Differential Sensitivity to 5-fluoro-orotic Acid as a Screen for Bait RNA-Independent False Positives in a Yeast Three-Hybrid System

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ABSTRACT

The yeast three-hybrid system presents a valuable tool for detecting and analyzing RNA-protein interactions in vivo. A major drawback of the use of such a transcriptional reporter-based assay in a library screen is the frequent occurrence of false-positive results due to bait RNA-independent activation of the reporter gene. To minimize the isolation of false positives in three-hybrid library screens, we incorporated a rapid and simple procedure based on differential sensitivity to 5-fluoro-orotic acid. The technique effectively eliminates bait RNA-independent false positives and thus greatly enhances the efficiency of the yeast three-hybrid system.

INTRODUCTION

In eukaryotic cells, posttranscriptional modifications play a critical role in gene expression. Primary transcripts are subjected to a multitude of processing reactions that typically include capping, splicing and polyadenylation. Processed mRNAs are transported into the cytoplasm, where their activity, stability and cellular localization are tightly modulated. These posttranscriptional events are properly executed through the concerted actions of a diverse set of RNA-binding proteins. Not surprisingly, RNA-protein interactions are crucial to many biological processes, including translational regulation, chromosome replication and development (4,5,11,12). RNA-protein interactions are also exploited by RNA viruses, including human immunodeficiency virus (HIV), hepatitis C virus (HCV) and picornaviruses, to modulate infectivity and replication (1,6–9). Thus, the identification and characterization of RNA-interacting factors have provided important insights into the molecular mechanisms of posttranscriptional control of eukaryotic gene expression.

The recent modification of the yeast two-hybrid system into a three-hybrid system has enabled the detection and analysis of specific RNA-protein interactions in vivo (10). The yeast three-hybrid technique has been used to screen and isolate cDNAs encoding RNA-interacting proteins from cDNA expression libraries (11,12). The system is based on the functional reconstitution of a transcriptional activator achieved through the association of a “prey” RNA-binding protein with its cognate “bait” RNA, as assessed by the activation of a reporter gene(s) in yeast (Figure 1), usually the dual-reporter system consisting of HIS3 and lacZ. However, a major shortcoming of the assay, particularly in a library screen, is the frequent appearance of false-positive results because of bait RNA-independent transcriptional activation of reporter genes (11–13). We and others have found that a significant portion of primary transformants that grow on histidine (His)-deficient medium and turn blue in a β-galactosidase assay did not require bait RNA for transcriptional activation of the reporter genes (5).

Since bait RNA-independent false positives affect the proficiency of the three-hybrid library screen, there is a need to devise a strategy to reduce the identification of such false positives before further characterization of putative, positive cDNA clones. A commonly used strategy is the counter-screen growth selection system based on 5-fluoro-orotic acid (5FOA) (13). Due to conversion of 5FOA to the toxic 5-fluoro-deoxyuracil (2), yeast cells expressing URA3 encounter severe growth inhibition on medium containing 5FOA. Since the plasmid encoding the bait RNA in the three-hybrid system carries the URA3 marker, cells that have lost the plasmid can be selected by culturing primary His+-transformants on 5FOA-containing medium. 5FOA-resistant cells devoid of the bait RNA plasmid could then be retested for their ability to grow on -His medium or lacZ expression to determine if the transcriptional activation of reporter genes indeed requires the bait RNA. However, this protocol is time-consuming and cumbersome (11). An alternative way to tackle the issue of a high frequency of RNA-independent positives occurring in the yeast three-hybrid system involves a colony color screen (12). In this system, the RNA plasmid contains an ADE2 gene. Because the strain is ADE2-defective, RNA plasmid-dependent activation of HIS3 can be scored by examining colony color in the absence of selection for the RNA plasmid. Thus, colonies that can activate the HIS3 reporter without the RNA plasmid would lose it, rendering them red or red-sec- tored, while colonies that require the RNA plasmid to activate HIS3 remain white. However, this system is also imperfect, with 80% of white colonies (1 of 6) being still RNA-independent; ultimately, the conventional 5FOA-based method had to be used to identify a true RNA-dependent clone (12).

To incorporate a faster and simpler procedure into the yeast three-hybrid method, we tested whether the differential sensitivity of primary His+ colonies to 5FOA treatment alone might serve as a reliable indicator for bait RNA-independent false positives. We reasoned that the multicopy RNA plasmid (carrying the URA3 marker) within an RNA-dependent clone is likely to be
amplified due to strong selective pressure for HIS3 expression during the library screen augmented by the addition of 3-aminotriazole (3AT) to the media. Consequently, such clones would be expected to be sensitive to 5FOA treatment as the URA3 protein enzyme accumulated accordingly. By comparison, RNA-independent clones, which presumably do not require the RNA plasmid to confer growth on -His medium, should be relatively resistant to 5FOA. To test the feasibility of this strategy, we performed a small-scale library screen. We have previously demonstrated that the 5′-untranslated region (UTR) of influenza virus nucleoprotein (NP) mRNA recruits and interacts with a set of cellular factors to regulate selective translation of the virus mRNA (9). We have thus used the yeast three-hybrid system to determine the identity of these NP UTR-interacting proteins within the context of a human cDNA library.

