Nonradioactive Detection of Differentially Expressed Genes Using Complex RNA or DNA Hybridization Probes

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BioTechniques 26:150-155 (January 1999)

ABSTRACT

The analysis of differential gene expression has become increasingly important in recent years. Typically, differentially expressed genes are identified in a primary screening procedure, yielding candidate genes whose differential expression has to be verified. We provide a highly sensitive, efficient and nonradioactive differential screening procedure to analyze numerous candidate genes in a single step. This comprises labeling of poly(A)⁺ RNA of the cell types analyzed with DIG Chem-Link and differential hybridization to the candidate genes fixed on dot blots. DIG Chem-Link allows, to our knowledge, for the first time efficient and direct nonradioactive labeling of RNA in vitro. Advantages of this method include extremely short exposure times and the feasibility to re-use the probes after prolonged storage. Using this procedure, we isolated several genes that are differentially expressed in maturing Langerhans cells.

INTRODUCTION

Differential gene expression is of central importance for many cellular activation and differentiation events. Techniques currently used to identify differentially expressed genes are: (i) the preparation of subtraction libraries (8), (ii) differential screening of cDNA libraries (10) and (iii) differential display (3). All these procedures require verification steps to prove differential expression of isolated cDNAs. Two different approaches can be pursued for verification: either cDNA-specific probes are generated for detection, e.g., by Northern blotting or by reverse transcription polymerase chain reaction (RT-PCR), or the selected cDNA clones are arrayed and analyzed by hybridization with discriminating probes. The latter approach allows for analysis of numerous

candidate cDNAs in a single step. Heretofore, mainly enzymatic labeling procedures have been available for generating the probes; however, the risk of using these procedures is that certain sequences may not be equally represented. Recently, a new nonenzymatic labeling technique, Chem-Link, has been made available by Boehringer Mannheim GmbH (Mannheim, Germany). DNA and RNA are labeled either by digoxigenin (DIG) or biotin through non-covalent binding to the N7 position of adenosine and guanosine nucleotides, mediated by *cis*-platinum complexes. Highly sensitive detection methods are available for both haptens. This method, to our knowledge, for the first time allows the in vitro preparation of highly sensitive detectable complex RNA probes, from total RNA or poly(A)⁺ RNA of the analyzed cell types. Further advantages of the nonradioactive labeling and detection are: (i) extremely short exposure times (minutes compared to hours to several days to radioactively labeled probes), (ii) the stability of labeled probes (at least one year compared to several days with radioactively labeled probes) and (iii) eliminating the inconveniences of working with radioactivity.

The longevity of the probes is especially important when the RNA for the screening procedure is limited as it is in our system. Applying the methods of differential screening and differential display, we isolate differentially expressed cDNAs of epidermal Langerhans cells (LC) to identify genes involved in primary stimulation of naive T cells (4,7). Dendritic cells (DC) are unique in their ability to efficiently activate naive T cells. LC, the DC of the skin, are especially suited for studying this process because of the following: they are uniformly immature, are not yet able to prime naive T cells (2) and they mature during in vitro culture in presence of keratinocytes into potent immunostimulatory DC (9). However, LC represent only 1%–3% of the epidermal cells and are dif-

difficult and expensive to isolate. Thus the mRNA amount available for the differential screening probes is strictly limited. Therefore, we introduced an amplification step during preparation of the differential screening probes (6) that allows for differential screening with as few as 100 ng of poly(A)⁺ RNA as starting material (4,7).

In this study, we performed differential screenings using poly(A)⁺ RNA and amplified complex cDNA probes, nonradioactively labeled with the DIG Chem-Link Labeling and Detection Set. The screening probes proved to be highly sensitive and reliable. Aliquots of 1 µg poly(A)⁺ RNA for RNA labeling and 100 ng poly(A)⁺ RNA for the amplified cDNA probes were sufficient for differential hybridization.

