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Background Priming during Reverse Transcription by Oligo(dT) Carried Over from mRNA Isolation

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The isolation of mRNA from total RNA is the first step in many experimental protocols such as the construction of cDNA libraries. Typically, mRNA isolation is accomplished using commercially available kits that provide some form of free or immobilized oligo(dT) to capture RNA with a poly(A) tail. The oligo(dT)/mRNA complex can be captured by different methods such as by streptavidin-biotin interaction on a magnetic particle or by physical retention on a cellulose matrix.

We observed that some of our plant mRNA preparations obtained using various mRNA isolation methods could be reverse-transcribed in the absence of any added primer. The yield and size distribution of the first-strand cDNA population synthesized without added primer were similar to that synthesized in the presence of added oligo(dT). Similar background priming was previously reported in total RNA preparations and attributed to the presence of small nucleolar RNAs that primed ran-

domly (1). Likewise, efficient cDNA synthesis in the absence of oligo(dT) primer is thought to occur in mRNA preparations because of contamination with fragments of DNA or RNA that can bind to mRNA at random sites and serve as a primer, in which case, a reselection for poly(A) RNA is recommended (2).

In our experiments, however, isolating poly(A) RNA using two rounds of purification did not prevent first-strand synthesis in the absence of a primer. The size distribution of the first-strand cDNAs obtained from these experiments suggested that the endogenous priming was occurring at the 3' end of the mRNA and not randomly. To demonstrate that the first-strand synthesis started from the poly(A) tail, we attempted to amplify two long full-length cDNAs, the *Arabidopsis thaliana* ferredoxin-dependant glutamate synthase, (4947 bp, GenBank® accession no. Y09667) and the *A. thaliana* histone acetyltransferase 13 (5811 bp, GenBank accession no. AF510669), using an oligo(dT) primer in combination with a specific 5'-end primer. Both full-length cDNAs were successfully amplified by RT-PCR from the purified first-strand population generated without the addition of oligo(dT) primer (data not shown). This indicates that at least some background priming of cDNA had originated at the poly(A) tail.

A possible explanation for this background priming is the presence of exogenous oligo(dT) carried over from the mRNA isolation process. To test this hypothesis, we compared *A. thaliana* mRNA preparations from four different methods for the presence of oligo(dT).

In method 1, 0.9 mg total RNA and 400 pmol of a 5'-biotinylated oligonucleotide GAGAt₂₅ [(gaga)₃ ctc gag t₂₅] were mixed to a volume of 1.16 mL and then heat denatured at 65°C for 10 min. Thirty microliters of 20× SSC were added, and the mixture was left at room temperature for 30 min. Streptavidin-magnetic particles (150 µL in 5× SSC; Roche Diagnostics, Laval, QC, Canada) were added, and the mixture was incubated for 10 min at room temperature before capturing the particles using a magnetic stand. The particles

were washed three times with 1 mL 0.1× SSC, and the mRNA was eluted twice with 100 µL water preheated to 65°C.

In method 2, 0.2 mg RNA and 150 pmol biotinylated oligo(dT) (Promega, Madison, WI, USA) in 0.5 mL water were heat-denatured at 65°C for 10 min. Thirteen microliters of 20× SSC were added, and the mixture was left at room temperature for 10 min before adding 0.6 mL in 5× SSC streptavidin-magnetic particles and incubating at room temperature for 10 min. The particles were captured and then washed four times with 0.3 mL 0.1× SSC, and the mRNA was eluted twice with 125 µL water preheated to 65°C.

In method 3, 0.2 mg total RNA in 200 µL water was heat-denatured for 2 min at 65°C before being mixed with the equivalent of 400 µL oligo(dT)₂₅-linked paramagnetic polymer particles (DynaL, Lake Success, NY, USA) that were concentrated to 200 µL in 20 mM Tris-HCl, pH 7.5, 1.0 M LiCl, 2 mM EDTA. The mixture was gently agitated for 5 min at room temperature. The particles were captured and washed twice with a buffer of 10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA before eluting the mRNA twice with 50 µL water preheated to 80°C.

