Short Technical Reports

Two-Hybrid Selection Assay to Identify Proteins Interacting with Polymerase II Transcription Factors and Regulators

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ABSTRACT

The RNA polymerase III-based two-hybrid system has been developed to detect interactions between proteins such as RNA polymerase II transcription factors and regulators that cannot be studied by the original RNA polymerase II two-hybrid system. This novel method appears to be most useful for a refined analysis of already known protein-protein interactions. However, the application of this system in library screenings has been impaired by the lack of a suitable assay for the selection of the activated pol III reporter gene in yeast. Here, we describe a novel selection assay for the pol III-based two-hybrid system that makes it readily usable for screening expression libraries to search for interacting partners. Our system utilizes a temperature-sensitive (*ts*) U6 snRNA, which is synthesized by RNA polymerase III from a mutated SNR6 gene in yeast. In this *ts* strain, interactions between hybrid proteins activate an artificial pol III reporter construct (UAS_G-SNR6), which controls expression of wild-type U6 snRNA. This wild-type U6 snRNA can suppress the *ts* phenotype and allow growth at the nonpermissive temperature of 37°C, thus providing a positive selection system for interacting proteins.

INTRODUCTION

The yeast two-hybrid system is a powerful technique to identify novel protein-protein interactions; this technology is widely applied in the new field of proteomics (12–14). One limitation of the original system (4), which is based on the selective activation of reporter genes transcribed by RNA polymerase II (pol II), is that it is not suitable for studying transcription factors and regulators of RNA pol II; in-

deed, most of these proteins activate the reporter genes on their own (i.e., in the absence of interacting partners) (2,10). A solution to this problem could be provided by an alternative two-hybrid system developed by Marsolier et al. (10), which is based on activation of a modified SNR6 reporter gene (UAS_G-SNR6) transcribed by RNA polymerase III (pol III). No pol II transcription factor has indeed been shown to be able to activate pol III genes (Reference 10 and unpublished data). The reporter construct of the pol III-based two-hybrid system, UAS_G-SNR6, bears 5 Gal4p-binding sites (UAS_G) in place of the original B block. This otherwise inactive UAS_G-SNR6 reporter gene can be specifically activated by tethering the pol III-specific transcription factor τ 138p to DNA through a fusion with the Gal4p DNA-binding domain (DBD) [Gal4(1-147)- τ 138p] (9). Activation of UAS_G-SNR6 can also be achieved by fusing τ 138p to a protein that interacts with a known partner fused to the Gal4pDBD (i.e., by a two-hybrid interaction) (10).

In the original method provided by Marsolier et al. (10), a genetic assay for U6 snRNA synthesis is used to indirectly monitor expression of the UAS_G-SNR6 reporter gene. These authors have constructed an *snr6* yeast strain harboring a wild-type SNR6 gene on a URA3-marked plasmid. Loss of this plasmid is tolerated by yeast cells when the UAS_G-SNR6 reporter gene carried by a second plasmid is activated, thus providing the cells with the essential U6 snRNA. Due to the presence of the URA3 marker gene, whose product turns 5-fluorotic acid (5-FOA) into a toxic compound, yeast cells that have lost the wild-type SNR6 plasmid can be identified for their ability to grow on agar plates containing 5-FOA. However, this selection assay for the expression of the UAS_G-SNR6 reporter gene turned out to be unsuitable for large-scale screening experiments because it gave a high background of yeast colonies growing on 5-FOA plates independently of activation of the UAS_G-SNR6 reporter gene (unpublished observations and see below). We do not have an evident explanation for such a high background. We have, however, observed that yeast transformation with

overexpressed cDNA libraries, as typically applied in a two-hybrid system, can generally cause reduced selectivity of the 5-FOA conditions and, consequently, to Ura3⁺ cells still capable of growing on 5-FOA (our unpublished observations). Thus, under these conditions, screening of expression libraries can lead to the isolation of a high number of false-positive clones. Moreover, 5-FOA selection is generally applicable only via replica plating, which makes large-scale screenings in any case extremely laborious. We have therefore developed a quick and reliable selection assay for the pol III-based two-hybrid system.

