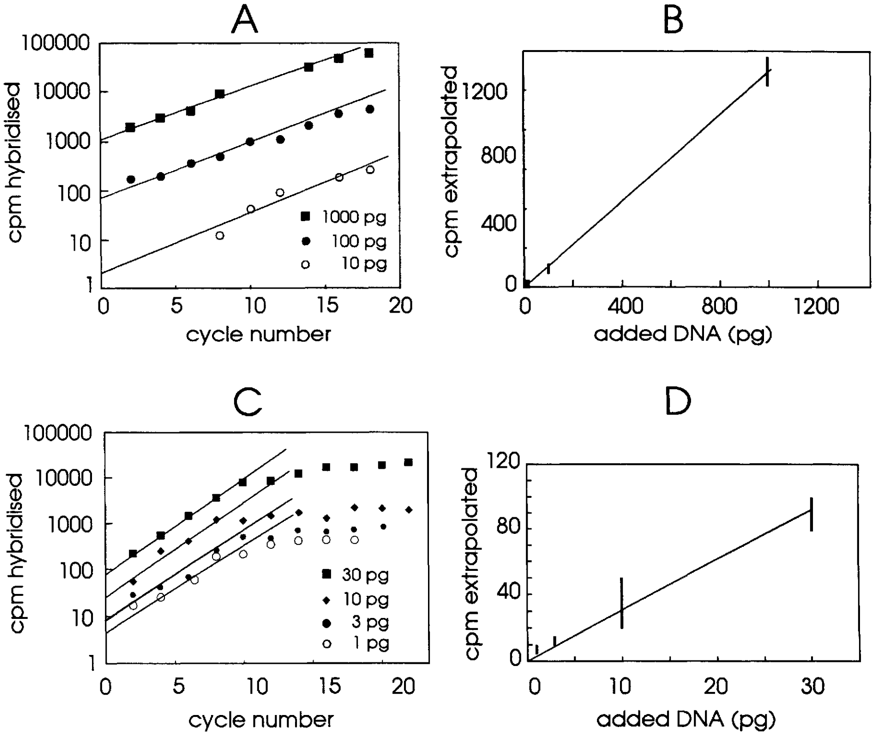

FIGURE 3 Quantitation assay with exogenous DNA. A 620-bp *P. anserina* fragment was ligated to the S/T linker and amplified as described in Materials and Methods (Preparation of the *P. anserina* DNA). Appropriate dilutions were added to 4 ng of S/T linker-ligated cortex cDNA. The amounts of *P. anserina* fragment in each amplification mixture are as indicated. Two rounds of amplification were performed. Aliquots were removed, dotted on Hybond N membrane (Amersham), and probed with [α-^{32}P]dCTP-labeled *P. anserina* sequence. After hybridization and washing, dots were cut out and counted by liquid scintillation. After correcting for pipetting and dotting variability (see Materials and Methods), results were plotted vs. cycle number on a semi-log scale (*A,C*). Extrapolations to cycle 0 were plotted vs. the amount of *P. anserina* DNA added, on a linear scale (*B,D*). Vertical bars were used to take into account the different possible extrapolation values.

