Direct Cloning of Differential Display Products Eluted from Northern Blots

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To identify novel or previously described mRNAs that are differentially expressed, numerous investigators have used differential display (DD) (3, 4). Briefly, DD involves the reverse-transcription (RT) of mRNA to single-stranded cDNA fragments followed by polymerase chain reaction (PCR) amplification. The PCR conditions and primers used generate a sub-population of cDNA fragments corresponding to the 3′ ends of mRNAs. Following gel electrophoresis of the radiolabeled PCR products and screening analysis, this method is not applicable.

Although DD protocols have been simplified and are being used by numerous investigators to successfully identify changes in gene expression, the PCR products that are amplified from the cDNA of specific tissues or experimental conditions must undergo a secondary screening to confirm that a differentially amplified cDNA fragment corresponds to a differentially expressed gene. Following the electrophoretic identification of a candidate band, the PCR products are reamplified and used as a hybridization probe directly (7) or cloned, and individual clones are used as hybridization probes against electrophoretically fractionated RNA. However, the identification of the individual PCR products that hybridize with mRNAs that are present in different steady-state levels between or among samples can be an arduous task (see Reference 7). To simplify the screening of differentially amplified cDNA fragments, we have developed a method using hybridization probes derived from the DD gels to probe a northern blot and clone only those cDNAs that anneal with the mRNAs from differentially expressed genes. After hybridization, single-stranded DNA molecules that hybridize with differentially expressed mRNA are re-amplified using PCR, cloned and sequenced. Therefore, even if the PCR product extracted from the denaturing gel contains more than one specific product, only differentially expressed cDNA fragments are cloned. This step reduces the number of northern hybridization and cloning reactions and eliminates the possibility that differentially expressed cDNAs are overlooked in the secondary screening analysis. If the band of interest is derived from an mRNA that is present in steady-state levels that cannot be detected by northern blot analysis, this method is not applicable.

The goal of this experiment was to identify transcripts that were present or absent in the heart tissue of a genetic model of cardiac hypertrophy before the onset of active heart tissue necrosis. We isolated total RNA from 30-, 60- and 90-day-old wild-type (WT) and cardiomyopathic (CM) hamster ventricles and used this material to initiate the synthesis of single-stranded cDNA. The cDNA was then subjected to DD RT PCR (5). A master mixture was prepared for each reaction as follows: 3.3 µL of double-distilled (dd)H₂O, 0.5 µL of 10× KlenTaq LA Polymerase Mix (CLONTECH Laboratories, Palo Alto, CA, USA), 0.05 µL of 5 mM dNTP, 0.05 µL [α-³²P]dATP, 0.25 µL of P primer (20 µM), 0.25 µL of T primer (20 µM) and 0.1 µL of 50× KlenTaq Polymerase Mix. We used the four P primers in combination with primer T2 (Table 1).

After mixing, 4.5 µL of the master mixture were added to 0.5 µL of each cDNA reaction. The PCR conditions were as follows: 5 min at 94°C, 5 min at 40°C, 5 min at 68°C, 2 cycles of [2 min at 94°C, 5 min at 40°C, 5 min at 68°C], 26 cycles of [1 min at 94°C, 1 min at 60°C, 2 min plus 4 s/cycle at 68°C], 7 min at 68°C, 0°C indefinitely.

Following the PCR amplification, 5 µL of denaturing loading dye (6) were added to each sample, the samples were heated at 92°C for 3 min, chilled on ice and electrophoretically fractionated on

| Table 1. Primers Used for Differential Display RT-PCR          |
|-----------------|-----------------|
| Primer | Sequence          |
| T2     | 5′-ATTATGCTGAGTGATCTTTTTTTTTTAC-3′ |
| P6     | 5′-ATTACCCCTCAGATTCTTTGTTG-3′    |
| P7     | 5′-ATTACCCCTCAGATTCTTTGTTG-3′    |
| P8     | 5′-ATTACCCCTCAGATTCTTTGTTG-3′    |
| P9     | 5′-ATTACCCCTCAGATTCTTTGTTG-3′    |
a denaturing acrylamide sequencing gel using the following conditions: 50°C, 3000 V, 100 W, 5 h. After electrophoresis, the gel was dried directly on the glass plate for approximately 10 min at 50°C until the urea crystallized. The gel was then rinsed with ddH2O to remove excess urea, transferred to 3MM paper (Whatman, Clifton, NJ, USA) and dried. After an overnight exposure to autoradiography film at room temperature, the gel and film were aligned, and bands that appeared to be differentially amplified were excised from the dried gel (Figure 1A).

