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Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization

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ABSTRACT

Differential display has been developed as a tool to detect and characterize altered gene expression in eukaryotic cells. The basic principle is to systematically amplify messenger RNAs and then distribute their 3′ termini on a denaturing polyacrylamide gel. Here we provide methodological details and examine in depth the specificity, sensitivity and reproducibility of the method. We show that the number of anchored oligo-dT primers can be reduced from twelve to four that are degenerate at the penultimate base from the 3′ end. We also demonstrate that using optimized conditions described here, multiple RNA samples from related cells can be displayed simultaneously. Therefore process-specific rather than cell-specific genes could be more accurately identified. These results enable further streamlining of the technique and make it readily applicable to a broad spectrum of biological systems.

INTRODUCTION

Current methods to distinguish mRNA in comparative studies rely largely on subtractive hybridization (1). This technique, although it has been used successfully in isolating a number of important genes (2, 3), is rather difficult to establish, irreproducible, and requires large amounts of RNA. We have recently developed an alternative approach, named mRNA differential display which circumvents these problems (4, 5). A similar approach utilizing only a single primer has recently been reported (6). The essence of differential display method is to use for reverse transcription an anchored oligo-dT primer which anneals to the beginning of a subpopulation of the poly(A) tails of mRNAs. The anchored oligo-dT primers consist of 11 or 12 T's plus two additional 3′ bases which provide specificity. These are used in conjunction with a decamer oligodeoxynucleotide of arbitrarily defined sequence for the subsequent PCR amplification, as has been used previously for detecting DNA polymorphism by PCR (7). Amplified cDNA fragments of 3′ termini of mRNAs were thus separated by size up to 500 bp on a denaturing polyacrylamide gel.

In this study we examined in more detail the specificity of the anchored oligo-dT primers, the sensitivity limit of the method in terms of the amount of input RNA, reproducibility from run to run; and we compared total RNA versus poly(A) RNA as templates. We describe and demonstrate that highly reproducible patterns of differential display can be achieved using conditions optimized here with DNA-free total RNA samples. In addition, we incorporate as a built-in control for reproducibility of amplified cDNA band pattern displays of more than two relevant RNA samples simultaneously. Therefore, genes specific to a process, as an example, cell transformation caused by cooperation of oncogene ras and inactivation of tumor suppressor gene p53, can be readily identified.

MATERIALS AND METHODS

Primer syntheses

Oligonucleotide primers Ltk3 (5′-CTTGATTGCC-3′), $T_{11}CA$, $T_{12}CA$ and degenerate anchored oligo-dT primers, $T_{12}MG$, $T_{12}MA$, T_{12} MT and $T_{12}MC$ were synthesized by the molecular biology core facility of Dana-Farber Cancer Institute. Some arbitrary 10mers, OPA1 to 20 were purchased from Operon Biotechnology Inc. (Alameda, CA).

Cell culture

Both normal mammary epithelial cells (76N) and breast cancer cells (21MT-2) were cultured at 37°C with 5% CO_2 in D medium (8) until reaching about 70% confluence before their total cellular RNAs were extracted. Murine fibroblast A31 cells were cultured in Dulbecco's minimal essential medium (DME) at 37°C with 10% CO_2 and 10% bovine calf serum until reaching 80% confluence. For growth arrest (G_0) of A31 cells by serum starvation, cells grown to 80% confluence were rinsed with DME and shifted to DME with 0.5% bovine calf serum and incubated for an additional 48 hours. Rat embryo fibroblast (REF) and its ras/p53 mutant doubly transformed derivatives, T101-4 and A1-5 cells (9) were cultured in DME with 10% fetal calf serum at 37°C with 10% CO_2 until reaching 80% confluence.

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RNA isolation and Northern blot analysis

The total cellular RNA isolation and Northern blot analysis were carried out essentially as previously described (10). The poly(A) RNA isolated from mouse fibroblast A31 cell was purified using Mini RiboSep™ kit, a mRNA isolation system from Collaborative Research Inc. (Bedford, MA). Reamplified or cloned cDNA probes were purified by agarose gel electrophoresis using QIAEX kit from QIAGEN (Chatsworth, CA). Extracted cDNA probes were labeled with ^{32}P using a Random-Prime DNA labeling kit from Boehringer Mannheim Biochemical (Indianapolis, ID) essentially as instructed except 1 μL of 10 μM corresponding $T_{12}MN$ primer was also included during the labeling.

