Differential Display Protocol With Selected Primers That Preferentially Isolates mRNAs of Moderate- to Low-Abundance in a Microscopic System

BioTechniques 20:1030-1042 (June 1996)

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ABSTRACT

A modified reverse transcription polymerase chain reaction (RT-PCR)-based differential display procedure with selected primers (SPR) was developed to increase the bias toward isolating moderate- to low-abundance transcripts that are differentially expressed during synapse formation in a microscopic neuronal system, the embryonic chicken ciliary ganglion. Major modifications, in comparison with available arbitrarily primed RT-PCR protocols, include the use of (i) *experimentally selected primer pairs (50% GC-rich 15–21-mers) that avoid the amplification of highly abundant ribosomal and mitochondrial transcripts;* (ii) *a higher PCR annealing temperature (50°C instead of 40°C);* (iii) *selection of sequencing gel bands that are dependent on the two primers for amplification;* (iv) *tests for reproducibility by SPR amplification of independent sets of RNA extractions and Southern blot analysis of the products with an isolated radiolabeled clone; and* (v) *quantitative RT-PCR, instead of Northern blot analysis, to confirm the differential expression of individual cDNAs. Thirty-six cDNAs were isolated and sequenced using SPR. None showed significant homology to highly abundant transcripts. In contrast, when no criterion for primer or band selection was applied, 22% of 55 cDNAs were identical to ribosomal and mitochondrial transcripts. Reproducible amplification of 9 out of 10 SPR-isolated cDNAs was established by Southern blot analysis. Differential expression was then confirmed for 4 selected sequences by quantitative RT-PCR. Thus, SPR is a reproducible and efficient procedure for identifying differentially regulated transcripts of moderate- to low-abundance in microscopic biological systems.*

INTRODUCTION

Identifying gene transcripts of low abundance that are differentially expressed in a microscopic system remains a technically challenging task. Changes in the expression of such transcripts underlie key developmental events, including synapse formation. Here we describe a modified reverse transcription polymerase chain reaction (RT-PCR)-based differential display protocol with selected primers (SPR) that dramatically increases the bias toward isolating moderate- to low-abundance transcripts. Moreover, SPR works effectively with small amounts of total RNA extracted from a microscopic biological system. We developed SPR to characterize changes in gene expression during synapse formation in the embryonic chicken ciliary ganglion (CG). The CG is uniquely well-suited for the study of synaptogenesis because of the feasibility of in situ surgical manipulations that prevent innervation or target tissue interactions (2,8,11,12). Quantitative RT-PCR studies show that transcripts encoding nicotinic acetylcholine receptors (AChRs), an identified synaptic component, are expressed at relatively low levels (ranging from 200 to 1000 transcript copies per cell), with 1.5-fold to 2-fold reductions occurring in these mRNA levels in CG neurons deprived of synaptic interactions (12). We now demonstrate that SPR provides the sensitivity and selectivity required to study the differential expression of mRNAs in total RNA equivalent to the amount extracted from a single CG (100 ng) from normal developing and operated embryonic day (E) 8 chickens.

SPR is a modified version of the two available arbitrarily primed RT-PCR methods: mRNA differential display (14) and RNA arbitrarily primed PCR (RAP-PCR; Reference 28). Both protocols compare RNA fingerprints by separating labeled RT-PCR products on a sequencing gel. However, the two methods differ in priming strategy. mRNA differential display uses oligo$(dT)_{12}$MN (M represents a degenerate base of A, C or G; N represents A, C, G or T) as an antisense primer to target the 3′ end of polyadenylated mRNAs and an arbitrary 10-mer (50% GC-rich sequence) as a sense primer (14). Recent modifications entail adding 10 base pairs to the primers, including a restriction site, in order to increase reproducibility, reduce false positives and aid cloning (15,29). In contrast, in RAP-PCR, a single arbitrary 20-mer

Table 1. Primer Pairs Tested with Total RNA and Standard RT-PCR

Pair Number	5′ Primer*	3′ Primer*	Target mRNA Sequence and Expected cDNA Size
A. Arbitrarily Designed Pairs			
1	catgagtggatcagaatcgtc	T_{13}	Unknown
2	acgctgttccttctt	acgatcaatgacgatggc	Unknown
3	acgctgttccttctt	aagtacgtggccatggt	Unknown
B. Specific Pairs That Target Identified Genes			
4	catgagtggatcagaatcgtc	cttgcccatttatgaataac	AChR α5 subunit (222 bp)[a]
5	ccggaattcatgaatgaggag	ctgcttcagtggaagga	GDP dissociation inhibitor (GDI-2; 373 bp)[b]
6	aatccgtgtcggagtcctca	tggcctctggagtgacatct	Gephyrin (257 bp)[c]
7	gatcacaataaacagctggag	gtgagatctgtgctggtgtc	Dystrophin (230 bp) or utrophin (218 bp)[d]
C. Arbitrary Combinations of Specific Primers			
8	Primer 5 or 6	Primer 4	Unknown

*All sequences are given in 5′ to 3′ orientation.

Primers were designed based on: [a]chicken AChR α5 sequence; [b]mouse GDI-2 sequence; [c]rat gephyrin sequence; [d]chicken dystrophin and human utrophin sequences.

