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A Rapid PCR Protocol for Identification of Differentially Expressed Genes from a cDNA Library

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This report describes the application of PCR to simplify and expedite the characterization of cDNAs identified by plus/minus differential screening. Differential screening of a cDNA library is a powerful method for identifying differentiation state-specific genes in a particular system; however, the process of purifying, confirming, and sequencing the potentially large number of candidate clones can be difficult and tedious. To shorten this procedure, we have used PCR to generate cDNA inserts rapidly and directly from the primary phage screens using the flanking λ vector sequences as primers. Generally, the most predominant amplified product is the differentially expressed cDNA, because the plus/minus screening is performed at relatively low densities. The PCR-amplified DNA can then be labeled radioactively and used as a probe to confirm the differential expression by RNA blot hybridizations, establish whether multiple cDNA inserts are related, obtain DNA sequence, and, finally, isolate the desired recombinant phage.

RESULTS AND DISCUSSION

As a model system for studying the mechanisms by which extrinsic cues influence neural crest differentiation, we used the CA77 thyroid C cell line.[1] When the CA77 cells are treated with the synthetic glucocorticoid dexamethasone, several morphological and biochemical changes can be observed within several days of treatment.[1,2] These changes are highlighted by increased transcription of the calcitonin/calcitonin gene-related peptide (CGRP) gene, increased roundness of the cells, partial retraction of neurites, an increase in the number of secretory vesicles, and a 10-fold decrease in the DNA synthesis rate. Interestingly, dexamethasone also causes a bias in the alternative RNA processing of the calcitonin/CGRP transcript to favor calcitonin hormone mRNA relative to CGRP neuropeptide mRNA production in the CA77 cells. The level of calcitonin mRNA increases about fivefold, while the level of CGRP mRNA increases only about twofold.

In an attempt to identify genes regulated upon dexamethasone-induced differentiation of the CA77 cells, we carried out differential screening of a CA77 cDNA library. Differential screening is a powerful technique for identification of genes important in the establishment and maintenance of cellular differentiation.[3] Glucocorticoid-induced genes were identified by differential hybridization of plus/minus cDNA probes to a CA77 thyroid C cell cDNA library prepared from cells that had been treated with 0.5 μM dexamethasone for 6 days. The library was constructed in LambdaGem-4 phage vector using the Riboclone cDNA Synthesis System (Promega). The packaged recombinant phage were plated at 2000–4000 plaque-forming units (pfu) per 150-mm plate on *Escherichia coli* LE392 cells. Radioactive plus/minus cDNA probes were synthesized from A^+ RNA of control (minus) or dexamethasone-treated cells (plus) CA77 cells using AMV reverse transcriptase and were hybridized to duplicate nitrocellulose filters containing phage plaques at 10^6 cpm/ml of a 50% formamide hybridization buffer[4] at 42°C for 72 hr. About 25% of the plaques show a hybridization signal with either the plus or minus probes. Autoradiographs of the control and dexamethasone cDNA-probed filters were compared to identify differentially hybridizing plaques (Fig. 1). Each differential plaque was cored with the small end of a pasteur pipette, and the phage were eluted into 500 μl of SM buffer[4] with 25 μl of chloroform. Approximately 10^6 pfu were recovered from each agar plug.

After a differential plus/minus hybridization signal had been identified, the next challenge was to isolate the cDNA insert to confirm differential expression. One fundamental aspect of differential screening is that a potentially large number of positive plaques are often identified on the primary screen. For example, we found 56 differentially hybridizing plaques on the primary screen of about 12,000 plaques using plus/minus probes. To know which candidate plaques to pursue, confirmation and characterization of the differential signals are required. To accomplish this, we initially followed standard protocols.[4] However, the secondary and tertiary screenings meant to confirm or discount differential expression were tedious and often were ambiguous and uninformative.

