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cDNA Library Screening Using the SOS Recruitment System

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ABSTRACT

The SOS recruitment system (SRS), a recently developed method for detecting protein-protein interactions, provides an attractive alternative to identify biologically important protein interactions. In SRS, the protein-protein interactions take place in the cytoplasm instead of the nucleus, as is the case in the conventional two-hybrid system. Although the SRS has overcome some of the disadvantages of the conventional two-hybrid system, it still has several problems and limitations. Here, we describe a new protocol for SRS library screening. A new combination of growth media to avoid the tedious step of replica plating greatly increases the number of independent colonies in a single library screening. Furthermore, we designed a pair of ras-specific primers and a one-step simple PCR to rule out the most abundant false positive, the mammalian ras cDNA, in SRS library screening.

INTRODUCTION

Since the introduction of the yeast two-hybrid technique by Fields and Song (7), hundreds of physiologically important protein interactions have been identified. The basic strategy of this method relies on the interaction of a fusion protein containing a transcriptional activation domain with one containing a DNA binding motif, and subsequent transcriptional activation of a reporter gene. The first generation of two-hybrid system used the DNA binding domain and the transcription activation domain of the yeast Gal4 transcription factor as bait and target, respectively. The β -galactosidase gene, *LacZ*, was used as the reporter gene to screen *LacZ* positive colonies. Later, several new reporter genes such as *HIS3* (5) or *LEU2* (10) were introduced to allow selection of positive clones and improved efficiency of screening. At the same time, other transcription fac-

tors such as LexA were used to replace Gal4 in the system (10). However, the problem of false positives or nonspecific interactions still persists, especially if the bait is a transcription activator or repressor or any other protein that can activate the transcription by itself. These false positives cause a high background and hamper efficient screens using the conventional two-hybrid system. Recently, the Son of Sevenless (SOS) recruitment system (SRS) was developed as a novel method for detecting protein-protein interactions to overcome these types of problems. In this system, protein interactions occur in the cytoplasm instead of in the nucleus (2). The SRS is based on the rescue of ras-mediated signal transduction. Basically, a *cdc25-2* temperature-sensitive yeast strain is transformed with a cDNA library and an SOS-bait fusion plasmid. A myristoylation peptide targets the library of proteins to the membrane. The interaction between bait and target protein recruits the SOS to the cytoplasmic membrane, allowing SOS to activate ras and the *cdc25-2* cells to grow at 37°C, which is the restricted temperature (2). The SRS provides some advantages to the conventional two-hybrid system. First, it can be used to identify proteins interacting with transcription factors without concern about the intrinsic transcriptional ability of the bait. Second, it may allow identification of protein interactions that require protein modifications that take place in the cell cytoplasm. In addition, it may detect weaker protein interactions due to the sensitivity of the ras signal transduction pathway. Using the SRS method, an Ap-1 repressor, JDP2, was found to interact specifically with c-Jun (2), and cIIP was found to interact with the carboxyl-terminus domains of BRCA1 (13). The most abundant false positives in the SRS screening are the cDNAs for ras and other components in the ras signal transduction pathway, which result in yeast growth at the restrictive temperature.

However, the major disadvantage of the published SRS protocol is the low number of independent colonies obtained in a single library screening. After cotransformation, *cdc25-2* cells have to grow on glucose plates first and are then replica plated onto galactose

plates. Direct plating of the yeast transformants onto galactose plate results in no colony growth. To make a clear replica plate, the number of colonies has to be stringently controlled. This limits the use of SRS in library screening, which usually requires millions of independent colonies in a single screening. Here, we describe a new SRS protocol that greatly increases the number of independent colonies in a single library screening. Also, we developed a simple PCR method to efficiently rule out ras cDNA as false positives.

MATERIALS AND METHODS

Plasmid and Library Constructions

A fragment of the full-length SOX9 open reading frame was generated by *Bam*HI and *Not*I digestion from pcDNA vector (12) and ligated to the carboxyl-terminus of SOS in the pYes2-SOS plasmid (2). The SOS-SOX9 fusion was excised with *Hind*III and *Not*I and ligated to the pADNS vector generated from pADNS-P110-SOS digested with *Hind*III and *Not*I, releasing the insert of p110-SOS (2). A polylinker encoding six glycine amino acids was inserted between the SOS and SOX9 through a *Bam*HI restriction site. The sequence of plasmid pADNS-SOS-SOX9 was confirmed by DNA sequencing. A cDNA library of primary chondrocyte was made using the Superscript™ Choice System (Life Technologies, Rockville, MD, USA). The cDNA was digested with *Eco*RI and inserted into the pYes-2 vector containing a v-Src myristoylation sequence. The function of the myristoylation peptide is to anchor the library proteins to the cytoplasmic membrane. Expression of the cDNAs that encode target polypeptides is under control of a galactose-dependent promoter. The total library contains about 1.5×10^6 independent colonies.

