
Improved PCR-Based Subtractive Hybridization Strategy for Cloning Differentially Expressed Genes

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ABSTRACT

An improved PCR-based subtractive hybridization strategy was used to clone apoptosis-related genes induced by all-trans retinoic acid (ATRA) from human promyelocytic leukemia cell line HL-60 cells. The protocol used the cap-finder method, long-distance PCR, streptavidin magnetic bead-mediated subtraction and spin column chromatography. Twenty-seven clones related to apoptosis were identified by reverse dot blot

assay. Seventeen were known genes, of which seven have been reported to be apoptosis related. The remaining 10 were unknown genes, five of which were sequenced and named *apr-1* to *apr-5*. *apr-1*, *apr-2*, *apr-3* and *TNF* were reidentified by reverse dot blot, and it is suggested that they might be related to apoptosis. The results suggest that this strategy might be efficient for large-scale cloning of differentially expressed genes in target cells.

INTRODUCTION

Studies of differentially expressed genes can be useful in understanding physiological or pathological processes. Subtractive hybridization (4) and differential display (DD) (5) are two widely used methods. Subtractive hybridization has been modified over the years, but there are remaining obstacles such as the large amount of mRNA re-

quired (6). DD has been widely used in recent years because it is easy and convenient. However, the DNA fragments obtained from this method are between 300 and 400 bp, and it is laborious to obtain a full-length sequence from cDNA libraries or by other methods. Furthermore, the false-positive rate is very high (11).

We decided to design a simple and efficient strategy for direct cloning of the novel genes expressed differentially in target cells. First, we used PCR-based subtractive hybridization to reduce the amount of mRNA required. We found it easier to obtain large amounts of driver cDNA for complete hybridization. Second, we reduced the population bias and the accumulation of shorter of PCR products by the following four steps: (i) using raw cDNA as a tester to hybridize with the PCR amplified driver; (ii) separating the cDNA hybrids using the biotin-streptavidin sys-

tem; (iii) using Sephacryl S-400 spin column (Promega, Madison, WI, USA) chromatography to eliminate small fragments from differentially expressed targets; and (iv) selectively cloning the differentially expressed genes from the electrophoretic gel after a few PCR cycles, excluding small fragments. Third and most important, we adopted the cap-finder technology (2), which not only could increase the population of full-length cDNA but also could ligate the cDNA with primer at the 5' and 3' termini when mRNA was reverse-transcribed. Fourth, we adopted long-distance PCR technology (1) to obtain longer PCR fragments. Finally, we used reverse dot blot analysis to identify the fragments produced.

RESULTS

Of the 34 white clones selected, 32 were identified with an inserted fragment. The 32 clones were then identified by reverse dot blotting to eliminate false-positive clones. The denatured plasmids from the above 32 clones were spotted on Hybond-N. The driver probe was prepared as described in step 6. There were five positive clones among them, which means these five clones were false positive in terms of differentially expressed mRNA, and the rest were suggested to be derived differentially from the apoptotic group (Figure 1).

The 27 clones were sequenced from one end, and the sequences were then analyzed by BLAST and Translate™.

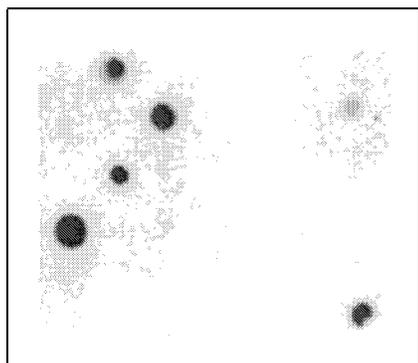


Figure 1. Identification of false-positive clones by reverse dot blot. The right-bottom dot is the positive control; the left-bottom dot is the negative control.