(50% GC content) is used as both sense and antisense primer (28). In both methods, cDNAs are amplified using a low-stringency PCR annealing temperature (40°–42°C) throughout (14) or only in the initial cycle (28,29). Differential expression of selected cDNAs is then confirmed by Northern blot analysis, which may result in the elimination of low-abundance transcripts. Four out of nine sequenced clones isolated by RAP-PCR are characterized as highly abundant (28). A future goal of these methods is to target low-abundance transcripts more efficiently (13,16).

SPR differs from these arbitrarily primed RT-PCR protocols by the use of *(i)* experimentally selected primer pairs that avoid the amplification of highly abundant transcripts, *(ii)* a high PCR annealing temperature (50°C) in all cycles, *(iii)* band selection from the sequencing gel based on their dependence on the two primers for amplification (in addition to their differential appearance in test and control tissue lanes, and dependence on the presence of reverse transcriptase), and *(iv)* PCR-based techniques to test for reproducible amplification and confirm differential expression. Taken together, these modifications improved reproducibility of the multiple band pattern with independent RNA extractions, favored the isolation of moderate- to low-abundance transcripts (that were not detectable by Northern blot analysis, with one exception) and allowed the study of their differential expression in a microscopic biological system.

MATERIALS AND METHODS

RNA Isolation

CGs were rapidly dissected from normal developing and operated embryos at the indicated times and immediately frozen on dry ice. The surgical micromanipulations, which prevent innervation or target tissue interactions in the CG, were performed as described previously (2,8,12). Typically, 4–5 CGs were pooled per tube. Total RNA was extracted by two different scaled-down guanidinium thiocyanate protocols (6,9,12) and used without DNase treatment.

Primer Sequences

Primers (13- to 21-mers) that were used are shown in Table 1.

RT-PCR Protocol

Total RNA was heat-denatured at 65°C for 5 min, centrifuged for 2 min at room temperature and kept on ice before cDNA synthesis. The RNA (100 ng or the amount present in a single E8 CG, in 2–4 µL diethylpyrocarbonate [DEPC] water) was reverse-transcribed in a total reaction volume of 20 µL containing 1× RT buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; and 3 mM $MgCl_2$), 1 U RNase inhibitor (Promega, Madison, WI, USA), 10 mM dithiothreitol, 20 µM deoxyribonucleoside triphosphates (dNTPs), 1 µM 3′ primer and 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV-RT; Life Technologies, Gaithersburg, MD, USA). The mixture was incubated at 65°C for 5 min, followed by 42°C for 10 min prior to adding reverse transcriptase, after which incubation was continued at 42°C for 50 min, followed by 94°C for 5 min, using a programmable thermal cycler (PTC-100™; MJ Research, Watertown, MA, USA). At the end of the cycle, the tubes were spun briefly to collect condensation.

cDNAs (2-µL aliquots), synthesized in the RT step, were amplified in a 20-µL reaction volume containing the following: 1× PCR buffer (50 mM KCl; 10 mM Tris-HCl, pH 9.0; and 0.1% Triton® X-100), 1.875 mM $MgCl_2$, 1 U *Taq* DNA Polymerase (Promega), 50 µM dNTPs, 1 µM of each primer and 1.25 µCi [α-^{32}P]dCTP (Du Pont NEN, Boston, MA, USA). The samples were heat-denatured at 94°C for 5 min and amplified for 30 cycles (94°C for 30 s; 50°C for 30 s; 72°C for 1.5 min), followed by 72°C for a final 5 min to complete extension. PCR controls included either the omission of reverse transcriptase in the RT step, or 5′ primer or DNA template (RT mixture) in the PCR step.

Band Selection and Reamplification

Radiolabeled PCR products were separated on a 6% sequencing gel (14). The dried gel was exposed to X-ray film (XAR5; Eastman Kodak, Rochester, NY, USA) overnight at room temperature. Bands were selected on the basis of three criteria: *(i)* differential appearance in test and control tissue lanes (bands bigger than 250 bp without marked differences were also sampled because of co-migrating species; see Results), *(ii)* dependence on both primers for amplification (see Figure 1) and *(iii)* dependence on the addition of reverse transcriptase. Selected bands were excised after careful alignment of the autoradiogram to the gel, and the DNA was recovered (13).

Cloning and Sequencing

Reamplified PCR products were cloned using the TA Cloning® kit (Invitrogen, San Diego, CA, USA). Plasmid DNA was isolated by the Wizard™ minipreps DNA purification system (Promega). Sequence analysis was done by the dideoxy chain-termination method (Reference 23; Sequenase® Version 2.0 DNA Sequencing kit [United States Biochemical, Cleveland, OH, USA]) with T7 and SP6 promoter primers (Promega). Nucleotide and predicted amino acid sequences were tested for homology to known sequences in the DNA and protein databases of the National Center for Biotechnology Information by BLAST search (1).

Test for Co-Migrating cDNAs

Since multiple cDNAs were often observed to co-migrate in a single band, 5 to 6 plasmid DNA minipreps were routinely prepared from the cloning of each band. After sequencing the DNA from one miniprep, inserts of the same size from the other minipreps were tested for sequence heterogeneity by restriction endonuclease mapping. Inserts were excised by *Eco*RI digestion and purified from a 1% low-melt agarose gel (Geneclean® kit; Bio 101, La Jolla, CA, USA). Inserts that were not cleaved by a selected enzyme, as well as those producing unexpected fragment sizes, were subsequently sequenced. Alternatively, *Eco*RI-digested plasmid DNA was Southern blotted, and the filter hybridized to the labeled clone.

