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Real-time RT-PCR analysis of mRNA decay: half-life of Beta-actin mRNA in human leukemia CCRF-CEM and Nalm-6 cell lines

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Abstract

Background: We describe an alternative method to determine mRNA half-life ($t_{1/2}$) based on the Real-Time RT-PCR procedure. This approach was evaluated by using the β-actin gene as a reference molecule for measuring of mRNA stability.

Results: Human leukemia Nalm-6 and CCRF-CEM cells were treated with various concentrations of Actinomycin D to block transcription and aliquots were removed periodically. Total RNA was isolated and quantified using the RiboGreen® fluorescent dye with the VersaFluor Fluorometer System. One μg of total RNA was reverse transcribed and used as template for the amplification of a region of the β-actin gene (231 bp). To generate the standard curve, serial ten-fold dilutions of the pBactin-231 vector containing the cDNA amplified fragment were employed, β-actin mRNAs were quantified by Real-Time RT-PCR using the SYBR® Green I fluorogenic dye and data analyzed using the iCycle iQ system software. Using this method, the β-actin mRNA exhibited a half-life of 6.6 h and 13.5 h in Nalm-6 and CCRF-CEM cells, respectively. The $t_{1/2}$ value obtained for Nalm-6 is comparable to those estimated from Northern blot studies, using normal human leukocytes (5.5 h).

Conclusions: We have developed a rapid, sensitive, and reliable method based on Real-Time RT-PCR for measuring mRNA half-life. Our results confirm that β-actin mRNA half-life can be affected by the cellular growth rate.

Background

Determination of mRNA half-life is important to our understanding of gene expression and mechanisms involved in the regulation of the level of transcripts in response to environmental changes or developmental cues. In addition, the stability of mRNA may determine how rapidly the synthesis of the encoded protein can be shut down after transcription ceases. mRNA half-life can be determined by densitometric analysis of *in situ* hybridization histochemistry [1] or by Northern blot analysis [2] of RNA samples removed from cells treated with transcriptional inhibitors such as actinomycin D (ActD) [3], α-amanitin [4], or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) [5]. Although reliable, these multi-step methods are laborious and time-consuming. The advent of new technologies such as the Real-Time PCR allows rapid and exact measurement of copy number of molecules present in the sample [6]. Real-Time Reverse Transcriptase PCR

(RT-PCR) allows precise and reproducible quantitative determination of the number of mRNA transcripts synthesized [7,8]. We have developed a rapid and reliable Real-Time quantitative RT-PCR approach to determine mRNA half-life based on the SYBR® Green I fluorogenic dye (Molecular Probes, Inc., Eugene, OR, USA) and relative to the amount of total RNA per cell samples.

To evaluate that approach, the β-actin gene was used as a reference molecule for mRNA stability. Actin proteins are components of the microfilament which play a crucial role in maintaining cell shape and motility. Expression of β-actin has been shown to be relatively constant as cells progress through the cell cycle [9] and has been used as a standard for an unchanging protein and mRNA in studies of gene regulation. In this study, we used the human leukemia cell lines CCRF-CEM (T-cell lineage, Acute Lymphoblastic Leukemia (ALL)) and Nalm-6 (B-cell precursor, ALL) that respond differently to antifolate drugs, such as methotrexate (MTX). Nalm-6 cells were shown to be more sensitive to MTX when compared to CCRF-CEM [10].

