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Overexpression of a novel gene, Cms1, can rescue the growth arrest of a Saccharomyces cerevisiae mcm10 suppressor

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ABSTRACT

MCM10 protein is an essential replication factor involved in the initiation of DNA replication. A mcm10 mutant (mcm10-1) of budding yeast shows a growth arrest at 37°C. In the present work, we have isolated a mcm10-1 suppressor strain, which grows at 37°C. Interestingly, this mcm10-1 suppressor undergoes cell cycle arrest at 14°C. A novel gene, YLR003c, is identified by high-copy complementation of this suppressor. We called it as Cms1 (Complementation of Mcm10 Suppressor). Furthermore, the experiments of transformation show that cells of mcm10-1 suppressor with high-copy plasmid but not low-copy plasmid grow at 14°C, indicating that overexpression of Cms1 can rescue the growth arrest of this mcm10 suppressor at non-permissive temperature. These results suggest that CMS1 protein may functionally interact with MCM10 protein and play a role in the regulation of DNA replication and cell cycle control.

Key words: *Saccharomyces cerevisiae, DNA replication, Mcm10, Cms1, cell cycle.*

INTRODUCTION

DNA replication is a fundamental process that must occur only once at each cell cycle. This restrict control appears to be achieved through the coordinated activities of numerous proteins. The budding yeast Saccharomyces cerevisiae provides an excellent eukaryotic model for study of proteins involved in the control of DNA replication.

In the budding yeast, minichromosome maintenance (MCM) proteins, MCM2-7, are a family of six sequence-related proteins that play crucial roles in regulation of DNA replication[1],[2]. Despite their sequence similarity, each of these proteins is indispensable for cell viability in S. cerevisiae. Some conditional alleles of mcm mutants have been shown to undergo cell cycle arrest at non-permissive temperature with predominantly unreplicated DNA, incomplete replication or a slowed rate of DNA synthesis [1].The MCM2-7 proteins interact with one another to form a large complex, which is a part of the pre-RC[2]. Cdc6 is required for the recruitment of the MCM complex to DNA replication origins[3],[4]. In addition to the role of MCM complex in initiation of DNA synthesis, it is also believed to be an active helicase that melts the origin DNA and then processively unwinds the growing forks[5-9].

Recently, a new minichromosome maintenance factor, Mcm10, has been identified[10]. However, this protein shares no sequence similarity with MCM2-7[2],[10]. Mcm10 is constitutively bound to chromatin[11]. Moreover, Mcm10 physically interacts with several members of the MCM2-7 family and is required for the association for MCM2-7 complex with replication origins[10],[11]. mcm10-1 mutant shows an ARS-specific Mcm defect and arrests at the restrictive temperature (37°C) with dumbbell morphology and 2C content[10]. Surprisely, mcm10-1 mutation also causes the pause of replication forks, suggesting it may involve the elongation of DNA replication[10]. In the present works, we

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have isolated a mcm10-1 suppressor strain, and identified a novel gene, Cms1, by high-copy complementation of the suppressor. In addition, we found that a novel open reading frame in S. pombe shares structural homology with the S. cerevisiae Cms1.

MATERIALS AND METHODS

Strains and media

Escherichia coli strain XL^1-Blue [supE44 hsdR17 recA1 endA1 gyrA46 thi relA^1 lac$^-$F' (proAB$^+$ lacIq lacZ△M15 Tn10(tet^r))] was used for plasmid construction and preparation. E. Coli strain DH12S [ø80dlacZ△M15 mcrA△(mrr-hsdRMS-mcrBC) ara△139 △(ara, leu)7697△lacX74 galU galK rpsL Sm^r deoR nupG recA1/F' proAB$^+$ lacI^{q}Z△M15] was used for electroporation. Yeast strains used are 8534-8C (MAT α ura3-52 leu2-3,112 his4-34), mcm10-2C (Mata his3 his4 leu2-3,112 ura3-52 mcm10-1) and WJ-1 (Mata his3 his4 leu2-3, 112 ura3-52 mcm10-1 unknown-suppressor). YPD Medium consists of 1% yeast extract, 2% peptone and 2% dextrose. SC-Leu and SC-Ura medium were prepared as described previously[12].

