

# Increased specificity of reverse transcription priming by trehalose and oligo-blockers allows high-efficiency window separation of mRNA display

Yosuke Mizuno<sup>1,3,4</sup>, Piero Carninci<sup>1,3</sup>, Yasushi Okazaki<sup>1,3</sup>, Minako Tateno<sup>1,3</sup>, Jun Kawai<sup>1,3</sup>, Hiroshi Amanuma<sup>2,4</sup>, Masami Muramatsu<sup>1,3</sup> and Yoshihide Hayashizaki<sup>1,3,5,\*</sup>

<sup>1</sup>Laboratory for Genome Exploration Research Project, Genomic Sciences Center (GSC) and Genome Science Laboratory and <sup>2</sup>Molecular Cell Science Laboratory, Riken Tsukuba Life Science Center, Institute of Physical and Chemical Research (RIKEN), Koyadai 3-1-1, Tsukuba, Ibaraki 305-0074, Japan, <sup>3</sup>Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Corporation, Koyadai 3-1-1, Tsukuba, Ibaraki 305-0074, Japan, <sup>4</sup>Institute of Biological Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-0006, Japan and <sup>5</sup>Institute of Basic Medical Sciences, University of Tsukuba, Tennohdai 1-1-1, Tsukuba, Ibaraki 305-0006, Japan

Received November 13, 1998; Revised and Accepted January 11, 1999

## ABSTRACT

We have developed a method for high-efficiency window separation of cDNA display by increasing the specificity of priming in reverse transcription. In the conventional method, two-base anchored oligo(dT) primers (5'dT<sub>16</sub>VN3', where N is any base and V is G, A or C) are used to make windows for the display of transcripts. However, reverse transcriptase often extends misprimed oligonucleotides. To avoid mispriming from dT<sub>16</sub>VN primers, we have developed two new technologies. One is higher temperature priming with reverse transcriptase thermoactivated by the disaccharide trehalose. The other is the use of competitive oligonucleotide blockers that hybridize to the non-selectively primed mRNAs, preventing the mispriming from the VN site. These methods were combined to improve restriction landmark cDNA scanning (RLCS), resulting in the elimination of the redundant signals that appear in different windows. This was achieved by the increased specificity of initiation of reverse trans-cription from the beginning of poly(A) sites. This method paves the way for the precise visualization of transcripts to allow expression profiles in individual tissues and at each developmental stage to be understood.

## INTRODUCTION

cDNA display technology is important for revealing expression patterns of both known and unknown transcripts and to detect mutations and polymorphisms. In higher organisms such as human and mouse, approximately 100 000 genes are differentially expressed in various tissues. It is very difficult to display these complex patterns of expression on a single profile owing to the

resolution limitations of analytical technologies such as electrophoresis. To overcome this problem, we introduce the concept of 'window separation' in an expression profile. The 'window' is defined as a set of transcripts with a certain sequence identity that are displayed together. Ideally, to give expression profiles of each tissue, each signal on the profile should show one-to-one correspondence to a transcript without redundancy.

Lots of effort has been used to develop methods for transcript visualization. These methods include differential display (DD; 1–3), arbitrary fragment length polymorphism (AFLP; 4), restriction landmark cDNA scanning (RLCS; 5) and molecular indexing (MI; 6,7). RLCS has advantages: it has the highest resolution, being able to show several thousand mRNAs in a single profile, and the intensity of the spots reflects the frequency of the transcripts. In contrast, because DD, AFLP and MI use PCR amplification to reduce the complexity, the intensity of bands does not reflect the frequency of the transcripts. Furthermore, DD and AFLP do not achieve one-to-one correspondence between a single signal and a transcript. In fact, several signals may be produced from a single mRNA owing to the use of arbitrary primers in the case of DD and the possibility that cDNAs have several restriction sites in AFLP. MI employs a combination of selective ligation at class IIS restriction sites [such as *FokI* (GGATGN<sub>9</sub>/N<sub>13</sub>)] and oligo(dT) priming, followed by one-dimensional electrophoretic separation. Because MI uses one-dimensional electrophoresis, it is necessary to divide cDNAs into 192 windows. In contrast, in RLCS, selection by two-base anchored oligo(dT) (5'dT<sub>16</sub>VN3', where N is any base and V is G, A or C) is sufficient on its own because the resolution shows up to several thousand spots in one profile. Here, theoretically, if separation using the two-base anchored oligo(dT) is perfect, each spot shows one-to-one correspondence with each mRNA. However, when we use RLCS, which should be separated into 12 windows, it frequently happens that many spots appear redundantly in several windows, thus decreasing the potential resolution power of the technique. This

