CMLS, Cell. Mol. Life Sci. 59 (2002) 1241–1245
1420-682X/02/081241-05

CMLS Cellular and Molecular Life Sciences

Differential display analysis of gene expression in yeast

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Abstract. RNA differential display (DD) is a powerful and straightforward method that employs random reverse-transcription polymerase chain reaction amplification of mRNA species with electrophoresis for comparative analysis of two or more transcriptomes. The small yeast genome represents a convenient model for studying basic functions of the eukaryotic genome and simultaneously provides valuable information towards further refinement of this technique. Several examples discussed below illustrate how DD coupled with classical yeast genetic approaches may be used for studying transcriptionally regulated genetic systems.

Key words. RNA differential display; clr4; yeast; differential expression.

Introduction

In the years since the RNA differential display (DD) technique was originally described by Liang and Pardee [1], the method was further improved [2, 3] and successfully used for a great variety of projects aimed at gene expression analysis. A random sampling approach in its essence, DD requires simultaneous handling of a considerable number of reactions. Automated, multiformat variations of the technique, therefore, are more efficient and versatile in bringing comprehensive screening of total mRNA transcripts (transcriptomes) to a practical level. However, the success rate of any particular DD application depends on the rationality of experimental design of the comparison and the efficiency of transcriptome coverage. From that point of view, the small yeast genome represents an attractive model for both technical refinement of DD and expression analysis per se. Genetic manipulations with the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* are distinguished with unparalleled knowledge of their genetics and the availability of an impressive set of genetic tools. The 12,000-kb budding yeast genome contains more than 6000 genes many of which have unknown functions [4]. Given that yeast may serve as a predictor of human gene function, both yeast species are convenient for studies of a number of crucial eucaryotic processes such as transcription regulation, splicing, cell cycle control, mitosis and meiosis, and cell metabolism.

Multiformat DD assay on *S. pombe* to identify Clr4p targets: technical considerations and results of the study

For our research on yeast we developed a multiformat DD assay (tables 1 and 2) based on the original procedure of Liang and Pardee [1]. This assay and the primer set proved to be versatile and reliable in our studies on human cancer cell lines [6] and rat kidney explants as a model of mesenchymal-epithelial transition during kidney embryogenesis [7]. Our major goal in the application of the DD assay to the yeast model was to assess transcription regulation activity of the clr4 gene, which encodes for a protein involved in silencing of yeast mating-type loci and centromeres via organization of repressive chromatin structure [8]. The differential display comparison was done between two isogenic *S. pombe* strains with a mutated or wild-type clr4 gene. Below we discuss in more detail some important steps that are critical in assay reliability.

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Table 1. Technical details of DD.

RNA isolation: Total RNA samples were obtained by heating and freezing of yeast cells in the presence of phenol and SDS [5]
DNAse treatment: 2–5 mg of each RNA sample was incubated with 2.5 units of Gibco BRL amplification grade DNAse I and 10 units of RNAse block (Stratagene, La Jolla, CA). After treatment, RNA samples were purified with the same RNeasy kit.
cDNA production: 0.8–1.2 µg of purified RNA was reverse transcribed in a 20-µl reaction mixture with MMLV or Superscript (Gibco BRL, Rockville, MD).
Primers for reverse transcription: $HT_{11}A$, $HT_{11}C$, and $HT_{11}G$, where H stands for the HindIII site.
Polymerase chain reaction: Multiformat PCR was done in Costar (Netherlands) 96-well microplates using multichannel automatic pipettes (Matrix Technologies, Hudson, NH). Each well contained 0.4 µl of RT mixture in 1X PCR buffer supplemented with 3 mM $MgCl_2$, 0.25 units of Taq DNA polymerase, 10 µM of each dNTP, 0.05 µCi of α-[^{33}P]-dATP, 4 pmol of an appropriate oligo-dT-anchored primer and 4 pmol of an upstream arbitrary primer (table 2). The cycling parameters were as follows: initial incubation at 94 °C for 3 min, then 94 °C for 30 s, 40 °C for 2 min and 72 °C for 30 s (40 cycles). The last cycle was followed by a 5-min extension at 72 °C.
Recovery and reamplification: PCR products were denatured in the 96-well microplates and loaded on a 5% sequencing acrylamide gel with an eight-channel syringe (Hamilton, Reno, NV). After separation, differentially expressed bands were excised and incubated in 100 µl of water at 90 °C for 15 min. Two µl of extracted cDNA were reamplified using the same primers and PCR conditions.
Cloning and sequencing: 0.5 µl of reamplified PCR product was used for ligation into PGEM-T Vector (Promega, Madison, WI). Clones were grown in 2 ml of LB media, and plasmids were isolated using Wizard Minipreps DNA Purification System (Promega). Sequencing was performed using a Dye Terminator Cycle Sequencing Ready Reaction kit and the Model 373A DNA Sequencing system (Applied Biosystems, Foster City, CA).