There were 17 known genes, and seven of these were reported to be apoptosis related, including ubiquitin-52, hsp-90, vimentin, α -tubulin, *tnf-r* and ferritin; the functions of the other 10 genes were not clear. These 10 were unknown genes, five of which had been sequenced and named *apr-1* to *apr-5*. The five sequenced genes were accepted by GenBank (accession nos. AF143235, AF143236, AF144055, AF144054 and AF144056). Three of the five genes had ORFs and may be related to apoptosis. The length of *apr-1* was 1474 bp and encoded 185 amino acids. BLASTn revealed that *apr-1* was not homologous to other genes, while BLASTp suggested *apr-1* was 70% homologous to Nectin, which is related to apoptosis (9). The length of *apr-3* was 1074 bp and encoded 208 amino acids. BLASTn showed *apr-3* was not homologous to other genes, while BLASTp suggested *apr-3* was 45% homologous to Notch4, a family reported to be related to apoptosis (3). The length of *apr-5* was 635 bp, which encoded 139 amino acids. BLASTn showed *apr-5* was not homologous to other genes, while BLASTp suggested *apr-5* was 53% homologous with Nef, which can up-regulate the expression of Fas and Fas-ligand and promote apoptosis (10). The details of these five genes can be obtained from GenBank.

The three new genes and *tnf* were reidentified as apoptosis related by reverse dot blotting (Figure 2). The 32 P-dATP-labeled tester DNA was used as a probe. A1, A2 and A3 dots were *apr-1*, *apr-3* and *apr-5*, respectively. B1 was a PCR product from the tester without 32 P-dATP, B2 was TNF and B3 was negative control.

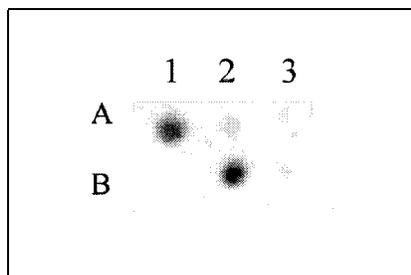


Figure 2. Identification of three new apoptosis-related genes by reverse dot blotting. A1, A2 and A3 dots were *apr-1*, *apr-3* and *apr-5*, respectively. B1 was negative control, B2 was TNF and B3 was PCR product from tester without 32 P-dATP.

