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Annealing control primer system for identification of differentially expressed genes on agarose gels

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We developed GeneFishing technology, an improved method for the identification of differentially expressed genes (DEGs) using our novel annealing control primer (ACP) system. Because of high annealing specificity during PCR using the ACP system, the application of the ACP to DEG discovery generates reproducible, authentic, and long (100 bp to 2 kb) PCR products that are detectable on agarose gels. To demonstrate this method for gene expression profiling, GeneFishing technology was used to detect genes that are differentially expressed during development using total RNAs isolated from mouse conceptus tissues at 4.5–18.5 days of gestation. Ten DEGs (DEG1–10) were isolated and confirmed by Northern blot hybridization. The sequence analysis of these DEGs showed that DEG6 and DEG10 are unknown genes.

INTRODUCTION

Techniques designed to identify differentially expressed genes (DEGs) in cells under various physiological stages or experimental conditions (e.g., during developmental or neoplastic differentiation or pharmacological treatment) have become pivotal in modern biology. The difficulty of identifying (or "fishing out") a gene responsible for a specialized function during a certain biological stage often arises because the gene is expressed at low levels, whereas the bulk of mRNA within a cell are highly abundant transcripts (1). To screen for DEG transcripts in low concentrations, PCR amplification is required. One screening method, differential display, requires PCR using short arbitrary primers and is described by Liang and Pardee (2). Although this method is simple, rapid, and only requires small amounts of total RNA, many investigators have experienced significantly high false-positive rates (1,3) and poor reproducibility of results (4) because of nonspecific annealing by short arbitrary primers. The use of short primers (10–13 bp) usually requires low annealing temperatures about 40°–45°C for all PCR cycles; these low temperatures cause nonspecific primer annealing. The use of longer primers (18–20 bp) make it possible to increase the annealing temperature to 60°C after 1–4 initial cycles at 40°–45°C (5,6). However, the additional tail sequences of longer primers are involved in nonspecific annealing to the cDNA template during PCR cycles at low annealing temperatures (7).

We recently developed the annealing control primer (ACP) system (7) that uses primers that anneal specifically to the template and allows only genuine products to be amplified, a process that eliminates false-positive results. The ACP system is based on two principles: the unique tripartite structure of the primers, which have distinct 3′- and 5′-end regions that are separated by a polydeoxyinosine [poly(dI)] linker, and the interaction of each region during two-stage PCR. We adapted the ACP system for the identification of DEGs involved in mouse development.

MATERIALS AND METHODS

First-Strand cDNA Synthesis

Total RNAs extracted from mouse conceptus tissues at different developmental stages [4.5, 11.5, and 18.5 days postcoitus (dpc)] were used for the synthesis of first-strand cDNAs by reverse transcriptase, as described by Hwang et al. (7). Reverse transcription was performed for 1.5 h at 42°C in a final reaction volume of 20 µL containing 3 µg of the purified total RNA, 4 µL of 5× reaction buffer (Promega, Madison, WI, USA), 5 µL of dNTP (each 2 mM), 2 µL of 10 µM cDNA synthesis primer, oligo$(dT)_{15}$, oligo$(dT)_{15}$ tail, or oligo$(dT)_{15}$ ACP (Table 1), 0.5 µL of RNasin® RNase Inhibitor (40 U/µL; Promega), and 1 µL of Moloney murine leukemia virus reverse transcriptase (200 U/µL; Promega). First-strand cDNAs were diluted by the addition of 80 µL of ultra-purified water.

ACP-Based GeneFishing PCR

Second-strand cDNA synthesis and subsequent PCR amplification were conducted in a single tube. Second-strand cDNA synthesis was conducted at 50°C (low stringency) during one cycle of first-stage PCR in a final reaction

Table 1. Primer Sequences Used in cDNA Synthesis and ACP-Based GeneFishing PCR

Use	Primer	Sequence
cDNA Synthesis Primer	Oligo(dT)$_{15}$	5′-TTTTTTTTTTTTTTT-3′
	Oligo(dT)$_{15}$ tail	5′-CTGTGAATGCTGCGACTACGATTTTTTTTTTTTTTT-3′
	Oligo(dT)$_{15}$ ACP	5′-CTGTGAATGCTGCGACTACGATIIIIITTTTTTTTTTTTTT-3′
Arbitrary Primer	10-mer	5′-GCCATCGACC-3′
	10-mer tail	5′-GTCTACCAGGCATTCGCTTCATGCCATCGACC-3′
	AP1	5′-GTCTACCAGGCATTCGCTTCATIIIIIGCCATCGACC-3′
	AP2	5′-GTCTACCAGGCATTCGCTTCATIIIIIAGGAGATGCG-3′
	AP3	5′-GTCTACCAGGCATTCGCTTCATIIIIICTCCGATGCC-3′