MATERIALS AND METHODS

Cells

LC were isolated from BALB/c Ann mice, bred in our animal facilities from breeding pairs originally obtained from the Zentralinstitut für Versuchstierkunde (Hannover, Germany). Epidermal cells were prepared from pelts of 2–5-months-old mice, and LC were isolated by immunomagnetic separation either directly (fLC) or following a 3-day cultivation period (cLC) as described previously (7).

mRNA Extraction and RT-PCR

Poly(A)⁺ RNA was isolated from fLC and cLC using the mRNA Isolation Kit (Boehringer Mannheim). Aliquots were used (i) for RT-PCR as described previously (5) using a Model 480 DNA Thermal Cycler (Perkin-Elmer, Ueberlingen, Germany), (ii) for direct nonradioactive labeling or (iii) to generate cDNA followed by amplification and subsequent labeling (see below).

Preparation and Amplification of cDNA Probes

Poly(A)⁺ RNA (200 ng) was reverse-transcribed using 200 U of SUPERSCRIPTTM II Reverse Transcriptase (Life Technologies GmbH, Eggenstein, Germany), and cDNA was amplified in a Model 480 DNA Thermal Cycler using the SMARTTM PCR cDNA Synthesis Kit (CLONTECH Laboratories, Palo Alto, CA, USA) following the manufacturer's recommendations.

Labeling of Probes

Aliquots representing 100–300 ng RNA or 2 µg DNA were labeled by incubation with 0.1–2 µL DIG Chem-Link in a 20-µL volume at 85°C for 30 min.

Plaque Filters, Dot Blots and Library Filters

For plaque lifts, neutral nylon membranes (Boehringer Mannheim) were used according to the manufacturer's recommendations. When replicate filters were taken, the first filter and the second filter were placed on the surface of the plate for 1 and 2 min, respectively, to allow efficient transfer despite the reduced number of phages.

Neutral nylon membranes were also used for dot blots.

The membrane was placed on Whatman 3-MM paper (Whatman International Ltd., Maidstone, Kent, England, UK), and 2 µL (or less-aqueous DNA solution) were dotted on the nylon membrane. The filters were air-dried and processed like plaque filters.

Total genomic P1 human library filters (library No. 700) were obtained from the Resource Center (Heidelberg, Germany) and used directly for hybridization.

Hybridization

Membranes were pre-hybridized with DIG Easy Hyb (Boehringer Mannheim) at 42°C for 1–3 h under slight agitation. The solution was replaced by the hybridization solution containing the denatured probe and incubated under slight agitation at 37°C (murine DNA probe), 42°C (human DNA probe) or 50°C (RNA probe) overnight in a water bath. Final concentrations of 20 ng/mL monospecific RNA or DNA probes, 40 ng/mL of complex RNA probes and 100 ng/mL of complex cDNA probes were used. When RNA probes were hybridized, or when arrayed library filters were used, we added 100 µg/mL of ultrasonic-fractionated carrier DNA (herring sperm DNA; Boehringer Mannheim) to the hybridization solution. The blots were washed twice with 2× standard saline citrate (SSC), 0.1% (wt/vol) sodium dodecyl sulfate (SDS) for 10 min at room temperature and then 2× with prewarmed 0.5× SSC, 0.1% (wt/vol) SDS for 15 min at 68°C.

Chemiluminescent Detection

Chemiluminescent detection was performed according to the recommendations of the manufacturer using CDP-StarTM (Boehringer Mannheim) with subsequent exposure to X-ray film or analysis and quantification using the Lumi-ImagerTM (Boehringer Mannheim).

RESULTS

Evaluating the Sensitivity of DIG Chem-Link

To address the question of whether slight variations in the ratio of mRNA/Chem-Link (e.g., due to differences in mRNA preparations) influence the outcome of the detection by the DIG Chem-Link system and to evaluate the sensitivity of the system, we used monospecific probes labeled by DIG Chem-Link before considering the use of complex RNA probes.

The amount of 100 ng neomycin-control RNA (Boehringer Mannheim), which is equivalent to the approximate amount of poly(A)⁺ RNA we generally isolate from 10⁶ LC, was subjected to five independent labeling reactions with increasing doses of DIG Chem-Link (0.1–0.5 µL). Each probe was used for hybridization with 1 µg neomycin plasmid DNA (Boehringer Mannheim) fixed on dot blots. In all cases, signals of similar intensity were obtained (Figure 1A), indicating that the amount of DIG Chem-Link was not a critical parameter within the range of interest.