\*To whom correspondence should be addressed at: Laboratory for Genome Exploration Research Project, Genomic Sciences Center (GSC) and Genome Science Laboratory, Institute of Physical and Chemical Research (RIKEN), Koyadai 3-1-1, Tsukuba, Ibaraki 305-0074, Japan.  
Tel: +81 298 36 9145; Fax: +81 298 36 9098; Email: yoshihide@rtc.riken.go.jp

means that two-base anchored oligo(dT) selection is not perfect. MI, DD and AFLP are also based on oligo(dT) priming for cDNA preparation. In this sense, two-base anchored oligo(dT) selection is elementary to all of these methods for the visualization of cDNAs. Therefore, we focused in this study on obtaining conditions that can achieve the highest efficiency of window separation.

The incompleteness of two-base anchored oligo(dT) selection is caused mainly by the ambiguity of initiation of reverse transcriptase: the mismatched 5'dT<sub>16</sub>VN3' primers can be extended at similar efficiency to the matched primers. This is analogous to results reported for HIV-1 and AMV reverse transcriptases, which allow mispaired primer extension and misincorporation to a high extent (8–11). To overcome this problem, we used higher temperature cDNA priming with reverse transcriptase thermoactivated by the disaccharide trehalose and competitive oligonucleotide blockers (oligo-blockers), which hybridize with all mRNAs that are expected not to be templates for first strand cDNA synthesis in a given window. The use of trehalose in this step is based on our finding that thermostabilization and thermoactivation of reverse transcriptase is achieved by the addition of trehalose (12,13). Trehalose seems to function as a chaperonin to protect and stabilize many enzymes, resulting in an increase in the optimal working temperature. This allows hot-start priming, achieving high-stringency conditions for primers to hybridize. To further improve the window separation, we introduced in a given window the use of 11 oligo-blockers that we anticipated would suppress non-specific annealing by competing with the extendable primer. Finally, we found the optimal conditions for high-efficiency window separation and confirmed these by RLCS pattern. These conditions can be applied to any method, such as DD, AFLP or MI, on which oligo(dT) priming is based.

## MATERIALS AND METHODS

### RNAs

The cDNA clones in a λZAP II cloning vector were picked up from a mouse kidney full-length cDNA library (14) and were sequenced to determine the 3' poly(A)-attached site. Clone length varied from 0.8 to 2.0 kb. Bulk-excised plasmid DNA was purified, digested with *Xho*I and transcribed *in vitro* with either T7 or T3 RNA polymerase (Gibco BRL, Grand Island, NY). Because a dT<sub>16</sub>VN primer mixture was used for making the cDNA clones, the prepared RNAs had a 16 base poly(A) stretch.

### dT<sub>16</sub>VN primers and oligo-blockers

dT<sub>16</sub>VN primers and oligo-blockers were synthesized with a DNA synthesizer (Perceptive, Framingham, MA). 3'-Amino-Modifier C3 CPG 500 (Glen Research, Sterling, VA) was used for the initial synthesis of the 3' site of the oligo-blockers. 5'-Biotinylated dT<sub>16</sub>VN oligonucleotides for RLCS were prepared using 5'-biotin phosphoramidite (Glen Research). The sequences were 5'-biotin-AGAGA-GAGAGTTTTTTTTTTTTTTTTVN-3' for the dT<sub>16</sub>VN primers and 5'-AGAGAGAGAGTTTTTTTTTTTTTTTTVN-Am-3' for oligo-blockers (Am represents an amino modifier). Synthesized dT<sub>16</sub>VN primers and oligo-blockers were purified by denaturing polyacrylamide gel electrophoresis containing 8 M urea (15). Eleven oligo-blockers (3 μg/μl each) and the specific dT<sub>16</sub>VN primer (3 μg/μl) were mixed in an equal volume and 12 sets of these mixtures (3 μg/μl total, 0.25 μg/μl for each primer and blocker) were prepared.

