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Nuclear Run-On Assay Using Biotin Labeling, Magnetic Bead Capture and Analysis by Fluorescence-Based RT-PCR

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ABSTRACT

In this report, we present a fluorescence-based approach to the assessment of cellular gene expression and transcription rates. Nuclear run-on was performed by supplying biotin-16-UTP to nuclei, and labeled transcripts were bound to streptavidin-coated magnetic beads. Total cDNA was then synthesized by means of random hexamer primed reverse transcription of captured molecules. To monitor transcript abundance in cDNA, both from nuclear run-on and total RNA, we propose a semiquantitative PCR approach based on the use of fluorescent primers.

INTRODUCTION

Quantitative analysis of mRNA species in different experimental conditions is crucial to the study of gene expression. The total amount of any mRNA is principally determined by the rate of new gene transcription and the stability of the mRNA. Therefore, besides analyzing total RNA levels of a specific gene, it is often useful to assess the rate of its nuclear transcription through the nuclear run-on assay (5,12). In the classical run-on technique, isolated nuclei are incubated with [α-^{32}P]UTP to label both nascent transcripts and elongated RNA molecules. Newly synthesized RNA is then used to detect specific transcripts by filter hybridization. This procedure guarantees sensitivity and accuracy. Nonetheless, it is often time consuming and requires the hazardous handling of a considerably high specific activity of α-^{32}P.

A modified nonradioactive technique using digoxigenin-labeled UTP and chemiluminescent detection was described (7). In this case, the impossibility of monitoring the filter washing conditions and the narrow range of exposure times dramatically reduce the protocol flexibility.

As an alternative to filter hybridization, a novel method was reported (9) in which the run-on reaction was performed either with or without nucleotides, and the overall transcript amount was detected by RT-PCR; the difference between PCR yields from samples incubated with and without nucleotides was taken as a measure of transcription rates. Considering the huge excess of RNA in nuclei before the reaction and the high variability among different nuclei aliquots, this approach might lead to inaccurate quantification.

Hence, we sought to develop a straightforward and safe approach to the assessment of cellular gene expression and transcription rates, which is presented in this report. For the synthesis of nuclear RNA, biotin-16-UTP was supplied to nuclei, and labeled RNA was captured by streptavidin-coated magnetic beads. RNA-binding beads were then used for random hexamer primed reverse transcription. A semiquantitative fluorescent PCR approach was applied to monitor transcript abundance in cDNA derived from both nuclear run-on and total RNA.

To set up proper conditions for the proposed techniques, we performed retinoic acid (RA) and cycloheximide treatments in neuroblastoma SK-N-BE cells and characterized the activation of the *RET* proto-oncogene (2,3) expression and transcription upon such treatments.

MATERIALS AND METHODS

Cell Lines and Growth Conditions

Human neuroblastoma IMR-32 and SK-N-BE cells, a gift of Prof. Della Valle (University of Bologna, Italy), were grown in RPMI medium (Hyclone Laboratories, Logan, UT, USA), supplemented with 10% fetal calf serum (FCS) (Life Technologies Italia Srl, Milano, Italy), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Where indicated, a 4-h treatment with 1 µM RA and 20 mg/mL cycloheximide was performed (1,8). All-*trans*-RA (Sigma-Aldrich Srl, Milano, Italy) was dissolved in ethanol and added to the

cell medium containing 10% charcoal-treated FCS. Cycloheximide (Roche Molecular Biochemicals, Monza, Milano, Italy) was dissolved in water. In control samples, ethanol alone (0.1% final concentration) was added to medium containing 10% charcoal-treated FCS.

Nuclei Preparation

Nuclei were prepared with minor modifications to standard protocols (11): 30–80 × 10^6 cells were collected by trypsination, centrifuged (4°C, 270× *g*) and washed twice with PBS devoid of calcium and magnesium. The pellet was resuspended in 4 mL cell lysis buffer [10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 150 mM sucrose and 0.5% Nonidet® P-40 (NP40)], and a 5-min incubation in ice followed. Nuclei were then collected by centrifugation (4°C, 170× *g*) and gently washed with cell lysis buffer devoid of NP40. After centrifugation, the pellet was resuspended in 100 µL freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA).

