

Optimization of Immunoblot Protocol for Use with a Yeast Strain Containing the CDC7 Gene Tagged with myc

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ABSTRACT

Several yeast cell division genes have human versions with putative roles in the formation of cancerous tumors. One of these cell division genes, *CDC7*, is also involved in meiotic division although its precise role in meiosis is unknown. One way to help elucidate *CDC7*'s role in yeast meiosis is to determine when Cdc7 protein is made using western analysis. Yeast strains were constructed that contain the *CDC7* gene tagged with a portion of the human myc gene. Antibodies were then obtained that recognize the human myc portion of the Cdc7-myc protein. Experiments were done to determine the appropriate antibody concentrations necessary to detect the tagged Cdc7 protein from mitotic cells on immunoblots. These conditions were then used to perform a western analysis of protein obtained from meiotic yeast samples. Although Cdc7 protein was detected, it was partially degraded in all meiotic samples, indicating that a protease is present whose activity must be blocked to continue this work.

INTRODUCTION

One of the ways being investigated to understand the spread of cancer is to determine how proteins affect normal cell growth processes. *Saccharomyces cerevisiae* or Baker's yeast is studied by thousands of researchers because yeast contains cell division proteins that are similar to those in human cells. One commonly studied yeast cell growth protein with a similar protein in humans is Cdc7 (Hess, *et al.*, 1998). This protein kinase is required for initiating DNA replication (S phase) during the mitotic cell cycle of yeast, although it is not understood how Cdc7 protein controls this initiation in yeast (Sclafani and Jackson, 1994).

Meiosis is a process similar but not identical to the mitotic cell cycle. It is worthwhile to examine the role of Cdc7 protein in meiosis to help understand its role in the mitotic cell cycle. Currently, very little is known about what *CDC7* does in meiosis although it is required for meiosis to occur properly in yeast (Schild and Byers, 1978). One way to examine the meiotic role of *CDC7* is to analyze Cdc7 protein levels throughout meiosis. In this report, we describe the construction of haploid a and a strains containing the *CDC7* gene tagged with myc. Since haploids cannot undergo meiosis but diploids can, the haploid strains were crossed together to produce the diploid. Using western analysis, it was determined that Cdc7 protein could be detected both in mitotic and meiotic cells using commercially available anti-myc antibodies, although the protein appears to be partially degraded when obtained from meiotic cells.

METHODS AND MATERIALS

Strain Construction

Both “a” and “ α ” haploid *CDC7^{ts}* strains were used to construct *CDC7-myc* haploids using the standard two-step gene replacement technique (Rothstein, 1991). Briefly, a plasmid containing nine copies of the human *myc* epitope cloned in frame at the 3’ end of the *CDC7* gene was cut with the restriction endonuclease *Mlu*I (Promega) and transformed into the *CDC7^{ts}* haploids using lithium acetate (Figure 1). Three individual colonies of each of the α and a transformants were grown as large patches on YPD medium which were then replica plated to medium containing the negative selection chemical 5-FOA (Angus Buffers and Biochemicals). The resulting popouts were tested for their temperature sensitivity to determine whether they had retained the non-temperature sensitive *CDC7-myc* allele.