### mRNA for RLCS

Total RNA was extracted from mouse (C57BL/6J) brain by the acid guanidium-phenol-chloroform method (16), then mRNA was purified with a poly(A) quick mRNA purification kit (Stratagene, La Jolla, CA).

### Conventional reverse transcription

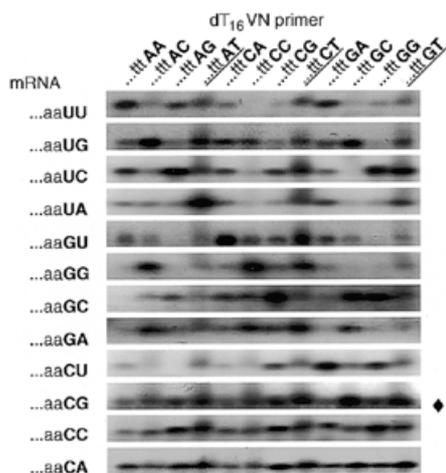
A 6.5 μl mixture including 200 ng of *in vitro* transcribed RNA as a template and 130 ng of single dT<sub>16</sub>VN primer was heated at 65°C for 10 min then put on ice for 5 min and heated again at 42°C. After 2 min, the reaction mixture [4 μl first strand buffer (5×; Gibco-BRL), 2 μl dithiothreitol (0.1 M), 1.3 μl dNTP mix (10 mM each), 0.3 μl [α-<sup>32</sup>P]dCTP (6000 Ci/mmol; Amersham Pharmacia Biotech, Aylesbury, UK), 100 U Superscript II reverse transcriptase, 0.5 μl bovine serum albumin (0.1%) and 6.5 μl water] was added and incubated for 1 h.

### Hot-start reverse transcription

A 6.5 μl mixture including 200 ng of template RNA, 0.52 μl of one of the 12 dT<sub>16</sub>VN primer-oligo-blocker sets (0.25 μg/μl for each primer and blocker) and 3.9 μl of 80% glycerol was heated at 65°C for 10 min, then cooled to 50°C. After 2 min, the reaction mixture [the same as for the conventional reverse transcription except that 6.5 μl of saturated trehalose (~80% w/v) was used instead of 6.5 μl water] was added after pre-heating at 50°C for 2 min. In Figure 2, trehalose, oligo-blockers or both were omitted from the reaction and water was substituted. Subsequently, the mixtures were cooled to various annealing temperatures (37–47°C) for 2 min. Finally, samples were heated again to 50°C and incubated for 1 h. To test the several annealing temperatures effectively and accurately, all reactions were done in parallel with a RoboCycler (Stratagene). After the reactions, part of each sample was loaded on an alkaline agarose gel (0.8% agarose) and run (15). Then the gel was dried and autoradiographic images were obtained by a BAS 2000 imaging analyzer (Fujix, Tokyo, Japan).

### RLCS

First strand cDNA was synthesized from 3 μg of mouse brain mRNA with 1.56 μg dT<sub>16</sub>VN primer-oligo-blocker mixture under either conventional or hot-start conditions. Then second strand reaction mixture (40 U *Escherichia coli* DNA polymerase, 20 U *E.coli* DNA ligase and 2 U RNase H) was added and incubated at 16°C for 2 h (17). To complete the second strand synthesis, additional enzyme mixture [10 U Ex-Taq polymerase (Takara, Tokyo, Japan), 40 U Ampligase (Epicentre, Madison, WI) and 1 U Hybridase (Epicentre)] was added and incubated at 65°C for 15 min (14). After complete digestion of the remaining mRNA by 15 U RNase I (Promega, Madison, WI) for 30 min, 10 μg proteinase K was added and incubated at 37°C for 30 min. To remove free dNTPs, 2 vol of cetyltrimethylammonium bromide (CTAB)-urea solution (1% CTAB, 25% urea, 10 mM Tris-HCl, pH 7.0, 4 μg *Escherichia coli* tRNA and 0.5 mM EDTA) (14) were added and incubated at room temperature for 1 h. After centrifugation at 15 000 r.p.m. for 10 min, the cDNA pellet was washed with 200 μl of 70% EtOH containing 0.2 M NaCl to remove the remaining CTAB. The cDNA was resuspended in 90 μl TE, 15 U RNase I was added and the mixture was incubated for 30 min at 37°C and extracted with phenol-chloroform. The aqueous phase was purified by Sephadex G75 (Amersham