Removal of chromosomal DNA contamination from RNA

50 μg of total cellular RNA was incubated for 30 min at 37°C with 10 units of human placental ribonuclease inhibitor (BRL, Gaithersburg, MD), 10 units of DNase I (BRL or Worthington Biochemical Corp., Freehold, NJ) in 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$. After extraction with phenol/$CHCl_3$ (3:1), the supernatant was ethanol precipitated in the presence of 0.3 M NaOAC and RNA was redissolved in diethyl pyrocarbonate-treated water.

Differential display

mRNA differential display was performed essentially as previously described (4), except routinely 0.2 μg of total RNA or 0.1 μg poly(A) RNA was used for reverse transcription in a 20 μL reaction volume. It is advisable to apply duplicate samples with the same or different amounts of RNA, in order to avoid the possibility of losing rarer mRNAs and to minimize errors in the PCR procedure which lead to spurious bands in one but not another lane. The cycling parameters are as follows: 94°C for 30 seconds, 40°C for 2 min., 72°C for 30 seconds for 40 cycles followed by 72°C for 5 min. The amplified cDNAs were then separated on a 6% DNA sequencing gel. AmpliTaq DNA polymerase was purchased from Perkin-Elmer Corp. (Norwalk, CT.). α-[^{35}S]dATP (1200 Ci/mmole) was obtained from New England Nuclear (Boston, MA.).

Recovery and reamplification of cDNA probes

The DNA sequencing gel was blotted on to a piece of Whatman 3mm paper and dried without methanol/acetic acid fixing. The autoradiogram and dried gel were oriented with either radioactive ink or needle punches. After developing the film, cDNA bands of interest were located by either marking with a clean pencil or cutting through the film. The gel slice along with the 3mm paper was incubated in 100 μL dH_2O for 10 min. After rehydration of the polyacrylamide gel, the cDNA was diffused out by boiling the gel slice for 15 min in a tightly capped microfuge tube. cDNA was recovered by ethanol precipitation in the presence of 0.3 M NaOAC, 5 μL of 10 mg/mL glycogen as a carrier and redissolved in 10 μL of H_2O. 4 μL of a eluted cDNA probe was reamplified in a 40 μL reaction volume using the same primer set and PCR conditions as used in the mRNA display except the dNTP concentrations were at 20 μM instead of 2−4 μM and no isotope was added. 30 μL of PCR samples were run on a 1.5% agarose gel and stained with ethidium-bromide. The remaining samples were stored at −20°C for subcloning.

Cloning and sequencing of cDNA probes

Reamplified cDNA probes were cloned into the PCRII vector using the TA cloning system from Invitrogen (San Diego, CA). Plasmid DNA sequencing of cloned cDNA probes with either T7 or SP6 primer was carried out using the Sequenase kit from United States Biochemicals Co (Cleveland, OH).

RESULTS AND DISCUSSIONS

The specificity of anchored oligo-dT primers

Theoretical consideration suggests that 12 possible anchored oligo-dT primers ($T_{12}MN$ where M may be dG, dA, or dC and N may be any one of the 4 deoxynucleotides) used in all