Northern and Southern Blot Analysis

Cloned cDNAs were labeled with [α-^{32}P]dCTP to high specific activity by random priming (Life Technologies, Gaithersburg, MD, USA) and purified using the CHROMA SPIN™-100 column protocol (CLONTECH Laboratories, Palo Alto, CA, USA). Total RNA or PCR products were separated by agarose gel electrophoresis (22), transferred to Zeta-Probe® membrane by alkaline blotting, hybridized and washed as specified by the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA).

Relative RT-PCR

To confirm the differential expression of specific transcripts by relative RT-PCR, primers and reaction conditions were optimized to obtain a single band corresponding to the target sequence on a 2% agarose gel stained with ethidium bromide. The 5′ primer was extended inwards or replaced with an internal sequence, and the PCR annealing temperature was increased to 63°C. The identity of the amplified product was confirmed by restriction endonuclease mapping. cβ4-tubulin mRNA was used as a standard since the levels of this transcript are not signifi-

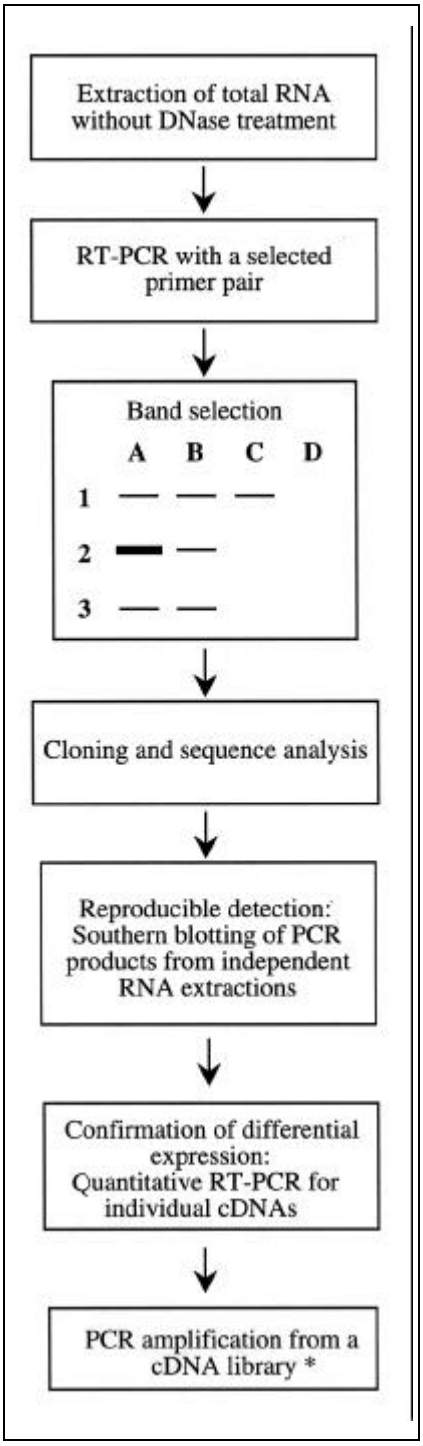

Figure 1. Schematic flowchart of the RT-PCR-based differential display protocol with selected primers (SPR). A detailed description of each step is given in the text. Total RNA from test and control tissue is amplified by RT-PCR with a selected primer pair, and the products are separated on a sequencing gel. A resulting autoradiogram is depicted to demonstrate the band selection approach. Lane A, control tissue; lane B, test tissue; lane C, the same as A except that the 5′ primer is not added in the PCR step; and lane D, the same as A except reverse transcriptase is omitted in the RT step. Band 1 is dependent on reverse transcription but appears in lane C, suggesting that it contains sequences that are amplified by the 3′ primer alone. Band 2 has a differential appearance in test and control tissue lanes, and is dependent on the two primers for amplification. Such bands were selected for reamplification, cloning, heterogeneity analysis and further testing to confirm differential expression of the individual cDNAs. Band 3 is similar in control (A) and test (B) lanes. Occasionally, such bands were also sampled to look for possible reciprocal changes in comigrating sequences that may mask an alteration in the intensity of the total signal. In particular, bands larger than 250 bp often contained comigrating sequences. *Optional step, included to establish that the differentially amplified cDNAs correspond to mRNAs that are indeed expressed in the embryonic chicken CG.

cantly affected by the surgical manipulations (12). Briefly, aliquots of the same CG RNA were amplified using the optimized primer pair or cβ4-tubulin-specific primers (12). Care was taken to remain in the exponential phase of amplification for each sequence (5). Twenty cycles were used for cβ4-tubulin amplification, whereas 30 cycles were typically used (sometimes with larger amounts of RT mixture) for the cDNAs of interest. The [α-^{32}P]dCTP-labeled PCR products were run on a 2% agarose gel, visible bands were dissected and their radioactivity counted. The ratio between the amount of cDNA in the test versus control sample was compared to the ratio for cβ4-tubulin. In cases where the cDNAs of interest were not visible on the agarose gel, Southern filters of the amplified product were hybridized to the radiolabeled cDNA clone. The ratio of the products in test and control CG lanes was then determined by densitometric scanning (pdi Discovery Series Model DNA 35™ Automated Scanning System, Protein DNA Image Ware Systems; Huntington Station, NY, USA) of the final autoradiogram.