The labeling reaction with 0.3 µL DIG Chem-Link was selected for further experiments. Dot blots were prepared with DNA target concentrations ranging from 20 000–0.02 pg and hybridized with the DIG-labeled probe. A final detection sen-

sitivity of 20 fg was achieved with neomycin plasmid DNA (Figure 1C). Dot blots with serial dilutions of murine β -actin cDNA that were treated likewise in parallel experiments served as a negative control. No signals were obtained with neomycin-specific RNA probes and nonhomologous β -actin samples (not shown). The high sensitivity encouraged us to use DIG Chem-Link for labeling of complex RNA probes, though dilution of a specific probe as part of a complex RNA mixture and dilution of the dotted DNA are not equivalent.

Differential Screening of Selected LC-Derived Genes Using DIG-Labeled RNA

Complex RNA probes were hybridized with known cDNA samples to test whether the correct expression pattern was obtained and whether moderately expressed genes were detectable. mRNA derived from 10^7 fLC and 10^7 cLC was pre-

pared as described, validated by RT-PCR with the 3-phosphoglycerate kinase (*PGK*) housekeeping gene (data not shown) and labeled with 2 μ L DIG Chem-Link. Equal amounts of PCR products from a gene, constitutively expressed in LC (*PGK*), one down-regulated gene (*pcLC115*) and two up-regulated genes (*pcLC36* and *fascin*) were fixed on two dot blots. One filter was hybridized with the fLC-specific RNA probe, and the other filter was hybridized with the cLC-specific RNA probe. Comparison of the two filters clearly shows that the signal intensities reflect the constant expression of *PGK*, down-regulation of *pcLC115* and up-regulation of *pcLC36* and *fascin* (Figure 2).

Differential Screening Using Amplified cDNA Probes

Poly(A)⁺ RNA was isolated from fLC and cLC. Approximately 200 ng were reverse-transcribed using the SUPERSCRIPT™ II Reverse Transcriptase (Life Technologies), and

Figure 1. Highly sensitive detection of cDNA using RNA probes labeled by DIG Chem-Link. (A) 1 μ g neomycin cDNA was fixed on dot blots and hybridized with probes containing 100 ng neomycin RNA labeled with 0.1, 0.2, 0.3, 0.4 or 0.5 μ L DIG Chem-Link. (B) Dots containing 20 000–2 pg neomycin DNA were hybridized with a neomycin RNA probe labeled with 0.3 μ L DIG Chem-Link; 5-min exposure. (C) 200–0.02 pg neomycin DNA hybridized with 100 ng neomycin RNA labeled with 0.3 μ L DIG Chem-Link; 30-min exposure.

Figure 2. Differential screening of cDNA samples using complex RNA probes labeled by DIG Chem-Link. Replicate filters containing equal amounts of PCR products of the 4 cDNA clones: 1, *PGK* (constantly expressed in LC); 2, *pcLC115* (partially down-regulated in cLC); 3, *pcLC36* (up-regulated in cLC); and 4, *fascin* (up-regulated in cLC), were hybridized with total mRNA derived from freshly isolated LC (d0) or 3-day cultured LC (d3).

Figure 3. Evaluation of amplified screening probes. (A) Aliquots of amplified total cDNA from freshly isolated (d0) or 3-day cultured (d3) LC were separated on a 1.4% agarose gel. (B) Aliquots of amplified total cDNA from freshly isolated (d0) or 3-day cultured (d3) LC were amplified with specific primers for the *PGK* housekeeping gene and the PCR products separated on a 1.4% agarose gel.

Table 1. Characterization of the Isolated cDNA Clones

Characteristics of Clones	No. of Clones
Isolated cDNA clones	112
Verified differentially expressed cDNAs	59
Up-regulated expression ^a	45
Down-regulated expression ^a	14
Independent cDNAs ^b	24
Frequency of isolation ^b	1–14 times
cDNAs isolated 10× or more	2
cDNAs isolated 2–3× ^b	6
Independent cDNAs corresponding to known murine genes	6
Independent cDNAs without homology to known sequences ^b	10

^acDNAs with up-regulated expression were preferentially selected.

^bcDNA clones were partially sequenced from one end. Due to length differences of the cDNAs at the 5' end, complete sequencing might reveal that some originate from the same gene.

