Use of *In Vitro*-Transcribed RNAs as Immobilized Targets During RNA:RNA Hybridization

ABSTRACT

We describe the use of in vitro*-transcribed complementary RNAs as filter-bound targets during nuclear run-on analyses. Use of these single-stranded reagents in high-stringency RNA:RNA hybridizations increases signal-to-background hybridization seen using DNA targets and allows efficient measurement of transcriptional rates across genes in either direction.*

INTRODUCTION

The immobilization of nucleic acids on membranes and subsequent hybridization with labeled probes is a basic technique in the detection and quantification of many RNA species (3). We have developed a method in which complementary RNA (cRNA) transcripts produced *in vitro* in bacteriophage RNA polymerase transcription reactions are fixed to membranes as homogeneous, single-stranded targets that are then hybridized to ^{32}P-labeled nuclear RNA. We have found this technique to be very useful in nuclear run-on analyses on isolated nuclei from cell lines stably transfected with synthetic immunoglobulin gene constructs. In these transfected cells, the synthetic gene may be integrated near cellular promoters so that transcription from the opposite strand can occur. Our procedure allows independent measurement of RNA produced from either direction on a gene in the absence of distracting DNA vector sequences.

MATERIALS AND METHODS

Generation and Quantification of cRNAs

The conditions for the generation of microgram quantities of cRNA transcripts from pGEM® vectors (Promega, Madison, WI) have been described previously (2,5). These vectors have tail-to-tail SP6 and T7 promoter sequences that flank a multicloning site, thereby allowing the generation of sense or antisense RNAs, depending on the orientation of the cloned fragment. Briefly, the reaction mixture to generate targets contains: 10 µl (2.2×10^7 dpm) of ^{3}H-UTP (1 mCi/ml; 15 Ci/mmol, Du Pont NEN, Boston, MA) (the ethanol storage solution was evaporated in a SpeedVac [Savant Instruments, Farmingdale, NY] before adding remaining components); 10 µl 5× transcription buffer (200 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl); 2 µl RNasin ribonuclease inhibitor (50 U/µl, BM Biochemical, Boehringer Mannheim, Indianapolis, IN); 2 µl 250 mM dithiothreitol (DTT) (freshly prepared, filter sterilized); 4 µl 1 mg/ml bovine serum albumin, RNase-free (Promega); 2 µl 0.25% Triton® X-100 (omitted from SP6 reactions); 4 µl 50 mM ATP; 4 µl 50 mM CTP; 4 µl 50 mM GTP; 3.8 µl 50 mM UTP (190 nmol, limiting nucleotide); 1 µl plasmid DNA template linearized with a restriction enzyme and stored at 1 µg/µl; and sterile H_2O to 50 µl final volume. A 1-µl sample was removed for a time zero precipitation with 10% trichloroacetic acid (TCA t_0). T7 RNA polymerase (New England BioLabs, Beverly, MA), 60 units, or 50 units of SP6 RNA polymerase were added, and the reaction was incubated at 37°C for 1 h. Then 0.5 µl of DNase I (2 U/µl; Boehringer Mannheim) was added, and the reaction was incubated at 40°C for 10 min.

Incorporation was quantified at time final (TCA t_f) by TCA precipitation of 1 µl of the 52.5-µl reaction. The total amount of ^{3}H radioactivity (^{3}H total) in reaction was determined by removing 1 µl and spotting directly onto a 2.4-cm glass microfiber filter. A wide-open ^{3}H window was used in a liquid scintillation counter. The reaction (51.5 µl) was extracted with an equal volume of phenol:chloroform. Sodium chloride was adjusted to 0.15 M, and the RNA was ethanol-precipitated and stored at -20°C until ready to fix to the membrane. Between 5 and 20 µg of cRNA per reaction are generally produced using this method.

The amount of cRNA transcript produced was calculated by determining the fraction of ^{3}H incorporated into cRNA from cpm generated from the above steps using the following formulas:

(A) $$\frac{\frac{\text{TCA } t_f}{1\ \mu l} \times 52.5\ \mu l - \frac{\text{TCA } t_0}{1\ \mu l} \times 50\ \mu l}{^3\text{H Total} \times \frac{52.5\ \mu l}{1\ \mu l}} = \text{fraction U incorporated}$$

(B) 190 nmol input U × fx ^{3}H U incorporated = nmol U incorporated

Nanomoles of cRNA transcript made can be precisely determined if the uridine composition of the transcript is known by using the following formula:

(C) $$\text{nmol uridine incorporated} \times \frac{1}{\text{fx of transcript as U*}} = \text{nmol cRNA}$$

*If not known, can estimate 0.25 for average gene transcript.