In Vitro RNA Synthesis and Purification

One volume of transcription buffer 2× [200 mM KCl, 20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 4 mM dithiothreitol (DTT), 4 mM each of ATP, GTP and CTP, 200 mM sucrose and 20% glycerol] was gently added to nuclei in ice, 8 µL biotin-16-UTP (from 10 mM tetralithium salt; Roche Molecular Biochemicals) was supplied to the mixture, which was incubated for 30 min at 29°C. Reaction was stopped by adding 6 µL 250 mM $CaCl_2$, 6 µL RNase-free DNase I (10 U/µL; Roche Molecular Biochemicals) and incubating for 10 min at 29°C. RNA purification of both nuclear run-on and total RNA was performed with TRIZOL® reagent (Life Technologies) according to the manufacturer's instructions. RNA was resuspended in 50 µL diethylpyrocarbonate (DEPC)-treated water.

RNA Binding to Magnetic Beads

Dynabeads M-280 (50 µL; Dynal, A.S., Oslo, Norway) (10) resuspended in binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 2 M NaCl) were mixed to an equal volume of run-on RNA and incubated 20 min at 42°C and 2 h at room temperature. Beads were separated by the magnetic apparatus supplied by Dynal, and a 15-min washing in 500 µL 15% formamide and 2× standard saline citrate (SSC) was performed twice, followed by a 5-min washing in 1 mL 2× SSC. Beads were then resuspended in 30 µL DEPC-treated water and stored at -20°C.

Semiquantitative RT-PCR

Random hexamer primed cDNA was prepared from 3–10 µL run-on RNA or 500 ng total RNA. A set of 8–16 progressive 1:2 dilutions of cDNA was prepared. Three microliters of each sample were used in 30-µL reactions with 6-fluoro amidite (6-fam) fluorescent forward primers. The human housekeeping *G3PDH* gene sequence was amplified with the forward primer, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, and the reverse primer, 5′-GCAGAGATGATGACCCTTTTGGCTC-3′, to give a 358-bp product (25 cycles). The human *RET* gene sequence was amplified with the forward IP18F primer, 5′-GGATTTCGGCTTGTCCCGAG-3′, and the reverse IP20R primer, 5′-CCATGTGGAAGGGAGGGCTC-3′, to give a 491-bp product (38 cycles) (2). One-tenth of each amplified sample was analyzed by polyacrylamide denaturing gel electrophoresis on a Model 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Product abundance was assessed through the area underneath the fluorescent peak, by means of the GeneScan® 672 software (Applied Biosystems).

Statistical Analysis

Lines correlating PCR yield to cDNA concentration were determined by the IGOR Pro software (WaveMetrics, Lake Oswego, OR, USA), and relative transcript abundance was evaluated through the ratio between the *RET* and the *G3PDH* line slopes (R value). In RA and cycloheximide treatments, different R values were determined both from independent treatments and, for each treatment, from independent groups of reactions. Differences between R values derived from treated and untreated cells were determined by means of the Student's *t*-test available online (Tools for Science, Statistics Web site, at www.physics.csbs.ju.edu/stats).

RESULTS

Setting Up Conditions for Proper Labeling and Purification of Run-On RNA

Eukaryotic nuclei used in run-on assays already contain a great amount of RNA, and new transcripts derived from in vitro transcription indeed represent a minor proportion of such molecules. Taking this into account, we sought to perform the run-on assay in such a way that newly synthesized RNA could be later separated from unlabeled molecules. In this line, biotin-16-UTP was supplied to nuclei in a standard reaction, RNA extracted by means of TRIZOL purification and Dynabeads used to trap the biotinylated molecules. In our approach, biotin-16-UTP is incorporated along the RNA molecules at any position, identically to what happens with radioactive [α-^{32}P]UTP, thus labeling both nascent molecules and elongated ones.

To monitor undesired RNA capture to Dynabeads, we set up control reactions in which UTP was added to the mixture in the place of biotin-16-UTP, and binding was performed. In this condition, if effective washing occurs, the following RT-PCR will give a blank product. Among the different washing conditions tested, the one reported in Materials and Methods allowed for thorough purification of run-on RNA.

