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Screening of differentially amplified cDNA products from RNA arbitrarily primed PCR fingerprints using single strand conformation polymorphism (SSCP) gels

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ABSTRACT

Arbitrarily primed PCR fingerprinting of RNA and differential display resolved on an acrylamide gel has been extensively used to detect differentially expressed RNAs. However, after a differentially amplified product is detected the next steps are labor-intensive: a small portion of the fingerprinting gel that contains the differentially amplified product is cut out, reamplified and the correct product is determined, typically by cloning and sequencing what is often a mixture of products of similar size. Here we use a native acrylamide gel to separate DNAs in the reamplified mixture based on single-stranded conformation polymorphisms. Reamplifications are performed for the region carrying the differentially amplified product and a corresponding region from an adjacent lane where the product is less prominent or not visible. Denaturation of the reamplified DNA followed by side-by-side comparison on an SSCP gel allows the classification of reamplified material into (i) those that can be directly cloned because the differentially amplified product is relatively pure, (ii) those that need to be reamplified from the SSCP gel before cloning and (iii) those that are too complex for further study. This screen should save considerable effort now wasted on directly cloning unsuitable products from RNA fingerprinting experiments. An example is presented of cloning a gene differentially expressed in *Trypanosoma brucei* life cycle.

INTRODUCTION

RNA arbitrarily primed PCR fingerprinting and differential display (1,2) are confounded if the product is not really differentially expressed. This particular problem is often due to variations in the fingerprints caused by slight differences in the quality or concentration of nucleic acid between samples, as originally demonstrated for DNA (3). This problem is largely controlled for by comparing fingerprints generated from two or more RNA concentrations for each sample, side by side. Those products that occur in only one concentration are eliminated from consideration. However even when intra-sample variation is controlled for, a labor-intensive, rate limiting step that remains is the isolation of differentially amplified products and their identification and confirmation as fragments from a differentially regulated RNA.

Methods to characterize differentially amplified products generally start by cutting out the product from the fingerprinting gel and reamplifying the product by PCR using the primers originally employed in the fingerprinting. The main stumbling block at this stage is the fact that the arbitrary fingerprint is always a mixture of products and the reamplification from a tiny portion of the gel usually generates multiple products, of almost identical size to the product of interest. Thus, in subsequent steps the reamplified material contains a mixture of desirable and undesirable DNA products.

Various strategies have been applied to distinguish the correct product from other products of similar size that are co-amplified. One strategy is to clone the reamplified cDNA mixture and then to sequence a number of independent clones, as originally shown for genomic DNA fingerprints (4,5). More than one sequence and its complement are frequently observed, demanding considerable work to find a statistically more abundant clone which is most likely to be the differentially amplified cDNA of interest. Then, the most frequent clone can be hybridized to a Southern blot of the original RAP–PCR gel to prove that the correct amplified product was cloned (6,7). The clone is generally confirmed as differentially regulated by hybridization to a Northern blot (8) or by RT–PCR (9). Another strategy avoids cloning until differential expression is confirmed. The amplified mixture from the original RAP–PCR gel is used directly for a Northern blot (10). If a discrete differentially hybridizing product is seen then this can be reamplified from the Northern blot using the original primers. This strategy has the virtue that the Northern blot essentially purifies the correct differentially expressed product. However, this method is only successful if the reamplified mixture is free of dispersed repeats that would obscure the data. Also, if a contaminating product in the mixture hybridizes to a mRNA that is more abundant than that targeted by the PCR product of interest, then the Northern blot will appear to show no differential expression. Furthermore, this strategy uses a lot of RNA, something that is not practical for many RNA sources.

The strategies described above can all be enhanced by the use of SSCP gels as described below.

