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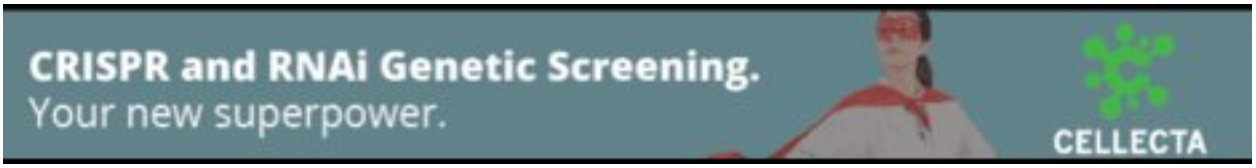

Detection and Differential Display of Expressed Genes by DDRT–PCR

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Analysis of gene expression is a central aim in most studies in molecular and cell biology. Interest in tissue-specific gene expression and, in particular, in changes in the expression patterns occurring in response to either mutations or transfected genes has stimulated the search for proper methods to identify the actual differences between two situations. For a long time, differential hybridization was the only method allowing for isolation of genes that were in either of two situations; there was no method available that would indicate the total number of genes subject to up- or down-regulation in a particular setting.

Pardee and co-workers were the first to show[1–3] that combinations of arbitrary primers (previously used for amplification of DNA polymorphisms[4,5] and for obtaining mRNA tags[6]) with anchored cDNA primers can be applied successfully to generate a set of fragments from cDNA derived from the total mRNA of a cell. Under the appropriate conditions, the pattern of fragments derived from one type of cells is reproducible and can be compared with that of another cell type.[1,3,7] Our theoretical calculations and experimental results confirmed that the method can generate patterns of bands that might represent almost all expressed genes in a particular cell and can also reproducibly detect differences in gene expression between two or more cell types or situations.[7,8] The method was named "differential display"[1] or DDRT–PCR.[7,8] This method has already been applied successfully by several groups using only a limited set of primers[9–11] and will probably become a standard method for studies on differential gene expression. Here, we present a detailed protocol that is applicable to any set of two or more comparable eukaryotic cell types and will result in reproducible patterns of PCR products that correspond to expressed genes.

OUTLINE OF THE METHOD

The strategy of the method consists of three basic and two additional steps, as shown in Figure 1: (1) Reverse transcription in fractions using a set of anchored primers, (2) amplification of cDNA species from each fraction using a set of arbitrary primers and anchored primers, (3) electrophoretic separation of the resulting fragments, (4) reamplification of fragments that are different in two situations, cloning and sequencing, and (5) confirmation of differential expression by an independent RNA analysis technique (Northern blotting, RNase protection, and/or nuclear run-on).

The method uses either cytoplasmic or mRNA as the starting material. The RNA is first reverse-transcribed to yield single-stranded cDNA. The step of reverse transcription is already an inherent part of the method. To be able to display as many different expressed genes as possible, a clever subdivision of the total number of mRNAs is required. Subdivision of the mRNAs should, at the same time, provide an anchor sequence for the subsequent PCR amplification. Liang and Pardee[1] originally suggested the use of 12 different primers of the type $T_{11}VN$, where V can be A, G, or C, and N can be any of the 4 nucleotides. By using these primers, one would generate 12 subfractions of cDNA that should represent almost equally one-twelfth of the expressed genes of a particular cell. Assuming that 15,000 genes are expressed in a cell at one time, one subfraction of cDNA would contain cDNA species representing 1250 different genes. Because the terminal 3′ base of the primer provides most of the specificity, Liang and co-workers[3] suggested reducing the number of cDNA subfractions to only four by using a T_{12} primer, where M is a degenerate mixture of A, G, and C. This reduces the number of cDNA species present in the pool. On the contrary, it reduces the theoretical chance of identifying cDNA species that is actually present. According to our experience, it is advisable to stick to the 12 fractions if one is interested in gaining

