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Direct identification of differentially expressed genes by cycle sequencing and cycle labelling using the differential display PCR primers

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ABSTRACT

Differential display PCR (DD-PCR) is an mRNA fingerprinting technique to identify differentially expressed genes by comparative display of arbitrarily amplified cDNA subsets. This attractively simple screening method was, however, followed by a labour intensive multistep identification procedure for DD-PCR products. In this report we demonstrate for the mouse mast cell protease 2 (MMCP-2) and the cytotoxic T-lymphocyte associated gene transcript CTLA-1 a streamlined approach by (i) direct cycle sequencing with the upstream differential display (DD) primer, followed by (ii) the PCR based generation of an antisense northern probe with the downstream anchor primer.

We have applied differential display PCR (DD-PCR; 1) to a murine tumor model in which transduction of the v-H-ras oncogene into IL-3 dependent non tumorigenic PB-3c mast cells gave rise to IL-3 autocrine tumors with an increased IL-3 mRNA stability. IL-3 mRNA expression from this autocrine tumor could be suppressed by somatic cell fusion with precursor cells implying a recessive alteration (2–4).

Culturing of the precursor cell PB-3c clone 20, the tumor line V2D1 and the hybrid 20/V2D1 has been described previously (3). Total cytoplasmic RNA was isolated from each cell line according to Gough (5) followed by DNAse I digestion (6). Integrity of the RNA was checked by agarose formaldehyde gel electrophoresis.

Comparison of precursor PB-3c clone 20 with the tumor V2D1 cells was performed by the DD-PCR technique essentially as described by Bauer *et al.* (7). For reverse transcription of an mRNA subset, 0.2 µg of RNA was mixed first with 50 pmol anchor primer, $dT_{12}VN$, (V representing A, G or C and N being A, C, G or T) in a volume of 7 µl, kept for 10 min at 65°C and immediately put on ice. Following addition of 300 U MMLV- reverse transcriptase® (Gibco Life Technologies) it was incubated for 1 h at 37°C in reverse transcription buffer (40 mM KCl, 50 mM Tris–HCl pH 8.3, 6 mM $MgCl_2$) containing 20 µM dNTP, 10 mM DTT and 40 U RNAsin® (Boehringer) in a total volume of 20 µl. After 5 min at 95°C the cDNA was stored at –20°C until use in DD-PCR.

DD-PCR was performed with 2 µl of 20 µl cDNA in a Perkin-Elmer Cetus thermal cycler 480 using PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl), 2.5 U AmpliTaq DNA Polymerase® (Perkin Elmer), 1.25 mM $MgCl_2$, 4 µM dNTP, 1 µCi [α-^{32}P]dCTP, 2.5 µM of the respective downstream anchor primer $dT_{12}VN$ and 0.5 µM of one upstream DD-primer from the set of 26 decameric oligonucleotide primers according to Bauer *et al.* (7) using the following conditions: 5 min incubation at 95°C followed by 40 cycles of 94°C for 30 s, 40°C for 2 min and 72°C for 30 s. PCR product (3 µl) was added to 2 µl sample buffer and incubated at 95°C for 5 min. The PCR products were resolved on a 6% polyacrylamide–7 M urea gel in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 0.025 mM EDTA). The gel was dried and analysed by a PhosphorImager® (Molecular Dynamics) (Fig. 1A and B).

The differentially displayed bands were cut out from a wet polyacrylamide gel after localisation on a X-ray film. The DNA was eluted with the QIAEX II Gel Extraction Kit® (QIAGEN) according to the protocol of the supplier. With 6 µl of the 20 µl eluate the DD-PCR was repeated without labelled dCTP but with 100 µM dNTP. The reamplification product was resolved on a 2% TBE agarose gel, stained with ethidium bromide (Fig. 1C), recovered with the QIAEX II Gel Extraction Kit and resuspended in 20 µl ddH_2O. This amplicon was used for (i) direct sequencing and (ii) generation of a labelled probe.

Direct sequence determination with the upstream DD-primer was done on the ABI 310 genetic analyzer® using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit® (Perkin Elmer). Gel purified reamplification product (6 µl) was mixed with 32 pmol upstream DD-primer and 8 µl terminator ready reaction mix® in a total volume of 20 µl. We modified the cycling conditions for the sequencing with short decameric DD-primers as follows: 25 cycles of 96°C for 30 s, 40°C for 15 s and 60°C for 4 min (480 Perkin Elmer Cetus Thermal Cycler®). The samples were prepared for the sequence analysis as described by the supplier using the ethanol precipitation protocol to remove unincorporated dye terminators (Fig. 1D and E). Sequence determination of 100–150 nt allowed the search in the gene bank and direct cycle labelling for northern analysis.

