# Determining transcript number using the polymerase chain reaction: *Pgk-2*, *mP2*, and *PGK-2* transgene mRNA levels during spermatogenesis

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## ABSTRACT

**We describe a technique that uses reverse transcription and the polymerase chain reaction (pcr) to rapidly quantitate numbers of specific mRNA transcripts from nanogram quantities of total cellular RNA. Linearity of input molecules to output signal was maintained by limiting the cycle number and the amount of input RNA and by minimizing the number of manipulations. Absolute levels of specific transcripts were determined by the inclusion of a separate standard curve composed of serially diluted *in vitro* transcribed RNA run alongside the experimental samples. This allowed rapid quantitation of many samples simultaneously. We applied this technique to measuring the expression of phosphoglycerate kinase 2 (Pgk-2) transgenes in the mouse testis during development. A human *PGK-2* transgene, a *PGK-2*/CAT transgene, and the endogenous *mPgk-2* gene all displayed similar patterns and levels of expression, consistent with the conclusion that peak RNA accumulation occurs in pachytene spermatocytes. Mouse protamine 2 (*mP2*) is expressed at a level approximately tenfold higher than *Pgk-2* and displays a different pattern of expression consistent with initiation of transcription occuring in haploid round spermatids.**

## INTRODUCTION

Quantitative analysis of RNA is central to the understanding of the mechanisms that regulate gene activity. Northern analysis is currently the most widely used method for analyzing RNA levels, however, northern analysis requires microgram amounts of RNA, is time consuming and is of limited quantitative use. Techniques based on solution hybridization of RNA are more sensitive and quantitative, however, the increased time and effort involved limit their widespread use. The advent of the polymerase chain reaction (pcr) (1) has resulted in the development of a number of quantitative (2–5) and semi-quantitative (6–8) procedures for the analysis of steady-state RNA levels. These protocols all include the addition of reverse transcriptase to the samples to convert the RNA into a DNA copy providing a suitable substrate for taq polymerase. The pcr approach allows increased sensitivity, ease of use, and requires only small amounts of RNA.

Protocols designed to quantitate the products of the pcr must account for the exponential nature of the cycling reaction. The product (N) of pcr amplification can be defined by the function $N=N_o(1+\text{eff.})^n$, and is initially linear with respect to input molecules ($N_o$). However, as cycle number (n) and total product (N) increase, the efficiency (eff.) of the pcr is reduced as the concentration of one of the substrates eventually becomes limiting, resulting in loss of linearity. Furthermore, small changes in the efficiency of the reaction result in large differences in the amount of total product. This leads to non-uniformity in the level of pcr product from tube to tube or experiment to experiment. Accordingly, efforts to use the pcr to measure RNA levels have included internal competitive or noncompetitive templates to control for such differences in amplification. Although useful for standardization, each of these templates requires additional cloning steps, and the use of an internal standard requires several reactions to measure each sample.

Another approach (9) takes advantage of the theoretical linearity of input molecules versus pcr product. Using this approach, the relative levels of testis-specific messages were measured by limiting both the cycle number and the amount of starting material such that the efficiency of the reaction remained constant. Although this approach is useful for determining the relative levels of a specific mRNA in different samples, it is not useful for comparing the level of different messages, and it is difficult to determine whether the message levels detected are of physiological importance (5,10). We have extended the utility of this approach through the use of an externally generated standard curve. The standard curve is used both to determine the range over which the reaction is linear and to determine the level of signal that corresponds to a specific number of RNA molecules. Here we present a novel quantitation protocol (modified from reference 9) that provides rapid and accurate analysis of absolute RNA levels from many samples and allows quantitative comparison of different messages.