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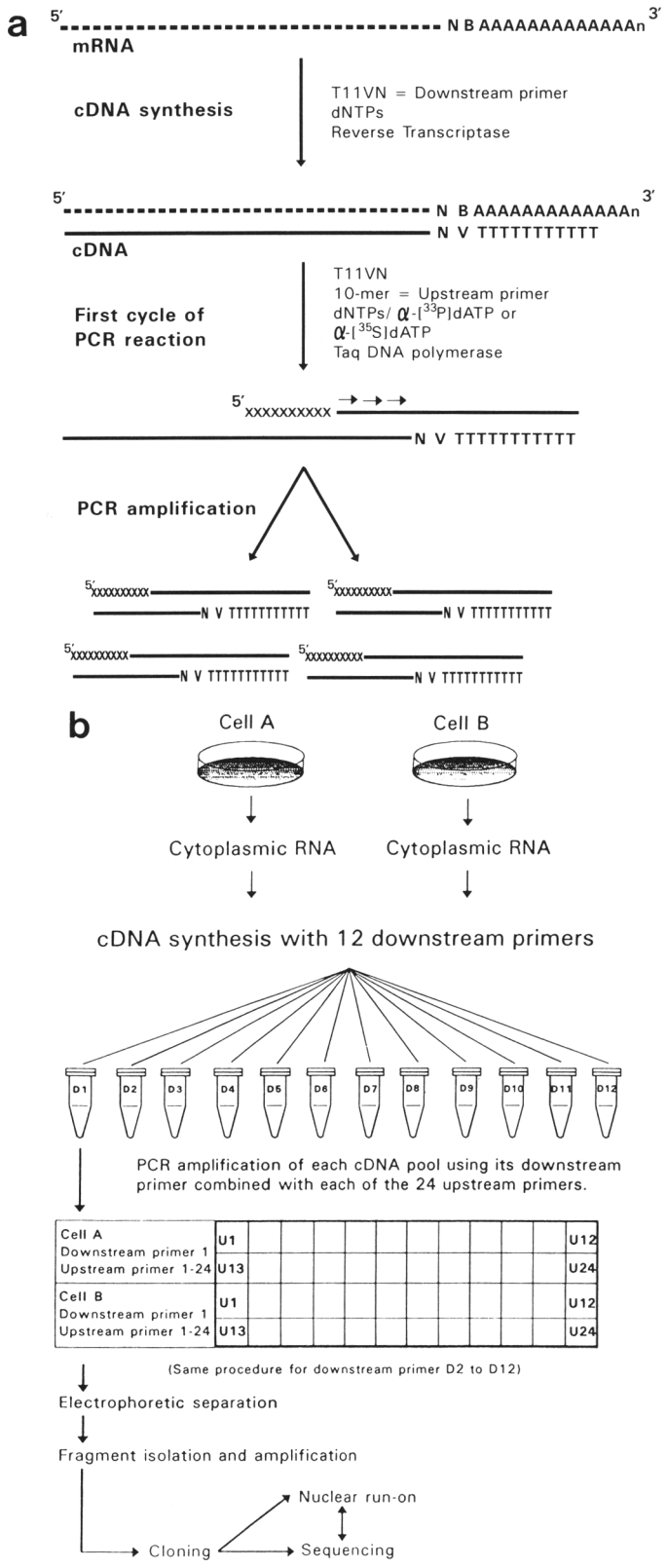

FIGURE 1 (*a*) Principle of the reactions underlying DDRT–PCR analysis. (N) Any of the four nucleotides G, A, T, or C. V can be any nucleotide besides T. (B) Any nucleotide besides A. (*b*) Flowchart of DDRT–PCR analysis with the subsequent confirmation of differential regulation and characterization of differentially expressed genes.

an almost complete picture of the expressed genes. However, reducing the number of fractions is useful if one is interested only in having a first glance at the situation or in identifing only new genes.

The next step is the actual amplification of as many of the cDNA species as possible in a PCR incubation. For this step, the anchored primers used for transcription serve as the downstream primers. The upstream primers are 10-mer oligonucleotides of arbitrary sequence; not every 10-mer is suitable, and each must be tested experimentally. Not only the lack of self-complementarity but also the same GC-content (50%) of all primers is important.[7] From our theoretical calculations, the chance for every mRNA species to be displayed requires that 24–26 different primers (Table 1) must be used in combination with every downstream primer; this amounts to 288 or 312 individual PCR reactions.[7] Optimal conditions for the annealing of the primers are required to obtain the maximal number of fragments displayed in the following electrophoretic separation.[3,7,8]

