Benchmarks

BENCHMARKS

Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

Differential Display Combined with a Regulated Transient Expression System

BioTechniques 26:1018-1020 (June 1999)

Differential display or RNA fingerprinting (6,9) is widely used to identify altered cellular gene expression at the mRNA level. We describe the differential display of RNAs synthesized from transiently transfected DNA—a technique not previously reported. Although we focus on the application of this methodology to analysis of a pool of RNAs in which cytoplasmic stability can be altered because of its mutant 3'-untranslated regions (UTRs), this methodology also allows the selection of RNAs from a pool of mutants having increased levels of abundance due to more efficient RNA processing or polyadenylation, or to enhanced nuclear export.

Analysis of RNAs with altered stabilities is greatly facilitated by synthesizing the RNAs in a pulse under the control of a transiently transfected, regulated promoter. Synthesizing the RNAs as a pulse allows them to decay for an appropriate period of time before their analysis using differential display. We used the tetracycline (Tet)-regulated promoter system (4), which is extremely powerful. Expression of a luciferase reporter gene after transient transfection of HepG2, human he-

patoma, cells with a Tet-luciferase plasmid (pUHC13-3; Reference 4) was 38-fold higher than luciferase expression from the strong cytomegalovirus promoter. Even in transient transfections, the Tet system maintained tight regulation. Expression from the Tet-regulated promoter was repressed 400-fold upon addition of Tet to the medium.

To test the use of differential display to analyze RNAs transcribed from transiently transfected DNAs, we constructed pools of random 3'-UTRs fused to the chloramphenicol acetyltransferase (CAT) reporter gene under the control of the Tet-regulated promoter (Figure 1). Since it was not feasible to synthesize a library of oligonucleotides >160 nucleotides (nt) long with approximately 130 nt of randomized sequences, we constructed a library of oligonucleotides with randomized sequences 64 and 65 nt in length (N64 and N65, respectively). The randomized sequences were flanked by both specific sequences that contained a *StyI* site, so that they could be ligated together, and *XbaI* and *BamHI* sites that allowed for insertion into the Tet-CAT plasmid (Figure 1). From this large library of mutants with different 3'-UTRs, two pools of mutants containing 1000 and 2000 independent clones, respectively (P1 and P2), were prepared and used in transfections.

HepG2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal

calf serum (FCS), and transfected (with a calcium phosphate method) with 10 μ g of one of the tetCAT-RM pools (P1 or P2), 5 μ g of the Tet-regulated transactivator expressing plasmid (pUHD15-1; Reference 4) and 5 μ g of pTZ18U carrier DNA per 100-mm plate. A 5 μ g amount of pUHD15-1 and 15 μ g of pTZ18U were used for mock-transfections. After a 4-h incubation, the medium was replaced and the cells were harvested, and RNA was extracted after 48 h. Total cellular RNAs were prepared using guanidinium thiocyanate extraction followed by centrifugation in cesium chloride (8). The extracted RNA was treated for 30 min at 37°C with 5 U of RQ1 DNase I in the presence of 40 U of RNasin® (both from Promega, Madison, WI, USA), (in 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂) in a 100- μ L reaction mixture, extracted with phenol/chloroform and ethanol-precipitated in the presence of 0.3 M NaOAc (pH 5.3). Total RNA (8 μ g) and 50 pmol of 3'-end-specific Tet I primer (5'-TTG-TTGTTAACTTGTTTA-3') were used for reverse transcription (RT) in a 25- μ L reaction volume in the presence or absence (mock RT) of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) used according to the supplier's instruction. After RT, Tet I primer was removed by using the Prep-A-Gene® DNA Purification System (Bio-Rad, Hercules, CA, USA). The

