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A dual-color reporter assay of cohesin-mediated gene regulation in budding yeast meiosis

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Summary

In this chapter, we describe a quantitative fluorescence-based assay of gene expression using the ratio of the reporter green fluorescence protein (GFP) to the internal red fluorescence protein (RFP) control. With this dual-color heterologous reporter assay, we have revealed cohesin-regulated genes and discovered a cis-acting DNA element, the Ty1-LTR, which interacts with cohesin and regulates gene expression during yeast meiosis. The method described here provides an effective cytological approach for quantitative analysis of global gene expression in budding yeast meiosis.

Keywords

fluorescence-based reporter assay; cohesin; Ty1-LTR; green fluorescence protein; red fluorescence protein; meiosis

1. Introduction

Regulation of gene expression in eukaryotic cells is achieved at different levels, including transcription, translation, and post-translational control. To determine the level of transcription, traditional assays include RT-PCR, Northern blot, microarray and RNA sequencing. To determine the level of protein production, Western blot and reporter protein assays can be used. However, these methods are either time-consuming or complicated in generating the internal controls. We describe here a dual-color fluorescence-based reporter assay for quantitatively analyzing the level of gene expression in yeast meiosis on the basis of protein production. Our method is similar to the *lacZ*-based reporter assay of gene expression in budding yeast (1), but we have incorporated an internal control, which allows us to determine the ratio of the reporter GFP to the level of an internal RFP control. This fluorescence-based approach is suitable for multi-well plates; therefore a large number of samples can be processed simultaneously for quantitative analysis of gene expression in yeast meiosis.

Emerging evidence suggests that cohesin regulates gene expression likely through mediating long-range chromatin interactions at the regulatory DNA elements, including promoters and enhancers (2, 3). Cohesin-mediated gene regulation appears to be conserved from lower to

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higher eukaryotes (4). In budding yeast, cohesin and its loader Scc2/Scc4 complex modulate the expression of *REC8*, which encodes a subunit of the meiotic cohesin complex (5). Mutations in NIBPL, the human homolog of yeast Scc2, and cohesin are linked to a developmental disorder called Cornelia de Lange Syndrome (CdLS) (6). Cells from CdLS patients have no obvious defects in sister-chromatid cohesion but display altered transcription profiles, further indicating cohesin's role in gene regulation (7).

We have identified a cis-acting DNA element, the Ty1-LTR (also called δ LTR), of which the expression is subject to cohesin regulation in budding yeast meiosis. Ty elements are retrotransposons and contain long-terminal repeats (LTR). One of them, Ty1, is composed of two ~330-bp LTRs at its ends and a ~5.3-kb central core domain (8). Solo Ty1-LTRs, arising from recombination of the intact element, are present in more than 100 copies in a typical yeast cell (8). Environmental stresses, including adenine starvation, can activate Ty1 transcription (9). Budding yeast meiosis is induced upon nitrogen depletion, and we have revealed that in the presence of Ty1-LTR, the expression level of the heterologous reporter increases in a cohesin-dependent manner during yeast meiosis (5). We therefore propose that cohesin interacts with Ty1-LTR to regulate downstream gene expression.

2. Materials

We used diploid cells from the budding yeast SK1 genetic background for meiotic analysis of cohesin-regulated gene expression. For global analysis of reporter gene activation, we used the yeast deletion collection library (ATCC, catalog#GSA-4), which is derived from the S288C genetic background. We followed the methods described by Guthrie and Fink, *Methods in Enzymology*, volume 194 (10) for yeast medium preparation and routine laboratory manipulations of yeast cells.

2.1. Yeast growth medium

1. YPD: 1% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) dextrose.
2. YPA: 1% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) potassium acetate.
3. YPG: 1% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glycerol.
4. Sporulation medium for the SK1 genetic background: 2% (w/v) potassium acetate (KOAc).
5. Sporulation medium for the S288C genetic background: 0.1% (w/v) yeast extract, 0.05% (w/v) glucose, 1% (w/v) potassium acetate.

