Identification of differentially expressed genes from limited amounts of RNA

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ABSTRACT

The identification of cellular RNA expression profiles by differential display (DD) involves the visualization of RT-PCR products from the RNA. Traditionally, DD protocols require 200–500 ng RNA for each RT reaction. Thus, the limiting factor in DD is the amount of RNA available and the sensitivity of the RT reaction. By replacing the type of reverse transcriptase in our method, the sensitivity of DD increased up to 100-fold. Very significantly, the cDNA species obtained are higher in molecular weight, increasing the chances of detection of differential display genes with less background bands. The false positives and background in general also decreased due to the utilization of *Taq* polymerase antibody to facilitated DNA synthesis in the PCR reaction step. The reverse transcriptases described here may have a greater priming capacity as well as strong processivity which would explain the higher sensitivity accomplished in comparison to more standard reverse transcriptases. Additionally, the application of a more sensitive DD to samples when the amount of RNA is limited would be highly recommended.

INTRODUCTION

Differential display (DD) is a powerful method used to detect and characterize altered gene expression in closely related cell lines or tissue types (1–5). It is applicable to a number of biological systems and has many advantages over other methods to distinguish differential expression of mRNA. DD results are easily reproduced and require only moderate amounts of RNA. Significant improvements have been made to minimize the number of false positives and facilitate the cloning of PCR products (3,4). However, the amount of total RNA required as starting material may limit the use of DD. In this study we examined a number of reverse transcription (RT) enzymes in relation to their sensitivity and reproducibility in DD, and present the enzyme and conditions found to provide the most sensitive detection.

MATERIALS AND METHODS

RNA preparation

LNCaP cells were maintained in RPMI containing 10% fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 µg/ml). Cells were harvested, centrifuged at 1000 r.p.m. for 5 min at 4°C and washed with PBS. MCF-7 cells were grown in DMEM medium, supplemented with 10% FCS, penicillin (50 U/ml) and streptomycin (50 µg/ml). Cells were treated for 24 h with 4 mM of the hydroxamic acid based hybrid polar compound suberoylanilide hydroxamic acid (SAHA), kindly provided by Victoria M. Richon and Paul A. Marks (Memorial Sloan-Kettering Cancer Center). SAHA was diluted in DMSO. Control cells were treated with an equal amount of DMSO. Total RNA was isolated from LNCaP or MCF-7 cells using Trizol[®] reagent (Gibco BRL, Grand Island, NY). The RNA was resuspended in diethyl pyrocarbonate (DEPC)-treated water for DD analysis. Total RNA was digested to eliminate residual DNA with DNase I using the Message Clean Kit (GenHunter, Nashville, TN).

Reverse transcription of RNA

RT was performed using four different reverse transcriptases and appropriate conditions. In all reactions, cDNA was synthesized in 20 µl from 2.0 µl of MCF-7 or LNCaP RNA that was diluted from concentrations of 0.1–100 ng/µl in DEPC water. Superscript II RT, MMLV RNase H⁻ (Gibco BRL) or MMLV (GenHunter) were used under conditions described by the RNImage kit (GenHunter). For each reaction, RT buffer (25 mM Tris-Cl, pH 8.3, 37.2 mM KCl, 1.5 mM MgCl₂, 5 mM DTT) and 20 µM dNTP, 200 nM H-T₁₁C anchor primer (5'-AAGC-TTTTTTTTTTTC-3') were used. Reactions were incubated at 65°C for 5 min, then 100 U RT enzyme was added and incubated at 37°C for 60 min and 75°C for 5 min. Reactions containing 2.0 µl of Omniscript or Sensiscript RT (Qiagen, Santa Clarita, CA), 2.0 µl 10× RT buffer, 2.0 µl 5 mM dNTP (final concentration of 500 µM), 2.0 µl of 10 µM H-T₁₁C (final concentration of 1 µM), 1.0 µl RNase inhibitor (10 U/µl) and 10.0 µl DEPC-treated water were incubated at 37°C for 60 min and then at 93°C for 5 min.