In method 4, 0.2 mg RNA in 450 µL water was heated for 5 min at 65°C and then mixed with 100 mg oligo(dT)₂₅ cellulose (New England Biolabs, Mississauga, ON, Canada) and 50 µL 5 M NaCl. After 5 min incubation at room temperature, the cellulose was pelleted, and the supernatant was heat-denatured and incubated again with the same cellulose for another cycle of selection. The cellulose was washed four times with 400 µL wash buffer (10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA), once with 400 µL low-salt buffer (10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA), and the mRNA was eluted twice with 200 µL 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, preheated to 70°C.

The mRNA preparations obtained with the four different methods were used to carry out reverse transcription reactions with and without the addition of exogenous primer. First-strand cDNA was prepared from 50 ng mRNA in 20 µL volume using 100 U

SuperScript™ reverse transcriptase (Invitrogen), 3 pmol [³²P]dCTP, and 1 mM dNTPs. Twenty-five picomoles of primer T₂₂VN (5'-AATCTAGAGGC-CGAGGCGGCCGTGT₂₂VN-3') were added to the reactions containing exogenous primer. The reverse transcription reactions were incubated for 30 min at 45°C. The results show that the reverse transcription reactions with mRNA preparations 1, 2, and 4 resulted in first-strand cDNA in the absence and presence of T₂₂VN primer. Only the mRNA isolated with method 3 was not primed in the absence of added primer (Figure 1A).

To detect and quantify the contaminating oligo(dT) within each mRNA preparation, we performed a dot blot (Figure 1B). RNA (0.5 µg) isolated from each of the methods 1–4, 0.5 µg RNA subjected to two successive rounds of poly(A) selection with method 4, and 0.5 µg *A. thaliana* total RNA were spotted onto an uncharged nylon membrane (Millipore, Etobicoke, ON, Canada) and probed with a radiolabeled oligo(dA)₂₂. The presence of carried-over oligo(dT) was repeatedly detected in mRNA from methods 1, 2, and 4. It was estimated to be in the range of 10–50 pmol oligo(dT)/0.5 µg mRNA for methods 1 and 2 and 1–5 pmol oligo(dT)/0.5 µg mRNA for method 4. No oligo(dT) could be detected in any mRNA preparation isolated with method 3 (Figure 1B). In an attempt to remove contaminating oligo(dT) from the mRNA, we designed the following experiments. First, 50 ng mRNA isolated with method 1 were heat denatured, quickly chilled on ice, and centrifuged through a Microcon-100® column (Millipore) at 4°C. Second, another aliquot of mRNA was first heat-denatured and then subjected to another round of capture on streptavidin-magnetic particles for 10 min at 15°C in 100 mM NaCl. These two methods did not remove the contaminating oligo(dT) or prevent the endogenous cDNA priming, indicating that the oligo(dT) was closely associated or that it quickly reassociated to its poly(A) target and thus co-purified with the mRNA (result not shown). In a third experiment, another aliquot of mRNA was purified on a denaturing formaldehyde agarose gel to separate

the small molecular weight component and extracted from the gel. The presence of contaminating oligo(dT) could no longer be detected in the re-extracted mRNA; however, it could not be reverse-transcribed, probably because of the presence of residual formaldehyde in the preparation (result not shown).

To examine further the proportion of endogenously primed cDNA in the presence of an added oligo(dT) primer, we performed a competition assay (Figure 2). Fifty nanograms of mRNA isolated with method 1 and containing

approximately 5 pmol contaminating biotinylated GAGAt₂₅ oligonucleotide were reverse-transcribed in the presence of an increasing amount of competing T₂₂VN primer (Figure 2A). The first-strand cDNA was synthesized using the same conditions as described above, before being captured on streptavidin-magnetic particles. Since the contaminating oligo(dT) from method 1 is biotinylated, the fraction of the cDNA that is not captured should cor-