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MATERIALS AND METHODS

Fingerprints

Total RNA was prepared from *Trypanosoma brucei brucei* GUTat 3.1 at different stages of the life cycle. The procyclic stage (Pc), was cultured *in vitro*, while the slender (Sl) and stumpy (St) bloodstream forms were grown in mice. Reverse transcription was performed on 500, 250 and 125 ng total RNA. The fingerprints were obtained as previously described (1,6–8,11), using either two different 10mer oligonucleotide primers of arbitrary sequences, or a combination of a 10mer arbitrary primer and a 11mer derived from the 5′ mini-exon sequence of the trypanosomes mRNA (12,13).

Reamplification

The region that contained the product of interest was cut out of the gel, as was the corresponding region in an adjacent lane where the product was apparently not present, or present at a significantly lower level of abundance. Products were eluted in 50–100 µl TE at 65°C for 2 to 3 h. The eluted solution was diluted by 20-fold in water, and 2 µl used in a 20 µl volume PCR reaction mixture containing 10 mM Tris–HCl pH 8.3, 10 mM KCl, 4 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µCi [α-^{32}P]dCTP and 2 U Ampli*Taq* polymerase Stoffel fragment (Perkin-Elmer-Cetus, Norwalk, CT) and 0.5 µM of each oligonucleotide primer (the same two primers used to generate the RNA fingerprint). Thermocycling was performed with a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer-Cetus), using 30 cycles of 94°C for 30 s, 35°C for 30 s and 72°C for 1 min. The PCR products (4 µl samples) were mixed with 18 µl formamide dye solution, heated to 92°C for 3 min and 1.2 µl was loaded onto MDE gel (HydroLink® MDE™ gel, J.T. Baker Inc., NJ), in 0.6× TBE buffer with 5% glycerol. Electrophoresis was performed overnight at 8 W for ~16 h. The gel was dried under vacuum and placed on a Kodak BioMax X-ray film for 20 h. Reducing the number of PCR cycles to 20 or 25 may better preserve differences between samples. However, in that case an intensifying screen may be needed in order to visualize the products on the SSCP gel.

Southern blotting, cloning and sequencing

Polyacrylamide gels were transferred by capillary action, overnight, onto nylon membranes (Hybond N+, Amersham, Buckinghamshire, UK) using standard conditions. Probes were labeled and hybridized to the membranes using the non-radioactive ECL™ direct nucleic acid labeling and detection system (Amersham) according to the manufacturer's instructions. Fragments were separated from dNTPs and primers on a low-melting-point agarose gel, and were cloned in pCR-Script™ SK(+) plasmid vector (Stratagene, La Jolla, CA) and Epicurian Coli® XL1-Blue MRF′ competent cells using standard conditions. Single-stranded phagemids were sequenced using the Sequenase DNA sequencing kit (US Biochemical, Amersham) and [α-^{35}S]dATP.

RT–PCR

The reverse transcription was performed on 250 ng RNA. RNA, 5 µl, was mixed with the same volume RT mixture for a 10 µl final reaction containing 50 mM Tris–HCl pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 0.2 mM of each dNTP, 13 U MuLV-reverse transcriptase, 2 µM primer (three different anchored-dT primers: $(T)_{12}$-G, $(T)_{12}$-A and $(T)_{12}$-C for Fig. 2D, lanes 1, 2 and 3, respectively). The reverse transcription was performed at 37°C for 1 h, the enzyme was inactivated by heating the samples at 94°C for 2 min and the cDNA obtained was diluted 4-fold in water. The PCR was performed using the primers 5′-AATGAAA-GTTACGATAGCGG and 5′-AAAGACAACGGAGATGGCA, chosen from the DNA sequence of the clone. Diluted cDNA, 5µl, was mixed with the same volume of PCR mixture for a 10 µl final reaction containing 10 mM Tris–HCl pH 8.3, 10 mM KCl, 4 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM of each primer and 2 U Ampli*Taq* polymerase Stoffel fragment (Perkin-Elmer-Cetus, Norwalk, CT). Thermocycling was performed with a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer-Cetus), using 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. Amplification products were run on a 2% agarose gel.