Amplification of cDNA

PCR of cDNA was achieved according to the RNImage protocol (GenHunter). For the PCR reactions 10% of the RT reaction was used including AmpliTaq DNA polymerase

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(Perkin-Elmer, Foster City, CA) or AdvanTaq Plus which includes the antibody against *Taq* DNA polymerase (#5400-1, Clontech, Palo Alto, CA). The PCR contained 200 nM of the primers, H-T₁₁C anchor primer with either H-AP3 (5'-AAGC-TTTGGTCAG-3') or H-AP5 (5'-AAGCTTAGTAGGC-3'), PCR buffer (10 mM Tris-Cl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂ and 5 mM DTT), 2 μ M dNTP mix, 20 Ci/mmol [α -³³P]dATP per reaction and 1 U *Taq* polymerase (Perkin-Elmer) in a total volume of 20 μ l. Reactions were cycled at 94°C for 15 s, 40°C for 2 min and 72°C for 30 s for 40 cycles follow by 72°C for 5 min. For AdvanTaq Plus, reactions contained PCR buffer (40 mM Tris-KOH, pH 8.0, 16 mM KCl, 4.5 MgCl₂, 3.75 μ g/ml bovine serum albumin) had 1 μ l of enzyme AdvanTaq, 200 μ M dNTPs and 200 nM primer mix and 20 Ci/mmol of [α -³³P]dATP per reaction.

Gel electrophoresis of cDNA fragments

PCR reactions were electrophoresed on extended-format denaturing 6% polyacrylamide gels using the programmable Genomix LR DNA sequencer (Beckman Coulter, Columbia, MD).

RESULTS

A total of four different reverse transcriptases were tested for their efficiency to generate DD patterns. Two of the four enzymes tested have been recently designed to generate higher efficiency RT *in vitro*. They were developed by Qiagen and named Sensiscript® and Omniscript®. Reverse transcriptases are usually derived from retroviruses such as avian myeloblastosis virus, Moloney murine leukemia virus (MMLV) or human immunodeficiency virus. The new Qiagen enzymes were isolated from a new source and optimized for RT. These recombinant enzymes were designed for RT using different amounts of RNA. For Sensiscript, the enzyme works from 0.2 to 50 ng with good linearity, and for Omniscript, the enzyme works from 50 ng to 2 μ g of RNA (Product profile, Qiagen and Quiagenews, No. 2, 1999). The other two enzymes tested, generally used in our laboratory for RT-PCR, were MMLV (GenHunter) and MMLV RNase H⁻ (Superscript®; Gibco BRL).

Figure 1A and B shows a LNCaP RNA sample, reverse-transcribed using Superscript, Sensiscript and Omniscript. For fragments larger than 600 bases the DD patterns of Sensiscript and Omniscript were very prominent, but not for Superscript. RT reactions containing 4 ng (Sensiscript) or 16 ng of total RNA (Omniscript) generated DD patterns indistinguishable from the 200 ng pattern. For MMLV Superscript, the pattern obtained was very faint at 200 ng of RNA, but indicated a >50-fold increase in detection levels for Sensiscript. On the same gel (Fig. 1B) the DD patterns obtained for fragments smaller than 400 bases were again much more prominent with Sensiscript than with Omniscript or Superscript. The increase in the detection level was ~50-fold for Sensiscript.

To find the sensitivity of Sensiscript, LNCaP RNA was diluted from 0.02 to 200 ng per RT reaction, and compared reversed transcriptase reactions were run as described in Figure 1, including Sensiscript, MMLV and Superscript RT. As low as 0.2 ng of cDNA from LNCaP cDNAs had a detectable signal whereas for MMLV or Superscript RT, 20 ng RNA was required to obtain a detectable signal (data not shown). This corresponded to a 100-fold increase in sensitivity.

