Nucleic Acids Research, Vol. 20, No. 19 4965–4970

Arbitrarily primed PCR fingerprinting of RNA

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Received August 20, 1992; Revised and Accepted September 2, 1992

ABSTRACT

Fingerprinting of RNA populations was achieved using an arbitrarily selected primer at low stringency for first and second strand cDNA synthesis. PCR amplification was then used to amplify the products. The method required only a few nanograms of total RNA and was unaffected by low levels of genomic double stranded DNA contamination. A reproducible pattern of ten to twenty clearly visible PCR products was obtained from any one tissue. Differences in PCR fingerprints were detected for RNAs from the same tissue isolated from different mouse strains and for RNAs from different tissues from the same mouse. The *strain-specific* differences revealed are probably due to sequence polymorphisms and should be useful for genetic mapping of genes. The *tissue-specific* differences revealed may be useful for studying differential gene expression. Examples of tissue-specific differences were cloned. Differential expression was confirmed for these products by Northern analysis and DNA sequencing uncovered two new tissue-specific messages. The method should be applicable to the detection of differences between RNA populations in a wide variety of situations.

INTRODUCTION

The arbitrarily primed PCR reaction uses a single arbitrarily selected primer (1,2) or two such primers (3) to produce a PCR fingerprint of a complex DNA such as a bacterial or eukaryotic genome. Polymorphisms that distinguish individuals can be used for genetic mapping (2,3,4,5,6), ecology (7), epidemiology (1,8) or phylogenetics (8). We show here that arbitrarily primed PCR can be performed on total cellular RNA to distinguish differences in RNA populations between different sources. Methods that allow the detection of differentially expressed genes are important for studying gene regulation and differentiation. The method described here could compliment other methods for studying differential gene expression (e.g. 9,10,11).

Genetic mapping of anonymous genomic sequence polymorphisms is a major application of arbitrarily primed PCR fingerprinting (2). Similarly, sequence polymorphisms in RNA could be genetically mapped. Such polymorphisms would represent anonymous genes known to be expressed in the tissue examined.

MATERIALS AND METHODS

RNA preparation

Total RNA was prepared from various mouse tissues using the guanidinium thiocyanate-cesium chloride method as described by Chirgwin et al. (12) .

cDNA production

Total RNA, prepared by the method above, was dissolved in water at a concentration of 0.02 μg/μl and ethanol precipitated using 0.3M sodium acetate, pH 6, and 2.5 volumes of ethanol. The resulting pellets were dissolved in water to a final concentration of 0.2 μg/μl, heated to 65°C for 10 minutes and placed on ice before cDNA synthesis. Arbitrarily chosen primers 'KZ', 5'-CCCATGTGTACGCGTGTGGG or the reverse sequencing primer 'RS' 5'-GGAAACAGTCATGACCATGA were used to initiate first strand cDNA synthesis, using Moloney reverse transcriptase in a buffer containing 50 mM Tris pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 100 μM each dNTP, 20 mM DTT, 1 μM primer and 0.5 U reverse transcriptase (Stratagene, La Jolla, CA) for 1 hr at 37°C in 20 μl reactions. The various amounts of RNA used at this step are described in the text and figures.

Polymerase chain reaction

Second strand cDNA synthesis was also initiated by arbitrary priming by adding to each first strand synthesis reaction an equal volume (i.e. 20 μl) of a mixture containing 10 mM Tris pH 8.3, 25 mM KCl, 2 mM $MgCl_2$, 1μM primer, 0.2 μCi/μl α-[^{32}P] dCTP and 0.1 U *Taq* polymerase (AmpliTaq, Cetus). The primer added in this step is the same as that used for the first strand synthesis. The final concentrations of buffer components after this addition are 30 mM Tris pH 8.3, 32.5 mM KCl, 3 mM $MgCl_2$, 50 μM each dNTP, 0.05 μCi/μl α-[^{32}P] dCTP and 1 μM primer. The reaction mixture was subjected to one cycle through the following temperature profile: 94°C for 5 min to denature, 40°C for 5 min for low stringency annealing of primer, and 72°C for 5 min for extension followed by 30 high stringency cycles:

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94°C for 1 min, 60°C for 1 min and 72°C for 2 min. The various amounts of cDNA used at this step are described in the text and figures. The single low stringency step can be performed at temperatures up to at least 48°C.