A resulting DD band (gel/3MM paper) cut from a single lane of the gel was placed in a tube with 40 μL of ddH2O, allowed to re-hydrate for 10 min at room temperature, then heated to 95°C for 5 min. The PCR for re-amplification included 10.75 μL of ddH2O, 8.5 μL of DNA in solution from the band of interest, 1.25 μL of each of the primers (20 μM) used for the original DD reaction, 2.5 μL of 10× Taq Buffer (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 0.5 μL of 5 mM dNTP and 0.25 μL of Taq (5 U/μL). The PCR conditions were as follows: 1 min at 94°C, 19 cycles of [30 s at 94°C, 30 s at 58°C, 2 min plus 4 s/cycle at 68°C], 7 min at 68°C, 0°C indefinitely.

A northern blot was prepared by electrophoretically fractionating total cellular RNA on a 1% denaturing formaldehyde agarose gel and transferred to a Zeta-Probe® membrane (Bio-Rad, Hercules, CA, USA) using standard methodology (6). The gel-purified, re-amplified PCR fragment was radiolabeled by random hexamer priming using [α-32P]dCTP (Amersham Pharmacia Biotech), and excess radionucleotides were removed by Sephadex® G-25 Spin Columns (Amersham Pharmacia Biotech). The hybridization conditions and post-hybridization washes used have been described previously (2).

A complex pattern of hybridization was observed on the autoradiogram of the northern blot (Figure 1B). Many of the RNA species that hybridized to the probe were common to WT and CM hamster ventricles. However, one low-molecular-weight RNA present in the samples derived from CM and the 90-day-old WT hamster ventricle hybridized with the probe. To isolate the hybridizing DNA, the nylon membrane was placed in direct contact with a piece of 3MM paper on which the outline of the hybridizing band had been drawn from the aligned autoradiogram. The 3MM paper was saturated with ddH2O, carefully aligned to the membrane containing the hybridizing band and placed in a sealed plastic container in a humidified chamber at 37°C. After 1 h, the 3MM paper corresponding to the hybridizing band was excised and placed in a sterile microcentrifuge tube. Following the incubation, remaining radiolabel was removed from the northern blot by incubating the membrane in stripping solution (6) for 2 h at 75°C. After ensuring that all radiolabel was removed from the membrane, the northern blot was stored at -20°C before being re-probed. Following the addition of 100 μL of ddH2O to the 3MM paper, a 10-min incubation at room temperature, heating at 95°C for 5 min and cooling on ice for 2 min, the eluted single-stranded DNA was subjected to PCR amplification. This amplification used the original primers and the conditions for re-amplification described earlier with the exception that 19.25 μL of