PCR Amplification of an Embryonic CG cDNA Library

A λgt10 cDNA library from E18 CG (kindly provided by Dr. Tom Boyd, Ohio State University) was plated at high density. Phage DNA was extracted from the plate lysates by the Prep-EZE™ Column kit (5 Prime→3 Prime, Boulder, CO, USA) and resuspended in 50 μL TE (10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, pH 8.0) per plate. Two microliters were used for amplification in the optimized PCR protocol.

A flow-chart summary of the procedures and band selection method is depicted in Figure 1.

RESULTS

RT-PCR Band Pattern with Different Primer Pairs

Three groups of primer pairs (Table 1) were tested in our standard RT-PCR protocol with total CG RNA in an amount equivalent to that extracted from a single ganglion (see Materials and Methods). The three groups included arbitrarily designed primer pairs (2 and 3), specific primers that target identified genes (pairs 4, 5, 6 and 7) and specific primers used in arbitrary combinations (pair 8). Individual primers had approximately 50% GC content. In addition, several combinations of T_{13} or T_{13}MN (14) and various 5′ primers (including arbitrary 10-mers) were also used (primer pair 1, for example). Multiple bands appeared in all experiments following separation of the radiolabeled PCR products on a sequencing gel and overnight exposure. Interestingly, multiple bands were even observed with specific primers that gave a single visible product on 2% agarose gels.

The multiple band pattern was primer sequence-dependent. The number of visible bands ranged from 10 to 80, with a mean of 34 ± 7 (SEM), based on RT-PCR amplification with 10 different primer pairs and overnight exposure of the autoradiograms (Figure 2). No differences were observed in the number of bands obtained with specific primer pairs as compared to arbitrary primers. Importantly, the band pattern was reproducible with different RNA samples, the same RNA sample in separate experiments and CG RNA that was extracted by two different techniques (see Materials and Methods), all amplified with the same primer combination.

Two general band patterns were distinguished on the autoradiograms. Specific primer pairs (4, 5 and 6) produced a predominant band of the expected size accompanied by a few weaker bands (Figure 2A). In all other cases, several bands of similar strength were observed (Figure 2B).

Band Selection, Heterogeneity and Sequence Analysis

Using all three types of primer pairs (Table 1), we isolated 55 cDNAs on the

basis of their differential appearance in synapse-deprived and control CG lanes and their dependence on the addition of reverse transcriptase. Analysis of these sequences resulted in an important observation. The majority (80%) of the highly abundant transcripts isolated (12 in total) were amplified by a single primer, and that primer had a high (50%–65%) GC content at the 3′ end. By applying primer and band selection, it is possible to avoid the isolation of high-abundance mRNAs.

Arbitrarily Designed Primer Pairs

Initially, we compared the sequence of cDNAs that were amplified with arbitrarily designed primers having 50% GC content (pairs 2 and 3; Table 1) to those amplified by oligo(dT)$_{13}$ and different 5′ primers having 50% GC content (for example, pair 1). Nineteen cDNAs were isolated using primer pairs 2 and 3. Fourteen of the cDNAs were dependent on the two primers for amplification. One sequence is identical to the first developmentally expressed exon of the embryonic chicken myosin heavy chain gene (17), whereas the remaining 13 cDNAs have little homology to known sequences. In contrast, the other 5 isolated cDNAs were amplified by the 3′ primer alone, and 4 of them are identical to mitochondrial and ribosomal sequences.

With primer pair 1, 12 cDNAs were isolated from the 4 major bands on the sequencing gel (Figure 2B), demonstrating that multiple cDNAs co-migrate in single bands. All of the cDNAs had the 5′ primer at both ends, and 6 were identical to ribosomal and mitochondrial sequences. Similar results were obtained using T_{13}MN and 5′ primers having 50% GC content (see also References 10 and 27).

To avoid highly abundant transcripts, we subsequently selected only PCR products that are dependent on the two primers for amplification. In total, 60% of the cDNAs that were amplified by a single primer are highly abundant transcripts (see also Reference 28). In contrast, only 5% of the products that were dependent on the two primers are highly abundant sequences. Thus, band