2.2. Reagents

1. Chemicals: lithium acetate (LiAc), polyethylene glycol (PEG, MW= 2,000), salmon sperm DNA, formaldehyde, and amino acids. These chemicals are purchased from Sigma-Aldrich.
2. Restriction enzymes, including AflII, BamHI, BglII, SalI, and StuI are purchased from New England BioLabs Inc.

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2.3. Equipment and supplies

1. Typhoon PhosphorImager FLA 7000 (GE Healthcare Life Sciences, Piscataway, NJ).
2. Fluorescence microscope, conventional.
3. Clear bottom black 96-well plates (Greiner Bio One, catalog# 655900).

3. Methods

The following procedures were carried out at room temperature unless otherwise specified. Yeast cells were incubated at 30°C.

3.1. Heterologous GFP and RFP reporter constructs

1. Constructing the heterologous P_{REC8}-*GFP* plasmid (pHG88): We cloned a 2158-bp fragment in front of the *REC8* open reading frame, and fused it in frame with the *GFP* open reading frame to generate P_{REC8}-*GFP*. The *GFP* open reading frame is followed by a 283-bp 3' UTR from the *SMC1* gene. The backbone of this plasmid was derived from pRS305, which harbors the *LEU2* selectable marker in yeast.
2. Constructing the heterologous *Ty1-LTR*-P_{DMC1}-*GFP* plasmid (pHG209): We first constructed P_{DMC1}-*GFP* plasmid pHG112, using a 1-kb fragment in front of the *DMC1* gene as its promoter, then fused it with the *GFP* open reading frame. The backbone of pHG112 was also derived from pRS305. By replacing the *GFP* sequence with that of *RFP*, we generated the P_{DMC1}-*RFP* plasmid pHG140, which harbors the *URA3* selectable marker in yeast and serves as an internal control for P_{DMC1}-*GFP*. Next, to construct *Ty1-LTR*- P_{DMC1}-*GFP*, we cloned a ~500-bp fragment of the Ty1-LTR in front of the *HIM1* locus, and inserted it into the SalI and BglII sites of pHG112 to generate pHG209.

3.2. Yeast transformation

We followed the high efficiency yeast transformation protocol developed by Gietz and Schiestl (11) to integrate P_{REC8}-*GFP* and *Ty1-LTR*-P_{DMC1}-*GFP* at the *LEU2* locus, and P_{DMC1}-*RFP* at the *URA3* locus by homologous recombination. Briefly, yeast cells are grown to the exponential phase, harvested, and heat shocked with LiAc/PEG at 42°C.

3.3. Induction of synchronous meiosis

To achieve synchronous meiosis, we modified a yeast culture procedure from Cha et al., (12), as detailed below. Because yeast strains from the SK1 background are prone to becoming petites, we patched yeast cells on the YPG plate for 2 days to remove petites, and then replicated these cells to the YPD plate before inoculation on Day 1.

1. Day 1: Inoculate a 5 mL YPD liquid culture and incubate yeast cells at 30°C on a rolling wheel overnight.

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2. Day 2: Set up a 25 mL YPA culture in preparation for synchronous meiosis. About 0.25 mL YPD culture is diluted 10 fold to 25 mL YPA in a 125 mL baffled flask, for an optical density (O.D.) around 0.2 at λ_{600}. Yeast cells are vigorously shaken at 30°C ~16 hours in a water bath. One can set up the YPA cultures around 5:00 p.m. on the first day and yeast cells will be ready to be transferred to the sporulation medium by morning of the second day.