(D) Nanomoles of cRNA transcript made × 330 ng/nmol = ng cRNA made.

When ^{32}P-labeled RNA transcripts were made, the reaction mixture was altered to contain 50 µCi of [α-^{32}P]-UTP (3000 Ci/mmol) instead of ^{3}H-UTP, and the unlabeled UTP was lowered to 20 µM final concentration. The ^{32}P-labeled RNA produced had a specific activity of approximately 7×10^7 cpm/µg.

Immobilization and Hybridization of cRNA Transcripts

We have found that nylon membranes become saturated at 0.5 µg of transcript per slot on a minifold apparatus (Hoefer Scientific, San Francisco, CA; 4.8 mm^2 surface area/slot). Therefore, we typically load 1 µg cRNA samples as generated above to ensure membrane saturation and target excess. Loading buffer is 200 µl of 25 mM $NaPO_4$, pH 6.5. Wells are washed with 100 µl of the 25 mM $NaPO_4$ prior to and after loading the cRNA. GeneScreen™ membranes (Du Pont NEN) are kept damp, and transcripts are UV-cross-linked to membrane by exposing membrane to a 254 nm UV light source, which gives an exposure at the membrane surface of 1200 µW/cm^2, for 2 min (1). Membranes can be stored at -20°C for several months.

The conditions for prehybridization and hybridization to ^{32}P-UTP-labeled nuclear run-on RNA and washing of the membranes have been previously

described (2,5). Briefly, nuclei from the mouse myeloma cell line K23, J558L or J558L transfected with the MPC11 immunoglobulin gene (4) were isolated and incubated in a transcription mixture containing 250 µCi of [α-^{32}P]-UTP (3000 Ci/mmol), 50 mM Tris-HCl, pH 7.9, 150 mM KCl, 6 mM $MgCl_2$, 1 mM $MnCl_2$, 0.6 mM DTT, 1 mM ATP, 0.6 mM CTP and 0.6 mM GTP. Incubation was for 15 min at 24°C. Labeled RNA was deproteinated, partially hydrolyzed and then hybridized (at 4×10^6 cpm/6 cm^2 filter) to cRNA targets on filters for 60 h at 56°C. Filters were then washed as indicated and exposed to x-ray film with a Cronex® Lightening Plus Intensifier screen at -70°C. Exposures in the linear response range of the film were used for the densitometric analyses.

The pGEM plasmids used in these experiments include: Ac = β-actin, the 1.15-kb *Pst*I fragment of mouse β-actin (6) cloned into the *Pst*I site of pGEM-4; probe b, pM1M2 which contains a 1.15-kb *Kpn*I–*Pst*I fragment including the gamma 2b membrane exons (5); probe c, pPSac which contains a 1.4-kb *Pst*I–*Sac*I fragment including the gamma 2b mb 3′-UT and poly(A) site (2) and b′ and c′ which are opposite orientations (not theoretically hybridizable to mRNA) of b and c.

RESULTS AND DISCUSSION

The K23 mouse myeloma cell line expresses the gamma 2a heavy chain immunoglobulin gene as well as the β-actin gene (2). RNA from nuclei of K23 cells was ^{32}P-labeled in a nuclear run-on assay and hybridized to nucleic acids immobilized on membrane as shown in Figure 1.

The signal obtained per unit time using antisense β-actin cRNA as a target was at least 2.8-fold higher than that obtained with the double-stranded actin insert DNA as a target for each of the various amounts of nucleic acid immobilized (see Figure 1, Panel D). In addition, in our hands, the mouse myeloma nuclear run-on RNA produces a high signal with the double-stranded DNA pGEM plasmids that contain no inserts (see Panel B, slot 1), perhaps because of repetitive sequences in the eukaryotic nucleic acid that cross hybridize