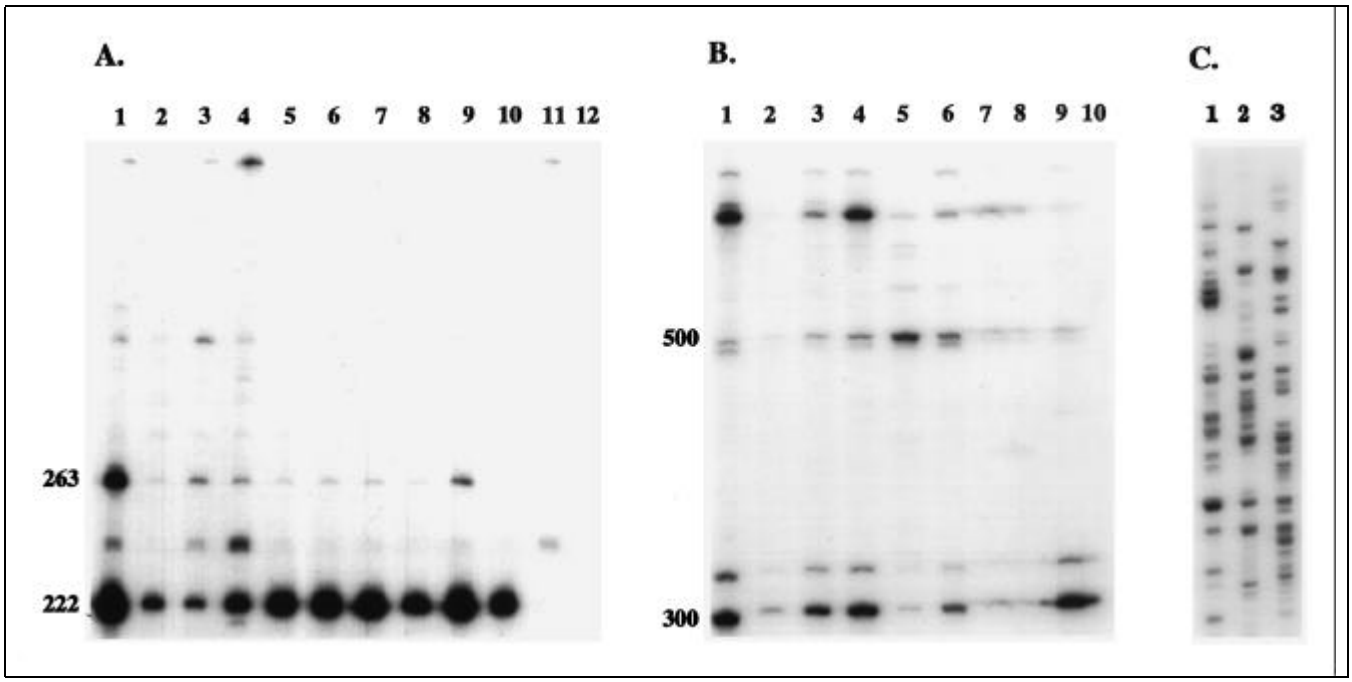

Figure 2. Autoradiograms demonstrating the band pattern obtained after RT-PCR with specific and arbitrary primer pairs, and separation of the [α-^{32}P]dCTP-labeled products on a 6% sequencing gel. Multiple bands were detected after overnight exposure. Although the autoradiograms in A and B have the fewest bands observed, they demonstrate the difference in the patterns obtained with specific vs. arbitrary primers. *Panel A*: RT-PCR with specific primer pair 4 (see Table 1) and total RNA from different tissues of E8 chickens: lane 1, normal developing CGs; lane 2, brain; lane 3, liver; lane 4, heart; lanes 5, 7 and 8, CGs without innervation (I); lanes 6 and 9, CGs without target (T); lane 10, CGs without I and T; lane 11, control without reverse transcriptase; lane 12, control, PCR without template (no RT mixture). The expected 222-bp band is the predominant product. It is 100% identical to the targeted AChR α5 subunit sequence. The 263-bp band was also isolated and sequenced. This clone, M691, has a single open reading frame, no significant homology to known DNA sequences and 53% similarity to the rod domain of human utrophin at the amino acid level. Overall, the appearance of the 222-bp band in the different lanes (after suitable shorter exposure of the gel) is in good agreement with quantitative analyses of α5 mRNA levels by competitive RT-PCR with a mutated α5 cRNA internal standard (12). Specifically, α5 is present at 200 transcript copies per E8 neuron, with a 1.6-fold reduction in input-deprived neurons. These findings establish the reliability and sensitivity of SPR, as well as demonstrating that comparable amounts of total RNA were used for PCR amplification in the different samples. *Panel B*: RT-PCR with primer pair 1 (T_{13} and an arbitrarily designed 5′ primer having 50% GC content) and total RNA from different tissues of E8 chickens: lanes 1 and 4, normal-developing CGs; lanes 2 and 5, CGs from embryos without I and T; lane 3, CGs without I; lane 6, CGs without T; lane 7, brain; lane 8, liver; lane 9, heart; lane 10, PCR without template. The 4 strong bands ranging from 300 to 1000 bp were excised. Twelve cDNAs were isolated from these bands and sequenced. All of the cDNAs were surrounded by the 5′-primer sequence. Five were identical to different fragments of the chicken mitochondrial genome, and one was identical to 28s ribosomal RNA. *Panel C*: RT-PCR with the arbitrary 3′ primer from pair 2 (see Table 1) and T_{13}, and total RNA from E8 chicken tissues: lane 1, normal developing CGs; lane 2, brain; lane 3, CGs without T. This autoradiogram is representative of the mean number of bands typically obtained in SPR.

selection dramatically increases the efficiency of isolating moderate- to low-abundance transcripts. This selection is accomplished by comparing the band pattern when PCR is also carried out in the absence of the 5′ primer. Avoiding products that are dependent only on the 5′ primer was not attempted because of the presence of excess 3′ primer in the RT mixture, and subsequently in the PCR reaction. Moreover, none of the cDNAs isolated with primer pairs having 50% GC content were dependent on the 5′ primer alone.

Specific Primer Pairs That Target Identified Genes

Results with arbitrarily designed primer pairs 2 and 3 suggested that specific primers may also be effective in amplifying multiple sequences, with the targeted sequence being the predominant product (Figure 2A). Fifteen cDNAs were isolated with specific primer pairs 4, 5 and 6. Three of them are the targeted sequences, 3 are homologous to other known sequences, whereas the remaining 9 have little homology to DNAs in the databases.

Specifically, the expected 222-bp fragment amplified by primer pair 4 is 100% identical to chicken AChR α5 subunit mRNA (12). The 373-bp cDNA amplified by primer pair 5 has 81% sequence identity to mouse Rab GDP-dissociation inhibitor 2 (GDI-2) mRNA (24). The 257-bp product from primer pair 6 is 92% identical to rat gephyrin mRNA (20).