MATERIALS AND METHODS

For yeast culture, synthetic minimal dropout media (BIO 101, Vista, CA, USA) containing 2% glucose were used. Synthetic minimal dropout media were designated by the amino acids that are left out (e.g., -Trp, -Leu and -His medium lacked tryptophan, leucine and histidine, respectively). All yeast three-hybrid procedures and assays were performed according to the manufacturer’s Yeast Protocols Handbook (PT3024-1; CLONTECH Laboratories, Palo Alto, CA, USA). The yeast host strain L40-coat [MATa, ura3-52, leu2-3112, his3A200, trp1A1::TRP1, ade2, LYS2::(lexAop)-HIS3, ura3::(lexAop)-LacZ] previously transformed with pIIIa/fem3-MS2 (which expresses the LexA DNA-binding domain fusion with MS2 coat protein LexA-MS2) was a generous gift from Dr. Dhruba J. SenGupta (University of Washington, Seattle) (5). The plasmid pIII/MS2-NP expressing a MS2-NP 5′ UTR hybrid RNA was constructed by inserting a 53-nucleotide, double-stranded oligonucleotide containing the influenza virus NP 5′ UTR into the XmaI site on plasmid pIII/MS2-1, which carries the URA3 marker. The orientation and sequence of the inserts were confirmed by sequencing. The plasmid pII/MS2-NP was introduced into L40-coat, and transformants were selected on -Trp, -Ura plates, resulting in the yeast strain L40-coat/NP (Trp+, Leu+, Ura+ and His+). Two hundred micrograms of a library of HeLa cDNA linked to the yeast GAL4 Transcriptional Activation Domain (CLONTECH) were introduced into L40-coat/NP. After being selected and amplified on -Trp, -Ura and -Leu plates, yeast transformants (Trp+, Leu+ and Ura+) were plated onto -Trp, -Leu, -His and -Ura plates containing 3 mM 3AT (Sigma, St. Louis, MO, USA) at a density of 0.5 × 10^6 colonies on 150-mm plates (Becton Dickinson Labware, Bedford, MA, USA) and incubated at 30°C for three days.

RESULTS AND DISCUSSION

A total of 120 primary His+ transformants were isolated from the library screen. To examine their bait RNA dependency, we plated the cells onto 5FOA-containing plates to select against the URA3 marker and thereby eliminate the RNA plasmid pIII/MS2-NP. Most 5FOA-treated His+ prototrophs (110 of 120 or 91.6%) grew on -Trp and -Leu plates containing 5 mM 5FOA (Figure 2A). Surprisingly, all 5FOA-resistant clones, which presumably had lost the RNA plasmid, were also able to grow on -His medium con-

![Figure 1. The yeast three-hybrid system and two classes of candidate clones: bait RNA-dependent and bait RNA-independent clones.](image-url)
containing 3AT (Figure 2B), indicating that these were bait RNA-independent false positives. To further verify that these 110 clones were indeed bait RNA-independent false positives, we isolated the library plasmids from twenty randomly selected 5FOA-resistant clones and reintroduced them into an L40-coat host strain that lacked the RNA plasmid. As expected, all resulting transformants retained their ability to activate HIS3 (data not shown).

We next examined the small group of clones isolated from the library screen that were sensitive to 5FOA (Figure 2A; arrows). Since these clones were 5FOA-sensitive, we cultured the cells on Ura-containing medium (-Trp or -Leu plates) to select for clones that had spontaneously lost the plasmid pIII/MS2-NP. As expected, spontaneous loss of bait RNA degraded the ability of this class of clones to grow in -His medium containing 3AT (Figure 2B), implying that these were bait RNA-dependent candidates. Consistent with the idea that the lack of growth was caused by amplification of the RNA plasmid resulting in increased accumulation of URA3 gene product, primary 5FOA-sensitive clones that had been cultured on +His medium (without HIS3+ selection) were able to develop resistance to 5FOA treatment (data not shown).

In contrast, when the selective pressure against the URA3 gene was relieved, all primary transformants that were initially sensitive to 5FOA treatment (a total of ten were isolated from the library screen) now grew in -His medium (Figure 2C, Panel S). Further, none were able to induce HIS3 expression (Figure 2D, Panel S). As a control, primary transformants that were resistant to 5FOA retained the ability to activate HIS3 (Figure 2D, Panel R). We also confirmed the bait RNA-dependency of all ten 5FOA-sensitive clones by isolating and reintroducing their library plasmids into L40-coat host strain lacking the RNA plasmid (data not shown).