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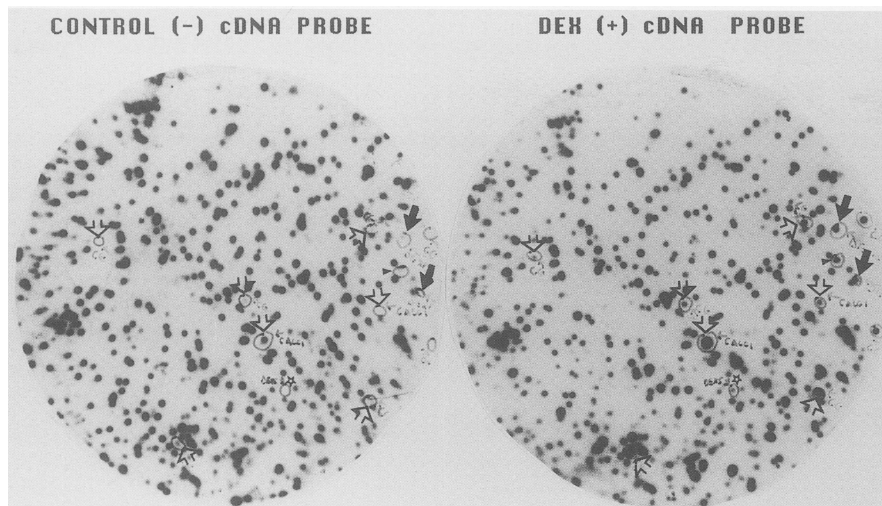

FIGURE 1 Identification of differentially expressed genes by plus/minus hybridization. Phage plaques from the CA77 dexamethasone-induced cDNA library were transferred onto duplicate filters and hybridized with cDNA probes prepared from either control cells (minus probe) or from dexamethasone-treated cells (plus probe). Approximately 500 of the 2000 plaques transferred to the filter yielded detectable signals with either probe. Representative plaques showing a greater hybridization signal with the plus probe are indicated.

tion with a specific probe and eluted from plates containing 20,000 pfu instead of the 2000–4000 pfu used for the plus/minus screening. As expected, the greater number of different phage yielded a larger number of bands that appeared as a smear following amplification and the desired cDNA band was not amplified preferentially (Fig. 2B). In contrast, when the PCR approach was used with a secondary plating of the isolated phage at 2000 pfu per plate, clear amplification products were observed that corresponded to the predominant phage species (Fig. 2B). This result confirms that the preferential PCR amplification is due to the relatively low density of plaques and hence the relative abundance of the desired phage isolated from the primary plate screening.

It should be emphasized that no further purification of the cDNA insert need be performed once it is contained in the low-melt agarose. It is sufficiently pure for radioactive labeling using the random priming method.[5] The low-melt agarose fragment was heated at 95°C for 10 min, and 5 µl of the melted agarose was added to the rest of the reagents preheated to 37°C (except for the Klenow enzyme). The reaction was incubated at 37°C between 2 hr and overnight. Immediately prior to hybridization, the labeled probe was heat denatured at 95°C for 10 min. To conserve reagents and reduce isotope usage, we found that cutting the standard 20-µl labeling reaction to 10 µl did not reduce the efficiency of labeling. This was particularly helpful when we were labeling 8–10 fragments at one time. To confirm that a cDNA insert was indeed differential, we used the PCR-generated probes to hybridize with RNA slot or Northern blots containing A^+ RNA from control and dexamethasone-treated CA77 cells (Fig. 3). Once a differential cDNA insert was confirmed, the labeled insert could also be used as a hybridization probe to isolate the desired recombinant phage to plaque purity. Furthermore, we used the labeled probes to establish whether any of the differential inserts were related to each other based on hybridizations with Southern or plaque lift filters.

In addition, we have found that the cDNA in low-melt agarose is an accept-

Therefore, we used PCR amplification to isolate cDNA inserts as described below to eliminate the need for further plaque screenings and to facilitate the rapid screening of a relatively large number of differentially expressed cDNA clones.

