Modified Differential Display Technique that Eliminates Radioactivity and Decreases Screening Time

Several techniques are available that detect variations in gene expression between cellular populations. These include subtractive hybridization (SH), differential colony hybridization (DCH) and mRNA differential display, all based on the analysis of mRNA. The first two techniques, however, are limited because they require large amounts of mRNA for SH or several rounds of screening for DCH. Differential display overcomes both of these limitations. However, the conventional differential display technique is plagued by false positives and is labor intensive. The identification of genes that are truly differentially expressed, therefore, becomes a formidable task. We describe a modified differential display technique that overcomes the limitations of the conventional technique. This new technique eliminates a source of false positives, decreases the time required to screen a set of primers and reduces the use of radioactivity.

INTRODUCTION

Differential display is a sensitive technique for detecting variations in gene expression between two cellular populations (2–4). However, if the populations being studied are genetically distant, then noise will be introduced into the data. This noise contributes to the high false positive rate in the conventional technique. In addition, standard differential display is also labor, time and resource intensive. We have made three modifications to the conventional technique. First, the reverse transcription (RT) of total RNA is primed with random hexamers. Second, PCR amplification products are separated on agarose and visualized with ethidium bromide. Because visualization is direct, excision of the target bands from the gel is more precise. Finally, the secondary amplification step in the conventional technique is eliminated. These modifications reduce the time required to screen a set of primers, reduce false positives from co-migrating bands and reduce the need for radioactive isotopes. Here, we report a demonstration of this modified technique using an animal model of prostate cancer metastasis—the Dunning model (1). Specifically, the non-metastatic AT.1 and the metastatic MAT-LyLu (MLL) tumors were compared.

MATERIALS AND METHODS

All reagents and cell culture products were obtained from Life Technologies (Gaithersburg, MD, USA) unless otherwise specified.

Cell Culture and Tumor Growth

MLL and AT.1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 U/mL penicillin, 1 µg/mL streptomycin and 0.25 µg/mL amphotericin B. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Tumors were initiated by injecting 1 × 10⁶ cells into both flanks of male Copenhagen rats. The tumors were grown until they reached a diameter of 1–2 cm by external palpation (2–3 weeks). The rats were then sacrificed using carbon dioxide, the tumors resected and flash-frozen in liquid nitrogen. The frozen tumors were stored at -80°C until used.

Total RNA Isolation

Approximately 500 mg of tissue were cut from tumors using sterile scalpel blades. The outer layer of tumor was scraped off to reduce contamination from non-tumor tissue and discarded. The tissue was placed into 7.5 mL of TRIzol™, homogenized, and total RNA was isolated following the manufacturer’s protocol. The total RNA was then digested with RNase-free, DNase I to eliminate possible contamination from genomic DNA. The digested RNA was extracted with phenol/chloroform, precipitated with ethanol, dissolved in nuclease-free water and stored at -80°C.

Differential Display

Total RNA was reverse transcribed using random hexamers to prime the reaction. Briefly, 2 µg of total RNA in a total volume of 22 µL was denatured at 65°C for 5 min and placed on ice. A master reaction mixture was prepared containing 10 mM dithiothreitol, 500 µM of each dNTP, 100 ng random hexamers (Roche Molecular Biochemicals, Indianapolis, IN, USA), 5× first-strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂) and 400 U M-MLV reverse transcriptase. Eighteen microliters of the master mixture were added to the total RNA solution and incubated using the following program: 22°C for 10 min; 37°C for 50 min; and 70°C for 15 min. The resultant cDNA was then amplified using 40 random 10-mers (Operon Technologies, Alameda, CA, USA). Each 30 µL reaction contained 2 µL of cDNA, 500 nM of one primer, 100 µM of each dNTP, 1.5 mM MgCl₂, 10× PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl) and 1 U Taq DNA polymerase. The reactions were incubated at 94°C for 2 min, then amplified for 40 cycles using the following program: 94°C for 2 min; 38°C for 2 min; and 72°C for 1.25 min. This was followed by an elongation step of 15 min at 72°C. Reactions were stored at -20°C until analyzed.