3. Day 3: Yeast cultures should have reached O.D. (λ_{600}) > 1.6 before moving forward with the following procedures.

   a. Collect yeast cells by centrifugation. Transfer cells from flask to a 50 mL conical tube, and centrifuge in a Beckman swing-buck centrifuge at 5 min × 2,000 rpm.

   b. Pour off the YPA medium gently. Resuspend cells with 25 mL autoclaved H_2O. Centrifuge as in step (a).

   c. Pour off water, resuspend cells in 25 mL 2% KOAc, and transfer them to a clean flask.

   d. Withdraw 1 mL yeast cells, and fix them with 1% (final concentration) formaldehyde at room temperature for 1 hour. This is the time zero (t=0) sample.

   e. Put the flask containing the remaining suspended cells in the shaker.

   f. Wash fixed samples once with PBS, then store in PBS at 4°C.

   g. Collect yeast samples at designed time points. For our analysis of cohesin's role in gene expression, we collected samples every 2 hours for a total of 12 hours.

3.4. Microscopy-based fluorescence detection

To determine GFP and RFP intensity in individual yeast cells, we performed fluorescence microscopy (Olympus, IX-71) with a 60× (NA=1.42) objective lens, using a microscope fitted with GFP and mCherry live cell filter sets (GFP: Excitation 470, Emission, 525; RFP: Excitation 572, Emission, 632, Chroma Technology Corp.). A cooled CCD camera (Photometrics, CoolSnap HQ2) was used to acquire fluorescence images. For quantitative evaluation of GFP production as discussed below in section 3.6, we usually acquired images of more than 200 yeast cells from each time point. Pixel intensity was set within the range of 200 to 3,000 counts to avoid saturation.

3.5. Scanner-based fluorescence detection

Complimentary to the microscopy assay described above, the scanner-based assay allows simultaneous analysis of a large population of yeast cells and multiple samples. We have access to a Typhoon PhosphorImager scanner and used clear-bottom 96-well black plates to collect yeast cells, according to the following procedure

1. Perform a 1:2 serial dilution of fixed samples in a 96-well plate. We typically diluted 4 times to reach 1:16 and loaded 100 μL samples in each well.

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2. Clean the surface and bottom of the plate with 70% alcohol.
3. Open the Typhoon scanner and set it to "Acquisition: Fluorescence."
4. Set up parameters: Pixel size at 200 μm and focal plane at +3 mm.

   For RFP: 580 BP 30 Cy3 (red channel); PMT: 600; laser: green (wavelength 532nm); sensitivity: normal.

   For GFP: 526 SP Fluorescein Cy2 (green channel); PMT: 600; laser: blue (wavelength 488nm); sensitivity: normal.

   Optimal PMT (photomultiplier tube) settings depend on the expression levels of RFP and GFP and may vary.
5. Scan. We always scanned the red channel first, because RFP is more sensitive to photobleaching.
6. Save and export acquired data for further analysis.

3.6. Quantification of fluorescence intensity

We used the IPLab measurement tool for quantitative analysis of fluorescence intensity. A script was developed in-house to expedite the measurement of microscopy-acquired GFP and RFP intensity from individual cells, (Section 3.4) and fluorescence scan-acquired population of cells (Section 3.5).

1. A circular region of interest is defined using the measurement tool.
2. The net fluorescence intensity is extracted by removing the background fluorescence.
3. The ratio of GFP/RFP is calculated and plotted as shown in Figure 1.
4. Other measurement tools, for example Image J, can also be used for fluorescence intensity quantification.

4. Notes

4.1. Fluorescence microscopy vs. fluorescence scanner

Fluorescence microscopy allows one to investigate individual yeast cells, and provides a resolution high enough to localize proteins of interest to subcellular compartments. On the other hand, fluorescence scanners, such as the Typhoon, are suitable for the analysis of large populations of cells; they can scan multiple samples simultaneously, and therefore can more easily be adjusted for automation in sample handling. With proper built-in controls, both methods are suitable for quantitative analysis of gene expression in meiosis.