10% of the cDNA solution were amplified in 100-μL volumes using the SMART system (CLONTECH). Five microliters of the amplified cDNA solution were separated on agarose gels, and 0.1 μL was amplified with PGK-specific primers and also separated on agarose gels. Comparable amounts of cDNA and PGK products were detected (Figure 3). The remaining 94.9 μL were phenol/chloroform-extracted, ethanol-precipitated, and the pellets were resuspended in 36 μL double-distilled water. Eighteen microliters containing approximately 2 μg of amplified cDNA derived from fLC or cLC were subjected to labeling with 2 μL DIG Chem-Link at 85°C for 30 min.

Approximately 20 000 clones of a cLC-derived cDNA library were plated, replicate filters were taken and hybridized with DIG-labeled, fLC-derived and cLC-derived probes. Figure 4A shows a typical result obtained with the cLC-derived probe. Strong and weak signals were detectable, indicating strong or weak expression, respectively, of the corresponding gene. Figure 4B shows small areas of replicate filters. Most plaques yielded signals of similar intensity, while some showed marked differences (arrows). Single plaques were isolated, phages were plated and replicate filters were hybridized again with fLC-derived and cLC-derived probes. Figure 4C shows replicate filters of a constantly expressed clone (pcLC168), an up-regulated clone (pcLC36) and a down-regulated clone (pcLC163).

In total, 112 differentially expressed cDNA clones were isolated. Re-screening revealed that 59 of these clones proved to be differentially expressed. All verified clones were partially sequenced with a vector-specific primer. Table 1 summarizes the results. The sequences obtained were compared with each other, with differentially expressed cDNAs we had isolated previously and with the DNA databases at the European Bioinformatics Institute (EBI) (Hinxton, UK). The analysis revealed that cDNAs corresponding to one and the same gene were isolated repeatedly (up to 14×). Among the remaining 24 unrelated sequences, 6 corresponded with known murine genes and 10 showed no homology to known sequences. Complete sequencing of these 10 cDNA clones will show if some of them are related with each other or with sequence entries of the accessible databases.

Screening of Arrayed Library Filters with DIG Chem-Link

Filters of 23 × 23 cm containing 27 648 independent, arrayed clones of a genomic human P1 library (1) were used directly for hybridization with differentially expressed murine cDNAs to isolate the homologous human gene. Figure 5A shows a positive signal of a cross-species hybridization between the human genomic DNA and the murine cDNA clone pcLC45. Each clone is dotted twice on a square to discriminate positive signals from background spots. Only a limited number of arrangements is realized, and the arrangement corresponding to the signal on the autoradiogram is shown enlarged in Figure 5B, lower left. The central spots of each square, which do not contain DNA, appear as white spots on the autoradiogram after hybridization with DIG Chem-Link.

DISCUSSION

In this study, we used the Nonradioactive DIG Chem-Link Detection System to label complex RNA or DNA probes, which we subsequently used for isolation and verification of genes differentially expressed in LC. The method is widely applicable since differential screening of cDNA libraries is an often used technique for isolation of differentially expressed genes. Candidate genes isolated by other techniques, e.g., differential display, can be verified by this method as well. In addition, it is possible to compare expression levels of already characterized genes using cDNA clones arrayed on nylon membranes. Plaque-filter screenings or screenings of arrayed cDNA libraries with monospecific probes are even less demanding because higher probe concentrations are achieved, and thus shorter exposure times can be used.

Alternatively, instead of using the chemiluminescent detection outlined above, a color detection was performed (data not shown) using the Multicolor Detection Kit (Boehringer Mannheim). Chemiluminescent detection was determined to be more sensitive than color detection. However, when strong signals are obtained, multicolor detection provides the advan-

tage of a simultaneous detection with two probes on the same filter and, therefore, a more straightforward readout. The probes can be labeled with DIG Chem-Link and Biotin-Chem-Link, respectively, and subsequently can be detected by different color substrates (e.g., red, green or blue). DIG-labeled probes yielded stronger signals than biotin-labeled probes, and mixed colors were easily detected on dot blots.

