
Identification of Host Factors Involved in 2 μ m Plasmid Maintenance in Yeast

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Abstract

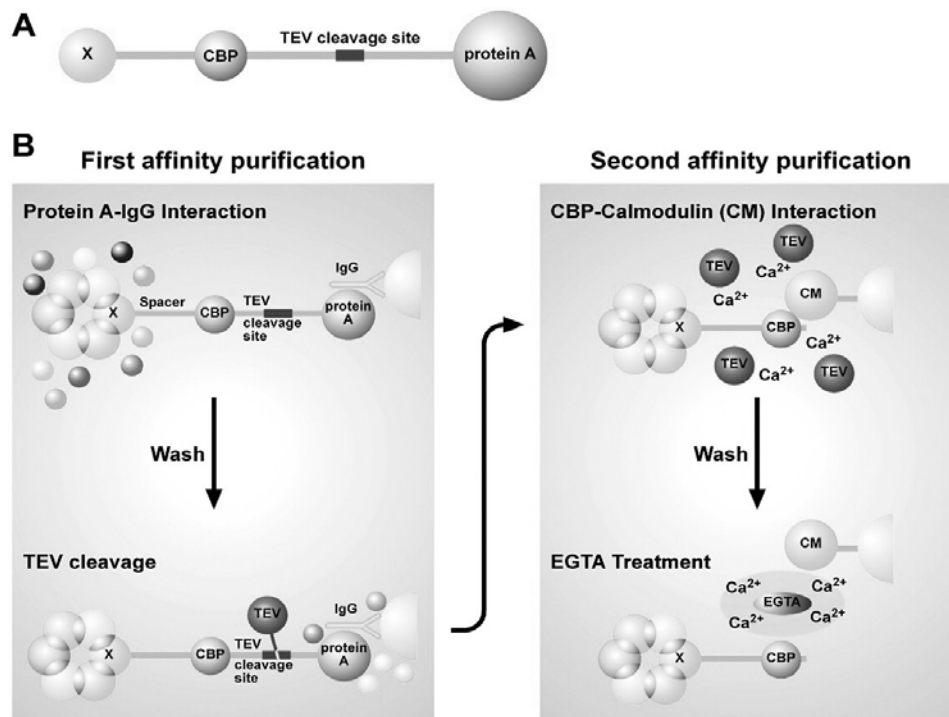
The yeast 2 μ m plasmid, a selfish genetic element, is a simple genome (4 genes) that is nonetheless able to maintain a stable copy number. By purifying complexes containing the 2 μ m- encoded proteins, we hope to learn which host proteins have been hijacked to facilitate plasmid partitioning. Our approach is to purify Tandem Affinity Purification (TAP)-tagged proteins through two independent affinity steps on IgG- and calmodulin-containing resins and to identify the 2 μ m plasmid associated proteins by mass spectrometry. We can then test whether purified proteins are important for segregation using genetic and biochemical methods such as partitioning in deletion strains, co-immunoprecipitation, and GST- pull-down assays. Using this unbiased approach, we can develop a testable model describing host proteins involved in segregation.

Identification of Host Factors Involved in 2 μ Plasmid Maintenance in Yeast

Introduction

Saccharomyces cerevisiae, more commonly known as baker's yeast, is identified today as the ideal eukaryotic microorganism for genetic and biochemical research. It has been especially useful for genetic studies because of the existence of both diploid and haploid cells, the ability to be cloned, rapid growth, mutant isolation, and more. The 2 μ plasmid of *Saccharomyces cerevisiae* is a simple genome that reflects much of the same characteristics of a chromosome; the only difference is that the 2 μ plasmid has the ability to maintain a controlled, high copy number and the ability to separate daughter cells uniformly during mitosis and meiosis without a centromere. The proteins of the 2 μ plasmid consist of FLP, REP1, REP2, and RAF1. FLP is the recombination enzyme, and REP1 and REP2 are vital components to segregation. However, the function of RAF1 is still unknown.

