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Cytoplasmic RNA Extraction from Fresh and Frozen Mammalian Tissues

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ABSTRACT

The quality of collections of expressed sequence tags and full-length cDNAs is adversely affected by the presence of "junk" clones derived from unspliced or partially spliced RNAs present in conventional total RNA preparations. One can overcome this problem by using intact cytoplasmic RNA to create cDNA libraries, but the methods in the literature that describe the preparation of RNA only work well for extracting cultured cells. Cell lines are not as diverse as one would like, and to clone comprehensive sets of human and model organism full-length cDNAs, libraries have to be prepared from tissue samples. Thus, we have developed a robust and inexpensive method that allows intact cytoplasmic RNA to be extracted from both fresh and frozen mammalian tissues. A mouse full-length, cap-trapped cDNA library prepared with RNA using this new procedure had excellent characteristics.

INTRODUCTION

The sequences of expressed sequence tags (ESTs) and full-length cDNAs have proven valuable, if not essential, for the analysis of genomic sequence data and for the validation of inferred coding regions (7,11). Unfortunately, ESTs are contaminated by cDNA sequences derived from incompletely spliced RNAs (immature mRNAs), and these contaminants are not always easy to recognize. In large cDNA collections (6,9), it is common to see clusters containing clones with A-rich portions of introns on their 3'-ends, included introns, or introns on their 5'-ends. These pose serious quality and cost problems because such cDNAs are often submitted for full-insert sequencing. To overcome these problems, one could use cytoplasmic

RNA, which is devoid of unprocessed HnRNA, as a starting substrate for cDNA library production. However, existing protocols are unsuitable for many fresh or most frozen tissues, and freezing microdissected specimens, which are useful for gene discovery, and post-mortem human samples is an essential part of collecting them. For this reason, we have developed a method for preparing cytoplasmic RNA from intact fresh and frozen animal tissues, which is based on ultra-mild lysis, and the complete inhibition of RNase, followed by the digestion of the enzyme.

MATERIALS AND METHODS

RNA Extraction

The solutions and instruments were precooled as required. For fresh samples, the tissue was quickly dissected, and the volume was roughly estimated. Ice-cold solution A (4.5 mL) was added (50 mM Tris-HCl, pH 7.5, 0.8 M sucrose, 150 mM KCl, 5 mM MgCl₂, 6 mM 2-mercaptoethanol) with 0.5 mL 200 mM vanadyl ribonucleoside complex (VRC; final concentration, 20 mM) (Sigma, St. Louis, MO, USA) to approximately 1 g tissue. The tissue was immediately transferred to a Potter homogenizer (GTR-1000; Eylea, Tokyo, Japan) (precooled on ice) and homogenized at 300–400 rpm, up and down five times on ice. Hard tissues may be briefly minced on ice before this step. The sample was quickly transferred to a clean, sterile tube on ice and centrifuged.

Tissues that cannot be processed immediately should be fast-frozen in liquid nitrogen and keep at -80°C. Just before the RNA is extracted, the tissue is powdered in a liquid nitrogen-cooled mortar (model 5912 Bio-Pulverizer for large tissues and model 5913 for small tissues; BioSpec Products, Bartlesville, OK, USA). Alternatively, the tissues can be powdered with a mortar and pestle in liquid nitrogen. The powdered tissue was transferred with the help of a sterile stainless-steel spoon (precooled in liquid nitrogen) into a clean, precooled tube, and ice-cold solution A was then added. Incomplete homogenization of the tissue results in genomic DNA con-

amination (and likely HnRNA contamination) from floating tissue fragments. For this reason, it is best to disrupt the sample quickly with a Potter homogenizer at 300–400 rpm, 4–5 times up and down, on ice. The homogenate was then transferred to a new tube and the next step begun immediately.

The tube containing the homogenized tissue was placed into a high-speed centrifuge precooled to 0°C and was centrifuged at 23 000× g at 0°C for 5 min. The supernatant was transferred to a clean 50-mL tube (Falcon®), and RNase-free double-distilled water was added to bring the final volume to 10 mL. SDS buffer (10 mL) (20 mM Tris-HCl, pH 7.4, 1% SDS, 200 mM NaCl), 40 mM EDTA, and 1 mg proteinase K (20 mg/mL) were added and incubated at 45°C for 20 min in a water bath. RNases will be rapidly degraded by the proteinase, and it is therefore less important to work quickly. Twenty milliliters of a mixture of Tris-buffered phenol:chloroform (1:1) were added, mixed, and centrifuged at 9500× g for 5 min at 20°C. Phenol extraction removes most of the VRC, which would otherwise inhibit the enzymes used to prepare libraries. The RNA was precipitated by the addition of 1.7 mL 5 M NaCl (final concentration, 0.2 M) and 1 volume of isopropanol, incubated at -20°C overnight, and centrifuged for 15 min at 9500× g. The pellet was completely dissolved in 4 mL RNase-free double-distilled water, and 1.3 mL 5 M NaCl were added to a final concentration of 0.3 M NaCl and 16 mL cetyltrimethylammonium bromide (CTAB)/urea solution (50 mM Tris-HCl, pH 7.0, 1% CTAB, 4 M urea, and 1 mM EDTA). The sample was incubated for 15 min at room temperature and then centrifuged for 15 min at 9500× g. CTAB precipitation allows polysaccharides and residual VRC to be removed. Because the CTAB precipitation poses yield problems at low RNA concentrations, for small quantities of RNA (small pellet after the isopropanol precipitation), one should reduce all the volumes downstream of the isopropanol precipitation to half or one-fourth and increase the centrifugation times to 30 min. The RNA precipitate, which is usually spread across a rather large surface of the wall of the tube, was resuspended in 4 mL 7 M guan-