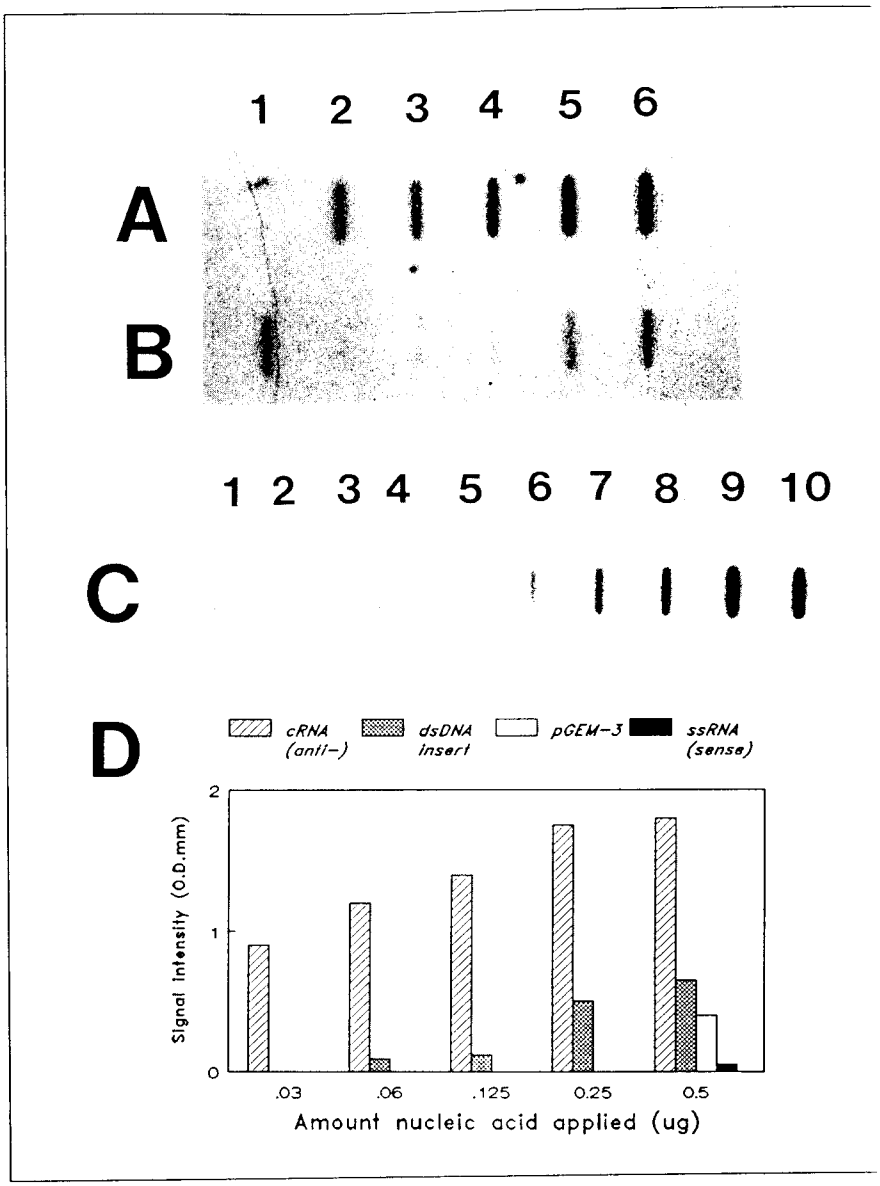

Figure 1. Hybridization of labeled nuclear run-on RNA to different immobilized nucleic a targets. Nuclei from the mouse myeloma cell line K23 were isolated and incubated in a nuclear run reaction containing [α-^{32}P]-UTP. Labeled nuclear RNA was isolated and partially hydrolyzed w NaOH prior to hybridization with filters containing a dilution series of either *in vitro*-transcrib β-actin cRNA or double-stranded DNA as indicated. Nylon membranes with immobilized cRNAs w prehybridized for 4 h in 10 ml of RNA hybridization buffer (50% formamide; 0.25 mM $NaPO_4$, pH 7 0.25 M NaCl; 1 mM EDTA; 7% sodium dodecyl sulfate [SDS]; 100 µg/ml sonicated salmon sp DNA) prior to hybridization. Membranes with cRNAs were washed in 250-ml washes: three 5-n washes at 60°C in 2× SSPE followed by one 20-min wash at 24°C in 2× SSPE + 0.25 µg/ml RNase one 10-min wash in 2× SSPE, 0.1% SDS, 60°C; one 10-min wash in 1× SSPE, 0.1% SDS, 60°C; 10-min wash in 0.1× SSPE, 0.1% SDS, 60°C; and one 10-min wash in 0.1× SSPE, 1.0% SDS, 6 (1× SSPE = 0.18 *N* NaCl; 10 mM $NaPO_4$, pH 7.2; 0.5 mM EDTA). Linearized plasmid pGEM-3 the gel-excised, β-actin 1.15-kb *Pst*I insert were denatured in 0.2 *N* NaOH at 80°C for 10 min be slotting onto membranes and then treated as above. Membranes were exposed to x-ray film for 2 with intensifying screens, and the autoradiograms were scanned with a densitometer in order to meas hybridization and signal intensities. **Panel A**, antisense β-actin cRNAs: slot 1, 0.5 µg previously trea with RNase A; slot 2, 0.03125 µg; slot 3, 0.0625 µg; slot 4, 0.125 µg; slot 5, 0.25 µg; slot 6, 0.5 **Panel B**, double-stranded DNA: slot 1, 0.5 µg linearized pGem-3; slots 2–6, beta actin insert (slo 0.03125 µg; slot 3, 0.0625 µg; slot 4, 0.125 µg; slot 5, 0.25 µg; slot 6, 0.5 µg). **Panel C**, single-stran β-actin RNAs: slots 1–5, sense RNA (slot 1, 0.03125 µg; slot 