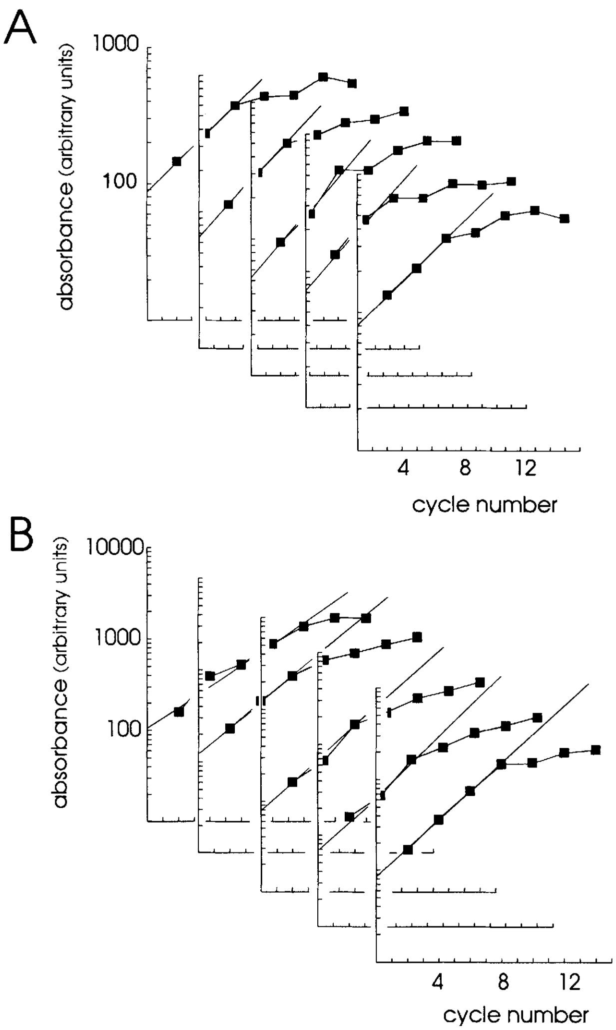

FIGURE 4 Reproducibility. Five parallel amplification kinetics with 1 pg of the *P. anserina* sequence added to 4 ng of rat cDNA were carried out as reported in Fig. 3. (*A*) PCR products were probed with G3PDH; (*B*) PCR products were probed with *P. anserina* sequence.

tivity content. A control dot from a DNA-free region was also counted, and the counts were subtracted as a background. The corrected values were plotted on a semi-log scale (Fig. 3A,C). Figure 3, B and D, shows that in the ranges used here, there is a proportional relationship between these values and the initial DNA quantity.

In Figure 3, the kinetics reached a plateau after 10 cycles with 1–10 pg of the *P. anserina* fragment but seems to be in the linear range after 15 cycles for 10–1000 pg of fragment. This is as expected, because (1) even 1000 pg is only a small quantity (~0.025%) of the cDNA to be amplified, and (2) the amplifications with 1–30 pg and 10–1000 pg, respectively, have been run in two different experiments with two different batches of *Taq* polymerase.

A more complete control might have to begin with increasing amounts of a specific RNA molecule. However, we wanted to focus our investigation on the essential amplification step to show that for a given sequence, the amount of amplified products was proportional to the starting amounts.

Reproducibility

The reproducibility of the procedure mainly depends on the reverse transcription and PCR steps. Because the reproducibility of the former step is widely established we focused our attention on the latter step. The efficiency of PCR is affected by variables such as incubation times, reaction temperatures, and substrate concentrations. A variation in the efficiency of PCR between individual tubes in an experiment would lead to unequal amplifications from tube to tube, thus making quantitation impossible. To check tube-to-tube uniformity, we performed a coamplification series similar to that reported above. Five replicate samples containing 1 pg of the *P. anserina* sequence and 4 ng of brain cDNA (see Fig. 3) were amplified, and aliquots were dotted and hybridized first to *P. anserina* and then to G3PDH probes. Curves presented in Figure 4 show that similar amplification efficiencies were obtained for both G3PDH (Fig. 4A) and *P. anserina* probes (Fig. 4B). When the five samples of a given sequence were compared, the standard variation among the five extrapolated values was ≤16.5% (Fig. 4). Thus, the reproducibility of the procedure seems to be reasonable.

Quantitation of Several mRNA Sequences in Normal and Trembler Mice

We then used this procedure to measure the expression levels of three mRNAs species in the well-characterized trembler mouse model. The trembler mouse suffers from a dominantly inherited autosomal mutation affecting the Schwann cell activities; this results in an abnormal myelinization of the peripheral nervous system.(21–23) Northern blot analyses have shown previously that the steady-state levels of mRNAs encoding myelin-specific proteins in normal and trembler mice are affected to various extents.(24–25)