Several co-migrating species were also isolated, with more sequences co-migrating with the larger products. Four other sequences, ranging in size from 366 to 373 bp, co-migrated in the expected 373-bp band. One additional sequence was isolated from the 257-bp band. This cDNA has a single open reading frame and is highly homologous to a clone isolated recently from a human fetal cDNA library (gb/T79520 and T79432). In comparison, the 222-bp band contained only the targeted sequence, as confirmed by restriction analysis, Southern blotting and sequencing of 6 minipreps.

Finally, 7 cDNAs were isolated from 4 other weaker bands on the sequencing gels. Two of them were amplified by primer pair 6, and are 75% and 78% homologous, respectively, to the 3′ end of the chicken POL-like gene (4).

However, not all specific primer pairs were useful. Primer pair 7, corresponding to the conserved 3′ end of dystrophin and utrophin mRNAs (Table 1), resulted in the amplification of several strong bands that were not the expected sizes. Two cDNAs were isolated that were dependent on the two primers for amplification, but are identical to mitochondrial transcripts. Thus, primer pairs must be tested experimentally in order to determine whether they avoid the amplification of highly abundant sequences.

Arbitrary Combinations of Specific Primers

Specific primers that avoid the amplification of abundant transcripts can also be used in arbitrary combinations, as established with primer pair 8 (for example, combining antisense primer 4 with sense primer 5 or 6). Seven cDNAs have been isolated so far. One is identical to chicken (Na^+/K^+)-ATPase β subunit mRNA (26). The remaining 6 have little homology to known sequences.

In summary, dramatically different outcomes were obtained using different primer pairs to amplify total CG RNA in our standard RT-PCR protocol. With primer pairs 1 and 7, mitochondrial and ribosomal sequences were frequently isolated. When selected arbitrary and specific primer pairs 2, 3, 4, 5, 6 and 8 were used, and only products that were dependent on the two primers were chosen, none of the 36 isolated sequences had significant homology with highly abundant known transcripts.

Reproducible Detection of Differentially Amplified Moderate- to Low-Abundance Transcripts

A subset of the isolated cDNAs were analyzed for reproducible amplification and differential expression during synapse formation. Initial experiments suggested that Northern blot analysis would not provide the sensitivity required to detect these transcripts because of their low abundance, particularly at E8, and limitations in amounts of total CG RNA imposed by the need

for embryonic surgeries. In particular, 10 cDNAs were labeled to high specific activity and hybridized to Northern filters with total RNA from E8 normal developing CGs (2–3 μg) and brain and liver (both at 5–10 μg). No appreciable signals were detected after 7 days of exposure with an intensifying screen at -70°C, with one exception. Blots hybridized to the 373-bp GDI probe showed a weak signal at 2.5 kb in CG and brain RNA. In contrast, when hybridized to a 340-bp 18s chicken ribosomal probe, the same filters revealed a strong signal after a 2-h exposure at room temperature. Moreover, AChR α5 subunit mRNA, included as a control since it is known to be present at 200 transcript copies per neuron (12), was not detected on these Northern filters.

To achieve greater sensitivity and establish reproducibility, radiolabeled cDNAs were hybridized to Southern filters of RT-PCR products that were amplified from independent RNA extractions with the same primer pair used to generate the cDNA clone. Nine out of the ten cDNAs resulted in a single band of the expected size. Four of these cDNAs were analyzed in multiple RT-PCR experiments, and differential amplification was reproducibly observed. As an example, in Figure 3A, the level of clone L2A appears to be reduced in target-deprived as compared to normal developing CGs. Equivalent amounts of total RNA from CGs of operated and control embryos were used, as demonstrated by RT-PCR with specific primers for cβ4-tubulin transcripts, which are not significantly affected by synapse deprivation (Figure 3C). Thus, SPR provides the sensitivity and selectivity required to reproducibly detect moderate- to low-abundance transcripts that appear to be differentially expressed.

Quantitative RT-PCR as a Confirmation of Differential Expression

Relative RT-PCR was used to confirm changes in the levels of 4 selected transcripts in response to synapse formation. Primers and reaction conditions were optimized to amplify only the targeted sequence, as determined by 2% agarose gel electrophoresis, restric-