MATERIALS AND METHODS

Yeast and Plasmids

The interaction assays were performed in the yeast strain MPy11 (*MAT α* , *ura3-52*, *lys2-801*, *ade2-101*, *trp1- Δ 63*, *his3- Δ 200*, *leu2- Δ 1*, *snr6- Δ 2*, *gal4::kan^r*). This strain is a derivative of MCM616 (11) in which the endogenous SNR6 gene is silent and the original plasmid carrying wild-type SNR6 has been replaced by a URA3-marked centromeric plasmid bearing the temperature-sensitive (*ts*) *snr6* A59C mutant. The *ts* point mutation A59C (8) was introduced into the SNR6 gene by PCR. The DNA fragment containing the *snr6* A59C locus from -139 to +313 was subcloned into the *KpnI/HindIII*-digested episomal vector YCplac33 (6).

To construct the UAS_G-SNR6 reporter pMP44, the HIS3-containing *EcoRI/XhoI* fragment of JJ215 was subcloned in pOT2, a plasmid carrying the CAT gene marker. The blunted *BglII/AccI* 2- μ m fragment of YEplac195 was inserted into the *EcoRV* site of the HIS3-containing pOT2 vector. The resulting HIS3 2- μ m shuttle vector was cut with *BamHI* and *EcoRI*, and a UAS_G-SNR6 *BamHI/EcoRI* fragment was cloned into it.

The plasmids expressing the τ 138 hybrid proteins listed in Table 1 are based on the *TRP1*-marked pGen- τ 138 vector (10), in which the *BamHI* site 5' of the τ 138 ORF has been deleted (a gift from M. Hottiger, University of Zürich) and a hemagglutinin (HA)-

epitope sequence has been inserted at the *Bam*HI site immediately downstream of the τ 138 ORF. Details of all the plasmids are available upon request.

Interaction Assays

The ts yeast strain MPy11 was transformed with the UAS_G-*SNR6* reporter plasmid pMP44 and the plasmid expressing the τ 138-hybrid protein (bait construct). This ts yeast strain was always kept at 25°C instead of 30°C to increase the temperature gap between selective and nonselective growth conditions. Four single transformants were picked, and about 10⁸ cells/transformant were plated and incubated at 37°C to test temperature sensitivity. The clone of choice (the one that gave the lowest number of colonies at 37°C) was transformed with the *LEU2*-marked plasmids expressing Gal4p(1-100) or the Gal4pDBD-fusion protein of interest according to the standard LiOAc protocol (5) with the following modifications: yeast cells were always kept at 25°C instead of 30°C and heat-shocked in a 42°C water bath for only 10 min. After quick centrifugation in a micro-

centrifuge, the cell pellet was gently resuspended in 200 μ L sterile water. Each cell suspension was split and spread on two selective plates (-His/Trp/Leu) that were incubated at 25°C for 24 h. One plate was then shifted to 37°C, while the other remained at 25°C to estimate transformation efficiency. Two to three days after the shift at 37°C, colonies of cells potentially expressing interacting proteins started to appear. These colonies were picked and sequentially streaked on plates containing 0.1% 5-FOA and on -His/Trp/Leu drop-out plates. Colonies growing on 5-FOA plates were picked and further processed to isolate plasmids (7).

RESULTS AND DISCUSSION

Figure 1 shows the principle of our novel selection technique to identify interacting proteins in a pol III-based two-hybrid system. The *SNR6* knockout strain MPy11 contains a ts U6 snRNA expressed from the mutant *snr6 A59C* gene that is carried by a *URA3*-marked plasmid (ts survival construct). This mutated U6 allows growth at 30°C

or less, but not at 37°C. A second plasmid carries the UAS_G-*SNR6* reporter gene (reporter). This reporter gene expresses wild-type U6 snRNA when RNA pol III transcription is activated by tethering τ 138p to DNA, for example, through an interaction between protein X fused to τ 138 and protein Y fused to the UAS_G-binding Gal4pDBD. Expression of the wild-type U6 snRNA suppresses the ts phenotype and allows cell growth at 37°C. Such a selection for growth at the nonpermissive temperature of 37°C is very tight; it is also technically easy and convenient because it does not require a replica-plating step. Moreover, since the ts survival construct carries the *URA3* gene marker, this system allows researchers to exploit growth selection on 5-FOA plates, not for the primary selection from millions of clones as in the original system, but for a second selection step to confirm interacting partners in individual cell clones. Those clones that can grow at 37°C because of true activation of the UAS_G-*SNR6* reporter gene can be selected for the loss of this plasmid by testing their ability to grow on 5-FOA plates. This second step is particularly