Isolation of mcm10-1 suppressors and identification of suppressor gene

mcm10-2C cells were grown to log phase at 30°C, plated on YPD agar plate, and incubated at 37°C for 4 d. From a total of 4×10^9 cells, we obtained ~ 300 temperature-sensitive suppressors. These temperature-sensitive suppressors were replica-plated and incubated at 37°C for 4 d and 14°C for 10 d. Colonies that grew at 37°C but not at 14°C were further analyzed and three temperature-sensitive suppressors were isolated. One of the three mcm10-1 suppressors, named WJ-1, is subjected to further study.

To identify the suppressor gene, WJ-1 was transformed with a yeast YEp13 genomic DNA library, and the transformants were spread on SC-Leu plate and screened for growth at 14°C. Plasmids were recovered from the yeast transformants and transformed into E. coli strain DH12S by electroporation, and the WJ-1-cold-sensitivity-complementing activity was confirmed by retransformation into the WJ-1 mutant. Thus, we finally isolated four types of plasmids based on restriction analysis. One type of plasmid with an insert of 8-kb was used for the following study here, and the other three types of plasmids will be described elsewhere. Based on the partial sequencing results of the two ends of the 8-kb insert, a search in Saccharomyces Genome Database (SGD) (http://genome-www.stanford.edu/Saccharomyces/) found that this insert contained three unknown ORFs. Subsequent subcloning and retransformation analysis indicated that one of the three ORFs, YLR003c, was the element responsible for the complementing activity.

Viability assay

Cells were grown to log phase at 30°C, diluted to a cell density of 3×10^6/ml, and then shifted to test temperatures. At each point of time, the cells were harvested and spread as a single cell on YPD plates at 30°C. After 3 d, colonies were counted. Percentage of viable is the number of colonies divided to the number of cells plated on YPD.

Plasmid construction

Plasmid YEpCms1 was generated by subcloning the 2.9-kb HindIII-SalI fragment, containing a full length of Cms1 gene (876bp), cut from the 8-kb insert of YEp13 into the HindIII-SalI site of YEp351 (2μ, LEU2). YCpCms1 was constructed by subcloning the 2.5-kb EcoRI fragment, containing a full length of Cms1 gene, cut from the same 8-kb insert into YCp50 (ARS1, CEN4, URA3).

Yeast transformation

Transformation of WJ-1 with yeast genomic library was performed by lithium acetate method[12]. General transformation of yeast was performed as described with the following modifications[13]. Yeast cells were grown to log phase at 30oC. For each transformation, 1 ml of cells was washed once with TE/LiAc (10 mM Tris-HCl pH7.5, 1 mM EDTA pH8.0, 100 mM LiAc), resuspended in 0.1 ml of TE/LiAc, added about 0.1 μg of ransforming DNA, 20 μmg of salmon carrier DNA and 0.7 ml of TE/LiAc/PEG (10 mM Tris-HCl pH7.5, 1 mM EDTA pH8.0, 100 mM LiAc, 40% (w/v) PEG4000) solution, The mixture was incubated at 30°C for 1 h. Then, the yeast cells were shocked for 15 min at 42°C and resuspended in 0.1 ml of water and spread onto appropriate plates.

Flow cytometry

3×10^6 yeast cells were collected and then fixed in 0.7 ml of 60% ethanol at 4°C for more than 2 h. Fixed cells were subsequently resuspended in 1 ml of 50 mM sodium citrate (pH7.4), sonicated briefly, and treated sequentially with 0.1 mg of RNase A and 1 mg of proteinase K at 50°C for 1 h each. An equal volume of 50 mM sodium citrate (pH7.4) containing 10 μg/ml propidium iodide was added to the cell suspension and subjected to flow cytometric analysis.

Sequencing and sequence analysis

Genomic DNA of WJ-1 strain was purified as described previ ously[14]. Genomic PCR of the Mcm10 and Cms1 genes was car ried out by using the following primers: 5' AGCACCGATGGA GCAAAAATAACA 3' and 5' CATTCGCCGGCACCATTAG G 3' ; 5' TTGACTGATATCGAAAACAGAAAT 3' and 5' A TCCCGGAATTATACTCTTATGAT 3' respectively. All the seq uencing was performed on ABI 377 automated sequencer.

The sequencing results were analyzed by using DnaStar pack age. Protein sequence analysis was performed by using software on the ExPASy web site[15]. Homolog search in the SwissProt a nd PIR was done with GCG SSEARCH program. CMS1 and SPB C23G7.07c protein sequences were aligned using the GCG GAP p rogram.