**Figure 1.** Reverse transcription was done with 12 different dT<sub>16</sub>VN primers for each of 12 RNAs differing in their VN sequence at the poly(A) site (144 reactions in total). Conventional conditions were used for the reaction (Fig. 2A). The three underlined primers showed especially strong mispriming. ♦, the strongest annealing mismatch with dT<sub>16</sub>Gt among the 12 RNAs.

Pharmacia Biotech) in a home-made spin column and ethanol precipitation was done. Recovered cDNA was resuspended in 7  $\mu$ l TE and the blocking reaction was done (5). The cDNA was then digested with *Xma*I and the restriction site was labeled by both [ $\alpha$ -<sup>32</sup>P]dCTP and [ $\alpha$ -<sup>32</sup>P]dGTP with Sequenase v.2 polymerase (Amersham Pharmacia Biotech) (5).

Ten micrograms of tRNA was added to 120  $\mu$ l of streptavidin-magnetic beads (CPG, Lincoln Park, NJ) for one reaction and left on ice for 30 min to suppress any non-specific cDNA interaction with the beads, then the cDNA was captured with the beads. The beads were washed twice with washing solution (2 M NaCl, 50 mM EDTA). The cDNA and magnetic beads were mixed for 15 min at room temperature, then the beads were washed twice with washing solution and twice with 0.2% SDS. The cDNA was released from the magnetic beads by the addition of releasing mix (40 ng/ $\mu$ l tRNA, 1.25% biotin, 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sodium *N*-lauroylsarcosinate) and incubated at 45°C for 2 h. The recovered cDNA fragments were extracted with phenol-chloroform and precipitated with ethanol. The fragments were then processed to produce a two-dimensional electrophoretic pattern (5). *Hinf*I was used for the in-gel digestion. At the end, the gel was processed, dried, and exposed for autoradiography (5,18).

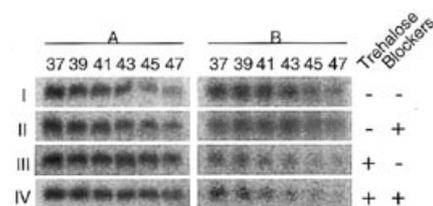
### Analysis of RLCS spots

The numbers of the RLCS spots were scored by visual inspection by eye. Two persons contributed independently to count the spots.

## RESULTS AND DISCUSSION

### The incompleteness of two-base anchored oligo(dT) selection

To understand exactly how two-base anchored oligo(dT) selection works under traditional conditions, we used reverse transcription with 12 different *in vitro* transcribed mRNAs and 12 different primers. These *in vitro* transcribed RNAs have different VN



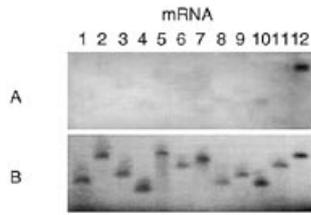
**Figure 2.** Comparison of the efficiency of priming using the dT<sub>16</sub>Gt primer for two single clones of *in vitro* transcribed RNA. ...aaCA RNA (A), which was complementary to the dT<sub>16</sub>Gt primer, and ...aaCG RNA (B), a representative RNA that showed the most non-specific priming, were used for reverse transcription. The reverse transcription was done under different conditions and at different annealing temperatures (37–47°C) in the presence or absence of trehalose and oligo-blockers: I, trehalose (–), oligo-blockers (–); II, (–, +); III, (+, –); IV (+, +). We achieved the best window separation in the presence of trehalose and oligo-blockers at higher annealing temperatures (45 and 47°C).