We are interested in gene expression during spermatogenesis, and have been studying the transcriptional control of the gene for Phosphoglycerate Kinase (*Pgk-2*), expressed only in meiotic

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and post meiotic male germ-cells. In the mouse, the initial stages of spermatogenesis occur synchronously and begin shortly after birth. Germ-cells first enter meiosis around day 12, and divide to form haploid round spermatids by about day 21. Thus, the age of the prepuberal mouse can be correlated with the developmental progression of germ-cells in the testis. Previously, we showed that a human *PGK-2* transgene is expressed in a tissue-specific and developmentally controlled manner in mice, and that a *PGK-2*/CAT fusion gene demonstrates tissue-specific CAT activity that is first detected at *post natal* day 13 (11). Due to the limits of quantitation and low yields of RNA from immature testes we were unable to accurately measure and compare RNA levels from these transgenes. Here, we have used this technique to show that the human *PGK-2* transgene, the *PGK-2*/CAT fusion gene and the endogenous mouse *Pgk-2* gene all share the same pattern of expression, and the mRNA levels vary less than fourfold among the three genes. Furthermore, the developmental expression profiles of the *PGK-2* genes are consistent with high levels of expression in pachytene cells, a cell-type that was previously thought to contain only low levels of *Pgk-2* message (12).

## MATERIALS AND METHODS

### RNA purification

Tissue samples were obtained from transgenic mice homozygous for either the *PGK-2*/CAT transgene or the human genomic *PGK-2* transgene. Animals were sacrificed on the days indicated (day 0 = date of birth), and the tissue was frozen at −70°C prior to RNA extraction. RNA was prepared by homogenization of the tissues using a polytron, and then precipitation in 3 M LiCl, 6 M Urea as described (13).

### RNA transcriptions

*In vitro* transcribed RNAs for standardization of the pcr were prepared with low specific activity according to the procedure for large scale transcriptions provided by the supplier (Bethesda Research Laboratories, Gaithersburg, MD). The concentration of the RNAs were determined by both TCA precipitable radioactivity and absorption at 260 nm. The concentration and integrity of the RNAs were confirmed by agarose gel electrophoresis in the presence of formaldehyde. All RNA transcriptions were performed using fragments of the genes of interest cloned into Bluescript pBPIIKS vectors (Stratagene, San Diego, CA) containing T3 and T7 promoters (Fig. 1). *Pgk-2*/CAT RNA was transcribed with T3 polymerase from a linearized vector containing the 780 bp CAT coding region cloned into the *Hind* III and *Bam* HI sites of the pBPIIKS polylinker. *PGK-2* RNA was transcribed with T7 polymerase from a linearized vector containing a 1.6 kb *Hinc* II fragment from the *PGK-2* coding region cloned into the *Hinc* II site. *Pgk-2* RNA was transcribed with T7 polymerase from a linearized template containing a 481 bp *Hinc* II fragment from the 3′ end of the *Pgk-2* coding sequence. Mouse Protamine 2 (*mP2*) RNA was transcribed with T3 polymerase from a linearized vector containing a 269 bp *Pst* I/*Eco* RI fragment from the 3′ untranslated region of *mP2* cloned into the same sites of pBPIIKS. All *in vitro* transcribed RNAs were diluted to $1 \times 10^9$ molecules per μl and stored as ethanol precipitates at −20°C.

### Pcr primers

All primers used for pcr amplification contained between 21 and 25 nucleotides and had calculated melting temperatures (Tm) of 64° C or above, as determined by Tm = (4× each cytosine or guanine + 2× each adenine or thymidine)°C. The *PGK-2*/CAT, human *PGK-2*, mouse *Pgk-2*, and *mP2* primers amplified regions of 281 bp, 358 bp, 356 bp, and 208 bp respectively. The sequences of each pair of primers are shown in Figure 1.

