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Controls for Validation of Relative Reverse Transcription-Polymerase Chain Reaction Assays

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Sequential use of reverse transcription-polymerase chain reaction (RT-PCR) has been employed for the measurement of mRNA levels because of its high sensitivity and specificity. Two types of RT-PCR assays are currently in use. The quantitative RT-PCR assay permits the determination of the number of mRNA molecules per cell while the relative RT-PCR assay allows the comparison of mRNA levels in different samples. Both methods yield comparable results⁽¹⁾ and the relative RT-PCR assay has been used in several quantitative studies in the past.⁽²⁻⁴⁾ The quantitative and relative RT-PCR assays are appropriate for use in different types of analysis. When quantitative determination of relative changes in mRNA levels are desired, the relative RT-PCR assay has the advantage of simplicity. In this study we present simple controls for validation of the conditions employed in the relative RT-PCR method.

MATERIAL AND METHODS

Cell culture and RNA isolation

MG63 cells (American Type Culture Collection, Rockville, MD) were cultured in 50% Dulbecco's minimal essential medium (MEM) and 50% Ham's F-12 containing 10% newborn bovine serum, 100 U/ml penicillin, and 100 µg/ml of streptomycin. Total RNA was isolated from subconfluent cultures using the method of Chomczynski and Sacchi.⁽⁵⁾

RT-PCR

The RT-PCR assay was performed in a single tube under optimized conditions that employ standardized stock reaction solutions as described previously.^(3,4,6,7) The primer sequences of the integrin α_2 -subunit and aldolase have been reported.⁽⁴⁾ The 5' end (sense) primers were end-labeled with [γ -³²P]ATP by T4 polynucleotide kinase (U.S. Biochemical Corp., Cleveland, OH) to a sp. act. of 2×10^7 dpm/µg, and 2×10^6 to 4×10^6 dpm was used in each PCR amplification. Aliquots of PCR products taken at different cycles, as indicated, were resolved on 5% polyacrylamide gels, visualized by ethidium bromide staining, excised from the gels, and counted with a liquid scintillation counter.

RESULTS AND DISCUSSION

To determine the ability of RT-PCR to measure differences accurately in mRNA

levels, a pooled total RNA sample was first serially diluted 1 : 2, 1 : 4, and 1 : 8. Integrin α_2 -subunit mRNA and a housekeeping gene (aldolase mRNA) in the diluted samples were determined by RT-PCR.⁽⁶⁾ The relative mRNA levels of α_2 or aldolase in these samples were, thus, expected to be 1 : 1/2 : 1/4 : 1/8. It was also expected that the α_2 /aldolase ratio in these samples would be identical. Murphy and co-workers⁽²⁾ have also shown that expected values are obtained when serial dilutions of an RNA sample are analyzed by the relative RT-PCR assay.

Control studies were carried as follows: 1, 0.5, 0.25, and 0.125 µg of the above RNA samples were converted to cDNA by avian myeloblastosis virus (AMV) reverse transcriptase and subsequently amplified by PCR for the number of cycles indicated, and the radioactivity in PCR products was compared (Fig. 1). PCR products of α_2 and aldolase increased exponentially over a 10-cycle range (cycles 18-28 for α_2 and cycles 14-22 for aldolase) (Fig. 1, A and C, respectively).

As anticipated, the expected ratios of PCR products of 1, 1/2, 1/4, or 1/8 were observed in the exponential range of amplification for α_2 and for aldolase (Fig. 1, B and D, respectively). As expected, the calculated α_2 /aldolase ratio was found to be nearly constant over the eightfold range of total RNA concentrations studied (Fig. 2). We conclude that relative RT-PCR studies carried out under the conditions described will permit accurate determination of relative differences in α_2 -integrin subunit mRNA concentrations.

Two types of RT-PCR assays are currently in use. In the quantitative RT-PCR assay initially described by Wang et al.,⁽⁸⁾ known amounts of a synthetic RNA (or cDNA) are coamplified in the same tube as a sample of cellular RNA. The quantitative RT-PCR assay allows the determination of the number of mRNA molecules per cell.^{1,8} In the relative RT-PCR assay,^(1,3,4) a housekeeping gene (actin, aldolase, etc.) and an experimental gene product (α_2 -integrin subunit, etc.) are amplified in separate tubes. Differences in the levels of a given species of mRNA in different samples are determined by comparing the ratio of experimental gene to housekeeping gene. The relative RT-PCR method allows one to determine differences in mRNA levels between different samples.