Determination of the Linear Range of Fluorescent RT-PCRs

Our RT-PCR approach is based on the assumption that, within the exponential phase of a PCR, there is a linear correlation between template (cDNA) concentration and reaction product (4). Up to 16 serial 1:2 dilutions are prepared from cDNA, derived either from nuclear run-on or total RNA. Reactions are set up with a fluorescent forward primer at a fixed cycle number, and the products are run on a DNA sequencing

apparatus. The amount of PCR product is calculated through the area underlying the fluorescent peak on the gel, peak areas plotted against cDNA concentrations and regression lines plotted. For any given cDNA and PCR, the range of sample dilutions allowing for linear correlation is to be determined. The GeneScan software gives a definite value of peak area only when the signal is above a certain threshold. Therefore, to avoid personal bias in determining the linear range, we decided to plot regression lines using the first point "visible" on the gel, the two following ones, plus the blank sample. Relative transcript abundance of any given gene can be evaluated through the ratio between its line slope and that of a housekeeping gene (R value). Figure 1 shows an example of curve fitting for the housekeeping *G3PDH* gene in cDNA derived from neuroblastoma IMR-32 cells.

An Application of the Proposed Technique

Experiments performed in different laboratories have shown that in neuroblastoma cells the *RET* proto-oncogene expression is enhanced by RA treatment (1,8,13). Bunone et al. (1) analyzed both total RNA and run-on transcripts upon treatment of SK-N-BE cells with RA and cycloheximide, thus collecting evidence that RA exerts its action by enhancing the level of the gene transcription.

To compare results collected by our techniques to those collected with standard procedures, we performed 4-h RA and cycloheximide treatments in neuroblastoma SK-N-BE cells. The *RET* gene RNA levels in treated and untreated cells were compared, using the *G3PDH* gene as an internal control of housekeeping gene expression. As shown in Figure 2, both total RNA expression and nuclear transcription are enhanced in treated cells, and the observed difference is statistically significant.

