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Rapid method for screening and cloning cDNAs generated in differential mRNA display: application of Northern blot for affinity capturing of cDNAs

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A promising new technique called differential mRNA display has been recently described and used to identify differences in subsets of mRNA samples (1,2). This technique enables one to analyze any mechanism which involves changes in specific mRNA levels. The key element of the technique is to use sets of anchored and arbitrary primers to generate cDNA fragments by reverse transcription, followed by polymerase chain reactions (RT-PCR). The cDNA fragments are resolved and compared on sequencing gels. The resulting cDNA patterns reflect differences in the mRNA levels and composition. Differentially displayed cDNAs are isolated, cloned, sequenced and used as probes on Northern blots to confirm differences in the particular mRNA level. The general strategy seems to be straightforward, although recent reports on refinements in methods suggest that the involved procedures are technically challenging (3,4).

We used the differential display technique as a part of our ongoing studies investigating the effect of basic fibroblast growth factor (bFGF) on human endothelial cells. We also encountered several technical difficulties. One problem, experienced by others as well (4,5), was the failure to clone the cDNAs that would reconfirm differences of mRNA levels demonstrated on the display. When Northern blots were probed with cloned cDNA fragments, frequently, no signal or no difference in message levels was detectable. The most probable reason for the failure could be the fact that DNA recovered from what appeared to be a unique band contained additional undetectable overlapping, or unresolved cDNAs. Contaminating cDNAs might have been copurified with the differentially displayed cDNA. In fact, our studies revealed that each band recovered from the sequencing gel contained 3 or more different cDNA fragments. To avoid the laborious screening of multiple cloned fragments we applied a different approach for cloning the appropriate differentially displayed cDNAs. We used Northern blots to affinity capture cDNA fragments that were radiolabeled in PCR prior to hybridization. Fragments of cDNAs which demonstrated differences in mRNA levels of the bFGF-treated and untreated cells were recovered from the membrane after hybridization, and cloned. Repeated Northern analyses, now using the cloned inserts as probes, confirmed that affinity-capturing is an effective and rapid method for selecting the appropriate cDNA fragments from differential display isolates.

Methods

Total RNA was isolated from control and bFGF-treated human endothelial cells using RNazol (Biotecx). RT-PCRs were performed in two independent experiments under conditions described in Figure 1. Fragments of cDNAs were separated and displayed on a sequencing gel (Figure 1A). The highly reproducible cDNA patterns demonstrated that samples originating from control and treated cells have similar RNA composition, with some obvious differences. One of the differentially displayed bands (DDB11), that was present only in samples of treated cells, was eluted from the sequencing gel as described (1), and subsequently reamplified in PCRs. Th PCR conditions and primers were the same as were used for the first generation of cDNA fragments, except that [α-^{32}P]dCTP was also included. The radiolabeled ~240 bp DDB11 PCR product was purified in 2% low melting agarose gel (6) and used to probe the Northern blots.

Northern blots were prepared according to standard protocols (6) using 10 μg of total RNAs. The RNA samples were transferred and UV-cross linked to NYTRAN filters (Schleicher and Schuell). One of the filters was prehybridized for 2 h, then incubated overnight with the labeled and denatured DDB11 probe at 42°C under standard conditions (6). Next, the filter was washed in 2×SSC and 0.1% SDS at room temperature for 15 min, then washed with 0.1×SSC and 0.1% SDS at 50°C for 30 min, and finally exposed to Kodak XAR film at −70°C for 12 hours. The autoradiogram demonstrated presence of an increased DDB11 specific mRNA level in the bFGF-treated samples (Figure 1B). A piece of the filter ~10 mm^2 in area containing the captured DDB11 probe was cut out, using the autoradiogram for band localization. The filter piece was stripped by boiling in 100 μl of water for 5 min. The eluted probe was recovered by precipitation (0.3 M NaOAc, pH 5.0; 75% ethanol) using glycogen (0.5 μg/μl) as carrier. The pellet was washed with 80% ethanol at −20°C, then reconstituted in water. An aliquot of the reconstituted sample was immediately reamplified in PCR using conditions described in Figure 1, except that no radioactive dNTP was included. The ~240 bp PCR fragment was resolved in 2% agarose gel (Figure 1C), purified, and then inserted into a *Sma*I linearized plasmid pUC19 to yield a vector designated p11-D.