Gel electrophoresis

10 μl of 80% formamide, with dye, was added to 2.5 μl of each PCR sample. The samples were heated to 65°C for 15 min and 2.5 μl of each was loaded on a standard 4% acrylamide, 50% urea sequencing gel prepared in 1×TBE, and electrophoresed at 1500 V until the xylene cyanol dye had reached the bottom of the gel.

Isolation of polymorphic PCR products

Isolation of PCR products was achieved in a manner previously described (5). The autoradiogram was aligned with the gel using hot ink dots and bands were cut from the gel using a razor blade. The piece of acrylamide was placed in a mircofuge tube and the DNA was eluted for at least 1 hr at room temperature into 50 μl TE. 5 μl of the eluent was PCR amplified in 50 μl using the standard PCR protocol (Perkin Elmer Cetus), 0.25 μCi α-[^{32}P] dCTP, and the KZ or RS primer. The amplified material was checked against the initial arbitrarily primed PCR reaction by running a second polyacrylamide gel. The products were blunt-end cloned by standard methods (13) into the pBSKII vector (Stratagene, La Jolla, CA), mobilized into single stranded phage. The ssDNA was sequenced using the Sequenase kit (USB, Cleveland, OH).

Northern blot analysis

For Northern blot analysis, samples (10 μg) of total RNA were resolved by electrophoresis on 1.2% agarose, 2.2 M formaldehyde gels and blotted onto nylon membranes (Duralon-UV, Stratagene). Blots were prehybridized with 50% formamide, 5×SSC, 10×Denhardt's solution, 0.1 mg/ml sonicated denatured salmon sperm DNA, 0.2% SDS, 0.05 M sodium phosphate buffer, pH 6.8, and 0.001 M EDTA for two hours at 42°C. Hybridization was performed for 16 hours at 42°C in 50% formamide, 5×SSC, 1×Denhardt's solution 0.02 M sodium phosphate buffer, pH 6.8, 0.1 mg/ml sonicated denatured salmon sperm DNA, 0.001 M EDTA, 0.2% SDS and 10% dextran sulfate with $2-5\times10^7$ cpm of ^{32}P-labelled probes prepared according to the method of Feinberg and Vogelstein (14). Filters were washed at 65°C with 2×SSC containing 0.5% SDS for 3 hours followed by 0.2×SSC and 0.5% SDS for 40 min before exposure to X-ray film at −70°C. Filters were stripped rehybridized to an oligonucleotide complementary to 28S ribosomal RNA as a control for gel loading and transfer. The sequence of the 28S ribosomal oligonucleotide is 5-AACGATG-AGTAGTGGTATTTCA (15) and the hybridization conditions were as described (16).

