A rapid and simple PCR-based method for isolation of cDNAs from differentially expressed genes

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ABSTRACT

Recently two techniques have been reported which use arbitrarily primed RT-PCR amplification of cDNA fragments from subsets of mRNAs to detect cDNA fragments from differentially expressed mRNAs. Here we report a simple and rapid PCR-based protocol to both detect and isolate cDNA fragments of up to 3000 base pairs from differentially expressed genes in two easy steps. To generate cDNAs from most mRNAs, the first step consisted of reverse transcription using a fully degenerate 6-mer oligonucleotide as primer. The second step consisted of PCR amplification of internal regions of the cDNAs with two or three longer primers with arbitrary but defined sequences. DNA fragments were easily displayed by agarose gel electrophoresis and then excised for direct use in cloning, sequencing, and Northern blot analysis. By repeating the PCR amplification (second step) on the same cDNA templates (first step) ten times with different sets of primers, over 170 discrete cDNA fragments were obtained from a single tissue. By combining the two-step procedure with 3'-RNA-anchored cDNA extension, additional DNA fragments can be generated from the same mRNA. The new procedure was used here to define 3600 bp of a new brain-specific mRNA.

INTRODUCTION

Changes in the levels and patterns of gene expression drive the major biological processes of living organisms. Several procedures have been proposed to identify differentially expressed genes by techniques such as subtractive hybridization of mRNAs and cDNAs (1). Recently, two general techniques have been reported which used arbitrarily primed RT-PCR amplification of cDNA fragments from subsets of mRNAs followed by separation on a DNA sequencing gel to detect cDNA fragments from differentially expressed mRNAs (2–4). The specific cDNA fragments were then eluted and re-amplified for further characterization. The techniques are useful, but they are laborious and time-consuming and provide DNA fragments of only 100–600 bp. Here we report a simple and rapid PCR-based protocol to both identify and isolate cDNA fragments of up to

3000 bp from differentially expressed genes in two easy steps. The first step consisted of reverse transcription of mRNAs using a fully degenerate 6-mer oligonucleotide as a primer under conditions that transcribed internal sequences of most mRNAs into cDNAs. The second step consisted of PCR amplification of internal regions of the cDNA with two or three longer primers with arbitrary but defined sequences under conditions in which 15–30 distinct bands of DNA fragments were easily displayed by agarose gel electrophoresis and then excised for direct use in cloning, sequencing and Northern blot analysis. By repeated PCR amplification of the cDNAs obtained in this first step with different primers for the second step, a large number of different DNA fragments were isolated from the same mRNA sample. In addition, the sequences of the 3'-ends of the internal fragments obtained with the two-step procedure were used to design primers for polyA-anchored PCR and thereby to generate additional DNA fragments from the same mRNA.

MATERIALS AND METHODS

Oligonucleotides were synthesized for us by Dr Kim De Riel (Temple University School of Medicine, Philadelphia, PA). Human polyA⁺ mRNA from brain, skeletal muscle, small intestine, trachea, fetal lung and adult lung were purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The commercially obtained polyA⁺ mRNA was isolated by the guanidinium isothiocyanate method (5) followed by polyA⁺ selection on columns of oligo(dT)–cellulose (6). The mRNAs were used to synthesize cDNAs using a commercial kit (SuperScript; GIBCO BRL, Grand Island, NY).

For the first step of the procedure (reverse transcription), a 40 µl reaction contained 520 ng of mRNA, 100 ng of fully degenerate 6-mer oligonucleotide, 500 µM of each of the four nucleotide triphosphates, 2.5 mM MgCl₂, 100 µg/ml BSA, 30 units of RNase inhibitor (Perkin-Elmer Cetus, Norwalk, CT) and 400 units of reverse transcriptase (SuperScript II RNase H⁻; GIBCO BRL, Grand Island, NY). The reaction was carried out at 20°C for 15 min, 42°C for 50 min, and 99°C for 6 min, and then at 95°C for 5 min. The sample was digested with ribonuclease H by adding 4 units (SuperScript kit) and incubating at 37°C for 30 min. For the second step (PCR amplification),

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a 100 μ l reaction contained 3 μ l of cDNA solution from the first step (about 40 ng), 5 units of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), 200 μ M of each of the four nucleotide triphosphates, 1.5 μ M $MgCl_2$ and 50 pmole of each of two or three oligonucleotide primers with a defined but arbitrary sequence (Table I). The thermocycling was at 94.5°C for 1 min, 34°C for 1 min and 72°C for 1 min. Typically, 45 cycles were employed, followed by 10 min 72°C.