4.2. Considerations for sporulation of yeast cells from the S288C background

We used 2% KOAc to induce yeast cells from the SK1 background to undergo meiosis at a very high efficiency (~90%). But the sporulation efficiency of yeast cells from the S288C background is notoriously poor compared to that of cells from the SK1 background. The sporulation medium we adapted improved the sporulation efficiency of S288C cells to

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~40%. This enhanced efficiency of sporulation is crucial for genetic screens that are based on meiotic gene expression using the deletion collection library, which is from the S288C background. To evaluate yeast sporulation efficiency, we used phase contrast microscopy to monitor dyad and tetrad formation. Yeast cells with the S288C background took 3–4 days at 30°C to achieve the highest efficiency of sporulation.

4.3. Confirmation of the level of GFP expression by Western blot

In addition to fluorescence-based evaluation of GFP production, Western blot provides a complementary tool for determining the level and stability of GFP produced during yeast meiosis. We used anti-GFP monoclonal antibody (Clontech, catalog# 632375) for Western blots.

4.4. Ty1-LTR in meiotic gene regulation

Transcription of Ty1-LTR regulates adjacent gene expression (9). We have found that noncoding RNAs are transcribed at the Ty1-LTR located at the *HIM1* locus in meiotic yeast cells (our unpublished data), and Ty1-LTR enhances the expression level of the P_{DMC1}-*GFP* heterologous reporter in a cohesin-dependent manner (Figure 1). Combined with a strong meiotic promoter, Ty1-LTR-mediated transcriptional activation is desirable for manipulating meiotic gene expression (13). For example, we have used *Ty1-LTR-*P_{DMC1} to overexpress genes of interest more than 10 fold compared to their endogenous expression level in yeast meiosis (5, 13).

4.5. Cohesin mutants

Using a promoter replacement approach (14), we have generated P_{CLB2}-*SMC1*, P_{CLB2}-*SMC3*, and P_{CLB2}-*SCC2* mutant alleles to deplete the cohesin subunit and its loading factor in budding yeast meiosis. On the basis of Western blot results, we have confirmed the efficiency of these conditional alleles (5, 15).

4.6. Use of the 96-well plate format for genetic screens

We used the 96-well plate format to screen mutants from the budding yeast deletion collection (ATCC, catalog#GSA-4) that regulate the expression of the heterologous reporter gene in meiotic cells. Our screening followed a 12-day cycle:

1. Day 1: Query yeast strain (P_{REC8}-*GFP::LEU2*, P_{STE5}-*URA3*, P_{DMC1}-*RFP::HIS5*) is mated to library strains in 96-well plates.
2. Day 2: Diploid yeast cells are selected with the SC-Leucine + G418 medium
3. Day 3: Yeast cells are sporulated in 0.1% yeast extract, 0.05% glucose and 1% potassium acetate. Sporulation takes about 4 days at 30°C.
4. Days 4–6: Haploids are selected on solid medium that is prepared in single-well plates (SC-Leucine + SC-Uracil + G418). The P_{STE5}-*URA3* construct has been reported previously by the Amon group (16). Plates are incubated at 30°C for 3 days.
5. Day 7: Haploids are transferred to the SC-Leucine + G418 medium to mate.

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6. Days 8–11: Homozygous diploids are selected in the 5-Fluoroorotic Acid medium for three days.
7. Day 12: Sporulation is carried out and fluorescence examined as described in Section 3.5.

Black plates with clear wells reduce the background fluorescence and minimize potential fluorescence from adjacent wells. Dual-color reporter-based screens can also be carried out on solid plates as reported previously (17).