Figure 1. Outline of the RNA pol III two-hybrid selection assay. In the *SNR6* knockout strain MPy11, the ts mutant *snr6 A59C* gene is carried by a *URA3*-marked plasmid (ts survival construct). A second plasmid carries the UAS_G-*SNR6* reporter gene (reporter). (A) The UAS_G-*SNR6* reporter gene is not activated if no interaction occurs between the fusion proteins τ 138-X and Z-Gal4p; as a consequence, yeast cells cannot grow at 37°C. (B) Activation of the UAS_G-*SNR6* reporter gene caused by the interaction between the fusion proteins τ 138-X and Y-Gal4p leads to expression of the wild-type copy of the U6 snRNA, which suppresses the ts phenotype (cell growth at 37°C). For simplicity, RNA polymerase III and transcription factors other than τ 138 have been omitted.

useful for library screenings because it eliminates cells that contain mutations or hybrid proteins that may suppress the ts phenotype but are still dependent on the presence of the *URA3*-marked ts survival plasmid.

We first tested whether expression of

Gal4(1-147)- τ 138p (10) would activate transcription of the UAS_G-SNR6 reporter gene to a level that is sufficient to sustain growth of the *snr6* ts mutant strain at 37°C. As shown in Figure 2 (plate b), expression of Gal4(1-147)- τ 138p was able to suppress the *snr6* ts

Figure 2. Suppression of the *snr6* ts phenotype by interaction-dependent expression of wild-type U6 snRNA from the UAS_G-SNR6 reporter gene. The *SNR6* knockout strain MPy11, which contains the *URA3*-marked ts survival construct and the UAS_G-SNR6 reporter plasmid, was transformed with vectors expressing the Gal4(1-147)- τ 138p fusion protein (b; positive control), τ 138-Gal11p wt plus Gal4p(1-100) (c), or τ 138-Gal11Pp plus Gal4p(1-100) (d). For each transformation, an aliquot of the cell suspension (1/3 of the volume) was spread in one of the three sectors of plate a, while the rest was spread on separate plates (plates b, c, and d). Plate a was incubated at 25°C for 4 days, while plates b, c, and d were incubated for 24 h at 25°C and then for 3 days at 37°C. (Plate a) Transformation controls incubated at the permissive temperature of 25°C. (Plate b) Expression of Gal4(1-147)- τ 138p suppressed the ts phenotype and allowed cell growth at 37°C. (Plate c) Co-expression of the noninteracting proteins τ 138-Gal11p wt and Gal4p(1-100) did not activate transcription of the UAS_G-SNR6 reporter gene, and cells could not grow at 37°C. (Plate d) The specific interaction between τ 138-Gal11Pp and Gal4p(1-100) was able to activate expression of wild-type U6 snRNA from the UAS_G-SNR6 reporter gene and allow cell growth at 37°C.

Short Technical Reports

Table 1. Leak Test for MPy11 Expressing Different τ 138p Hybrid Proteins

Hybrid Protein	False Positives in 10^8 Transformed Cells	
	exp. 1	exp. 2
τ 138-mBRCA1 (aa 1455–1812)	0	3
τ 138-mBARD1	1	0
τ 138-hSRB7	0	0
τ 138-yGal11Pp	0	1
τ 138-yGal80p	0	0

The selectivity of our assay was tested by transforming the strain MPy11 containing the UAS_G-*SNR6* reporter plasmid with vectors expressing five different τ 138p hybrid proteins. For each τ 138p hybrid protein, two independent experiments (exp. 1 and exp. 2) were performed. The observed growing colonies, the total number of which is presented for both sets of experiments, are referred to as false positives since no interaction partners fused to Gal4p(DBD) were provided.