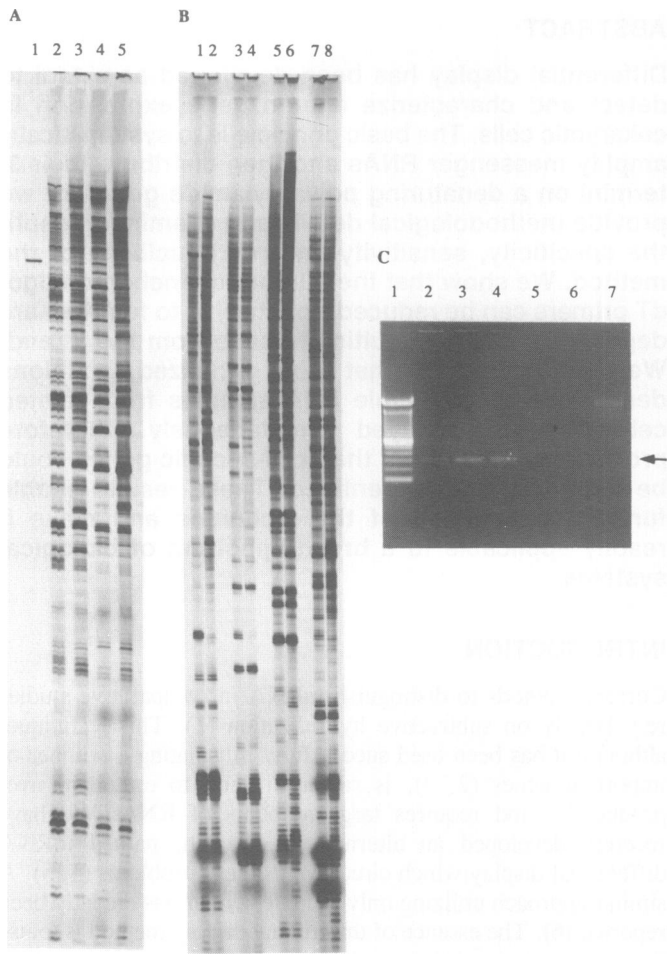

Figure 1. Specificity of anchored oligo-dT primers. **(A)** 0.2 μg of total RNA from A31 cells before (lanes 2 and 4) and after serum starvation (lanes 3 and 5) were differentially displayed with either $T_{12}CA$ (lanes 2 and 3) or $T_{12}MA$ (lanes 4 and 5) in combination with Ltk3 primer. Lane 1 used TK cDNA plasmid pAMTK as template, amplified by primer set $T_{12}CA$ and Ltk6. **(B)** 0.2 μg of total RNA from normal mammary epithelial cell 76N (lanes with odd numbers) and breast cancer cell 21-MT2 (lanes with even numbers) were displayed using a 10mer, OPA18 (AGGTGACCGT) in combinations with either $T_{12}MG$ (lanes 1 and 2), $T_{12}MA$ (lanes 3 and 4), $T_{12}MT$ (lanes 5 and 6) or $T_{12}MC$ (lanes 7 and 8). **(C).** 10 ng of TK cDNA plasmid pAMTK was amplified with Ltk3 primer in combinations with either $T_{11}CA$, $T_{12}CA$, $T_{12}MA$, $T_{12}MG$, $T_{12}MC$ and $T_{12}MT$ (lanes 2 to 7, respectively). Lane 1 was a 100 bp ladder as molecular weight standard (BRL).

combinations with at least 20 different arbitrary 10mers are necessary to display 10,000 of the mRNA species that are present in a mammalian cell (4). To study in more detail the specificity of the anchored $T_{12}MN$ primers, $T_{12}CA$ and $T_{12}MA$ (M represents either G, A or C) were compared in combination with the Ltk3 primer to display a pair of mRNAs samples from murine fibroblast cells which were harvested before and after serum starvation (Figure 1 A). If each M base could provide specificity one would predict that there would be three times as many bands with $T_{12}MA$ as compared to $T_{12}CA$. But the result showed rather surprisingly similar patterns of displays with similar number of bands (Compare lanes 2 and 3 with 4 and 5). Consistent with these findings was an observation that a differentially expressed cDNA cloned from a cDNA library by the differential display method showed a nucleotide mismatch with the $T_{12}GC$ primer at the M base (5). This suggests that the penultimate base from the 3′ end of the $T_{12}MN$ primer may exhibit considerable degeneracy during priming in the reverse transcription step.