Table 2. Oligonucleotides used in DD PCR reaction.

1. XGTCACAC	21. XCATAGCC
2. XAGTGAGC	22. XCTTGATG
3. XCTATGGC	23. XCCAGTAC
4. XCTGTGTC	24. XCGCATTG
5. XACGGACG	25. XCTCCGTC
6. XATGCACG	26. XTAAAGGG
7. XAGCAGCT	27. XCATGGTC
8. XTGAGCGT	28. XTGGCTCC
9. XGCGCAAC	29. XTTCGCAG
10. XGAAGCGT	30. XCTAAGCG
11. XTGGCATG	31. XCTGACAC
12. XGCGAGGT	32. XCTAACCG
13. XGTCTAAA	33. XATTGGTC
14. XCTATTTC	34. XACCAATC
15. XTGAATTC	35. XCAATCGC
16. XAAATCGA	36. XGTCATAG
17. XTTATTCG	37. XCTGACTG
18. XGTTATAG	38. XATACAGG
19. XAGTTATC	39. XAACGAGG
20. XCAAGTTT	40. XCAAGTCC

X, *XbaI* site.

DNAse treatment

DNA carryover in RNA samples represents one of the major sources of false-positive bands in DD gels. Genomic DNA contamination that can greatly influence reproducibility of DD profiles [1] can be easily tested by running polymerase chain reactions (PCRs) with arbitrary primers on RNA samples without reverse transcription (routine PCR is DNA specific, [9]). The DNA carryover can be minimized by using an appropriate RNA isolation kit along with an optimized procedure of DNAse treatment (see [1] and table 1 above).

Selecting suitable primers

Comigrating of several different DNA sequences within a band on DD gels constitutes the second major cause of false-positive signals. Heterogeneity within a band in our study was assessed by the amount of different clones produced from the same band. As could be expected based on the small size of yeast transcriptome, sequence heterogeneity within one band in our DD assay was on average relatively low, as compared with human or rat genome (data not shown). Nevertheless, in no case has a 100% pure band been reported, suggesting that the procedure can be further optimized. For example, a band's complexity can be reduced by optimization of both downstream and upstream primers [2]. Careful consideration of primer annealing properties, appropriate anchored primers and conditions of electrophoresis [2, 9] helps in producing more consistent DD profiles and minimizes the number of transcripts analyzed in the verification step. However, as homogeneity of any particular band can never be guaranteed, assessment of its content by molecular cloning or single-strand confornational polymorphism (SSCP) analysis is highly recommended [9].

Multiple sampling and Northern verification

Since inconsistent PCR conditions may be another source of false-positive bands, we used triplicate RNA isolation and sampling during reverse transcription (RT) and PCR steps. Triplicate PCR reactions on both *S. pombe* strains with 120 primer pairs were done in 96-well plates (720 PCR samples in total). Final PCR products were separated on 10 denaturing gels from which 14 bands representing either up- or downregulated genes were isolated (fig. 1 and table 3). The bands were further analyzed by cloning and sequencing. Differentially expressed bands that showed consistent expression in each of the three samples were isolated and analyzed, while inconsistent bands were considered false positives (fig. 1).

As a result of Northern hybridization on the clr4$^-$ and clr4$^+$ strains complemented by studies on integration of clr4$^+$-expressing plasmid in the clr4-deficient yeast genome, we characterized four new differentially ex-