### Reverse transcription/pcr

Both reverse transcription and pcr were performed in the same tube in a single buffer. Quantitation of experimental RNA samples was as follows: a suspension of 100 ng ethanol precipitated RNA was aliquoted to a 0.67 ml centrifuge tube, glycogen (20 μg, Boehringer Mannheim, West Germany) was added and the sample was centrifuged in a microfuge at 12,000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 1× pcr buffer (50 mM KCl, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin; Cetus, Emeryville, CA) containing dNTPs at 200 μmole each, 2 μCi alpha $^{32}P$ labeled dATP (3000 Ci/mmole, Amersham, Arlington, IL), and 100 pmole each primer. Each sample was then incubated at 65°C for 3 minutes and then placed on ice for 2 minutes. Moloney Murine Leukemia Virus Reverse Transcriptase (20 units, Pharmacia, Sweden), Amplitaq Polymerase (5 units, Cetus), and RNasin (35 units, Promega, Madison, WI), were mixed and then added to the sample, and 50 μl mineral oil was layered on top. Each set of tubes was immediately transferred to an Ericomp Thermocycler (Ericomp, San Diego, CA) and incubated for 10 minutes at 37°C, then 19−25 cycles of 92°C for 20 seconds, annealing temperature for 10 seconds, and 72°C for 30 seconds. Annealing temperatures were set at the melting temperature of the lowest Tm oligonucleotide.

*In vitro* transcribed RNA standards were treated similarly, except that the ethanol precipitated T3 or T7 RNA transcripts

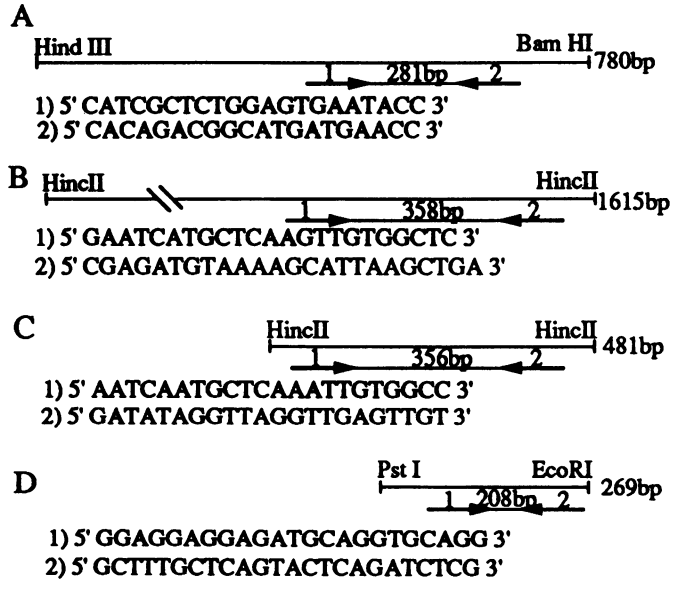


**Figure 1.** Schematic representation of subclones and sequence of primers used to quantitate RTpcr. All subclones are flanked by restriction sites used to clone into pBluescript BPIIKS vectors. The arrows denote the approximate positions of the 5′ and 3′ pcr primers. The sizes of the subclones and the pcr products are indicated, and the sequences of the 5′ and 3′ oligonucleotide primers (1 and 2 respectively) are shown below each subclone. Subclones are oriented with the start of transcription to the left. A, *PGK-2*/CAT; B, human *PGK-2*; C, mouse *Pgk-2*; D, *mP2*.

were first resuspended in diethyl pyrocarbonate treated $H_20$, serially diluted as required, then reprecipitated in the presence of 20 μg glycogen and 100 ng liver RNA. After the pcr, formamide loading buffer (0.1% w/v xylene cyanol, 0,1%w.v bromophenol blue, 10 mM $Na_2EDTA$, 95% v/v formamide) was added, the samples were heated to 65°C for 3 minutes, and then electrophoresed in a 6% acrylamide/6 M urea gel at 200 volts for 1.5 hours. The gel was then dried down and exposed to a Molecular Dynamics Phosphorimager plate (Molecular Dynamics, Sunnyvale, CA) for one to ten hours and quantitated using the phosphorimager. Similar results were obtained by excising the bands from the dried gel and quantitating by liquid scintillation counting.