ACP, annealing control primer.
The polydeoxyinosine [poly(dI)] linkers are underlined. I represents deoxyinosine.

volume of 49.5 µL containing 3–5 µL (about 50 ng) of the diluted first-strand cDNA, 5 µL of 10× PCR buffer plus Mg (Roche Applied Science, Mannheim, Germany), 5 µL of dNTP (each 2 mM), 1 µL of oligo(dT)$_{15}$, oligo(dT)$_{15}$ tail, or oligo(dT)$_{15}$ ACP (10 µM), and 1 µL of 10 µM arbitrary primer (Table 1, 10-mer, 10-mer tail, or 10-mer ACP) preheated to 94°C. The tube containing the reaction mixture was held at 94°C while 0.5 µL of *Taq* DNA Polymerase (5 U/µL; Roche Applied Science) was added to the reaction mixture. The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. After second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94°C for 40 s, followed by 65°C for 40 s, 72°C for 40 s, followed by a 5-min final extension at 72°C. The amplified PCR products were separated in 2% agarose gel stained with ethidium bromide. The differentially expressed bands were extracted from the gel by using a GENECLEAN® II Kit (Qbiogene, Carlsbad, CA, USA), directly cloned into the pGEM®-T Easy vector (Promega) without reamplification of the recovered bands, and sequenced with ABI PRISM® 310 Genetic Analyzer (Perkin Elmer, Boston, MA, USA).

Northern Blot Analysis

Northern blots of full-stage mouse conceptus tissue (4.5–18.5 dpc) and of multiple adult mouse tissues (Seegene, Seoul, South Korea), each lane containing 20 µg of total RNA, were hybridized overnight with the ^{32}P-labeled cDNA probe in QuikHyb® solution (Stratagene, La Jolla, CA, USA) as previously described (8). The probe cDNA fragments for each DEG were prepared by PCR using their corresponding clones as templates. The fragments were amplified by using the universal (tail) sequences of oligo(dT)$_{15}$ ACP and arbitrary ACPs JYC5 (5′-CTGTGAATGCTGCGACTACGAT-3′) and JYC4 (5′-GTCTACCAGGCATTCGCTTCAT-3′).

RESULTS AND DISCUSSION

Principle of GeneFishing Technology

To apply the ACP primer system to differential display, first-strand cDNAs are synthesized by reverse transcription using oligo(dT)$_{15}$ ACP as a primer. This method requires only a single cDNA synthesis for each different RNA sample, in contrast to the multiple cDNA reactions required for differential display methods (2,9). Using first-strand cDNAs as templates, second-strand cDNAs are synthesized during one cycle of first-stage PCR using an arbitrary ACP primer and an initial annealing temperature (50°–53°C). In other protocols, the presence of residual oligo(dT) primers used in first-strand cDNA synthesis results in high background noise because oligo(dT) can potentially anneal to all cDNAs in the reaction mixture. In our approach, oligo(dT)$_{15}$ ACP primer and arbitrary ACP coexist in the same reaction tube, but the 3′-end core region [(dT)$_{15}$] of oligo(dT)$_{15}$ ACP cannot anneal to the first-strand cDNAs at the initial annealing temperature due to the lower annealing temperature required. However, such annealing temperature does permit the 3′-end core sequence (10-mer) of the arbitrary ACP to anneal to a specific template site. This is one of the unique features of GeneFishing technology; because of the selective hybridizing ability of the ACP system during first-stage PCR, background from residual oligo(dT) primers can be eliminated. Second-strand cDNAs are then amplified during second-stage PCR at a second annealing temperature (65°C), which are high-stringency conditions, using the sequences at the 3′ and 5′ ends of the second-strand cDNAs as the templates for the amplification priming sequences. During the second-stage PCR, the 3′-end core region sequences alone of the oligo(dT)$_{15}$ ACP or the arbitrary ACP primer cannot anneal to the cDNA templates in such high-stringency conditions, another selective hybridization feature of GeneFishing technology. Consequently, second-strand cDNAs can be amplified at almost the theoretical optimum of a 2-fold increase in product for each cycle of second-stage PCR amplification.