Technical Tips

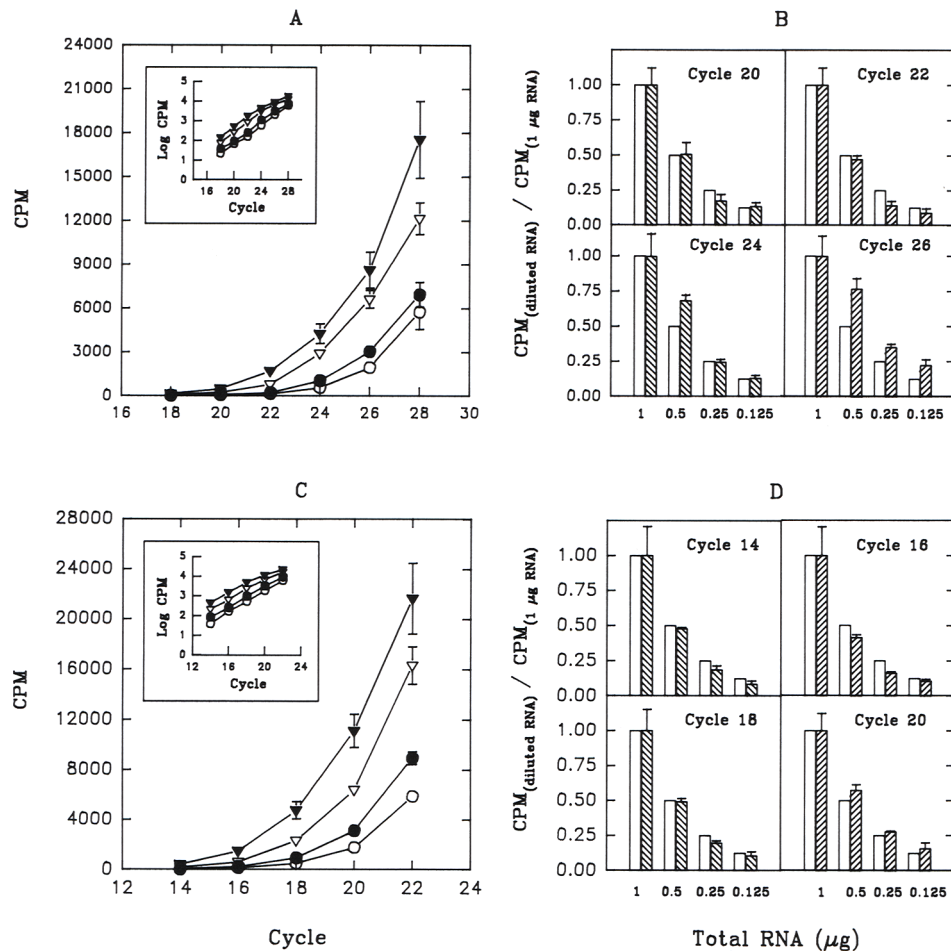


FIGURE 1 Measurement of α_2 -integrin subunit and aldolase mRNA levels with the relative RT-PCR assay. (A) α_2 -Integrin subunit mRNA amplification: A pooled RNA sample from MG-63 osteosarcoma cells was serially diluted, and 1 (\blacktriangledown), 0.5 (∇), 0.25 (\bullet), and 0.125 (\circ) μg of RNA were amplified by RT-PCR using ^{32}P -end-labeled sense primers. Aliquots were withdrawn at the cycle number indicated. Following PAGE separation, the radioactivity in appropriate bands was counted. α_2 -Integrin subunit-PCR products accumulated in an exponential fashion as indicated by the linear semilog plot (*inset*). The data presented are the mean of triplicate assays \pm s.d. (B) Comparison of expected (open bars) with observed (hatched bars) values. The results presented in A are displayed such that the mean cpm generated by 1 μg of RNA is given a relative value of 1 and the expected (0.5, 0.25, and 0.125) and observed values for each dilution are then presented. Error bars represent the s.d. Calculations were performed as follows. The observed RT-PCR data (hatched bars) are compared with expected results (open bars) based on the initial dilution of the RNA samples. The expected ratio of $\text{cpm}_{(\text{diluted RNA})}/\text{cpm}_{(1 \mu\text{g RNA})}$ (open bars) was calculated by dividing the cpm of the 1- μg RNA sample by the known dilution factor. In a similar fashion, the observed $\text{cpm}_{(\text{diluted RNA})}/\text{cpm}_{(1 \mu\text{g RNA})}$ ratio (hatched bars) was obtained by dividing the experimentally determined cpm for a given diluted sample by the cpm of the 1- μg sample. The results indicate that expected and observed values agree well for each dilution of the RNA sample over a wide range of PCR cycles. (C,D) The same serially diluted total RNA used in A and B was amplified for aldolase by RT-PCR (see A, above, for description of symbols), and the results are presented in a fashion identical to A and B above. As in the case of the α_2 -integrin subunit, aldolase cDNA amplified exponentially (see C *inset*) and observed values (hatched bars) agreed well with expected values (open bars) (D).

