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A fluorescent assay for the genetic dissection of the RNA polymerase II termination machinery

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Abstract

RNA polymerase II is a highly processive enzyme that synthesizes mRNAs and some non-protein coding RNAs. Termination of transcription, which entails release of the transcript and disengagement of the polymerase, requires an active process. In yeast, there are at least two multi-protein complexes needed for termination of transcription, depending upon which class of RNAs are being acted upon. In general, the two classes are relatively short non-coding RNAs (*e.g.* snoRNAs) and relatively long mRNAs, although there are exceptions. Here, a procedure is described in which defective termination can be detected in living cells, resulting in a method that allows strains with mutations in termination factors or *cis*-acting sequences, to be identified and recovered. The strategy employs a reporter plasmid with a galactose inducible promoter driving transcription of green fluorescent protein which yields highly fluorescent cells. When a test terminator is inserted between the promoter and the fluorescent protein reading frame, cells fail to fluoresce. Mutant strains that have lost termination capability, so called *terminator-override* mutants, gain expression of the fluorescent protein and can be collected by fluorescence activated cell sorting. The strategy is robust since acquisition of fluorescence is a positive trait that has a low probability of happening adventitiously. Live mutant cells can easily be cloned from the population of positive candidates. Flow sorting is a sensitive, high-throughput detection step capable of discovering spontaneous mutations in yeast with high fidelity.

Keywords

transcription termination; RNA polymerase II; fluorescence activated flow sorting; yeast genetic screen

1. Introduction

The process of terminating transcription has been studied for many DNA-dependent RNA polymerases across many eukaryotic organisms (reviewed in [1–4]). The mechanisms of termination by RNA polymerases I, II, and III are very different and turn out to be more complicated than would have been expected from the simple model first elucidated in

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prokaryotes with a single RNA polymerase and a relatively simple set of termination sequences [5]. The various transcripts these polymerases synthesize are handled by different protein machineries. RNA polymerase II itself turns out to have more than one termination mechanism, depending upon the destiny of the primary transcripts and how they will be post-transcriptionally modified [1][2]. Genetic approaches have been invaluable in identifying both the cis-acting sequences and *trans*-acting proteins involved. As a result, the use of model organisms with their facile molecular genetic features has led the way in this regard.

Most of the reported genetic screens and selections have employed the genetically tractable yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and the worm *Caenorhabditis elegans*; these studies have identified important components for terminating transcription by RNA polymerase II [6–16]. The majority of efforts have focused on the baker's yeast *S. cerevisiae* which is probably the organism in which RNA polymerase II's termination mechanisms are best understood [2, 17, 18].

This article describes a genetic screening method in *S. cerevisiae* in which cells contain a reporter plasmid with a model RNA polymerase II terminator that expresses green fluorescent protein (GFP) conditionally, depending upon the upstream transcription terminator's function. Since the assay asks for fluorescent mutant cells to emerge from a population of non-fluorescing cells, it is a strong positive screen that can be used in an unbiased manner with respect to candidate *trans*-acting genes that operate the terminator. Fluorescence-activated cell sorting (FACS) can be used to collect the positives with potentially very high throughput and therefore, is ideal for the detection of low frequency events. The basic method should be applicable to termination by other RNA polymerases, at a variety of termination sequences, and in other sortable cell types, microbial or mammalian. The identification of new alleles of genes known to be involved in termination, as well as altogether new genes involved in the process, should advance our understanding of the unusually complex process of terminating transcription.

2. Methods

2.1. Yeast strains and plasmids.

Yeast strains DY1513 (MAT α *his3* *1 leu2* *0 met15* *0 ura3* 0[pGal-GFP kan *URA3*]) and DY1514 (MAT α *his3* *1 leu2* *0 met15* *0 ura3* 0[pREFGFP kan *URA3*]) contain the plasmids pGAL-GFP and pREF-GFP, respectively, and have been described previously [15]. Strains were grown on SC *ura*[−] dropout solid medium [19] with either 2% (w/v) glucose or 2% (w/v) galactose, as described. For liquid growth, cells were inoculated into SC *ura*[−] with 2% raffinose to which 2% galactose was added for induction. All incubations were carried out at 30°C.