## RESULTS

The protocol for the combined reverse transcription and polymerase chain reaction (RTpcr) is predicated on the theoretical linear relationship of input molecules to total product. By limiting the amount of starting material and the number of cycles of pcr, we could keep all substrates in excess, insuring uniform amplification. Because the final yield of the pcr is sensitive to small differences in efficiency, another consideration was to minimize the number of manipulations involved. By combining all components of both the reverse transcriptase and the pcr in a single reaction mixture (pcr buffer, dNTPs, primers, reverse transcriptase and taq polymerase) we could achieve amplification of the RNA. It was then essential to demonstrate that the reaction was linear, sensitive and reproducible.

### Linearity of RTpcr

To examine the linearity of the RTpcr, eleven samples of twofold dilutions of T3 transcribed CAT RNA were mixed with 100 ng C57B6/DBA2 mouse liver RNA. The range of the experiment was from 4,900 molecules to 5,000,000 molecules of CAT RNA. Each sample was prepared as described in Materials and Methods and both the pcr mix and the enzyme mix were aliquoted from a common stock. Samples with fewer input molecules required more cycles to obtain a detectable signal. Accordingly, samples containing from 4,900 to 78,000 CAT RNA molecules were run for 25 cycles, and samples containing 78,000 to 5,000,000 molecules were run for 21 cycles. The signals from the 25 cycle reactions were normalized to the 21 cycle values using the overlapping 78,000 molecule samples. Approximately half of the resulting products were run on a 6% polyacrylamide gel, dried, and quantitated using the Molecular Dynamics phosphorimager system. As shown in figure 2A, there is a linear relationship between input RNA molecules and signal when the number input RNA molecules is between 19,000 and 5,000,000. The signal begins to level off as the concentration of CAT RNA molecules is decreased below this range. A control reaction containing no added CAT RNA molecules also gives a faint signal (data not shown), suggesting that this constant signal at very low RNA concentrations is due to contamination of the pcr solutions with a total of approximately 10,000 CAT RNA or DNA sequences per reaction.

### Variability between experiments

To determine the variability of the RTpcr, CAT RNA dilution curves were repeated over a period of one month, using 21 cycle reactions and identical thermocycler times and temperatures, but using different prepared solutions, different tubes of enzyme stock, and different lots of $^{32}P$ labeled dATP. Figure 2B shows three such CAT dilution series, each normalized at the 78,000 molecule point to account for differences in exposure time. Although each experiment shows a linear relationship between input molecules and signal, the slopes of the three lines vary as much as twofold; the slope of each line representing the relative efficiency of amplification. It is important to note that although the efficiency of the reaction may vary from day to day (presumably due to differences in the concentrations or specific activities of the components), in any given experiment, linearity of input molecules to signal is observed. Because a high degree