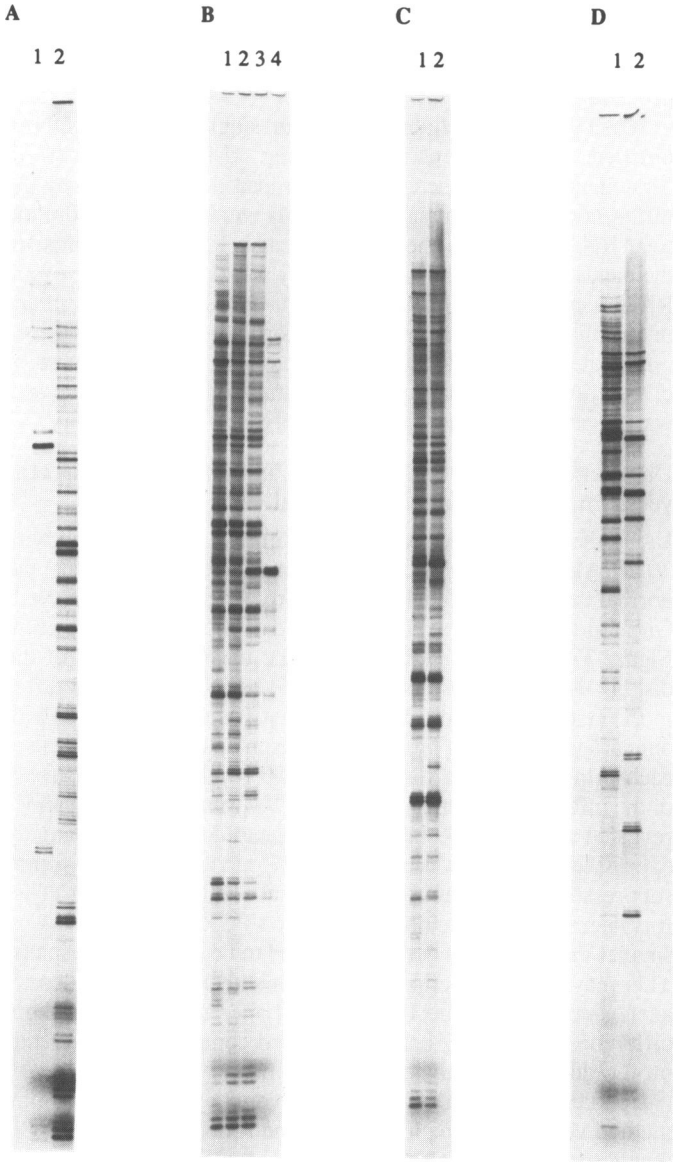

Figure 2. Optimizing conditions for differential display. **(A)** Dependence of differential display on the reverse transcription reaction. 0.2 μg of DNase I treated total RNA from 76N cell was incubated in a 20 μL reverse transcription reaction using $T_{12}MC$ primer in the absence or presence of reverse transcriptase. 2 μL of non-reverse transcribed (lane 1) and reverse transcribed RNA (lane 2) were subsequently amplified and displayed. **(B)** Sensitivity of differential display as a function of amount of input RNA. 2, 0.2, 0.02 or 0.002 μg of total RNA (lanes 1, 2, 3 and 4) from 76 N cell were independently reverse transcribed with T12MC primer and amplified in combination with OPA18 primer. **(C)** Comparison of total cellular RNA with poly(A) RNA by differential display. 0.2 μg of total RNA (lane 1) or 0.1 μg of poly(A) RNA (lane 2) from A31 cell were reverse transcribed and amplified with primer set $T_{12}MC$ and OPA 18. **(D)** Comparison of total cellular RNA with a cDNA library by differential display. 0.2 μg of total RNA of 76 N cells (lane 1) or 1 μg of single stranded cDNA phagemid rescued from a directional cloning cDNA library of 76 N cell (lane 2) were compared by differential display with primer set $T_{12}MA$ and Ltk3.

To test if the N base can provide specificity, four different degenerate primers $T_{12}MG$, $T_{12}MA$, $T_{12}MT$ and $T_{12}MC$ were compared when individually used in combination with a single arbitrary 10mer to display a pair of RNA samples from normal human mammary epithelial versus metastatic breast cancer cells (Figure 1B). The results showed that band patterns obtained with the four $T_{12}MN$ primers that differ in the 3′ end base 'N' differ much more than those obtained with primers changed only at the 'M' base. Furthermore, when primers $T_{11}CA$, $T_{12}CA$ and T_{12} MA were compared with $T_{12}MG$, T12MT and $T_{12}MC$, each in combination with the Ltk3 primer to amplify the 3′end of the murine thymidine kinase (TK) gene using a cDNA plasmid (11), only the first three primers were able to specifically amplify the target TK cDNA template which has a TG(A)n tail (Fig. 1C).

It is worthwhile noting that an oligo-dT primer without anchoring 3′ bases such as T_{12}, when used in combination with an arbitrary primer, gave a total smear on the gel (12). This may be explained as the T_{12} primer being able to anneal to any part of the poly(A) tail for a given mRNA species to initiate the reverse transcription, so the resulting amplified cDNA product would be heterogeneous. In fact, when a high concentration of poly(A) RNA was used as template, the high background smear generally observed (12) might also be attributed to contamination of the oligo-dT primers coming from the oligo-dT column during poly(A) RNA purification. Also, a longer primer set such as $T_{18}MG$ and an arbitrary 16mer gave a very high background and many fewer discrete bands on the differential display as compared with $T_{12}MG$ and a 10mer. Increasing the annealing temperature to 48°C helped to reduce the background but at a cost of losing even more bands (12).