sequences at the poly(A) site and the primers have different VN sequences at the 3'-end. By using 12 sets each of template RNA and primer, 144 reactions were done in total (Fig. 1). Ideally, only 12 reactions, where a primer and an RNA are complementary, should produce specific signals. However, as shown in Figure 1, several mismatched primers were extended and showed strong signals. Primers that have thymine at the 3'-end tend to be extended even if the 3'-end is mismatched (19). We confirmed the same tendency (primers ...ttAT, ...ttCT and ...ttGT), although the reverse transcription was also elongated with other mismatched primers. This suggests significantly higher ambiguity of reverse transcriptase than with the *Taq* polymerase. These results show clearly that two-base anchored oligo(dT) selection is incomplete under conventional conditions (Fig. 1).

### High-efficiency window separation using trehalose and oligo-blockers

We thought that the incomplete window separation under conventional conditions was due to the lower annealing temperature. Running the reaction at a higher temperature allows the primer to hybridize to template RNA more specifically. However, under normal conditions, the reaction would not be efficient because the activity of reverse transcriptase would decrease. Recently we discovered that in the presence of a disaccharide, trehalose, reverse transcriptase can be thermostabilized and thermoactivated (12,13). Trehalose seems to function as a chaperonin-like molecule (12). Furthermore, high temperature reverse transcription in the presence of trehalose can melt the strong secondary structure of mRNA, achieving the synthesis of full-length cDNA effectively. Thus, we used hot-start reverse transcription with trehalose, annealed primers and mRNAs at a higher temperature. To further increase the specificity of the priming, we also tested the addition of oligo-blockers to suppress non-specific annealing by competition with the mismatched annealing. Oligo-blockers have an amino group at the 3'-end instead of a hydroxyl group; enzymatic polymerization will not start from this group. Eleven other oligo-blockers added to the extendable primer would compete with the given primer to anneal to a mismatched mRNA.

To evaluate the effect of trehalose and oligo-blockers, we used a representative set of template RNAs and a primer. As shown in Figure 1, the mismatched primer dT<sub>16</sub>Gt produced the highest signal when ...aaCG RNA was used as a template. We therefore



**Figure 3.** Improved reverse transcription. Using the dT<sub>16</sub>GT primer (Fig. 2), reverse transcription was done for all 12 RNAs. (A) Optimal conditions found in Figure 2. RNA and primer including oligo-blockers were denatured at 65°C for 10 min then cooled to 50°C. After 2 min, the remaining reaction mixture containing trehalose (heated in advance to 50°C for 2 min) was added. The mixture was cooled to 45°C for annealing for 2 min and then incubated at 50°C for 1 h. The following RNAs were used: lane 1, ...aaUU-; lane 2, ...aaUG-; lane 3, ...aaUC-; lane 4, ...aaUA-; lane 5, ...aaGU-; lane 6, ...aaGG-; lane 7, ...aaGC-; lane 8, ...aaGA-; lane 9, ...aaCU-; lane 10, ...aaCG-; lane 11, ...aaCC-; lane 12, ...aaCA-. (B) Conventional conditions. The mismatched reverse transcription was promisingly decreased in (A) (lanes 1–11). The much higher specific reverse transcription for a single RNA was obtained in the high temperature reaction containing trehalose and oligo-blockers (lane 12).

decided to use dT<sub>16</sub>GT primer with ...aaCG RNA as a mismatched pair and ...aaCA RNA as a matched pair. We considered the best condition to be achieved when the matched pair produced the highest signal and the mismatched pair produced the lowest.