Northern blotting

Total RNA was isolated from yeast cells by extraction with hot acidic phenol assay[16], and 10 μg total RNA was subjected to Northern blot analysis. The probe used was a 0.9-kb MunI/Eco47-3 fragment containing the entire open reading frame encoding Cms1 from pUC-Cms1. The probe of actin mRNA was used as an internal control.

RESULTS AND DISCUSSION

Isolation of mcm10-1 suppressor strains

To gain further insights into the role of Mcm10 in the regulation of DNA replication, we sought to isolate mcm10-1 suppressors. A mcm10-1 mutant, mcm10-2C (a gift from Dr. Tye, B.K.; see ref. 10), was subjected to a genetic screen (see Materials and Methods). Three kinds of mcm10-1 suppressors were isolated, and one of them, named WJ-1, was chosen for the studies in this report. The strain WJ-1 can rescue the growth arrest of mcm10-1 at 37°C, whereas WJ-1 showed cold-sensitivity (Fig 1A), indicating that WJ-1 is a temperature-sensitive suppressor of the mcm10-1 mutant. Furthermore, the sequencing result of the gene mcm10 of WJ-1 showed that the mcm10 mutation in WJ-1 genome was a proline to leucine substitution at amino acid 269 (data not shown), which was identical to that of mcm10-2C, indicating that the suppression of mcm10-2C is not due to the reversional mutation of mcm10-1.

To investigate whether WJ-1 cells could recover from the cell cycle arrest, we compared the relative viability of wild-type 8534-8C, mcm10-2C and WJ-1. The yeast cells were treated first at either 37°C or 14°C for particular time, and then collected and spread out on plates. After three-day incubation at 30°C, the colonies of the yeast cells were counted and the viability was calculated. The results showed that the viability of wild-type cells was not affected under the condition of either 37°C or 14°C treatments (Fig 1B). However, the viability of mcm10-2C cells was significantly affected when the cells were treated at 37°C but not at 14°C (Fig 1B). The viability of the mcm10-2C cells got worse and worse when the incubation time at 37°C was increased (Fig 1B). In contrast, the effects of the restrictive temperatures on the viability of WJ-1 cells were reversed as it was compared with mcm10-2C. The viability of WJ-1 cells was reduced when the cells were treated at 14°C, whereas the viability of them treated at 37°C was similar to that of wild-type cells (Fig 1B). The loss of the viability of WJ-1 cells was also in a linear fashion (Fig 1B). These data indicate that WJ-1 cells can compensate the growth suppression of mcm10-2C at 37°C, while WJ-1 itself is still a temperature-sensitive strain when it grows at low temperature.

Interruption of WJ-1 cell cycle at non-permissive temperature

To examine the cell cycle of WJ-1 cells, the DNA contents of WJ-1, mcm10-1 mutant and wild-type yeast cells were measured by flow cytometry at various time points after a shift from the permissive temperature to the non-permissive temperatures. The results showed that the DNA content of wild-type yeast cells was a typical profile of exponential growth at all three temperatures (Fig 2). After the temperature was shifted up to 37°C, most mcm10-1 cells contained an approximate 2C DNA, suggesting the cell cycle of mcm10-2C was arrested at G2/M phase