The plasmid pGAL-GFP kan was assembled by inserting a PCR product of the GFP reading frame amplified with primers 5'-tctagactgcagatggctagcaaaggagaagaact-3' and 5'-tgaatttcgagtttagcagccggtatcctttgtata-3' and digested with *Pst*I (blunted) and *Xho*I into the plasmid pYES2 cut with *Hind*III (blunted) and *Xho*I. The kanamycin resistance gene was excised from pFA6a-kanMX4 [20] with *Eco*RI and *Bam*HI (both blunted) and inserted into

this construct's ampicillin resistance gene's unique *Bgl*I site. This converts the plasmid's antibiotic resistance from ampicillin to kanamycin. The plasmid pREF-GFP was assembled by a three-way ligation of a PCR product encoding the yeast intergenic terminator found upstream of *IMD2* [21, 22] (*Hind*III-*Pst*I cut product made with primers 5'-ggggtagcaagcttttcgtattctattctattccttc-3' and 5'-cgctgcagaacaaatcgctttatgacagtt-3') into the plasmid pYES2 (Invitrogen; cut with *Hind*III and *Xho*I) along with a PCR product encoding the GFP open reading frame (*Pst*I-*Xho*I cut PCR product made with primers 5'-tctagactgcagatggctagcaaggagaagaact-3' and 5'-tgaattctcgagttagcagccggatcctttgtata-3'). The kanamycin resistance gene from pFA6a-kanMX4 (32) was excised by digestion with *Eco*RI and *Bam*HI, filled in with DNA polymerase, and inserted into the blunted *Bgl*I site of the ampicillin resistance gene in pREF-GFP to yield pREF-GFP kan.

2.2. Visualizing fluorescent colonies and cells.

Parental strain DY1514 was grown overnight at 30°C in SC ura⁻ with 2% galactose. Cells were resuspended to a density of 1.33 OD/ml in 1.5 mL and were sorted at 488 nm using a FACSaria II (Becton-Dickinson) with FACSDiva software. Sorted GFP-positive cells were plated onto SC ura⁻ plates incubated at 30°C and replica plated onto SC ura⁻ containing galactose. Fluorescent colonies were identified by illumination with a Dark Reader™ lamp (models SL7S, SL10S) which has a broad emission profile of 400-500 nm light and filter glasses which pass light >520 nm (Clare Chemical). Isolates were independently cultured and subjected to further analysis. Flow cytometry analysis employed the LSRII machine (Becton-Dickinson) with a 488 nm excitation wavelength and a long pass filter (>505nm) and a band pass filter for 515-545 nm light.

2.3 Whole genome sequencing

Genomic DNA was purified and subjected to high-throughput sequencing by FliSeq2000 (Illumina Inc.). In brief, the purified DNA was sheared to an average fragment size of 300 bp using acoustic focusing. The SPRI-works platform was used to create libraries, which were sequenced on an Illumina HiSeq2000 with a read length of 50 bases plus 7 bases for the multiplex tags. The resulting sequence was extracted and de-multiplexed using Illumina's SCS2.8 software. The data were then analyzed using the CLC Genomics Workbench package. Imported reads were trimmed to remove low quality sequence as well as any reads of <36 bases in length. Reads were mapped to the published yeast genome obtained from the Saccharomyces Genome Database, yielding an average coverage of 95× across the genome (it is likely that less coverage would suffice, but the lower limit has not been explored). Variations (SNPs and InDels) were called using a minimum Q score of 20 and a coverage minimum of 25X. This yielded over 500 SNPs and 350 InDels per strain. We focused on SNPs in protein-encoding sequences to look for differences between strains. Genomic DNA from the starting parental strain was run in parallel. This greatly reduced the many background incidental changes between lab strains seen versus the Saccharomyces Genome Database reference sequence. Variations that were common, were not in open reading frames, or were otherwise unlikely to be related to the phenotype were removed from consideration. All variations in the reduced subset were manually checked to ensure that the calls were accurate and each was independently re-sequenced by Sanger sequencing.

3. Setting up the reporter and yeast strain.

The basic premise of this method is to provide haploid yeast with a plasmid from which expression of a fluorescent protein is dependent upon readthrough of a transcriptional terminator located upstream of the fluorescent protein reading frame (Fig. 1) [15]. Readthrough can result from inactivating mutations of the DNA sequences of the terminator itself (*cis*-acting mutations), or loss of function mutations in *trans*-acting termination factors generated from other genes. Expression can be scored with a handheld benchtop illuminator and emissions filter (Fig. 1), or in a more sensitive, high-throughput manner using flow cytometry (Fig. 2).

In one version of this approach, a high copy 2 μ plasmid with the strong inducible *GAL1* promoter was employed to insure a high level of expression of GFP in order to maximize the sensitivity of detecting small levels of terminator readthrough [15]. The plasmid was modified for selection in bacteria or yeast by the addition of a kanamycin resistance gene and the *URA3* nutritional marker. Sequences encoding GFP were inserted into the polylinker downstream of the promoter, and when cells were grown on galactose, strongly fluorescent colonies were detectable by handheld illumination with an appropriate lamp (Fig. 1, left). When a test terminator was inserted between the promoter and GFP, expression of GFP was strongly quenched returning GFP expression to background (Fig. 1, right). Mutations in proteins needed to operate the terminator were expected to restore fluorescence to cells. The initial subject [15] was an intergenic terminator found between the *PHO12* and *IMD2* open reading frames. This terminator acts as an attenuator in the regulation of *IMD2* (an enzyme involved in guanine nucleotide metabolism) by GTP, and yields short non-coding RNAs referred to as cryptic unstable transcripts (CUTs) that originate downstream of *PHO12* and end at the terminator upstream of the *IMD2* reading frame [16, 22–24]