For northern analysis, an antisense probe was generated by cycle labelling with the downstream anchor primer. Gel purified reamplification product as template (5 µl) was incubated with PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl), 25 pmol of the respective $T_{12}VN$, 0.04 mM dNTP, 50 µCi [α-^{32}P]dCTP and 2.5 U AmpliTaq DNA Polymerase® (Perkin Elmer) in a total

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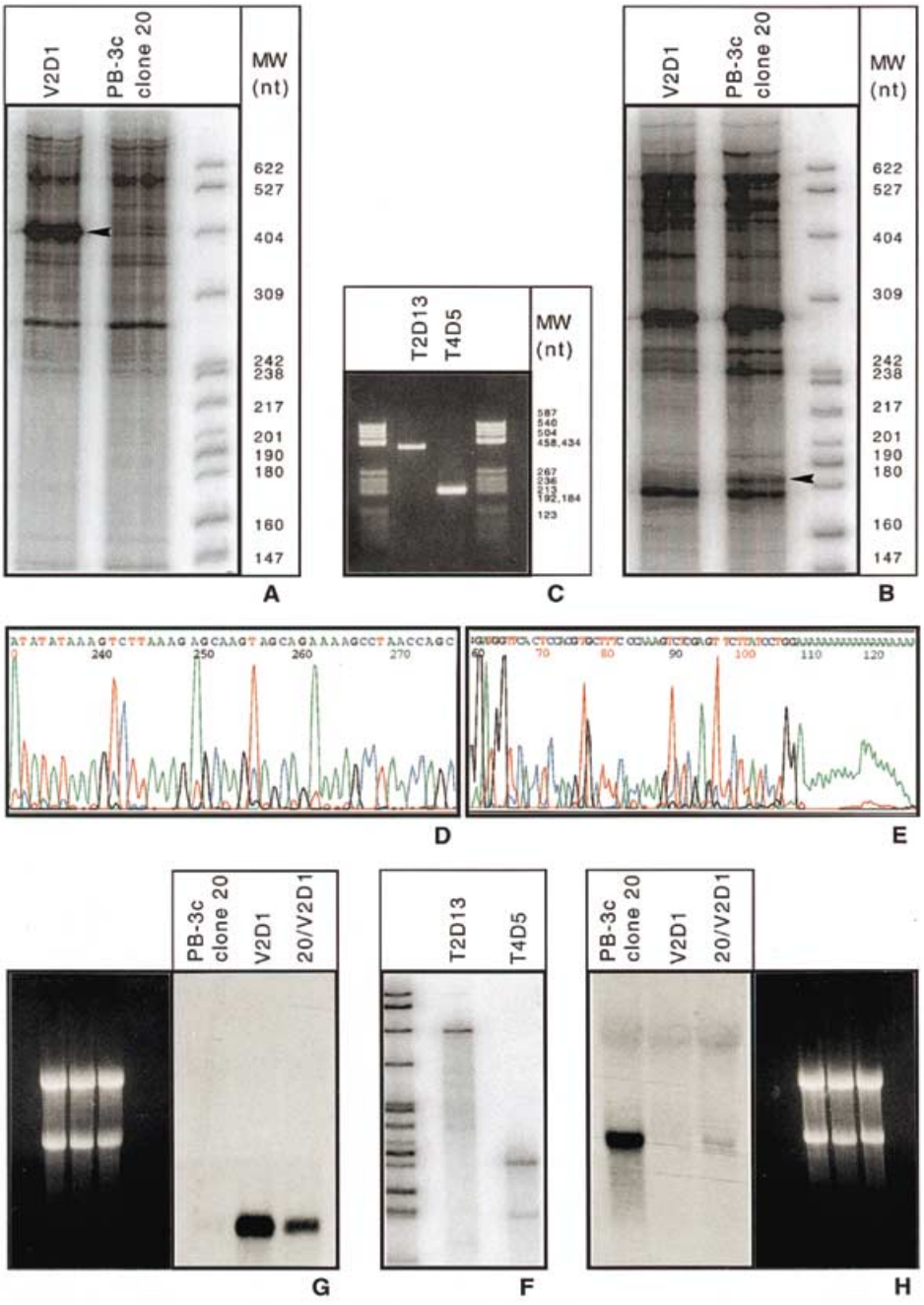