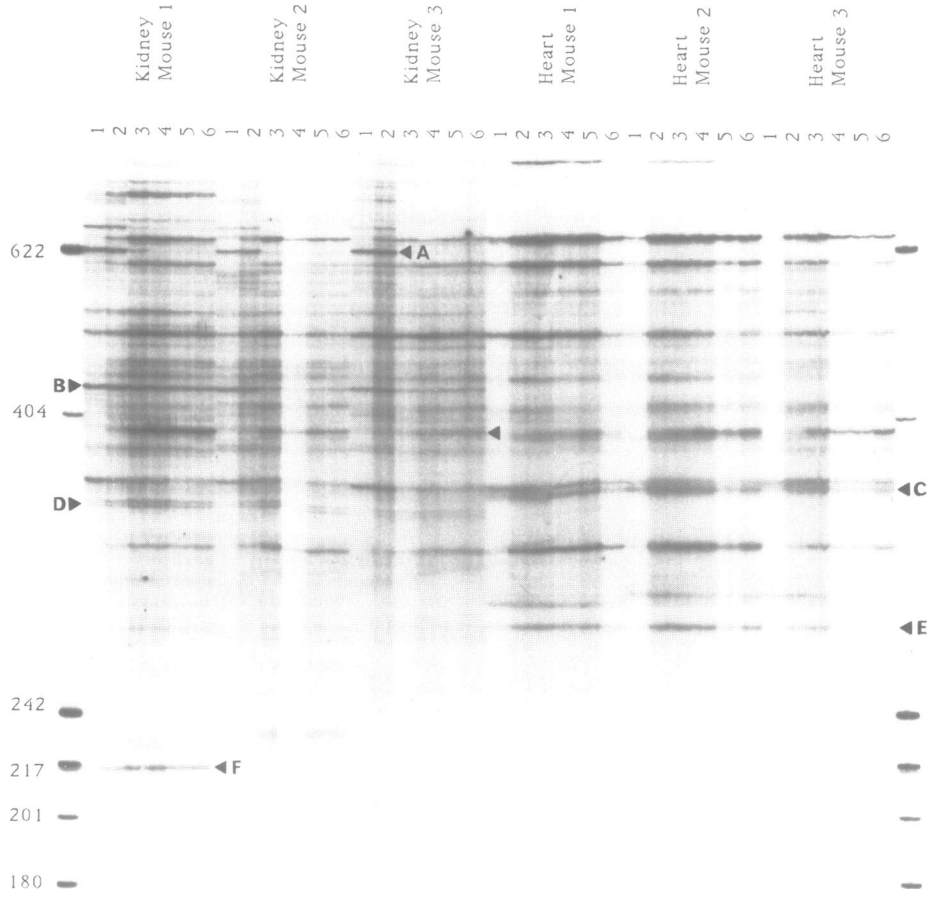

Figure 1. Arbitrarily primed PCR with titration of cDNA. Four cDNA populations were derived using an arbitrary primer and total RNA from the kidney and heart of two mice (the products of complex crosses of C57BL/6J and CBA). Each group of six lanes is for cDNA derived from to 2.5 μg, 1 μg, 0.2 μg, 0.04 μg, 0.008 μg, and 0.0016 μg of starting RNA, respectively. Each of the four RNA preparations was amplified by arbitrarily primed PCR. Some of the polymorphisms between mice or between tissues are indicated by arrows. Molecular weight markers are indicated on the left.

RESULTS AND DISCUSSION

As a simple model to test the feasibility of arbitrarily primed PCR on cDNA, we compared the RNA populations of various easily isolated organs from the mouse. In brief, total RNA was isolated from these tissues by guanadinium/CsCl ultracentrifugation (12) which largely frees the RNA of other cellular components, particularly genomic DNA. First strand cDNA was produced by Moloney reverse transcriptase using an arbitrary 20 base primer. Extension of primers that match imperfectly has been demonstrated for several polymerases, and is anecdotal for reverse transcriptase. Apparently, primer extension occurs most favorably at better matches and becomes progressively less likely at worse matches. The arbitrarily primed first strand cDNA was heat denatured and subjected to arbitrarily primed second strand synthesis using Taq polymerase at low stringency. The products that result have the primer sequence at both ends and were then PCR amplified at high stringency with simultaneous radiolabelling. The products were separated by polyacrylamide gel electrophoresis and visualized by autoradiography. The resulting fingerprint patterns are highly reproducible.