Figure 1. Enrichment of high molecular weight cDNAs. LNCaP RNA was diluted and used in RT reactions as suggested by RNAimage Kit (GenHunter) but using three different reverse transcriptases and oligo dT primer HT11-C. Primers HT11-C and H-AP5 were used to generate cDNA pools and *Taq* polymerase was used in the PCR step. The resulting cDNAs at high (A) or low (B) molecular weight fragments generated after DD are shown. To determine the sensitivity of Sensiscript, additional LNCaP RNA was serially diluted and RT reactions were achieved using Sensiscript and MMLV reverse transcriptases. HT11-C primer and H-AP3 were used instead of the H-AP5 primer to generate cDNA pools. *Taq* polymerase was used in the amplification step. Under these conditions, the limit of detection of Sensiscript was 0.2 ng of total RNA in the RT reaction.

In order to test if Sensiscript or Omniscript reverse transcriptases maintain their high sensitivity of detection using other RNA templates, the source of RNA and the PCR conditions were changed. We prepared RT reactions using MCF-7 RNA

Figure 2. Differentially displayed genes are detected at low RNA levels. MCF-7 RNA was used to prepare cDNA with Omniscript reverse transcriptase. DD patterns were obtained with two different DNA polymerases, with higher (AdvanTaq Plus) and lower (AmpliTaq) fidelity. The upper arrow indicates differentially displayed bands identified when MCF-7 cells were treated with the histone deacetylase inhibitor SAHA compared to control MCF-7 cells (up-regulated in treated cells). The lower arrow indicates a down-regulated cDNA upon treatment with SAHA. Its presence is seen in the untreated cells. In this experiment the limit of detection was 0.8 ng of total RNA. Note that both polymerases detected the differentially displayed bands.

from cells treated with or without the histone deacetylase inhibitor SAHA for 24 h. Two different DNA polymerases were used to generate cDNA products using Sensiscript or Omniscript. The results with the two reverse transcriptases were similar, but the patterns obtained using different polymerases differed. The detection level was 0.8 ng of total RNA using Omniscript and 0.2 ng of total RNA using Sensiscript. Figure 2 shows that the differentially expressed bands were preserved. The drug treated cells up-regulated an ~500 bp fragment (Fig. 2, upper arrow). A lower molecular weight band was found only in the control RNA and not in the SAHA-treated RNA, suggesting a down-regulation of that transcript (Fig. 2, lower arrow).

With AdvanTaq Plus amplification, fewer background bands were obtained compared to AmpliTaq amplifications. AdvanTaq Plus was chosen due to its property of reducing background products during PCR (CLONTECHniques, October, 1988, Vol. 13, No. 4). AdvanTaq Plus DNA polymerase includes TaqStart antibody (#5400-1, Clontech), with decreased background by providing a 'hot start'. Even though both of the DNA polymerases were able to detect specifically the differentially expressed bands between control MCF-7 RNA and SAHA treated MCF-7 RNA, we recommend the antibody-mediated DNA polymerases to reduce potential false positive signals.

DISCUSSION

The detection level between Sensiscript and MMLV or MMLV H⁻ Superscript RT enzymes was >50-fold (Fig. 1A and B). To determine the sensitivity of DD, total RNA quantities were varied from 0.2 to 200 ng, and four different RT enzymes and conditions were tested. The use of Sensiscript with the appropriate RT conditions resulted in an ~100-fold increased

cDNA detection by DD (Fig. 2). We have also found that larger cDNA fragments were obtained when Sensiscript reverse transcriptase was used (Fig. 1A).

Before using these Qiagen reverse transcriptases, the number of cDNA bands greater than 500 bases was not very high. However, by utilizing Sensiscript or Omniscript, larger than 700 base cDNA fragments were easily visualized with 0.2–16 ng of RNA; greater amounts of RNA (>16 ng of total RNA) did not increase the level of detection. These results corroborate those recently described for Omniscript and Sensiscript in which 0.2 ng of RNA can generate visible gene-specific fragments by ethidium bromide gel visualization (Product Profile, Qiagen).