Figure 1. Illustration of the direct cycle sequencing and cycle labelling strategy for DD-PCR products with two differentially expressed genes as described in the text.

volume of 100 µl (5 min at 95°C, 40 cycles of 1 min at 96°C, 2 min at 35°C, 1 min at 72°C). The labelled product was extracted with the QIAquick PCR Purification Kit® (QIAGEN) and the integrity and size of the probe was visualised on a 6% polyacrylamide–7 M urea gel (Fig. 1F). Hybridisation was done with 6×10^6 c.p.m. in a volume of 5 ml. Northern blots were prepared as described previously with 20 µg RNA per lane (8). The filters were prehybridised in 5 ml prewarmed hybridisation solution (50% formamide, 5× SSC, 5× Dehnhardt's solution, 5 mM EDTA, 0.5 mg/ml yeast tRNA, 0.1 mg/ml heparin, 0.2% SDS) for 2 h at 45°C in a rotating hybridisation oven. After adding the heat denatured probe, the filter was hybridised overnight. Washing was done twice with prewarmed 2× SSC, 0.1% SDS at 55°C for 30 min. The filter was analysed by a PhosphorImager (Fig. 1G and H).

To illustrate this approach we show the identification of one upregulated (Fig. 1A) and one downregulated band (Fig. 1B) which were found during DD-PCR comparison of the tumor line V2D1 and the precursor line PB-3c clone 20 with the following primer pairs: anchor primer $T_{12}CG$ (T2) versus DD-primer D13 (GTTTTCGCAG) and anchor primer $T_{12}CC$ (T4) versus DD-primer D5 (GGAACCAATC). As shown in Figure 1D and E, the reamplified DD-PCR products could be directly sequenced using DD-primers D13 or D5, respectively. The sequences could be read over a stretch of more than 100 nt in sense direction at the

end of which the complementary sequence to the respective downstream anchor primer could be identified (Fig. 1E). Comparison of both sequences with entries in GenBank/EMBL/PDB databases identified a 100% match with the mouse mast cell protease 2 (MMCP-2) (9) and the mouse cytotoxic T lymphocyte associated gene transcript CTLA-1 (10), respectively. Differential expression of both genes was confirmed by northern analysis using the antisense probe generated from the sequenced fragments. MMCP-2 is expressed in the tumor cell V2D1, but is not expressed in the precursor cell. The expression of MMCP-2 in the hybrid 20/V2D1 is at least 10 times downregulated compared to the tumor cell (Fig. 1G). Ethidium bromide stained gels served as loading controls. Initially identified in Ki-ras transformed mast cells, MMCP-2 is a marker for intermediate differentiation stages between the bone marrow derived and the fully mature mast cells (9). In contrast CTLA-1 is expressed in the precursor cell PB-3c clone 20 but is missing in the tumor V2D1 and in the hybrid cell (Fig. 1H). This serine protease belongs to the granzyme group predominantly expressed in cytotoxic T-cells but it has also been found in mast cells, yet is lacking in the mastocytoma P815 (10).

Direct cycle sequencing with decameric DD-primers and direct generation of an antisense probe by cycle labelling is a streamlined approach to identify a differentially expressed gene represented by a DD-PCR fragment. It avoids the conventionally used labour intensive subcloning procedure, which consisted of ligation into a plasmid vector, transformation of *Escherichia coli* cells and identification of a clone corresponding to the differentially expressed gene before the sequence could be determined. The costs for the modification of the DD-PCR product by primer extension in order to use classical sequencing primers for direct sequencing can also be saved (11). In the optimal case the sequence and the confirmation of differential expression can be obtained within 3 days. If the reamplified DD-PCR product consists of more than one template or was primed by the same primer on both sides subcloning cannot be avoided, which was the case in ~20% of the 30 differentially displayed bands detected during our systematic DD-PCR screening. Furthermore, the direct identification of DD-PCR products generated with different primer combinations but arising from the same differentially expressed gene avoids unnecessary further analysis. If northern hybridisation does not yield a signal due to low abundance of the transcript, the sequence may provide the information for other more sensitive methods to confirm differential expression.

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