These data taken together suggest that the two 3′ bases MN are absolutely essential to provide $T_{12}MN$ primers with specificity for anchoring to the beginning of the poly(A) tails. But the penultimate base M can exhibit considerable degeneracy and it is the last base from the 3′ end that provides most of the specificity. Therefore instead of having to use all 12 possible $T_{12}MN$ primers, four such degenerate primers that differ only in the last base can be used to discover many of the poly(A) tails of mRNAs. This significantly cut down the number of reverse transcription reactions to be performed for finding differences between RNA samples.

Improvements and refinements of the method

One of the most important factors for a successful differential display is to use DNA-free RNA samples. Often RNA isolated by either GIT/CsCl gradient centrifugation or using a simplified method described by Chomczynski and Sacchi (13) still contains

residual amount of chromosomal DNA contamination (see Materials and Methods). Routinely our RNA samples were cleaned from DNA contamination before performing differential display. When RNA was badly contaminated with chromosomal DNA, the display pattern would largely become independent of the reverse transcription (12). One way to find out if the RNA sample has chromosomal DNA contamination is to perform the PCR reaction without reverse transcription. If the RNA sample is free from DNA contamination, the display should be totally dependent on the reverse transcription step (Figure 2A). Often a couple of bands were seen that were independent of reverse transcription, even though the RNA sample was treated with DNase, but these band generally did not show up after the reverse transcription reaction. One explanation is that there could still be a residual amount of DNA contamination after DNase treatment of the RNA, but after reverse transcription the amplified cDNA competes for limited dNTPs substrates much more efficiently.

To determine the sensitivity limit of differential display in terms of input RNA concentrations, DNA-free total RNA was varied from 2 μg to 0.002 μg in a 20 μL RT reaction (Figure 2B). The result indicated that the amount of input RNA can be as low as 0.02 μg which is equivalent to RNA from about 200 cells, and that the method can be scaled down. The pattern was much sparser with 0.002 μg RNA.

By and large, the majority of the bands did not exhibit much change in pattern with RNA input between 2 and 0.02 μg. But some bands were differently amplified or expressed at these different concentrations; the intensity of some bands even exhibited an inverse relationship with input RNA. This suggests that under the PCR conditions used the differential display method is not very quantitative.

The other aspect of the sensitivity limit of the method is how rare a messenger RNA can be detected. This has not been well determined, and raises the question of sensitivity limits, particularly for mRNAs amplified from (degenerate) primer misfits. Although we previously showed that TK mRNA which belongs to the rare message class (<50 copies per cell) could be seen (4), the 5′ primer used had perfect matches in all 10 bases. Given the fact that arbitrary 10mers hybridize in a degenerate fashion with 2 to 3 mismatches (4, 5), more differential display experiments such as using 5′ primers that have 2 or more mismatches to the TK gene or other genes that are known to be expressed at low level are needed to address the important issue of whether rare messages can be fully represented by the method. A caution should also be made that because there do exist some minor differences in amplified cDNA pattern across the RNA concentrations from 2 to 0.02 μg used as templates, some of these difference could be either due to the noise level intrinsic to the procedure or reflecting the loss of detection of rarer mRNAs when RNA concentration is lowered.

It was also very interesting to observe that when the same set of primers, T_{12}MC and OPA18, were used to display RNA either from human mammary epithelial (Figure 2B) or mouse fibroblast cells (Figure 2C) on the same gel, a totally different pattern of species or tissue-specific displays were obtained. Therefore this kind of mRNA finger printing may be useful also for identification and classification of the RNA sources.

One important aspect of the differential display method as described before is that either poly(A) RNA or total cellular RNA can be used as templates (4), and the resulting cDNA patterns displayed by a given primer set were nearly identical (Figure 2C). This indicated that T_{12}MN primers preferentially select the mRNA over the abundant ribosomal RNA and transfer RNA during the reverse transcription reaction. Total RNA was routinely used in most of our experiments, since it is generally much easier to purify than poly(A) RNA; this also avoids the risk of oligo-dT contamination from the latter's purification procedure.

