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A yeast assay for high throughput screening of natural anti-viral agents

Rashi Srivastava and Sunil K. Lal*

Virology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Road, P.O. Box 10504, New Delhi 110067, India

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Abstract

Over the last decade the yeast *Saccharomyces cerevisiae* has become a popular organism for studying heterologous gene expression and in vivo protein–protein interactions. Many variations of these basic systems have originated over the years. Besides these vast and varied applications of the yeast expression system, *S. cerevisiae* has also been used extensively in fundamental research as a model simple eukaryote. We have used the *S. cerevisiae* system to design a high throughput screen for anti-viral agents from natural sources. The design of the assay rests on the ability of the L-A helper virus and the M_1 satellite virus to detect small variations in −1 ribosomal frameshifting. A minor change in frameshifting efficiencies can be detected and clearly shown phenotypically in terms of zones of clearing on an agar plate. Using such a process, we have initiated a high throughput screening process for natural anti-viral agents.
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During translation of mRNA, the ribosomes maintain precisely the correct translational reading frame. This is a fundamental property that provides the basis to the integrity of the protein translational machinery and eventually to cell growth and viability. This machinery operates at an extremely low-error rate in frameshifting [1–3]. Most double-stranded RNA (dsRNA) and non-segmented (+) stranded RNA viruses, including Retroviruses, use programmed −1 ribosomal frameshifting as a fundamental process in their replication within the host cell [4]. What makes the whole process a more interesting target for anti-viral intervention is the fact that as yet there are no reports of any eukaryotic cellular mRNAs that show −1 ribosomal frameshifting.

The basic rationale behind the development of this assay is that many viruses that cause disease in humans, animals, and plants utilize programmed ribosomal frameshifting to regulate the production of their structural and enzymatic proteins. Since the altering of this molecular phenomenon disrupts the virus life cycle and eliminates/reduces virus production, it would serve as an excellent target for screening of compounds for their anti-viral properties [5]. This phenomenon is aimed at developing a yeast-based assay system to identify compounds that may have anti-viral properties [4].

In viruses that utilize programmed −1 frameshifting, the open-reading frame (ORF) encoding the major viral structural protein (typically Gag protein) is located at the 5′ end of the mRNA, whereas the ORFs encoding proteins with enzymatic function (typically Pro and Pol) are located at the 3′ end of the transcript and out of frame with the Gag ORF. It is a ribosomal frameshift event that results in the production of the minority enzymatic proteins while the majority of the protein produced is Gag [6–8]. The importance in maintenance of this delicate yet appropriate ratio of the Gag and Pol proteins, which is a direct function of the efficiency of −1 ribosomal frameshifting has been well established [9]. In an attempt to use this approach to screen for anti-viral agents, we have developed this assay and used it for our initial screening. Programmed −1 ribosomal frameshifting has been developed to be an excellent target for compounds that function as anti-viral agents, because this molecular process is predominantly utilized by these viruses to regulate their gene expression. This demonstrates the great need for identifying agents that function to affect programmed −1 ribosomal frameshifting and thus reduce viral titer.

The L-A helper virus-infected yeast host with M_1 superinfection was used for the described assay. A −1

* Corresponding author. Fax: +91-11-616-2316.
E-mail address: sunillal@icgeb.res.in (S.K. Lal).

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ribosomal frameshift event was responsible for the correct expression ratios of Gag and Pol, both encoded in the L-A helper virus [2,8]. The killer trait of the L-A + M_1-infected yeast strain failed to show up when YPD and SD (synthetic dextrose) media were used at natural pH. Since the killer toxin that is produced by this infected yeast strain is stable between pH 4.6 and 4.8, buffered methylene blue medium (MBM) was used for the assay [10–12]. Phosphate–citrate buffer, made by adjusting the pH of citric acid (final concentration 1 M) to 4.5 with K_2HPO_4, was used to lower the pH of the media to 4.7. After sterilization of YPD medium in an autoclave, 100 ml of the phosphate–citrate buffer was added to 900 ml of the medium. Methylene blue, a stain for dead yeast cells, was then incorporated into the medium at a final concentration of 0.003% [13,14].

The clear zone forming phenotype of the superinfected yeast strain was observed when a suspension of this strain was grown in YPD liquid medium, pelletted, and placed over low-pH MBM plates that have previously been spread with a culture of sensitive yeast Y526 strain cells. After incubation for two days at 22 °C, the superinfected yeast strain was surrounded by a zone of clearing fringed with a deep blue color, indicating the death of the sensitive cells. This clear zone phenotype is indicative of the L-A virus and M_1, processing their RNA inside the yeast cell by producing exactly the right amount of ribosomal slippage for production of the correct ratio of Gag and Pol proteins. The correct ratio of Gag and Pol results in the production of the toxin so as to kill the sensitive strain around it. This killing of the sensitive strain is what results in clearing of the lawn of cells (Fig. 1).