The amplified cDNA was separated on a 14×11 cm gel of 2% DNA Typing Grade Agarose in 1× TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 8.0) and visualized with ethidium bromide. A 100 bp ladder was included as a size reference. The DNA Typing Grade agarose was able to resolve bands separated by 50 bp. For each primer tested, three AT.1 tumors were compared to three MLL tumors to reduce the probability of differences occurring by chance. The entire experiment, from the RT step through the separation, was repeated three times, and only consistent differences were chosen for further study. The criteria used to identify differences in mRNA expression between the AT.1 and MLL tumors were: (i) similar band intensities within a tumor type, (ii) similar between run band intensities and (iii) a difference in absorbance of at least two between the AT.1 and MLL bands. Overex-
pressed bands that met these criteria were excised from the gel(s), and the cDNA was extracted using the QIAquick™ Gel Extraction Kit (Qiagen, Valencia, CA, USA). The cDNA was then ligated into the pGEM®-T Easy vector (Promega, Madison, WI, USA) and used to transform bacteria. The insert was sequenced by the University of Michigan DNA Sequencing Core and used as a reagent in additional studies.

Relative Quantitative PCR and Northern Analysis

Relative quantitative PCR (RQ-PCR) uses expression of a ubiquitous gene to normalize the amount of cDNA used in subsequent PCR experiments to assess the relative expression of a target transcript. Briefly, 50 ng of cDNA were amplified with β-actin primers (Clontech Laboratories, Palo Alto, CA, USA) following the manufacturer’s protocol. Sets of reactions were run over a range of cycles (e.g., 22, 24, 26 and 28). The products were separated on 2% agarose, and the band intensities were compared. The band intensities were compared in a set of reactions with the same cycle count and where no band was saturated. The amount of cDNA used in subsequent reactions was adjusted according to these intensities. For example, if the intensity of band A at 24 cycles was twice the intensity of band B, then 100 ng of B cDNA and 50 ng of A cDNA were used in future reactions. The same procedure was then used to verify differential expression of the target gene using sequence specific primers. Differential expression of the target gene was confirmed if the band intensities were similar within one tumor type for all cycles and of greater absorbance in the tumor from which the original cDNA was derived.

Differential gene expression was also verified by poly(A)+ Northern analysis. Briefly, poly(A)+ RNA was isolated from 500 µg of total RNA using the PolyATtract® System IV (Promega), following the manufacturer’s protocol. Then, 2–3 µg of poly(A)+ RNA were separated on a 1.5% agarose gel containing 18% formaldehyde. RNA markers were used as size standards. After separation, the RNA was transferred to a Nytran® membrane using the Turbo-Blotter™ System (Schleicher & Schuell, Keene, NH, USA) and following the manufacturer’s protocol. The membrane was cross-linked and dried at 80°C for 0.5–2 h in a vacuum oven.

The membrane was prehybridized and hybridized in ExpressHyb™ (Clontech Laboratories), following the manufacturer’s protocol. The probe was random-prime labeled with α-32P-dCTP and purified on a SuperSelect® G-50 column (5 Prime → 3 Prime, Boulder, CO, USA). The membrane was then
washed and sealed in plastic. The hybridization was visualized by autoradiography. β-actin was used as a control.

RESULTS

Figure 1 is a representative gel from a differential display experiment. The band indicated by the arrow meets the criteria for differential expression outlined above. Although not shown, this band pattern was consistent when repeated. Using 40 random 10-mers as amplification primers, we identified 13 differentially expressed cDNAs. One of these, M-C1-700, was cloned and sequenced. A search of the GenBank® database revealed its sequence to be unique. Figure 2 shows the β-actin PCR products, grouped by tumor, separated on 2% agarose. The bands representing 30 cycles were compared, and the amount of cDNA used in the sequence specific amplification was adjusted according to their relative intensities. Primers specific to the M-C1-700 sequence were used to amplify cDNA from six tumors for 24, 26, 28 and 30 cycles. Figure 3 shows the products of these amplifications, grouped by cycle set, separated on 2% agarose. It can be seen that the band corresponding to the M-C1-700 gene is consistently overexpressed in the MLL tumors and that expression is similar within a tumor type. Figure 4 shows the Northern analysis confirming the overexpression of the M-C1-700 gene in the MLL tumor. Size markers (not shown) were used to estimate the size of the primary transcript at approximately 7 kb. β-actin expression is equal in both tumors.

