Western blot immunodetection with a human anti-centromere serum as shown in Figure 2B (right panel, lane 2). We included as positive controls HeLa and CHO protein extracts (Figure 2B, left panel, lanes 3 and 4) to show that the mammalian CENP-B protein runs at 80 kDa (Figure 2B, right panel, lanes 3 and 4).

This report proposes an alternative approach for expressing a significant amount of a toxic protein in bacteria by using the pET expression system, avoiding or limiting the IPTG addition and reducing the incubation time when other restricted conditions (e.g., BL21 (DE3)pLysS, antibiotic replacement or original plating method) do not work properly.

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Increased Efficiency in Screening Large Numbers of cDNA Fragments Generated by Differential Display

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Differential display is a powerful screening technique used for the detection of differentially expressed genes (4). This method possesses several advantages over conventional differential or subtractive hybridization techniques (6). For reasons known and unknown, differential display generates a high percentage of false positives (5), making it necessary to screen many fragments to identify those few that are truly differentially regulated. Although numerous reports of improvements to the differential display technique have been published (5), including ways to decrease the number of false positives, very little has been described to allow for the rapid confirmation of altered expression of the potentially large number of fragments that are identified. We describe here a time-saving and labor-reducing protocol using 96-well technologies for handling large numbers of fragments generated by differential display. The protocol includes a number of improvements to the processing of differential display fragments: (i) amplification of the differential display fragment directly from the gel; (ii) modifications to increase the number of ligations that can be performed with the popular TA Cloning® Kit (Invitrogen, San Diego, CA, USA); (iii) screening transformed bacteria for cloned fragments of the correct size by colony polymerase chain reaction (PCR); and (iv) confirmation of altered expression by dot-blot differential hybridization. With this protocol, the cloned fragments can be assessed for responsiveness without the need for plasmid purification.

A flowchart that outlines the procedures for handling large numbers of differential display fragments is shown in Figure 1. After the differential display reactions are performed, the fragments are resolved on denaturing

Benchmarks

sequencing gels. Gels are soaked briefly in water and dried. Differentially expressed fragments are cut from the gel and amplified directly using as a template a small piece of the dried gel. The amplified fragments are cloned into a T-tailed vector (pCR™ II; Invitrogen) with the modifications described

below. Competent bacteria are transformed with the ligation reactions and plated onto media containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and an antibiotic. White colonies are picked, transferred to 150-mm plates pre-marked in a 96-well format and used directly as a source of

template in colony-PCR. Inserts of the correct size are identified by agarose gel electrophoresis. Those colonies that contain the fragments of interest are spotted individually onto a 150-mm master plate, and the amplification is repeated. Equal amounts of the amplified fragments are then dot-blotted onto duplicate filters that are probed with 32 P-labeled cDNAs from control and treated tissues. Filters are then compared for changes in the intensity of the dots after exposure.

Amplification of differential display fragments. Currently, most investigators separate the differential display fragments by denaturing gel electrophoresis in the presence of urea. The bands of interest are cut from the gel and eluted from the polyacrylamide by a time-consuming procedure requiring boiling and precipitation with glycogen (4,5). The purified cDNA fragments are then used as templates for amplifying microgram quantities of the fragment to clone and sequence. We have found that removal of most of the urea from the polyacrylamide gel by soaking in water before drying allowed us to use a piece of the dried gel (ca. 2 mm $\times$ 2 mm) containing the fragment of interest directly in the amplification reaction. The urea was removed by immersing the gel sitting on one glass plate with approximately 2 cm of distilled water for 15–20 min. The intensity of the cDNA bands was not appreciably affected by this treatment. To amplify the fragments from the dried gel, we used 40 μ L of an amplification mixture containing the same primers that were used in the differential display as detailed in Reference 4. Almost all of the fragments (ca. 80%) could be amplified after 40 cycles of PCR to approximately 5–50 ng fragment/ μ L. All but a few of the remaining fragments (<5%) could be amplified by adding a fresh aliquot of *Taq* DNA polymerase to the amplification mixture and repeating the amplification. To prevent evaporation of the amplification mixture, care was taken to ensure that the gel piece did not protrude through the oil overlay. We have had only limited success amplifying cDNAs from denaturing gels that were not soaked in water, most likely because of the urea in these gel pieces. For those amplifications that

Figure 1. Confirmation of altered expression of large numbers of fragments generated by differential display. The procedures for amplification, cloning and confirmation of altered expression of large numbers of differential display fragments are described in the text.

Benchmarks

generated fragments in addition to the one desired, cloning all the fragments and screening with the method outlined below was more efficient than first gel-purifying the desired fragment to be cloned.

Cloning the amplified fragments.

