Use of the Polymerase Chain Reaction in the Quantitation of *mdr*-1 Gene Expression

Lizabeth Deutsch Murphy, Cynthia Elaine Herzog, Jonathan Brett Rudick, Antonio Tito Fojo, and Susan Elaine Bates*

Medicine Branch, National Cancer Institute, Bethesda, Maryland 20892

Received February 12, 1990; Revised Manuscript Received August 10, 1990

ABSTRACT: The ability of the polymerase chain reaction (PCR) to quantitate expression of mRNA was examined in the present study. The model chosen was expression of the multidrug resistance gene *mdr*-1/Pgp in two colon carcinoma cell lines which express *mdr*-1/Pgp at levels comparable to those found in many clinical samples. PCR was utilized to evaluate differences in *mdr*-1/Pgp expression in the two cell lines after modulation by sodium butyrate. Thus, comparisons were made across a range of *mdr*-1/Pgp expression as well as comparisons of small differences. The PCR was found to be both sensitive and quantitative. Accurate quantitation requires demonstration of an exponential range which varies among samples. The exponential range can be determined by carrying out the PCR for a fixed number of cycles on serial dilutions of the RNA reverse transcription product, or by performing the reaction with a varying number of cycles on a fixed quantity of cDNA. By quantitation of the difference in PCR product derived from a given amount of RNA from the sodium butyrate treated and untreated cells, the difference in mRNA expression between samples can be determined. Normalization of the results can be achieved by independent amplification of a control gene, such as β_2-microglobulin, when the latter is also evaluated in the exponential range. Simultaneous amplification of the control and target genes results in lower levels of PCR products due to competition, which varies from sample to sample. The PCR is thus a labor-intensive but sensitive method of quantitating gene expression in small samples of RNA.

Use of the polymerase chain reaction (PCR)[1] method to detect expression of mRNAs in cells derived from various sources has been described (Murakawa et al., 1988; Noonan & Roninson, 1988). Overexpression of the *mdr*-1/Pgp gene has been shown in vitro to mediate multidrug resistance, and expression of the gene has been found in human tumors and tissues (Pastan & Gottesman, 1987). Studies with patient samples, particularly those obtained from patients in relapse, are often limited to the small quantities of tissue obtained by needle aspiration or biopsy. RNA in situ hybridization and immunohistochemistry can be used with small samples, but quantitation by these methods is difficult, and evaluation of gene expression in total RNA is often desired. Amplification by PCR of cDNA obtained from mRNA is theoretically suited to such situations. We sought to determine whether sensitive and accurate quantitation of *mdr*-1 mRNA could be achieved. In this procedure, mRNA is converted to cDNA by reverse transcription, and subsequently the cDNA is amplified by Taq polymerase using oligonucleotide primers directed against portions of the *mdr*-1 cDNA (Murakawa et al., 1988; Noonan & Roninson, 1988; Saiki et al., 1988). Thus, a linear relationship between the quantity of mRNA and the final PCR product requires both faithful conversion to cDNA and exponential amplification.

We evaluated this question using two human colon carcinoma cell lines established from tumors in untreated patients. The lines express *mdr*-1 mRNA and respond to sodium butyrate, a differentiating agent, by increases in *mdr*-1 gene expression (Mickley et al., 1989). We used two different methods for radiolabeling the PCR product: end-labeling the 5′-oligonucleotide primer with T4 polynucleotide kinase and addition of [^{32}P]dCTP to the reaction mixture. Two methods were used to quantitate the radiolabeled product: scintillation counting of bands excised from a gel guided by ethidium staining and densitometric scanning of bands after autoradiography. Finally, two methods were compared to determine the range over which amplification was exponential: serial dilution of the cDNA after reverse transcription and termination of the PCR reaction after sequential cycles. The results demonstrate that PCR is both sensitive and quantitative and that it can occur exponentially, but over a range which must be determined and which varies from sample to sample.

Experimental Procedures

The human colon carcinoma cell lines SW620 and HCT-15 were obtained from the American Type Culture Collection, routinely cultured, and treated with 2 mM sodium butyrate (Sigma) as previously described (Mickley et al., 1989). Total cellular RNA was isolated by the guanidinium isothiocyanate/cesium chloride method and Northern analysis performed as previously described (Maniatis et al., 1982). In each experiment, water was used as a negative control for contamination. Reverse transcription of 1 μg of total RNA using 10 pmol of specific 3′ primer was performed for 1 h at 37 °C using 150 units of M-MLV reverse transcriptase (Bethesda Research Laboratories) in 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 unit/μL RNasin (Promega), and 1 mM each of dATP, dGTP, dCTP, and dTTP (Murakawa et al., 1988; Noonan & Roninson, 1988). The samples were then heated at 95 °C for 5 min to terminate the reverse transcription. The resulting cDNA was serially diluted in water

* Author to whom correspondence should be addressed at Building 10, Room 12N226, National Institutes of Health, Bethesda, MD 20892.