Figure 1. First-strand cDNA reaction with or without added primer. (A) Alkaline gel electrophoresis of ³²P-labeled first-strand cDNA primed with T₂₂VN primer (indicated by “+”) and without added primer (indicated by “-”). RNA template: 50 ng *A. thaliana* poly(A) RNA isolated with methods 1–4. Poly(A)-tailed RNA ladder (Invitrogen) is used as marker (M). The different intensities of the first-strand cDNAs reflect the varying ratio of poly(A) RNA to ribosomal RNA obtained from the four different methods: the more ribosomal RNA is present in the mRNA preparation, the less poly(A) RNA is available as template for the reverse transcription. The molecular weight in kilobases of each marker cDNA band is indicated. (B) Dot blot probed with ³²P-labeled oligo(dA)₂₂. Top row: 0.08, 0.4, 2, 10, and 50 pmol T₂₂VN oligonucleotide, each spotted in 1 µL 100 mM NaOH. Bottom row: 0.5 µg poly(A) RNA extracted from *A. thaliana* tissue using methods 1–4 (dots 1–4), poly(A) RNA obtained after two cycles of poly(A) RNA isolation using method 4 (dot 5), and 0.5 µg total RNA (dot 6). All samples were alkaline-treated, then concentrated to 1 µL, and spotted. The images were acquired using the Storm™ 820 Gel and Blot Imaging system and analyzed with the ImageQuant 5.2 software (Amersham Biosciences, Baie d’Urfé, France).

Figure 2. Competition assay between the added T₂₂VN primer and the contaminating GAGAt₂₅ primer. (A) Alkaline gel electrophoresis of ³²P-labeled first-strand cDNA from mRNA obtained with method 1 primed with increasing concentration of T₂₂VN primer. No primer (lane 1), 0.05 pmol (lane 2), 0.5 pmol (lane 3), 5 pmol (lane 4), 50 pmol (lanes 5, 7, and 8), or 500 pmol (lane 6). RNA/primer mixtures were heat-denatured for 5 min at 65°C (lanes 1–6), for 10 min at 75°C (lane 7), or not heat-denatured (lane 8). (B) Alkaline gel electrophoresis of the first-strand cDNA from (A) that was not captured on streptavidin-coated magnetic particles. (C) Quantification of the total cDNA (black) and the cDNA not captured (dashed) using the Storm 820 Gel/Blot Imaging system and the ImageQuant 5.2 software volume analysis. Percentages of cDNA not captured are indicated for each lane.

respond primarily to the cDNA primed from the added T₂₂VN primer. Most of the cDNA synthesized in absence of exogenous primer was captured (Figure 2B, lane 1), indicating that it was synthesized from the biotinylated GAGAT₂₅ oligonucleotide carried over from the mRNA isolation step. Less than 49% of the cDNA was primed from the T₂₂VN primer when 50 pmol of this primer, which is the recommended amount for a standard reverse transcription reaction, were added. The percentage of newly primed cDNA reached 67% when 500 pmol T₂₂VN primer were added (Figure 2). Also, a heat denaturation of mRNA and primer for 10 min at 75°C before reverse transcription produced the same level of background priming when compared to a reaction that was not heat-denatured (Figure 2, lanes 7 and 8).

In an attempt to prevent endogenous priming of the mRNA isolated with method 1, 500 pmol oligo(dA)₂₂ were included in the reverse transcription reaction; however, this reduced endogenous priming by only 50% (data not shown). That, along with the previous observations, suggests that the contaminating oligo(dT) is either stably associated or quickly reassociated with the mRNA poly(A) tail. Because the contaminating oligo(dT) is not anchored, it can hybridize over the entire length of the mRNA poly(A) tail and prime at multiple sites. Since reverse transcriptase is a processive enzyme capable of strand displacement (3), we hypothesize that the contaminating oligo(dT) could be a more efficient primer than any anchored oligo(dT) primer.