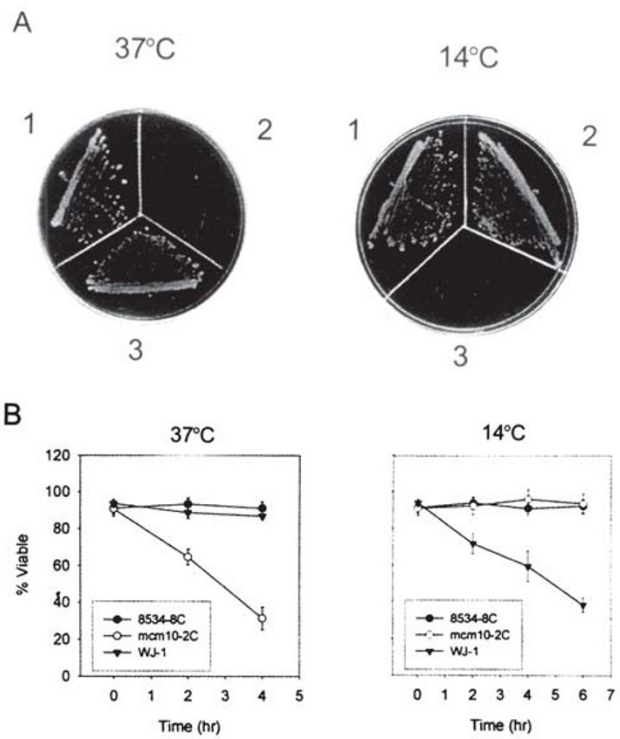

Fig 1. Isolation and characterization of WJ-1 as a mcm10-1 suppressor
(A) The growth properties of yeast strains. 8534-8C (1), mcm10-2C (2) and WJ-1 (3) were grown on YPD plate at 37°C for 3 d (left) or at 14°C for 10 d (right). (B) Measurement of viability of yeast cells. 8534-8C, mcm10-2C and WJ-1 strains were treated first at either 37°C (left) or 14°C (right) for particular time (X-axis), and then collected and spread out on plates. After three-day incubation at 30°C, the colonies of the yeast cells were counted and the viability was calculated (Y-axis). At least three independent viability experiments are averaged, and data are expressed as the mean ±SD.

at non-permissive temperature (Fig 2, see also ref. 10). However, WJ-1 cells showed a normal cell cycle profile at 37°C (Fig 2), indicating that the effect of the cell-cycle arrest due to mcm10-1 mutation was suppressed in WJ-1 cells.

Interestingly, more and more WJ-1 cells had a DNA content of greater than 2C and the peak of WJ-1 DNA content moved to the > 2C position when the cells were exposed longer and longer to 14°C (Fig 2). These data suggest that WJ-1 cells arrest at the non-permissive temperature and might undergo either partially re-replication of chromosomal DNA or continuously mitochondrial DNA replication. The DAPI staining of nuclei showed that some of WJ-1 cells had distinctly increased nuclear volumes, supporting the view of partially re-replication of chromosomal DNA (data not shown).

Cloning of Cms1 gene and sequence analysis of CMS1

WJ-1 cells were transformed with a yeast YEp13-based genomic library and screened at 14oC (Materials and Methods). One plasmid was found to partially complement the growth defect of WJ-1. An 8-kb fragment neighboring the centromere at No. 12 chromosome of budding yeast in the database was