[1] Abbreviations: PCR, polymerase chain reaction; *mdr*-1, multidrug resistance-1; Pgp, P-glycoprotein; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; DTT, dithiothreitol; BSA, bovine serum albumin.

from 1:2 (500 ng of RNA) to 1:65 536 (0.015 ng of RNA).[2] Added to these dilutions were 70 μL of 1× PCR buffer (50 mM KCl, 40 mM Tris, pH 8.3 at 37 °C, 2.5 mM $MgCl_2$, and 100 μg/mL BSA), 0.2 mM each of dATP, dGTP, dCTP, and dTTP, 50 pmol each of the 5′ and 3′ primers, and 1.5 units of Taq polymerase (Perkin-Elmer Cetus) plus 10 μCi of [^{32}P]dCTP or 10^6 cpm of 5′ primer ^{32}P-labeled by T4 polynucleotide kinase by standard methods (Murakawa et al., 1988; Noonan & Roninson, 1988; Saiki et al., 1988; Maniatis et al., 1982). The final reaction volume was 100 μL. Primers were selected from two exons separated by one or more long intronic sequences which allowed identification of amplification of contaminating genomic DNA. Sequences (cDNA) used were as follows: 5′ (410–441 bp); 3′ (664–695 bp) of the *mdr*-1 gene (Chen et al., 1986). The resulting PCR product from cDNA was 286 bp, and from genomic DNA was 827 bp. For β_2-microglobulin, primers were 5′ (1477–1504 bp) and 3′ (3511–3537 bp), giving rise to a 201 bp PCR product (Gussow et al., 1987). The reaction mixture was overlaid with 100 μL of mineral oil, and heated at 95 °C for 6 min. Amplification was performed in sequential cycles at 94 °C, 1 min, 15 s; 55 °C, 1 min, 15 s; and 72 °C, 2 min. In later experiments, nonspecific PCR product was reduced by shortening the extension time at 72 °C to 1 min, 30 s in the first 20 cycles. Unless otherwise specified, amplification was carried to 30 cycles. After the last cycle, all samples were incubated for an additional 10 min at 72 °C. The products were extracted with 100 μL of chloroform and electrophoresed in 1× Tris–borate electrophoresis buffer with 2 mM EDTA on 2% NuSieve/1% agarose gels. The gels were washed in fresh electrophoresis buffer, stained with 2 μg/mL ethidium bromide, photographed, and exposed overnight at 4 °C for autoradiography. Bands were cut from the gel, guided by ethidium bromide staining, along with comparable-size fragments above and below for determination of background levels of ^{32}P. Scintillation counting was performed in 20 mL of Aquasol (DuPont). Duplicate gels were exposed for further autoradiography at −70 °C. Autoradiograms were analyzed by scanning densitometry using the Beckman DU-8 spectrophotometer, and the area under the curve was calculated for each peak. These results were plotted on a log–log scale against the quantity of RNA present in the PCR reaction diluted from the reverse transcription reaction. The PCR reaction was considered to be exponential if 2-fold amplification was detected. For each graph, an ideal line (thin or dashed line in each figure) was also plotted, based on a doubling of reaction product by serial dilution or by sequential cycle.

Results

We have previously reported increased *mdr*-1/Pgp gene expression after treatment of colon carcinoma cell lines in vitro with sodium butyrate, including the SW620 and HCT-15 cell lines (Mickley et al., 1989). Figure 1 demonstrates the induction in these two lines by Northern analysis. Figure 2 compares the two methods used to quantify the amount of PCR product. Sensitivity was comparable whether densitometry was performed on autoradiograms, or the gel bands were excised and counted. In the SW620 cell line, which expresses low levels of *mdr*-1 mRNA, an exponential relationship was seen across a range of serial 1:2 dilutions of the RNA. At concentrations greater than the 1:8 dilution (representing 125 ng of RNA), the amplification begins to fall from the exponential range. Figure 3 shows the results obtained by quantitation of PCR product in the HCT-15 cell line with and without sodium butyrate treatment. This cell line has higher levels of *mdr*-1/Pgp mRNA than SW620. As a result, exponential ranges were demonstrated, but plateaus were reached in more dilute samples than in the SW620 cell line. In order to determine the relative amounts of mRNA in the two samples, points in the exponential phase must be utilized. When