Electrophoresis can be done on sequencing gels[1–3] or on nondenaturing gels.[7] We recommend the latter type because nondenaturing gels reduce the artificial complexity of the patterns often observed with sequencing gels as a result of strand separation and incomplete addition of a terminal A by certain thermostable DNA polymerases. Also, the bands are much easier to process using nondenaturing gels. Radioactive nucleotides are the preferred labeled substrates; thus gels are first exposed to autoradiography with X-ray film. The bands of interest are cut out from the gel and reamplified, and the fragments are then cloned into a suitable vector. Because more than one cDNA species could be present within one band, isolation and further characterization of several colonies are advisable. We also recommend repeating the DDRT–PCR analysis with the pairs of primers that led to the detection of differences,

TABLE 1 List of 10-mer Upstream Primers Used for DDRT-PCR

No.	Sequence (5′ → 3′)
1.	T ACAACGAGG
2.	T GGATTGGTC
3.	C TTTCTACCC
4.	T TTTGGCTCC
5.	GGAACCAATC
6.	A AACTCCGTC
7.	T CGATACAGG
8.	T GGTAAAGGG
9.	T CGGTCATAG
10.	GGTACTAAGG
11.	T ACCTAAGCG
12.	C TGCTTGATG
13.	GTTTTCGCAG
14.	GATCAAGTCC
15.	GATCCAGTAC
16.	GATCACGTAC
17.	GATCTGACAC
18.	GATCTCAGAC
19.	GATCATAGCC
20.	GATCAATCGC
21.	GATCTAACCG
22.	GATCGCATTG
23.	GATCTGACTG
24.	GATCATGGTC
25.	GATCATAGCG
26.	GATCTAAGGC

In the standard setup only the first 24 primers are used.

ideally once with the same RNA and once or twice with a new RNA preparation. This procedure increases the certainty of detecting real differences in gene expression.

Characterization of the cloned fragments should be done in two ways. First, the fragments of interest should be analyzed to determine whether they correspond to a known or an unknown gene. This requires sequencing of the clones. However, one also has to ensure that the fragment is differentially regulated and not just an artifact. This requires application of another method for detection of differences in gene expression. Northern blot analyses or RNase protection assays are the most obvious choice. Disadvantages of these methods are not only their labor-intensive and time-consuming performance but also the requirement for labeling of multiple probes corresponding to the differentially displayed cloned candidates. Our preference is to verify the differential expression of the PCR-obtained fragments by nuclear run-on assays, which require only one labeling reaction for every cell type studied and can incorporate an almost unlimited number of samples. We recommend including six clones from every fragment of interest in the nuclear run-on analysis. From this analysis, one can determine which clone derived from a particular fragment actually corresponds to the regulated gene. Sequencing is carried out afterwards only for the clones that are derived from differentially expressed genes.

The whole DDRT–PCR analysis of two cell types, including 576 PCR incubations and 12 gels with 48 lanes each, can be carried out by one person within 8 working days. Repetition of DDRT–PCR with selected primer pairs takes another 2–6 days. The time required for analysis of the bands by nuclear run-on and subsequent sequencing is hard to estimate. To introduce the beginner to the method and to provide the advanced user with a handy guide, we provide an easy-to-follow, day-by-day protocol.

PROTOCOLS

Cytoplasmic RNA Preparation

Solutions

1. Extraction buffer

0.14 M NaCl
10 mM Tris-HCl (pH 7.5)
1.5 mM $MgCl_2$

2. Solution D:[12] Guanidinium thiocyanate lysis solution. Disolve 100 grams of guanidinium thiocyanate (Fluka) in 117 ml of water, add 7.0 ml of 0.75 M sodium citrate (pH 7.0) and then 10.5 ml of 10% Sarkosyl, dissolve at 65°C, and store at room temperature for several months. Before use, add 72 µl of concentrated β-mercaptoethanol to 10 ml of lysis solution.