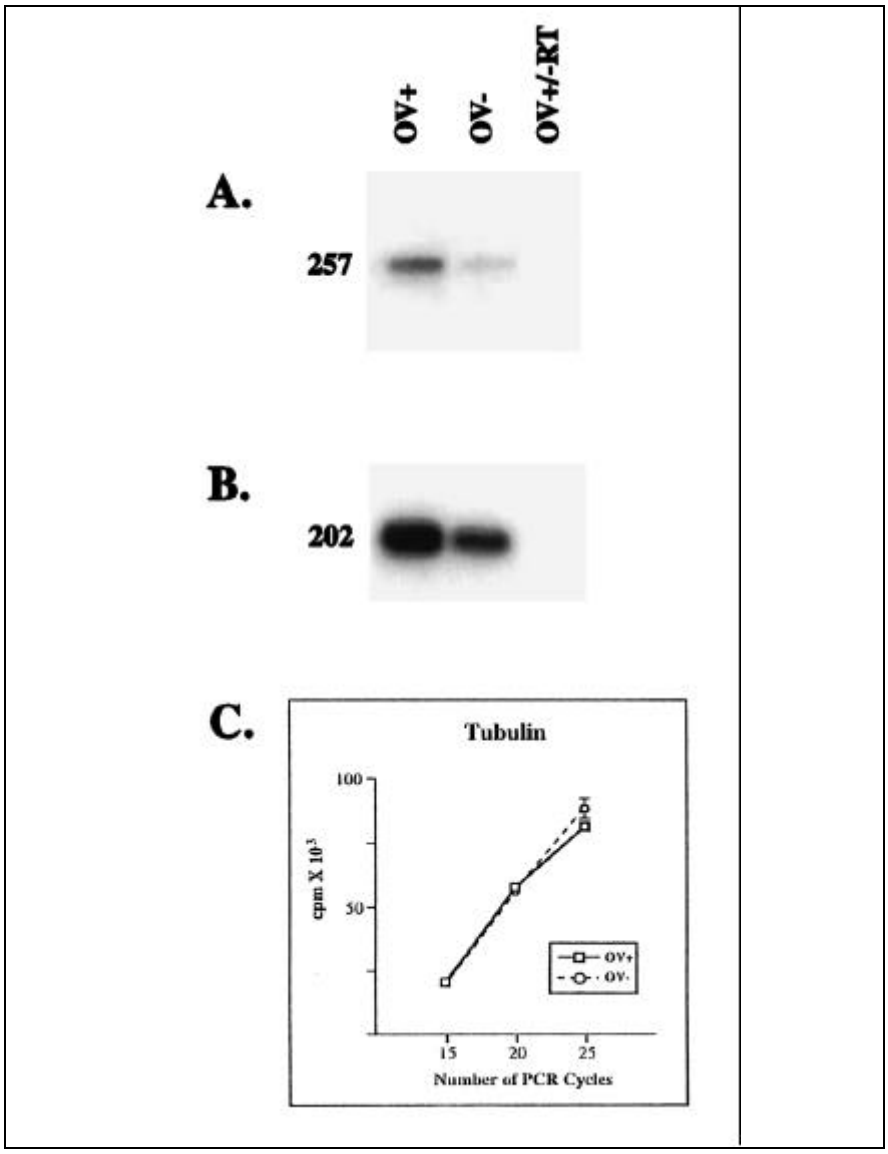

Figure 3. Differential expression of two transcripts in target tissue-deprived CGs. *Panel A*: Amplification and differential appearance of clone L2A was reproducibly observed using multiple independent RNA extractions and Southern blot analysis. Target tissue-deprived (OV-) and contralateral control (OV+) CGs were dissected from 4–5 E8 embryos. Aliquots of total RNA, equivalent to the amount present in a single ganglion, were amplified by RT-PCR with primer pair 6. The products were blotted, and the Southern filter hybridized to the radiolabeled L2A clone, which was isolated in an earlier SPR experiment with the same primer pair. Reproducible amplification of the L2A transcript is established by the presence of the expected 257-bp band, including confirmation of its identity by restriction endonuclease mapping (not shown here). The absence of the band in the control lane without reverse transcriptase (-RT) demonstrates that it is amplified from mRNA, not genomic DNA. The decline in L2A mRNA levels in target tissue-deprived as compared to contralateral control CGs was consistently observed, suggesting that it is differentially expressed. *Panel B*: Differential expression of clone M691 was confirmed by relative RT-PCR using cβ4-tubulin mRNA as a standard. Aliquots of the same RNA samples as in 3A were subjected to optimized RT-PCR, resulting in the amplification of a single band of the expected size (202 bp) on a 2% agarose gel. Optimization included the use of a new internal sense primer, based on the sequence of the cloned 263-bp cDNA (see Figure 2A), a higher PCR annealing temperature (63°C as opposed to 50°C), 4 μL of RT mix instead of the routine 2 μL and amplification in the exponential range (30 PCR cycles). M691 mRNA levels are lower in CGs deprived of target-tissue interactions. *Panel C*: In contrast, cβ4-tubulin mRNA is present in equal amounts in aliquots of the same RNA samples, as determined by radioactive counting of dissected cβ4-tubulin cDNA bands after 15, 20 and 25 cycles of specific RT-PCR amplification in the presence of [α-^{32}P]dCTP (see Materials and Methods for experimental details). Each value represents the mean ± SEM for triplicate samples. This result confirms previous observations that cβ4-tubulin mRNA levels in the CG are not significantly affected by synapse formation (12) and establishes the specificity of reductions in M691 transcript levels.

tion endonuclease mapping and sequence analysis. Figure 3B shows the decrease in clone M691 transcript levels in target-deprived as compared to the contralateral control E8 CG, as an example. cβ4-tubulin mRNA was used as a standard, and both transcripts were amplified in the exponential range from aliquots of the same total RNA sample (Figure 3C).

Amplifying Isolated Sequences From an Embryonic CG cDNA Library

To establish that the transcripts of interest are indeed expressed in the embryonic CG, optimal PCR conditions were used to amplify these sequences from phage DNA that was extracted from a plated λgt10 E18 CG cDNA library. Three out of four selected sequences were detected, as confirmed by restriction endonuclease mapping and Southern blot analysis.

