Nucleic Acids Research, 1996, Vol. 24, No. 4 797–799

Efficient isolation of differentially expressed genes by means of a newly established method, 'ESD'

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Received November 17, 1995; Revised and Accepted December 21, 1995

We have developed a new method for isolating differentially expressed genes, and named it 'ESD' after three methodological key procedures; Equalization of cDNAs (1), Subtractive hybridization (2) and Differential display (3). The main features of our method are that (i) equalized tracer is used to improve the efficiency of subtraction, (ii) gel analysis is employed to analyze the outcome of subtraction, and (iii) a self-subtractive line is adopted, by which we can isolate candidate genes without ambiguity on the gel analysis even when the subtraction is not fully completed.

Here we report the isolation of differentially expressed genes which are specifically up-regulated in P19 embryonal carcinoma cells after 48 h retinoic acid (RA)-treatment which ultimately lead them to differentiate into neuronal cells, but absent or expressed at low levels in undifferentiated or dimethyl sulfoxide (DMSO)-treated P19 cells which differentiate into muscle cells.

The ESD strategy is outlined in Figure 1. As the first step, mRNA were prepared from undifferentiated (N-), 48 h RA-treated (R-), and 48 h DMSO-treated (D-) P19 cells using a mRNA Separator Kit (Clontech). Five μg each of N-, R- and D-mRNA were converted to double-stranded cDNA using a Time Saver cDNA synthesis kit (Pharmacia). The double-stranded cDNA (~500 ng) was fragmented by digestion with *Alu*I. Then, the resultant R-cDNA was ligated to 10 μg T-23/21 adapter (T-23; 5′-TCGGATCCTATCGTAGCTTCTCA-3′, T-21; 5′-AGAAGCTACGATAGGATCCGA-3′) at both ends and used as a 'tracer'. For comparative representatives, each of the N-, R- and D-cDNA was ligated to D-23/21 adapter (D-23; 5′-CTAAGCTTGCGATGGTACGACAG-3′, D-21; 5′-GTCGTACCATCGCAAGCTTAG-3′) and used as a 'driver'. Ligation mixtures were diluted to 1 μg/ml, and multiple PCRs were performed to obtain any amount of tracer and driver cDNAs necessary for the following subtraction step. Each PCR reaction mixtures contained 2 μl of the diluted ligation solution (2 ng of template), 10 mM Tris–HCl (pH 9), 50 mM KCl, 4 mM $MgCl_2$, 0.25 mM each dNTP, 2.5 U *Taq* DNA polymerase (AmpliTaq, Takara) and 1 μg primer (T-21 or D-21) corresponding to the template (final volume: 100 μl). PCR was carried out for 25 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 1.5 min, followed by a final 3 min extension step at 72°C. The PCR products were combined, extracted with phenol/chloroform, ethanol precipitated, and used subsequently to prepare equalized tracer and biotinylated drivers (step 1 in Fig. 1).

In the conventional subtraction method, abundant common cDNAs are not fully eliminated so that the enrichment of candidate cDNAs can be inefficient. To overcome this difficulty and make the subtraction more efficient, we equalized cDNA species in the R-tracer prior to the subtraction by a previously reported procedure (1). Briefly, 100 μg of the R-tracer was dissolved in 10 μl of dH_2O in 0.5 ml Eppendorf tubes and combined with 10 μl of 2× hybridization buffer [0.24 M NaH_2PO_4 (pH 6.8), 1.6 M NaCl, 2 mM EDTA, 0.2% SDS]. The samples were overlaid with mineral oil and boiled for 10 min. Reassociation was then performed at 66°C for 48 h. The separation of single- from double-stranded cDNAs was performed by a standard hydroxyapatite gel chromatography method (4). The single-strand fraction, in which different cDNA species were progressively more equal, was then amplified and converted into double-stranded cDNAs by PCR using T-21 as the primer. By equalizing the tracer species, we could obtain an effective starting material in which the relative abundance of common species had already been reduced.