RESULTS AND DISCUSSION

In an effort to improve the ease and effectiveness of the characterization and confirmation of PCR-reamplified products we have investigated single-stranded conformation polymorphism (SSCP) gels as a method of purifying the cDNA product of interest away from other products of different sequence but of similar size.

Single-stranded DNAs fold to form stable and metastable secondary structures that affect their mobility in a native acrylamide gel. Hayashi exploited this phenomenon to distinguish between point mutations in molecules that were otherwise identical (14). RAP–PCR fingerprinting product mixtures are much easier to distinguish than point mutations because, while the contaminating products are generally about the same length as the product of interest, they are of completely different sequence. Thus, they can be expected to have distinct mobilities on an SSCP gel.

The SSCP procedure was applied to products isolated from an experiment involving different stages in the development of *Trypanosoma brucei*, the unicellular eukaryote responsible for sleeping sickness in Africa. The RNAs investigated were prepared from the procyclic form of the parasite that divides in the midgut of the tsetse fly vector, the slender form which divides rapidly in the blood of the mammal host and the stumpy form that has stopped dividing and is primed for recycling into the biting fly.

In each case, the region of the RAP–PCR gel that contained the differentially amplified cDNA product of interest were reamplified and simultaneously radiolabeled, as was the corresponding region in an adjacent lane where the product of interest was apparently not present, or was present at a significantly lower level of abundance. These are referred to as the 'experimental' and the 'control'.

Each PCR product can be expected to produce two SSCP bands, one for each of the two cDNA strands. In Hydrolink® MDE gels it is rare that one strand will migrate as two different confomers. Occasionally these strands will have the same mobility and produce only one band. Our results showed that it was almost impossible to obtain only one or two bands on the SSCP gel, even if the same samples, run an agarose gel, gave only one band. This underlines the utility of this method for purification. There were four classes of patterns that were seen in the SSCP gels.

Class 1

The PCR product exhibits one to three bands of very low intensity, in addition to two bands of very high intensity. These two strong bands were present only in the experimental lane, in the case of a presence versus absence of the eluted band in the RAP–PCR gel (Fig. 1A), or present in both experimental and control lanes, in the

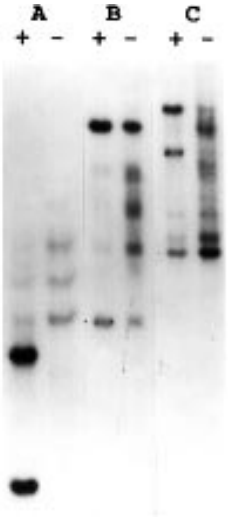

Figure 1. Examples of patterns generated by an SSCP gel after reamplification of differentially amplified products from RNA fingerprinting gels. (**A**), (**B**) and (**C**) represent three different products isolated from different fingerprints. For each product, the reamplification of the band from the RAP–PCR lane where it was of high abundance (+) and from a lane where it was of low abundance or undetectable (–) were loaded side-by-side on the gel. The two single strands of the product can be resolved, almost pure of contamination (A and B), or clearly distinguishable from the contaminants and candidates for a second elution and reamplification (C). This last pattern is the most frequently observed in our experiments. The data presented showed differences in mobility ranging ~20% (4 cm range in a 20 cm run).

case of a difference of intensity of the eluted band (Fig. 1B). In this last case, the two bands visible in the control are from a low level of the differentially amplified product that was able to amplify because it was purified away from competing products in the same fingerprinting lane. In both these cases the original amplification can be used for cloning or for Southern or Northern blots.

Class 2

One or two prominent products in the experimental lane are shared with the control and one or two others are not. An example is shown in Figure 1C. In this case the product(s) unique to the experimental lane can be further cut and reamplified from the SSCP gel (as shown later). The pattern then obtained exhibits only one or two bands corresponding to the two single strands. This purified product can now be cloned or hybridized. We have performed this enrichment step for seven products that have been tested and confirmed as the correct differentially amplified products. An example will be presented later in this paper.