dine hydrochloride. Two volumes of ethanol were added to precipitate the RNA. The pellet was incubated at -20°C for 1–2 h and centrifuged for 15 min at 9500× *g* at 4°C. The pellet was then washed with 10 mL 75% ethanol and centrifuged again for 3 min at 9500× *g*, the supernatant was discarded, all of the residual ethanol was carefully removed, and the RNA was dissolved in 200–1000 µL RNase-free double-dis-

tilled water. The “standard” cytoplasmic RNA extraction has been previously described by Sambrook et al. (8) and others (5), with or without the addition of RNase inhibitors (Figure 1).

PCR Amplification of γ -Actin Transcripts

The RT-PCR quantitation of the intronic contamination of RNA prepara-

tions was performed as follows: reverse transcription was performed either with SUPERScript™ II (spleen) (Invitrogen, Carlsbad, CA, USA) or M-MLV reverse transcriptase (RNase H; Promega, Madison, WI, USA), following the manufacturer's protocol, which begins with 1 µg RNA and 250 ng of the random dN₆ primer. Aliquots of the first-strand product (Figure 2) were amplified in a thermal cycler (PTC-200 DNA Engine™; MJ Research, Waltham, MA, USA) using 2.5 U *Ex Taq*™ (Takara Bio, Tokyo, Japan)/reaction, *Ex Taq* buffer, and the indicated primers at a concentration of 5 µM, in a final volume of 50 µL. Exon-exon amplification was performed with primers 5'-GC-CATTCAGGCGGTGCTGTC-3' and 5'-GTGCTAGGTGCTAGGGCTGT-3', with denaturation at 94°C for 1 min, amplification at 94°C for 30 s, 58°C for 30 s, 72°C for 60 s, repeating the cycles as indicated (Figure 2), and a final extension at 72°C for 3 min. The exon-intron PCR was performed with primers 5'-GCCATTCAGGCGGTGCTGTC-3' and 5'-CCTTGGCTCCTAAATCATGT-3', with denaturation at 94°C for 1 min, amplification at 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, repeating the cycles as indicated (Figure 2), and a final extension at 72°C for 3 min.

Figure 1. Denaturing agarose gel electrophoresis of cytoplasmic RNAs extracted in the course of this study. (A) Frozen spleen, standard cytoplasmic extraction protocol. (B) Fresh spleen, standard protocol with the addition of VRC. (C) Frozen spleen, standard cytoplasmic extraction protocol with the addition of VRC. Notice that the RNA looks intact, but genomic DNA contamination indicates nuclear membrane damage. This suggests that the preparation is likely to be contaminated HnRNA as well. (D) Frozen spleen, new protocol described here. Frozen tissues (lanes E–H) and fresh tissues (lanes I–M), with our protocol: lanes E and J, brain; lanes F and K, testis; lanes G and L, kidney; lanes H and M, lung; lane I, spleen (comparable to lane D); lane N, TK-1 cells (ATCC no. CRL-2396) extracted using the protocol described in Reference 5. These data are representative of more than 100 extracted samples.

Figure 2. Evaluation of residual intronic contamination by exon-exon and exon-intron quantitative RT-PCR of γ -actin RNA. (a) A schematic representation of the RT-PCR strategy. (b) Quantitative RT-PCR, total RNA extracted with standard method. (c) Quantitative RT-PCR, cytoplasmic RNA extracted using this method. The number of PCR cycles and the quantity of RNA (ng) template used are indicated. M, molecular weight markers (50 bp ladder; Invitrogen). Splenic RNA made from a frozen sample. RNA prepared from fresh spleens gave nearly identical results (data not shown). The renal RNA was extracted from fresh tissue. The results shown are typical of at least two replicate experiments.

Sequence Analysis

To estimate the fraction of cDNAs that were full-length in the library we prepared, we compared the clones it contained to those in a database extracted from GenBank®, using the following keywords: mRNA AND mouse AND complete CDS (10). We used the following parameters for the sequence alignments: E-value < 1.0 e-20; identity threshold ≥ 90%; overlap threshold ≥ 50 bp; and maximum gaps allowed, 5.