DISCUSSION

A modified differential display technique was described. This new technique has several advantages over the conventional technique. First, by using random hexamers to prime the RT, the resultant cDNA is more likely to include coding regions because it is not locked to the poly(A) tail of the messenger RNA. Second, separation on agarose and fluorescence detection reduces the need for radioactive isotopes. Also, reactions can be stored and analyzed for convenience because the method of detection will not decay. Finally, the modified method offers a time advantage. Starting with total RNA, 40 primers can be screened in less than one week. Additionally, since there is no secondary amplification step following band identification and isolation, any contaminating (i.e., co-migrating) bands will not be a significant source of false positives. The design of the modified technique results in a higher probability of the target cDNA being selected. The target cDNA will be present at much higher levels than any contaminating cDNA. Thus, during

Figure 1. Representative modified differential display gel. Arrow indicates a differentially expressed cDNA (M-C1-700) with an approximate size of 700 bp. The left-most lane is a 100 bp DNA ladder.

Figure 2. β-actin relative quantitative PCR gel. cDNA from three AT.1 and three MLL tumors has been amplified with rat β-actin primers. For each time point: (i) the band intensities for an individual tumor increase with cycle number; and (ii) the bands from the MLL tumors have a higher absorbance than those from the AT.1 tumors. The left-most lanes are 100 bp DNA ladders.

Figure 3. M-C1-700 sequence-specific relative quantitative PCR gel. For each time point: (i) the band intensities for the AT.1 tumors are similar; (ii) the band intensities for the MLL tumors are similar; and (iii) the band intensities for the MLL tumors are greater than those of the AT.1 tumors. The left-most lanes are 100 bp DNA ladders.
ligation into a plasmid and colony selection, it is more probable that the target cDNA will be selected. As an example, in general, we submit three plasmids from separate colonies to be sequenced. To date, seven differentially expressed inserts have been sequenced, and no major differences between the three submitted plasmids were found. Therefore, contamination from co-migrating bands has not been a problem.

Although this modified technique offers several advantages over conventional differential display, it has several drawbacks. First, because ethidium bromide detection is less sensitive than autoradiography, more total RNA is required—2 µg as compared to 50 ng. Verification of gene expression by Northern analysis, however, requires between 10 and 20 µg of total RNA, so this increased requirement has not been a limiting factor. Second, the presence of co-migrating bands can be a greater problem in this modified technique because the resolution of agarose is less than polyacrylamide. However, the above arguments lessen the impact of co-migrating bands. Also, separation of the excised bands on an 8% polyacrylamide gel revealed only single bands (data not shown). Finally, the use of random hexamers in the RT may lead to cDNA derived from ribosomal RNA or genes common to both populations. In the modified technique, this would be manifested by a band of similar intensity and size in all lanes (data not shown). We have excised, cloned and sequenced such a band, and a search of the GenBank database showed it to be homologous to the mouse Major Histocompatibility Locus class II region (GenBank Accession No. AF027865).

A modified differential display technique has been developed that overcomes several of the disadvantages of the conventional technique. The modifications made to the standard differential display technique eliminate a source of false positives, reduce the use of radioactivity and decrease the time required to screen a set of primers.

REFERENCES


This work was supported by a National Institutes of Health Special Project of Research Excellence for Prostate Cancer (1P50 CA69568). It was also supported by a fellowship from the American Foundation for Urologic Disease (Hoechst Marion-Roussel Scholar). Address correspondence to Dr. Eric D. Schwab, Research Investigator, University of Michigan, 1500 East Medical Center Dr., 7410 CCGC, Ann Arbor, MI 48109-0946, USA. Internet: eschwab@umich.edu

Received 25 June 1999; accepted 14 October 1999.

Kenneth J. Pienta and Eric D. Schwab
University of Michigan
Ann Arbor, MI, USA

Figure 4. Northern analysis of AT.1 and MLL poly(A)+ RNA using M-C1-700 cDNA as a probe. Overexpression of M-C1-700 is confirmed by a greater absorbance in the MLL lane when compared with the AT.1 lane. β-actin was used as an experimental control.