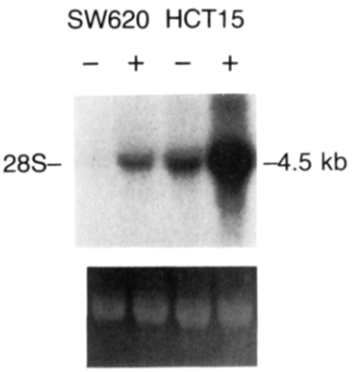

FIGURE 1: Northern analysis of *mdr*-1 gene expression in two human colon cancer cell lines before and after 2 mM sodium butyrate treatment. Eight micrograms of RNA was electrophoresed in a 6% formaldehyde gel and transferred to nitrocellulose using standard methods (Maniatis et al., 1982). A 1.4-kb cDNA fragment encoding the middle third of *mdr*-1/Pgp subcloned into a pGEM 4 vector was utilized to make an antisense riboprobe as previously described (Chen et al., 1986; Melton et al., 1984; Bates et al., 1989). Hybridization and washing conditions were as previously described (Mickley et al., 1989; Bates et al., 1989). Autoradiography was performed at −70 °C. In the lower panel, the photograph of the ethidium bromide stained gel is shown to demonstrate integrity of RNA and comparability of loading.

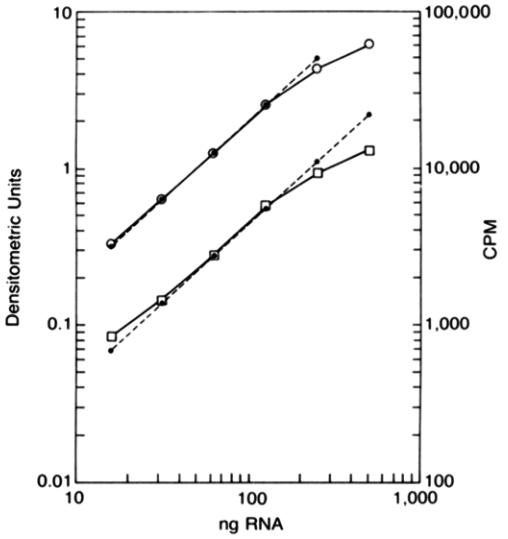

FIGURE 2: Quantitation of PCR product after amplification of *mdr*-1 mRNA in the SW620 cell line. PCR was performed on serial 1:2 dilutions of the reverse transcription product (from 500 ng of RNA) with 10 μCi of [^{32}P]dCTP/dilution. Twenty microliters of each resulting 100-μL reaction mixture was analyzed by electrophoresis on agarose/NuSieve gels. Autoradiography was performed overnight, and the bands were scanned on a densitometer. The area under the curve for each band was calculated and plotted on the graph (○). Bands on a duplicate gel were excised; plus a matching region was excised above and below to determine background cpm. The background cpm were averaged for each lane and subtracted from the counts in the band of interest, and the results were plotted on the graph (□). Both densitometry and scintillation counting give exponential results over a comparable range. For this graph, as well as those following, an ideal PCR (thin line) was plotted based on a doubling of reaction product with each dilution.

[2] In this paper, when a quantity of RNA is indicated as being used for PCR, this refers to the primer-specific reverse transcriptase generated cDNA derived from this amount of RNA.

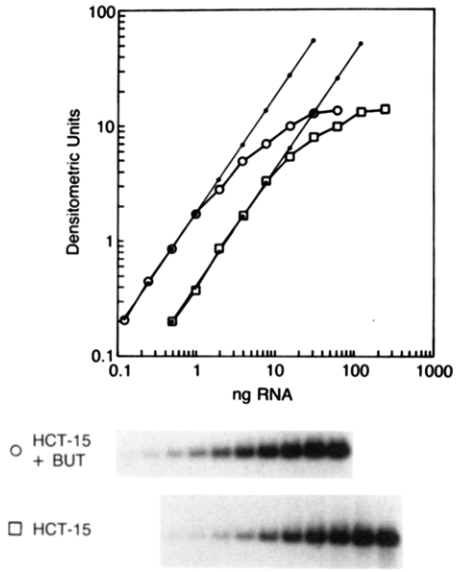

FIGURE 3: PCR measurement of increased *mdr*-1 expression in the HCT-15 cell line after sodium butyrate treatment. For this analysis, cDNA derived from the reverse transcription was serially diluted. Shown in the figure are the results from gels beginning at two different dilutions so that a longer exponential range could be shown with each treatment. Serial dilutions plotted for HCT-15 treated with butyrate (○) begin with 62.5 ng of RNA, while those for HCT-15 without butyrate (□) begin with 250 ng of RNA. End-labeled primers were utilized to quantitate the PCR product, with 16 μL of the product analyzed on each gel. Autoradiograms were exposed for differing lengths of time for each segment of the curve, to be certain that the linear range of the autoradiographic film was being utilized for densitometry. All values were normalized to those of the overnight exposure, which is shown in the panel below the graph. When the values for 0.49 ng of RNA, which is in the exponential range of both curves, are compared, the level of *mdr*-1 mRNA expression in the sodium butyrate treated cell line is 4.5-fold higher than in the untreated cells.