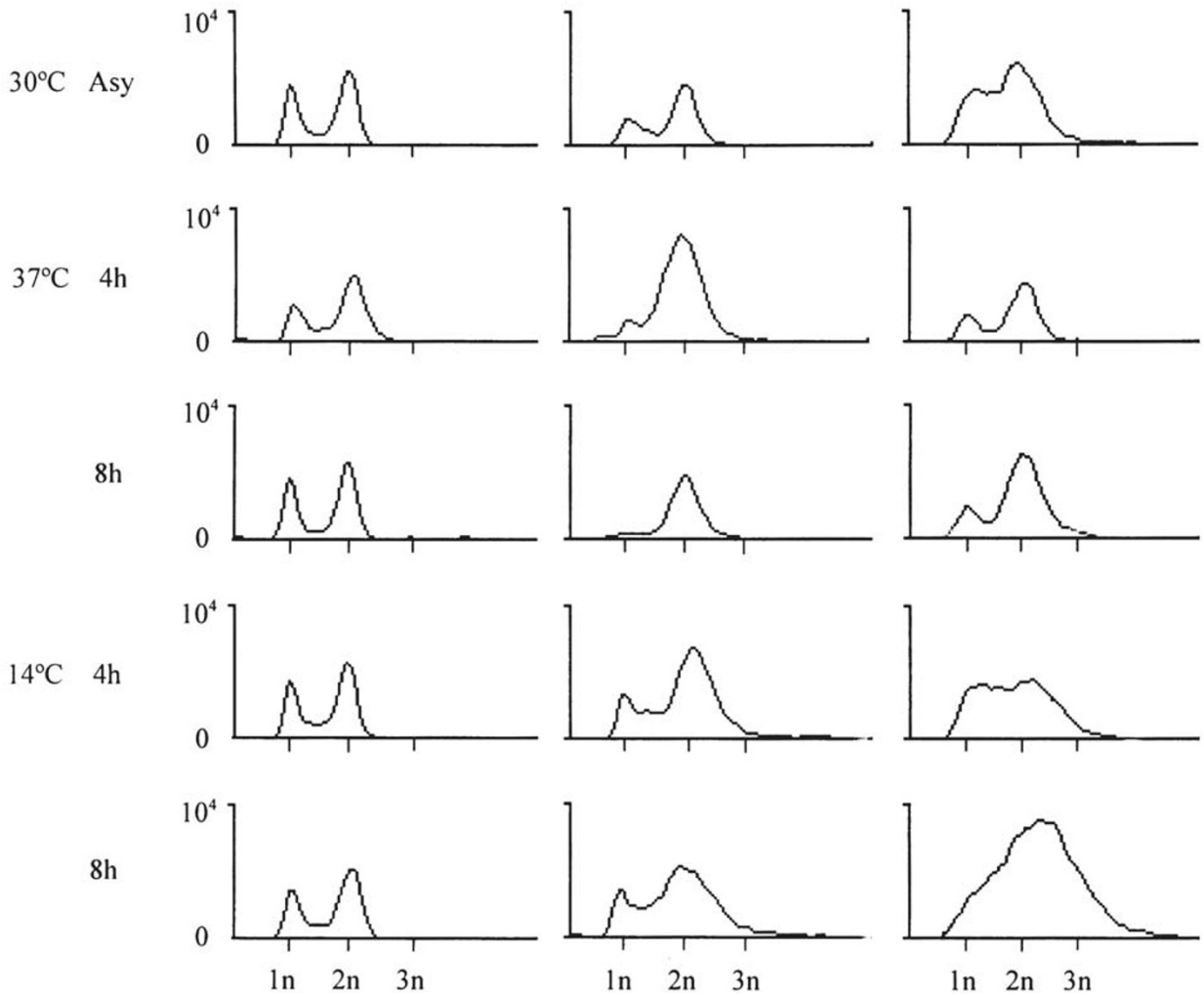

Fig 2. FACS analysis of the DNA contents of asynchronous yeast cells
8534-8C, mcm10-2C and WJ-1 cells were grown at particular temperature. The yeast cells were collected at variant time points, and DNA content was measured by FACS as described in Materials and Methods.

found to match the two ends of the insert exactly, which was further confirmed by restriction mapping. This 8-kb insert contains three functionally unknown open reading frames (ORF), YLR001c, YLR002c and YLR003c. Subsequent subcloning and retransformation analysis demonstrated that only one of them, YLR003c (876 bp), was the right one that partially complemented the growth arrest phenotype of WJ-1 at 14°C (see Fig 4B and 4B'). We renamed YLR003c as Cms1 gene (Complementation of Mcm-10 Suppressor).

Cms1 gene encodes a putative protein of 291 amino-acid residues. An aspartic acid-rich region is at residues 5-62 (Fig 3). The bipartite nuclear localization signal (NLS) lies at residues 69-86 (Fig 3). A short peptide near the C-terminus of the aspartic acid-rich region, DGKK (residues 51-54), was predicted as a potential amidation site by PrositeScan program analysis in ExPASy (Fig 3). Using the same method, two potential tyrosine kinase phosphoryla-