Development of the RNA fingerprinting protocol

To determine the most robust fingerprinting conditions, we tested several of the reaction parameters including magnesium concentration, input RNA and cDNA concentrations. The magnesium optimum was determined to be about 4 mM (data not shown), as previously determined for AP-PCR (1). To determine the amount of RNA needed, 5 μg of RNA was used to produce first strand cDNA. The product of the first strand synthesis was then serially diluted over a 1500-fold range, corresponding to cDNA from 2.5 μg to 0.0016 μg of input total RNA, and second strand arbitrarily primed PCR amplification was performed (Figure 1). About ten to twenty products were detected by each primer from total tissue RNA using this method. The arbitrarily primed PCR fingerprints produced over this range of concentrations of input cDNA were almost identical, except for a few differences at the higher end of the range. At greater than 0.04 μg of input RNA extra products appeared, for instance, at about 620 bases in the kidney RNA lanes, labelled A in Figure 1. This slight concentration-dependence is also observed in arbitrarily primed PCR fingerprinting of genomic DNA (1,2). Serial dilution of the RNA in the range 2.5 μg to 0.012 μg per 20 μl reaction before cDNA synthesis also revealed a broad concentration optimum, over this 500-fold range (Figure 2). The method is reproducible over a wide range of RNA concentrations down to a few nanograms of total RNA and possibly lower.

Fingerprints generated in this manner were RNA-dependent and not due to contaminating genomic DNA. The pattern was insensitive to prior treatment of the input nucleic acid with DNase and sensitive to RNase (data not shown). Heat inactivation of the reverse transcriptase before the first strand synthesis reaction resulted in the complete elimination of the fingerprint. In some cases, a very faint pattern of products (not shown) unrelated to the RNA-directed fingerprints resulted.

Genomic DNA contamination is tolerated

Arbitrarily primed PCR fingerprinting of DNA requires at least two low stringency annealing steps on denatured DNA, permitting initial priming events to occur in opposite directions, and thereby

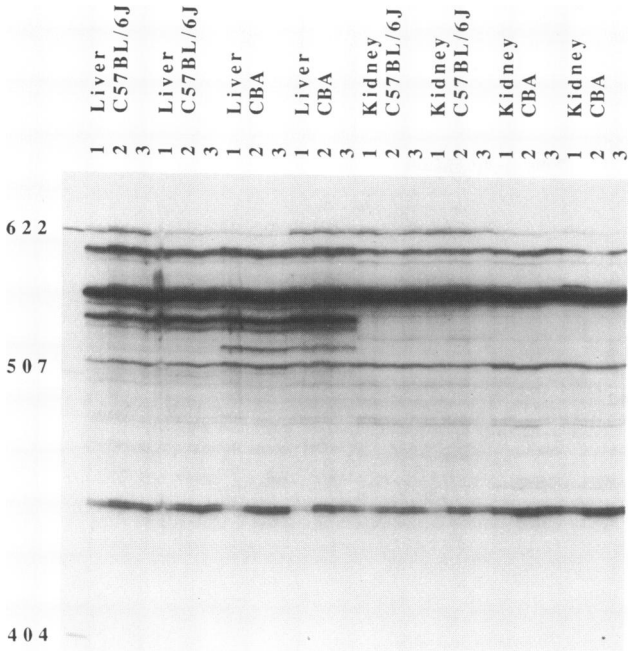

Figure 2. Arbitrarily primed PCR with titration of RNA. cDNA populations were derived using an arbitrary primer and total RNA from the liver and kidney of two mice each from the inbred lines C57BL/6J and CBA. Each group of three lanes is derived from 0.2 μg, 0.05 μg, and 0.012 μg of starting RNA. Molecular weight markers are indicated on the left.

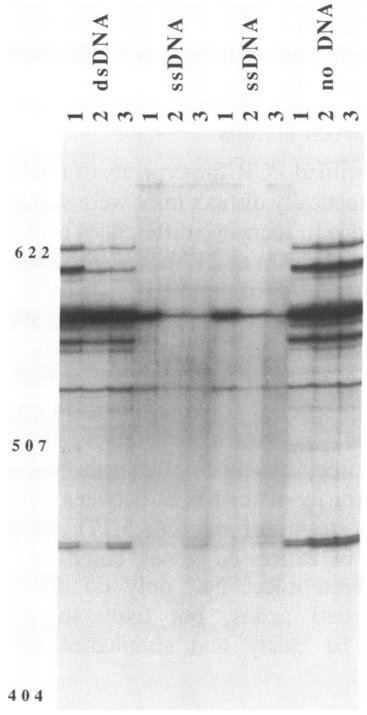

Figure 3. Low levels of dsDNA contamination has little effect on RNA fingerprinting. Total liver RNA from a C57BL/6J mouse was PCR fingerprinted using the RS primer. Each group of three lanes display fingerprints from 0.2 μg, 0.05 μg, and 0.012 μg of starting RNA. In the first set of lanes, 25% (by mass) of mouse genomic double stranded DNA was added. In the second and third group of lanes, 25% (by mass) of mouse genomic single stranded DNA was added. The fourth group of lanes is a control with no extra DNA added. Molecular weight markers are indicated on the left.