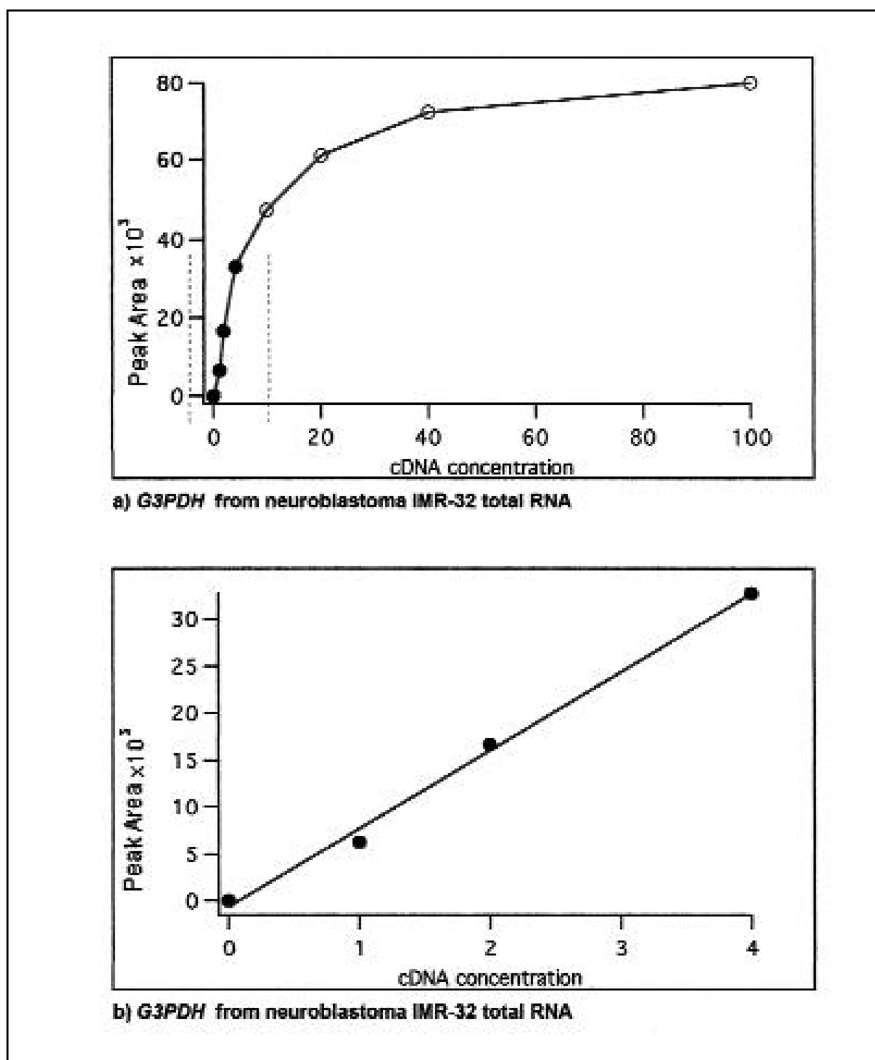

Figure 1. An example of curve fitting. The *G3PDH* gene was amplified using cDNA from IMR-32 cells. (a) Depicts all points of amplification, the curve reaching a plateau with the most concentrated samples, while (b) represents the linear range used in regression analysis (filled circles). Peak area refers to the absolute value of fluorescent PCR product, while cDNA concentration is a relative value, 1 referring to the most diluted sample and 0 to the blank sample.

DISCUSSION

In this report, a novel approach to the run-on transcription assay is presented. Experiments performed with the described protocol led to the same results as compared to published data obtained with standard procedures. Statistical

analysis on independent experiments was undertaken. Thus, reliability and reproducibility of the proposed technique were assessed. Our approach shows some intriguing advantages. First, it is safe and easily performed. Second, purified RNA can be used for different independent cDNA synthesis and stored for later use. The sensitivity of PCR allows detection of rare transcripts. Thus, by carefully adapting dilution conditions, comparisons can be made between cells or cellular states in which highly variable levels of a given transcript are to be detected. One major drawback of filter hybridization in standard run-on is nonspecific hybridization, especially when homologous genes share sequence similarities. In our approach, this difficulty can be circumvented by carefully designing PCR primers.

Our semiquantitative RT-PCR approach is considerably straightforward. Nonetheless, as any empirical end-point PCR-based quantification, it requires correction for experimental variations in individual RT and PCR efficiencies (4), which may be very time consuming. As an alternative, new procedures using real-time online quantification [LightCycler® or TaqMan® (6)] could be combined to the proposed run-on protocol, thus acquiring improved standards of quantification.

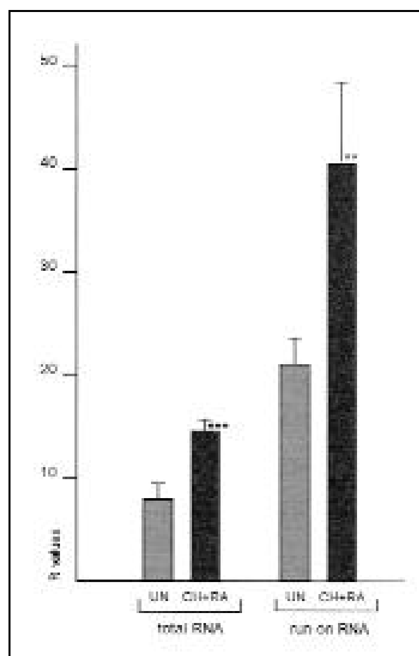

Figure 2. An application of the proposed protocols. SK-N-BE cells were treated with RA and cycloheximide for 4 h (1,8), and the *RET* gene expression and transcription levels were determined using the *G3PDH* gene as an internal control. The histogram depicts R values from total and nuclear run-on RNA in treated (RA+CH) and untreated (UN) cells. Mean values and error bars refer to six independent R calculations. Asterisks indicate significance of results; three asterisks indicate $P < 0.01$, while two asterisks indicate $P < 0.05$.

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