To circumvent secondary and tertiary screenings of over 50 plaques showing differential hybridization, we PCR-amplified the various cDNA species directly from the eluted phage particles using primers based on the vector sequences. The 50-µl PCR reaction contained 20 mM Tris-HCl (pH 8.6), 50 mM KCl, 2.5 mM $MgCl_2$, 30 µg/ml bovine serum albumin, 10 nmoles of each dNTP, 50 pmoles each of the SP6 primer (5′-GATTTAGGTGACACTATAG-3′) and the T7 primer (5′-TAATACGACTCACTATAGGG-3′), 1.25 units of AmpliTaq DNA Polymerase (Perkin-Elmer Cetus), and ~10^4 pfu of the eluted phage (5 µl). Amplification cycles were: 1 min at 94°C, 1 min at 55°C, 2 min at 72°C for 30 cycles followed by 72°C for 5 min (Perkin-Elmer Cetus Thermal Cycler). An aliquot (20 µl) of the PCR reaction was loaded into wells (4.5 mm wide) in a 2% NuSieve GTG (FMC Inc.) low-melt agarose gel containing 1x TBE[4] and 0.5 µg/ml ethidium bromide. The gel was visualized under long-wave UV light and major bands in each lane were excised, with an attempt to get the smallest amount of agarose as possible (<75 µl). In this way, we were able to resolve and isolate multiple cDNA fragments quickly (Fig. 2A). Although we sometimes observed up to four different amplification products from a single pool of eluted phage cored from the primary screen, there was almost always a major band on the gel.

We have noticed that in general the prominent amplification product from the PCR reaction represents the differentially expressed cDNA clone. The PCR bias is useful because the signal intensity can be used as an initial criteria for choosing which cDNA insert to analyze further by RNA blot hybridizations, although all PCR-amplified cDNA bands should be excised from the agarose gel and stored for further analyses if needed. The basis for the preferential amplification is an inherent result of PCR amplification of primary phage plaques identified by plus/minus screening because the plaque density must be relatively low for plus/minus screening. To demonstrate this point, we performed the PCR using phage pools of two different complexities. The PCR protocol was performed with vector primers using phage identified by hybridiza-

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able template for DNA sequencing using a modification of the protocol of Kretz et al.[6] To isolate the PCR fragment, 90 µl of the reaction was loaded into wells (15.5 mm wide) in a 1.0% NuSieve GTG low-melt agarose gel and electrophoresed as described above. The desired band was excised from the gel, usually resulting in a 100–150 µl, and heated to 70°C for 10 min. The sequencing reactions were then performed using the Sequenase Version 2.0 Kit (IBI) according to the manufacturer's protocol, with the following exceptions. For the annealing step, 10 µl of the DNA (contained in the melted agarose), 1 µl of the primer (50 ng/µl), and 2 µl of reaction buffer (provided in the Sequenase kit) were boiled for 2 min, then allowed to cool in a 37°C heat block for 30 min. After annealing, the reaction tubes were kept at 37°C to prevent the agarose from resolidifying.