To examine the above features of the ACP system using differential display, we compared ACPs with the conventional short or longer primers. The conventional longer primers have the same structure of the ACP except for the poly(dI) linker. The conventional short oligo(dT)$_{15}$ and arbitrary (10-mer) primers were not stable enough to generate any products under conditions such as the above (Figure 1, lanes 1–3). The use of the conventional longer oligo(dT) primer was confounded with high background (Figure 1, lane 4). Further, the results with the conventional longer primers showed a quite different pattern from the results generated with ACP primers (Figure 1, lanes 6 and 9). According to data obtained from the cloning and sequencing of the amplified products, the universal (tail) sequence of the conventional longer primers was involved in primer annealing in an unpredicted manner (data not shown). Our results are consistent with the results from the amplification of a target nucleotide sequence previously described (7), in which nontarget universal sequences of the longer conventional primers are involved in primer annealing,

which results in numerous nonspecific products. Current differential display methods have problems of the anchor/anchor products or arbitrary/arbitrary products. However, the ACP system eliminated these problems because PCR products were generated by only the combination of oligo(dT)$_{15}$ ACP and arbitrary ACP, but not by either oligo(dT)$_{15}$ ACP or arbitrary ACP alone (Figure 1, lanes 7–9).

Identification of Genes Expressed Differentially During Mouse Conceptus Development

GeneFishing technology was used to detect genes that are expressed differentially during mouse conceptus development by using total RNAs isolated from 4.5 to 18.5 dpc [embryonic day (E); E4.5–E18.5] mouse conceptus tissues. From the differential expression levels of mRNA fragments as observed on agarose gels, 10 DEGs (DEG1–10) were isolated from E4.5, E11.5, and E18.5 tissues using 3 arbitrary ACPs (Figure 2A). The expression patterns of the DEGs were confirmed by Northern blot analysis (Figure 2B). Northern blot analysis showed a different expression pattern for DEG10 from the results seen on agarose gel because the low resolution of the agarose gel did not allow differentiation between two different fragments, one of which was DEG10, which were placed side by side on the agarose gel.

The use of agarose simplifies the process but generates low resolution. Such a low resolution of agarose gel does not allow the PCR products of similar size to be distinguished. However, as shown in Table 2, our approach generates PCR products with 9- to 10-mer base pair matches when the 3′-end core sequence (10-mer) of arbitrary ACP hybridizes to the first-strand cDNA. In contrast, the current differential display generates PCR products with 6- to 8-mer base pair matches (10). Accordingly, our approach minimizes the possibility of accidentally generating PCR products of similar size.

Sequence analysis showed that 5 of these 10 DEGs were known genes (Table 2). The other 5 DEGs have not been characterized although their full-length cDNA or expressed sequence tag (EST) sequences were deposited in a National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) (Table 2). Northern blot and sequence analyses suggest that DEG2 is an isoform of β-tropomyosin 2. EST analysis of DEG5 and DEG7 showed homology to caldesmon 1 and U6 small nuclear RNA (SnRNA)-associated spliceosomal-like protein LSM6, respectively. However, because the expression patterns of these DEGs during these embryo developmental stages have not been characterized in detail, further study is needed.

NCBI database searches using the full-length sequence of the predicted proteins products of DEG6 and DEG10 revealed that DEG6 has significant homology with human decidual protein induced by progesterone, but that the predicted protein product of DEG10 is not homologous with any protein