When the total cellular RNA and single stranded cDNAs purified from a directional cloning cDNA library (from Strategene) of the same cell were compared as templates by the differential display, many more bands were observed from the total RNA than from the cDNA library (Figure 2D). Most of the bands seen from the cDNA library were also seen in the total RNA display; a few appeared to be enriched in the cDNA library or originated from the plasmid vector. Some expressed genes might therefore be under represented or lost in the course of cDNA library construction. Differential display may serve as a way of monitoring the general quality of a cDNA library.

Differential display of mRNAs from human breast cancer cells versus normal mammary epithelial cells

Using the four degenerate T_{12}MN primers in combination with 20 arbitrary 10mers, RNA from human breast cancer cells and normal mammary epithelial cells were differentially displayed. One fourth of the data, representative of the rest, are shown in Figure 3. The result indicated that the banding patterns of the display were dependent on both the T12 MN primers that differ in the last 3′ base and on the arbitrary 10mers, consistent with our earlier result using limited primer combinations (4). In general each primer set could display 50−100 bands. A visual survey of the mRNA differential display with 80 primer set combinations revealed less than 1% bands that appeared to be differentially expressed in both senses (putative 'oncogenes' and 'tumor suppressor genes') between the normal and breast cancer cells. It is interesting that the displayed cDNA PCR products that were smaller than 300 bp generally appeared to be doublets or triplets. This may be explained as a result of either additional deoxyadenosine added by the Taq DNA polymerase (14) or the slight difference in mobility between the two complementary strands on the denaturing polyacrylamide gel.

Up to 90% of the cDNA bands recovered by the simple boiling method could be reamplified after one round of 40 cycle PCR reaction so they can be used as probes for either Northern blot analysis or cloning. Of 15 bands analyzed by Northern blot analysis 5 were confirmed to be differentially expressed, another 6 failed to detect any signals and the remaining ones were false positives. This agrees well with the results from our other application as well as from others who have been using our procedure (15, 16).

Reproducibility and multiple display

A troublesome aspect of the method is that the 'noise level' of false positives, though a few percent, can be very appreciable relative to the truly different bands between cells. One way to