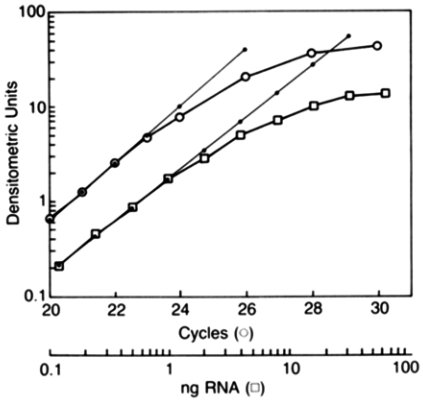

FIGURE 4: Detection of the exponential range by serial dilution and by termination at sequential cycles in RNA from HCT-15 cells treated with butyrate. The product of the PCR reaction after 30 cycles of amplification of serially diluted cDNA (□) is compared to termination of amplification at sequential cycles (○). Serial 1:2 dilutions were performed beginning with 62.5 ng of RNA, as shown in the graph, and amplified 30 cycles. 125 ng of RNA was used for each of the sequential cycle PCR reactions. Thus, corresponding steps of PCR product are found at 28 cycles/31.25 ng, 26 cycles/7.8 ng, 24 cycles/1.95 ng, 23 cycles/0.98 ng, etc. At 23 cycles and 0.98 ng, the PCR reaction reaches a plateau, indicating that either method can be used to determine the exponential range of amplification.

two such points from identical amounts of input RNA are compared, a 4.5-fold difference in densitometric units is observed, indicating that *mdr*-1 expression in these cells increased 4.5-fold after sodium butyrate treatment.

Figure 4 compares the results obtained with serial dilutions of RNA amplified 30 cycles to the results found when

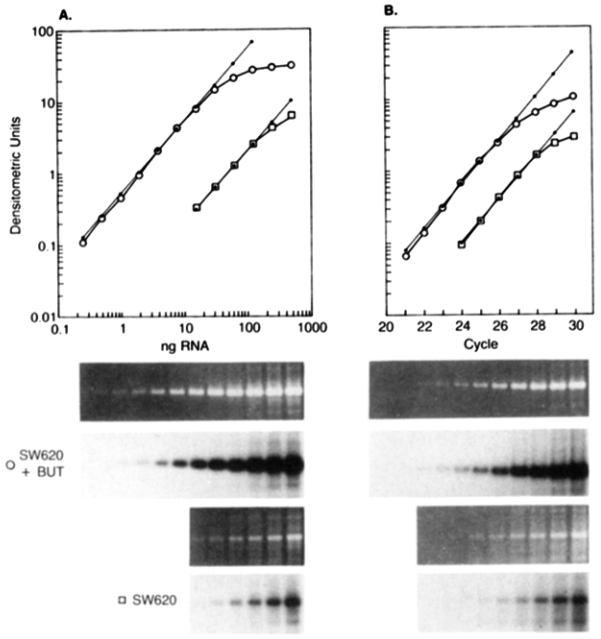

FIGURE 5: Analysis of increased expression of *mdr*-1 mRNA in SW620 cells by two methods for detection of the exponential range. RNA from SW620 cells with (○) and without (□) sodium butyrate treatment was analyzed by PCR. Results from analysis of serial dilutions and sequential cycles are shown in the left and right panels, respectively. Photographs of the ethidium bromide stained gels and the autoradiograms are shown beneath the corresponding graphs. All serial dilutions were carried to 30 cycles. Sequential cycles were initiated with 500 ng of RNA in untreated cells, and with 125 ng of RNA in the treated cells, so that a correction factor of 4 exists between the two lines. A 25–28-fold difference can be seen in densitometric units, if a point from the exponential range for both methods is compared.

equivalent RNA aliquots were amplified with varying numbers of PCR cycles. As shown, the plateau in PCR amplification occurred at a comparable point with both approaches. Thus, the amount of product is doubled with each successive cycle until the plateau is reached, just as the product of 30 cycles doubles when successive 1:2 dilutions are compared until a plateau is reached. The plateau is reached at a comparable point even though the reactions were performed several weeks apart, and quantitated from exposures on different autoradiograms. When the same experiment was performed with the SW620 cell line, the plateau was again achieved at a comparable point by both serial dilution and sequential cycle methods as shown in Figure 5.

