Isolation of Differentially Expressed Genes by Combining Representational Difference Analysis (RDA) and cDNA Library Arrays

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ABSTRACT

*The difference products (DP) of representational difference analyses (RDA) were used as hybridization probes on cDNA arrays. The effectivity of RDA products obtained with increasing driver/tester ratios (DP 1 = 100:1, DP 2 = 800:1 and DP 3 = 400 000:1) to isolate differentially expressed genes was compared with the effectivity of conventional differential hybridizations. Pacreatic cancer and control tissues were used as a test system to isolate differentially expressed genes. The use of RDA products as hybridization probes showed two major advantages: (*i*) a reliable identification of true differential signals; and (*ii*) only one autoradiograph had to be analyzed, which eliminated the need for a laborious subtraction of signal intensities obtained with different cDNA probes. Increasing driver/ tester ratios in iterative rounds of RDA delivered more specific results, though the total yield of differential clones was gradually reduced. In this situation, the intermediate RDA product DP 2 provided the best compromise.*

INTRODUCTION

cDNA library arrays have been successfully used for the analysis of complex genomes. Hybridizations of cDNA library arrays with labeled cDNA probes from human tissues allows the measurement of tissue- or cell-specific gene expression of individual cDNA library clones (1–4,8,9). However, despite the improvement of protocols for probe generation, probe competition and hybridization and the availability of sophisticated image-analysis systems, the analysis and interpretation of hybridization results remains a challenge. New image-analysis systems are costly, and their use will be restricted to a small number of specialized laboratories. In addition, hybridizations with labeled cDNA probes preferably detect mRNA species expressed at middle to high abundance, thus often missing rare transcripts such as tissue-specific transcription factors and putative tumor suppressor genes. The isolation of low-abundant genes requires the use of an enriched probe that lacks the abundantly expressed genes. To produce such a probe, representational difference analysis (RDA) appeared to be the ideal technique. RDA, originally developed for genomic DNA to isolate differences between two complex genomes (7), is a combination of subtraction and kinetic enrichment coupled to subsequent amplification. Later it was adapted for use with cDNA to study differential gene expression between two mRNA populations (6) and was recently used for the detection of genes specifically expressed in pancreatic cancer (5). The standard cDNA RDA protocol is ideally suited to rapidly reduce the number of candidate genes in a highly specific manner, thus allowing focus to be placed on a small number of differential genes. This high specificity of cDNA RDA led us to test the use of RDA products as probes on cDNA library arrays. The aim of this approach was to provide a straightforward and reliable protocol that eliminates the problems usually encountered during standard differential hybridizations with cDNA probes. The efficiency of hybridizations with RDA probes and conventional differential hybridizations was compared using pancreatic cancer as a test system.

MATERIALS AND METHODS

RNA Sources and Extraction

Human pancreatic tissue (pancreatic cancer tissues, chronic pancreatitis tissue and normal pancreas from organ donors) was obtained from the Institute of Experimental Medicine in Budapest, Hungary and from the Department of Visceral Surgery at the University of Berne, Switzerland. All tissue samples were obtained after approval by the local Ethics Committees.

Total RNA from shock-frozen pancreatic tissues was prepared using standard guanidinium thiocyanate extraction followed by centrifugation in a cesium chloride gradient (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Poly(A)$^+$ RNA was selected using Dynabeads® (Dynal, Lake Success, NY, USA) as described by the manufacturer. cDNA was prepared with the SUPERSCRIPT™ Choice System (Life Technologies, Gaithersburg, MD, USA) following the manufacturer's instructions.