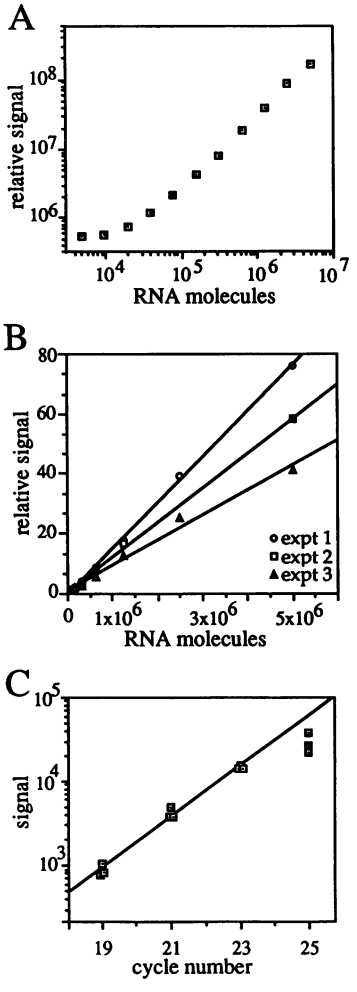


**Figure 2.** Quantitation of Reverse Transcription/pcr. A) Linearity of RTpcr. Eleven samples of twofold serial dilutions of T3 transcribed CAT RNA were mixed with 100 μg liver RNA and subjected to the RTpcr protocol. Half of the reaction mixture was run on a 6% polyacrylamide gel, dried, quantitated using a Molecular Dynamics phosphorimager and plotted as signal versus RNA molecules. Samples containing 4,900 to 78,000 molecules were run for 25 cycles; samples containing 78,000 to 5,000,000 molecules were run for 21 cycles. The two sets of points were normalized to the 21 cycle curve by the overlapping 78,000 molecule values. B) Experiment variability. Over a period of one month, three sets of serial twofold or fourfold serial dilutions of T3 transcribed CAT RNA molecules were mixed with 100 ng liver RNA, subjected to RTpcr for 21 cycles and quantitated as in A. A line drawn through each set of points describes the linear fit of the data by the least squares technique. C) Tube to tube uniformity. Triplicate samples of 5 million molecules of CAT T3 RNA mixed with 100 ng liver RNA were subjected to RTpcr for the number of cycles indicated and quantitated as in A. A line drawn through the 19, 21, and 23 cycle points describes the exponential increase in product with increasing cycle number.

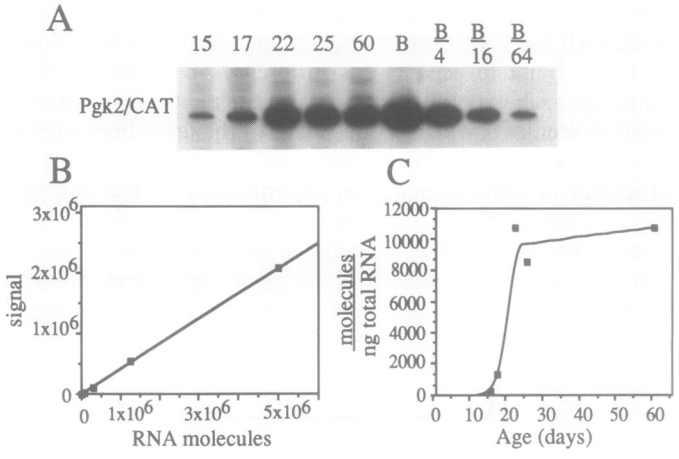


**Figure 3.** Quantitation of *Pgk-2*/CAT transgene RNAs in the testis. A) Autoradiograph of a dried 6% polyacrylamide gel showing the products of RT pcr from a developmental timecourse. Total RNA samples (100 ng) from the testes of *Pgk-2*/CAT transgenic mice of the ages indicated were amplified for 21 cycles using CAT primers. On the right is a standard curve of fourfold serial dilutions of T3 transcribed CAT RNA run concurrently to allow absolute quantitation of the *Pgk-2*/CAT transgene RNAs. The letter B = $5\times10^6$ molecules. B) Plot of signal versus CAT RNA molecules of the standard curve in A. The equation of the line drawn through the points allows the conversion of the developmental timecourse signal into numbers of RNA molecules. C) Appearance of *Pgk-2*/CAT RNA during development. The signal in A was converted to numbers of CAT RNA molecules using the standard curve and is presented as molecules CAT RNA per ng total RNA as a function of development.

of variability was seen, we were unable to use a single standard curve for multiple experiments, and were required to include a new standard curve to run alongside each set of experiments.

## Uniformity among reaction vessels

The efficiency of the pcr is affected by variables such as incubation time, reaction temperature, and the concentrations of the substrates. Variation in the efficiency of pcr among individual tubes in an experiment would lead to unequal amplification from tube to tube, making quantitation impossible. The linearity of the standard curves presented above (each data point representing a separate tube) suggests that each sample is amplifying with the same efficiency. To test this directly, triplicate samples of 5,000,000 molecules of CAT RNA were each amplified for 19, 21, 23 or 25 cycles and processed as described above. Figure 2C shows a semilog plot of signals corresponding to the 12 samples. The variation in signal among triplicate samples is 20% or less. The efficiency of amplification (calculated by $N=N_o(1+\text{eff.})^n$) at 19, 21 and 23 cycles is nearly 100%, however, by 25 cycles the efficiency of amplification is clearly decreasing. Thus, we can conclude that while in the exponential range of the reaction, the efficiency of amplification is uniform, both for adjacent tubes at a particular cycle number and for reactions amplified for sequential numbers of cycles.