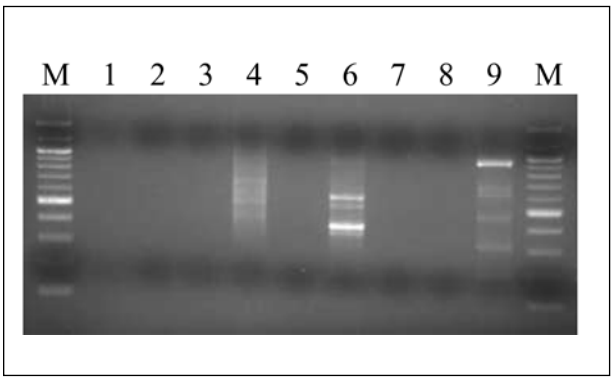

Figure 1. Comparison of conventional primer and annealing control primer (ACP) systems in differential display method. The first-strand cDNAs are synthesized from the total RNAs of mouse conceptus tissues at 18.5 [days postcoitus (dpc)] by using either (lanes 1–3) oligo(dT)$_{15}$, (lanes 4–6) oligo(dT)$_{15}$ tail, or (lanes 7–9) oligo(dT)$_{15}$ ACP. A two-stage PCR amplification was conducted using a single primer or a pair of primers. Lane 1, oligo(dT)$_{15}$; lane 2, 10-mer; lane 3, oligo(dT)$_{15}$ plus 10-mer; lane 4, oligo(dT)$_{15}$ tail; lane 5, 10-mer tail; lane 6, oligo(dT)$_{15}$ tail plus 10-mer tail; lane 7, oligo(dT)$_{15}$ ACP; lane 8, 10-mer ACP; lane 9, oligo(dT)$_{15}$ ACP plus 10-mer ACP. M represents a 100-bp size marker (generated by Forever 100-bp Ladder Personalizer; Seegene).

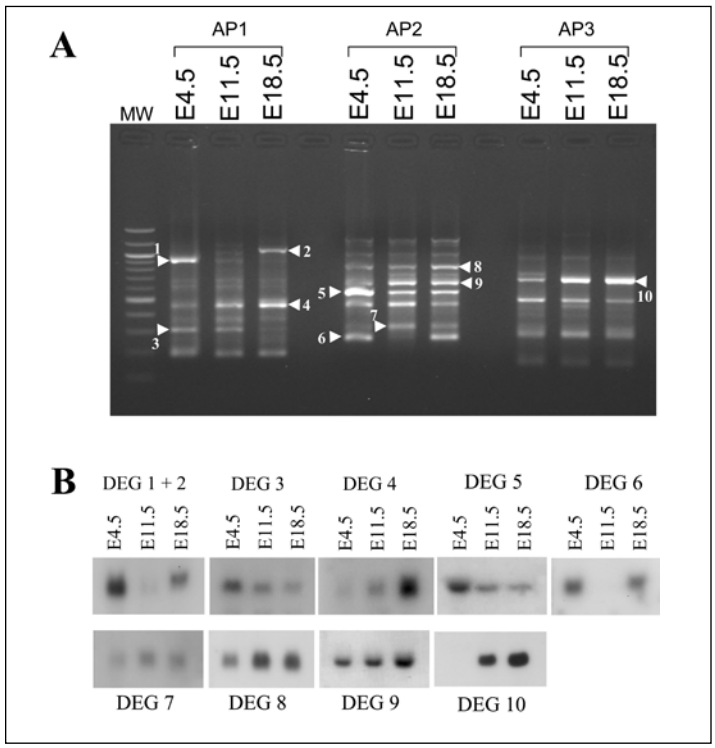

Figure 2. Results of GeneFishing PCR for the identification of differentially expressed genes (DEGs) during mouse conceptus development. (A) RNA fingerprinting on agarose gel. The two-stage PCR amplification was performed using 1 of the 3 arbitrary 10-mer ACPs in combination with oligo(dT)$_{15}$ ACP as indicated. Arrows indicate DEGs during mouse conceptus development. MW represents a 100-bp size marker generated by Forever 100-bp Ladder Personalizer. (B) Northern blot analysis of the 10 DEG clones shows their expression patterns during mouse conceptus development. Arrows indicate DEGs during mouse conceptus development. The loading controls (the lower part of each panel) show each gel before blotting, stained with ethidium bromide and photographed in UV light, and demonstrate the presence of similar levels of 18S and 28S rRNA. E, embryonic day.