introducing the primer sequences at two ends and facilitating subsequent high stringency PCR amplification of the bounded sequences. In this RNA fingerprinting protocol, contaminating chromosomal DNA would be double stranded during the first strand cDNA synthesis step, and therefore should not be able to participate in the first low stringency step. Subsequent to first strand synthesis, only a single round of denaturation and low stringency annealing need occur (with primers >15 bases). Because of this regime, the arbitrary primer is never introduced into a genomic sequence twice and in opposite directions, and is therefore not an efficient substrate for PCR amplification. To test this possibility, denatured or non-denatured mouse genomic DNA was included in the first strand cDNA reaction. In Figure 3, the presence of denatured genomic DNA almost completely eliminates the RNA-dependent pattern and results in an largely uniform background smear, presumably due to promiscuous priming in the first step under these low stringency conditions. However, when 20% of the input nucleic acid is non-denatured genomic DNA the PCR fingerprints are by and large unaffected over a wide range of concentrations. That the presence of moderate amounts of dsDNA does not adversely affect the fingerprinting of RNA suggests that the rigorous density centrifugation method used here for RNA production may not be necessary. This insensitivity to moderate dsDNA contamination combined with the observation that only a few nanograms of RNA is needed per lane could allow application to tissues or cells which are difficult to obtain in large amounts. In cases where many mRNAs can be expected to differ, the method should reveal a sample of such messages. However, because each primer samples only a small subset of the RNA population, the method would be inappropriate in situations where one was looking for differences in a single message or only a very small number of messages.

Differences between strains

The arbitrarily primed PCR fingerprints of total RNA from the same tissue of genetically distinct mice were similar, but reflected polymorphisms due to sequence differences between strains. For instance, a polymorphism at 217 bases (product F) in Figure 1 revealed in kidney RNA of mouse 1 is absent in mice 2 and 3. Similarly, in Figure 2 a polymorphism at about 550 bases distinguishes CBA liver from C57BL/6J liver. Repeat experiments gave identical results. One would generally not expect such strain-specific polymorphisms in one tissue type to reflect differential gene expression. Rather, such differences are more likely to reflect sequence polymorphisms, similar to those detected by arbitrarily primed PCR between genomic DNAs of different inbred strains of mice (3,5,17). In principle, such polymorphisms in expressed genes could be mapped using recombinant inbred mice. Not only do the polymorphisms represent expressed genes, but tissue-specific patterns of expression can be easily and simultaneously explored, as described below.

Differences between tissues within a strain

Differences between arbitrarily primed PCR fingerprints of RNA from different tissues reflect tissue-specific gene expression. Fingerprinting of RNA from mouse liver, kidney, and heart revealed numerous differentially expressed genes, reflecting the complex genetic makeup of these tissues (Figures 1 and 2).

Experiments with several other arbitrarily chosen primers also have revealed differences between tissues reflecting differential gene expression. We have also used ten base long primers to generate tissue-specific patterns (Figure 4). The protocol for use of the ten-mers was essentially the same as with the longer primers, except that a 35°C annealing step was introduced, and the ramp time for the transition to 72°C was increased to 30 sec.