Results and Discussion

The β-actin mRNA half-life was determined from CCRF-CEM and Nalm-6 cell lines treated with 0.5 μM, 1 μM, and 5 μM ActD (Sigma-Genosys, Woodlands, TX, USA). Under these conditions, Naml-6 cells were more sensitive than CCRF-CEM cells as measured by viable cell counts. It has been shown that ActD inhibits cell proliferation by forming a stable complex with single-stranded DNA and blocking the movement of RNA polymerase that interferes with DNA-dependent RNA synthesis [11]. Based on their respective IC50 for ActD, β-actin mRNA half-life was evaluated from Nalm-6 cells treated with 0.5 μM and 1 μM ActD, and from CCRF-CEM cells treated with 1 μM and 5 μM ActD. Twenty-four h before treatment, the cells were transferred to a tissue culture flask at a concentration of 6×10^5 cells/ml, and aliquots of 3×10^6 cells were collected every 2 or 4 h for a period of 8 to 24 h. Total RNA was isolated and its concentration determined as described in Materials and Methods. For each sample, the amount of β-actin mRNA was quantified relative to 1 μg of total RNA by Real-Time RT-PCR. First-strand cDNA was synthesized using 1 μg of total RNA (DNase-treated) and a region of the β-actin mRNA was amplified using primers BA67 and BA68 [12]. Serial ten-fold dilutions (10^4 to 10^9 molecules) of pBactin-231 were used as a reference molecule for the standard curve calculation (Figure 2). All Real-Time PCR quantitations were performed using the BIO-RAD iCycler iQ system (BioRad, Hercules, CA, USA). A representative example of RT-PCR amplification plots is shown in Figure 1. A fluorescence threshold value (Ct) was calculated for each samples. For each standard curve, the correlation coefficients ranged from 0.988 to 0.995 indicating a high degree of confidence for the measurement of the copy number of molecules in the samples. As shown in Figure 2, the number of β-actin mRNA molecules ranged between 10^7 to 10^8 molecules/μl of cDNA for most of the cDNA preparations. Melting curves confirmed the presence of a single amplification product. Amounts of β-actin mRNA molecules were calculated for each time-point and plotted as a function of time (Figure 3). In Nalm-6 cells, the β-actin mRNA exhibited a half-life of 6.6 h. This result is comparable to the previously reported published value of approximately 5.5 h [13] using the traditional Northern blot procedure. A significant higher half-life value of ~13.5 h was obtained with the CCRF-CEM cells. It is interesting to observe a two-fold difference for β-actin mRNA half-life between these two cell lines. This difference cannot be explained by the concentrations of the ActD used to block transcription because similar half-life values were obtained with different ActD concentrations (see Figure 3). However, since the expression of the β-actin gene is regulated as a function of the cell cycle with transcription in the G_1 phase and mRNA decay in G_2 phase [14,15], it is possible that this difference in β-actin mRNA half-life could be explained by their respective cellular growth rate. Indeed, CCRF-CEM and Nalm-6 cells exhibited different cellular growth rate with generation times of 24 and 36 h, respectively. Taken together, this suggests that cells growing at a slower rate such as Nalm-6 would process the β-actin mRNA at a faster rate in order to respond to its cytoskeleton requirement. Future experiments will address this issue.

Conclusions

In summary, we described an alternative method using Real-Time RT-PCR to determine the rate of mRNA degradation by accurately measuring the number of mRNA molecules relative to total RNA. Using this approach, we obtained a values for the β-actin mRNA half-life in Nalm-6 cells that are comparable to those estimated from Northern blot studies using normal human leukocytes [13]. Therefore, Real-Time RT-PCR is a reliable method for measuring mRNA half-life. Because of its sensitivity, half-life of mRNAs expressed at very low level can be determined in cases in which Northern blots may not be sensitive enough. The length of the amplified fragment is important to determine the mRNA half-life because incomplete or degraded mRNA can interfere with the measurement of the actual mRNA half-life. Ideally, full-length cDNA molecules should be amplified to ensure integrity and identity of the mRNA species. The use of the fluorogenic SYBR Green I dye limits the length of the amplified product (cDNA recommended to be less than 200 bp). However, the use of TaqMan® (Applied Biosystems, Foster City, CA, USA), molecular beacons (Molecular Probes, Inc., Eugene, OR, USA), or fluorescence resonance energy transfer (FRET) probes (Roche Molecular Biochemicals,