For analysis of the PCR products, the samples were electrophoresed in a 2% gel prepared either with a low melting or regular agarose containing 2–5 μ g of ethidium bromide per 100 ml. DNA bands were visualized under long-wavelength ultraviolet light and cut from the gel. The DNA from the gel pieces was isolated either by heating the low melting gel at 65°C for 10 min and adding 3 volumes of H_2O or by extraction of the regular agarose by freezing and thawing at –20°C followed by filtration through low binding 0.45 μ M cellulose acetate centrifuge filter units (Spin-X™; Costar, Cambridge, MA).

To clone the PCR products eluted from the gels, a commercial kit (TA Cloning™; Invitrogen, San Diego, CA) was employed. Clones were screened by blue–white changes. Positive clones were grown in 1 ml of LB media and plasmid DNA was isolated and then sequenced by the dideoxynucleotide method (Sequenase 2.0; USB, Cleveland, OH) or used for Northern blotting (6).

Tissue specificity of isolated cDNA fragments was assayed by Northern blotting (6) using commercial filters. One Northern blot contained approximately 2 μ g of polyA⁺ mRNA per lane from eight different human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas), and a second Northern blot contained polyA⁺ mRNA from spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocyte (MTN blots I and II; Clontech Laboratories, Inc., Palo Alto, CA).

The sequence of the 3'-end of cDNA was obtained with a commercial kit for 3'-RNA-anchored cDNA extension (3'-RACE system; GIBCO BRL, Grand Island, NY). The primers were [adapter primer GGCCACGCGTCGACTAGTAC(T)₁₇], and [reverse primer CUACUACUACUAGGCCACGCGTCGACTAGTAC]. Forward primers were RACE-1-GGCTCACTTAGCATTAGGATA and RACE-2-CACAGAAGAGTGTGAACTGG. Reverse transcription was carried out at 37°C for 60 min. The PCR thermocycling was at 94.5°C for 1 min 10 sec, 54°C for 1 min, and 72°C for 2 min 30 sec for 25 cycles followed by 15 min at 72°C.

RESULTS

In preliminary experiments we tested the procedures of Liang and Pardee (2) and of Welsh *et al.* (4) to isolate cDNAs from differentially expressed genes. Although the methods were useful, we encountered several limitations. (a) Because the DNA fragments were separated on a DNA sequencing gel, the procedures were limited to fragments of less than about 600 bp. (b) The initially isolated PCR products had to be re-amplified once or twice (2,4) in order to generate adequate amounts for Northern blot assays, cloning, sequencing, etc. We found, however, that re-amplification did not succeed for many fragments and re-amplification by PCR frequently generated multiple bands of cDNAs. (c) The procedures of elution of DNA fragments from DNA sequencing gels, re-amplification, verification and re-purification of DNA fragments required use of radioisotopes and were relatively time consuming. For these

Figure 1. Agarose gel electrophoresis of RT-PCR products obtained with the two-step procedure from mRNA isolated from fetal lung, adult lung, trachea, small intestine, skeletal muscle and brain. The primers used for PCR amplification were a mixture of three 10 nt primers (BS52-CAAGCGAGGT, BS54-AACGCGC-AAC and BS55-GTGGAAGCGT). Arrows indicate brain-specific fragments. Arrow marked C indicates brain-specific fragment of about 1080 bp.

Figure 2. PCR amplification of the same cDNA templates from lung with ten different sets of primers. Lane 1: primers BS52 and BS54; Lane 2: BS54 and BS55; Lane 3: BS52, BS54 and BS55; Lane 4: BS57 and BS58; Lane 5: BS61 and BS62; Lane 6: BS70 and BS73; Lane 7: BS71 and BS74; Lane 8: BS76 and BS78; Lane 9: BS77 and BS79; Lane 10: BS72 and BS75 (see Table I).

reasons, we developed a two-step procedure whereby cDNAs of up to 3000 bp were readily isolated by agarose gel electrophoresis and the yields of the products were sufficient so that DNA bands of interest could be used directly for Northern blot analysis, cloning and nucleotide sequencing.