We are interested in learning the mechanism by which the 2 μ plasmid can efficiently segregate during cell division in the absence of a centromere. We believe that since it encodes only 4 proteins, it may hijack cellular proteins to achieve its purpose. To identify these putative host proteins, we are using the tandem affinity tagging method. One technique of purification is tandem affinity purification (TAP) which efficiently separates epitope-tagged protein complexes from crude extracts as seen in diagram B. The TAP tag consists of the calmodulin-binding peptide (CBP) and two immunoglobulin G (IgG)-binding domains of the *Staphylococcus aureus* protein A as seen in diagram A.



The epitope types are separated by a spacer region and a cleavage site for tobacco etch virus (TEV) protease. TAP is attained by binding the tagged protein to the IgG column, releasing the protein by TEV protease cleavage, binding the CBP-tagged protein to a calmodulin column, and eluting the bound protein by a buffer lacking calcium, which prevents the CBP/calmodulin interaction. The two advantages of the TAP techniques include its yield and its nondenaturing conditions. The tagging method includes PCR followed by yeast transformation. The PCR can obtain the DNA segment of interest, and then it can be introduced in the yeast by transformation.

To detect protein in a given sample, the technique Western blot (Immunoblot) can be utilized. Western blot uses gel electrophoresis to separate denatured proteins by mass. Then the proteins are transferred to a membrane (usually nitrocellulose) where they are probed using antibodies specific to that protein. Thus, the amount of protein in a given sample can be determined and compared to other groups. In order for the protein to be visible, the technique silver staining is sometimes applied to confirm that similar amounts of total protein were loaded. In order to ascertain which host proteins may be important for plasmid partitioning, the complexes containing 2 μ proteins must be purified. The purified proteins can later be tested whether they are vital for segregation using the methods like partitioning in deletion strains, co-immunoprecipitation, and GST- pull-down assays.

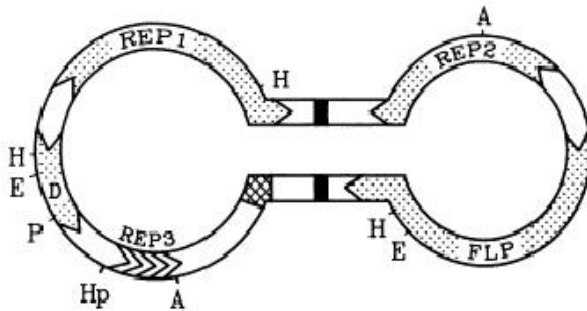


Diagram 1: 2 μ Plasmid

Procedure

2 μ Miniprep:

The cells were grown overnight at 30°C in 5 mL of YPD, collected in a centrifuge at 2000 rpm for 5 minutes, and the supernatant was then discarded. Then the cells were resuspended in .5 mL of 1 M sorbitol and 1 M Na₂EDTA (pH 7.5). Next, the cells were transferred to a 1.5 mL microcentrifuge tube. Zymolyase 100T (.02 mL of a 2.5 mg/mL) was added, incubated for 1 hour at 37°C, and centrifuged in a microfuge for 1 minute. Then with the QIAGEN kit, the bacterial cells were resuspended in 250 μ L of Buffer P1 and transferred to a microcentrifuge tube. Buffer P2 (250 μ L) was added and mixed thoroughly by inverting the tube 4-6 times. Then 350 μ L of Buffer N3 was added and mixed again by inverting the tube 4-6 times. The cells were centrifuged for 10 minutes at 13,000 rpm in a table-top microcentrifuge. Next, the supernatant was collected and

pipetted into the QIAprep spin column. The flow through was once again centrifuged for 30-60 seconds, and the supernatant was discarded. By adding .5 mL of Buffer PB, the QIAprep spin column was washed. Then the spin column was centrifuged for 30-60 seconds, and the flow through was disposed. The QIAprep spin column was rinsed again by adding .75 mL of Buffer PE and was spun down for 30-50 seconds. Immediately, the flow-through was discarded, and the tube was centrifuged again to remove the residual wash buffer. Finally, the QIAprep column was placed in a clean 1.5 mL microcentrifuge tube. To elute DNA, 50 μ L of Buffer EB was added (10 mM Tris-cl, pH 8.5) to the center of each QIAprep spin column. Then it stood at room temperature for one minute and was centrifuged for an additional minute.