Basically, any kind of RNA preparation can be used. However, we have obtained the best results using DNA-free cytoplasmic RNA preparations, which are carried out as follows:

1. Wash ~4×10^6 cells with PBS (lacking Ca^{2+}).
2. Resuspend cells gently in 450 µl of extraction buffer.
3. Add 50 µl of 5% NP-40 dropwise and mix; leave for 2 min on ice.
4. Spin at 550*g* for 5 min at 4°C.
5. Transfer supernatant into a fresh tube containing 4.5 ml of solution D and mix; leave for 10 min on ice.
6. Add 450 µl of 2 M Na-acetate (pH 5.0) and 4.5 ml of acid phenol, shake for 5 min, and then add 1.5 ml of chloroform/isoamylalcohol (49:1).

7. Spin for 10 min at 4000*g*.
8. Take the upper phase and add 5.7 ml of isopropanol; leave for 20 min on ice.
9. Spin at 4500 rpm for 30 min, take off supernatant carefully, and discard.
10. Resuspend the pellet in 0.3 ml of solution D,[12] transfer to an Eppendorf tube, and incubate for 15 min on ice.
11. Precipitate the RNA with 1 ml of 96% ethanol at −70°C.
12. Spin at full speed for 10 min, wash the pellet with 1 ml of 70% ethanol, and spin again.
13. Remove supernatant and resuspend the pellet in 200 μl of DEPC-treated water. Add 10 μl of 1 M Tris (pH 7.5), 2 μl of 1 M $MgCl_2$, 2 μl of RNase-free DNase (Boehringer Mannheim), and 0.5 μl of RNasin (Promega), and incubate for 15 min at 37°C.
14. Add 200 μl of phenol/chloroform (1:1), vortex, and spin to separate the two phases.
15. Take the supernatant and add 20 μl of 2 M Na-acetate (pH 4.0) and 1 ml 96% ethanol, cool down to −70°C for 10 min, spin for 10 min, and wash the pellet with 70% ethanol.
16. Resuspend the pellet in 10–20 μl of DEPC-treated distilled water, and measure the concentration.
17. RNA should be stored in ethanol/acetate at −70°C.

RNA preparation and cDNA synthesis can normally be done within 1 day

Single-strand cDNA Synthesis

The cDNA synthesis reaction is designed for running 6×96 PCR incubations, which gives the complete DDRT–PCR analysis for two cell types using 12 downstream and 24 upstream primers. From each cell type, there will be 12 cDNA pools, which will be incubated with 24 different upstream primers. For two cell types, 2×12 cDNA reactions must be prepared. The volumes in the master mix should be adjusted accordingly.

cDNA Master Mix

150 μl of 5× first-strand cDNA synthesis buffer (BRL)
75 μl of 0.1 M DTT (BRL)
150 μl of 100 μM dNTP mix (Pharmacia)
19 μl of 40 U/μl of RNasin (Promega)
19 μl of sterile DEPC-treated water

cDNA Synthesis

For cDNA synthesis, 24 reaction tubes are prepared as follows:

1. Add 3.0 μl of 25 μM downstream primer to each tube. Because there are 12 different downstream primers, 12 tubes are set up per cell line: 1–12 for cell line A and 13–24 for cell line B.
2. Add 3.0 μl (100–300 ng) of cytoplasmic RNA from A to each of tubes 1–12 and RNA from cell B to each of tubes 13–24.
3. Add 7.5 μl of sterile water to all 24 tubes.
4. Heat for 10 min at 70°C and place on ice immediately.
5. Add 16.5 μl of the cDNA master mix to all 24 tubes.
6. Incubate for 2 min at room temperature.
7. Add 1.5 μl (300 units) of SuperScript RNase H-minus reverse transcriptase (BRL).
8. Incubate for 8 min at room temperature followed by 1 hr at 37°C, heat

to 95°C for 5 min, place on ice immediately, and store at −70°C for further use.

DDRT–PCR and Gel Electrophoresis of DNA Fragments

This PCR protocol is designed for 96 PCR reactions, which allows the analysis of two cDNA subfractions from two different cell types with two dT11VN downstream primers and 24 upstream primers within 1 day (see Fig. 1b). (This has to be repeated five times within the next days using the other 10 downstream primers.)