Class 3

Both the experimental and the control lanes exhibit the same profile of a few bands with no strong differences in intensity. To determine which band(s) is the product of interest, a larger strip of gel, including the band differentially amplified and one or two conserved bands, can be cut from the original fingerprint and reamplified as well as the corresponding control. The difference between the experimental and the control is often preserved because each reaction now has other products to amplify in addition to the differentially amplified product of interest. The DNA mass in the control lane is distributed to these products rather than merely erasing differences in the product of interest. Also, limiting the number of cycles to 20, or less, will better preserve the stoichiometry of products between samples. Limited experiments indicate that this may be the case (data not shown). If this strategy is used then it is necessary to cut and reamplify the product of interest from the SSCP gel, as in Class 2.

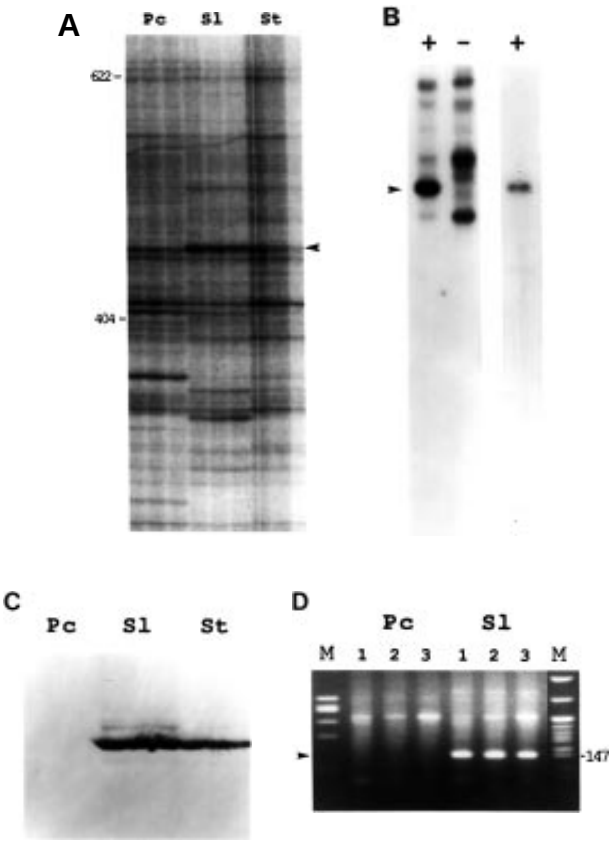

Figure 2. Different steps involved in the purification and confirmation of a differentially expressed gene in the life cycle of *T.brucei* (Protozoa). (**A**) Fingerprints of RNA extracted from three different stages, procyclic (Pc), slender (Sl) and stumpy (St). Three different concentrations for each RNA were reverse transcribed using the arbitrary primer 5′-GGGGCACCAG and amplified using the mini-exon primer 5′-GTACTATATTG. The arrow indicates the differentially amplified product, present in samples Sl and St but absent in sample Pc, that was cut, eluted from the gel and reamplified. (**B**) Electrophoresis on a SSCP gel of the reamplification products of this band, cut out from sample Sl (+, on the left side), and the corresponding region cut out from sample Pc (–). The arrow indicates the product of interest, absent in the lane (–), that was further purified by a second cut and reamplification (lane + on the right). (**C**) Southern blot of a part of a polyacrylamide gel hybridized with the purified fragment. The differential hybridization confirms that the fragment purified corresponds to the band that was differentially amplified on the original fingerprint. (**D**) Products of RT–PCR run on an agarose gel confirming the differential expression of the gene between the procyclic and the slender forms of the parasite. RNAs were reverse transcribed using three different anchored oligo-d(T) primers. Amplification was performed using two specific primers determined after sequencing of the fragment (see Materials and Methods). The arrow indicates the expected product of 147 bp, confirming the presence of the mRNA in the slender forms, and its absence in the procyclic forms. M, molecular weight markers (ΦX174/*Hae*III on the left, and pBR322/*Msp*I on the right).