The TA Cloning Kit has become one of the most popular methods for cloning differential display fragments. Despite its ease of use, the cost to clone many fragments can be prohibitively high. The protocol provided by the kit suggests using 50 ng of pCR II plasmid vector in a 10- μ L ligation, transformation of competent bacteria with 1 μ L of the ligation mixture and then plating out only 5%–20% of the transformation mixture. Thus, a large percentage of vector is never used. We have reduced the amount of vector required in a 5- μ L ligation reaction to 12.5 ng. For a new lot of vector or a large set of fragments to be cloned, we set up a series of ligations with different amounts of a test fragment and 12.5 ng of vector to determine the optimal ratio of fragment to vector. After ligation, we transformed either 25 μ L (24 μ L of cells plus 1 μ L 0.5 M β -mercaptoethanol) of competent bacteria provided in the TA Cloning Kit or 50 μ L of chemically competent cells (7) with 2 μ L of the ligation mixture. After heat shock and recovery, the entire transformation mixture was plated out. At the optimal ratio of fragment to vector, about 300 colonies were observed, with 10%–90% of them containing plasmids with inserts. Once the conditions for ligation had been optimized, 4 μ L of a stock ligation reaction mixture (as outlined in the TA Cloning Kit) containing 12.5 ng of vector were added, using a multichannel pipettor, to 1 μ L of fragment in a 96-well plate. The plate containing the ligations was then carefully floated in a 14°C water bath overnight. Two microliters of the ligation mixtures were moved to another 96-well plate, which was then placed on ice. Competent cells were added, allowed to incubate for 30 min and then heat-shocked at 42°C following the protocol provided with the TA Cloning Kit. The bacteria were transferred to individual tubes containing pre-warmed SOC medium, allowed to recover for 30–60 min and then plated according to instructions in the TA Cloning Kit on

YT plates containing ampicillin (50 μ g/mL) and X-gal. These modifications accelerate the cloning of large numbers of fragments while reducing waste of the expensive vector.

Screening for inserts by colony

PCR. Colonies containing vectors with cloned inserts were screened using a modification of the colony-PCR technique (2). PCR tubes (MicroAmp®; Perkin-Elmer, Norwalk, CT, USA) were set up immediately before amplification and contained 15 μ L of an amplification mixture (200 μ M dNTPs, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 5% glycerol, 0.1 μ g/ μ L cresol red, 0.6 U *Taq* DNA polymerase and 15 pmol each of the M13 forward [5'-GTTTTCCCAGTCACGACGTTG-3'] and M13 reverse [5'-CAGGAA-CAGCTATGACCATG-3'] primers). Glycerol and cresol red were added to the reaction mixture to replace Ficoll® and bromophenol blue in the sample loading buffer. At these concentrations, they do not interfere with the amplification. A sterile pipet tip was used to touch each colony and spot a 150-mm plate containing YT plus kanamycin pre-marked in a 96-well format. The pipet tip was then placed into the corresponding PCR tube containing the amplification mixture and removed before amplification. The amplification was performed in a PCR System 9600 Thermal Cycler (Perkin-Elmer) as follows: 1 cycle at 94°C for 5 min; and 25 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by a 5-min extension at 72°C. The samples were then removed four at a time by a multichannel pipettor and applied directly to 1.5% agarose gels (20 cm $\times$ 20 cm; Pharmacia Biotech, Piscataway, NJ, USA) containing 3 or 4 rows of 22 lanes. The primers used in the amplification anneal to sites in the pCR II vector that are approximately 100 bp on each side of the cloning site. Thus, colonies that contained plasmids with no inserts gave a fragment of 205 bp, and colonies that contained plasmids with inserts gave a band of 205 bp plus the size of the insert (data not shown). Given that multiple cDNA species of the same size can be amplified from the same differential display band (8), we routinely screened 12 colonies from each ligation and chose at least 2

colonies that produced fragments of the correct size to be screened for differential expression.

Dot-blot differential hybridiza-

tion. The procedure for screening cloned cDNA fragments for altered expression consisted of immobilizing the amplified fragments on nylon membranes using a dot-blot apparatus (Schleicher & Schuell, Keene, NH, USA) and hybridizing the membranes to ³²P-labeled cDNAs from control or treated tissues. Those colonies that were to be screened were spotted onto a 150-mm master agar plate and allowed to grow overnight at 37°C. A multichannel pipettor was used to simultaneously touch 12 colonies in a row, which were subsequently ejected into 70 μ L of the reaction mixture (described above) separated into aliquots into each PCR tube. The amplification of the cloned fragments was repeated as described above, and 7 μ L of the amplification mixture were resolved by agarose gel electrophoresis to confirm amplification. The rest of the amplification solution was transferred to a 96-well microplate. The fragments were denatured by adding 7 μ L of 3 M NaOH and pipetting up and down to ensure that the solution was well mixed. The covered plate was then incubated at 65°C for 1 h. The solution was neutralized by the addition of 30 μ L of 20 $\times$ standard saline citrate (SSC). Equal volumes (35 μ L) of the DNA fragments (ca. 100–500 μ g) were pipetted onto prewashed nylon membranes (HyBond®-N⁺; Amersham, Arlington Heights, IL, USA), which had been overlaid with 200 μ L of 6 $\times$ SSC to achieve even distribution of the fragment across the dot. The membranes were processed according to the recommendations of the manufacturer of the membrane. The DNA was photocross-linked to the membrane using a Stratalinker® (Stratagene, La Jolla, CA, USA). Membranes were prehybridized, hybridized with the ³²P-labeled cDNAs and washed according to a standard protocol (7). Starting with poly(A) mRNA from the livers of two control rats or two rats treated with a peroxisome proliferator (WY-14,643 [WY] at 0.1% in the diet for 22 weeks), cDNAs were made using a kit (cDNA Synthesis Kit; Pharmacia Biotech)