The increase in *mdr*-1 mRNA in the SW620 cell line by sodium butyrate treatment is shown in Figure 5. Both the ethidium bromide stained gel and the autoradiogram are shown for PCR product analyzed both by the serial dilution and by the sequential cycles methods. The quantity of RNA analyzed by sequential cycles is 500 ng in the untreated cells, the same quantity used in the first sample of the dilution series. Thus, the points at 30 cycles and at 500 ng of RNA are identical for each treatment. With both methods, the amplification of cDNA from untreated SW620 cells ceases to be exponential two points below the final cycle or dilution. In the SW620 cell line treated with sodium butyrate, sequential cycles were performed on 125 ng of RNA, while serial dilutions begin with 500 ng of RNA. The values plateau at a similar point whether analyzed by sequential cycles or by serial dilutions. Determination of the difference between the two samples requires comparing two points from the same amount of input RNA within the exponential range of each sample. The 25-fold difference between the exponential portion of the two curves from the serial dilution method in the left panel reflects the

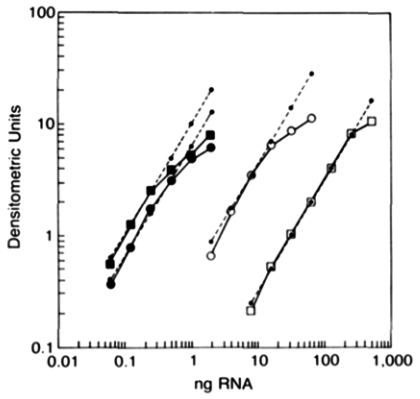

FIGURE 6: Utilization of β_2-microglobulin mRNA amplification in normalizing PCR amplification products. RNA from treated (○, ●) and untreated (□, ■) SW620 cells was analyzed for expression of β_2-microglobulin (●, ■) by PCR and compared to the result obtained with *mdr*-1 (○, □) amplification. Reverse transcription reactions and the PCR amplifications were performed separately for each gene. PCR amplification of serial 1:2 dilutions of RNA was performed beginning with 500 and 62.5 ng for the *mdr*-1 amplification in RNA from SW620 cells without (□) and with (○) sodium butyrate treatment, respectively. For β_2-microglobulin amplifications, PCR was performed on serial dilutions from 1.95 ng of RNA. Plateau is reached after 30 cycles in the two β_2-microglobulin-initiated amplifications with 0.24 ng of RNA. If points from the exponential range of each are compared, and normalized by the 1.6-fold difference in β_2-microglobulin level, *mdr*-1 expression differs 22-fold.

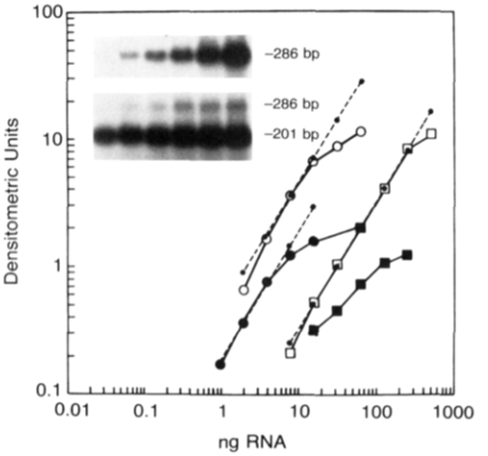

FIGURE 7: Competition between β_2-microglobulin and *mdr*-1 primers for amplification. In order to quantify mRNA and eliminate variables due to "tube effects", some investigators add a known quantity of internal standard to each PCR reaction. This experiment demonstrates competition from the internal standard for exponential amplification of *mdr*-1. Reverse transcriptions were carried out separately on 1 μg of total RNA. Then 250 ng of RNA from the *mdr*-1 reverse transcription was combined with 31.25 ng of RNA from the β_2-microglobulin reverse transcription. Serial 1:2 dilutions were obtained, and PCR amplification was carried out. The results from these reactions (●, ■) are compared to the results obtained by the independent amplification (○, □) shown in Figure 6. Levels of PCR product for the SW620 cells treated with butyrate (●) plateau 1–2 dilutions earlier than in the previous experiment, while PCR products from untreated SW620 cells (■) in the exponential range are not detectable. The upper panel shows the results of the independent *mdr*-1 amplification of serial dilutions, beginning with cDNA equivalent to 250 ng of input RNA from untreated SW620 cells. In the lower panel, the amplification product is shown for *mdr*-1 (286 bp) when PCR is carried out on the identical dilutions used in the upper panel, but amplified simultaneously with β_2-microglobulin (201 bp) as described above.

increase in *mdr*-1 mRNA after butyrate treatment. A 7-fold difference is observed between the exponential ranges by the sequential cycle method in the right panel. With a correction factor of 4 for the amount of initiating RNA in the sequential cycle amplification, a 28-fold difference in *mdr*-1 expression is deduced. Thus, both methods demonstrate a comparable increase in *mdr*-1 mRNA from butyrate-treated SW620 cells, similar to the 20–25-fold increase observed by Northern analysis (Mickley et al., 1989).