## *PGK-2*/CAT RNA developmental timecourse

Having demonstrated that we can select conditions under which the reaction is linear with *in vitro* transcribed standards, we next applied the technique to experimental samples. We have shown previously that CAT activity expressed from a *PGK-2*/CAT transgene is found only in the mouse testis and is first detected at *post natal* day 13 (11). To analyze the developmental pattern

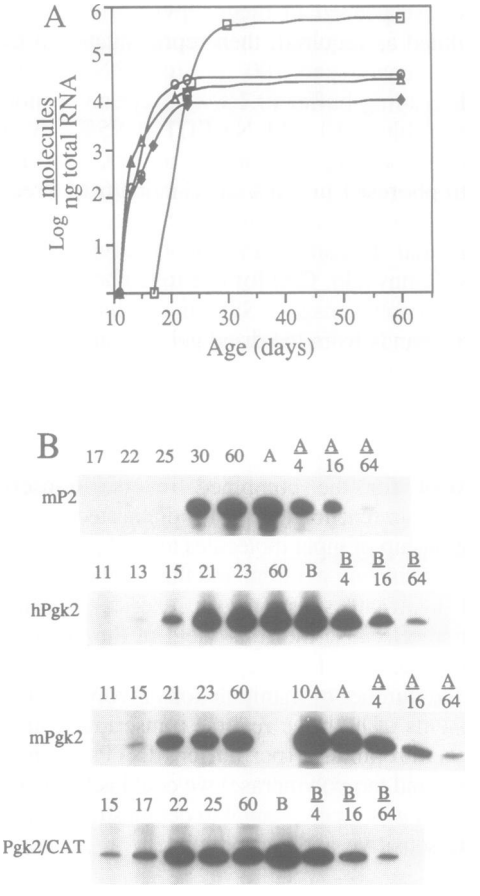


**Figure 4.** Developmental accumulation and quantitation of *Pgk-2*, *mP2*, *PGK-2* transgene and *PGK-2*/CAT transgene RNAs. One hundred ng total testis RNA isolated from mice at various days was subjected to the RTpcr protocol for various cycles. A) Semilog plot of the message levels of the four genes analyzed. *mP2*, open squares; *PGK-2*, open circles; *mPgk-2*, open triangles; *PGK-2*/CAT, closed diamonds. B) Autoradiographs of pcr products used to derive points in A. The target gene amplified in each experiment is indicated. Numbers refer to the developmental age in days of each RNA sample. The letters refer to the number of *in vitro* transcribed RNA molecules used in each standard curve. A, $1\times10^7$ in the *mPgk-2* experiment, $1\times10^8$ in the *mP2* experiment; B, $5\times10^6$. Note that in the *mPgk-2* experiment, the 10A sample ($1\times10^8$ molecules) is out of the linear range and was not used in the standard curve calculation.

of expression, CAT RNA levels were measured in samples of testis RNA isolated from transgenic *PGK-2*/CAT mice of various ages. *Post natal* days 15 to day 25 were monitored closely as this represents the period of most dynamic change in the expression of the *PGK-2* promoter.

In a single experiment, 100 nanogram aliquots of RNA from mouse testes at *post natal* days 15, 17, 22, 25, and 60 were amplified at 21 cycles using primers corresponding to sequences in the CAT coding region. Included alongside the experimental points in separate tubes was a standard curve consisting of fourfold serial dilutions of T3 transcribed CAT RNA starting at 5,000,000 molecules. The standard curve was amplified simultaneously using the same primers under identical conditions. Figure 3A shows an autoradiogram of the resulting pcr products. The points of the standard curve, designed to encompass the range of the experimental points, were quantitated, and demonstrated that the reaction was in the linear range (Figure 3B). The standard curve was used to convert the signal from the experimental points (d15-d60) to molecules of CAT RNA per nanogram total RNA.