Table 1. Number of Clones Classified as Differentially Expressed in Pancreatic Cancer Tissues

Method	No. Differential Clones
Differential hybridization[a]	109
Hybridization with RDA products[b]	
DP[c] 1	172
DP 2	99
DP 3	27

[a]Standard differential hybridization.
[b]Hybridizations with RDA products with a gridded array containing 576 cDNA clones.
[c]DP = RDA difference products.

cDNA Arrays

Arrays of 576 selected cDNA clones were used for the experiments: 458 different cDNA clones from a pancreatic cancer cell line (4), 16 known, cancer-specific fragments (5), 43 clones containing vector only and 59 cDNA clones of known human genes, including human oncogenes (e.g., *ras*, *myb* and *myc*) and housekeeping genes (e.g., cytochrome oxidase and actin). These clones were spotted in duplicate on Hybond®-N+ membranes (Amersham Pharmacia Biotech) using a BioGrid™ robotic device (BioRobotics, Cambridge, England, UK). Vector clones were spotted at the same position in each 4×4 clone array in the first three rows of each grid to control for unspecific hybridization.

Northern Blots

Thirty micrograms of total RNA from pancreatic cancer, pancreatic

control and chronic pancreatitis tissues were size-fractionated on 1.2% agarose, 8% formaldehyde denaturing gels and transferred to Hybond-N+ membranes.

Generation of Probes for Differential Hybridizations

For conventional differential hybridizations, poly(A)$^+$ RNA pooled from 10 pancreatic cancer tissues, 10 samples of chronic pancreatitis and 10 healthy control pancreas tissues, respectively, were used. First-strand cDNA was generated using 4 µg poly(A)$^+$ RNA, Oligo(dT) (Life Technologies) and SUPERSCRIPT Reverse Transcriptase (Life Technologies). About 1 µg of each first-strand cDNA (3,4) was eventually used for labeling as described below.

Generation of RDA Probes

RDA probes were prepared following the protocol of Hubank and Schatz (6). Poly(A)$^+$ RNA of the twenty samples of chronic pancreatitis and healthy pancreas was mixed, eventually to form the driver. The tester was isolated from the pooled cancer samples. Each cDNA was restriction-digested with *Dpn*II and ligated to DNA adapters, different in sequence for the tester and driver. Subsequent to PCR amplification, the driver product was again *Dpn*II-digested for the removal of the adapter cassette. The first difference product (DP 1) was produced by mixing driver and tester at a 100:1 ratio. After denaturation and reannealing at 67°C for 48 h, polymerase chain reaction (PCR) was performed with the tester-specific primer. DP 2 (ratio 800:1) and DP 3 (400 000:1) were prepared accordingly (5), using 200 ng of each product.

The sequences of the adapter cassettes and the detailed reaction conditions have been previously described (5,6).

Hybridizations

Hybridizations were performed as described earlier (3,4) and labeling was by random hexamer priming incorporating [α-^{33}P]dATP (Amersham Pharmacia Biotech). Probes were competed with 1.25 mg/mL sonicated human placenta DNA (Sigma Chemical, St. Louis, MO, USA) and 500 mg/mL poly(U) homopolymer (Amersham Pharmacia Biotech) for 2 h. Hybridization was in 6× sodium saline citrate (SSC) (900 mM NaC1, 90 mM sodium citrate), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 100 µg/mL yeast tRNA, 50 µg/mL sonicated human placenta DNA, 10 µg/mL poly(A) homopolymer and 50% formamide at 42°C for at least 48 h. Filters were washed at high-stringency conditions (0.25× SSC/0.1% SDS at 65°C).

Data Analysis

Autoradiographs were analyzed with an image-analysis system consisting of a ScanJet™ 4c/t laser scanner (Hewlett-Packard, Palo Alto, CA, USA) and the Optimas® software (Stemmer, Puchheim, Germany). In the differental hybridization approach, clones were classified as differential when the signal intensity determined in the hybridization with the cancer cDNA probe was at least 3-fold greater than the intensities obtained with both control tissue cDNA probes for the same clone. When using RDA products as hybridization probes, all clones with a signal intensity of at least 3-fold over background were classified as differential.