If priming specificity of Omniscript and Sensiscript is higher than that of other reverse transcriptases, longer transcripts may be generated by the specific priming of poly(A) tails in favor of the lower specificity of internal priming sites. Internal priming is less specific and results in shorter cDNA products, as was seen for MMLV reverse transcriptases (Fig. 1A). To guarantee RT of every template, and therefore obtain a higher detection level, robust enzymes that do not discriminate RNA species due to their secondary structure would be needed. One explanation of why there are more molecules of cDNA with Sensiscript reverse transcriptases, and therefore higher detection levels of DD, is that difficult templates may inhibit the progression of the reverse transcriptases. If secondary structures are present, such as stable hairpin loops, denaturation of the RNA at high temperatures may not help, due to the fast re-formation of such structures. Omniscript or Sensiscript may be more robust enzymes with the capacity to read hairpin structures. This could explain why larger transcripts are found using Omniscript and Sensiscript [besides decreasing the priming on internal poly(A) tracks]. Indiscriminantly reverse transcribing all RNA equally well would supply a more authentic repertoire of the cDNAs and would contribute to the higher output production of cDNA observed in the experimental data. The higher priming capacity probably present in these new reverse transcriptases could rescue the expression of very low abundant RNA species. This has always been one of the problems difficult to avoid in screening DD results. Generally, the amplified bands correspond to highly to moderately abundant transcripts, with low chances of obtaining a very low abundant RNA transcription and amplification. By using new specific enzymes, such low abundant RNAs become more competitive, increasing their success of being detected by DD. One possibility is that higher specific enzymes used with primers containing more than one base at the 3' of the anchor primer could generate a greater selection of cDNAs. So far, two base-variation at the 3' end primer have not provided higher specificity in a number of base combinations (6). If 12 different combinations of anchor primers instead of three can be used efficiently, the cDNA libraries generated would have greater fidelity.

For specific gene amplification in RT-PCR, Sensiscript and Omniscript RT was linear over a wide range of concentrations of RNA (from 1 µg to 0.2 ng). This capacity would allow reproducibility of differentially displayed RNAs.

The usual amount of total RNA traditionally required for DD was 200 ng (we recommend decreasing this value to 20 ng if Omniscript is used) and between 2 and 10 ng if Sensiscript is used. Our results indicate consistent patterns with these recommended amounts and reveal high molecular weight cDNA fragments.

These simple enzymatic replacements in the DD procedure allow the application of DD to samples where RNA may be limited. Increasing the sensitivity of DD, as well as reducing background levels during PCR, allows expression of a rare population of cells studied, such as in urine, amniotic fluid or tissue biopsies, in which the target population of cells to be studied represent a small percentage of the total. One application is when tumor cells circulate in blood as the result of metastasis (7) or when endothelial cells circulate in blood under pathological condition (8). To study tumor markers or cell activation markers in such under-represented cell populations, the use of reverse transcriptases with high sensitivity will be crucial.

Theoretically, at least 300 combinations of primers are required to include the majority of mRNAs in a mammalian cells (5), and 6000 ng of total RNA would be required if 200 ng of RNA is needed per RT reaction and its 10 subsequent PCR reactions. With the modified RT procedure, as low as 0.2–2 ng of RNA would be sufficient for each RT reaction generating 10 PCR reactions. Therefore, ~6–60 ng of total RNA would be required to visualize the repertoire of genes in a given eukaryotic cell. Using current RNA isolation procedures, ~10 µg of RNA per 1×10^6 cells can be recovered. In turn, 600–6000 cells would be necessary to generate visible DD patterns that could encompass the entire expression repertoire. This number of circulating endothelial cells has been isolated from 10 ml of blood with the use of immunomagnetic procedures. For metastatic cancer, the circulating epithelial cells in circulation can reach similar levels. In either case, DD can provide a feasible way of

understanding gene expression and contribute to define disease markers. In addition, previously reported enhancing modifications for DD which were not done with potent reverse transcriptases (5,6) could be tested using these newer enzymes.

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