Figure 1. Construction of the random 3'-UTR library and schematic of the primers used in RT and PCR. The tetCATXB vector is derived from the Tet-regulated expression vector pUHD10-3 (3) and contains Tet operator (Tet O) sequences located upstream of a minimal promoter sequence. A pool of synthetic mutant 3'-UTRs was created as follows. An *XbaI* site, a *BamHI* site and a simian virus 40 (SV40) late polyadenylation site were introduced immediately downstream of the CAT coding sequence. Oligonucleotides containing specific 5' and 3' sequences containing *XbaI* and *StyI* sites for N64, and *StyI* and *BamHI* sites for N65, and a central core of random nucleotides 64 (N64) or 65 (N65) nt in length was synthesized, PCR-amplified, ligated together at the *StyI* site, PCR-amplified and ligated into the *XbaI*-*BamHI* site in the 3'-UTR of the tetCATXB plasmid. After transformation, DNA was isolated from a total of approximately 1000 independent clones to create pool 1 and 2000 independent clones for pool 2. Primer annealing sites for Tet Q and Tet I are shown by arrows.

Table 1. Sequence of a PCR Product Obtained after Differential Display Analysis

*Xba*I
5'-**TCTAGAGTGCTGCGAGCGTGGTTTTCCACCTGGTAGAAGT**GCGACGCGAGGAGGA-
*Sty*I
GACTATTTATTGTACCCTGGCAGCCATAGCGTTCGATGTTTGGCTACTTGCCTTCAAGGACACT-3'

Note: The *Xba*I and *Sty*I sites are noted, and N64 is in boldface.

final cDNA volume was 20 μ L. A 3- μ L aliquot of the cDNA, 20 pmol of 5' [32 P]-end-labeled Tet Q primer (5'-ACAGTACTGCGATGAGTG-3', specific activity of ca. 5×10^6 cpm/ μ g) and 20 pmol of the arbitrary primer (AP: 5'-CGGGAAGCTTGCTAAGACTAGC-3'; Reference 7) were added to the 25 μ L PCR mixture. The PCR cycles were

basically as described by Zhao et al. (10) and begin with one low-stringency cycle (94°C for 1 min, 40°C for 4 min and 72°C for 1 min) followed by 35 high-stringency cycles (94°C for 1 min, 55°C for 1 min and 72°C for 1 min). PCR products were analyzed on an 8% polyacrylamide/7 M urea gel.

To minimize detection of cellular

RNAs in the differential display, we carried out RT using a primer that anneals specifically to RNAs transcribed from the transfected plasmid (Tet I) as described. In addition, the cDNAs were polymerase chain reaction (PCR)-amplified using a 5'-end-labeled primer specific to the 3' end of the CAT coding sequence (Tet Q) and an arbitrary primer as described.

The longest possible RT-PCR product from the transiently expressed genes was the approximately 200-nt-long PCR product generated by the Tet I and Tet Q primers (Figure 2A, lane 2). The expected size of the products using the Tet Q primer and an arbitrary primer is approximately 45–200 nt. Despite using primers specific to the construct for RT and for one PCR primer, we still observed some bands from RNAs present in non-transfected or mock-transfected cells (Figure 2A, lanes 7 and 9). Since the primer we used for RT (Tet I) contained part of the polyadenylation signal, which is common to most mRNAs, the primer could have annealed to the 3' ends of some cellular mRNAs. In addition to the bands observed in mock- and non-transfected cells, the cells transfected with pool 2 (Figure 2B) showed numerous additional PCR-amplified bands (arrowheads in Figure 2B, lane 1). Additional evidence that the PCR bands are derived from the transcribed RNAs comes from the observation that when different pools of 3'-UTRs were used, the PCR-amplified DNAs seen on the gel were different (Figure 2A, compare lanes 3 and 5).

One common problem in differential display is DNA contamination of the RNA (5). We observed only a very slight background of PCR products without RT even in the presence of the large excess of transfected DNA (Figure 2A, lanes 4, 6, 8 and 10).

