Quantitative Nuclear Run-Off Transcription Assay

BioTechniques 33:1016-1017 (November 2002)

The nuclear run-off transcription assay is currently the most sensitive technique to measure the in situ transcription of specific genes. It provides information on the synthesis of a specific gene that occurs as a function of cell state and is unaffected by potentially confounding posttranscriptional events such as RNA processing, transport, editing, and mRNA degradation (2). Briefly, this assay takes advantage of the high incorporation rates of radiolabeled uridine 5′-triphosphates into both newly synthesized RNAs and those transcripts that already have been initiated. Characteristically, both nascent and elongated RNA molecules are detected by solid support techniques such as filter hybridization, in which in vitro synthesized transcripts bind to immobilized DNA oligonucleotides or templates (3). In principle, hybridization onto a solid support can be limited by the efficiency and specificity of the hybridization event. While alternative approaches such as RT-PCR have been suggested for the measurement of RNA transcripts (5,6), in the context of a nuclear run-off assay this technique may not be ideal because of the inability to account for inefficiencies in the PCR process along with varying fidelity and reproducibility.

Significantly, the ribonuclease protection assay (RPA) as a solution hybridization method is more specific and 10–100 times more sensitive than filter hybridization, and, typically, less radioactive material is required. Moreover, RPA is associated with a wider linear relationship between mRNA amount and band intensity than solid support hybridization techniques, providing a method for RNA quantitation. The use of multiple probes also allows the simultaneous detection and measurement of several different transcripts, including an internal control from the same test-tube reaction (1,4,7). As applied to a nuclear run-off transcription assay, an RPA-based approach may also allow specific regions of the transcript to be investigated, which may be important in the investigation of very long genes. Here we have substituted RPA for filter hybridization as an alternative modification to the current protocol of nuclear run-off transcription assay.

We were interested in the effect of mechanical force on vascular syndecan-4 gene expression in a rat pulmonary artery smooth muscle cell line (PAC-1). Cells at a density of 5×10^7/10-cm dish were plated on the flexible silicon membrane and subsequently exposed to cyclic strain at 10% strain amplitude and 1 Hz for 1 and 24 h. Cyclic strain is an in vitro mechanical stimulus used to mimic the circumferential deformation of the vessel wall in vivo. The cell monolayer was then extensively washed in DEPC-treated PBS and mechanically removed from the dish by sterile scrapers. Cells were harvested by centrifugation at 500× *g* for 5 min at 4°C, resuspended in 4 mL lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% Nonidet P-40), and thoroughly vortex-mixed and incubated on ice for 10 min. A complete release of nuclei from cells was verified by a light microscope.

Nuclei were collected by centrifugation at 500× *g* for 5 min at 4°C, washed in lysis buffer once, and suspended in 300 µL DEPC-treated water. An aliquot of this nuclear fraction was removed to determine the protein concentrations by BCA protein assay (Pierce Chemical, Rockford, IL, USA). For each sample, approximately 150 µg nuclear proteins were used for the further assay. The nuclear fraction was then mixed with an equal volume of 2× reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, and 20 µM UTP), and transcription was initiated by the addition of 15 µL 10 mCi/mL [α-^{32}P]UTP and 1 U/100 µL RNase inhibitor (6 U/sample; Roche Applied Science, Indianapolis, IN, USA). Incubation was carried out at 30°C for 2 h with gentle shaking. DNase I was prepared in 1 mL 10 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$ at a concentration of 40 µg/mL, and 0.6 mL of this solution were added to the reaction to terminate transcription. After a 10-min incubation at 30°C, protein digestion was initiated at 42°C for 30 min by the addition of 20 µL 5% SDS, 0.5 M Tris-HCl, pH 7.4, 0.125 M EDTA, and 10 µL 20 mg/mL proteinase K (200 µg/sample). Radiolabeled RNAs were sequentially extracted twice with an equal volume of ice-cold phenol:chloroform:isoamyl al-