Yeast Transformation and Library Screening

The yeast strain, *cdc25-2*, has the genotype of MATa, *ura3*, *leu2*, *trp1*, *lys2*, *his200*, *cdc25-2*, *ade101*, GAL⁺. All media were made according to Aronheim et al. (2). The bait plasmid

(pADNS-SOS-SOX9) was transformed into the *cdc25-2* cells and then grown on glucose minus leucine plates at 25°C for several days. Colonies were picked and tested to exclude spontaneous revertants that would grow at 37°C. On the first day, 10 fresh

colonies containing the bait plasmid were inoculated into 75 mL glucose minus leucine medium and shaken vigorously at 25°C overnight. On day 2, the cells were diluted with 300 mL pre-warmed yeast peptone dextrose (YPD) medium to a density of 2×10^6 /mL and

Figure 1. Glucose-coated (70 mg/mL) galactose plates fully support the growth of library transformants and do not suppress the expression of the pYes-2 plasmids. (A) Varying concentrations of glucose solution were spread onto the galactose plates just before plating. The library transformants were plated onto a glucose plate and galactose plates with different concentrations of glucose coatings and incubated at 25°C until colonies appear. The colonies were counted and calculated as a percent of colonies that grew on glucose plates. (B) Three independent colonies, which had been transformed with different combinations of bait and target plasmids, were picked and replica plated onto glucose plates, galactose plates, and galactose plates coated with 70 mg/mL fresh glucose solution. All the plates are minus leucine and uracil. The plates were incubated at 25°C or 37°C for three days. The plasmids of SOS-Jun, m^{#2}, mSOS, and ADNS were described in (1,2). SOS-SOX9 is the bait plasmid constructed, and # 564 is one of the positive plasmids, which encodes PKA- α and specifically interacted with SOX9 in SRS. M represents V-Src myristoylation sequence. (C) Cells transformed with pADNS-SOS-SOX9 and # 564 plasmids were collected from different plates (1, galactose plates; 2, galactose plates coated with 70 mg/mL glucose solution; 3, glucose plates), and cell lysates were extracted. A western blot analysis was done using the PKA- α antibody at a dilution of 1:1000.

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shaken at 25°C until the cell density reached 1.0×10^7 /mL. Cells were spun down and washed with double-distilled water and subsequently 0.1 M LiAc. Cells were resuspended with 1.5 mL 0.1 M LiAc and then aliquoted to 30 1.5-mL Eppendorf® tubes. For each tube, 240 µL polyethylene glycol (PEG) (50%), 36 µL 0.1 M LiAc, 25 µL denatured salmon sperm ssDNA (2 µg), 50 µL double-distilled water, and 2 µg library DNA were added sequentially. The mixture was vortex mixed vigorously and then incubated at 25°C for 1 h followed by heat-shock at 42°C for 25 min. Cells were spun down again, and the supernatant was removed. Double-distilled water (300 µL) was added into each tube, and the cells were resuspended gently. The suspended cells were plated onto galactose minus leucine and uracil plates (150 × 15 mm), which had been coated with a thin layer of 300 µL glucose solution (70 mg/mL) just before plating. The plates were incubated at 25°C for 2–3 days until small colonies were visible and then transferred immediately to 37°C and incubated for 3–4 days. The colonies were picked and allowed to grow on glucose minus leucine and uracil plates at 25°C. To test the galactose dependency, colonies were replica plated onto glucose and galactose minus leucine and uracil plates and incubated at 37°C. Only those colonies showing growth on galactose plates, but not on glucose plates, at 37°C were selected for further characterization.