Selection of primers

In initial experiments we used the same 10- or 18-mer primers in both reverse transcription and subsequent PCR. Only a few

Figure 3. *EcoRI* digestion of 11 plasmids recovered after cloning of brain specific DNA fragment C (see Fig. 1).

Figure 4. Northern blot hybridization using as a probe a cloned brain-specific cDNA (upper band C of about 1080 kb in Fig. 1).

discrete bands with low yield and very high background were seen when the products were analyzed by electrophoresis in agarose gels, apparently because all the cDNAs obtained by reverse transcription contained the sequences of the primers and, therefore, were amplified in the subsequent PCR. As a result, too many fragments were generated. To generate discrete DNA bands, we chose to use a short 6-mer primer with a fully degenerate sequence for the first step (reverse transcription) and then to use larger 10- to 23-mer primers with arbitrary but defined sequences for the second step (PCR amplification). The criteria used to design the primers for PCR amplification of the cDNAs were: (a) no stop codon was present in any possible reading frame in both directions; (b) the sequences had a minimal tendency to form internal loops; (c) the sequences included the most commonly employed codons in mammals; (d) there was a minimal overlap in sequences among primers; (e) the GC content should be 60–70%; and (f) there was a minimal number of glycine codons so as to avoid over-representations of the collagens that are abundant in many mammalian tissues. The criteria were readily met for primers that were 13-mer or less (Table I). They were more difficult to satisfy with longer primers.

Optimal conditions for the second step

In optimizing conditions for the procedure, our goal was to generate 15–30 discrete DNA bands with RNAs from different tissues so that the DNA fragments were easily resolved by agarose gel electrophoresis. Therefore, we varied the length of the primers used for the second step (PCR amplification). Also, we tested combinations with one, two or three different primers in each PCR reaction. As is indicated in Table I, combinations of two

10 nt primers or two 13 nt primers gave 15–29 major distinct bands. The size of the DNA fragments varied from 100 to 3000 bp. The yields of the DNA fragments were high enough for direct use of the DNA in cloning or Northern blotting. Under the same conditions, employing three 10-mer primers instead of two 10-mer primers increased the number of discrete bands from 15 to 26 (see results for primers BS52-, BS54- and BS55- in Table I). Primers of 18–23 nt appeared to yield a smaller number of discrete bands than shorter primers of 10–13 nt. In effect, use of two or three primers of 10 nt or 13 nt were optimal if the aim was to generate 15–30 discrete fragments.

With three 10-mer primers with cDNA templates from six different tissues, 14–26 DNA fragments were readily obtained from each tissue (Fig. 1). From one to six bands were generated from each tissue that were tissue-specific in that they were found in one tissue but not in others. A number of other bands were partially tissue-specific in that they were found in several tissues but not in others. Also, comparison of patterns from fetal and adult lung revealed some bands present in fetal and absent in adult lung, or the reverse. Therefore, the procedure identified development-specific mRNAs. In addition, many bands were found to be of different intensity in different tissues, suggesting that they identified mRNAs whose levels varied in a tissue- or development-specific manner. Repeating the experiments under the same conditions with the same primers generated the same bands (not shown). Contamination with chromosomal DNA did not present any apparent problem, since no bands were detected when the experiments were repeated without reverse transcriptase. Also, the DNA fragments appeared to arise from different mRNAs, since only the brain-specific fragment (marked C) hybridized when the fragment was eluted, cloned and used as a probe for Southern blotting (data not shown) of all the fragments obtained from brain (right-hand lane in Fig. 1).

To generate larger numbers of DNA fragments with RNA from the same tissue, the same cDNA templates obtained in the first step (reverse transcription) were amplified with different primers in the second step (PCR amplification). As indicated in Figure 2, about 15–24 new fragments were readily obtained each time the same cDNAs from lung were amplified with different primers. Samples prepared with one or more common primers generated many of the same bands (lanes 1, 2, and 3 in Fig. 2). However, over 170 apparently discrete bands were obtained by repeating the second step ten times under the conditions described in Figure 2. As discussed by Sobral and Honeycutt (7), it is possible to control the number and size of PCR products by varying primer lengths, different polymerases and other parameters of the reaction.