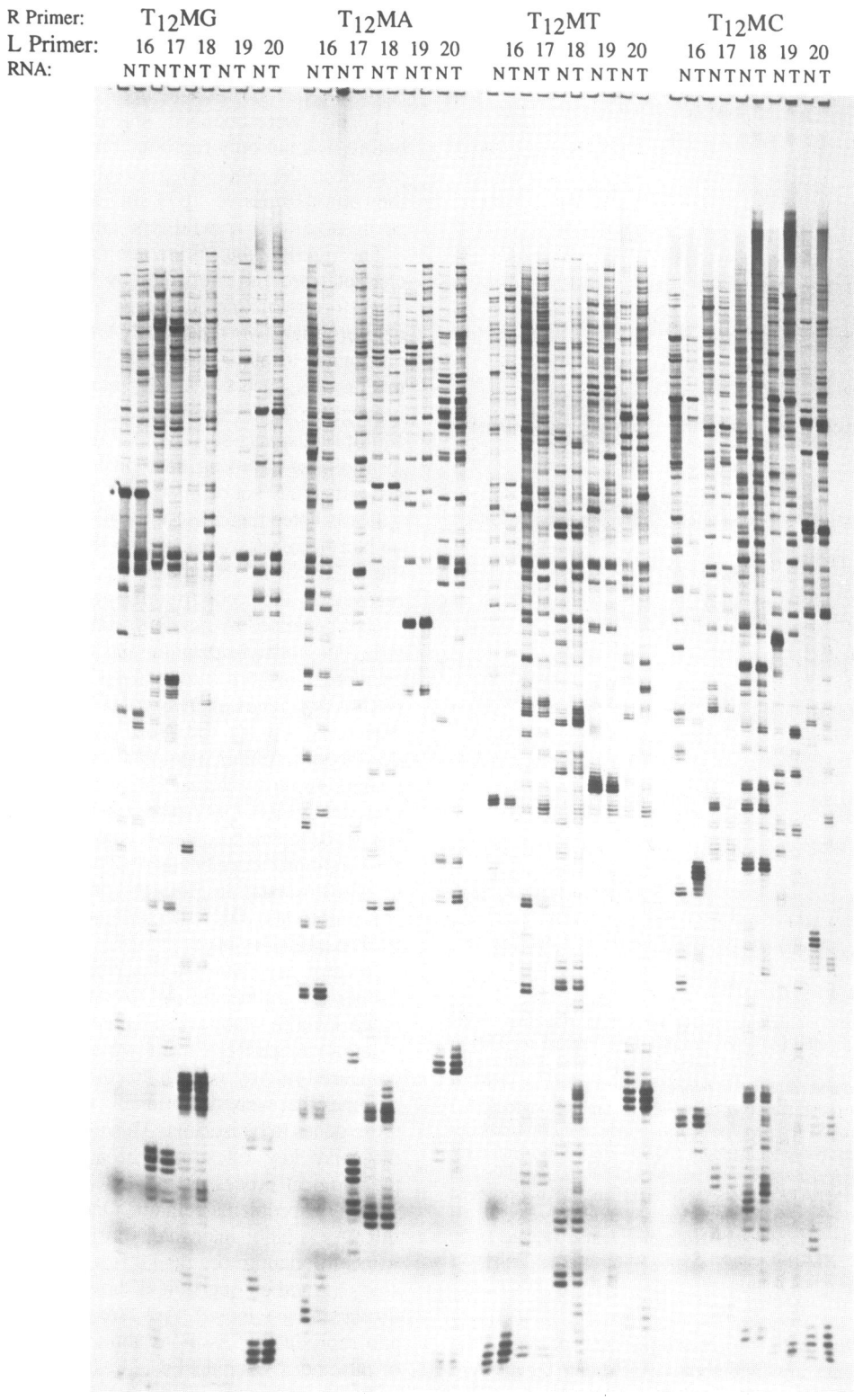

Figure 3. Differential display of mRNA from normal mammary epithelial cell 76 N and a breast cancer cell line 21MT-2 using 20 combinations of primer sets made of $T_{12}MG$, $T_{12}MA$, $T_{12}MC$, $T_{12}MT$ and a group of 5 arbitrary 10mers. N stands for Normal cell and T stands for breast cancer cell. The five arbitrary 10mers used were OPA16 (AGCCAGCGAA), OPA17 (GACCGCTTGT), OPA18 (AGGTGACCGT), OPA19 (CAAACGTCGG) and OPA20 (GTTGCGATCC).