RESULTS AND DISCUSSION

We set out to determine whether lysing fresh or frozen tissues under very mild conditions would allow us to extract cytoplasmic RNA, without activating endogenous RNase, and to stabilize and remove nuclei to eliminate unprocessed RNAs. Initially, we screened homogenization protocols simply by

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staining homogenates prepared from frozen mouse kidney samples with Tripan Blue dye and examining the products under a microscope. We discovered that complete cell lysis could be achieved in solutions that appeared to leave the nuclei intact. We settled on the simplest procedure possible—the disruption of tissues with a Potter homogenizer in the presence of 0.8–1.5 M sucrose and physiological salt concentrations, without detergents such as Nonidet™ P-40, which is commonly used to prepare cytoplasmic RNA from cultured cells (8). Since this seemed promising, we next addressed the issue of RNase inhibition, choosing VRC because it is effective and relatively inexpensive. Finally, we optimized the sucrose concentration. The concentration chosen (0.8 M) seems to protect the nuclei and gives good RNA yields as well (yields fall when sucrose concentrations greater than or equal to 1.2 M are used; data not shown). Figure 1 shows that the method described works well for fresh and frozen tissues. For example, the RNA isolated from spleen, a notoriously difficult tissue to extract, has intact 28S and 18S rRNA in the absence of detectable genomic DNA contamination. Furthermore, VRC appears to perform as well as or better than expensive protein-based RNase inhibitors. To date, we have used the method successfully to prepare RNA from 58 mouse tissues and embryonic samples with consistently good results.

To discover whether intron-containing RNA species were effectively eliminated by our procedure, we studied γ -actin transcripts by means of exon-exon or exon-intron quantitative RT-PCR, comparing our new method to a standard RNA extraction procedure (4). Intron-exon PCR, which amplifies a product from partially spliced RNA, gives little signal when template is used that was prepared with our new technique (Figure 2). In frozen spleen and fresh kidney RNA samples, contamination by HnRNA was reduced 40- and 10-fold, respectively. Because the average contamination of libraries by cDNAs representing partially processed transcripts has been 5%–10% in our experience, using our method should allow one to decrease the contamination by such clones to 0.125%–1%. This should dra-

matically reduce the number of junk cDNAs in full-length libraries, including noncoding clones and intron-containing cDNAs that are sometimes annotated as new genes in large-scale sequencing projects.

To test this prediction, we used our technique to prepare cytoplasmic RNA from 16 adult and mouse embryonic tissues, pooled them, and used the pool to make a cap-trapped normalized cDNA library (1,2,3). One-pass sequencing of the 5'-ends of 32 716 clones from this library revealed 12 466 known genes, 86.2% of which contained the first ATG. (Materials and Methods section and Reference 10). We were able to use an automated method to assign gene ontology terms to about 10% of these clones. We estimate that a minimum of 8%–10% of these are likely to encode plasma membrane proteins or secretory products, which are synthesized by membrane (i.e., endoplasmic reticulum)-bound polysomes. Polysomal mRNA is usually thought to comprise 10%–15% of the total, in good agreement with the number we found. Thus, it appears that the centrifugation method described in our protocol does not precipitate rough endoplasmic reticulum, which would result in a loss of membrane-associated mRNA species.

In addition to examining the 5'-ends of cDNA in the library we created, we sequenced and studied the 3'-ends of 24 864 clones. Of 6843 cDNAs corresponding to known transcripts, 96.9% contained the annotated termination codon. We looked for the six most common polyadenylation signals (i.e., AAUAAA, AUUAAA, AAUUAA, AAATAA, AGTAAA, and AATATA) 50 or fewer bases upstream of the putative A-tail of the unknown transcripts and found that one of these was present in 84.5% of these clones. This is undoubtedly an underestimate of the number of clones with intact 3'-ends because a substantial number of mRNAs have alternate poly(A) signals or no recognizable signal at all.

Finally, we attempted to determine the proportion of cDNAs that were truly full-length. To do this, we examined the 5'- and 3'-ends of all the cDNAs representing known transcripts in the collection sequenced. A majority

(83.5%) had intact 5'- and 3'-ends; 2.6% had intact 5'- but not 3'-ends (these may have initiated internally); 13.3% had intact 3'- but not 5'-ends (presumably these failed to be reverse transcribed completely); and 0.5% of the cDNAs lacked both the known 5'- and 3'-ends.

As suggested above, we feel that we can now prepare cDNA libraries comprised of clones, most of which are full-length and free from introns. This will facilitate the characterization of the human and mouse transcriptomes and those of other species. In addition, our method can undoubtedly be used to reduce the number of false positives in the differential analysis of transcripts with alternative splicing (DATAS) libraries (<http://www.exonhit.com/texts/technology/index.html>).

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