4. Flow sorting.

Plating candidate terminator override (abbreviated *tov*) strains to examine them for GFP expression by benchside illumination can be done for hundreds of cells. However, FACS can be used to screen 10^6 - 10^7 candidates with high sensitivity and the only limit to how many cells can be put through the machine is the time it takes to do so. Live fluorescent cells can be quickly selected in an automated fashion and clones picked by dilution and plating. Calibration of this assay using two populations of cells; one with a terminator-containing reporter plasmid and the other with a terminator-free reporter plasmid, shows a dynamic range of about four logs of GFP intensity on a per cell basis (Fig. 2). Yeast have a low level of intrinsic autofluorescence from endogenous fluorophores that gives a small signal at the detection wavelength used here [25], so even strains lacking GFP-coding plasmids give non-zero readings [15].

Here, $\sim 10^7$ cells of the parental strain were sorted into a gate with a cutoff of 10^3 units of GFP fluorescence intensity in a search for strains that contained spontaneous mutations affecting terminator function [Fig. 3]. A second round of post-sorting enrichment yielded approximately 5×10^3 candidates. A manageable population was obtained by plating these onto solid galactose-containing medium and visually screening this set for the strongest

GFP-positive colonies using handheld illumination. Given this frequency of spontaneous mutation, mutagenesis will not generally be necessary. If a rare event is being sought, very large numbers of cells could be examined with little difficulty. Plating is necessary to enable the picking of individual clones for further analysis such as re-running the cloned population on a cell analyzer to confirm stable, high level GFP expression. In an example shown here, compare the fluorescence of the *tov9* mutant population (blue line) vs. that of the starting wildtype strain (orange line; Fig. 4).

Identification of specific mutations can be readily achieved with whole genome sequencing. In this example, a single nucleotide deletion in a tract of five cytidines was found in the termination helicase *SEN1* that resulted in a frame-shift after proline²¹⁹⁰. Including this example, genomic sequencing of eight independent *tov* isolates identified six single base substitutions, an insertion, and a deletion [15]. These fell into five genes involved in termination: *NAB3*, *TRF4*, *SSU72*, *PCF11*, and *SEN1*. The mutation rate under these conditions was estimated to be at least 10^{-6} .

5. Validation & Troubleshooting.

There are a number of options for the validation that GFP positive isolates are defective in termination. An initial assay of a number of candidates can use northern blotting or reverse transcriptase-PCR to score for readthrough of the terminator into the GFP reading frame in the reporter plasmid. By probing or amplifying the GFP mRNA, a group of possible candidates can be examined without the need to probe individual candidates for specific endogenous transcripts, the termination or stability of which may vary from mutant strain to mutant strain. Subsequently, isolates can be examined for readthrough of a specific panel of endogenous genes to assess the generality of the mutation's effect. Whole transcriptome analysis would be another valuable assessment when the investigator settles on a set of mutants of interest.

Since it is possible for mutations to accumulate in the terminator sequence itself, strains should be cured of the reporter plasmid and retransformed with fresh reporter plasmid to insure the defect is chromosomally inherited.

Once there is confidence that a candidate mutation has been identified, a wildtype version of the gene can be introduced into the strain that also harbors the reporter plasmid to restore termination and reverse expression of GFP. This step helps confirm that the mutated gene is causally involved. In the example shown here, *SEN1* rescues *tov9* (red line; Fig. 4). While only a recessive mutation would be rescued this way, this has been the only type of mutation identified with this approach thus far [15]. Introducing the newly obtained mutant version of the candidate gene can provide evidence that the candidate mutation is causal since it should not be effective in reversing GFP expression if the mutation is recessive, but could do so if the mutation is dominant. This complementation approach should be accompanied by a correction in the profile of any endogenous transcripts' termination defects that may have been observed. Causality of a candidate mutation can be further confirmed by re-introducing the change revealed by genomic sequencing into the chromosome of a wildtype starter strain.