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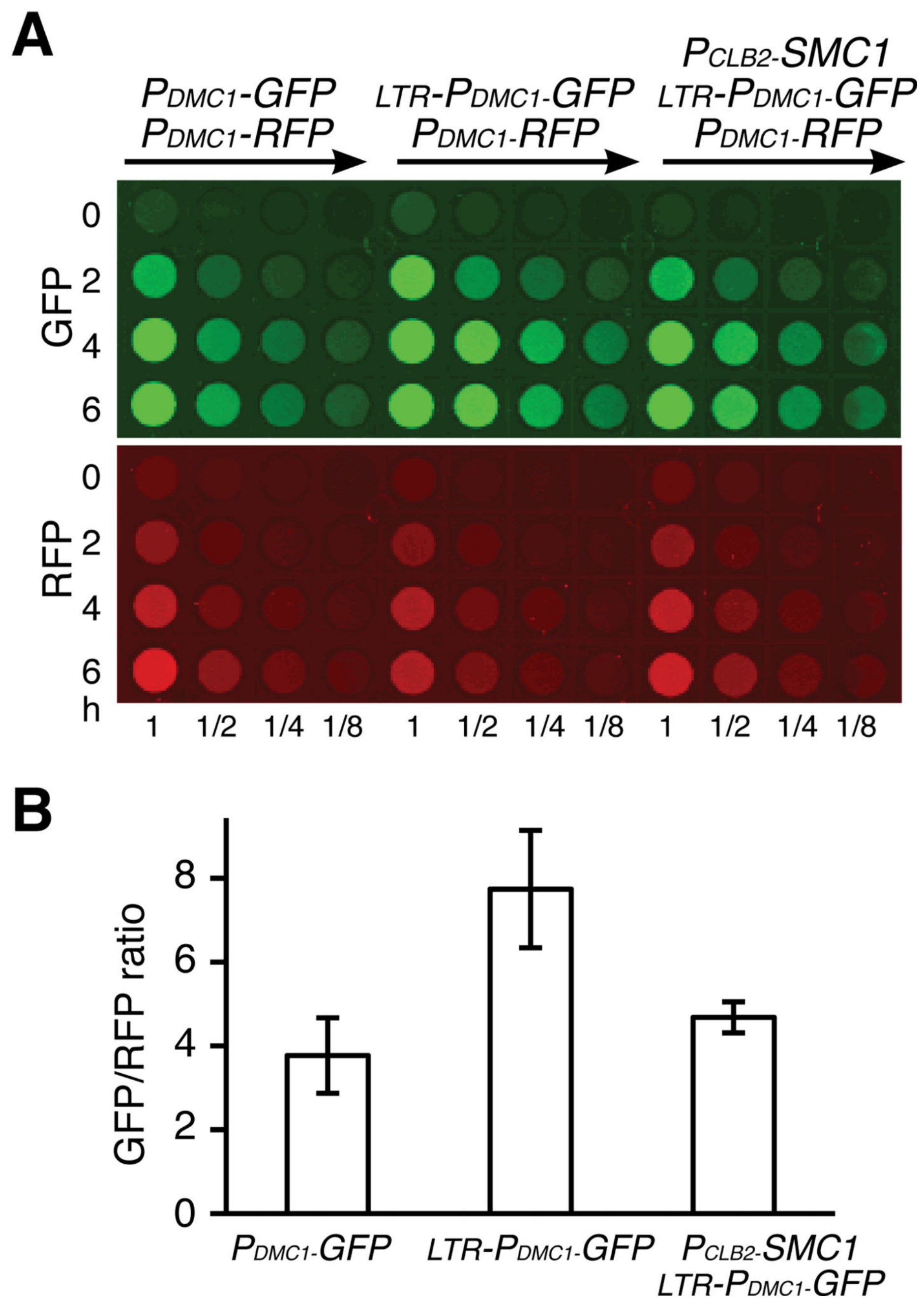

Figure 1.

Enhancement of the *DMC1* promoter activity by Ty1-LTR and cohesin during yeast meiosis. (**A**) Heterologous reporter assay of GFP and RFP production. Cells were collected at indicated time after induction of yeast meiosis, diluted, and scanned for fluorescence intensity. The P_{CLB2}-*SMC1* allele depletes Smc1p specifically in meiotic cells. (**B**) Quantitative measurement of GFP and RFP output in representative strains as shown in A. Error bars represent standard deviation.