Here, we wanted to check whether the relative level of expression of mRNA encoding Po, MBP, and MAG determined by our measurements could be correlated with the results of previous Northern analyses. Total RNA was extracted from a single sciatic nerve of 8-day-old normal or trembler mice. The two RNA samples (≅1 μg each) were processed as described in Materials and Methods. The dotted PCR products were then hybridized successively with MBP, Po, MAG, and G3PDH probes. Quantitation was achieved by densitometry of the dot blots (Figs. 5 and 6) A ubiquitous mRNA probe, the human G3PDH corresponding to a key gene in glycolysis, was used as an internal control to normalize for errors in RNA measurements and variability in pipetting and blotting. This mRNA is believed to have constant expression in most tissues and was also

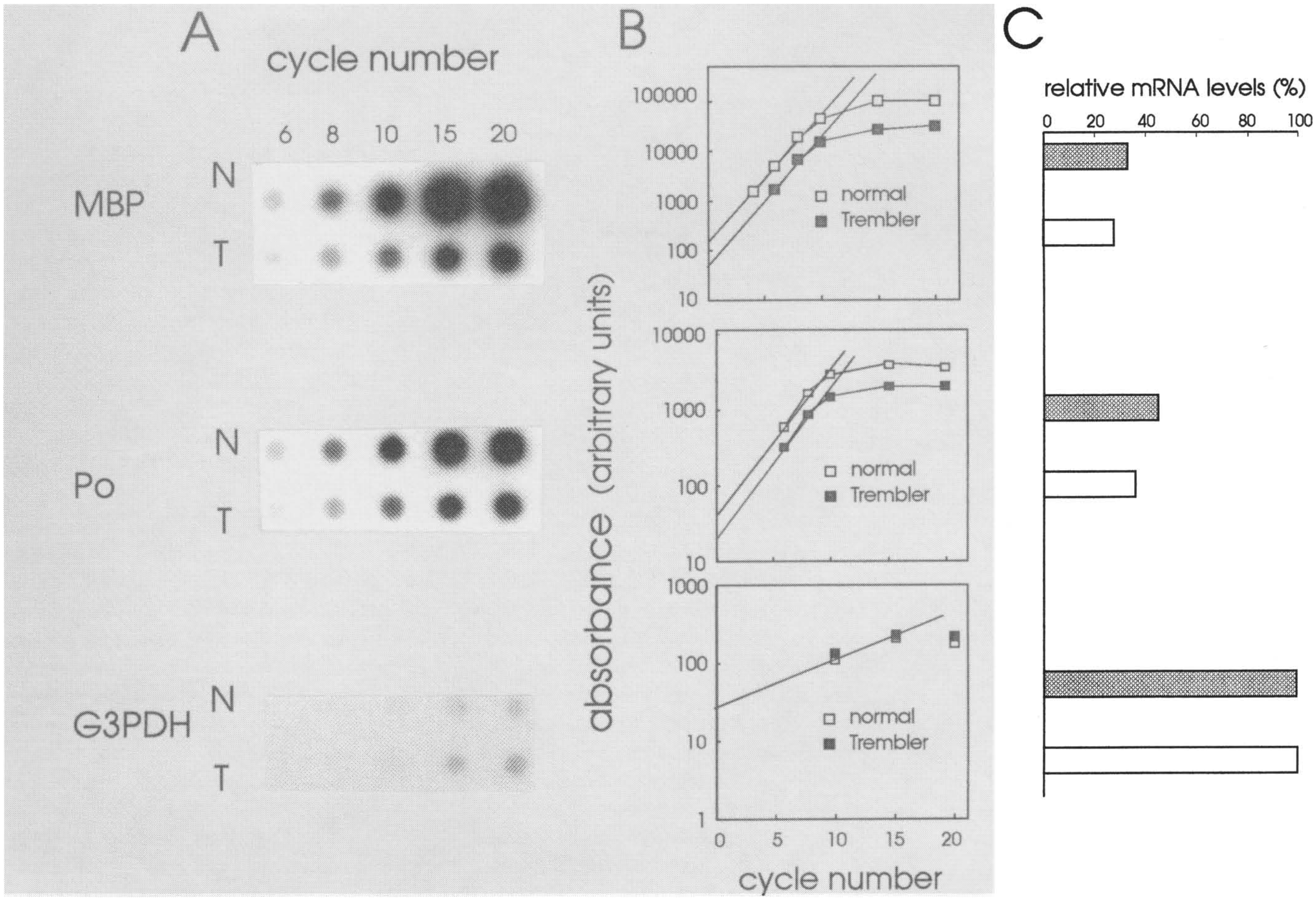

FIGURE 5 Relative quantitation of mRNA in normal and trembler mice. Total RNA purified from a single sciatic nerve from normal and trembler mouse was processed as described. cDNAs were ligated to S/T linker and used for the amplification. Kinetics were carried out at second-round PCR. Aliquots were taken at cycles 4, 6, 8, 10, 15, and 20 and dotted on Hybond N membrane (Amersham). The blot was then hybridized consecutively to MBP, Po, MAG, and G3PDH probes. (*A*) Representative autoradiographs of dot blots obtained with the different probes; (*B*) curves obtained for MAG, MBP, and Po after scanning blots in *A* using a Samba 2005 image analyzer; (*C*) comparison of the results of this paper with those of Bascles et al.(24) (Shaded bar) RT–PCR; (open bar) Northern hybridization. These results are presented as the percentage of the maximal level of specific mRNA found at 8 postnatal days.