RESULTS AND DISCUSSION

The study of differential gene expression in pancreatic cancer has been hampered by the strong desmoplastic reaction present in most of pancreatic cancer tissues. Because stromal tissue components make up to 90% of the individual tumor samples, differential screening approaches are bound to detect genes of stromal origin. Pancreatic cancer and control tissues are thus ideally suited to test the efficiency and reliabilty of differential screening methods. We have recently shown that the use of chronic pancreatitis tissue samples as an additional control helps to reduce the number of differential genes of stromal origin; as stromal tissue displays a similar degree of fibrosis and inflammation as pancreatic cancer tissues (3,5). In our presented approach, cDNA from chronic pancreatitis tissues was either used as a hybridization probe or

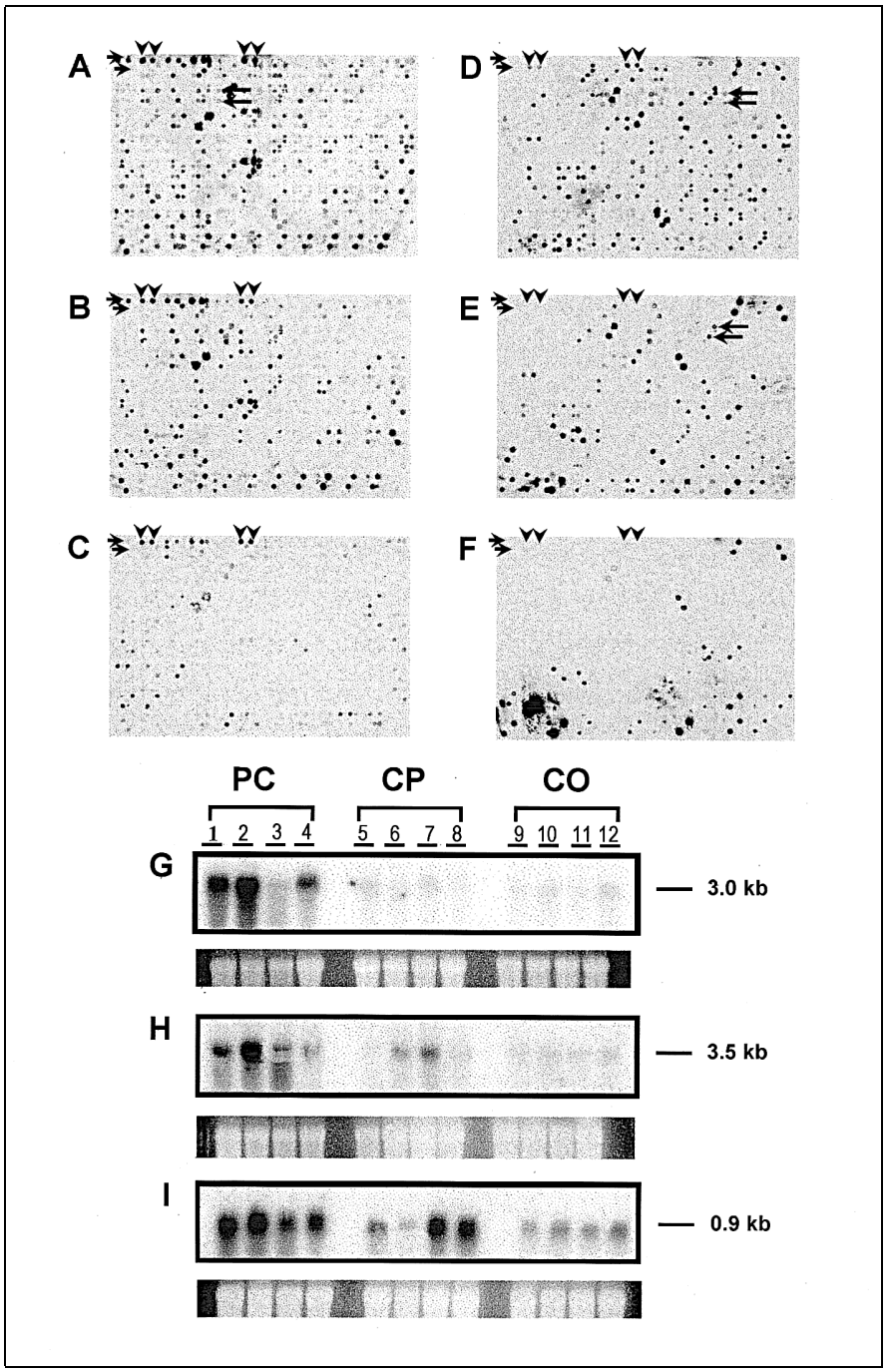

Figure 1. Typical hybridization patterns obtained with tissue cDNA probes isolated from (A) pancreatic cancer, (B) chronic pancreatitis and (C) healthy pancreas tissues. Clones were classified as differentially expressed by subtracting signal intensities in Panels B and C from those in Panel A. Hybridizations of RDA products are shown in Panels D (DP 1), E (DP 2) and F (DP 3). All clones were spotted in duplicate. The positions of a selection of clones spotted as controls are highlighted on all autoradiographs by arrowheads (two mitochondrial genes) and small arrows (vector-only clone). Vector clones were spotted at the same position in each 4×4 clone array in the first three rows of each grid to control for unspecific hybridization. Note that none of the vector clones gave a specific signal in any of the hybridizations. The two housekeeping genes gave strong signals with all tissue cDNA probes (A–C) and with the DP 1 product (D), whereas they gave no signal in the hybridizations with the DP 2 or DP 3 products (E and F, respectively). In Panels G–I, Northern blots with representative results are shown for three selected genes. The clone used in Panel G was identified with the DP 2 product (see large arrows in Panel E), the clone used in Panel H was isolated with the DP 1 product (see large arrows in Panel D) and the clone used in Panel I was isolated by differential hybridizations (see large arrows in Panel A). PC: pancreatic cancer; CP: chronic pancreatitis; CO: normal pancreas.