One complication of this method is the side effect of plasmid loss. Flow cytometry is an excellent way to measure the occurrence of plasmid loss [26, 27]. Frequencies of 10^{-2} for *CEN*-based plasmids have been detected [26]. This can manifest as the accumulation of up to 20% of the cells in a culture that have lost the plasmid [27]. As a result, a reporter-containing, GFP-positive culture can yield a small background of negative cells with a bimodal flow cytometry profile characteristic of a mixed population (blue line, note small peak at 10^2 , Fig. 4). Similarly, providing a strain with a plasmid that contains a wildtype gene to rescue a recessive mutation, may not completely extinguish a termination defect when a subset of cells lose the rescuing plasmid (red line, shoulder at 10^3 - 10^4 , Fig. 4). Thus, plasmid loss is a source of population heterogeneity. This complication may be outweighed by the ease of introduction of plasmids into yeast *versus* the alternative, integration of the reporter into the chromosome. An advantage of integration of the reporter is an improvement in the penetrance and consistency of GFP expression across the population since all cells contain only one copy, although there is a reduction of the level of fluorescent protein on a per cell basis, likely due to the higher copy number of plasmids [15].

The sensitivity of this method is due in part to the ability of cells to accumulate a high level of stable GFP over time. Thus, even a low level of terminator override can lead to a strong signal after hours of galactose induction. A corollary of this feature is that once the ceiling of fluorescence is achieved, there will be a loss of responsiveness to continued readthrough. Overall, the dynamic range between the peaks of the 'on' and 'off' populations is two to three logs of fluorescence intensity (Fig. 4).

Using a galactose-inducible promoter enables a high level of expression in a controllable burst. However, some transcription factor or elongation mutants can be defective in galactose-induction of the *GAL1* promoter, and these will be lost. A reporter with a strong constitutive promoter could provide an alternative in this regard. The strength of the terminator employed is probably also an important factor; an efficient terminator will keep the background of GFP low and yield high signal to noise ratios when comparing a mutant to its parental strain.

6. Concluding Remarks and Potential Expanded Uses.

A schematic of the workflow from starting strain to *toV* mutants is presented in Fig. 5. By exploiting a fluorescent reporter and FACS, it has proven rapid and easy to carry out an unbiased screen for yeast termination mutants. Readthrough of a potent terminator driven from an inducible promoter can be easily detected. Analysis of a large number of cells means mutagenesis is not necessary and random changes can be found in good numbers. Using this approach for the intergenic *IMD2* terminator/attenuator, loss of function mutants were identified with no apparent false positives [15]. Whole genome sequencing was an efficient way to identify candidate genetic changes. The facile molecular biology of yeast allowed the quick and efficient confirmation of candidates and re-creation of the mutations in a fresh genetic background to demonstrate causality, without the need to perform genetic crosses and segregation tests. With appropriate reporter plasmids, this approach can easily be applied to other RNA polymerase II terminators or to terminators driven by other RNA polymerases. With some ingenuity, a similar high throughput screening approach using

fluorescent reporter proteins combined with the power of FACS could also be adapted for genetic studies of the translation process.

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Abbreviations

FACS	fluorescence activated cell sorting
CUTS	cryptic unstable transcripts
GFP	green fluorescent protein

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Highlights

- Transcription termination by the processive RNA polymerase II enzyme uses specific proteins.
- Mutation of termination factors and *cis* sequences can lead to readthrough of termination signals.
- Yeast are given a reporter plasmid with a fluorescent protein downstream of a test terminator.
- Sorting fluorescent cells provides a strong positive selection for terminator-override mutants.
- Spontaneous mutants can be found and validated quickly and efficiently.

Figure 1.

Yeast strains DY1513 (left) and DY1514 (right), containing a plasmid with the *GAL1* promoter upstream of GFP (pGAL-GFP kan), or a plasmid with a terminator between the promoter and GFP (pREF-GFP kan), respectively [15], were grown at 30°C on SC ura⁻ selective medium with 2% galactose (w/v) in place of glucose. Plates were photographed under white light (top) or using a Dark Reader™ illumination lamp and filter to image GFP (bottom).

Figure 2.

Flow cytometric detection of GFP expression differences between strains DY1513 (GAL-GFP) and DY1514 (GAL-STOP-GFP), which have plasmids lacking or containing a terminator between the promoter and GFP. The y-axis is a measure of forward scatter which is generally proportional to yeast cell size. The x-axis is GFP fluorescence intensity units.

Figure 3.

FACS analysis of a yeast strain (DY1514; *from Loya et al. 2012*) from the first round of screening showing a sample of the pre-sorted population (red; 10⁴ cells) and a post-sorted sample (blue; 150 cells) of GFP positive candidates. The y-axis is a measure of forward scatter which is generally proportional to yeast cell size. The x-axis is GFP fluorescence intensity units.

Figure 4. Histogram representation of flow cytometric analysis of three yeast strains: 1) the parent wildtype strain (DY1514; orange) that bears the plasmid containing a terminator between the *GAL1* promoter and GFP (pREF-GFP kan), 2) a terminator override (*tov9*) mutant derivative selected from DY1514 and cloned from the GFP positive population which contains a mutation in *SEN1* (blue), and 3) the *tov9* mutant strain complemented with a plasmid containing wildtype *SEN1* (red).

Figure 5.
Workflow for fluorescent screen of recessive termination override mutants.