Table 2. Sequence Alignment of DEGs with Known Full-Length cDNAs or ESTs

GenBank® Accession No. DEGs	Alignment of Arbitrary 10-mer ACP and Oligo(dT)$_{15}$ ACP Sequences[a]	Identity	cDNA
M87635 DEG1	145 1018 5′-**G**CCATCGACC-3′---------- poly(**A**) tail 5′-GCCATCGACC-3′----------oligo(dT)$_{15}$ ACP	β-Tropomyosin 2	Full-length
AK003186 DEG2	183 1024 5′-**G**CCATCGACC-3′----------poly(**A**) tail 5′-GCCATCGACC-3′----------oligo(dT)$_{15}$ ACP	A new isoform of β-Tropomyosin 2	Full-length
NM_011570 DEG3	1471 1711 2675 5′-**G**CCATCGACC-3′----------poly(**A**) region---------poly(**A**) tail 5′-GCCATCGACC-3′----------oligo(dT)$_{15}$ ACP	Testis-derived transcript	Full-length
NM_011619 DEG4	714 1112 5′-**G**CCATCGACC-3′----------poly(**A**) tail 5′-GCCATCGACC-3′----------oligo(dT)$_{15}$ ACP	Troponin T2	Full-length
BB609848 DEG5	210 710 5′-**A**GGAGATGCG-3′---------poly(**A**) region---------- 5′-AGGAGATGCG-3′---------oligo(dT)$_{15}$ ACP	Homology to caldesmon 1	EST
BC031533 DEG6	1093 1279 5′-**A**GGAGATGCG-3′----------poly(**A**) tail 5′-AGGAGATGCG-3′----------oligo(dT)$_{15}$ ACP	Uncharacterized	Full-length
CB589336 DEG7	373 614 5′-**c**GGAGATGCG-3′----------poly(**A**) 5′-aGGAGATGCG-3′----------oligo(dT)$_{15}$ ACP	Homology to LSM6	EST
BC043018 DEG8	85 769 5′-**A**GGAGATGCG-3′----------poly(**A**) tail 5′-AGGAGATGCG-3′----------oligo(dT)$_{15}$ ACP	T-complex testis expressed 1	Full-length
NM_153064 DEG9	1029 1608 5′-**A**GGAGATGCG-3′----------poly(**A**) tail 5′-AGGAGATGCG-3′----------oligo(dT)$_{15}$ ACP	NADH dehydrogenase Fe-S protein	Full-length
XM_129567 DEG10	1826 2436 5′-**C**TCCGATGCC-3′----------poly(**A**) tail 5′-CTCCGATGCC-3′----------oligo(dT)$_{15}$ ACP	Uncharacterized	Full-length

DEG, differentially expressed gene; EST, expressed sequence tag; ACP, annealing control primer.
[a]Numbers above the sequence indicate the position (bolded) of the nucleotide sequences of each cDNA. The 1-bp mismatch between the arbitrary 10-mer of DEG7 and the target sequence is indicated by lowercase letters.

sequences in any available sequence database. Interestingly, the corresponding genomic sequence analysis of DEG10 also showed no homologous structures in any available genomic sequence database, which included rat genomic sequence databases.

The ACP system detects fewer bands per reaction compared to current differential display methods (which generate 50–100 bands), which is a drawback that can be overcome by using more arbitrary ACPs. However, generating fewer bands and using a high concentration of dNTP (200 μM) allows researchers to use ethidium bromide-stained agarose gel to detect differentially expressed products. Current differential display methods use insufficient amounts of starting material and an insufficient concentration of dNTP (2–5 μM) to detect different banding patterns; these factors are also responsible for the low reproducibility of differential display results (11,12). In addition, because the cDNA fragments obtained from differential display are short (typically 100–500 bp) and correspond to sequences at the 3′ end of the gene that primarily represent the 3′ untranslated region, the fragments do not usually contain a large portion of the coding region. Therefore, labor-intensive screening of full-length cDNA is required, unless significant sequence homology (information for gene classification and prediction of function) is detected.

Differential display methods generally use denaturing polyacrylamide gels and radioactive detection techniques, which restricts the use of these methods to laboratories with the appropriate equipment. Relatively long exposure times and difficulty in isolating interesting bands from the polyacrylamide gels are additional drawbacks of differential display techniques. Although nonradioactive differential display methods have recently been described, including silver staining (13), fluorescence-labeled oligonucleotides (14), and the use of biotinylated primers (15) and ethidium bromide-stained agarose gels (16–19), these methods have met with only limited success. GeneFishing technology reaction products can be detected on ethidium bromide-stained agarose gel, and the results are reproducible and reliable, all of which greatly increases the speed of DEG analysis while avoiding the use of radioactivity and expensive methods of detection.

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Y.-J.K., C.-I.K., and Y.-Y.G. contributed equally to this work.

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