mixed with normal pancreas cDNA to form the driver used for cDNA RDA.

The isolation of clones from hybridizations with cDNA probes derived from pancreatic cancer tissues required the subtraction of hybridization intensities obtained with pancreatic cancer and both chronic pancreatitis and healthy pancreas (Figure 1). This subtraction led to the identification of 109 clones that were classified as differential. Of these, 38 clones exhibited only very weak differences in signal strength between the individual tissue cDNA probes. Northern blot analyses revealed that these clones were overexpressed in both chronic pancreatitis and pancreatitic cancer tissues, indicating that the subtraction of hybridization results was not sufficient to completely eliminate differential genes of stromal origin (e.g., Figure 1, A and I, and Figure 2). In addition, data analysis was complicated by

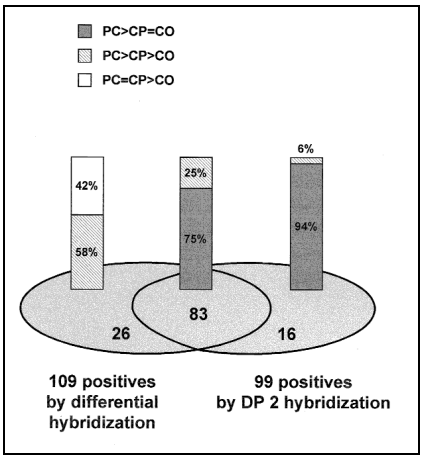

Figure 2. Graphical representation of the results obtained by standard differential hybridizations and by hybridizations with the DP 2 RDA probe. 109 cDNA clones were classified as differentially expressed in pancreatic cancer after subtraction of the hybridization results obtained with tissue cDNA probes. 99 cDNA clones hybridized with the DP 2 RDA probe. 83 cDNA clones were detected with both methods. 16 cDNA clones were only classified as differential by the DP 2 product and were shown to contain genes selectively overexpressed in pancreatic cancer. 26 cDNA clones were only detected in the differential hybridization approach. These genes were expressed in both, pancreatic cancer and chronic pancreatitis. The bars show the results of Northern blot hybridizations done with selected clones from each group to verify the differential expression pattern. A total of 50 Northern blots were done. PC: pancreatic cancer; CP: chronic pancreatitis; CO: normal pancreas; >: higher expression level on Northern blots; =: similar expression level on Northern blots.

the strong signals obtained for highly abundant genes, such as housekeeping genes, with cDNA probes from all pancreatic tissues. In contrast, the majority of clones classified as differential gave only weak signals (Figure 1, A–C).