Characterization of differentially expressed mRNAs

Several fingerprint products from differentially expressed messages in the kidney and heart were purified from the gel in Figure 1, reamplified and used as probes on Northern blots. Of five products from the PCR fingerprints tested in this way four hybridized primarily or exclusively to the expected tissue in the Northern blot. One such PCR product (product B, Figure 1) was abundant in kidney and undetectable in the heart. Another (product E, Figure 1) was about four-fold more abundant in the heart than in the kidney. The hybridizations with probes from products B and E are presented in Figure 5. Two others (products

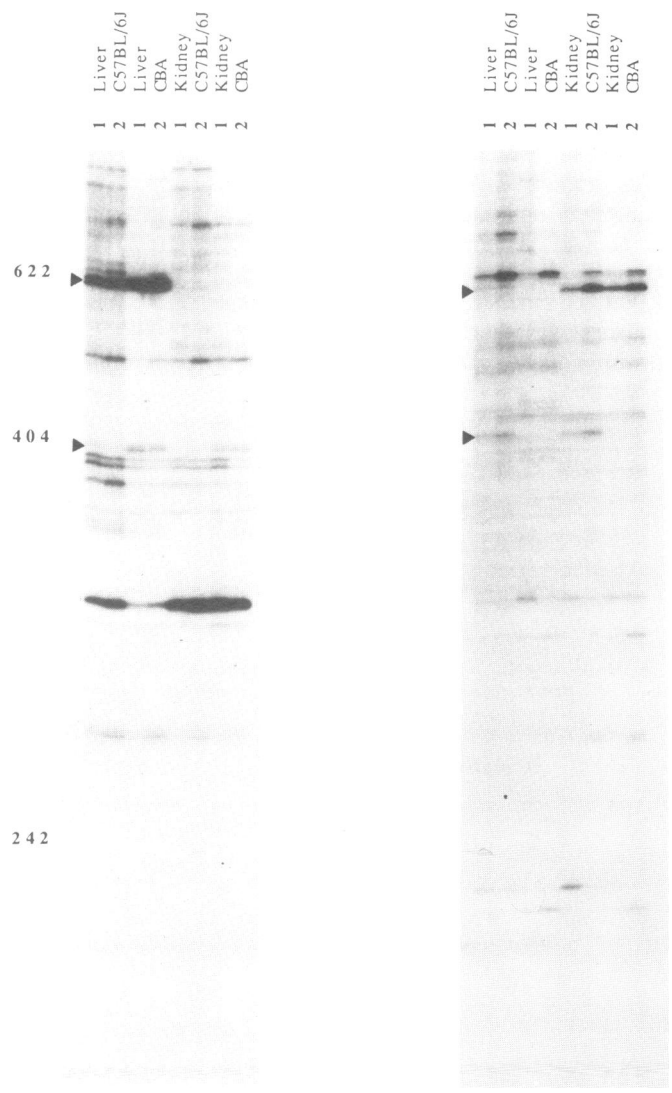

Figure 4. Fingerprinting using primers ten bases in length. 0.2 μg and 0.05 μg of total RNA were fingerprinted using the primers 5′-AGGGGTCTTG and 5′-AATGCGGG (Operon, Alameda, CA) using a protocol adjusted for ten-mers. A few of the polymorphisms between mice or between tissues are indicated by arrows. Molecular weight markers are indicated on the left.

D and F, Figure 1) were of moderate abundance in the kidney and undetectable in the heart. Finally, product C hybridized to multiple mRNAs in both tissues, some of which were differentially expressed.

Five of the fingerprint products representing five differentially expressed genes from the kidney and heart, described above, were cloned and sequenced. No structural RNA sequences were represented in this small sample and four contained open reading frames. The sequences were compared to Genbank v.70 database. Product C is from near the middle of the reading frame for an α-actin mRNA that is confined to vascular smooth muscle. Its size indicated that it was from a spliced message. This mRNA is highly homologous to other members of the α-actin family, which explains the multiple products seen on Northern blots. Product E present in both kidney and heart in a one to four ratio proved to be a region of α-1-globin mRNA and spans a splice junction, indicating that it was from a spliced message. This message presumably originates from reticulocytes present in all tissues at low frequency. The sequences of the other probes were not homologous to any sequence in the database. Thus, over 50% of the products isolated from this one experiment (products D, F and probably B) are new differentially expressed genes.