Highly expressed genes such as β_2-microglobulin or β-actin are used to confirm the comparability of RNA loading on gels in Northern analysis. The utility of β_2-microglobulin in assessing comparability of "loading" in PCR was evaluated. In preliminary experiments, it was apparent that an exponential range could be determined, but PCR of 1 μg of RNA reached a plateau by the 18th cycle for β_2-microglobulin, when *mdr*-1 mRNA was still undetectable. Figure 6 demonstrates the value of β_2-microglobulin in normalization of results from *mdr*-1 mRNA amplification. PCR of serial dilutions beginning with 1.95 ng of RNA was performed for the β_2-microglobulin quantitations. For PCR of *mdr*-1 mRNA, serial dilutions began with 500 and 62.5 ng of RNA for SW620 cells without and with sodium butyrate treatment, respectively. This range of input RNA was chosen in order to achieve amplification in the exponential range. The *mdr*-1 PCR product from 7.8 ng of RNA, expressed in densitometric units, is 0.25 for untreated SW620 and 3.5 for treated SW620 cells. For β_2-microglobulin, the plateau occurs in the reactions initiated with more than 0.24 ng of RNA, after 30 cycles of amplification. The β_2-microglobulin PCR product from 0.12 ng of RNA, again expressed in densitometric units, is 1.26 for untreated SW620 and 0.79 for treated SW620 cells. This 1.6-fold difference in the level of PCR product obtained with β_2-microglobulin reflects variability in input RNA rather than a true difference in *mdr*-1 mRNA expression. Normalization of the *mdr*-1 densitometric units by the β_2-microglobulin densitometric units, $3.5/0.79 = 4.4$ and $0.25/1.26 = 0.20$, results in a true difference in *mdr*-1 expression of $4.4/0.20 = 22$-fold, analogous to the result obtained without normalization in a similar experiment shown in Figure 5, where comparable RNA amounts were compared.

A number of reports have appeared showing normalization of the mRNA of interest to an internal standard utilizing the same or different primer sets (Wang et al., 1989; Noonan & Roninson, 1988). The internal standard is coamplified in the same tube with the mRNA of interest. We evaluated this approach for β_2-microglobulin in the RNA from the SW620 cells. Because β_2-microglobulin expression is over 1000-fold more than *mdr*-1 expression in these cells, the *mdr*-1 signal would not have been detectable if cDNA from 1 μg of total RNA had been amplified in the presence of both primer sets. Thus, unequal amounts of the two reverse transcription products were coamplified. These results were compared to those achieved with amplification of the two mRNA species separately. RNA from each treatment condition was aliquoted once, diluted, and then split for separate analysis of each gene. Reverse transcription reactions were performed separately on 1 μg of total RNA. Serial dilutions were performed from a mixture of 31.25 ng of input RNA for β_2-microglobulin and 250 ng of input RNA for *mdr*-1. As shown in Figure 7, simultaneous amplification results in premature attenuation of the exponential phase of the PCR reaction. This attenuation is different for the two *mdr*-1 expression levels. For example, a plateau is reached in RNA from untreated SW620 cells such that PCR products in the exponential range are undetectable, as demonstrated in Figure 7. For treated SW620 cells, the exponential range is detectable, but the plateau occurs in more dilute samples than when amplification is performed independently. Thus, competition with an internal standard varies with the amount of the mRNA of interest in the initiating

sample, thus rendering quantitation by comparison to an internal standard variable.

Discussion

The present study demonstrates that the polymerase chain reaction can be utilized to quantitate gene expression. Accurate measurement of PCR products can be achieved by radiolabeling the PCR product by addition of either end-labeled primer or radiolabeled nucleotides. After the products are subjected to gel electrophoresis, either densitometry of bands on autoradiograms or scintillation counting of excised bands can be used to arrive at a numerical value for the PCR product. Determination of an exponential range of amplification can be achieved either by serially diluting the amount of cDNA in the PCR or by terminating the PCR at sequential cycles. PCR in both cases ceases to be exponential at comparable points. Assay by termination of the reaction at sequential cycles requires greater amounts of input RNA. Since the strength of the PCR method is in its sensitivity in small samples, serial dilution of the reverse transcription product is favored in settings where the RNA amount is limiting.