Figure 1. Identification and isolation of a transcript expressed in CM ventricle tissue using DD and northern blot analysis. (A) Comparison of the products generated by DD RT-PCR using single-stranded cDNA of 30-, 60- and 90-day-old WT and CM hamster ventricles as a template and primers P8 and T2. The age of the animals in days is indicated above the aligned lanes of the DD gel (A) and northern blots (B,C). The PCR products generated were fractionated on a denaturing polyacrylamide gel. A portion of the autoradiogram is shown. The band of approximately 350 bp (arrow), in the samples derived from CM hamster ventricles, was selected for further analysis. Northern blot analysis of total RNA isolated from WT and CM hamster ventricles using the 350-bp band of interest (B) or the insert of a single clone (C) as a hybridization probe. 10 μg of total RNA were fractionated on a denaturing formaldehyde gel, transferred to Zeta-Probe membrane and stained with methylene blue to assess the relative amount of total RNA transferred to the membrane. After pre-hybridization, the blot was allowed to anneal with 1× 106 cpm/mL of radiolabeled PCR product. This PCR product was generated by re-amplifying the resulting band that was isolated from the acrylamide gel. Numerous transcripts ranging in size from >9 to 0.2 kb annealed with this probe (B). To isolate the DNA that specifically annealed with the low-molecular-weight RNA in the CM hamster samples (indicated by an arrow pointing to lane 4 of Panel B), the blot was placed in direct contact with an ddH2O-saturated 3MM filter paper, incubated for 60 min and then the portion of the 3MM filter paper that corresponded to the differentially hybridizing band on the northern blot was excised. After a second round of PCR re-amplification, the 350-bp PCR product was cloned. The insert of a single clone designated CM1.3 was radiolabeled and used as a probe in northern hybridization (C). A transcript of approximately 520 bases present in the RNA isolated from ventricles of 30-, 60- and 90-day-old CM and 90-day-old WT hamsters annealed with the CM1.3 probe. The steady-state level of the 520-base RNA that hybridized with CM1.3 was greatest in the 60-day-old CM hamster sample. The relative mobility of RNA molecular weight standards (0.24–9.8-kb RNA ladder; Life Technologies, Gaithersburg, MD, USA) is indicated on the left of each northern blot.
DNA in solution were used instead of 10.75 µL of ddH2O and 8.5 µL of DNA in solution. The re-amplified eluted band was electrophoresed, gel-purified and cloned into a pGEM®-T vector (Promega, Madison, WI, USA). We then identified clones containing recombinant plasmids, isolated plasmid DNA and used the insert of an individual clone as a hybridization probe in northern blot analysis. A single hybridizing mRNA of approximately 520 bases was identified in the lanes corresponding to the CM and, to a lesser extent, 90-day-old WT hamster ventricles (Figure 1C).

By using the re-amplified band of interest as a hybridization probe against northern blots, we were able to isolate, clone and identify a differentially expressed cDNA despite the fact that numerous cDNA species were present in the original band obtained. The ability to isolate and clone DNA that has hybridized to specific mRNA species should increase the speed of the secondary screening following DD and should eliminate the possibility that positive bands are not discarded simply because the specific clone is overlooked in the secondary screening analysis.

REFERENCES

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Simple Protocol for Extracting Nuclear DNA from Single Embryos of a Marine Snail

The analysis of genetic polymorphisms in groups of organisms leads to an understanding of the processes that structure populations, and, increasingly, DNA polymorphisms are the subject of study. Workers may be interested in organisms as research tools for understanding either evolutionary processes, the impact of pollutants or the sustainability of harvesting regimes. Marine animals figure prominently in all of these fields, but the extraction of DNA that is suitable for polymerase chain reaction (PCR) from small marine embryos is complicated by the small quantities of tissue initially present. A recent investigation using our model organism [Littorina saxatilis (Olivi)] recommended the use of the relatively expensive Chelex (12). The technique we describe here extracts DNA from individual whole embryos by incubating them in a simple lysis buffer, thus allowing DNA from individual embryos to be used in both mitochondrial and nuclear investigations (nuclear investigations need a higher-quality DNA extraction product than do mitochondrial investigations). This single-step procedure is relatively inexpensive and yields DNA that is directly suitable for PCR, whereas extractions using toxic reagents (8,14) and involving several handling steps (7,16) are cumbersome when very small amounts of tissue are involved.

The embryos of the intertidal snail Littorina saxatilis are found in a mother’s brood pouch (13). A method of extracting DNA from the adults is a phenol/chloroform-extraction (6); however, this is a difficult technique to apply when very small amounts of tissue are involved. An alternative is simply to amplify after adding an embryo to the PCR mixture, but this often fails to produce reliable amplifications (see Figure 1). In this paper, using both nuclear and mitochondrial PCR primers, we compare the success rates for two different extraction techniques: (i) directly adding whole