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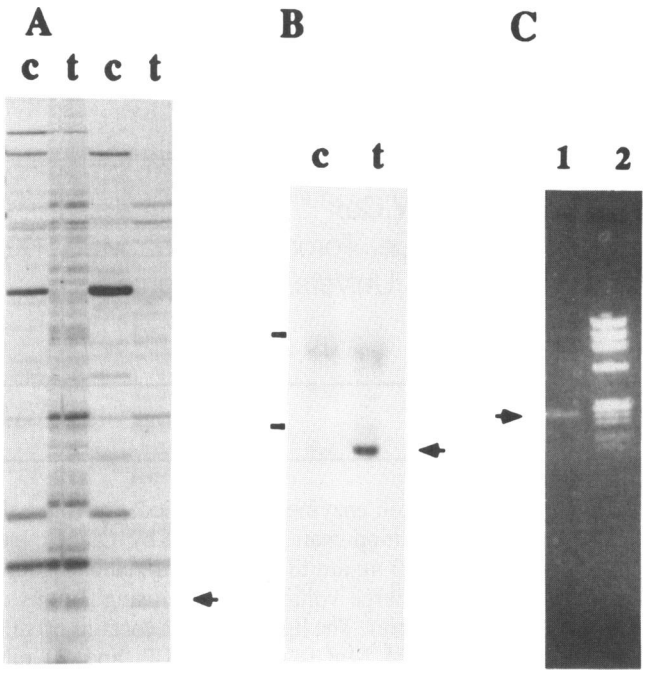

Figure 1. Affinity-capture of cDNA from differential display isolate. (A) Sequencing gel showing ^{35}S-labeled cDNA fragments generated in RT-PCR from RNA of control (*c*) and bFGF- treated (*t*) endothelial cells. For cDNA synthesis 2 μg of total RNA was incubated with 200 units of MMLV reverse transcriptase (BRL) in the presence of 2.5 μM primer (5′T_{12}CC3′), 20 units RNAsin (Promega) and 20 μM dNTPs in a final volume of 25 μl at 35°C for 60 min. The reaction was stopped by heat inactivation at 95°C for 5 min. In a final volume of 40 μl PCR mix, 1 μl cDNA was amplified in the presence of 2.5 μM 5′T_{12}CC3′ and 0.5 μM 5′CACTCTCCTC3′ primers, 2 μM dNTPs and 0.5 μM [α-^{35}S]dATP (1200 Ci/mmol) (Amersham) using 2 units of Taq polymerase (Perkin-Elmer). PCR was performed for 40 cycles with 94°C for 30 s, 42°C for 60 s, 72°C for 30 s, with a final elongation cycle for 5 min at 72°C. Autoradiogram of a 6% sequencing gel resolving 4 μl aliquots of the PCR mixtures is shown. Independently performed amplification yielded almost identical patterns. Differential display band 11 (indicated by arrow) was cut out from gel, and reamplified in two consecutive PCR reactions using the same condition as above except that in the second round PCR 20 μM dATP, dGTP, dTTP, 2 μM dCTP and 25 μCi of [α-^{32}P]dCTP (3000 Ci/mmol) (Amersham) were included. Labeled DDB11 was used on Northern blot (shown in *B*) as probe. (B) Northern analysis was performed as described in the Methods. The blot was hybridized with DDB11 that was ^{32}P-labeled in PCR. The positions of DDB11-specific mRNA, and 28S and 18S rRNAs are indicated. (C) PCR analysis of affinity-captured cDNA fragment. DDB11-specific cDNA probe, stripped from the Northern filter, was reamplified under the same PCR conditon as above without adding labeled dNTP. Ethidium bromide stained and UV visualized cDNA fragment (lane 1) and φX174 marker (lane 2) separated in 2% agarose gel is shown.

The cloned insert released from p11-D was random prime labeled (Boehringer Mannheim) and used as a probe on an intact Northern blot. The Northern analysis resulted an almost identical autoradiogram (not shown) to that obtained previously with labeled DDB11 PCR products (Figure 1B), confirming that plasmid p11-D carries the insert which generated the positive signal on the first Northern blot.

Our protocol describes an application of Northern blots for affinity capturing cDNA. This provides a rapid and effective method for screening, selecting and cloning cDNAs obtained by a wide variety of techniques, including differential mRNA display and RNA fingerprinting (7).

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