PCR Confirmation:

The cells were resuspended in 30 μ L of .2% SDS, vortexed for 15 seconds, heated at 95°C for 4 minutes, and spun down in a centrifuge for 1 minute. Then a master mix was created as shown in the table below.

Master Mix for Rep 1 or *Rep 2	1x (μL)
10x Pfx buffer	5
10x Enhancer	5
10mM dNTP's	1.5
MgSO ₄	1
SG98	1
SG91 or * SG93	1
ddH ₂ O	32.5
Pfx	1

Table 1: Master Mix for PCR

24.5 μ L of master mix and .5 μ L of sample were then placed into the PCR tubes. The samples were then loaded into thermal cycler for about 2.5 hours, where the temperature cycled from 94⁰C for 30 seconds, 52⁰C for 30 seconds and 68⁰C for 2.5 minutes for 35 cycles. 5 μ L of loading dye containing glycerol and bromophenol blue was added to each sample for visibility. All the samples and 7 μ L of 500 base pair ladder were loaded onto a 1.2% agarose gel for electrophoresis. After completion, it was stained with ethidium bromide and then photographed with UV light.

Yeast Transformation:

Overnight cultures were subcultured in 10 mL of YPD at 30°C for 6 hours. The cells were then spun down and washed with sterile ddH₂O. Each pellet was then resuspended in 200 μ L of ddH₂O, and 100 μ L was transferred into Eppendorf tubes. The tubes were then centrifuged, and the supernatant was subsequently removed. For the (-) DNA control, nothing was added. But for the rest, 10 μ L of plasmid was then added. For each tube 240 μ L of 50% PEG (polyethyleneglycol), 36 μ L of 1 M lithium acetate, 10 μ L of 2 mg/mL ss DNA, and 42 μ L of ddH₂O were added. All the tubes were then vortexed and incubated at 30°C for 30 minutes, 42°C for 20 minutes, on ice for 5 minutes. The cells

were spun down and resuspended in 100 μ L of ddH₂O. All were then plated on the appropriate selective plates at 30°C for about 3 days.

Protein Extraction:

3 mL of yeast cells were grown overnight in an appropriate medium. Then 1 mL of culture was pipetted into a sterile, labeled microfuge tube and chilled on ice for 10 minutes. Next, 150 μ L ice cold Solution A was added (2N NaOH + 8% 2-ME) and mixed by inverting the tube several times. Then it incubated on ice for 10 minutes. Later, 150 μ L of ice cold Solution B (50% TCA) was added, mixed by inverting tube several times, and incubated on ice for 10 minutes. Subsequently, the tube spun for 2 minutes in microfuge, and the pellet was washed with 1 mL of ice cold acetone, spun for 2 minutes, and the supernatant was aspirated. Afterwards, the pellet was dried at room temperature for 5 minutes. Next, the pellet was twice resuspended in 100 μ L sample buffer. Finally, it was heated at 95°C for 5 minutes, and 10 μ L were loaded on the protein gel.

Western Blot (Immunoblot)

The proteins were transferred to nitrocellulose membranes by electroblotting at 100V for 1 hour. Then the gel was carefully separated from the membrane. Next, the membrane was placed in about 30 mL of BLOTTO (TBST + 5 % Dry milk), and then it was shaken for 1 hour. The liquid was poured off and washed twice for 3 minutes in 40 mL of 1 X TBST. Meanwhile, 40 mL antibody solution (40 mL BLOTTO and 10 μ L antibody) was mixed. After pouring off the last wash, the antibody mix was added and then shaken for 1 hour. Then the mixture was poured off and was washed 3 times for 15 minute durations in 1 X TBST. Afterwards, chemiluminescent detection was performed, and the TAP tagged construct should be approximately 20 kD larger than the calculated MW of the protein (total MW approx 65 kD for Rep1 and 60 kD for Rep2).

Silver Stain

The gel was first washed in fixative (40% methanol/10% acetic acid) for a minimum of 30 minutes. Then it was washed in oxidizer (100 mL 1:10 dilution stock) for 5 minutes. The gel then had several water washes, specifically, five 1 minute washes and two 5 minute washes. After the water washes, the silver reagent (100 mL of 1:10 dilution stock) was poured and washed for 20 minutes. The gel then had a quick water rise for a maximum of 30 seconds, and then it was washed with the developer for another 30 seconds. The silver staining was then stopped by washing the gel with 5 % acetic acid for 15 minutes.