The resulting plot of the developmental accumulation of *PGK-2*/CAT RNA (Figure 3C) shows the level of message increasing from 250 molecules/ng total RNA at day 15 to 10,000 molecules/ng at day 60. The majority of the RNA accumulates between days 17 and 25 and correlates with the appearance and proliferation of pachytene spermatocytes in the developing testis (14). The observation that the level of specific message per nanogram total RNA remains at a high level from day 25 to day 60, a period during which the proportion of pachytene cells in the testis is decreasing, suggests that *PGK-2*/CAT RNA is also being expressed at significant levels in spermatids, the predominant cell-type in the adult mouse testis (14).

### Mouse *Pgk-2*, Protamine 2 (*mP2*), and human *PGK-2* developmental accumulation

To determine if the *Pgk-2*/CAT transgene expression reflects that of the human transgene and endogenous *Pgk-2* genes, we analyzed the expression patterns of both the human genomic *PGK-2* transgene and the endogenous *Pgk-2* locus and compared the expression levels and patterns to that of *PGK-2*/CAT. A measure of developmental accumulation of these genes would determine more precisely the regulation of the *Pgk-2* genes in the testis and would uncover any differences in the regulation of the three genes. *mP2* was also analyzed, as its expression pattern begins in the haploid round spermatid stage of spermatogenesis (15). The expression pattern of *mP2* is important as it will delineate the appearance and proliferation of round spermatids, and will thus serve as a reference point for the *Pgk-2* expression patterns.

Each of the three genes, mouse *Pgk-2*, *mP2*, and the human *PGK-2* transgene were analyzed by RTpcr using a standard curve for each obtained using known amounts of T3 or T7 transcribed RNAs. The standard curves demonstrated linearity of amplification and allowed absolute quantitation of the expression levels as described above. RNA isolated from mice containing the human *PGK-2* transgene was used for both the endogenous *mPgk-2* gene and the *PGK-2* transgene. RNA isolated from mice containing the *PGK-2*/CAT transgene was used for the *mP2* experiment. Figure 4 shows developmental RNA accumulation curves for all four genes analyzed. Among the Pgk-2 genes, the highest level of expression is found in the human *PGK-2* transgene, and the lowest in the *PGK-2*/CAT transgene. Levels of expression of transgenes are in part a function of their site of chromosomal integration, and therefore, correlating the levels of expression with the presence or absence of transcriptional signals is difficult. The transgenic lines chosen for this analysis were the highest expressing lines tested (11), and despite concerns over position of integration effects, the three Pgk-2 promoted RNA levels varied less than fourfold. More importantly, examination of the RNA accumulation of the three genes demonstrates that all three genes are expressed with very similar developmental timecourses. In contrast, the *mP2* developmental analysis shows a very different pattern of RNA accumulation. Expression of *mP2* is first detected at day 22 and accumulation reaches 65% of maximum levels by day 30; this is consistent with *mP2* gene expression inititating in round spermatids.

## DISCUSSION

Here we describe a procedure for the quantitative analysis of RNA message levels using reverse transcription and the polymerase chain reaction. We have shown that by careful selection of cycle number and quantity of input RNA we can readily quantitate RNA levels. Because we have demonstrated uniform amplification from tube to tube, we are able to include a standard curve alongside the experimental points to determine absolute levels of mRNA. This protocol is rapid and simple: up to twelve samples of RNA have been quantitated in less than seven hours with sufficient resolution to detect at least twofold differences in the amount of RNA. Incorporation of $^{32}P$ labeled dATP into the pcr product increases sensitivity, allows simple quantitation, and eliminates the need for Southern transfer. The linear range of the assay is demonstrated here to be greater than two orders of magnitude. By using less input RNA or fewer cycles, this linear range can be extended greatly. At very low levels of input RNA the signal begins to level off as the number input molecules continues to decrease, and we believe this is due to contaminating RNA or DNA. We have not attempted to stringently eliminate contamination in our system, although, by reducing the level of contamination we would expect to increase the sensitivity of the assay.