We used as our model for these studies expression of *mdr*-1/Pgp, utilizing cell lines with levels of *mdr*-1/Pgp in the range commonly found in clinical samples, from the very low level in the uninduced SW620 cells to the higher levels found in the HCT-15 line after sodium butyrate treatment. PCR was used successfully to quantitate expression, detecting even the modest increase seen in the HCT-15 cell line after the addition of sodium butyrate. Determination of the exponential range, a very labor-intensive process, is an absolute requirement. Since the exponential range extends consistently only a few dilutions or cycles above the level of detection of signal, a broad range of points will need to be examined in clinical samples, which vary widely in *mdr*-1 expression. Determination of differences between samples requires analyses such as those found in Figures 5 and 6. Previous studies which have reported an inability to directly quantitate concentrations of mRNA over a broad range have failed because the analyses were performed after plateau (Becker-Andre & Hahlbrock, 1989; Gilliland et al., 1990). However, reliable determination of differences by the PCR methodology described here provides a clinical tool which is comparable to Northern analysis in accuracy, but greatly exceeds its sensitivity. Furthermore, when quantities of RNA are very low, PCR provides the only method of analysis.

Traditional methods of RNA quantitation such as Northern analysis have utilized analysis of a control gene such as β_2-microglobulin or β-actin to verify comparability of RNA loading, transfer, or hybridization between samples. Although the perfect control has never been found, β_2-microglobulin and β-actin have proven useful, with some limitations. Their chief value lies in being endogenous to the RNA preparation from the beginning. Normalization of PCR results by analysis of β_2-microglobulin, as in our model system, provides a means whereby errors in RNA measurement can be corrected. However, measurements of the control gene must also be performed in the exponential range. Since these control genes are expressed at higher levels than most messages under study, and plateau quickly, a false appearance of comparable loading can occur. Figure 6 describes normalization by β_2-microglobulin PCR. When points are compared from the exponential range of each dilution series, β_2-microglobulin is detectable at a level 1.6 times greater in the sample from untreated cells than in that from treated cells. One can infer that 1.6 times more total RNA was utilized in the reverse transcription and PCR initially. The assumption underlying such a statement is that β_2-microglobulin is expressed comparably in the two treatment conditions.

Recent studies have suggested alternative methods for quantitation of mRNA without utilizing an endogenous standard. Two reports utilized a competitive method which is not cycle dependent, and is evaluable after plateau (Becker-Andre & Hahlbrock, 1989; Gilliland et al., 1990). A standard template utilizing the same primers is added in serial dilutions to aliquots of cDNA resulting from the reverse transcription reaction. The standard can be separated by size from the PCR product of the mRNA of interest. Since the amount of template added is known, it is assumed that when the PCR products resulting from the standard and the endogenous mRNA are equal that the amount of mRNA in the original sample is equal to the amount of template added. Thus, a standard curve is generated by the dilutions of template added, which allows assignment of an absolute value to the mRNA of interest; this method does not require evaluation of the efficiency of the reaction itself. However, errors in comparison between samples are not controlled by this method at several points: (1) error in total RNA or template measurements; (2) error in aliquoting; and (3) errors arising from incomplete digestion and heteroduplex formation when restriction digestions are utilized to separate the internal control from the PCR product.

A second method of quantitation is to add a known amount of a standard template and perform serial dilutions similar to the approach in Figure 7 (Wang et al., 1989). Addition of the known amount of template again allows construction of a standard curve from which the amount of mRNA of interest can be deduced. If the two reactions are proceeding in the exponential phase simultaneously, this is a reasonable method to interpret the quantity of mRNA in the sample being assayed. However, errors in RNA measurement, in template measurement, and in aliquoting are not controlled in this method. This method would be equivalent to adding a known standard to a total RNA sample being analyzed by Northern analysis and deducing the amount of mRNA in the sample from the intensity of the two hybridization signals. Variables in transfer, hybridization, and autoradiography would be controlled, but not the source of the most variability: RNA measurement.

On the other hand, if the reactions are not proceeding in the exponential phase simultaneously, assay of either an exogenous or an endogenous standard together with the mRNA of interest by PCR will cause failure of quantitation. Simultaneous amplification of control genes which are expressed at higher levels than the mRNA of interest leads to premature and variable plateau in the latter. In Figure 7, had the PCR been performed on comparable levels of reverse transcription product for β_2-microglobulin and *mdr*-1, the expression in the untreated SW620 RNA could never have been detectable. Even when the combined PCR was initiated with $^1/_8$th the amount of β_2-microglobulin cDNA as *mdr*-1, the detectable *mdr*-1 product is in a prematurely reached plateau. Such competition, which varies with the amount of mRNA of interest in the sample, renders quantitation by comparison to a standard within the same tube of restricted utility.