phenotype, thus allowing colony formation at 37°C. A similar result was obtained by co-expressing two known interacting proteins (i.e., τ 138-Gal80p and Gal4p), which had been previously shown to specifically activate UAS_G-*SNR6* transcription (10) (data not shown). To investigate sensitivity and specificity of our selection assay, we exploited the defined interaction between a fragment of the dimerization domain of Gal4p (residues 58–97) and Gal11Pp, a mutant form of the pol II holoenzyme component Gal11p carrying a single amino acid substitution (Asn342 to Ile) (1). From plasmon resonance experiments, this interaction is known to have a K_d of 10^{-7} M (3). Since Gal4p only interacts with Gal11Pp but not with the wild-type form of Gal11p (Gal11p wt), these proteins provide an ideal model for testing the pol III two-hybrid selection system. Two τ 138 fusion proteins were made, the first bearing Gal11p wt and the second bearing Gal11Pp. Equal levels of expression of these fusion proteins were detected by western blot analysis (data not shown). As a UAS_G-binding interaction partner, we expressed Gal4p (1-100), which contains the dimerization domain of Gal4p that interacts with Gal11Pp but not with Gal11p wt (2). Figure 2 shows that yeast ts cells expressing the fusion protein that contained the Gal11Pp mutation (τ 138-Gal11Pp) were able to grow at 37°C, while no growth was detected for cells

expressing the wild-type form of Gal11p fused to τ 138p (τ 138-Gal11p wt) (plates c and d). The lack of growth at 37°C of yeast cells expressing τ 138-Gal11p wt is clearly dependent on the nonpermissive temperature because aliquots (1/3 of the volume) of each transformation mixture were able to form colonies at 25°C (Figure 2, plate a).

Conditions mimicking a screening experiment were tested as follows: the plasmid expressing Gal4p(1-100) was mixed with its parental “empty” vector in defined ratios, and the derived plasmid mixture was used to transform the ts yeast strain that already contained the τ 138-Gal11Pp fusion protein described above. Only yeast cells expressing Gal4p(1-100) are expected to grow at 37°C due to the interaction with τ 138-Gal11Pp that activates UAS_G-*SNR6*. According to the transformation efficiency for each individual plasmid in cells growing at 25°C, the expected number of colonies expressing Gal4p(1-100) was computed and referred to as 100%. On the average, the actual number of colonies growing at 37°C was about 75% of this value. This difference is mostly due to a lower transformation efficiency of cells growing at the nonpermissive temperature of 37°C because the same type of experiment with a plasmid carrying the complete wild-type *SNR6* gene gave similar values. Of all the colonies growing at 37°C, 60 were randomly picked and subjected to

Short Technical Reports

PCR analysis to assess the frequency of the Gal4p(1-100) plasmid. All of these cells contained the Gal4p(1-100) plasmid (data not shown). The recovery rate and the frequency of the Gal4p(1-100) plasmid indicate that our selection assay is very sensitive and tight.

To further investigate the selectivity of our assay, five τ 138p hybrid proteins (baits) were constructed, four of which carried protein domains that can activate transcription by pol II (data not shown), and tested for potential spontaneous suppression of the ts phenotype ("leak test"). To this purpose, these hybrid proteins were expressed in the ts strain containing the UAS_G-SNR6 reporter plasmid. For each τ 138p hybrid construct, and in two independent experiments, about 10^8 transformed cells were plated and then incubated at 37°C. Four days later, the number of growing colonies was counted (referred to as false positives). Zeros to a maximum of three false positives per 10^8 transformed cells were observed (Table 1). We then compared the selectivity of our

assay with the original 5-FOA method (10) by replica-plating cells expressing one of the τ 138p hybrid proteins (τ 138-Gal11Pp) on media containing 0.1% 5-FOA and counting the number of growing colonies; application of the 5-FOA selection step gave an average of three false positives per 10^4 transformed cells (i.e., 30 000/ 10^8 cells) (data not shown). Therefore, the selectivity of the pol III-based two-hybrid system was dramatically improved.

We have so far performed one screening experiment to completion with a mouse embryonic cDNA library fused to the DNA-binding Gal4p(1-147) and τ 138-mBRCA1 as a bait. For the pol III-based two-hybrid system, it is generally most convenient to fuse the bait protein to τ 138 and the library to the Gal4p DNA-binding domain. Of a total of 2.5 million transformed cells, 272 colonies grew at 37°C, 188 of which were confirmed by growth on 0.1% 5-FOA plates. After plasmid recovery, 183 of these 188 clones were shown to activate UAS_G-SNR6 in a bait-dependent manner. These 183 clones could be arranged in 14 complementation groups (details of these results will be published elsewhere). The results obtained through this series of screening experiments confirmed that our novel selection assay is indeed very efficient and reliable.

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