The linearity achieved with this method may be attributed to several factors. First, limiting cycle number and input RNA molecules keeps all other substrates in excess. Designing the protocol to use one tube, one buffer for all enzymatic reactions also minimizes variability. The use of the 3′ pcr primer (as opposed to a poly dT oligomer) to prime the reverse transcriptase reaction eliminates the potential for differential priming efficiencies of certain messages and insures uniform cDNA synthesis. Additionally, maintaining the annealing temperature at the Tm of the primers reduces the level of background bands, and may therefore increase linearity by not depleting the substrates.

Although this protocol uses unaltered *in vitro* transcribed RNA molecules as the template, these molecules differ from the target messages not only in length but in the absence of modifications that natural messages contain ( 5′ capping and 3′ poly A tail). These differences may affect the efficiency of amplification in early steps of the pcr and could affect the accuracy of the quantitation. Additionally, care must be taken to assure that all reactions amplify with equal efficiency. Therefore, a standard curve in the linear range that brackets the experimental points is essential for accurate quantitation. Another variable that could affect the efficiency of the reaction is the source and preparation of the RNA sample. We have amplified standard amount of CAT RNA in samples of total RNA from various mouse tissues and shown that all samples amplify with similar efficiencies (data not shown). However, RNA preparations of different purity or residual contaminants might be expected to have different amplification efficiencies.

Several other pcr protocols have been published for quantitating RNA levels. These protocols rely on internal standards that must be modified to produce an amplified product that may be distinguished from the target pcr product. Such modifications include addition of a restriction site (2,4), addition of an intron (4) and the construction of an artificial amplification template (3). The use of such internal standards requires not only the additional steps to prepare these altered template, but also necessitates performing several pcr reactions to quantitate each sample of RNA.

In our analysis of the transcriptional control of the *Pgk-2* gene during spermatogenesis, RTpcr has enabled us to readily measure the steady-state levels of *Pgk-2* RNA throughout the development of the germ-cell. By determining the initiation of transcription

and quantitating the accumulation of message of the three genes, we show here that the *PGK-2*/CAT transgene, the *PGK-2* transgene, and the mouse *Pgk-2* gene all function in an identical manner. We assume therefore that the transcriptional machinery in the developing germ cell recognizes the same cis-acting sequences in the 1.4 kb human 5′ sequence as in the endogenous locus.

The developmental curves presented here also serve as markers for the appearance and proliferation of specific cell-types in the developing testis. As the appearance and accumulation of *mP2* RNA is a marker for the appearance and proliferation of round spermatids, we suggest that the peak of *Pgk-2* expression occurs earlier, during the pachytene stage of germ cell development. Noting that the proportion of pachytene cells peaks at approximately day 21, then decreases in the adult (14), the continued high levels of expression of *Pgk-2* RNA during development suggest that continued expression occurs in haploid round spermatids. This conclusion is supported by *in situ* data (16 and our unpublished observations).

Singer-Sam et. al. (9), using a related technique with separated spermatogenic cell populations, showed that round spermatids had the highest level of *Pgk-2* RNA. This apparent discrepancy may be reconciled by the observation that round spermatids contain roughly one fourth as much RNA as pachytene spermatocytes (17), causing the *Pgk-2* specific RNA to appear higher in round spermatids when calculated as a function of total RNA. Analysis on a per cell basis (as done in *in situ* experiments), or as a function of total testis RNA, however, shows the highest levels of *Pgk-2* RNA to be in pachytene cells.

This type of analysis is very useful for determining the accumulation of specific messages during development and correlating those messages with the appearance and proliferation of specific cell-types. These expression curves allow us to plot coordinate regulation of both messages and protein products as a function of development and therefore to learn more about the control of development in the testis.

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