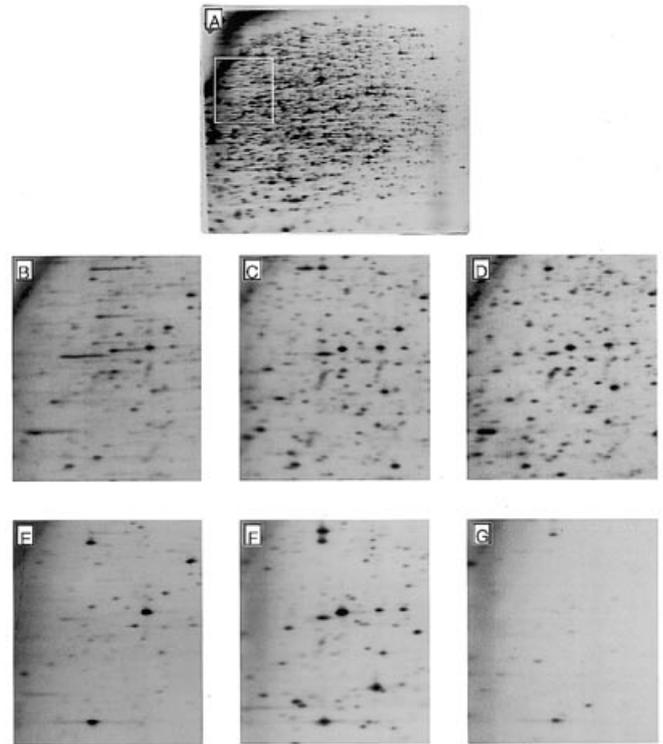
Various different conditions (with and without trehalose or oligo-blockers and at different annealing temperatures) were tested with a matched and a mismatched pair (Fig. 2A and B, respectively). Although the annealing temperatures of several primers were estimated to be ~35°C, a range of 37–47°C was tested to obtain higher specificity. In the absence of trehalose (Fig. 2, I and II), cDNA could be synthesized from both matched and mismatched RNA at almost the same level and no specificity could be observed. The addition of trehalose (Fig. 2, III), however, seems to improve specificity; this may be caused by the increased fidelity of reverse transcriptase (unpublished data). Mismatched cDNA synthesis was suppressed efficiently at the higher annealing temperature. By comparing IV in Figure 2A and B, we determined that the best annealing temperatures were 45 and 47°C with trehalose and oligo-blockers.

### Best conditions for high-efficiency window separation

To confirm whether the best conditions above (Fig. 2) also work for other RNAs, we used reverse transcription with the same primer (dT<sub>16</sub>GT). Figure 3 shows the reverse transcription under improved (Fig. 3A) and conventional conditions (Fig. 3B). Here we could clearly see that with trehalose and oligo-blockers and annealing at a higher temperature (45°C), only matched pairs could produce a specific signal (Fig. 3A). Under conventional conditions (Fig. 3B), the mismatched primer could extend and produced non-specific signals.

### Evaluation of the best conditions using RLCS

To confirm whether the conditions found in the previous experiment using one mRNA and one primer at a time were also best for tissue mRNAs where various mRNAs are expressed, RLCS was performed using mouse brain mRNAs. Because we used the

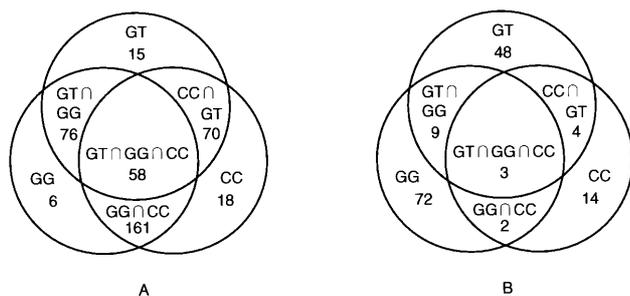


**Figure 4.** RLCS profiles using window-separated mouse brain mRNAs. (A) Twelve dT<sub>16</sub>NV primers without competitors were used for reverse transcription, followed by RLCS. (B–G) Three representative sets of dT<sub>16</sub>NV, dT<sub>16</sub>GT (B and E), dT<sub>16</sub>GG (C and F) and dT<sub>16</sub>CC (D and G), were chosen. Part of the RLCS profile boxed in (A) is expanded. (B–D) Conventional conditions. (E–G) dT<sub>16</sub>GT, dT<sub>16</sub>GG and dT<sub>16</sub>CC, respectively, were used with oligo-blockers and trehalose at thermoactivated condition.

biotinylated oligo(dT)<sub>16</sub> VN primer for the first strand cDNA synthesis, RLCS ideally can produce one signal from a transcript (one-to-one correspondence). Because 30 000 genes at most are expressed in a tissue, we considered 12 window separation to be reasonable when RLCS, whose resolution is >2000 spots, is used for visualization of mRNAs.