Using the equalized R-tracer (R′-tracer), we then performed subtractive hybridization (step 2 in Fig. 1). In this step, the R′-tracer was subtracted by the unequalized population, i.e. R′ minus R (R′–R), in addition to the conventional processes, R′–N and R′–D. In the conventional subtractions, the candidate genes in the R′-tracer were enriched, while with R′–R subtraction they were removed as well as commonly expressed genes. Subtraction was performed as follows. One μg each of R′-tracer was mixed with 10 μg of biotinylated N-, R-, or D-driver which were prepared as described previously (2). The mixtures were co-precipitated with ethanol, and resuspended in 20 μl 1× hybridization buffer. The solutions were overlaid with mineral oil, heated at 98°C for 10 min to ensure complete denaturation, and hybridized for 48 h at 65°C. After hybridization, 100 μl HEPES–EDTA buffer (50 mM HEPES, pH 7.5, 2 mM EDTA, 150 mM NaCl) was added to the samples. Seventy microliters of streptavidin (Gibco-BRL; 1 mg/ml in HEPES–EDTA buffer) was mixed with the hybridized cDNA solution and incubated at room temperature for 30 min to form complexes with biotinylated cDNAs. Protein

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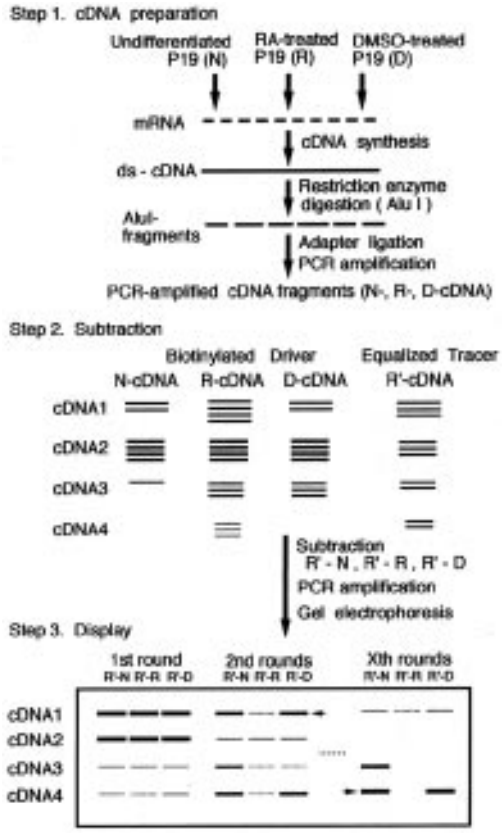

Figure 1. Schematic outline of ESD strategy. Number and thickness of bars indicates relative content of four representative cDNAs (cDNA 1–4). Arrows indicate the bands representing the candidates that are differentially expressed in RA-treated cells.

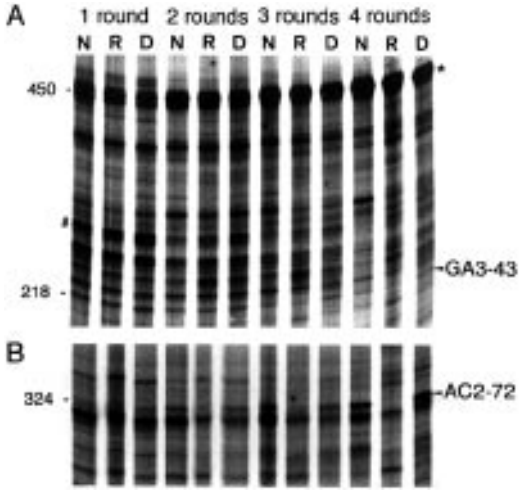

Figure 2. Representative portions of autoradiograms. The different lanes represent: (N), R′–N; (R), R′–R; and (D), R′–D lines. The nested primer used in (**A**) was T-AluI-GA, and that in (**B**) was T-AluI-AC.

and protein–DNA complexes were removed by extraction with an equal volume of phenol/chloroform. The streptavidin binding and phenol/chloroform extraction steps were repeated until there was no visible protein–DNA complex at the interface between the organic and aqueous phases (usually it took four or five repeated extractions). Small aliquots (2 µl) were then sampled for the next 'display' analysis step, and the remaining aqueous phase was subjected to an additional round of subtraction. In this study, these subtraction steps were repeated four times.