Other conditions for the two-step procedure

We found it was important to completely digest the products of reverse transcription with RNase H before the PCR. Without complete digestion, high backgrounds and low yields of fragments were observed. Also, 'hot' start by adding of Taq polymerase into the PCR tube that was pre-heated at 35°C or higher decreased the background. In addition, the yields of PCR fragments were increased by using 200 μ M concentrations of each NTP instead of the 2 μ M concentrations employed by Liang and Pardee (2) and 50 μ M concentrations used by Welsh *et al.* (4). Because of the limited resolution of agarose gel electrophoresis, bands eluted from the gel can contain cDNAs from more than one mRNA. Three of six tested were shown by Northern blot analysis to hybridize to a single band of mRNA. Results of Northern blot

Figure 5. Partial nucleotide sequence of cloned brain specific fragment C. RACE-1 and RACE-2 indicate primers specific for sequence of fragment C which were used to amplify 3'-end of brain-specific mRNA (see Fig. 6).

Figure 6. Amplification of cDNA from 3'-end of the brain specific mRNA (product C) using 3'-RACE system. Lane 1, MW marker; Lane 2, amplification with primer RACE-1 and UAP; Lane 3, amplification with primer RACE-2 and UAP (see Fig. 5); Lane 4, 0.5 μ l of PCR product with primer RACE-1 (lane 2) were re-amplified with primer RACE-2 and UAP for 15 cycles. The PCR product in lane 2 is slightly smaller than the PCR product in lane 1 and the PCR product in lane 3 is slightly shorter than the PCR product in lane 1.

for one of the three bands (band A, Fig. 4) are shown in Figure 8. The three remaining DNA fragments gave multiple bands or smears, but one of the three (band C in Fig. 1) gave a single band after the fragment was cloned (see Fig. 4). Some of the bands of different size may originate from the same mRNA, but this was not encountered in the experiments here.

Identification of a new brain-specific mRNA

In further experiments, a specific DNA band of about 1080 bp obtained from brain with a set of three 10 nt primers (band C in Fig. 1) was characterized. The band was cut out from the gel and the DNA was cloned. Five of 11 clones isolated contained an identical insert of about 1080 kb (Fig. 3). Northern blotting analysis using one of the five identical clones as a probe demonstrated that an mRNA hybridizing to the probe was present in brain but not in 15 other tissues (Fig. 4). The approximate size of the mRNA band detected in brain was about 14 kb. The clone of 1080 bp was partially sequenced (Fig. 5). Sequences from both ends of about 150 bp was obtained that consisted of an open reading frame. The sequences were not found in GenBank.

A longer cDNA for the new brain-specific mRNA was isolated by using the internal sequence from the first DNA fragment (Fig. 5) to design primers for polyA-anchored cDNA extension (3'-RACE). The mRNA from brain was reverse transcribed with an adaptor primer to the polyA sequence. The 3'-end was amplified using an antisense primer to the sequence in the adaptor primer and two different sense primers specific for sequences

Figure 7. DNA fragments generated when the second step (PCR amplification) was performed with two 18 nt primers (BS77-CTGGTCACACTGGCTCTC and BS79-CACAGTGAGCTTTGGGCT) specific for the Clara cell 10 kDa secretory protein. Arrows indicate some of the tissue-specific bands. Arrow marked as A indicates a skeletal muscle-specific band of about 1250 bp. Arrow marked as B indicates the lung and trachea-specific 249 bp fragment from Clara cell 10 kDa secretory protein.

Figure 8. Northern blot hybridized with DNA isolated directly from gel piece containing muscle specific band A of about 1250 kb (see Fig. 7).

in product C (Fig. 5). One of the specific primers (RACE-2) was internal to another (RACE-1). Therefore, primer RACE-2 was expected to amplify a 3'-end cDNA fragment that was 39 bp shorter than the fragment amplified with primer RACE-1. With primer RACE-1 a product of about 2500 bp was amplified from brain (Fig. 6). With primer RACE-2 another fragment was generated which was slightly shorter than the product with primer RACE-1, indicating that both primers amplified the 3'-end of brain-specific mRNA for product C. To prove further that the 2500 bp fragment represented the 3'-end of the brain specific mRNA, the PCR product generated with primer RACE-1 was re-amplified with primer RACE-2. If the 2500 bp fragment with primer RACE-1 was indeed the 3'-end of message for product C, it must contain an internal sequence recognized by primer RACE-2. As expected, primer RACE-2 re-amplified the 2500 bp

Table I. Primers with arbitrary but fixed sequences used for the second step (PCR amplification)