The consequences of the presence of contaminating oligo(dT) in an mRNA preparation are numerous. Most of the protocols for the production of cDNA libraries require the presence of a restriction site within the oligo(dT) primer for subsequent directional cloning of the cDNA, in which cases, additional priming by contaminating oligo(dT) could reduce the cloning efficiency. In addition, many protocols for cDNA synthesis propose the use of an anchored oligo(dT)VN to prevent the reverse transcription of long poly(A) tails that can be an important cause of background in DNA sequencing or hybridizations (4). The presence of non-

anchored oligo(dT) in the reverse transcription will not only increase the proportion of cDNA clones with long poly(A) tails, but it is also likely to increase the internal priming at A/T-rich regions, thus increasing the occurrence of both 5' and 3' truncation in the cDNA population (5). Finally, the presence of a contaminating oligo(dT) that is biotinylated, as it is the case for the methods 1 and 2, would be the source of major background problems for any downstream application that involves the biotin-streptavidin interaction. For example, the Cap Trapper protocol for the cloning of full-length cDNA (6) could not be performed efficiently on mRNA contaminated with biotinylated oligo(dT). In our hands, only mRNA isolated with method 3 did not contain carried-over oligo(dT). This result is consistent with other observations regarding the stability of these types of beads in applications such as the isolation of mRNA directly from crude tissue and the construction of solid-phase cDNA libraries, whereby the beads are typically exposed to harsh conditions (heat, detergents, etc.) without any loss of the oligo(dT) (7–12).

REFERENCES

1. Frech, B. and E. Peterhans. 2003. RT-PCR: 'background priming' during reverse transcription. *Nucleic Acids Res.* 22:4342-4343.
2. Sambrook, J. and D.W. Russel. 2001. *Molecular Cloning: A Laboratory Manual*. CSH Laboratory Press, Cold Spring Harbor, NY.
3. Kelleher, C.D. and J.J. Champoux. 1998. Characterization of RNA strand displacement synthesis by Moloney murine leukemia virus reverse transcriptase. *J. Biol. Chem.* 273:9976-9986.
4. Shibata, Y., P. Carninci, K. Sato, N. Hayatsu, T. Shiraki, Y. Ishii, T. Arakawa, A. Hara, et al. 2001. Removal of poly-A tails from full-length cDNA libraries for high-efficiency sequencing. *BioTechniques* 31:1042-1049.
5. Nam, D.K., S. Lee, G. Zhou, X. Cao, C. Wang, T. Clark, J. Chen, J.D. Rowley, and S.M. Wang. 2002. Oligo(dT) primer generates a high frequency of truncated cDNAs through internal poly(A) priming during reverse transcription. *Proc. Natl. Acad. Sci. USA* 99:6152-6156.
6. Carninci, P., A. Westover, Y. Nishiyama, T. Ohsumi, M. Itoh, S. Nagaoka, N. Sasaki, Y. Okasaki, et al. 1997. High efficiency selection of full-length cDNA by improved biotinylated cap trapper. *DNA Res.* 4:61-66.
7. Jacobsen, K.S., E. Breivold, and E. Hornes. 1990. Purification of mRNA directly from

crude plant tissues in 15 minutes using oligo dT microspheres. *Nucleic Acids Res.* 18:3639.

8. Rainieri, I., C. Moroni, and H.P. Senn. 1991. Improved efficiency for single-sided PCR by creating a reusable pool of first-strand cDNA coupled to a solid phase. *Nucleic Acids Res.* 19:4010.
9. Lee, Y.H. and V.D. Vacquier. 1992. Reusable cDNA libraries coupled to magnetic beads. *Anal. Biochem.* 206:206-207.
10. Sharma, P., A. Lsvnneborg, and P. Stougaard. 1993. PCR-based construction of subtractive cDNA library using magnetic beads. *BioTechniques* 15:610-611.
11. Lambert, K.N. and V.M. Williamson. 1993. cDNA library construction from small amounts of RNA using paramagnetic beads and PCR. *Nucleic Acids Res.* 21:775-776.
12. Aasheim, H.C., A. Deggerdal, E.B. Smealand, and E. Hornes. 1994. A simple subtraction method for the isolation of cell-specific genes using magnetic monodisperse polymer particles. *BioTechniques* 16:716-721.

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