To test the effectiveness of the Tet-

Figure 2. Differential display of transiently expressed mRNAs containing random 3'-UTRs. (A) RNAs from the transfected cells (P1: lanes 2–4; P2: lanes 5 and 6; mock-transfection [M]: lanes 7 and 8) or RNAs from the untransfected cells ([–]: lanes 9 and 10) were reverse-transcribed (lanes 2, 3, 5, 7 and 9) or mock-reverse-transcribed (RT[–]: lanes 4, 6, 8 and 10) and PCR-amplified using 5'-end-labeled Tet Q and Tet I primers (lane 2) or Tet Q and AP primers (lanes 3–10). The PCR samples were then analyzed by electrophoresis. DNA size markers (lane 1) and their sizes are shown on the left of the panels. (B) Cells were transfected with P2 (lanes 1 and 2) or mock-transfected (lanes 3 and 4) and either treated with (Tet[+]: lanes 2 and 4) or without (Tet[–]) tetracycline from the start of the transfection, and the RNAs were analyzed using the same method as in Panel A. The arrowheads identify PCR-amplified bands specific for RNAs transcribed from transiently transfected DNAs.

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regulated promoter system in our differential display system, we compared the PCR-amplified DNAs produced in the absence and in the presence of Tet (Figure 2B). When transcription was blocked with Tet, the PCR-amplified bands specific for the transfected cells were absent (Figure 2B, lanes 1 and 2, arrowheads). We cut out several bands corresponding to PCR-amplified DNAs seen in transfected cells maintained in the absence of Tet, PCR-amplified them, recloned them and sequenced them. These DNAs had the configuration we designed (CAT, *Xba*I site, N64, *Sty*I site and a part of N65; see Figure 1).

In one application of this method, for the identification of 3'-UTR sequences that are potentially important in RNA stability, we (i) made the RNA by maintaining cells in the absence of Tet for 48 h, then (ii) stopped transcription by adding Tet and (iii) allowed the RNAs to decay for 12 h before differential display. We then cut out a specific band for

PCR amplification cloning and sequencing to obtain the sequence shown (Table 1). In addition to this sequence, clones with two other sequences were obtained from this band (data not shown). Additional studies in a separate experimental system will clearly be required to unequivocally demonstrate that the isolated clone exhibits mRNA stability properties of interest.

The data demonstrate that the specific PCR-amplified DNAs we observe derived from RNAs transcribed from the transiently transfected DNA, under the control of the Tet-regulated promoter. The procedure was reproducible with band intensity increasing when amounts in the range of 1–12 µg of RNA were used as starting material.

An important feature of the differential display approach we used is that it allows direct visual identification of the RNAs having levels that changed in response to the regulatory stimulus under study. In this work, we used mutant pools consisting of the *cat* gene fused to 3'-UTRs containing random sequences. We used specific sequences for primer annealing in both the coding region and at the end of the 3'-UTR. The approach we describe can also be applied to a cDNA library generated from cellular mRNAs with tagged primer sequences at the 5' and 3' ends of the RNAs, and the cloned DNAs transcribed under the control of the Tet-regulated promoter. The cells could then be transfected, treated with regulatory molecules and, after an interval for RNA decay, the RNAs could be analyzed by this differential display method. Synthesizing the RNAs in this way as a pulse from a regulated promoter would eliminate artifacts that arise when actinomycin D is used to inhibit cellular RNA synthesis and to identify mRNAs whose stability is regulated (1,2).

In this work, we describe for the first time the differential display of RNAs synthesized from transiently transfected DNA. This method coupled with our use of a regulated promoter allows identification of RNAs from mutant pools, or natural RNAs that have increased levels of abundance due to changes in RNA processing, polyadenylation, nuclear exit or cytoplasmic degradation.

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This research was supported by NIH Grant No. DK-50080. Address correspondence to Prof. David Shapiro, Department of Biochemistry, B-4 RAL, University of Illinois, 600 South Mathews Avenue, Urbana, IL 61801, USA. Internet: djshapir@uiuc.edu

Received 17 August 1998; accepted 10 March 1999.

Hiroshi Kanamori and David J. Shapiro
*University of Illinois
Urbana, IL, USA*