In an elegant analysis of parameters involved in the quantitative RT-PCR assay, it was shown clearly that the relative and quantitative RT-PCR assays yielded comparable results.⁽¹⁾ Because of the unique advantages of each method, the

relative and quantitative RT-PCR assays are appropriate for use in different situations. Whereas the quantitative RT-PCR assay has the advantage of yielding the absolute number of mRNA molecules per cell, the relative assay has the

advantage of simplicity. It should be noted that both the quantitative and the relative RT-PCR assay require the determination of the level of a housekeeping gene to correct for errors in pipetting or RNA quantitation.⁽¹⁾ Whereas the quantitative RT-PCR assay can be used over a wide range of PCR cycles, the relative RT-PCR assay is restricted to use in the exponential range of amplification. This is a minor advantage of the quantitative assay because it is quite easy to determine the PCR cycle numbers that are in the exponential range of amplification. The technical disadvantages of the quantitative assay are the necessity (1) to prepare synthetic mRNAs containing the primer set employed, (2) to determine the relative efficiency of the reverse transcription reaction, and (3) to carry out determinations at several ratios of known-to-unknown mRNA.

Whereas only the quantitative RT-PCR assay is capable of determining the number of mRNA molecules per cell, the relative RT-PCR assay is simple and quite adequate when comparison of mRNA levels in different samples is desired. The control studies described here provide a simple means to establish the validity of measurements made with the relative RT-PCR assay.

ACKNOWLEDGMENTS

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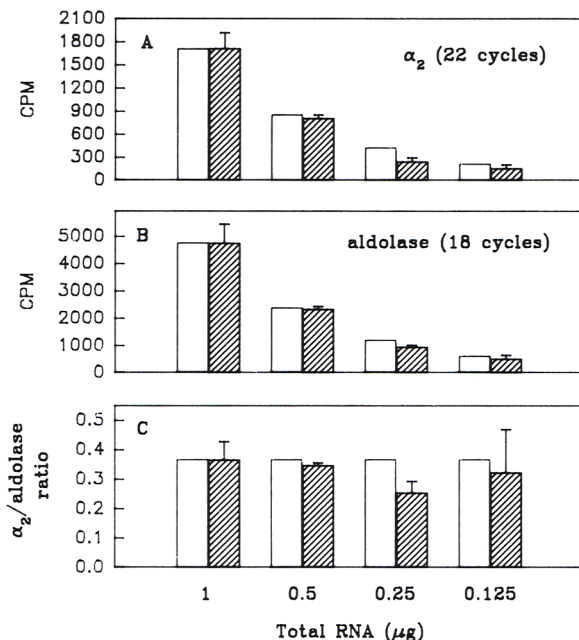


FIGURE 2 Comparison of relative α_2 -integrin mRNA levels determined by the relative RT-PCR assay. Data from Fig. 1 for the α_2 -integrin subunit (A, cycle 22) and aldolase (C, cycle 18) were used to calculate the α_2 /aldolase ratio presented. The data used to calculate the α_2 /aldolase ratio is given in A and B. The results indicate that when PCR products are harvested in the exponential range of amplification (cycles 20–24 for α_2 , cycles 16–18 for aldolase), the expected (open bars) and observed (hatched bars) results are in good agreement. The ratio of α_2 -subunit mRNA/aldolase mRNA remains essentially constant over the eightfold range of RNA concentrations amplified by RT-PCR (C). Thus, even fairly large pipetting errors would not be expected to significantly alter the integrin mRNA/aldolase ratios derived by the relative RT-PCR assay.

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