DDRT–PCR Master Mix

234.0 µl of 10× PCR buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C), 0.1% gelatin, 1% Triton X-100]
140.4 µl of 25 mM $MgCl_2$ (to be optimized depending on the *Taq* polymerase)
11.7 µl of [^{33}P]dATP, 10.0 mCi/ml (Dupont NEN)
46.8 µl of 100 µM dNTP mix (Pharmacia)
22.0 µl of *Taq* polymerase (5 U/µl)
949.1 µl of sterile water

From this master mix, 324 µl are transferred to each of four tubes, which are supplemented with downstream primer and cDNA as follows:

DDRT–PCR Setup

Tube number	1	2	3	4
Total number of reactions	24	24	24	24
Master mix (µl)	324	324	324	324
Downstream primer number	1	1	2	2
Downstream primer (µl, 25 µM)	54	54	54	54
cDNA from cell type	A	B	A	B
Amount of cDNA (µl)	27	27	27	27
Total µl	405	405	405	405

Ninety-six PCR mixes are prepared in a microtiter plate or in tubes arranged in a microtiter format (Perkin-Elmer) as follows:

1. Distribute 15 µl of the premixed components from each of the four tubes into 24 wells or tubes.
2. Add 5 µl (2 µM) of the different upstream primers using a 12-channel micropipette. Each of the 24 wells or tubes will only receive 1 of the 24 different upstream primers.
3. Spin briefly in a centrifuge that holds microtiter plates to mix the upstream primers with the premixed components.
4. Run PCR reactions in a suitable thermocycler (e.g., GeneAmp PCR system 9600, Perkin-Elmer) equipped with a heated lid.

Amplification Protocol

30 sec at 94°C
60 sec at 40°C
60 sec at 72°C
40 cycles
5 min at 72°C

The gels can be prepared during the PCR incubations. For 96 reactions, we run two gels with 48 samples each, covering exactly all of the reactions from one cDNA subfraction of two cell types to be compared. If more cell types are to

be compared, it is advisable to run the related reactions (generated using the same pair of primers) side by side.

Electrophoresis

Solutions

1. 20× TTE

215 grams of Tris base
71.3 grams of Taurine
20 ml of 0.5 M EDTA
Make up to 1 liter with water

2. 6% gel solution

14.5 ml of 40% stock 19:1 acrylamide/bisacrylamide
5.0 ml of 20× TTE
80.5 ml of water
Pass through a 0.2-μm filter

Use 50 ml of the 6% gel solution for a 35×40×0.2-cm gel; add 40 μl of TEMED and 200 μl of 10% ammonium persulfate.

Concentrate half of the DDRT–PCR incubation mixture (10 μl) by vacuum and heat (10 min) to 2 μl, adjust with glycerol to 5%, xylene cyanol FF, and bromphenol blue. Load 2 μl onto a prerun 6% polyacrylamide gel without urea and run in TTE or TBE buffer at 50 W. The run is stopped when the bromphenol blue runs out of the gel. Dry the gel on filter paper and expose to X-ray film overnight. A typical result is shown in Figure 2.

Reamplification, Cloning, and Sequencing of DNA Fragments

Cut out the bands of interest (e.g., those differing between the patterns from control cells and the test cells) from the filter paper and transfer to Eppendorf tubes. Add 100 μl of DEPC-treated water and boil for 10 min. Take out and discard the filter paper.

Reamplification PCR

26.5 μl of eluted DNA
5 μl of 10× PCR buffer (as above)
3 μl of 25 mM $MgCl_2$
5 μl of 500 μM dNTP mix
5 μl of 2 μM downstream primer*
5 μl of 2 μM upstream primer*
0.5 μl of *Taq* polymerase (5 U/μl)

*Both primers should be the same as the ones used to generate the original PCR product.

Amplification is carried out with the cycle protocol given for DDRT–PCR. A total of 10 μl of the reaction mixture is run on a 2% agarose gel to check the size. Fragments of interest are cloned using TA cloning kit (Invitrogen) using 1–3μl of the reamplification solution. Generally, six white colonies are picked from each cloned fragment and plasmid minipreparations are made. Plasmids are cut with *Eco*RI and checked for inserts by agarose gel electrophoresis. At this point, the inserts could be sequenced. However, because it is still unclear which of the potentially different cDNA species contained within one band actually corresponds to a regulated gene, if there is a regulated one at all, it is advisable to confirm the actual difference in expression by another RNA