In this paper we also report for the first time three improvements to RLCS. First, we used CTAB precipitation to remove free dNTPs that were used for the first and second strand cDNA synthesis. Second, we performed additional ligation in the second strand cDNA synthesis step at a higher temperature to achieve more efficient extension and ligation with thermostable RNase H (Hybridase), thermostable ligase (Ampligase) and Ex Taq polymerase. Third, to increase the efficiency of cDNA recovery from the streptavidin beads, we used excessive free biotin in guanidine thiocyanate to exchange biotinylated cDNAs with free biotin. This exchange reaction, in the presence of a chaotropic agent, allowed more efficient release of the biotinylated cDNA fragments. With these improvements, we reproduced the RLCS pattern more efficiently and produced spots that we considered to be derived from longer cDNAs.

Figure 4 shows the resulting pattern of RLCS using high-efficiency window separation. Figure 4A shows the whole RLCS profile with a mixture of 12 dT<sub>16</sub>VN primers without trehalose or oligo-blockers. As representative cases of window separation, we show three different windows using three sets of dT<sub>16</sub>VN primes:



**Figure 5.** Analysis of the number of spots appearing in RLCS. The number of the spots in the boxed area (Fig. 4) was counted. CC GG, for example, is the number of spots present in both  $dT_{16}CC$  and  $dT_{16}GG$  windows. (A) Conventional conditions. (B) Thermoactivated condition with trehalose and oligo-blockers.

$dT_{16}GT$ ,  $dT_{16}GG$ , and  $dT_{16}CC$ . Figure 4B and E is derived from the  $dT_{16}GT$  window, Figure 4C and F from the  $dT_{16}GG$  window and Figure 4D and G from the  $dT_{16}CC$  window. Here, Figure 4B–D was produced under conventional conditions and Figure 4E–G under improved conditions. The box in Figure 4A is magnified and shown in Figure 4B–G. In Figure 4B–D, 58 spots overlapped (Fig. 5A), whereas in Figure 4E–G only three overlapped (Fig. 5B). These results clearly show that using the trehalose and oligo-blockers allows window separation to be performed more efficiently.

Spots whose intensity did not change between conventional and improved conditions were likely to be derived from genes whose sequences were complementary to the  $dT_{16}GT$  primer. This result suggests that the decrease in number of spots did not derive from a decrease in the intensity of the whole film.

It was reported that the polyadenylation of mRNA *in vivo* occurs preferentially after the CA sequence, which lies 10–30 bases after the polyadenylation signal, AAUAAA (20,21). Accordingly, the last base of mRNAs, just before poly(A), would be a C. If the RLCS films using cDNA synthesized under improved conditions reflect this distribution, the number of spots in the windows using  $dT_{16}GA$ ,  $dT_{16}GC$ ,  $dT_{16}GG$  and  $dT_{16}GT$  should be greater than in windows using the other  $dT_{16}VN$  primers. In fact, the number of spots in the windows using  $dT_{16}CC$  primer was lower than those in the windows using  $dT_{16}GG$  and  $dT_{16}GT$  primer, but not absent. Although this agrees partly with a previous report (20,21), C is not the only base to be a last base of the mRNA.

Figure 5 shows a detailed analysis of the numbers of spots in this area. The total number of spots appearing in three windows under conventional conditions was 404. Of these, only 39 (9.6%) appeared in only one window. In contrast, under improved conditions, 134 of 152 spots (88.1%) appeared in only one window, thus suggesting greatly increased specificity. However, some spots still show redundancy between three windows even under improved conditions: we could consider this as partial failure to separate. Alternatively, because only a few spots overlap between windows under improved conditions, we could consider that these overlaps derive from variations in the cleavage or polyadenylation site. Multiple alignment sequence analysis of

ESTs and the Body Map (22) suggests several variations in the cleavage site for some mRNAs (data not shown).

