1832–1833 Nucleic Acids Research, 1995, Vol. 23, No. 10

Differential display by PCR: novel findings and applications

M. Jorge Guimarães*, Frank Lee+, Albert Zlotnik and Terrill McClanahan

DNAX Reseach Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA 94304, USA

Received February 27, 1995; Accepted March 30, 1995

The differential display by PCR (DD-PCR) technique (1,2) was conceived to allow the identification and molecular cloning of differentially expressed genes. This technique was devised to amplify messenger RNAs and display their 3′ termini on polyacrylamide gels.

We have simultaneously compared the gene expression patterns of 12 different populations representing various tissues or *in vitro*-derived hematopoietic cells in order to identify genes which are regulated during hematopoietic development. The results of this gene search will be published elsewhere (3). In this report we document several important considerations in the use of DD-PCR. We have optimized the conditions to detect more bands per run and have demonstrated that DD-PCR allows the cloning of portions of mRNAs other than just their 3′ termini, due to priming by one or both primers. Importantly, we have shown that DD-PCR allows the molecular cloning of very low abundance genes. We demonstrate that DD-PCR can be applied, in a reproducible manner, to the study of questions related to temporo-spatial patterns of gene expression requiring comparisons among a large number of mRNA populations.

ES cells and embryoid bodies were prepared (4), the fibroblastic STO cell line (American *Type Cell* Culture, ATCC CRL 1503), the neuronal Neuro-2a (American *Type Cell* Culture, ATCC CCL 131) and the multipotential hematopoietic precursor cell line FDCPmixA4 (5) were cultured (American *Type Cell* Culture, 4), and mouse embryos were dissected as described (6). RNA was isolated using RNAzol solution (Tel-test, Inc., Friendswood, TX) and DNase treatment was performed using the MessageClean Kit (GenHunter Corporation, Brookline, MA) following manufacturer's instructions. RNA samples were quantitated by absorbance at 260 nm and evaluated on formaldehyde gels. High quality RNA was found to be imperative for the success of the subsequent steps. For reverse transcription, PCR and polyacrylamide gel electrophoresis, the manufacturer's recommendations were followed (RNAmap Kit, GenHunter Corporation). Primers AP3, AP5 and T12MC, synthesized using the sequence information provided in the RNAmap kit (DNAX), AmpliTaq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT) and ^{35}S-dATP (Amersham, Arlington Heights, IL) were used. PCR tubes were introduced in the thermocycler when the block was either at room temperature (cold start) or after pre-warming the block to 94°C (hot start). A duplicate reverse transcription reaction and PCR were performed for each sample and run side by side in the same polyacrylamide gel. A radioactively labelled DNA ladder was prepared (7). Denaturing polyacrylamide gels (Gel-Mix 6 solution, Gibco-BRL) were run (sequencing gel apparatus model S2, Gibco-BRL), dried and exposed as recommended (GenHunter Corporation). PCR products were isolated from long electrophoretic runs. DNA was extracted from the dried gel slice and reamplified by PCR (GenHunter Corporation). Reamplified PCR products were cloned (TA Cloning kit, Invitrogen, San Diego, CA). To exclude the possibility of more than one PCR product being represented as a single band in the polyacrylamide gel, three independent clones derived from each polyacrylamide gel slice were sequenced using the 70750 Reagent Kit For Sequencing With Sequenase T7 DNA Polymerase and 7-deaza-dGTP (Amersham, Cleveland, OH) and run in polyacrylamide gels as above. Poly(A) RNA was selected from total FDCPmixA4 RNA (Oligotex-dT mRNA kit, QIAGEN) and from day 8.5 yolk sac (FastTrack mRNA Isolation Kit, Invitrogen) following manufacturer's instructions. Five μg of poly(A) RNA were used for the cDNA synthesis (Superscript Plasmid System, Gibco-BRL). Lambda *NotI–SalI* Arms (Gibco-BRL) were ligated to the cDNA and packaged (λ Packaging System, Gibco-BRL). Libraries were screened as described (7).

PCR reactions were performed in duplicate using cDNAs generated in independent cDNA synthesis reactions. The results shown in Figure 1A confirm the reproducibility and reliability of the results obtained by DD-PCR.

DD-PCR should allow the display of all of the estimated 15 000 unique species of mRNA transcribed in the cell at any given time (1). The number of primer combinations necessary to visualize all of the different messages as bands in polyacrylamide gels has been suggested to range from 240 (1) to 312 (7). Using conditions previously described for DD-PCR (2) we observed an average of 45–55 bands per lane indicating the need to perform a minimum of 300 primer combinations. We sought to increase the number of bands visualized while keeping a reduced number of reverse transcription reactions (2). By increasing the concentration of the 5′ primer from 2 to 30 μM, using 32°C annealing temperature, a cold start and 40 PCR cycles (Fig. 1B), an average of 66 bands per lane were easily detected, without a significant increase in the background signal, representing a 25% decrease in the total number of primer combinations required to visualize 15 000 bands.