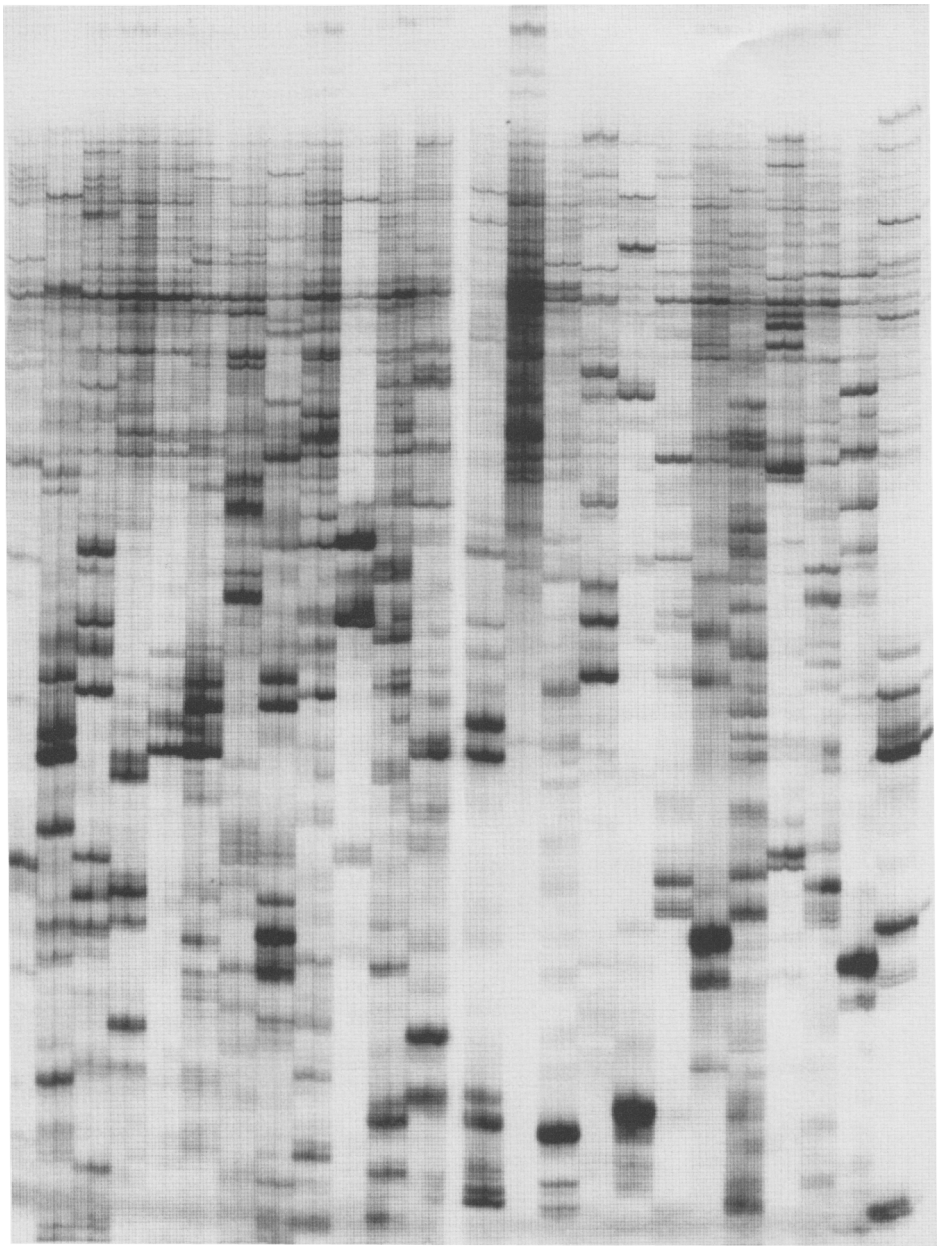

FIGURE 2 Electrophoretic pattern of one nondenaturing gel containing the PCR samples obtained with one downstream primer and 24 upstream primers.[7] The 24 pairs of samples represent cDNA from a retinoblastoma (*Rb*)-gene deficient tumor cell line (*left* side of each pair) and the same cell line after transfection of the *Rb* gene (*right* side of each pair).

detection method. Using nuclear run-on analysis, multiple fragments (the six clones isolated of each fragment) can be processed at the same time using only one labeling reaction per cell type. If the primary interest is to find out as quickly as possible whether the fragment corresponds to an unknown gene of special interset, one would sequence the DNA immediately. DNA sequence analysis can be carried out using an fmole sequencing kit (Promega) or a cycle sequencing kit (U.S. Biochemical).