To abolish the clear zone forming phenotype of the superinfected yeast strain, various concentrations of antibiotics that disrupt −1 ribosomal frameshifting were used with the superinfected strain. Spotting the superinfected strain after antibiotic treatment or mixing the superinfected strain with antibiotic solution did not give any positive results (data not shown). The superinfected yeast strain was then grown in the presence of the antibiotics in concentrations ranging from 50 to 200 μg/ml in YPD broth. The period of incubation and the volume of inoculation to be plated on the lawn of sensitive strains on MBM plate were then standardized. It was found that a concentration of 50 μg/ml was too low for the killer and the sensitive strains. Sparsomycin did not have any effect in the range used for the test. It was found that within the concentration range of 100–200 μg of anisomycin and cycloheximide/ml in YPD broth, when the superinfected yeast strain was grown for two overnights, 25 μl of this suspension was successful in eliminating the killer phenotype, which appeared as a loss of the zone of inhibition (Fig. 2). Since these antibiotics specifically disrupt ribosomal frameshifting, it is clear that when this assay system is used for screening of natural anti-viral agents, the assay will function as well and show detectable phenotype.

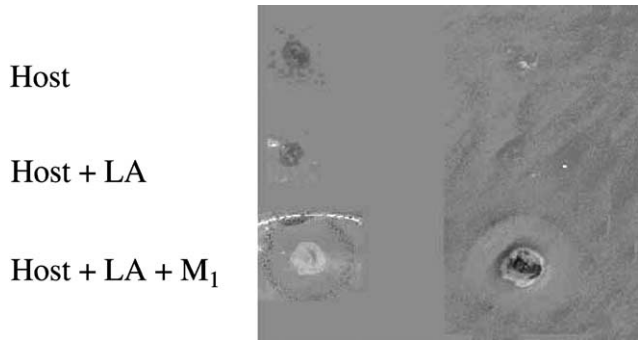

Fig. 1. The development of a superinfected *S. cerevisiae* yeast strain which can harbor L-A and M_1 viruses. The zone of clearance formed can be seen clearly around the spotted superinfected strain.

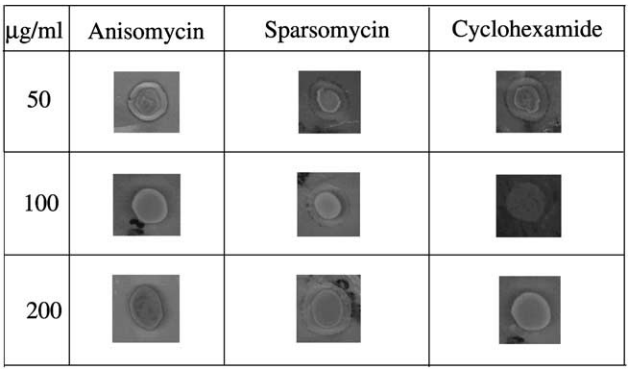

Fig. 2. Effects of varying concentrations of anisomycin, sparsomycin, and cyclohexamide on −1 ribosomal frameshifting efficiencies of the L-A and M_1 virus. Small amounts (100 g/ml) of anisomycin and cycloheximide were able to show disappearance of the zone of inhibition created by the superinfected yeast strain. Similar dissapearance of zones of inhibition would be expected by the usage of this assay during anti-viral screeing.

Materials and methods

Intact L-A virions were infected into the yeast host strain SL321(MATa *his*(3,4), *leu2*, *lys2-801 ade2-10 trp1-Δ1*) superinfection of M_1 followed. Yeast strains L40a and Y526 showed good sensitivity with sharp zones of inhibition and thus were used as the sensitive strain for subsequent experiments. Sparsomycin, anisomycin, and cycloheximide were purchased from Sigma Chemical, USA. These antibiotics were used in the detection assays and the exact amounts were determined per petri dish in order to get reproducible results with the screening.

The L-A double-stranded RNA virus is endogenous to yeast cells along with the M_1 satellite dsRNA virus. The M_1 satellite virus secretes a killer toxin and is encapsidated and replicated using the Gag and Gag–Pol gene products synthesized by the L-A virus inside the infected yeast cell. Yeast cells harboring both L-A and M_1 viruses secrete the toxin and are immune to its action whereas virus-free cells are sensitive to the toxin. A ring of growth inhibition is indicative of the killer activity of the superinfected yeast cells harboring both L-A and M_1 viruses.