Primers combination	Length (nt)	Number of fragments in single tissue ¹	GC content
BS52-CAAGCGAGGT	10	15	60%
BS54-AACGCGCAAC	10		60%
BS52-CAAGCGAGGT	10	16	60%
BS55-GTGAAGCGT	10		60%
BS54-AACGCGCAAC	10	15	60%
BS55-GTGAAGCGT	10		60%
BS52-CAAGCGAGGT	10	26	60%
BS54-AACGCGCAAC	10		60%
BS55-GTGAAGCGT	10		60%
BS57-GGAAGCAGCT	10	29	60%
BS58-CAGTGAGCGT	10		60%
BS61-G(C)TCACGGACG ²	10	26	70%
BS62-G(C)CCATGCACG	10		70%
BS70-GAGCTATGGC	10	26	60%
BS73-AGCCTGTGTC	10		60%
BS76-CTGGTCACAC	10	15	60%
BS78-CACAGTGAGC	10		60%
BS71-GAGCTATGGCATG	13	27	54%
BS74-AGCCTGTGTCTGA	13		54%
BS77-CTGGTCACACTGGCTCTC ³	18	16	60%
BS79-CACAGTGAGCTTTGGGCT	18		56%
BS72-GAGCTATGGCATGCTCTAT ³	19	14	47%
BS75-AGCCTGTGTCTGAACCACT	19		53%
BS64-GCTGCACCTTGGACGCCATGAA ³	22	10	60%
BS65-CAGTGGTAGGTGATGTTCTGGGA	23		52%
BS39-GCTGCACCTTGGACGCCATGAA ³	22	9	60%
BS40-CAGTGGTAGGTGATGTTCTGGGA	23		52%

¹Number of major distinct band suitable for direct cloning or Western blotting is indicated for one of six tissues with largest number of bands.

²Degenerate primers with both a G and a C in the 5'-position.

³Non-optimal primers because they generated relatively few discrete bands of DNA with high background and low yield. Also, they did not rigidly meet all the criteria for selecting primers for the second step of the procedure (see text).

product of primer RACE-1 into a slightly shorter product (Fig. 6). Thus the total length of the cDNA sequences recovered for brain specific mRNA in two steps was about 3600 bp. The cDNA is currently being characterized further.

Amplification of a known tissue-specific protein

As a further test of the two-step procedure, two oligonucleotide primers of 18 nt were prepared to specifically amplify the cDNA for the Clara cell secretory protein (CCSP) of 10 kDa that was found in epithelial cells lining the respiratory and terminal bronchials of the lung (8). With the two primers, the expected band of 249 bp (product B) was obtained from samples of lung and trachea (Fig. 7). The band was shown to be specific for the mRNA of 10 kDa protein in that it was cleaved into fragments of 197 bp and 48 bp by the restriction endonuclease *Ava*I and 159 bp and 86 bp by *Pvu*II (not shown), the predicted size for fragments based on the cDNA sequence (8). The 249 bp band was not detected in other tissues (Fig. 7), which correlates with published data on tissue specificity of the CCSP (8).

Although the primers amplified a CCSP cDNA, they were not totally specific under conditions for random PCR with an annealing temperature of 34°C, since several other tissue-specific cDNA fragments were generated: seven specific for brain, two for skeletal muscle and two in lung that appeared to be development-specific (Fig. 7). For example, a DNA fragment of about 1250 bp (product A) was found in skeletal muscle but not in five other tissues. The band of 1250 bp was directly used as probe in Northern blot analysis. As indicated in Figure 8, the

probe hybridized to an mRNA of about 17 kb in skeletal muscle. The same probe did not hybridize with any mRNA in 15 other tissues, except for heart, where a very weak band was observed after long exposure.

DISCUSSION

A series of recent reports have demonstrated the power of suitably designed RT-PCR procedures for generating large numbers of cDNA fragments from the multiple mRNAs of cells (2–4,10). The procedure described here is similar to the two procedures described by Welsh *et al.* (4) and Liang and Pardee (2), but has several advantages. Liang and Pardee (2) designed their procedure to generate and display 50–100 amplified cDNA fragments on a DNA sequencing gel. To generate up to 100 cDNA fragments, they used an anchored primer with the sequence 5'-T₁₁NN so as to synthesize single-stranded cDNAs from a large number of mRNAs. They then amplified the cDNAs by PCR with the same primer together with a second primer of a 10-mer with an arbitrary but defined sequence. The limitations of the procedure are that short sequences of less than 500 bp were generated and most originated from the 3'-end non-translated ends of mRNA (see Table II). Also, the DNA fragments had to be eluted from the DNA sequencing gels and re-amplified to obtain adequate amounts for Northern blot and other assays. In our experience here, elution and re-amplification of the DNA bands was inefficient, time-consuming and frequently unsuccessful. Welsh *et al.* (4) designed their procedure to generate 10–20 different