Nuclear Run-on Analysis

To confirm differential regulation of individual candidate bands, we suggest

using nuclear run-on assays instead of Northern blot hybridizations. Because vector sequences can create considerable nonspecific signals, we prefer to probe with the cloned fragments. The DNA fragments to be tested are amplified from 1 ng of fragment using two primers specific for flanking sequences in the TA vector (5′-TAGTAACGGCCGCCAGTGT and 5′-GCCGCCAGTGTGATGGATA) in a standard PCR reaction using the Perkin/Elmer 9600 machine (30 sec at 94°C, 30 sec at 65°C, 60 sec at 72°C, 40 cycles, 5 min at 72°C). Here, we give our standard method,[13] which is relatively fast and can be carried out within 3 days.

Solutions

1. Lysis buffer
10 mM Tris-Cl (pH 7.4)
5 mM $MgCl_2$
10 mM KCl
0.5% NP-40

2. Nuclear freezing buffer
50 mM Tris-CL (pH 7.4)
5 mM $MgCl_2$
40% glycerol
0.5 mMDTT

3. Transcription buffer
25 mM Tris-CL (pH 8.0)
12.5 mM $MgCl_2$
750 mM KCL
1.25 mM NTP (without CTP)

4. 10× STE
10% SDS
0.1 M Tris-Cl (pH 7.5)
50 mM EDTA

5. 20× SSPE
3.6 M NaCl
200 mM NaH_2PO_4 (pH 7.4)
20 mM EDTA (pH 7.4)

Preparation of Nuclei

1. Approximately 5×10^7 cells are required for one assay. Harvest the cells, wash with PBS, and resuspend im 10 ml of lysis buffer.
2. Move dounce homogenizer (type B) up and down eight times in the cell suspension.
3. Spin the extract at 2000 rpm for 10 min, and remove the supernatant completely.
4. Resuspend the nuclei in 210 μl of nuclear freezing buffer and mix by pipetting up and down several times with a yellow tip.
5. Freeze immediately at −70°C, even if you continue with the next steps. Upon thawing, the quality of the nuclei becomes obvious. The nuclei should be dispersed easily by pipetting with a yellow tip. Big clumps that are difficult to disperse indicate bad quality.

Nuclear Transcription

1. Add 60 μl of transcription buffer and 30 μl of [^{32}P]CTP (300 μCi) to 210 μl of nuclei.

2. Vortex briefly and gently and incubate for 30 min at 30°C; shake every 10 min.

Preparation and Labeled RNA

1. Add 10 μl of DNase (230 units, RNase-free) to the nuclear transcription reaction and incubate for 30 min.
2. Add 36 μl of 10× STE buffer and 10 μl of proteinase K (10 mg/ml), and then heat to 65°C for 3 min; incubate at 37°C for 45 min.
3. Extract with 360 μl of phenol/chloroform, reextract the interphase with 100 μl of STE buffer, combine aqueous phases (460 μl), and add 203 μl of 7.5 M ammonium acetate (pH 5.0); add the same volume of isopropanol, put in dry ice for 20 min, spin for 10 min, and reprecipitate the RNA with ethanol.
4. Resuspend the pellet in 180 μl of 10 mM of Tris (pH 7.5)–1 mM EDTA, add 20 μl of 2 N NaOH, and place the tube on ice for 5 min. After adding 200 μl of 0.48 M HEPES (free acid), precipitate the RNA with 900 μl of ethanol at −20°C overnight.