used as an internal standard in previous Northern analyses.(23,24) G3PDH blots showed (Fig. 5A) that there is nearly no difference in DNA loading between the samples under study (normal and trembler). Kinetics corresponding to Po, MBP, and G3PDH probes are displayed in Figure 5B. Because, from the above results, one can expect that a given sequence is always amplified with the same efficiency, we systematically used parallel straight lines for extrapolations. The extrapolation (Fig. 5B) ratios between normal and trembler MBP, and Po mRNA levels were calculated and compared to the previous values given by hybridization experiments (Fig. 5C). Both approaches show that MBP and Po mRNA levels decrease three and two times, respectively. Figure 6 shows the results of five parallel experiments for MAG. Because of a low signal-to-noise ratio, the data are scattered. However, they indicate that the ratio of MAG expression in the two samples is close to 1. This compares well to the 1.5-fold decrease determined by Northern blot experiments.

It is noteworthy that this experiment addresses itself to various mRNAs abundance, as Po and MPB mRNAs are highly abundant and MAG mRNA is expressed at a much lower level.

DISCUSSION

Several protocols using RT–PCR for measuring the level of a given mRNA species in total RNA have been described in the literature.(1–13) This type of assay leads to very high sensitivity, allowing the quantitation of very low abundance mRNA species in minute amounts of tissues. However, each sequence to be quantitated necessitates preliminary calibration studies and at least one or several amplification assays.

Alternatively, our procedure allows the quantitation of many mRNA species from the products of a single two-round amplification reaction. Other protocols using the amplification of populations of DNA or cDNA have been applied to the preparation and the screening of cDNA libraries.(16,17) In these cases, the representation of the amplified products was checked and found to be reasonable.