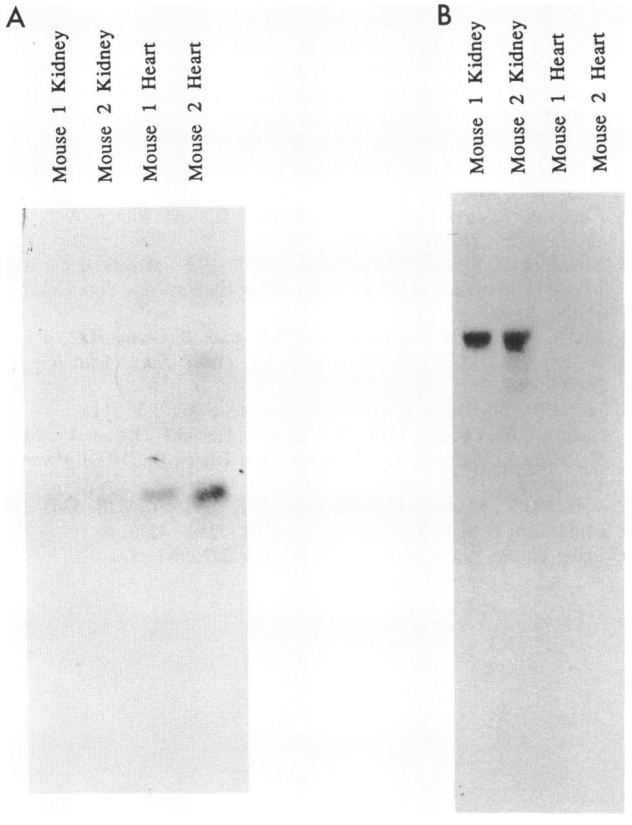

Figure 5. Northern blots of differentially amplified PCR products. Each panel contains an autoradiogram as described in the materials and methods. Panel A. Probed with a 300 base PCR product 'E' found more prominently in the heart than in the kidney. This DNA was later found to encode part of the α-1-globin mRNA. Panel B. Probed with a 430 base PCR product 'B' found in the kidney and not detectable in the heart. The function of this RNA is unknown.

The two sequences that were in the database allowed us to assess the match between the primer and the RNA in the reverse transcription step (first strand synthesis) and between the primer and the cDNA in the low stringency *Taq* polymerase step (second strand synthesis). In three of four cases, the primers matched the sequence in the database perfectly or almost perfectly at the seven 3′ nucleotides (Table 1). However, the second strand synthesis on α-1-globin had an extremely poor match. Since the α-1-globin product was only found in one strain of mouse it is possible that there is a sequence polymorphism at one of the primer annealing sites that distinguishes the α-1-globins. Such a mutation could considerably improve the match with the primer.

Complexity sampled in RNA fingerprints

The representation of each message in an arbitrarily primed PCR fingerprint is a function of several variables including the efficiency of the interaction with the primer (in two places and in opposite directions) and its abundance. Better matches can be found in the more complex classes of sequences, by mere virtue of their complexities. However, when the amplifications of two sequences are initiated by equally good matches, the sequence from the more abundant RNA may predominate at the end of the PCR amplification. The fingerprints are therefore only partly normalized with respect to abundance. This issue is of importance in both gene mapping and the detection differentially expressed genes. Many differentially expressed genes of interest are in the low abundance class. That relatively abundant messages are included in the sample is demonstrated by four differentially expressed products, all of which were detectable on Northern blots, which are relatively insensitive to messages of very low abundance. Also, two clones were from known moderately abundant messages. However, three clones were for previously unknown messages. The various clones and control probes used in these experiments gave Northern hybridization signals that ranged over an order of magnitude while giving nearly equivalent Southern hybridization signals. Also, the matches between the

Table 1. Homology of primer with successful annealing sites.

