0023-6837/03/8305-759$03.00/0
LABORATORY INVESTIGATION

Vol. 83, No. 5, p. 759, 2003
Printed in U.S.A.

BRIEF METHOD

Rapid Identification of Differentially Expressed Genes by Combination of SSH and MOS

Kerstin Boengler, Frederic Pipp, Wolfgang Schaper, and Elisabeth Deindl

Max-Planck-Institute for Physiological and Clinical Research, Department of Experimental Cardiology, Bad Nauheim, Germany

Suppression subtractive hybridization (SSH) is a technique especially designed for the detection of rare transcripts, which vary in their expression pattern between two experimental setups (Diatchenko et al, 1996). SSH, which allows a synchronous normalization and subtraction of two cDNA pools in one step, shows several advantages compared with other techniques. In contrast to DNA-microarrays, SSH does not rely on the availability of specific sequences. Like differential display, SSH also allows the identification of unknown genes, but the identified cDNA fragments are longer and often located in the coding region, thereby facilitating the assignment to a certain gene. One drawback of SSH, however, is the occurrence of background molecules in the subtracted library. They can arise via unspecific annealing during the ligation of the adaptor-molecules or represent redundant cDNA fragments that were not subtracted during the two hybridizations. These cDNAs give positive results in the first screening process, but their differential expression cannot be confirmed by independent techniques like Northern blot hybridizations. Mirror orientation selection (MOS) (Rebrikov et al, 2000) makes use of the fact that background molecules are not amplified by PCR and therefore have only one relative orientation to the adaptor molecule. Target genes, however, have many predecessor molecules as a result of the amplification, and are represented by both orientations relative to the adaptor. The technical procedure involves the detachment of one adaptor, heat-denaturation, and re-annealing of the sample, as well as an exponential amplification of the target molecules by PCR. We describe a shortening and simplification of the screening process for differentially expressed genes in subtracted libraries making use of slot-blot analysis and the elimination of background molecules by MOS.

DOI: 10.1097/01.LAB.0000069520.16101.73

Received February 11, 2003.
Address reprint requests to: Dr. Kerstin Boengler, Max-Planck-Institute, Department of Experimental Cardiology, Benekestr. 2, D-61231 Bad Nauheim, Germany.

Differential gene expression was studied via SSH in a rabbit model of arteriogenesis (growth of preexisting collateral arteries) (Ito et al, 1997). Collateral arteries were isolated from the surrounding muscle tissue 24 hours after induction of arteriogenesis by femoral artery occlusion as well as of sham-operated animals. Total RNA was isolated as described previously (Chomczynski and Sacchi, 1987) and 10 μg were treated with DNase. The mRNA was extracted (Oligotex Mini Kit, Qiagen, Hilden, Germany), amplified using the SMART-technique (SMART PCR cDNA Synthesis Kit, BD-Clontech, Palo Alto, California), and subjected to SSH (PCR Select cDNA Subtraction Kit, BD-Clontech). In the forward hybridization, the cDNA derived from collateral arteries 24 hours after femoral occlusion served as tester and the cDNA of collaterals 24 hours after sham operation as driver (and vice versa for the reverse subtraction). The subtractions were performed according to the manufacturers protocol. PCR products were cloned in pGEM-Teasy (Promega, Madison, Wisconsin). To screen for differentially expressed genes we performed slot-blot analysis, hybridizing clones from the forward subtracted library with forward and reverse subtracted cDNA pools.

Bacteria transformed with cDNA fragments of the forward subtracted library were grown in 100 μl LB+ampicillin at 37° C overnight and 1 volume of phenol/chloroform was added. After mixing and centrifugation (14,000 g, 2 minutes) the aqueous phase was transferred to a fresh tube. Saline sodium citrate, ×20 (SSC; 3 M NaCl; 0.3 M sodium citrate), was added to a concentration of ×6 SSC in a total volume of 400 μl. The samples were boiled for 10 minutes and cooled on ice. A total of 175 μl of the solutions, corresponding to approximately 5 ng plasmid-DNA, were applied to nylon membranes (Duralon UV membrane, Stratagene, La Jolla, California) using a slot-blot apparatus. Two identical filters were created, which were incubated for 15 minutes in denaturing solution (1.5 M NaCl; 0.5 M NaOH), for 15 minutes in renaturing solution (1.5 M NaCl; 0.5 M Tris/HCl pH 8.0), and UV-crosslinked (UV-Stratalinker, Strat-

agene). Subsequently, the blots were hybridized with fluoresceine-11-dUTP labeled cDNA probes (ECF random prime labeling kit, Amersham Biosciences, Freiburg, Germany) generated from forward or reverse subtracted cDNAs at 60° C overnight. After washing (60° C with ×2 SSC, 0.1% SDS for 30 minutes) the signals were amplified with the ECF signal amplification module (Amersham Biosciences) and detected with a Storm imaging system using ImageQuant software (Molecular Dynamics, Sunnyvale, California). Bacteria transformed with the vector pGEM-Teasy served as negative control. In this assay, up-regulated genes hybridize only with the forward subtracted probe. Figure 1A shows the increased expression of the actin-depolymerizing factor cofilin2, which was used as an example for a gene being up-regulated on mRNA and on protein level in the early phase of arteriogenesis (Boengler et al, 2003). The negative control displayed no hybridization signal.