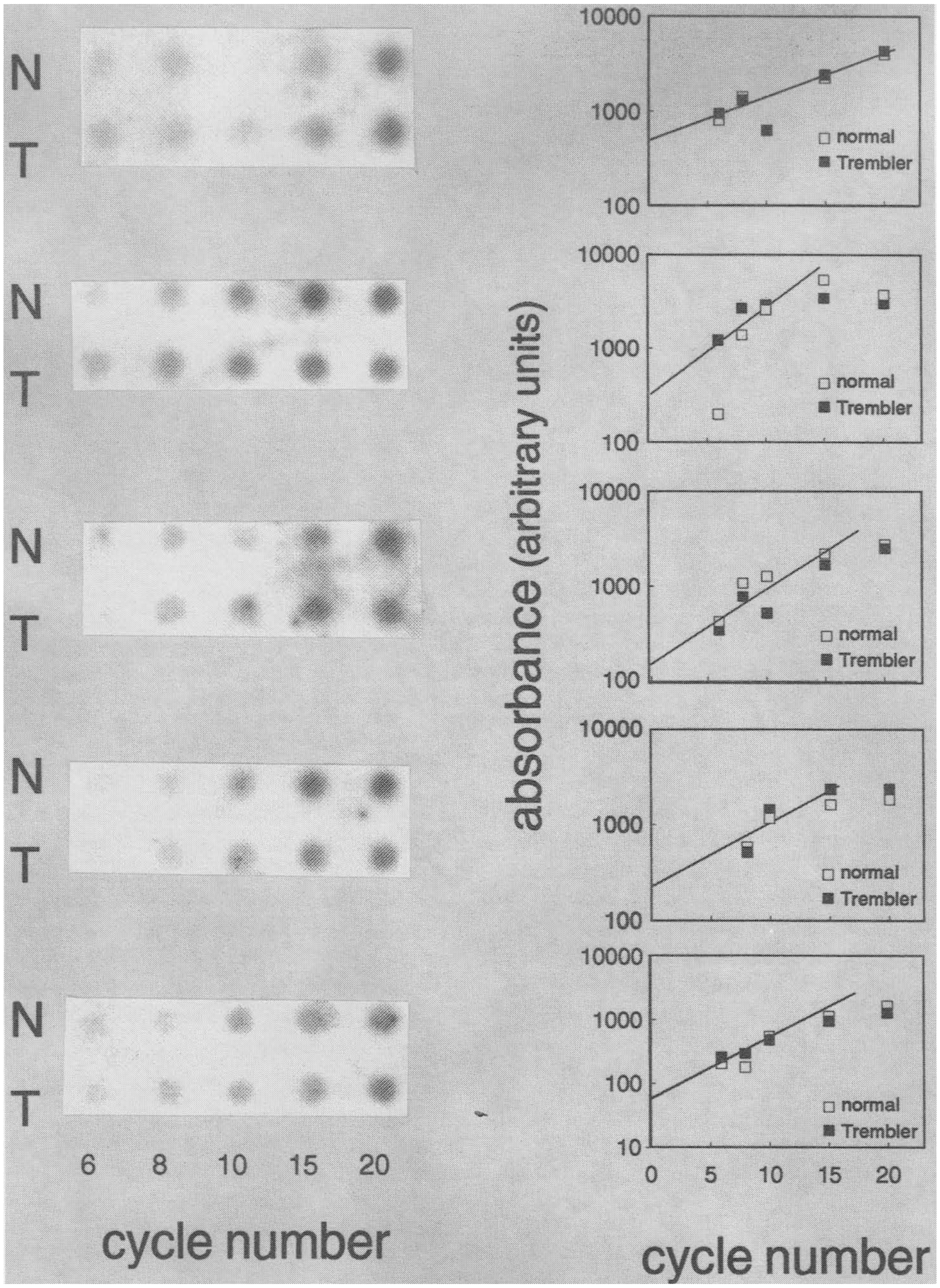

FIGURE 6 Comparison of MAG mRNA levels in normal and trembler mice. Five different kinetics of amplification were done as reported in Fig. 5. The dots were hybridized to the MAG cDNA probe. (*Left*) Autoradiographs; (*right*) results of the densitometric analysis.

However, our main purpose is not to compare a given species to another within the same population but to monitor relative changes of one or several mRNA species between different mRNA populations. In that case, it is necessary only that the ratio between amplification efficiencies of the sequences studied remains independent of the population considered. Two sets of controls suggest that this is the case. First in a model system, when *P. anserina* sequence was added to 4 ng of a cDNA population, the mass of the resulting PCR product was proportional to the initial amounts (Fig. 3). Second, we determined the relative levels of expression for the normal versus trembler mouse of mRNAs encoding Po, MBP, MAG (Figs. 5 and 6). Our results are in good agreement with those measured by Northern blot.(24)

However, further experiments are necessary to see whether our procedure is reliable enough to detect variations less than twofold. While this approach cannot detect and quantitate very low-abundance mRNA species, only minute amounts of tissue are necessary and there is no need to know the sequence of the mRNA under study or to synthesize specific oligonucleotides to be used as primers, as a sequence-independent single primer is used in the amplification reaction. Nor is there any need to synthesize or clone an internal standard, as the method uses internal controls present in the RNA population, thus avoiding preliminary calibration constraints that are expensive and time consuming.

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