Class 4

The experimental lane may give a complex pattern or a smear that may or may not differ from the control. This category represents

failures and should be rejected. However, even in these cases SSCP has served well by giving the investigator an easy assay to identify those mixtures that would be very difficult to pursue further. Without this assay much time would be wasted on this class of products.

Class 1 is observed most often when the background is very low (less contaminant cut out of the RAP–PCR gel with the band). Classes 2 and 3 are most often observed when the background is high on the RAP–PCR gel.

In total, four different pairs of primers were used for cDNA fingerprinting. These fingerprints detected ~200 cDNA products. Of these, 22 were clearly differentially amplified between two of the three stages of *Trypanosoma* development. These 22 products were reamplified and screened using SSCP. Of these, 13 gave potentially usable SSCP patterns, two from class 1, six from class 2 and five from class 3. So far nine products have been cloned, sequenced and confirmed on Southern blots. Four of them have also been confirmed for differential expression using RT–PCR. An example will be presented below.

Note that when the reamplified material from the fingerprinting gel is resolved by SSCP it is possible to assess which reamplifications are likely to yield a clear majority of one fragment when cloned. Even when the SSCP indicates a simple pattern of one or two bands (as in Fig. 1A and B) this does not preclude the infrequent cloning of other products from the mixture as well. Thus, sequencing of a few clones can be performed, though, after a purification from the SSCP gel and a second reamplification, as shown in Figure 1C, the chance of sampling two different sequences is very low. A major clone can be found by sequencing no more than four clones.

As an example of the characterization procedure, Figure 2 presents data for the different steps of isolation, purification and confirmation of the differential expression of a gene. RNA fingerprinting of the three different stages of *T.brucei* is shown in Figure 2A. One differentially amplified fragment, that was present in the two bloodstream forms only, was reamplified and resolved on a SSCP gel twice in a row. Figure 2B illustrates reamplifications of this product which belongs to class 2. The cDNA fragment from this second step of reamplification was cloned into the pCR-Script vector, as described in Materials and Methods. The four sequenced clones had the same sequence. One clone was used as a probe on a Southern blot against a polyacrylamide fingerprint gel (Fig. 2C), confirming the right fragment was cloned. This product was apparently absent in the procyclic but present in the two bloodstream forms. Primers were derived from the sequence of this clone and used in RT–PCR. Differential expression was so extreme that even after 35 cycles the difference between the two different stages was preserved (Fig. 2D). Extra PCR products in this reaction, which act as internal positive controls for amplification, are presumably due to arbitrarily primed PCR because the annealing temperature used was 5–10°C below the T_m of the primers. A search of the GenBank database indicated strong homology to an expression site-associated gene, ESAG 1 (15,16), a member of a gene family associated to the expression sites of *T.brucei* variable surface genes (VSGs), which are known to be expressed in the bloodstream stages of the parasite and not generally in the procyclic stage. This product has been deposited in the GenBank database (accession number U49237). A detailed description of the developmental profile of some of the novel genes discovered in these experiments is in preparation, including their distribution in other stages of development and under various treatments.

In summary, SSCP represents a rapid method to screen for contaminating products in reamplifications from RNA fingerprints. By comparing reamplifications from fingerprinting lanes where the desired product is of high abundance with the equivalent position from lanes in which the desired product is absent or of low abundance, it is possible to identify the desired product in the mixture. If necessary, the enriched product of interest can be purified by reamplification from the SSCP gel. This strategy greatly aids in selecting suitable reamplification mixtures for the labor-intensive step of purifying and identifying the differentially amplified product of interest.

In the future it should also be possible to use the SSCP method directly on DNA eluted from the RAP-PCR fingerprinting gel without reamplification. Elution is sufficiently efficient and DNA can be sufficiently labeled to allow detection after SSCP. This direct method has the advantage that it will preserve stoichiometry from the fingerprinting gel.

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