Phosphate–citrate buffer (PCB) was prepared by dissolving 192 g of citric acid to 1 L of distilled water and adjusting the pH with K_2HPO_4 to 4.5. The solution was autoclaved before use.

Methylene blue stock solution (MBSS) contained 5% Methylene blue dye. Methylene blue media (MBM) contained 600 μl MBSS (0.003%), 100 ml PCB, and 900 ml of liquid YPD (agar).

Liquid cultures of the sensitive yeast strain Y526 were grown with shaking at 300 rpm at 30 °C overnight. The following day, this strain was spread-plated on the MBM plates as described above. Another liquid culture of the superinfected yeast strain containing both the L-A and M_1 viruses was grown overnight in liquid YPD medium with shaking at 300 rpm at 30 °C. [If antibiotic assay need to be done, add 50/100/200 μg/ml to the shake tubes now.] Spin down and harvest the superinfected yeast cells for the assay. Spot these cells on the spread-plated sensitive cells on the MBM plate. Incubate the plate for 2 overnights at 22.5 °C. The lawn of sensitive cells starts to grow and the zone of clearing begins to show after the second overnight incubation. To screen for natural anti-viral agents the same protocol may be used with the candidate anti-viral agent being tested getting added in place of the antibiotics used in the experiments above.

Results and discussion

The yeast-based zone of clearing, due to toxin production by the superinfected yeast strain, has been developed as an anti-viral screening assay. Typically this assay is composed of the L-A helper virus and the M_1 satellite virus. The dsRNA genome of L-A contains two ORFs, the 5′ *Gag* gene encoding the major viral coat protein (Gag) and the 3′ *Pol* gene encoding a multifunctional protein that includes the RNA-dependent RNA polymerase and a domain required for viral RNA packaging. A −1 ribosomal frameshift event within the host cell is responsible for the production of the L-A-encoded Gag–Pol fusion protein. The M_1 satellite dsRNA genome is encapsidated and replicated inside the icosahedral 39 nm L-A viral particle. Changes in the efficiency of programmed −1 ribosomal frameshifting along the L-A mRNA result in a rapid loss of encapsidated M_1, This phenotype is evident by replica-plating colonies of test cells on a lawn of uninfected yeast cells that are sensitive to the killer toxin. Superinfected cells maintaining the M_1 virus secrete the killer toxin, creating a characteristic ring of growth inhibition.

Fig. 1 shows clearly the formation of the zone of clearing when the host yeast cells are superinfected with L-A and M_1. This zone of clearing of the sensitive yeast cells is missing when the spotted strain contains only L-A or no infection. The fact that programmed −1 ribosomal frameshifting appears to be virus-specific makes it an attractive target to identify agents that affect the efficiency of this process and, consequently, disturb the equilibrium of viral maintenance. It is of great interest to mention that small changes in the −1 ribosomal frameshifting efficiencies will have large effects on virus production. Increasing or decreasing minutely, the efficiency of programmed −1 ribosomal frameshifting within the yeast host cells will hence result in an amplified response showing a large inability of yeast cells to maintain the M_1 "killer" satellite virus of L-A.

Peptidyl-transferase inhibitors (anisomycin and sparsomycin) were used to further confirm the specificity of phenotype due to −1 ribosomal frameshifting in yeast superinfected cells [13]. These drugs represent a well-studied class of small molecules that affect the protein-synthetic machinery at the step at which the −1 ribosomal frameshifting is postulated to occur. We have shown (Fig. 2) that at concentrations as low as 100 μg/ml, there is no effect on the viability of the host cell or on the rates of protein translation (data not shown). However, the efficiency of programmed −1 ribosomal frameshifting was affected, which resulted in the disappearance of the zone of clearing around the spotted superinfected host.

Upon screening for anti-viral agents, candidate compounds that change the efficiency of programmed −1 ribosomal frameshifting efficiencies at concentrations that do not drastically inhibit the translational machinery would be the ideal candidates for further investigations. These compounds are potential agents for therapy at low-drug concentrations. Since these candidate compounds will have significantly low-toxic effects on the host, such candidates will carry greater potential for anti-viral drugs.

Most conventional anti-viral strategies target a virus-specific protein, for example, nucleoside analogues and protease inhibitors. The most commonly used classes of anti-viral agents, both target gene products encoded by the viral pathogen. This strategy targets a host-cellular process rather than a viral gene, it minimizes the ability of viruses to evolve drug-resistant mutants.

Besides all the above advantages that contribute to making this approach an important one, thousands of candidate anti-viral candidates can be quickly screened for anti-viral activity. The yeast phenotype being used in the screen for anti-viral activity is easily detectable and many candidate anti-viral agents can be screened on a single plate. This makes it a low-recurring cost and efficient screen to quickly screen thousands of candidate anti-viral compounds.

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