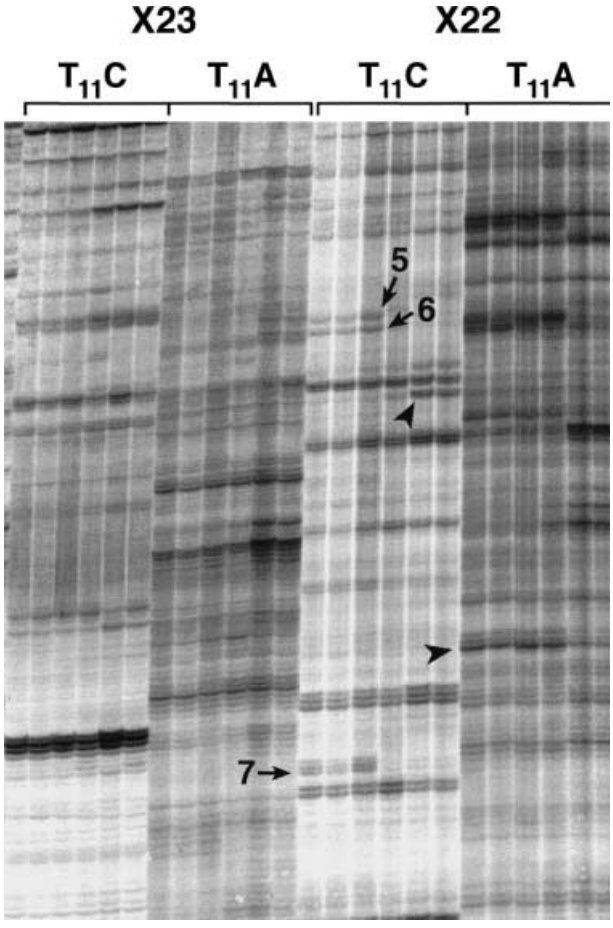

Figure 1. Identification of transcripts differentially expressed in clr4⁻ (three left lanes in each set of six) and clr4⁺ (three right lanes) strains of *S. pombe.* Each lane represents a separate RNA isolation and RT-PCR reaction performed in a single multiformat experiment. Oligo-(dT) anchored and upstream primers are shown on the top. Arrows and numbers indicate reproducible differentially expressed bands that were isolated and further analyzed (table 3). Arrowheads show false positives.

pressed yeast DNA sequences (table 3). Two redundancies were found among the sequences. Each of these redundant pairs, apparently, represented separated strands of a single DNA fragment. Three out of four identified genes were characterized as upregulated by the clr4 gene, and one as downregulated. Only one of these sequences, GeneBank accession number AF095794, represented a known gene, ubi4, which encodes for polyubiquitin. Interestingly, this gene was recently shown to be essential for meiosis in *S. pombe* [10]. Overall, the data obtained by RNA DD on *S. pombe* demonstrated for the first time that clr4 can mediate both trancriptional activation and repression of different genetic loci.

Table 3. Characterization of differentially expressed bands.

Band no.	clr4 affect[a]	Hetero-[b] geneity	Northern[c]	GenBank accession no.
1	+	4	+	AL035548
2	+	2	+	Z69627
3	–	?	?	
4	+	2	–	
5	–	2	–	
6	–	4	–	
7	–	4	–	
8	–	2	–	
9, 10	–	2	+	Z98951; AL031546
11	+	2	+	AF095794
12	–	2	–	
13, 14	–	2	?	

[a] Upregulation, +; downregulation, –.
[b] Number of different cDNA species identified from the band reamplification.
[c] Northern confirmation: difference detected, +; no difference detected, –; uncertain results, ?.

Present and future of DD applications to yeast

A number of successful applications of DD to yeast that complemented classical genetic approaches have been reported recently in the fields of RNA polymerase II transcription complex function, stress response and meiosis regulation. In all these cases DD techniques similar to the one reported above were employed; however, many utilized two-base oligo-(dT) anchored primers instead of one-base anchored primers, and native polyacrilamide gels were used instead of denaturing gels for separation of PCR products.

Shen and Green [11, 12] developed and described a detailed DD protocol which was used for identification of yeast targets of the TATA-binding-protein-associated factor $yTAF_{II}145$. Out of approximately 50 differentially expressed bands the authors reported isolation of two ribosomal subunit protein (RPS) genes, RPS5 and RPS30, and the inorganic pyrophosphatase gene PPA1. While RPS genes encoding for the 40S ribosome were previously known to be $yTAF_{II}145$ dependent, PPA1 represents a newly described target of this transcription factor.

Han et al. [13] used the DD approach with the Delta RNA Fingerprinting Kit (Clontech, Palo Alto, CA) to identify genes regulated by the *Saccharomyces cerevisiae* multisubunit Mediator complex – one of the two major RNA polymerase II coactivators. As a result, the authors isolated 87 complementary DNA (cDNA) bands overexpressed in the wild-type strain and 50 bands overrepresented in two mutant strains. Out of these 137 bands, 5 genes showed a 5- to 10-fold expression reduction in Mediator mutant strains revealed by Northern blot analysis. Three of them, ARG4, HIS4 and LYS20, are amino acid biosynthesis genes; YGR260W encodes for a protein ho-

mologous to allantotate permease and YGL117W is a new gene. Other studies aimed at uncovering molecular structure and target specificity of various components of the same transcription complex also used DD as a convenient and efficient approach [14, 15].