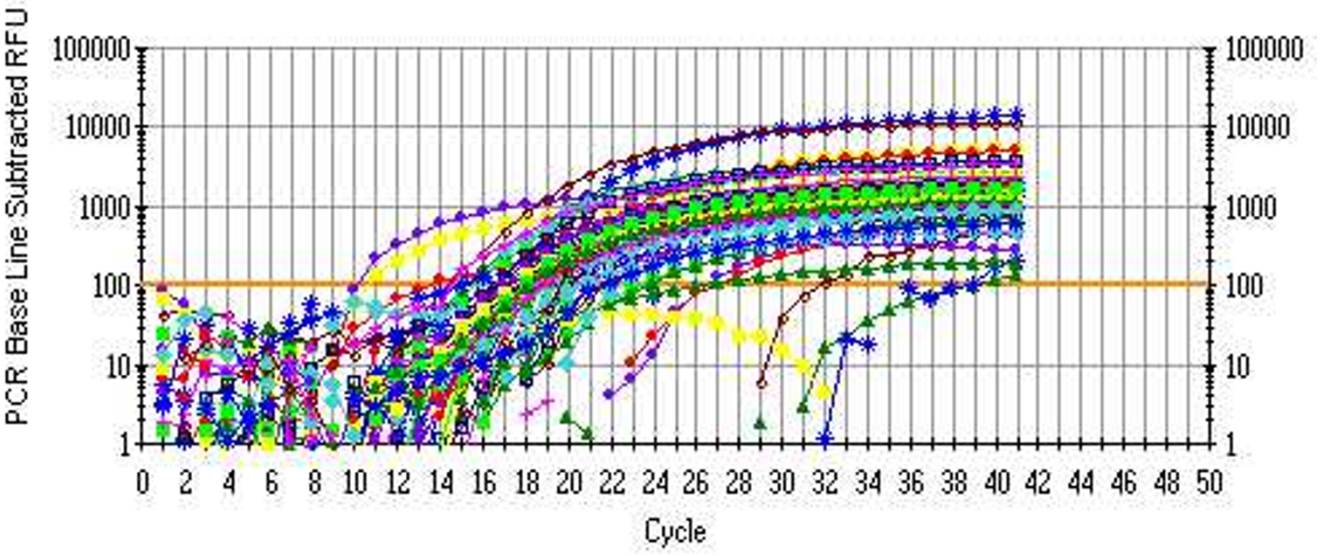

Figure 1
Real-Time RT-PCR amplification plot of β-actin mRNA. Representative RT-PCR plot resulting from the amplification of β-actin cDNA templates (1/20 volume) synthesized from 1 μg of total RNA (see Materials and Methods for details). For each time point, the cDNA was generated and quantified from three independent experiments and run in triplicates. The calculated cycle threshold (Ct = 99) provides an arbitrary cut off point at which a Ct value is assigned for each sample.

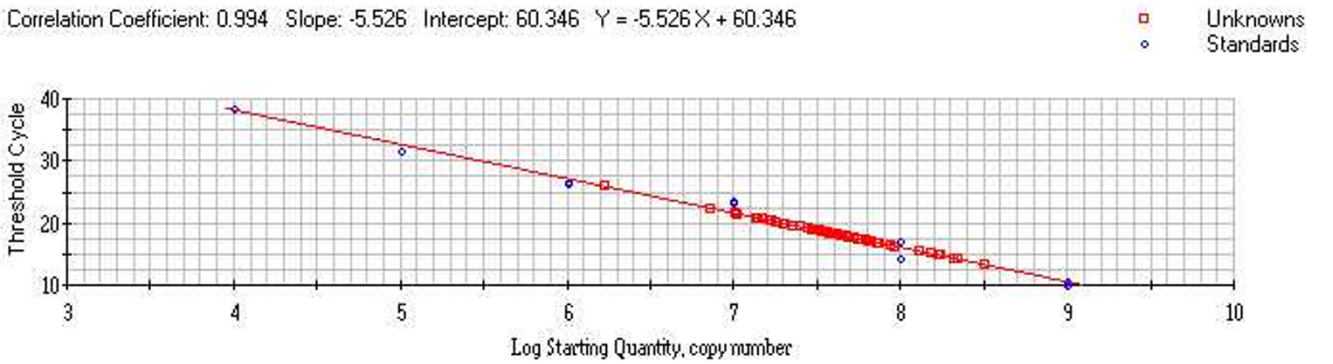

Figure 2
Real-Time RT-PCR quantification of β-actin mRNA. Standard curve showing amplification efficiencies of ten-fold serial dilutions of the pBactin-231 template. Real-Time PCR amplifications were performed using β-actin specific primers BA67 and BA68. The calculated Ct values were plotted versus the log of the initial amount of pBactin-231 molecules (10^4 to 10^9) to generate the standard curve. Squares and circles represent β-actin standards and mRNA samples, respectively.

Indianapolis, IN, USA) could overcome this limitation and allow amplification of longer PCR products. In addition, since multiplex Real-Time RT-PCR can be achieved in the same reaction tube using different fluorogenic dyes, this method could be modified to simultaneously estimate mRNA half-life of several genes. Thus, our approach represents a rapid and sensitive assay to determine mRNA half-life.

Materials and Methods

Leukemia cell Lines

The human leukemia cell lines CCRF-CEM (T-cell, ALL) and Nalm-6 (B-cell precursor, ALL) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and DSMZ (Braunschweig, Germany), respectively. Both cell lines were grown in RPMI 1640 (Sigma-Genosys, Woodlands, TX, USA) supplemented with 10% fetal bovine serum at 37°C under a 5% CO_2 atmosphere.