In the final step, the residual R′-tracer species obtained after each round of the three subtractions were PCR-amplified in the presence of [α-^{32}P]dCTP (see below). The resultant PCR products were separated side-by-side on a 4% DNA sequencing polyacrylamide gel, and detected by autoradiography (step 3 in Fig. 1). As illustrated in the figure, two types of bands representing the candidates would be observed; (i) a stepwise reduced band, which disappeared predominantly in the R′-R lane as the subtraction cycle was repeated, and (ii) a stepwise enriched band, in which the signal intensity increased gradually only in the R′-N and R′-D lanes. Thus, by comparing the outcome of R′-R self-subtraction with those of the conventional lines, we can identify and isolate candidates without ambiguity even when the common species were not fully subtracted out. First, the T-21 primer was used to amplify the remaining R′-cDNAs for the gel analysis. However, a discrete banding pattern did not appear on the gel; the banding pattern was smeared (data not shown). This indicated that too many tracer cDNA species still remained and thus could not give rise to discernible bands visible on the gel. For this reason, we constructed nested primers by which the residual tracer cDNAs were selectively amplified to represent a subfraction of them. Here, four kinds of nested primers, T-AluI-GA (5′-CGATAGGATCCGACTGA-3′), T-AluI-GT (5′-CGATAGGATCCGACTGT-3′), T AluI-TG (5′-CGATAGGATCCGACTTG-3′) and T-AluI-AC (5′-CGATAGGATCCGACTAC-3′), were constructed and used. In this step, the PCR reaction mixtures contained 1 µl of the sampled solution, 10 mM Tris–HCl (pH 9), 50 mM KCl, 4 mM $MgCl_2$, 2.5 µM each dNTP, 4 µl [α-^{32}P]dCTP (>3000 Ci/mM, Amersham), 50 ng of a nested primer (Fig. 2) and 2.5 U *Taq* DNA polymerase (AmpliTaq, Takara) (final volume: 20 µl). PCR was carried out for 40 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 1.5 min, followed by a final 3 min extension step at 72°C.

By using the nested primers, we obtained discrete banding patterns. Examples are shown in Figure 2. Two representative candidate bands with the expected patterns mentioned above were observed; the band intensity of clone GA3-43 disappeared gradually only in the R′-R lane as the subtraction cycle was repeated (Fig. 2A), while that of clone AC2-72 was enriched only in the R′-N and R′-D lanes (Fig. 2B). On the other hand, bands other than the candidates were observed: the intensities of most of the bands remained unaffected even after four rounds of subtraction (an example is shown by the symbol * in Fig. 2), whereas a few bands disappeared gradually in all lines (shown by # in Fig. 2). The former bands with no change in intensity probably represent remaining R-tracer cDNAs which were removed. These would obscure the library screening and impair isolating candidates in conventional subtraction procedures. In contrast, our ESD method made it possible to isolate candidates even when such hindrances were present.

Consequently, eleven bands fitting the criteria of candidates were isolated and subcloned into the TA-cloning vector pGEM-T (Promega) for further analysis. To confirm whether these candidates were really up-regulated in RA-treated cells, we performed Northern blot analysis. Probes were prepared from

subcloned fragments of all eleven candidates. Northern blot analyses revealed that all of the candidates were up-regulated in RA-treated cells committed to neural differentiation and absent or expressed at low levels in undifferentiated and DMSO-treated cells (data not shown). These results strongly demonstrate the reliability of the ESD method because no false positives were isolated. In summary, our ESD method adopts advantages in the previously reported procedures: (i) equalization of DNA species (1); (ii) the PCR-coupled subtraction (2,5); (iii) gel analysis of differential gene expression (3,6). Overall, the ESD method seems to be similar to representational difference analysis (RDA) (6). However, the specific features of our method is important to improve the efficiency and reliability of gene screening: equalized tracer cDNAs served as effective starting materials for efficient subtraction, and adoption of self-subtractive line made it possible to isolate candidate genes reliably on the gel analysis even when subtraction was not fully completed.

The ESD method should provide a useful way to isolate and study genes that are differentially expressed during various biological processes of interest.

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