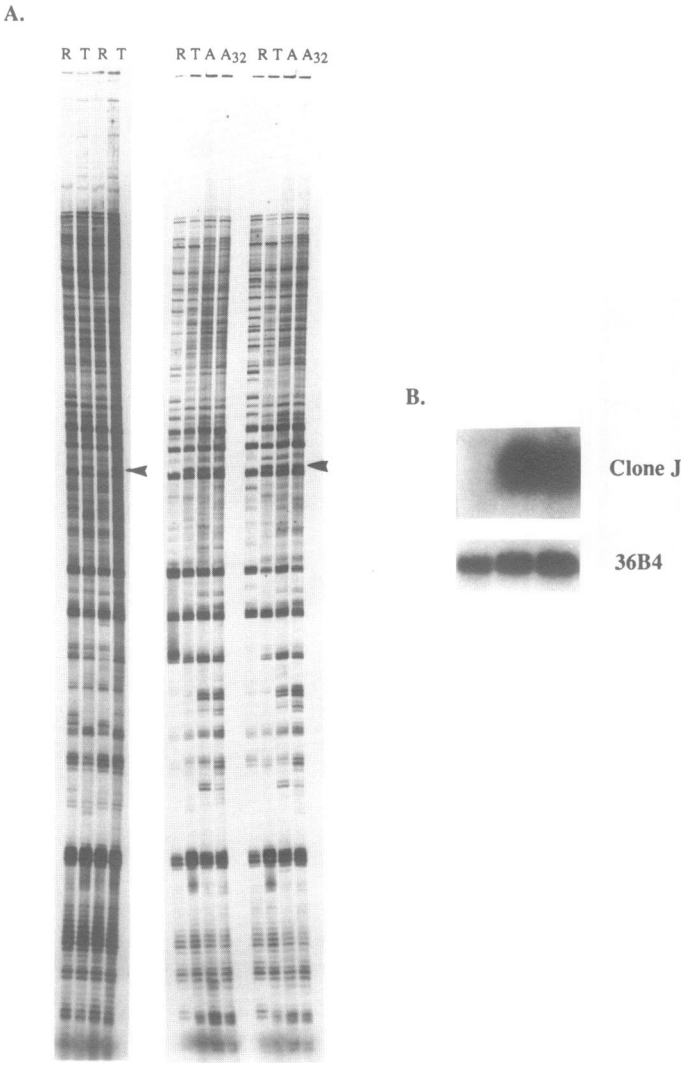

Figure 4. Reproducibility and multiple display of mRNAs from normal versus ras/p53 mutant transformed cells. **(A)** RNA samples from normal rat embryo fibroblasts REF (R) and its ras/p53 doubly transformed derivative T101-4 cells (T) were reverse transcribed and amplified in duplicate with T12MA and OPA17 primers (left four lanes). In a separate experiment, RNA samples from REF (R), T101-4 (T) and another ras/p53 temperature-sensitive mutant transformed cell line A1-5 grown at non-permissive-temperature (A) and shifted to permissive-temperature for 24 hours (A_{32}) were reverse transcribed and amplified in duplicate with T12MA and OPA17 primers (right eight lanes). An arrowhead indicates a reproducible difference between normal and transformed cells. **(B)** Northern blot analysis of this reamplified cDNA probe (named as clone J). 20 mg of total RNA from REF, T101-4 and A1-5 cells were analyzed. 36B4 was used as a probe for RNA loading control.

minimize false positive bands is to run duplicates, or repeat the experiment for those lanes in which putative candidate bands were identified (Figure 4A, left panel). In this experiment, the same RNA samples from normal REF cells and its ras/p53 mutant transformed derivative, T101-4 cells, were reverse transcribed and amplified in duplicate with T12MA and OPA17 primers. The pattern of the mRNA displayed as shown here are highly reproducible (> 95% the same by counting discrete bands) between duplicate experiments. One reproducible difference between REF and T101−4 cells was observed (Figure 4, indicated by arrowhead).

To see if this cDNA band is also present in other ras/p53 mutant transformed cells, multiple RNA samples from normal REF cells and transformed cells, T101-4 and A1-5, which contains a temperature-sensitive p53 mutation different from that of T101-4, were reverse transcribed and amplified in duplicate (Figure 4A. right two panels). Again, highly reproducible display patterns in general were obtained, and showed that the difference seen before was not only reproducible but also this cDNA band was present in the other A1-5 transformed cells whether grown at the non-permissive (p53 in mutant conformation) or the permissive (p53 in wild-type conformation) temperature.

The reproducible difference on the display was then further characterized and confirmed by Northern analysis (Figure 4B). Therefore by taking advantage of the side by side comparisons of more than two related RNA samples that are possible with differential display, differentially expressed genes that are unique to a process, such as ras/p53 mutation caused cell transformation, here given as an example, can be more accurately isolated. Multiple displays such as this also provide an internal control to minimize choosing false positive, or individual cell-specific genes that may not be relevant at all to the system under the study.

It was noted that most of the probes that failed to detect signals on Northern blots were smaller than 200 bp in length. Therefore the failure of the Northern blot could be due to the inefficient recovery of the probe from the agarose gel, much shorter labeled probes generated by random-prime labeling method, and too high stringency of hybridization and washing conditions used in the standard Northern blot analysis. It was also possible that the mRNAs of genes expressed at very low levels were below the detection limit of the Northern analysis. A recent report described a similar method for detection of RNAs using only a single arbitrary 20mer (6). Using DNA-free RNA and single arbitrary 10mers for both reverse transcription and PCR, we have not yet been able to obtain a consistent RNA amplification under the conditions described here. This difference could be attributed to differences in length of arbitrary primers, dNTP concentrations, DNA contamination in RNA preparations, or PCR parameters used.

In summary, here we described in more detail methods that further streamline the mRNA differential display technique to make it more readily applicable to a wide range of biological systems. When RNA from two or more relevant cell types were compared side by side, the method could reveal a small fraction of genes that were differentially expressed, and allow probes to those genes to be isolated. This make it possible to identify genes that play key roles in a broad spectrum of biological and pathological processes.

Several problems remain. One is that finding a majority of mRNAs is labor intensive; short cuts need to be devised if one wants to obtain more than a few mRNA differences for further study. Another question is how efficiently rare mRNAs are displayed (see above). Are rare mRNAs revealed as efficiently and reproducibly as more abundant ones? Finally the problem of false positives must be addressed in order to reduce the labor of further screening. Undoubtedly, future work should focus on these issues to make differential display a more effective and reliable tool for biomedical researchers.

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