Assay of a control gene (as in Figure 6) does not eliminate these variables, but allows one to control for them, and allows normalization in small samples. We chose this approach for quantifying RNA by PCR because in our experience the major error in RNA quantification lies in the measurement and aliquoting of the RNA itself. Comparably loaded Northerns require precision, and PCR, in utilizing smaller quantities of

RNA, requires even greater precision.

The advantage cited for the other methods is that the internal standard provides a control for the reaction efficiency and that an absolute value results. Our method concentrates on a reliable determination of differences. The reaction efficiency is proven by showing that the PCR product is doubling every cycle. However, addition of ^{32}P-labeled dCTP to the PCR substrates does allow one to calculate the amount of PCR product made. The products must be separated by gel electrophoresis, and the bands excised and counted in a scintillation counter. From these data, the quantity and specific activity of the [^{32}P]dCTP, and the sequence of the PCR product, one can calculate the number of molecules of specific mRNA in the total RNA assayed.[3] Thus, an absolute value can be determined for each product. For our purposes, reliable determination of differences between samples is more important than having a determination of the exact number of molecules of mRNA.

Although expression of *mdr*-1/Pgp was analyzed in this study, the results can be applied to other genes in other systems. PCR is a feasible, but labor-intensive, approach to quantitation of gene expression in tumors, tissues, or cell lines. Although further studies will be required to determine the best gene for normalization of results, PCR appears to be quite reproducible and accurate. Its strength lies in its sensitivity, and its capacity to accurately reflect mRNA expression in small samples.

[3] Assuming 6000 cpm resulting from 25 ng of total RNA amplified 30 cycles without plateau, PCR = $2^{30} = 1.07 \times 10^9$ amplifications at 2 per cycle for 30 cycles, PCR product = 286 bases × 2 = 572 bases, ratio of cold/hot dCTP = 0.2 mM cold dCTP/33 nM [^{32}P]dCTP = 6060, and specific activity of [^{32}P]dCTP = 3000 Ci/mmol = 3×10^3 cpm/fmol, then 6000 cpm divided by 3000 cpm/fmol = 2 fmol of [^{32}P]dCTP, 2 fmol × 6060 (cold dCTP/hot dCTP) = 12120 fmol of dCTP, 12120 fmol of dCTP/119 dCTP's per PCR product = 102 fmol of PCR product, 6.02×10^{23} molecules/mol × 102 fmol = 6.14×10^{10} molecules of product, 6.14×10^{10} molecules of product/1.07×10^9 amplifications in 30 cycles = 57.4 molecules of input mRNA, and 57.4 molecules of input mRNA/25 ng of input RNA = 2.3 mRNA molecules/ng of input total RNA.

ACKNOWLEDGMENTS

We thank Ruth Morsillo for assistance with typing the manuscript. We thank Silvia S. Fojo for technical assistance and advice.

Registry No. Sodium butyrate, 156-54-7.

REFERENCES

Bates, S. E., Mickley, L. A., Chen, Y.-N., Richert, N., Rudick, J., Biedler, J. L., & Fojo, A. T. (1989) *Mol. Cell. Biol. 9*, 4337–4344.

Becker-Andre, M., & Hahlbrock, K. (1989) *Nucleic Acids Res. 17*, 9437–9446.

Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., & Roninson, I. B. (1986) *Cell 47*, 381–389.

Gilliland, G., Perkin, S., Blanchard, K., & Bunn, H. F. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2725–2729.

Gussow, D., Rein, R., Ginjaar, I., Hochstenback, F., Seemann, G., Kottman, A., & Ploegh, H. L. (1987) *J. Immunol. 139*, 3132–3138.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, pp 187–207, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Melton, D. A., Kreig, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., & Green, M. R. (1984) *Nucleic Acids Res. 12*, 7035–7056.

Mickley, L. A., Bates, S. E., Richert, N. D., Foss, F., Rosen, N., & Fojo, A. T. (1989) *J. Biol. Chem. 264*, 18031–18040.

Murakawa, G. J., Zaia, J. A., Spallone, P. A., Stephens, D. A., Kaplan, B. E., Wallace, R. B., & Rossi, J. J. (1988) *DNA 7*, 287–295.

Noonan, K. E., & Roninson, I. B. (1988) *Nucleic Acids Res. 16*, 10366.

Pastan, I., & Gottesman, M. (1987) *N. Engl. J. Med. 316*, 1388–1393.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., & Erlich, H. A. (1988) *Science 239*, 487–491.

Wang, A. M., Doyle, M. V., & Mark, D. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9717–9721.