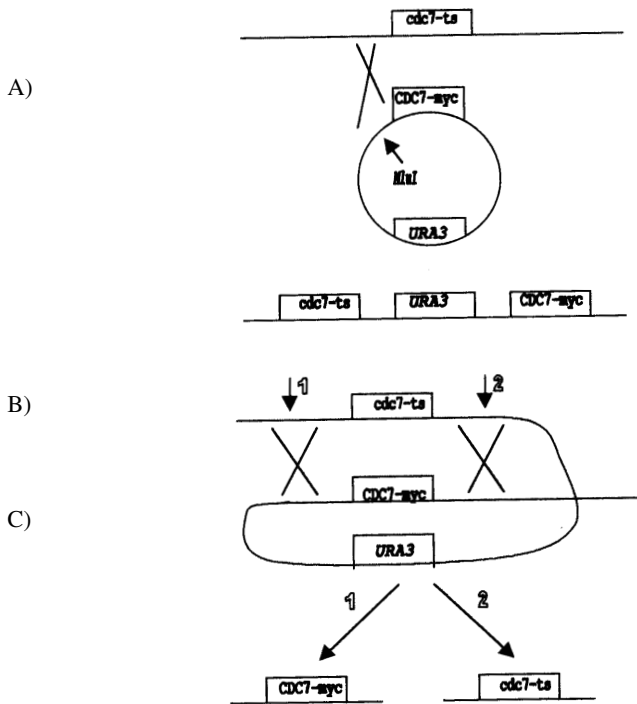


Figure 1. Construction of yeast strains containing *CDC7* tagged with *myc*. A) A plasmid containing the *CDC7-myc* construct and the selectable marker *URA3* was cut with the restriction endonuclease *Mlu*I and transformed into both an “a” and “ α ” *CDC7^{ts}* strain. B) This plasmid integrated into the yeast chromosome by homologous recombination, resulting in strains that had both the *CDC7^{ts}* and *CDC7-myc* alleles at the chromosomal locus with an intervening *URA3* gene. C) After transfer to nonselectable YPD medium, spontaneous intrachromosomal recombination resulted in the loss of the *URA3* gene and one of the two *CDC7* alleles. If the crossover occurred on the left side of *CDC7^{ts}* (#1) then the “popout” contained the desired *CDC7-myc* construct.

Protein Analysis

Haploid or diploid cells were grown mitotically to exponential phase (2×10^7 cells/ml) in liquid YPD medium. Samples of 5ml of cells were pelleted and resuspended in 500ul of protein lysis buffer (50mM tris, 50mM NaCl, 0.1% triton, 0.1% tween-20, 1mM PMSF) to which 0.5g of 0.5mm glass beads was added. The cells were shaken vigorously using a bead beater (Biospec Products), spun in a microfuge at 4°C, and the supernatant transferred to another tube. Loading buffer was added to each sample and the protein extracts were boiled for 5'. The protein was subjected to SDS-PAGE using 20ul of sample per lane. After transferring to nitrocellulose, the membrane was incubated in 5% milk plus 200ng/ml anti-myc antibody graciously provided by Bob Sclafani. After washing with PBS and BBS-tween, the membrane was incubated in 5% milk plus 10ng/ml anti-mouse secondary antibody (Pierce). After PBS and BBS-tween washes, Supersignal West Pico Chemiluminescent Substrate (Pierce) was added to the membrane which was then exposed to X-ray film.

RESULTS AND DISCUSSION

Two haploid temperature sensitive *CDC7* mutant yeast strains, an “a” and an “ α ”, were transformed with a plasmid containing the wild-type *CDC7* gene with nine copies of a portion of the human *myc* gene fused in frame to its 3' end (Figure 1). Transformants contained the *CDC7^s* mutation, the *CDC7-myc* allele, and the selectable marker *URA3*. Transformants of each of the two haploids were grown on non-selective YPD medium to allow intrachromosomal recombination to occur, resulting in the loss of the *URA3* gene and one of the two copies of the *CDC7* genes (Figure 1C). These YPD plates were replica plated to plates containing 5-FOA, allowing growth of these Ura- strains. If the crossover occurred to the left of *CDC7^s* (Figure 1C, option #1), the resulting “popout” would contain the *CDC7-myc* gene, the desired genotype.

To distinguish between the two possible genotypes, a genetic test was performed. Haploids that retained the *CDC7^s* allele were temperature sensitive and unable to grow at the restrictive temperature of 30°C. However, haploids that contained the *CDC7-myc* construct were no longer temperature sensitive and would grow at the restrictive temperature. Table 1 shows that 8 out of 13 “a” popouts and 4 out of 4 “ α ” popouts tested were no longer temperature sensitive, indicating that they contained the desired *CDC7-myc* construct.

Pairs of “a” and “ α ” *CDC7-myc* haploids were crossed together to form diploid strains in which both copies of *CDC7* were tagged with the human *myc* epitope. These diploid strains were grown mitotically to exponential phase, and protein was isolated as described in Methods and Materials. The protein was subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and incubated with antibodies against the human *myc* epitope. As shown in Figure 2A, Cdc7 protein of the appropriate estimated size (70 kD) was detected from two independent diploids (lanes 7 and 8) as well as from the haploid parents of those diploids (lanes 4 and 6). No Cdc7 protein was detected from a control diploid that did not contain the *CDC7-myc* construct (lane 1). No other bands were observed on the X-ray film from this experiment (data not shown), indicating that the antibody was very specific.