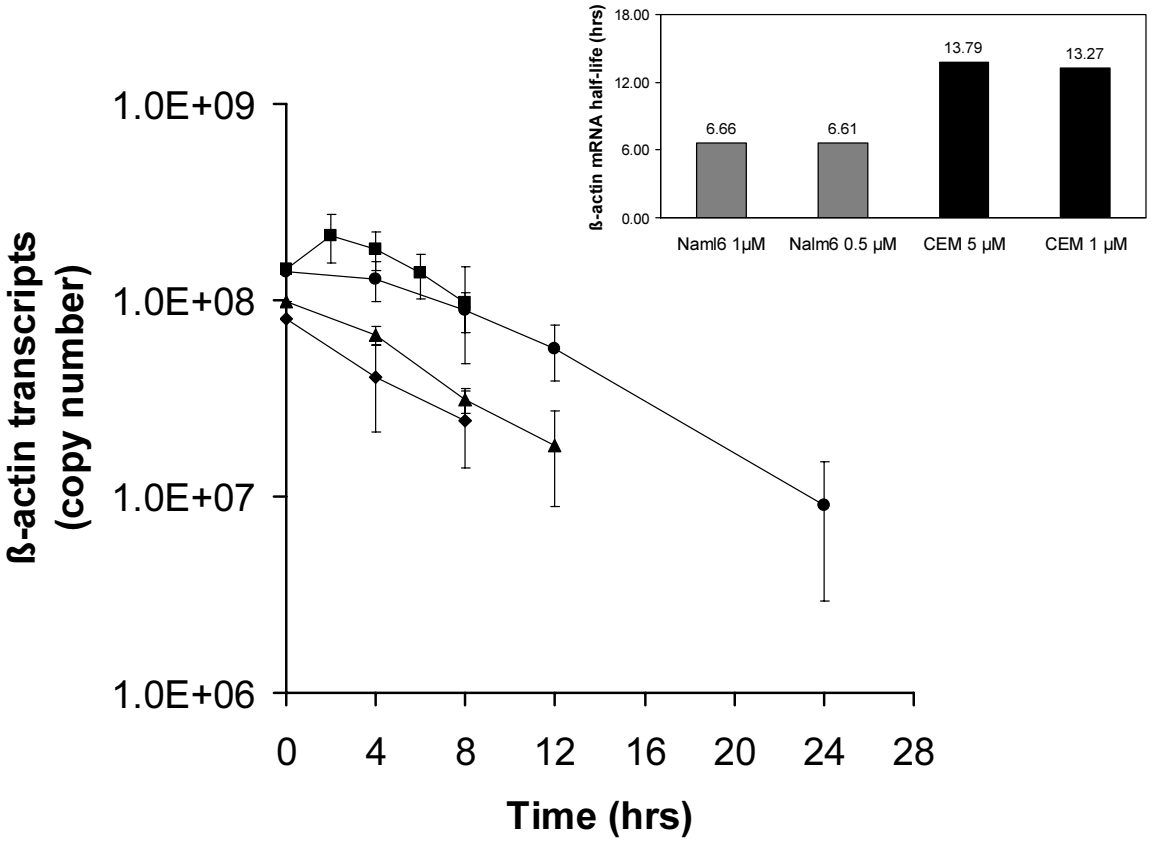

Figure 3
Half-life of β-actin mRNA in Nalm-6 and CCRF-CEM cells. The CCRF-CEM cells were treated with 5 μM (squares) or 1 μM (circles) Act-D for various times to block mRNA synthesis. Similarly, the Nalm6 cells were treated with 1 μM (lozenges) or 0.5 μM (triangles) Act-D. Total RNA, cDNA, and Real-Time PCR amplification were performed as described in the text. The values represent the mean (±) the standard deviation (SD) of the β-actin RNA copy number per μg of total RNA from three independent experiments. Triplicate determinations were averaged at each data-point. The inset shows the calculated half-life for the β-actin mRNA in each cell line for each treatment.

Culture medium was changed according to standard tissue culture techniques to insure cellular integrity. Trypan blue exclusion methodology was used to assess cell viability.

RNA isolation and Real Time RT-PCR

Total RNA was isolated using the RNeasy kit (Qiagen, Inc., Valencia, CA, USA) and its concentration determined using the RiboGreen® fluorescent dye (Molecular Probes, Inc., Eugene, OR, USA) with the VersaFluor Fluorometer System (BioRad, Hercules, CA, USA). Quality and integrity of total RNA was assessed on 1% formaldehyde-agarose gels. First-strand cDNA was synthesized using 1 μg of total RNA (DNase-treated) in a 20 μl reverse transcriptase reaction mixture as described by Leclerc and Barredo [16]. A region of the β-actin mRNA was amplified using primers BA67 (5'-GCGGGAAATCGTGCGTGACATT) and BA68 (5'-GATGGAGTTGAA GGTAGTTTCGTG), as described by Lenz et al. [12]. The cDNA amplified fragment (231 bp) was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) to generate the plasmid pBactin-231 (4139 bp). Serial ten-fold dilutions (10^4 to 10^9 molecules) of pBactin-231 were used as a reference molecule for the standard curve calculation (Figure 2). All Real-Time PCR reactions were performed in a 25 μl mixture containing 1/20 volume of cDNA preparation (1 μl), 1X

SYBR Green buffer (PE Applied Biosystems, Foster City, CA, USA), 4 mM $MgCl_2$, 0.2 μM of each primers (BA67 and BA68), 0.2 mM dNTPs mix and 0.025 Unit of AmpliTaq Gold® thermostable DNA polymerase (Applied Biosystems, Foster City, CA, USA). Real-Time quantitations were performed using the BIO-RAD iCycler iQ system (BioRad, Hercules, CA, USA). The fluorescence threshold value was calculated using the iCycle iQ system software.

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