Reverse transcription step:																				
primer	5′	C	C	C	A	T	G	T	G	T	A	C	G	C	G	T	G	T	G	G	G
		〃	–	′	〃	–	′	〃	′	–	′	〃	′	〃	〃Δ	〃	〃	〃	〃	′	〃
vsm α-actin	5′	C	A	T	A	g	A	T	A	g	G	C	A	C	Gt	T	G	T	G	A	G
		〃	–	〃	〃	′	〃	′	′	Δ	′	〃	–	′	〃	〃	〃	〃	〃	〃	〃
α-1-globin	5′	C	a	C	A	C	G	C	A	–	G	C	t	T	G	T	G	T	G	G	G
Low stringency PCR step:																				
primer	5′	C	C	C	A	T	G	T	G	T	A	C	G	C	G	T	G	T	G	G	G
		〃	–	–	〃	–	′	–	′	′	〃	–	–	′	′	〃	〃	〃	〃	〃	〃
vsm α-actin	5′	C	a	a	A	a	A	g	A	C	A	g	c	T	A	T	G	T	G	G	G
		–	〃	〃	〃	〃	〃	Δ	〃	〃	′	〃	–	〃	Δ	〃	–	〃	〃	〃	〃
α-1-globin	5′	a	C	C	A	T	G	–	G	T	G	C	t	C		T	c	T	G	G	G

primer and the mRNA were excellent in three of four cases we examined (Table 1). Such good matches would be rather rare. For example, matches of eight bases in opposite strands situated less than 1000 bases apart are calculated to occur about once every 10,000,000 bases ($4^8 \times 4^8/1000$). Nevertheless, for the moment, it is not clear how efficiently we are sampling the low abundance, high complexity class and this issue is still a question actively under investigation.

Further adaptations and applications of the method

In the development of this method, there are many variables yet to be explored. Our preliminary experiments using oligo-dT in the reverse transcriptase step followed by the addition of the arbitrary primer at the second low stringency PCR step (data not shown) yielded products that are, on average, larger than those seen when an arbitrary primer is used at both low stringency steps. This may reflect the fact that most messages in liver and kidney have poly-A at the 3′ end. This protocol will be biased against unprocessed RNAs or structural RNAs containing no poly-A stretches but will be selective only in the second priming step, perhaps adversely affecting 'abundance normalization'. Another potential disadvantage of the oligo-dT primed reverse transcription step is that it does not sample non-polyadenylated mRNAs including, for example, 50% of rodent brain cytoplasmic RNAs (18,19), hnRNA and mRNAs in eubacteria. These mRNAs would be sampled if an arbitrary primer is used in both initial steps.

Among other parameters that can be explored are higher annealing temperature and lower ionic strength, and shortening the length of the low stringency steps, any of which might be expected to require better matches for the initial priming events and thus might improve the abundance normalization. Alternatively, the method could be applied to abundance fractionated RNA. Other parameters include, for example, the primer sequence. Primers that carry sequence motifs for particular classes of messenger RNA or sequences from dispersed repeats may bias the fingerprint to a particular class of messages, although it is hard to assess to what extent.

In summary, the data presented suggests at least two possible applications. First, sequence polymorphisms in RNAs could be a source of polymorphisms for mapping genomes. These polymorphisms, by virtue of being genes, might be more interesting than entirely anonymous DNA polymorphisms. Second, differences between two RNA populations during development, or due to different *in vitro* treatments, could be assessed and a sample of differentially expressed mRNAs cloned directly, even if this sample is confined to only the more abundant mRNAs. For instance, cell cultures could be treated with various reagents and the populations of RNA from treated and untreated cells could be studied. Tissues that can be dissected could be compared to each other.

Note added

After submission of this manuscript, a protocol appeared using arbitrarily primed PCR fingerprinting of RNA which uses $T_{11}CA$ in the first step and an arbitrary ten-mer in the second step (20).

ACKNOWLEDGEMENTS

We thank Rhonda Honeycutt and Bruno Sobral for critical reading of the manuscript. This work was supported by NIH grants 2R01HG00106, 1RO1HG00456 to MM, 1R29AI32644 to JW and 2RO1GM38731 to KC.

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