Western Blot Analysis

Cells transformed with pADNS-SOS-SOX9 and # 564 plasmids were collected from different kinds of plates (Figure 1C) and suspended in 30 mL ice-cold buffer containing 150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM sodium azide, pH 8.0, and then centrifuged at 3000 rpm at 4°C for 10 min. Cells were resuspended in 500 µL ice-cold RIPA buffer containing 10 mM sodium phosphate (pH 7.0), 1% Triton® X-100, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, and 0.1 mM sodium orthovanadate. After being boiled for 5 min, cells were bead beaten and then cooled on ice. Cell lysate were removed and protein

concentration was determined. Fifty micrograms of total extract of each sample was loaded on a SDS 10% polyacrylamide gel and western blot analysis was done as described previously (10) with PKA-Cα antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:1000.

PCR to Identify *ras* cDNA as False Positives

The galactose-dependent colonies were picked and mixed with 20 µL pre-mixed PCR solution [200 ng each mouse *ras*-specific primers (see the sequences in Figure 2A), 2 µL 10× PCR buffer, and double-distilled water) and heated at 100°C for 10 min. The mixture was then quickly spun down, and the supernatant was changed to a new tube. Five microliters of PCR mixture (0.5 µL 25 mM dNTP, 0.4 µL *Taq* DNA polymerase (Promega, Madison, WI, USA), and 4 µL double-distilled water) were then added, and PCR was done for 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. PCR product (10 µL) was run on a 1% agarose gel.

RESULTS AND DISCUSSION

New Combination of Glucose and Galactose Increases the Efficiency of SRS Library Screening

The main advantage of the SRS is that the protein interactions take place in the cell cytoplasm instead of in the nucleus. Thus, the SRS is more appropriate for screening proteins that are transcription factors and those that function physiologically in the cytoplasm. SOX9 is a typical transcription factor that is required for chondrocyte differentiation and cartilage formation (3,12). To identify interacting partners of SOX9 in chondrocytes and to avoid the transcriptional activity of SOX9, we used the SRS instead of the conventional two-hybrid system in a screen of a chondrocyte cDNA library. To prevent interference between each domain in the fusion protein, we incorporated a flexible spacer of six glycine amino acids in-frame between SOS and SOX9. The chondrocyte cDNA library contained a total of approximately $1.5 \times$

10^6 independent colonies. In the original SRS protocol, it would be tedious to screen such a large number of colonies because yeast cells would have to be plated on glucose plates first until the colonies appear and then replica plated onto galactose plates to induce expression of the library cDNAs. The step of replica plating restricts the number of independent colonies one can easily screen in SRS. To overcome this problem, we used a combination of glucose and galactose, which allowed us to skip replica plating of a large number of independent colonies. Since the existence of glucose in the media may suppress the galactose-dependent expression of the library cDNAs, we optimized the minimum amount of glucose that would support the growth of yeast transformants but not suppress the expression of library cDNAs. The results show that a thin layer of glucose on top of galactose plates fully supports the growth of yeast transformants at a concentration as low as 70 mg/mL, whereas lower concentrations of glucose decreased the number of transformants with complete inhibition of transformants growth on galactose (Figure 1A). To test whether a thin layer of 70 mg/mL glucose on top of galactose plates suppressed expression of the pYes2 library plasmids that were galactose dependent, we compared the growth of two previously identified galactose-dependent colonies (1,2) and one positive colony (# 564) encoding the catalytic subunit of cAMP-dependent protein kinase A (PKA-Cα) that we obtained from the library screening using SOX9 as bait (7). The yeast colonies, which had been transformed with different combinations of bait and target plasmids, were replica plated onto glucose plates, galactose plates, and galactose plates with 70 mg/mL glucose coating. All plates were minus leucine and uracil. The results showed no repression of growth by the 70 mg/mL glucose on top of the galactose plate, whereas plates containing only glucose completely inhibited the growth of colonies (Figure 1B). To directly demonstrate that the thin layer coating of glucose would not inhibit the expression of library cDNAs, we performed a western blot analysis comparing the expression of clone #564, which encodes the PKA-Cα, in

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Figure 2. PCR to rule out the false positives of mammalian *p21-ras*. (A) DNA sequences of the 5' and 3' *p21-ras*-specific primers. (B) PCR was done directly with the putative galactose-dependent colonies picked from fresh plates. The two *p21-ras*-specific primers generated a 525-bp PCR fragment (arrow). Lane 12 represented markers; lanes 1, 3, 5, 9 and 10 were positives for *p21-ras*.