Table II. Comparison of present procedure with previously published procedures

Liang and Pardee (3)		Welsh <i>et al.</i> (4)		Present procedure	
1.	cDNA with anchored (dT) _n primer	1.	cDNA with one arbitrary primer	1.	cDNA with fully degenerate hexamer
2.	PCR amplification with same primer plus one arbitrary primer and 35S-dNTPs	2.	PCR amplification with same primer and 32P-dNTPs	2.	PCR amplification with 2 or 3 arbitrary primers
3.	Separation on DNA sequencing gel and autoradiography	3.	Separation on DNA agarose gel and autoradiography	3.	Separation on sequencing and ethidium bromide staining
4.	Electro-elution and PCR reamplification with ³⁵ S-dNTPs	4.	Elution and PCR reamplification with ³² P-dNTPs	4.	Elution of 0.1 to 3 kb fragment for cloning, sequencing, and 3'-RACE to generate additional sequences
5.	Verification of products on DNA sequencing gel	5.	Verification of products on DNA sequencing gel		
6.	Separation on agarose gel	6.	Separation on agarose gel		
7.	Elution of 0.1 to 0.6 kb fragment for cloning, sequencing etc.	7.	Elution of 0.1 to 0.6 kb fragment for cloning, sequencing etc.		

cDNA fragments for display on a DNA sequencing gel. They used a single 20-mer oligonucleotide with a defined but arbitrary sequence to reverse transcribe internal sequences of mRNAs. For PCR amplification of the single-stranded cDNAs, they used an [α -³²P]-labeled deoxynucleotide together with the same single 20-mer oligonucleotide used in the reverse transcriptase step. As a result, the subset of single-stranded cDNAs with primer sequences at both ends were efficiently amplified. The procedure had the advantage of providing fragments with internal sequences of the mRNAs instead of the less-informative 3'-sequences. The procedure, however, had some of the same limitations as the procedure of Liang and Pardee (2) in that only fragments of up to 600 bp were generated, and the yields were low so that the fragments had to be eluted from the DNA sequencing gel and re-amplified before further analysis.

The procedure here used a fully degenerate oligonucleotide of only 6 nt for the first step (reverse transcription) so as to synthesize a relatively large number of single-stranded cDNAs primarily from internal sequences of most mRNAs. The second step consisted of PCR amplification with longer oligonucleotides of 10–13 bases to generate 15–30 DNA fragments in relatively large yield so that it was readily possible to detect and isolate the fragments by agarose gel electrophoresis for further analysis without re-amplification and without the use of radioisotopes. Conditions were developed to generate no more than 30 distinct DNA bands because the presence of larger number of bands made it difficult to isolate homogeneous fragments in high yield. However, about 170 distinct DNA bands were readily generated from the same sample of single-stranded cDNA obtained in the first step by repeating the second step ten times using ten different pairs of primers of a defined but arbitrary sequence. Obviously, the second step would have to be repeated with over 1000 different pairs of primers in order to obtain cDNA fragments from most of the 15,000 or so different mRNAs in a cell. The two-step procedure was relatively rapid in that cDNA fragments of up to 3000 bp and in high enough yield for analysis can be obtained from a sample mRNA in about 9 h. Some isolated

fragments probably contain disproportionate amounts of sequences from minor mRNAs, but since there is no need for re-amplification by PCR, minor DNAs do not become major products during the isolation procedure. As indicated, DNA fragments containing additional sequences from the same mRNA can be obtained using the 3'-sequences from the fragments obtained in the two-step procedure for a 3'-RACE synthesis. The method here detects fewer mRNA species in each PCR than the method of Liang and Pardee (2) and is probably biased in favor of more abundant large mRNAs. However, it is simpler and faster to perform than other methods. Also, it has been successfully employed to obtain three cDNAs ranging in size from 1 to 3.5 kb derived from mRNA that are differentially expressed in osteoblasts during mineralization (11). Although not tested here, it may be possible to obtain more sequences from the same cDNA by carrying out 5'-RACE (12) in addition to 3'-RACE.

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