Table 1. Results of testing “popouts” for temperature sensitivity.¹

“a” popouts	YPD 22°C	YPD 30°C
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	o
8	+	o
9	+	o
10	+	o
11	+	+
12	+	+
13	+	o
“α” popouts	YPD 22 °C	YPD 30 °C
1	+	+
2	+	+
3	+	+
4	+	+

¹ “+” indicates growth; “o” indicates no growth

The two independent diploid strains were then induced to undergo meiosis by incubating them in potassium acetate starvation medium. Samples were taken after 0, 15, and 16 hours of meiosis and protein was isolated followed by electrophoresis and immunoblotting. As shown in Figure 2B, a single Cdc7p band was observed in samples of protein taken from mitotic cells (0 hrs., lanes 3 and 6). However, several degradation products were observed in addition to that band from all of the meiotic samples (lanes 4 and 5, 7 and 8). In future experiments, several different types of protease inhibitors will be used to try to eliminate this degradation problem.

In summary, “a” and “α” haploid yeast strains were engineered to contain the *CDC7* gene tagged with a portion of the human *myc* gene to enable the detection of *CDC7* protein in meiosis using commercially available anti-myc antibodies. Both a genetic assay and western analysis confirmed the presence of this construct in both the haploids and the resulting diploids. These diploid strains will be used in future experiments to carefully determine the timing of Cdc7 protein expression during meiosis to correlate that expression with landmark events of meiosis such as DNA replication and the two nuclear divisions.

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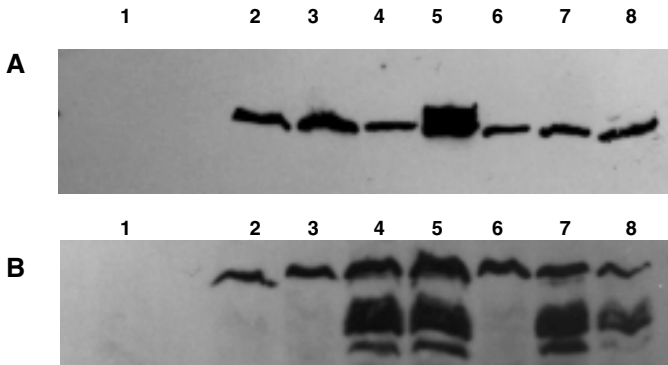


Figure 2. Results of *CDC7-myc* immunoblotting experiments. A) Yeast strains were grown mitotically, and protein isolated and subjected to SDS-polyacrylamide gel electrophoresis. After transferring the protein to a nitrocellulose membrane, an immunoblot was performed using antibodies specific to the myc epitope. Lane 1: negative control; Lane 2: positive control; Lane 3: haploid "a" transformant before popping out; Lane 4: haploid "a" popout; Lane 5: haploid "α" transformant before popping out; Lane 6: haploid "α" popout; Lane 7: *CDC7-myc*-containing diploid; Lane 8: independent *CDC7-myc*-containing diploid. B) Two independent diploids were grown mitotically and then were induced to undergo meiosis. Protein was isolated and an immunoblot was performed using antibodies specific to the myc epitope. Lane 1: mitotic negative control; Lane 2: mitotic positive control; Lane 3: diploid #1 at 0 hrs.; Lane 4: diploid #1 at 15 hrs.; Lane 5: diploid #1 at 16 hrs.; Lane 6: diploid #2 at 0 hrs.; Lane 7: diploid #2 at 15 hrs.; Lane 8: diploid #2 at 16 hrs.

REFERENCES

- Hess, G.F., R.F. Drong, K.L. Weiland, J.L. Slighthorn, R.A. Scalfani, and R.E. Hollingsworth (1998). A human homolog of the yeast *CDC7* gene is overexpressed in various tumors and transformed cell lines. *Gene* 211:133.
- Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast in *Guide to Yeast Genetics and Molecular Biology*, C. Guthrie and G.R. Fink, eds.
- Schild, D. and B. Byers (1978). Meiotic effects of DNA-defective cell division cycle mutations of *Saccharomyces cerevisiae*. *Chromosoma* 70:109.
- Scalfani, R.A. and A.L. Jackson (1994). Cdc7 protein kinase for DNA metabolism comes of age. *Molecular Microbiology* 11:805.