Results

Figure 1: PCR of Rep 1 and Rep 2

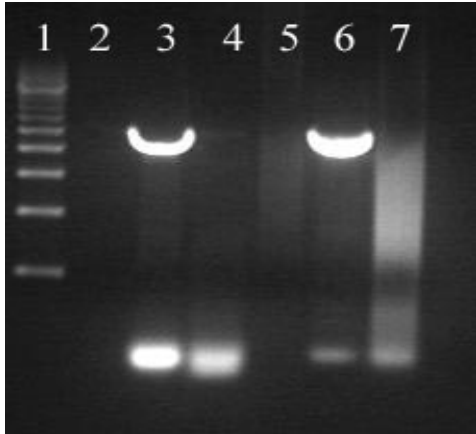


Figure 1 Legend:

Lanes	Strains
1	500 base pair ladder
2	(-) DNA
3	Rep 1#1
4	Rep 1#2
5	Rep 2#1
6	Rep 2#2
7	Parent Strain

Figure 2: Western Blot

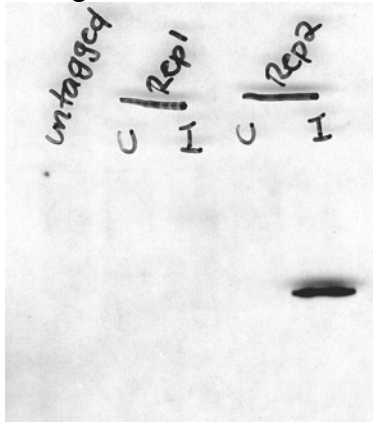


Figure 2 Legend:

Lanes	Strains	Name	Description
1	Wild Type Untagged	Untagged	Cir+ strains grown in sRaff (u)
2	Rep 1 Uninducing		
3	Rep 1 Inducing	U	Rep1 or Rep2 TAP tagged strain grown in sRaff
4	Rep 2 Uninducing	I	Tagged strains grown in YPRG overnight
5	Rep 2 Inducing		

Figure 3: Silver Stain

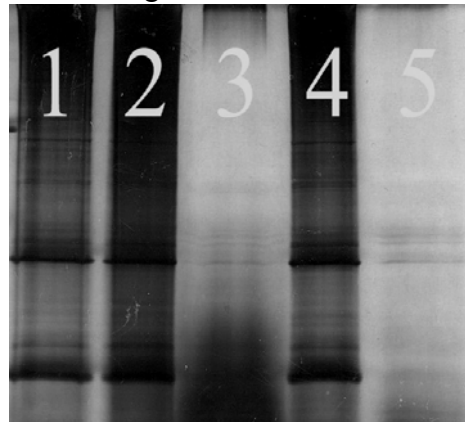


Figure 3 Legend:

Figure 4: Protein Molecular Weight Curve

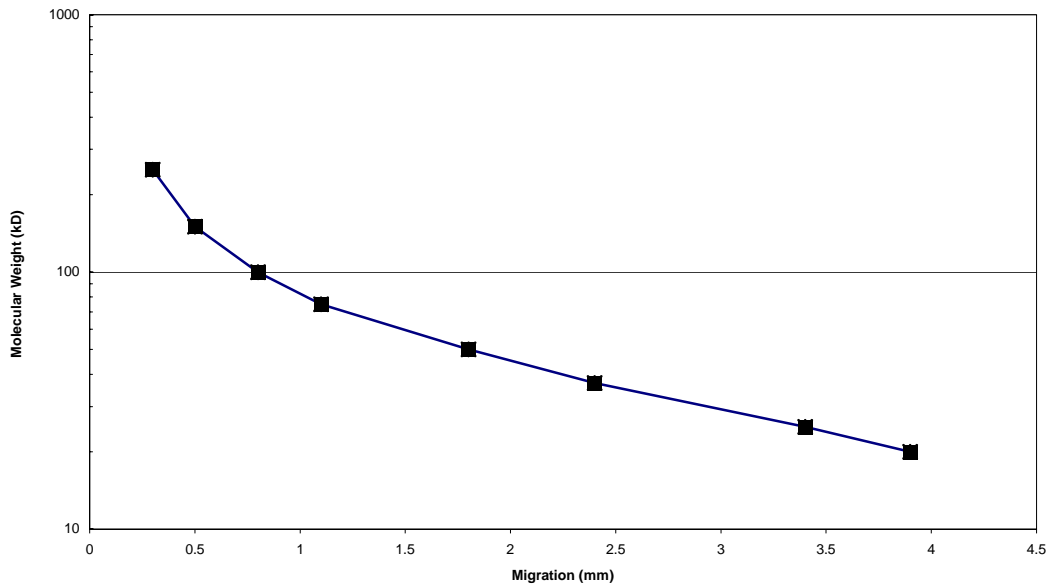


Figure 1: PCR Confirmation of TAP Cassette in Rep1 and Rep2 Genes: DNA was extracted from yeast cells transformed with TAP-Rep1 or TAP-Rep2 cassettes and was subjected to PCR using primers specific for each integration. The resulting PCR products were run on a 1.2% agarose gel and visualized by staining with ethidium bromide.

Figure 2: Western Blot of Rep1 and Rep2 (uninduced and induced): Rep1 and Rep2 were expressed as both uninducing (in Raffinose) and inducing (transferred to Galactose). The proteins were then transferred to a membrane and then were probed using antibodies specific to protein. According to the data, TAP-Rep 1 is apparently not expressed. Therefore, additional PCR positive strains must be screened for expression.

Figure 3: Silver Staining of Rep1 and Rep2 Genes: Silver staining was applied to the proteins in order to confirm that similar amounts of total protein were loaded. Rep 1 and Rep 2, uninducing (raffinose) and inducing (galactose transferred), were tested along with the wild type strain. The silver staining confirmed that it is suitable to proceed with purification.

Figure 4: Protein Molecular Weight Curve: The molecular weight of a molecule has been calculated by taking the log of the molecular weight of a known molecule (like the markers) and comparing it with its distance because the log of a molecule's molecular weight is proportional to the distance the molecule has migrated. According to the graph, the TAP-Rep2 weighs about 63 kD because it migrated about 1.5 mm. Therefore, it confirms that it is correct size.

Conclusion

The TAP tag was amplified by PCR and transformed into yeast; the integration was verified by PCR, and the expression was confirmed by the Western blot. As the cells grown in Raffinose were split into factions of uninduced (in Raffinose) and induced (transferred to Galactose), the samples were run on protein gels for western blot and silver staining. The process of western blotting confirmed the expression of protein A epitope tag and the silver staining confirmed that similar amounts of total protein were loaded and were the correct size in Rep2. It also revealed that they were Gal-inducible (not expressed in Raff, but expressed in Raff and Gal). Therefore, it is suitable to proceed with purification. However, Rep1 will require additional screening as shown in the Western blot. After the proteins are purified, they can be tested in future experiments whether they are vital for segregation using the methods like partitioning in deletion strains, co-immunoprecipitation, and GST- pull-down assays.

References

1. Bauer, Andreas; Kuster, Bernhard. 2003. Affinity purification-mass spectrometry: Powerful tools for the characterization of protein complexes. *Eur J Biochem.* 270: 570-578.
2. Burke, Dan; Dawson, Dean; Stearns, Tim. *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*. Cold Spring Harbor Laboratory Press. 2000 ed. 2000.
3. Schimanski, Bernd; Nguyen, Tu N.; Gunzl, Arthur. 2005. Highly Efficient Tandem Affinity Purification of Trypanosome Protein Complexes Based on a Novel Epitope Combination. *Eukaryotic Cell.* 4: 1942-1950.
4. Velmurugan, Soundarapandian; Ahn, Yong-Tae; Yang, Xian-Mei; Wu, Xu-Li; Jayaram, Makkuni. 1998. The 2 μ m Plasmid Stability System: Analyses of the Interactions among Plasmid- and Host-Encoded Components. *Mol. Cell. Biol.* 18: 7466-7477.
5. Volkert, Frederic; Wilson, Duncan; Broach, James. 1989. Deoxyribonucleic Acid Plasmids in Yeasts. *Microbiological Reviews*, 53: 299-316.

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