DISCUSSION

This study demonstrates that SPR is useful for preferentially identifying differentially expressed transcripts of moderate- to low-abundance in small amounts of total RNA extracted from a microscopic biological system. SPR combines the primer design (50% GC content) and reaction conditions of specific RT-PCR with the sensitivity of PCR product separation on a sequencing gel developed for arbitrarily primed RT-PCR differential display. Reproducible amplification and differential expression of specific transcripts is then confirmed by RT-PCR-based techniques.

Comparison with Other RT-PCR-Based Differential Display Approaches

SPR is a modification of the arbitrarily primed RT-PCR methods for identifying differentially expressed mRNAs (13,28) that were developed as a simpler and faster alternative to the screening of subtracted cDNA libraries (18,19). SPR differs from the two original RT-PCR-based differential display protocols in the use of *(i)* a higher PCR annealing temperature, *(ii)* selected primers (see below), *(iii)* the selection of bands that are dependent on both primers for amplification and *(iv)* quantitative RT-PCR to confirm differential expression.

The use of a higher annealing temperature increased the selectivity of PCR amplification and improved reproducibility of the band pattern. However, it resulted in a diminished number of bands, as previously observed (3). Although fewer products are obtained, SPR dramatically increases the efficiency of isolating moderate- to low-abundance transcripts.

Primer and band selection resulted in the isolation of 36 cDNAs that are not homologous to highly abundant genes. In RAP-PCR, 4 out of 9 isolated cDNAs are highly abundant and 33% of the sequences have a single open reading frame (28). In SPR, 65% of the unknown sequences have a single open reading frame. Limited cDNA sequence analysis or abundance information is available for differentially expressed cDNAs isolated by mRNA differential display. However, 9 out of 15 bands are detected by Northern blot analysis (14), suggesting that a portion of the products correspond to transcripts of moderate- to high-abundance (but see also Reference 10). In contrast, in SPR no signal was detected by Northern blot analysis with 9 out of 10 tested cDNAs. Included as a control, the 222-bp AChR α5 cDNA, present at 200 transcript copies per E8 CG neuron, was not detected (12). Although the sensitivity of our Northern blot analysis was limited by the amount of RNA and the size of the probes, signals were readily detected with a similarly sized cDNA of ribosomal origin. These results suggest that SPR favors the isolation of moderate- to low-abundance transcripts. For this reason, only RT-PCR-based techniques were used to establish reproducible amplification and differential expression of these sequences.

Necessity for Experimental Selection of Primer Pairs

Primer selection is the key variable that increases the bias toward amplifying moderate- to low-abundance transcripts in SPR. Both arbitrary and specific primers that have 50% GC content

were effective. In particular, primers with a relatively low GC content (<50%) at the 3′ end worked best, and were even effective as a single primer in amplyfing only moderate- to low-abundance sequences [compare the 3′ ends of the reverse primers from pairs 4, 5 and 6 to those from 2, 3 and 7 in Table 1; also, data not shown; (21)].

Overall, our results demonstrate that primers that avoid the amplification of highly abundant transcripts must be selected experimentally. Theoretically, suitable primers may be designed that lack significant homologies to highly abundant known sequences using available computer programs. However, specific primer pair 7, which targeted the conserved end of dystrophin and utrophin mRNAs, amplified mitochondrial transcripts. Moreover, analysis of isolated cDNA sequences shows that only a 6-bp perfect match between the 3′-end of the primer and the sequence is sufficient for priming, even with the greater selectivity achieved by using a high PCR annealing temperature [50°C, this study; (25)]. The large 16 775-bp intronless sequence of the chicken mitochondrial genome greatly increases the likelihood of such matches (7). Thus, experimental testing of primers is necessary. However, specific primers already known to avoid the amplification of highly abundant sequences can be readily used in target-predicting or arbitrary combinations.

PCR Amplification of Non-Predicted Sequences with Specific Primers

The ability of specific primer pairs to amplify several unexpected cDNAs, in addition to the targeted sequence, is surprising in light of the accepted high specificity and selectivity of RT-PCR. It should be noted that only a single visible band was detected after separating these PCR products on a 2% agarose gel stained with ethidium bromide. The same holds true when the radiolabeled products are separated on a 6% sequencing gel and exposed to X-ray film for a short period of time to avoid saturating the major signal. The weaker bands only appeared after overnight exposure, with more bands being visualized after longer exposures. The cDNAs were dependent on the presence of reverse transcriptase and were reproducibly amplified in different RNA samples. These weak bands should therefore be attributed to the limits of primer specificity under the conditions of our RT-PCR protocol. Our successful use of specific primers in arbitrary combinations supports this conclusion. Thus, the identification of non-predicted, differentially expressed genes appears to be a highly reproducible extension of specific RT-PCR applications. Moreover, in the case of

specific primer pairs, the targeted sequence serves as an internal standard for quality and quantity of RNA, which is important when small amounts of total RNA are used (Figure 2A).

In summary, SPR is a useful modification of the available differential expression methods and has two distinguishing features—the predominant sampling of moderate- to low-abundance transcripts and the applicability to microscopic biological systems.

ACKNOWLEDGMENTS

We thank Drs. H. Bayley and A. Ross for critically reading the manuscript; Dr. A. Shisheva for GDI-2 primers and discussions; Dr. M. Schwartz Levey for discussions and help throughout this study; and our summer students K. My Ly, M. Kulesa, M. Kostov and L. Schoening for technical assistance and successful use of the protocol. This work was supported by NIH Grant NS-21725.

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Received 30 June 1995; accepted 7 November 1995.

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