2, 0.0625 µg; slot 3, 0.125 µg; slot 4, 0 µg; slot 5, 0.5 µg). Slots 6–10, antisense RNA: slot 6, 0.03125 µg; slot 7, 0.0625 µg; slot 8, 0.125 slot 9, 0.25 µg; slot 10, 0.5 µg. **Panel D**, signal intensities from densitometric scans were plotted function of amount of nucleic acids immobilized on the filters.

with the plasmid. The hybridization signal seen in Panel C, slots 6–10, is specific for message-sense RNA as demonstrated by the lack of hybridization to cRNAs designed to measure transcription from the opposite strand as shown in Panel C, slots 1–5. The use of a double-stranded DNA as target does not allow for measurement of transcription in both directions on a gene, but the quantitation of opposite-strand transcription in isolated nuclei is an important consideration in the analyses of run-on data, especially with synthetic gene transfectants and their random integrations.

Hybridization of ^{32}P-labeled, run-on RNA to the excised 1.15-kb β-actin DNA insert alone on nitrocellulose, under hybridization conditions optimal for DNA:RNA reactions (Reference 1 and data not shown) did not produce a signal comparable with that obtained with the cRNA targets. This, furthermore, resulted in a higher level of background hybridization, presumably because of the lower stringency conditions required for the RNA:DNA hybridization here as opposed to RNA:RNA hybridization with the cRNAs.

To further investigate the cRNA target technique, RNA from ^{32}P-labeled nuclei of J558L cells was hybridized to membranes with various cRNAs immobilized. The J558L cells produce actin but lack immunoglobulin (Ig) heavy chain genes (4). As shown in Figure 2, Panel A, hybridization of J558L RNA to the actin signal is at least 20 times more than that seen with the immunoglobulin-specific targets (sense and antisense) on the membrane, indicating that there is little nonspecific hybridization with the cRNA targets.