 Taken together, our results demonstrate that primary His+ clones isolated from a yeast three-hybrid screen can simply be tested for their sensitivity to lower-affinity, RNA-protein interaction (between 1 and 10 nM), the HIV Tat protein and its RNA target (TAR) were selected (3). As shown in Figure 3, when grown on minimal -His medium supplemented with 3AT, clones co-expressing IRP-1 and IRE or Tat and TAR were sensitive to 5FOA treatment, as opposed to RNA-independent clones. Thus, our method should be applicable to the identification of both strong and weak RNA-protein interactions.

To further demonstrate a general utility of our 5FOA sensitivity-based procedure in the yeast three-hybrid assay, we examined the RNA-protein interaction of two well-characterized known interacting partners. To test an RNA-protein interaction of high affinity (between 0.01 and 0.1 nM Kd), we chose the iron regulatory protein 1 (IRP-1) and its RNA target, the iron-responsive element (IRE) in the 5′ UTR of ferritin mRNA (5). For analysis of a lower-affinity, RNA-protein interaction (between 1 and 10 nM), the HIV Tat protein and its RNA target (TAR) were selected (3). As shown in Figure 3, when grown on minimal -His medium supplemented with 3AT, clones co-expressing IRP-1 and IRE or Tat and TAR were sensitive to 5FOA treatment, as opposed to RNA-independent clones. Thus, our method should be applicable to the identification of both strong and weak RNA-protein interactions.

Taken together, our results demonstrate that primary His+ clones isolated from a yeast three-hybrid screen can simply be tested for their sensitivity to lower-affinity, RNA-protein interactions. As shown in Figure 3, when grown on minimal -His medium supplemented with 3AT, clones co-expressing IRP-1 and IRE or Tat and TAR were sensitive to 5FOA treatment, in contrast to RNA-independent clones. Thus, our method should be applicable to the identification of both strong and weak RNA-protein interactions.
5FOA treatment to determine if they are bait RNA-dependent or -independent candidates; sensitive clones are likely to be authentic bait RNA-dependent ones. While other recent variations of the three-hybrid system have been described, our procedure incorporates a technically simple and straightforward step into a commonly used three-hybrid system, therefore allowing researchers to continue using the system with higher efficiency. Coupled with other modifications, such as the colony color-selection scheme described by Zhang et al. (12), our method is expected to increase the efficiency and reliability of the three-hybrid system in library screens.

REFERENCES


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Rapid Genetic Screening for Hemochromatosis Using Automated SSCP-Based Capillary Electrophoresis (SSCP-CE)

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ABSTRACT

Hereditary hemochromatosis (HHC) is an autosomal recessive disease in which increased iron absorption causes iron overload and progressive tissue damage. Clinical manifestations include diabetes, cardiomyopathy, hypogonadotropic hypogonadism, arthritis and liver cirrhosis, which might ultimately lead to hepatocellular cancer. HHC represents one of the most common genetic diseases in Northern Europe and the UK, with an estimated carrier frequency between 1:8 and 1:10 (12). Conventional diagnosis of the disease in its symptomatic stage is based on a combination of clinical, biochemical and histological parameters (4), as described in detail in Materials and Methods.

However, these parameters lack specificity and fail to detect HHC in its asymptomatic stage, which is important since iron removal by phlebotomy is an effective means to prevent irreversible tissue damage. Importantly, an association between HHC and mutations in the HFE gene, a major histocompatibility complex class I-like gene located on chromosome 6p, has been described recently (5,7,8). The majority of patients turned out to have the same point mutation changing a cysteine at amino acid position 282 to tyrosine (C282Y). This mutation is most prevalent in Northern Europe, North America and Australia and was detected in 91% of HHC patients in the UK (6) but only in 64% of Italian patients (3). A second mutation, changing the histidine at position 63 to aspartatic acid (H63D), was detected in a minority of patients (2,5,8). However, involvement of this mutation in the full clinical manifestation of HHC has not yet been formally confirmed. Different clinical phenotypes have been associated with different types of mutations (C282Y homozygosity vs. C282Y and H63D compound heterozygosity) and the most common ancestral haplotype, which is linked to HLA-A3 on chromosome 6p (1,9,10).

In summary, these studies have provided a reliable genetic tool to detect HHC early during its presymptomatic course and thus prevent all clinical manifestations. Therefore, an easy, cost-effective test for the detection of HFE mutations will facilitate population-based screening. Here, we describe a protocol that adapts single-strand conformation polymorphism (SSCP) analysis for capillary electrophoresis and that is suitable for large-scale, cost-effective genetic testing.