Significant differences in gene expression may be undetectable with 40 cycles of PCR due to template saturation. By reducing the

* To whom correspondence should be addressed

+Present address: Millenium Pharmaceuticals, 640 Memorial Drive, Cambridge, MA 02139, USA

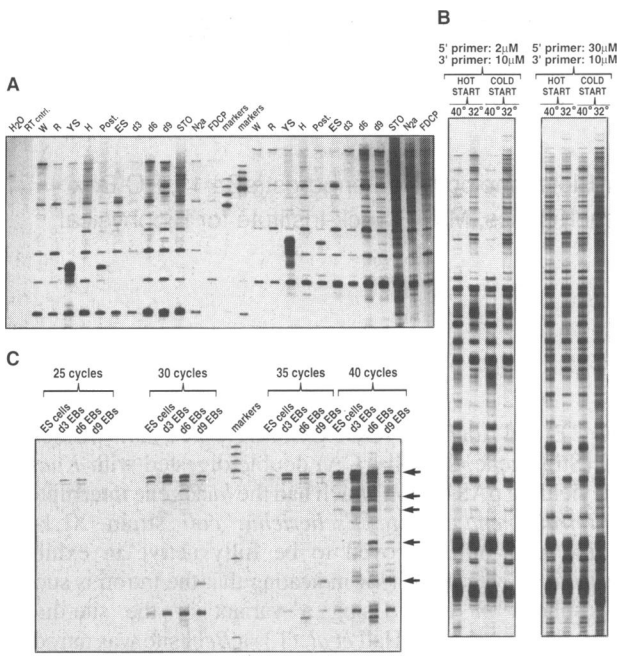

Figure 1. Patterns of gene expression obtained by DD-PCR. (**A**) Twelve populations compared simultaneously in duplicate PCR reactions: W, early day 8.5 day embryo; R, late day 8.5 day embryo; YS, day 8.5 yolk sac; ES, embryonic stem cells; d3, day 3 embryoid bodies (EBs); d6, day 6 EBs; d9, day 9 EBs; STO, fibroblastic cell line; N2a, neuronal cell line Neuro-2a; FDCP, hematopoietic cell line FDCPmixA4 (5). Arrow shows a PCR product specific to day 8.5 yolk sac identified as the mouse homologue of Calbindin-D9K (3). (**B**) Direct comparison between previously described (2) (left column) and optimized PCR conditions (right column). (**C**) Effect of cycle number on the pattern of bands obtained by DD-PCR. Bands are shown for which a decrease in the number of cycles disclosed evident quantitative differences (arrows).

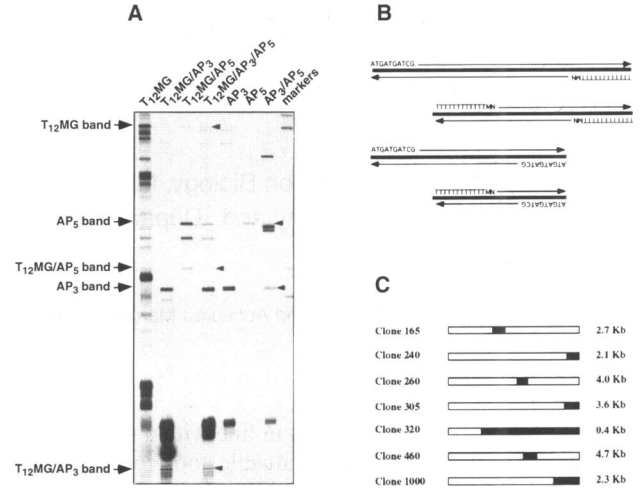

Figure 2. Reformulation of the theoretical principle of DD-PCR. (**A**) Bands can result from the amplification of a cDNA sequence primed by only one of the primers. Designations refer to primer combinations used. STO cell line derived cDNAs were used in this experiment. (**B**) Schematic representation of products generated from any combination of two primers. (**C**) Schematic representation of the relative location and size of the PCR fragments generated by DD-PCR within their respective full length cDNAs. Shadowed areas represent the PCR products. White areas represent the full length cDNAs with the size shown on the right (3).

number of cycles, we increased the detection of differentially expressed bands (Fig. 1C). This semi-quantitative analysis aids in the identification of interesting bands for further study.

We demonstrated that, contrary to the theoretical principle of the technique, a significant number of bands observed arose as a result of the amplification of cDNA sequences primed by only one of the two primers used in the PCR (Fig. 2A, detailed in Fig. 2B). These bands are reproducible (Fig. 2A). This may result from the A–T rich nature of the 3′ untranslated region of many genes, which could be recognized by the T12MN primer. Additionally, a 10 bp random sequence and its inverse-complement may exist in the same cDNA, resulting in the amplification of a region of cDNA between these two positions. Consequently, the longer the cDNA the more likely it is that it will be primed by a particular primer combination.

As shown in Figure 2C, we found that the PCR fragments generated by DD-PCR originated from any location within the full length cDNA, not just at the 3′ end, in contrast to what would be expected from the use of a polyT-containing 3′ primer.

Upon extraction and cloning of 15 single bands, three independent clones for each candidate were sequenced. We found only two cases where one of the three cloned products was not identical to the others. In both of these cases, the products did not contain the sequence of the primers used in the PCR, suggesting that they were cloning artifacts. Generally, one band represents only one gene.

Furthermore, we found the technique to be biased toward the cloning of low abundance genes, most of which are represented by long (>3 kb) mRNAs (Fig. 2C). The majority of the genes had a frequency ≤1/15 000 and almost half of them had a representation as low as 1/60 000.

We have provided conditions to increase the number of bands per run, to perform a semi-quantitative analysis of bands and demonstrated that DD-PCR is not always biased to the 3′ termini of genes. We have shown that DD-PCR is capable of identifying very low abundance mRNAs and can be applied to the study of complex biological questions involving differential gene expression.

ACKNOWLEDGEMENTS

M. Jorge Guimarães is supported by a grant from JNICT, Portugal (CINCIA/BD/2685/93). DNAX Research Institute of Molecular and Cellular Biology, Inc., is supported by the Schering-Plough Corporation.

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