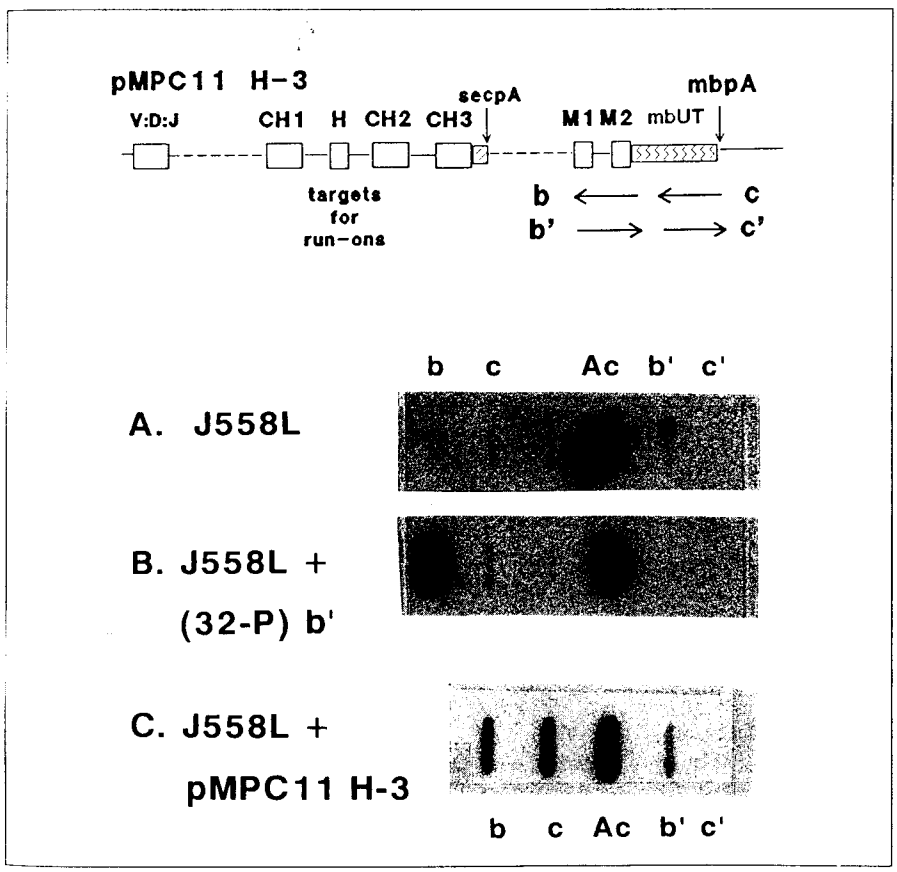

Figure 2. Hybridization of different RNA preparations to cRNA targets. Nuclei from the indicated cells were isolated, ^{32}P-labeled in a nuclear run-on reaction and hybridized to filters containing 1 μg per slot of the indicated cRNA targets. The orientation and location of the cRNAs are indicated in the drawing. Filters were washed as described in Figure 1. Film exposures were for 72 h. **Panel A**: J558L-labeled nuclear RNA, 4×10^6 cpm. **Panel B**: J558L-labeled nuclear RNA as in Panel A plus 2000 ppm *in vitro*-transcribed, ^{32}P-labeled b′ cRNA mixed in prior to the hybridization step. **Panel C**: labeled nuclear RNA from J558L cells tansfected with the mouse MPC11 immunoglobulin gamma 2b heavy chain gene, clone #3 (4).

To quantitate what percentage of the labeled nuclear material the β-actin probe represents, we added various quantities of synthetic ^{32}P-labeled Ig heavy chain sequence (in mRNA sense) to the hybridization between untransfected J558L ^{32}P-labeled nuclear RNA and the filters. As shown in Figure 2, Panel B, the exogenously added, synthetic Ig mRNA produces a signal comparable to that seen with the J558L nuclear-derived actin mRNA when the synthetic Ig RNA represents approximately 2000 parts per million or 0.2% of the input counts.

When J558L cells are transfected with the intact Ig heavy chain gene, they produce large amounts of Ig mRNA (4). We used the cRNA target technique to measure transcription in the run-on assay from J558L cells transfected with the Ig MPC11 gamma 2b heavy chain gene. As shown in Figure 2, Panel C, the hybridization to the Ig mRNA complementary cRNA targets (b and c) was substantial, while hybridization to the opposite sense strand (c′) was negligible. Hybridization to the target b′ was about 20% of that seen with target b in this experiment. These results indicate that in this transformant, most of the transcription is from the Ig coding strand of the transfected gene. A comparison of the hybridization seen with b′ in Panels C vs. A indicates that there is minimal transcription over background in the non-coding direction from the transfected gene. Note that the low b′ signal in Figure 2, Panel B, could be attributed to carryover of unlabeled b′ encoding DNA, added inadvertently with the b′ probe. Comparison of the signal obtained with the b and c target cRNAs vs. the β-actin signal in Figure 2, Panel C, indicates that the Ig mRNA sequences represent about 25% to 50% as many labeled molecules as the β-actin precursors or approximately 0.05% to 0.1% of the input counts, using the calibration described in Figure 2B.

The technique we have described allows measurement of transcription from either direction on a gene with higher signal-to-background hybridization than when using double-stranded DNA targets. With the described mixing experiments, it should also be possible to quantitate the amount of a transcript being produced in a new experimental system.

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