Filter Hybridization

1. Amplify the inserts out of the cloning vector using primers specific for flanking vector sequences. Denature 2 μg of each fragment (per filter) with 0.25 N NaOH for 10 min at room temperature; apply via individual slots to a Hybond N filter (Amersham) in a slot–blot device (Schleicher & Schuell). Wash slots immediately with 1 M ammonium acetate (5–10 volumes) to neutralize the DNA solution. Prepare identical strips with multiple samples for each hot RNA probe.
2. Hybridize the filters with the total lot of hot RNA (1×10^7 to 5×10^7 cpm) at 55°C for 2 days in the SSCP buffer system (10 ml). Use the same amount of label for each nuclear RNA preparation.
3. After washing, either expose the filters to X-ray film for ~1 week or analyze preferentially on a PhosphorImager.

A typical result from our analysis of genes regulated by the retinoblastoma protein is given in Figure 3. It shows clearly that 9 of 10 candidate genes are expressed at different levels in the two cell lines compared. None of these candidates appeared to be differentially regulated by Northern blot analysis.

DISCUSSION

DDRT–PCR allows generation of highly reproducible patterns of bands from a particular cell line. We have used DDRT–PCR in both of our laboratories for 1.5 years for studies of liver regeneration and for the analysis of genes regulated by tumor suppressors. We have identified a large number of differentially regulated genes in both projects.[14,15] We would like to stress that, although confirmation of differently displayed bands as such by repetition of the DDRT–PCR analysis is advisable, confirmation of the actual differential regulation by another method, such as nuclear run-on analysis, is required. In our experience, a considerable number of the differently displayed bands of one analysis are not reproducible (20–40%). However, if a particular difference is reproduced once, it is 90% certain that it can be reproduced in every other repetition. Confirmation of differential regulation by Northern blot hybridization is often not successful. We suceeded in only 5 of 50 attempts in the detection of different expression levels in the two cell lines analyzed. In some cases, no mRNA was detectable at all, and in most cases, the differences were hard to see on film. In the situations that we examined, the regulated genes could be expressed at constitutive levels and may only be induced for

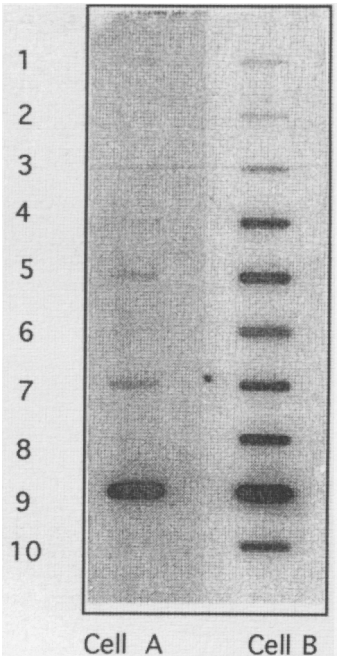

FIGURE 3 Nuclear run-on analysis of fragments differently displayed on nondenaturing gels in the DDRT–PCR analysis of *Rb*-regulated genes. Hybridized filters were analyzed using a PhosphorImager. Cell A is the *Rb*-deficient tumor cell line BT549; cell B is BT549 ttransfected with the Rb gene.[14]

a very short period of time in response to an inducing signal without great influence on the total pool of the respective mRNA. When confirming the expression differences by nuclear run-on assays, we detect particularly the induced activities. Basic expression at relatively low levels may appear as background. Thus, this method is well suited to confirm differential regulation.

It should be mentioned that DDRT–PCR is not quantitative. Even drastic differences between two situations (e.g., a strong band from one cell type and absence of this band in the control cell) can occur when the actual difference of expression is only fivefold, and, more often, slightly different intensities in a particular band position are related to dramatic differences in gene expression. The latter is often the result of the simultaneous presence of a constitutively expressed RNA fragment in the same gel position.

In summary, DDRT–PCR is the most flexible and comprehensive method available for the detection of almost all genes expressed in a particular cell and for the identification of differences in gene expression between different cell types. This technique has at least three advantages over differential hybridization or differential libraries: (1) It allows simultaneous display of all differences, (2) it detects up-regulation and down-regulation of genes at the same time (the latter may be difficult in the case of transient transfection assays), and (3) it is faster. Improvements of the technique are imaginable, particularly with regard to the easier detection of weak, differentially expressed bands. Attempts toward automatic analysis[7] will be continued. The method is already established in numerous basic research laboratories, and we anticipate its use in applied research and diagnostic laboratories in the near future.

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