Benchmarks

according to the manufacturer's instructions. cDNAs were labeled with [α - 32 P]dCTP using the Rediprime™ DNA Labeling Kit (Amersham). All control cDNAs, except bifunctional enzyme, were amplified with the same reaction mixture as described above, except that plasmid DNA was used as the template. Bifunctional enzyme was amplified using the primers Pst1 (5'-TTAATAGTTTGC GCAACG-3') and Pst2 (5' GACGAGCGTGACACCA-CG-3') in place of M13F and M13R primers.

We used dot-blot differential hybridization to confirm altered expression of differential display fragments from the livers of rats fed a control diet and those fed a diet containing WY, a

hepatocarcinogen. To determine if conditions were appropriate for detecting differential gene expression, we first examined the expression of several genes that we expected to be altered after treatment, as well as three genes that we expected would not change after treatment. The expected changes in three up-regulated genes, malic enzyme (dot 4), *CYP4A3* (dot 6) and bifunctional enzyme (dot 7) and a down-regulated gene, transthyretin (dot 5), were observed (Figure 2, top). The loading controls, β -actin (dot 1), glyceraldehyde phosphate dehydrogenase (GAPDH, dot 2) and albumin (dot 3), had variable levels of expression compared to one another but remained unaltered (β -actin and GAPDH) or were down-regulated

Figure 2. Dot-blot differential hybridization. *Top:* Duplicate membranes containing the cDNAs of loading controls, β -actin (dot 1), GAPDH (dot 2) and albumin (dot 3), and the positive controls, malic enzyme (dot 4), transthyretin (dot 5), *CYP4A3* (dot 6) and bifunctional enzyme (dot 7), were probed with identical quantities of 32 P-labeled cDNAs from the livers of control and WY-treated rats. *Bottom:* Duplicate membranes containing immobilized fragments amplified from colonies possessing the cloned cDNAs were probed with the 32 P-labeled cDNAs as described above. A number of fragments identified on the blots were shown to be differentially expressed.

Benchmarks

(albumin) after exposure. The expression of 48 putative WY-responsive genes before and after exposure is shown in Figure 2 (bottom). Several fragments exhibited down-regulation after exposure, including 53B, 58A, 58B, 60B, 63B, 70B and 73B. Clones 61A, 72A and 72B exhibited up-regulation. A significant number of the fragments did not appear to hybridize to any of the labeled cDNAs. This phenomenon was also observed when differential display fragments were used as probes of Northern blots (8). In our case, the high percentage of false positives may be partly because we chose many fragments that were only marginally altered and had weak expression so that we would not miss any true differentially expressed cDNAs. In addition, we screened only two clones from each ligation. Because many cDNA species can be amplified from one differential display fragment (8), we may not have screened enough fragments to find the one that was responsive. All fragments that exhibited altered expression after exposure were sequenced. Only clone 60B was a known gene ($\alpha 2u$ -globulin) previously shown to be down-regulated by peroxisome proliferators (1). In summary, starting with 414 cloned fragments using 26 unique primer combinations, we confirmed altered regulation of 30 fragments.

The protocol described here for screening differential display fragments has unique advantages over the conventional screening method of probing Northern blots with individual fragments: (i) greater numbers of fragments can be handled at once with a significant decrease in the time it takes to confirm altered expression. A total of 414 cloned fragments were screened two times in approximately 6 weeks; (ii) less RNA is needed to perform the analysis. Typically, 5 μ g or less of poly(A) RNA is needed for each cDNA reaction. Many times this amount would be necessary if screening multiple Northern blots at one time; (iii) unlike in earlier reports where the uncloned amplified cDNAs were used as probes, only one fragment is examined at a time, allowing for an unambiguous assessment of expression; (iv) once the responsive fragments have been identified and the colonies carrying them

have been spotted onto master plates, additional time-saving manipulations can be performed. Cultures of the bacteria can be grown and then stored at -80°C on 96-well plates. Filters containing a library of differentially expressed fragments can be probed with known genes that are candidates for altered regulation; and (v) the colonies are in a convenient format to perform colony-PCR sequencing (3) or to isolate the plasmid DNA using 96 sample plasmid miniprep kits. The improvements outlined here will help investigators more quickly identify differentially expressed fragments and begin the major challenge of understanding the role that altered regulation of these genes plays in biological processes.

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