Because of limited RNA amounts for probe generation, we preferred chemiluminescent detection. Chemiluminescent detection of small amounts of DNA samples using DIG-labeled RNA probes proved to be very sensitive. Two picograms of DNA were detectable after a 5-min exposure, and even 20 fg were detectable after 30 min of exposure. The kinetics of DNA-DNA or DNA-RNA hybridization and, as a result, of signal intensity, depend on sample and probe concentration. Low probe concentrations were also determined to be non-

critical. We detected correct expression levels of all analyzed genes, including moderately expressed genes such as *PGK* using 1 µg of total poly(A)⁺ RNA labeled with DIG Chem-Link. The nonenzymatic labeling of RNA using Chem-Link promises to be especially reliable, because favored or disfavored labeling methods of certain mRNAs, due to either mRNA secondary structures or sequence preferences of enzymes, can be excluded. However, when mRNA amounts for probe generation are limited, it is possible to reverse-transcribe the poly(A)⁺ RNA to amplify the cDNAs as we describe here [or alternatively, as we described in earlier studies (6)] and to label the amplified cDNAs with DIG Chem-Link. Highly expressed and moderately expressed genes are detectable, and nearly all recombinant plaques yielded signals using these probes (not shown). Furthermore, signal intensity obtained with these cDNA probes reflects expression levels as shown by differential screening of already characterized genes fixed on dot blots (data not shown). Replicate filters from re-plated single plaques yielding differential signals in the first screening often yielded differential signals in the second screening as well, and all plaques of the same cDNA clone on the same filter yielded signals of similar intensity. Furthermore, cDNAs of differentially expressed genes identified in previous studies (7) were isolated again.

Applying this method, approximately 100 000 clones can be screened, starting with as few as 200 ng poly(A)⁺ RNA. This estimate implies that the same hybridization solution is used up to three times. Probes can be re-used after months or even years of storage, while radioactively labeled probes are lost after a few weeks. Because all DNA molecules of the probe contribute to the same signals, monospecific probes can be re-used even more frequently (10–15 times).

DNA probes yielded stronger signals than RNA probes and avoid the inconveniences of working with RNA. When using amplified cDNA probes, a test hybridization with known differentially expressed genes is suggested.

Approximately 50% of the isolated clones yielded differential signals in the second screen, indicating the reliability of the method. Thirty-nine cDNAs of differentially expressed

Figure 4. Differential screening with complex cDNA probes labeled by DIG Chem-Link. (A) Section of a plaque-filter screening of a cLC-derived cDNA library with amplified, cLC-specific cDNA probes labeled by DIG Chem-Link. (B) Replicate filters of a cLC-derived cDNA library hybridized with fLC-derived (d0) and cLC-derived (d3) probes. Differential signals are marked by arrows. (C) cDNA clones were re-plated, replicate filters were taken and hybridized with fLC-specific (d0) or cLC-specific (d3) cDNA probes labeled by DIG Chem-Link. pcLC168: constantly expressed; pcLC36: up-regulated; pcLC163: down-regulated during maturation.

Figure 5: Screening of an arrayed genomic library using cDNA labeled by DIG Chem-Link. (A) The murine cDNA pcLC45 was labeled by DIG Chem-Link and hybridized with arrayed clones of a human genomic P1 library fixed on nylon membranes. (B) Schematic of the 12 spotting patterns used to dot each clone twice on a square of the membrane. The spotting pattern corresponding to the positive signal on the filter is enlarged. The central spot of each square appears brighter, allowing positioning of the filter.

genes were isolated repeatedly. The repeated isolation of cDNAs of the same gene indicates high expression levels. The most frequently isolated cDNAs correspond to an as-yet unknown gene that is currently under investigation. Thirty-two cDNAs had been isolated before, and 14 additional cDNA clones were isolated during this study. On the other hand, most genes were isolated only once or twice, indicating that there are still a lot of differentially expressed genes to be identified. The high number of 10 newly isolated unrelated cDNAs without homology to genes within the DNA databases shows that there are many new genes among them. In addition, some genes that had previously been characterized to be differentially expressed in maturing LC, have not yet been isolated, though they yield differential signals with our screening probes (e.g., intracellular adhesion molecule [ICAM]-1) (6). Because the cLC-derived cDNA library is highly representative and contains approximately 18×10^6 independent cDNA clones, it should be possible to isolate nearly all genes differentially expressed in maturing LC.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft Kn 120/6-3.

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