Figure 3. A new protocol for SRS library screening. The bait plasmid and cDNA library were transformed sequentially into the *cdc25-2* yeast cells. Transformants were plated directly onto galactose plates coated with a thin layer of glucose solution at 25°C until colonies were visible. The plates were then transferred to 37°C for another 3–4 days. Large colonies were picked to test for galactose dependency and rule out *p21-ras* positives.

different plates as indicated in Figure 1C. While the glucose plate completely inhibited expression of the protein, the galactose plate and galactose plate coated with glucose showed the same expression levels for this protein (Figure 1C). Overall, our data suggest that, during library screening, the thin layer of glucose on top of galactose plates was preferentially consumed during the first several days of yeast growth and that the trace amounts of remaining glucose did not suppress the activation of the GAL promoter in the pYes2 library plasmids (8). This assumption is also supported by the observation that the number of mammalian *ras* cDNAs among the galactose-dependent positive colonies obtained using this method was comparable to that of the previous protocol (data not shown). Moreover, the fact that we detected interactions between SOX9 and the catalytic subunit of camp-dependent protein kinase A (6), which was likely to be transient and weak, further demonstrates that this new library screening protocol would not discriminate against detection of weak interactions. The new combination of media should support expression of any library encoded proteins. We also have tried to mix glucose directly with galactose in the medium before pouring the plates, but this method inhibited the expression of library proteins probably because of the glucose still remaining in the medium (data not shown). We have successfully used our improved method, which skips the most cumbersome step of replica plating from the original SRS protocol, in a screen of polypeptides interacting with SOX9. It greatly increased the number of independent colonies that could be screened and simplified the procedure. Although this glucose overlay technique was designed for the SRS library screening, it may have more general application in other yeast techniques involving the galactose-dependent expression of cDNAs.

PCR to Rule Out *ras* as a False Positive

In comparison to the conventional two-hybrid system, the SRS eliminates the false positives due to the nonspecific transcriptional activation of a

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reporter gene, but it retains the problem of false positives due to nonspecific interactions. For example, in our SRS library screening, we found collagens, heat shock proteins, rRNA, and hnRNP proteins, which are also the common false positives in the conventional two-hybrid library screening. However, the most abundant false positive in the SRS is the mammalian *p21-ras* cDNA, which bypasses the temperature sensitivity of the *cdc25-2* mutant. Cotransformation of the mammalian GAP gene (mGAP) may reduce the isolation of *ras* (1), but introduction of an irrelevant plasmid in the *cdc25-2* cells may cause other problems during the library screening. Yu et al. (13) described a DNA dot blot method to eliminate *ras*, but it was based on a relatively tedious DNA hybridization, and it only eliminated the HBL-100 cell-specific *ras* protein. A new approach of *ras* recruitment system was introduced, which efficiently eliminates the isolation of *ras* false positives. However, it introduces false positives of SOS proteins, which appear in the SRS as well, and still needs the cotransformation of mGAP (4). Here, we developed a one-step simple PCR that can efficiently identify the mouse *p21-ras* cDNA in the putative galactose-dependent positive colonies. Two specific primers of *p21-ras* were both designed corresponding to sequences in the 5' part of the mouse *p21-ras* cDNA sequence (Figure 2A). A similar pair of primers can be easily designed to detect other *ras* proteins. PCR of the galactose-dependent colonies with two mouse *p21-ras* primers generated a 525-bp fragment of the *p21-ras* cDNA (Figure 2B). Although occasionally cDNAs for *K-ras*, *R-ras*, *N-ras*, *Rit* (1), *ERBA* (11), and *Ras* guanyl releasing protein (6), which are close relatives of the *p21-ras* cDNA and seem to have the same functions, cannot be excluded by this PCR method. *p21-ras* constituted about 90% of *ras* false positives in our SRS library screening.

The SRS provides an attractive alternative approach to screen for protein interactions. In contrast to the conventional two-hybrid system, the SRS system still needs to be improved before it can be widely applied. The new protocol we described here (Figure 3) bypassed the tedious step of replica plat-

ing and introduced a simple PCR method to identify cDNA for *p21-ras*. Our modified SRS procedure greatly improved the efficiency in cDNA library screening and potentially increased the number of positive clones by reducing the colony loss caused by incomplete colony lifting in the replicating step of the previous protocol. With this modified SRS library, the screening protocol takes about 20 days to complete and obtain the DNA sequences of final positive colonies. In a typical library screening with our new SRS protocol, 1.6×10^6 independent colonies generated 30–40 galactose-dependent colonies, among which 10–20 corresponded to *p21-ras*.

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