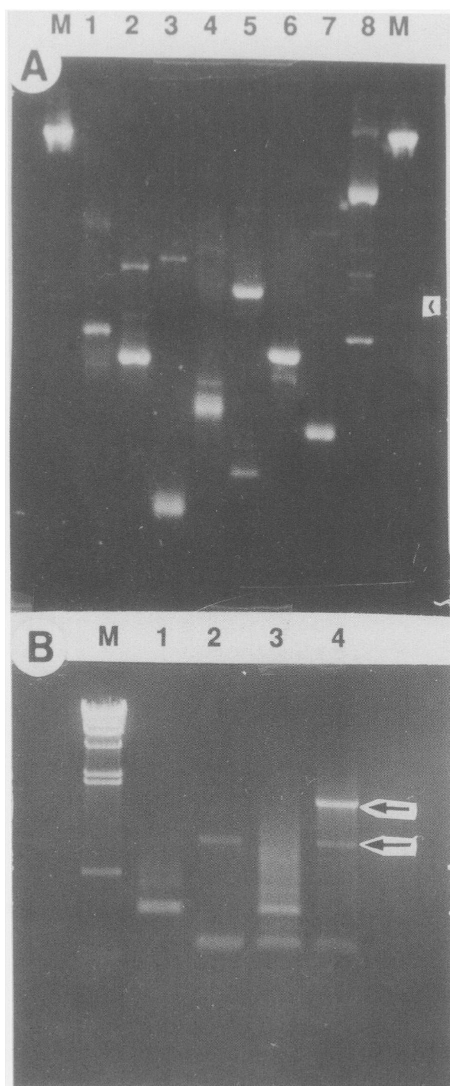

FIGURE 2 Agarose gel electrophoresis of PCR-amplified cDNA inserts. The inserts were amplified from recombinant phage particles eluted from the agar plugs. (*A*) A 2% agarose gel of PCR products (20 µl) from different phage plugs (lanes *1–8*) taken from primary plus/minus screening plates. (*B*) A 1.0% agarose gel of PCR products (10 µl) from two different phage plugs taken from primary plates with 20,000 pfu (lanes *1* and *3*) that were identified by a specific probe. The PCR products of phage identified by the same probe following secondary screening at 2000 pfu of the phage used for lanes *1* and *3* are shown in lanes *2* and *4*, respectively. The major amplification products in lanes *2* and *4* are indicated by the arrows. λ *Hin*dIII DNA fragments were used as molecular weight standards (lane *M*). (>) 500-bp marker on the 2% gel.

The procedure described in this report for isolating cDNA inserts using PCR is similar to that of Goueli and Ahmed,[7] but with notable advantages that are particularly applicable to the plus/minus screening technique. We were able to PCR amplify cDNA inserts directly from pools of multiple phage particles rather than from purified DNA. This is an important feature because it eliminates the need for secondary and tertiary screens using plus/minus cDNA probes to isolate the desired recombinant phage to plaque purity. The multiple phage inserts present in the eluted phage from primary plaques can be resolved by gel electrophoresis of the PCR amplification products. A major advantage of the protocol described here is that these PCR-generated fragments from the primary plaque screening can then be used to confirm differential expression quickly by RNA blot hybridizations. In addition, the PCR-generated probes can be used to establish whether multiple candidate clones are related based on cross-hybridization. Once candidate clones are confirmed, the PCR-generated probes can then be used for subsequent purification of the desired recombinant phage. These steps minimize wasted time and effort spent on clones that are not actually differential or are related. Finally, no further purification of the cDNA insert is necessary for rapid sequence determination. Overall, these modifications greatly facilitate the analysis of multiple candidate cDNA clones identified by plus/minus differential hybridization.

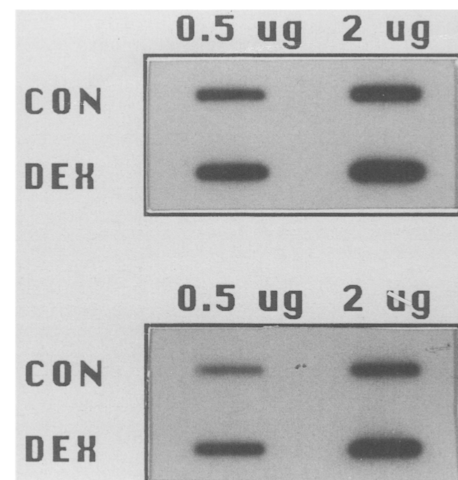

FIGURE 3 RNA slot blot hybridization using radiolabeled PCR-generated probes. PCR-amplified cDNA inserts were ^{32}P-labeled and hybridized to A^{+} RNA (0.5 µg and 2.0 µg) isolated from control and dexamethasone-treated CA77 cells and immobilized on nylon filters. Differential expression of two separate cDNA clones was confirmed by these autoradiographs.

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