Protocol

1. Isolate polyA mRNA from 10^7 HL-60 cells and apoptotic HL-60 cells induced with 10 μ M ATRA for 12 h using the PolyAtract[®] System 1000 (Promega, Madison, WI, USA).
2. Prepare driver cDNA. Driver mRNA (from HL-60 cells) was reverse-transcribed as follows: 0.5 μ g mRNA was reverse-transcribed with oligo-dT₍₁₅₋₁₈₎ in a 20- μ L reaction volume by the usual method (7) and then labeled with biotin by PCR. The components in the reaction were 1 μ L reverse-transcribed product, 20 μ M biotin-21-dUTP (Clontech Laboratories, Palo Alto, CA, USA), 200 μ M dNTPs, 1 μ g primer (oligo-dT₍₁₅₋₁₈₎) and 1 μ g 10-base random oligonucleotides in a 50- μ L reaction volume. The PCR parameters were 40 cycles of 95°C for 20 s, 40°C for 5 min and 72°C for 3 min.
3. Prepare tester cDNA. Tester mRNA (from apoptotic HL-60 cells) was reverse-transcribed as follows: 0.5 μ g tester mRNA was reverse-transcribed in a 10- μ L volume using the SMART[™] PCR cDNA Synthesis kit (Clontech Laboratories), but the 3' primer provided was replaced by the oligo-dT anchor primer 5'-TACGGCTGCGAGAAGACGACAGAAT₃₀(A/G/C)(A/G/C/T)-3'. In this kit, the primer for capturing the 5' cap sequence of mRNA is 5'-TACGGCTGCGAGAAGACGACAGAA-3'.
4. Separate differentially expressed genes by hybridization. The 5- μ L tester reverse-transcribed product was added to 100 μ L PCR products of the driver cDNA. This mixture was then denatured at 98°C for 5 min and hybridized at 65°C for 12 h in 6 \times standard saline citrate (SSC) and 0.5% SDS. After hybridization, 20 μ L hybridization liquid was added to the Sephacryl S-400 spin column; the liquid was recovered by centrifugation at 1600 \times g so that the salts and shorter fragments were removed. Then, 20 μ L recovered liquid was added to 0.1 mL Streptavidin MagneSphere[®] paramagnetic particles (Promega) in 0.1 \times SSC, prepared from 1 mL original particle. This mixture was incubated at room temperature for 2 min and then at 65°C for 5 min. The particles were removed by centrifugation at 12 000 \times g. The supernatant served as the PCR template. The supernatant (containing the unhybridized tester cDNA) was amplified with the Advantage[™] cDNA PCR kit (Clontech Laboratories) in the presence of 200 μ M dNTP, 400 nM primer 5'-TACGGCTGCGAGAAGACGACAGAA-3', 40 μ L subtractive supernatant in a 50- μ L reaction volume. The PCR parameters were 15–25 cycles of 95°C for 15 s and 68°C for 5 min. Although one round of subtractive hybridization was enough, more rounds could be added. In the repeated subtraction, the PCR products of the previous subtraction were used as a template instead of the tester cDNA. The total number of PCR cycles should be limited to no more than 60 after three rounds of subtraction to reduce random mutation caused by PCR.
5. Clone the amplified PCR product of differentially expressed targets after electrophoresis. The PCR products amplified from the subtraction were separated on a 1.5% agarose gel, the smear between 0.6 and 1.5 kb as extracted by the Nucleo Trap[™] Gel Extraction Kit (Clontech Laboratories) and fragments (or products) inserted into the T vector to construct a subtractive cDNA library for DNA sequencing. The plasmids were purified by the plasmid mini kit (Qiagen, Valencia, CA, USA).
6. Get rid of false positive clones by dot blotting. A denatured plasmid (0.1 μ g) was spotted onto Hybond[®]-N nylon membranes, (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The driver labeled with ³²P-dATP was prepared as described in step 2 and Molecular Cloning (8).
7. Sequence differentially expressed fragments on ABI 377 by means of ABI Prism[™] BigDye[™] terminator cycle sequencing ready reaction kit (PE Biosystems, Foster City, CA, USA).
8. Analyze sequences by computer using the GenBank[®] database.
9. Identify that the three new genes were apoptosis-related by reverse dot blotting. Denatured TNF DNA (0.1 μ g), which was used as a positive control, and plasmids of three new genes were dotted on Hybond-N. The tester DNA labeled with ³²P-dATP was used as a probe and prepared as described in step 2 and Molecular Cloning (8).

DISCUSSION

When we cloned novel genes by DD, we found that there were too many bands on the gel, and sometimes the bands looked just like a smear if the sensitivity of the film was not high. It was then difficult to cut the differentially expressed bands free from other unwanted bands because the fragments were often too small, between 300 and 500 bp, although sometimes 700-bp fragments could be obtained. By improved PCR-based subtractive hybridization, we produced (or cloned) 20 of the 27 fragments

using the anchor primer and cap-primer. Among the 32 clones we selected randomly, 27 were identified as differentially derived from apoptotic HL-60 cells, and the other five were false positive. Of the 27 clones, at least seven fragments were reported as apoptosis related, and three of the five new genes appeared to be new members of different apoptosis-related gene families.

Several useful techniques were used in this hybridization method to obtain the best results, such as cap-finder cDNA synthesis, long distance PCR, biotin-streptavidin magnetic isolation and

Sephacryl S-400 chromatography. The combination of these techniques might lead to a more efficient, simpler and easier way to obtain longer or full-length differentially expressed genes than by using DD and subtractive cDNA hybridization. Using raw cDNA as a tester was employed to hybridize with PCR-amplified driver, thus greatly decreasing the possibility of false positives. The false-positive clones were easily eliminated by reverse dot blotting. These results suggest that this method has potential for use in large-scale scanning of cDNA-specific target cell expression.

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