With a large-scale DD assay, yeast represents a convenient model to study stress-related gene expression. Miura et al. used 117 primer sets to produce 38 candidate PCR bands, out of which 9 genes were described as either up- or downregulated in organic-solvent-tolerant strains of *S. cerevisiae* [16]. Garay-Arroyd and Covarrubias isolated three new *S. cerevisiae* genes strongly responsive to osmotic stress (GRE1-3) [17] that represent an unidentified hydrophilic protein, a putative dihydroflavanol reductase, and a putative xylose reductase. These genes possess a post-diauxic shift (PDS) element in their promoters known to be involved in gene activation during diauxic growth, stationary phase and in response to heat shock. The authors also provide evidence that all three GRE genes are positively regulated by the high osmolarity glycerol (HOG) pathway.

Gross and Watson, while seeking budding yeast genes involved in heat shock response, reported some inconsistency in the DD fingerprints with repetitive rounds of analysis [18]. We also experienced this problem (data not shown) and concluded that the reason for the inconsistency was subtle fluctuations of PCR parameters from experiment to experiment that are beyond control. Therefore, multiple sampling in one PCR experiment is a better tool against false positives than repeated rounds of PCR. In addition, use of the same thermal cycler and making master mixes can reduce PCR inconsistencies. This inconsistency, however, was not crucial for Cross and Watson's study since they isolated only bands highly reproducible from strain to strain. Out of 30 bands, 12 failed to produce any signal during Northern analysis. Eventually, three genes with well-known protein products were confirmed and characterized: HSP90, HXK1 and STA1. Among those open reading frames (ORFs) that did not produce signals in Northern hybridization, two were identified as a putative transcription factor and a hypothetical protein from the family of multi-drug-resistant translocases.

Nag and Axelrod, using four oligo-(dT)-anchored and six AP primers isolated 21 genes differentially expressed during early and late stages of *S. cerevisiae* meiosis [19]. Among these genes IME1, DMC1 and 18S ribosomal DNA (rDNA) were already known to be meiosis induced, while TIF5, POM152, MOT2 and others appeared to be novel meiosis-associated genes. Interestingly, in this study several genes were detected more than once with different sets of primers. Such redundancy may be due either to very high levels of gene expression or extensive coverage of the transcriptome by the set of primers used in the study. Unfortunately, no data about intensity of the targets' expression during meiosis is provided in the paper. Working on sporulation regulation in budding yeast, Mai and Breeden confirmed transcription repression properties of the Xbp1 protein that are induced by stress and starvation during the mitotic cycle [20]. With the set of arbitrary primers purchased from Operon Technologies (Aladema, CA), the authors characterized three cyclin genes, CLN1, CLN3 and CLB2, previously known as Xbp1 targets. Two other targets isolated in the same study are CYS3 (cystathione γ-lyase), which catalyzes the biosynthesis of cysteine, and SMF2, whose function is unknown. Loss of XBP1 expression resulted in derepression of these targets and delayed sporulation for several hours. While deletion of CLN1 also resulted in sporulation delay, Δ*cln1*Δ*xbp1*cells showed no further delay in sporulation, indicating that CLN1 may be the critical target of Xbp1.

All these examples of successful DD application to yeast models confirm the rationality and practical significance of the assay and provide valuable information on yeast genome function. It is also evident from the works cited above that automated high-throughput DD applications are needed to ensure high reproducibility and efficient transcriptome coverage. Fluorescent differential display technology (FDD) puts together a rationale design of primers and PCR conditions with an automated radiation-free gel-scanning stage. This version of DD is compatible with various fluorescent detection systems and has been developed during the last several years [3, 9, 21]. Miura et al. [22] tested this technique on yeast to identify new targets of the transcription factor Pdr1p. As a result, 80 reproducibly displayed candidate bands related to Pdr1p activity were isolated. After the identities of 72 out of the 80 bands were revealed and their apparent redundancy was accounted for, 23 independent messages remained. In 19 out of these 23 cases, differential expression was confirmed by adapter-tagged competitive PCR [23].

In the field of emerging technologies of yeast functional genomics DD represents a rather practical and relatively inexpensive assay affordable for most molecular biology laboratories. Such drawbacks of the method as sensitivity to DNA contamination and low reproducibility of the expression profiles are considerably reduced in recent modifications, making the DD technique even more attractive than before.

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