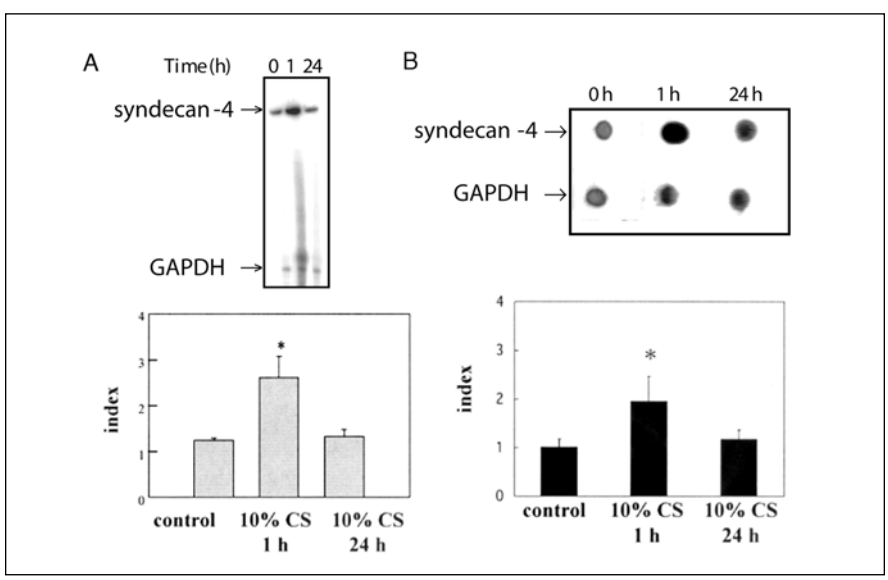

Figure 1. Nuclear run-off transcription assay. Arterial smooth muscle cells were exposed to 10% cyclic strain (CS) for 1 and 24 h, after which nuclei were isolated from stretched and unstretched (control) cells. In vitro transcription was initiated, and syndecan-4 mRNAs were examined by the modified RPA (A) or filter hybridization (B). Results from three independent experiments of A and two of B were computed and expressed as $\bar{x} \pm$ SD. ANOVA (single factor) was performed for the statistical analysis. CS, cyclic strain; * $P = 0.018$ (control vs. 10% CS 1 h).

cohol followed by chloroform extraction. RNA was precipitated in the presence of 0.1 volume of 3 M sodium acetate (pH 5.2) and two volumes of ethanol on ice for 30 min, washed once in 75% ethanol, and dissolved in DEPC-treated water.

RPA was conducted using RPAII™ Ribonuclease Protection Assay kit (Ambion, Austin, TX, USA) under the manufacturer's recommended conditions. Using Riboprobe® System-T7 kit (Promega, Madison, WI, USA), nonradiolabeled antisense RNA probes of syndecan-4 and GAPDH were synthesized against 50 ng each gene template, and 150 ng either syndecan-4 or GAPDH probe were included in the hybridization reaction. The templates were derived from the linearized pTrack/syndecan-4 (full-length, 699 bp) constructs and pPMG/GAPDH (96 bp) vectors (BD Biosciences Pharmingen, San Jose, CA, USA). The protected RNA hybrids were denatured at 95°C for 4 min and separated by 5% acrylamide gel electrophoresis in Tris-Borate-EDTA buffer, after which the gel was exposed directly to a Kodak® BioMax MS film (Eastman Kodak, Rochester, NY, USA) at -80°C for 2 h. Radioactivities of protected RNA were recorded by a Umax scanner and subjected to digital analysis by Scion Image software (Figure 1A). In comparison with a standard curve generated by the scanned intensities as a function of the employed radioactivities, syndecan-4 and GAPDH signals were quantified, and the ratio of syndecan-4 to GAPDH was expressed as $\bar{x} \pm SD$ (Figure 1A). Using 5 µg linearized plasmid DNA, we also performed a filter hybridization in accordance with a standard procedure (1) to compare and validate the changes in the experimental samples. The result of this assay is shown in Figure 1B.

The modified nuclear run-off transcription assay reported here represents a direct approach to quantify RNA transcripts produced in vitro. The length of detected RNA fragments is limited by the size of the probe used. Therefore, only those with an identical size after RNase digestion will be included in the final assessment. Moreover, radioisotope management is less labor intensive in the current protocol. In the context of the nuclear run-off transcription assay, we believe that RPA provides a potentially useful alternative to filter hybridization.

REFERENCES

1. **Green, M.E. and T.P. Bender.** 2000. Identification of newly transcribed RNA. 4.10.1-4.10.11. *In* F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (Eds.), Current Protocols in Molecular Biology. John Wiley and Sons, New York.
2. **Greenberg, M.E. and E.B. Ziff.** 1984. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature *311*:433-438.
3. **Grimes, A., H.J. McArdle, and J.F. Mercer.** 1988. A total extract dot blot hybridization procedure for mRNA quantitation in small samples of tissues or cultured cells. Anal. Biochem. *172*:436-443.
4. **Ilekis, J.V., B.C. Stark, and B. Scoccia.** 1995. Possible role of variant RNA transcripts in the regulation of epidermal growth factor receptor expression in human placenta. Mol. Reprod. Dev. *41*:149-156.
5. **Patrone, G., F. Puppo, R. Cusano, M. Scaranari, I. Ceccherini, A. Puliti, and R. Ravazzolo.** 2000. Nuclear run-on assay using biotin labeling, magnetic bead capture and analysis by fluorescence-based RT-PCR. BioTechniques *29*:1012-1017.
6. **Rolfe, F.G. and W.A. Sewell.** 1997. Analysis of human interleukin-5 gene transcription by a novel nuclear run on method based on the polymerase chain reaction. J. Immunol. Methods *202*:143-151.
7. **Zinn, K., D. Dimaio, and T. Maniatis.** 1983. Identification of two distinct regulatory regions adjacent to the human β-interferon gene. Cell *34*:865-879.

Supported by National Institutes of Health grant no. NIH RO1HL60963.

Address correspondence to Dr. Elliot L. Chaikof, 1639 Pierce Drive, Rm 5105, Emory University, Atlanta, GA 30322, USA. e-mail: echaiko@emory.edu

Received 8 January 2002; accepted 26 August 2002.

Lei Li and Elliot. L. Chaikof
Emory University
Atlanta, GA, USA