Differential Display Assay and Analysis

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Differential display analysis, first described by Liang and Pardee (3), is a powerful procedure for quantitative detection of differentially expressed genes. The advantage of this method is that it permits simultaneous identification of genes that are up- or down-regulated under different conditions. The procedure is based on the polymerase chain reaction (PCR) amplification of mRNA of cells or tissues, using a short 5' arbitrary primer, an oligo(dT)-NN 3' primer and radiolabeled deoxyribonucleoside triphosphate (dNTP). Differential gene expression is visualized by autoradiography after electrophoretic separation. The cDNA bands are then

recovered from the gel, reamplified by PCR and molecularly cloned for sequencing and further identification. Although straightforward, the method suffers from two main drawbacks. First, the yield of the cDNA products is often of low quality and is associated with a nonspecific background detected as smear of DNA on the gel. The second drawback stems from the fact that usually more than one cDNA species is present within one amplified band. Because of this, recovering a unique radiolabeled DNA species is technically challenging and often results in the failure to isolate and clone the cDNA of interest (2,3,6). The present report describes two improvements to the assay: (i) the use of an RNase H-deficient reverse transcriptase (RT), which enables a remarkably efficient presentation of the differentially expressed genes; and (ii) the use of a restriction-enzyme fingerprinting approach that enables a rapid grouping of the various cDNA species existing within one amplified DNA band.

We used the differential display method to identify the genetic effects of the *tat* gene of equine infectious anemia virus (EIAV) on the regulation of cellular gene expression. Canine fetal thymus (Cf2th) cells were stably transfected with Tat-expressing cDNA (4) or with the pCEV21 vector, as previously described (5). Total RNA was extracted using the guanidinium isothiocyanate method (1). Five micrograms of total RNA were reverse-transcribed at 37°C for 60 min in a 50- μ L reaction mixture that contained 7.5 μ M of a 5'-T₁₀GG oligonucleotide as anchor primer, employing the StrataScript™ cDNA Syn-

thesis Kit (Stratagene, La Jolla, CA, USA). This kit contained 50 U of Moloney murine leukemia virus (M-MLV) RNase H- RT, an RNase H-deficient reverse transcriptase. Alternatively, 5 μ g of total RNA were reverse-transcribed at 42°C for 60 min in the presence of 7.5 μ M of a 5'-T₁₀GG oligonucleotide primer, using avian myeloblastosis (AMV) RT (Promega, Madison, WI, USA) according to the manufacturer's recommendations. From each of the resulting cDNA reaction mixtures, 1 μ L was used as template in a 20- μ L PCR mixture that contained 0.5 μ M of an arbitrary primer (5'-GTGAGCTCC), 2.5 μ M of T₁₀GG primer, 1 U *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN, USA), 20 μ M of each dNTP, 2 μ L of 10 $\times$ PCR buffer (100 mM Tris-HCl pH 8.3; 500 mM KCl; 15 mM MgCl₂; 0.01% gelatin) and 1 μ L of [³⁵S]dATP (10 mCi/mL) (Amersham International, Little Chalfont, Bucks, UK). Forty cycles of PCR were performed with cycle times of 30 s at 94°C, 1 min at 42°C and 30 s at 72°C. Six microliters of each of the PCR reactions were loaded on a 6% polyacrylamide sequencing gel, electrophoresed at 1500 V for 4–5 h, dried on filter paper and exposed to autoradiographic film. Figure 1 shows a comparison between the differential display products using two different RT enzymes: the M-MLV RNase H- RT and the AMV RT. As can be seen, the use of the RNase H- RT (Figure 1A) allowed generation of highly reproducible patterns of RT-PCR bands, as opposed to the low sensitivity and poor resolution obtained by the use of AMV RT (Figure 1B). Thus, the use of

Figure 1. The differential display patterns using two different RTs for cDNA synthesis. Two preparations of total RNA obtained from canine cells stably transfected with Tat-expressing cDNA (lanes c and d) or with pCEV21 vector as control (lanes a and b) were reverse-transcribed using either M-MLV RNase H- RT (A) or AMV RT (B). Following PCR as described in the text, the [³⁵S]-labeled cDNA products were analyzed on a 6% polyacrylamide sequencing gel. The arrowheads at the right point to the up-regulated genes, and the arrowheads at the left point to the down-regulated genes.

Figure 2. The restriction enzyme fingerprinting of the cloned cDNAs. Following *Sau*3AI digestion, DNAs were separated on a 4% agarose gel and stained with ethidium bromide.

Benchmarks

M-MLV RNase H⁻ RT enabled the detection of RT-PCR products that were up- or down-regulated in canine cells stably transfected with Tat-expressing cDNA (Figure 1A, lanes c and d), as compared to pCEV21 vector stably transfected cells (Figure 1A, lanes a and b).

One of the differentially displayed bands was sliced out of the gel, placed in a 1.5-mL microcentrifuge tube and rehydrated with 50 μ L of distilled water. After the sample was centrifuged to eliminate gel fragments, 5 μ L of the supernatant were taken to prime a 100- μ L, second nonradioactive PCR, which included the same upstream and downstream oligonucleotide primers. Ten microliters of the PCR were blunt-ended by the Klenow fragment of *E. coli* DNA polymerase (Boehringer Mannheim) and cloned into a pBluescript[®] II SK vector (Stratagene) digested with *EcoRV*. The ligation mixture was transfected into *E. coli* XL-1 blue (Stratagene), positive clones were selected using the blue/white phenotype and DNA plasmid was extracted by the alkaline lysis method (7). DNA from each clone was digested with *HindIII* and *BamHI* (Boehringer Mannheim) to ensure the existence of a DNA insert. Since the same expected length of DNA insert was detected in each of the clones obtained from a single band (data not shown), our approach to group the various clones was to use a tetra-nucleotide recognition site enzyme, *Sau3AI*, for restriction mapping. Digested DNAs were separated on a 4% NuSieve[®] (FMC BioProducts, Rockland, ME, USA) agarose containing 0.5 μ g/mL ethidium bromide. According to this restriction mapping (Figure 2), the various cDNA clones obtained from a single band could be grouped into four distinct species: A (lanes 1, 2, 8), B (lanes 3, 5, 6, 9, 11), C (lanes 4, 7) and D (lane 10). Nucleotide sequence analysis confirmed that clones with the same restriction enzyme fingerprinting were identical (data not shown). Accordingly, it could be deduced that cDNA species B might represent an up-regulated gene in Tat-expressing canine cells.

This rapid, nonradioactive and inexpensive approach to cDNA characterization significantly reduces the number

of clones that need to be further analyzed by nucleotide sequencing for identification, as well as by nuclear run-on or Northern analysis for confirming the deregulated expression of the suspected gene.

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