The success of SSH is hampered by the fact that background molecules are present in the subtracted library, which gives false-positive results in the slot-blot analysis. Therefore, we used MOS, a technique designed for the elimination of these cDNAs.

MOS was performed as described previously (Rebrikov et al, 2000). Briefly, the PCR products after SSH were digested with *Xma*I to remove one adaptor, then 5 ng of the purified products were heat-denatured and hybridized at 68° C for 14 hours. The ends of the resulting PCR products were filled in and amplified with the Advantage Polymerase Mix (BD-Clontech) using the primer, 5'-GGTCGCGGCCGAGGT-3'. The PCR conditions were 27 cycles 95° C, 10 seconds; 62° C 20 seconds; 72° C 2 minutes.

To analyze the result of MOS, equal amounts of unsubtracted, subtracted PCR products, and of the resulting fragments after MOS were size-fractionated on an 1.5% agarose gel (Fig. 1B). The unsubtracted and subtracted cDNA pools showed a more or less similar appearance, a few bands and a smear of cDNA. The application of MOS resulted in a reduction of smearing cDNAs, leading to recognizable distinct bands. The agarose gel was transferred to a nylon membrane according standard procedures (Ausubel et al, 1987), and hybridized with a ^{32}P-dCTP random primed labeled 830 bp cDNA (rediprime II, Amersham Biosciences) specific for rabbit cofilin2. The blot was hybridized overnight at 42° C and washed with ×0.1 SSC, 0.1% SDS at 65° C for 1 hour. The 830 bp cofilin2 signal was already detected in the unsubtracted and in the subtracted sample, but a strong enrichment was achieved by the application of MOS (Fig. 1C).

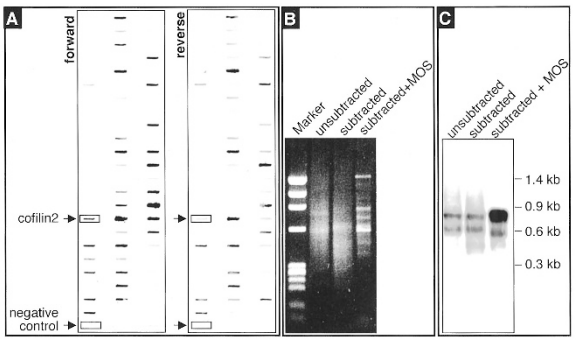

Figure 1.

Identification of differentially expressed genes by combination of SSH and MOS. Crude bacterial DNA extracts were transferred to nylon membranes and hybridized with forward and reverse subtracted cDNAs (A). Positions of cofilin2 and the negative control are indicated. Unsubtracted, subtracted, and subtracted cDNA pools after performance of MOS were size-fractionated on an 1.5% agarose gel (B). The subjected to electrophoresis cDNA was transferred to nylon membrane and hybridized with a cofilin2-specific probe (C).

We sequenced approximately 150 clones after the slot-blot analysis and approximately 50 randomly chosen clones after the performance of MOS. A total of 2.6% of the clones after the slot-blot analysis represented cofilin2. In contrast, after the performance of MOS, 15% of the clones were identified as parts of the cofilin2 gene. The use of MOS, therefore, led to a nearly 6-fold enrichment of cofilin2 fragments in the generated library.

In this study we investigated the differential gene expression in growing collateral arteries 24 hours after femoral occlusion by SSH. To identify truly differentially expressed genes, usually the cloned PCR products are amplified by PCR, size-fractionated on agarose gels, blotted on membranes, and hybridized with forward and reverse subtracted cDNAs. In this study we showed that it is possible to shorten this screening process. Transferring crude bacterial DNA extracts directly to membranes via slot-blot apparatus is sufficient to achieve reproducible signals in the differential hybridization. Omitting the PCR amplification and the Southern transfer results in lower costs and time savings for the screening process for positive clones.

The success of SSH can be hampered by the abundance of false-positive clones. We used MOS, a technique designed for the elimination of such background molecules, which was previously demonstrated only as proof of principle. For the first time we showed that application of this method on subtracted libraries derived from complex tissues resulted in an elimination of background molecules. This led to an enrichment of differentially expressed genes, as shown here for cofilin2.

In summary, our study demonstrated a reduction of background molecules in a subtracted library and a shortening and simplification of the screening process for differentially expressed genes using an experimental approach combining both SSH and MOS.

References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K (1987). Current protocols in molecular biology. New York: Greene Publishing Associates/Wiley and Sons.

Boengler K, Pipp F, Broich K, Fernandez B, Schaper W, and Deindl E (2003). Identification of differentially expressed genes like cofilin2 in growing collateral arteries. Biochem Biophys Res Comm 300:751–756.

Chomczynski P, and Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Diatchenko L, Lau Y-F, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskayqa N, Sverdlov ED, and Siebert PD (1996). Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA 93:6025–6030.

Ito WD, Arras M, Scholz D, Winkler B, Htun P, and Schaper W (1997). Angiogenesis but not collateral artery growth is associated with ischemia after femoral artery occlusion. Am J Physiol 273:H1255–H1265.

Rebrikov DV, Britanova OV, Gurskaya NG, Lukyanov KA, Tarabykin VS, and Lukyanov SA (2000). Mirror orientation selection (MOS): A method for eliminating false positive clones from libraries generated by suppression subtractive hybridization. Nucl Acids Res 28(20):e90.