Since RDA allowed production of a single driver by combining cDNA from chronic pancreatitis and normal pancreas and subtraction of tester and driver prior to hybridization, only one autoradiograph had to be analyzed in the approach using RDA probes. The vast majority of signals obtained (>80%), even at low driver/tester ratios, were clearly above background (Figure 1, D–F), which facilitates analysis. With the DP 1 probe, 172 clones were classified as differentially expressed (Table 1). Northern blot analyses demonstrated that a large number of these genes, besides being overexpressed in pancreatic cancer, were also moderately overexpressed in chronic pancreatitis (Figure 1, D and H). DP 2 and DP 3 experiments identified 99 and 27 clones, respectively, all of which were from transcripts that were found to be expressed at a higher level in pancreatic cancer than in both controls (Figure 1G). Whereas some highly abundant, nondifferential genes, such as housekeeping genes, were still detected with the DP 1 probe, they were eliminated by iterative rounds of RDA in the DP 2 and DP 3 products (Figure 1). None of the vector control probes gave a signal with any of the DP products. The fraction of pancreatic cancer-specific expressed genes increased with rising driver/tester ratios; whereas, in parallel, the total yield of differential clones decreased. In the presented approach, the hybridization with the DP 2 product, obtained after the second round of RDA at a driver/tester ratio of 800:1, represented the best compromise between yield and specificity. The yield was comparable to standard differential hybridizations, whereas the fraction of genes selectively overexpressed pancreatic cancer tissues was higher (Figure 2). Other applications, such as the identification of growth factor target genes in cell lines, might require other driver/tester ratios to generate the ideal DP 2 probe.

CONCLUSIONS

We have presented an alternative technique for the isolation of genes differentially expressed in cancer tissues by combining the advantages of gridded library arrays and cDNA representational difference analysis. This technique is superior to conventional differential hybridizations with gridded arrays because it provides a higher specificity and produces hybridization results that allow reliable and convenient data analysis with an automated

system or even by eye. As compared with conventional cDNA RDA, which is highly specific to isolate a small number of differentially expressed genes, our technique facilitates the study of the expression patterns of arrayed cDNA clones or whole libraries. We are convinced that in the future, hybridizations with cDNA-RDA probes and gridded library arrays will represent a valuable technique to study differential gene expression in a variety of biological systems without needing to purchase expensive equipment.

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Fission Yeast Expression Vectors Adapted for Positive Identification of Gene Insertion and Green Fluorescent Protein Fusion

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ABSTRACT

A pYZ series of fission yeast expression vectors, derivatives of the pREP series, was designed to allow positive identification of cloned gene insertion and fusion to the green fluorescent protein (GFP) gene for in vivo analysis of gene expression. To validate this new vector system, the human immunodeficiency virus type 1 (HIV-1) vpr *gene of viral isolate pNL4-3 was expressed in the pYZ1N vector. Vpr-induced phenotypic changes were the same as those observed with* vpr *expressed from pREP1N. Consistent with observations in mammalian cells, a Vpr-GFP fusion protein localizes on the nuclear membrane of fission yeast cells. Additionally, we were able to detect a naturally occurring mixture of* vpr *genes from a plasma sample of an HIV-infected pediatric long-term surviving patient. These pYZ vectors expedite gene cloning for general purposes and are particularly suited for large-scale random gene screening.*

INTRODUCTION

The pREP series of expression vectors (11,12) is widely used in fission yeast research. The advantageous feature of these vectors is the thiamine repressible **n**o **m**essage in **t**hiamine (*nmt1*) promoter, which is useful for demonstrating gene-specific effects in media either containing or lacking thiamine. Furthermore, the effect of gene dosage can also be tested, since the set of three expression vectors, pREP1, pREP41 and pREP81, have different sequences in the TATA box and consequently express the same mRNA at different levels (1,6). The pREP vectors have been modified for various purposes. The pRIP series were derived from these vectors by deleting the *ars1* origin of replication sequence and are