We conclude that window separation using trehalose and oligo-blockers can greatly improve the specificity of RLCS window separation. This method can easily be extended to any method that is based on oligo(dT) priming, such as DD, AFLP or MI.

## ACKNOWLEDGEMENTS

We all thank Noriko Kikuchi, Sumiharu Nagaoka, Mari Itoh, Kazuhiro Shibata and Yuko Shibata for giving us the cDNA clones, and Nobuya Sasaki and Mamoru Kamiya for helpful discussions. This study was supported by a Research Grant for the Genome Exploration Research Project from Core Research for Evolutional Science and Technology (CREST) and Special Coordination Funds for promoting Science and Technology from the Science and Technology Agency of the Japanese Government, a Grant-in-Aid for Scientific Research on Priority Areas and the Human Genome Program from the Ministry of Education and Culture, Japan and a Grant-in-Aid for a second Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan to Y.H.

## REFERENCES

- Liang,P. and Pardee,A.B. (1992) *Science*, **257**, 967–971.
- Liang,P., Averboukh,L. and Pardee,A.B. (1993) *Nucleic Acids Res.*, **21**, 3269–3275.
- Welsh,J., Chada,K., Dalal,S.S., Cheng,R., Ralph,D. and McClelland,M. (1992) *Nucleic Acids Res.*, **20**, 4965–4970.
- Vos,P., Hogers,R., Bleeker,M., Reijans,M., van de Lee,T., Hornes,M., Frijters,A., Pot,J., Peleman,J., Kuiper,M. *et al.* (1995) *Nucleic Acids Res.*, **23**, 4407–4414.
- Suzuki,H., Yaoi,T., Kawai,J., Hara,A., Kuwajima,G. and Wantanabe,S. (1996) *Nucleic Acids Res.*, **24**, 289–294.
- Kato,K. (1995) *Nucleic Acids Res.*, **23**, 3685–3690.
- Kato,K. (1996) *Nucleic Acids Res.*, **24**, 394–395.
- Perrino,F.W., Preston,B.D., Sandell,L.L. and Loeb,L.A. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 8343–8347.
- Mendelman,L.V., Petruska,J. and Goodman,M.F. (1990) *J. Biol. Chem.*, **265**, 2338–2346.
- Creighton,S., Huang,M., Cai,H., Arnheim,N. and Goodman,M.F. (1992) *J. Biol. Chem.*, **267**, 2633–2639.
- Zinnen,S., Hsieh,J. and Modrich,P. (1994) *J. Biol. Chem.*, **269**, 24195–24202.
- Carninci,P., Nishiyama,Y., Westorver,A., Itoh,M., Nagaoka,S., Sasaki,N., Okazaki,Y., Muramatsu,M. and Hayashizaki,Y. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 520–524.
- Carninci,P. and Hayashizaki,Y. (1999) *Methods Enzymol.*, **303**, 19–44.
- Carninci,P., Kvam,C., Kitamura,A., Ohsumi,T., Okazaki,Y., Itoh,M., Kamiya,M., Shibata,K., Sasaki,N., Izawa,M., Muramatsu,M., Hayashizaki,Y. and Schneider,C. (1996) *Genomics*, **37**, 327–336.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.
- Gubler,U. (1987) *Methods Enzymol.*, **152**, 330–335.
- Hatada,I., Hayashizaki,Y., Hirotsune,S., Komatsubara,H. and Mukai,T. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9523–9527.
- Gelfand,D.H. and White,T.J. (1990) In Innis,M.A., Gelfand,D.H., Sninsky,J.J. and White,T.J. (eds), *PCR Protocols: A Guide to Methods and Applications*. Academic Press, San Diego, CA, p. 129.
- Sheets,M.D., Ogg,S.C. and Wickens,M.P. (1990) *Nucleic Acids Res.*, **18**, 5799–5805.
- Chen,F., MacDonald,C.C. and Wilusz,J. (1995) *Nucleic Acids Res.*, **23**, 2614–2620.
- Okubo,K., Hori,N., Matoba,R., Niyama,T., Fukushima,A., Kojima,Y. and Matsubara,K. (1992) *Nature Genet.*, **2**, 173–179.