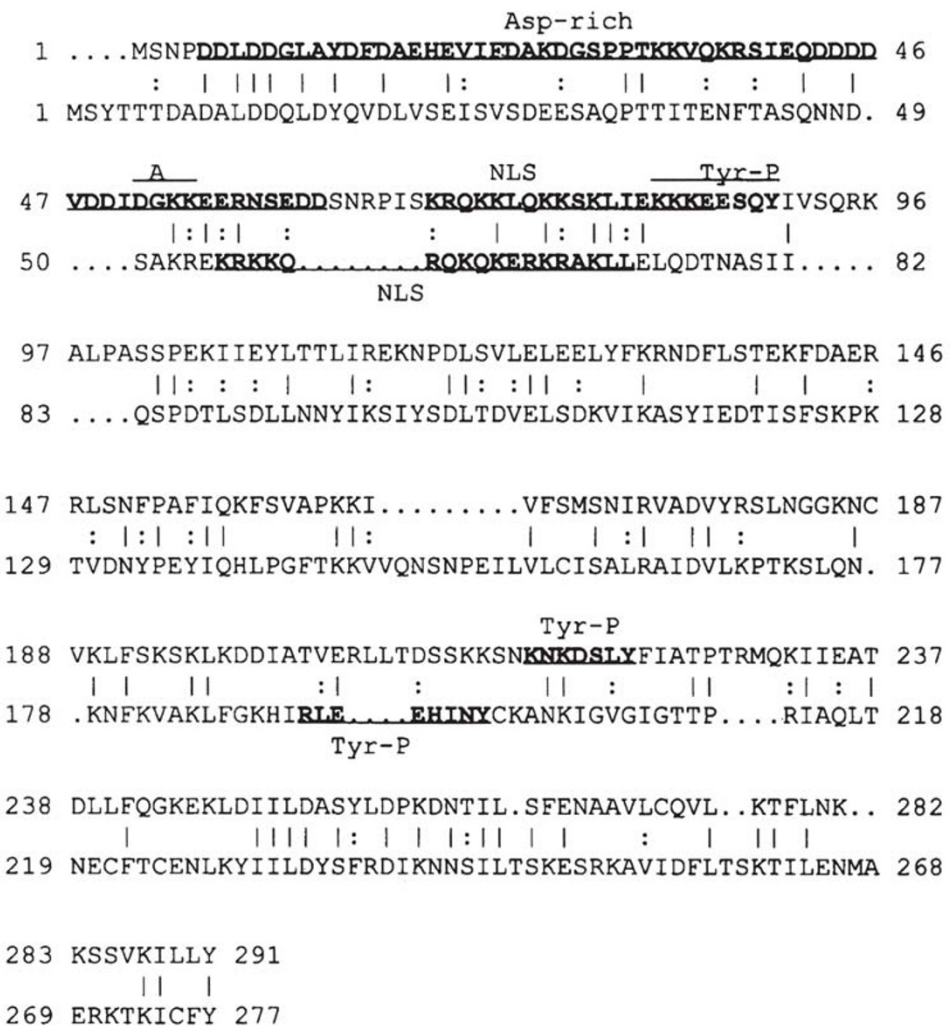

Fig 3. Comparison of CMS1 with a fission yeast protein sequence
CMS1 (upper) and SPBC23G7.07c (lower) predicted protein sequences were aligned using the GCG program. Identical residues are indicated by vertical lines (1) and similar residues by colons (:). The nuclear localization signal (NLS) is shown; the potential tyrosine phosphorylation sites (Tyr-P), the amidation site (A) and the aspartic acid-rich region (Asp-rich) are blacked and marked by lines. The CMS1 protein sequence is obtained by translation of the YLR003c genomic open reading frame. The SPBC23G7.07c protein sequence is taken directly from Protein Information Resource (PIR), accession number T39953.

tion sites were predicted to be present at residues 83-90 (KKKEESQY) and 216-222 (KNKDSLY) (Fig 3).

Comparison of the Cms1-encoded sequence with other sequences in the SwissProt and PIR databases revealed only one open reading frame (ORF) of unknown function in fission yeast Schizosaccharomyces pombe, SPBC23G7.07c with weak similarity (expect value = 2.4e-06). The alignment in Fig 3 shows that there is 29% identity and 39% similarity between the two predicted proteins. SPBC23G7.07c is supposed to encode a protein of 277 amino acids. SPBC23G7.07c also contains a NLS at similar position (55-72) near the N-terminus (Fig 3). SPBC23G7.07c was predicted to contain one potential tyrosine kinase phosphorylation at site 191 to 198 (Fig 3). Furthermore, both of these two proteins sequences have similar distribution patterns of the regions of predicted secondary structures. The Cms1 and SPBC23G7.07c protein sequences also share many conserved residues and have similar predicted molecular weights (33.4 and 31.6 kDa, respectively), isoelectric points (8.2 and 8.4, respectively), amino-acid compositions and hydropathy profiles (Fig 3).

Overexpression of Cms1 gene can partially complement the cold-sensitive phenotype of WJ-1

The expression levels of Cms1 mRNA of the wild-type strain, mcm10 mutant and its suppressor at different growth conditions were analyzed by Northern blotting. The results showed that the Cms1 mRNA of wild-type 8534-8C was expressed at either 37°C or 14°C (Fig 4A), suggesting that Cms1 plays a role in the process of the cell growth. In contrast, it was not found the correlation of the level of mRNA expression of Cms1 to the cell growth of both mcm10-2C and WJ-1 strains (compare Fig 4A with Fig 1A), suggesting that Cms1 gene is not critical one for the cell growth.

Since Cms1 gene was functionally screened by using YEp plasmid library containing 2 μ origin of DNA replication which produce high-copy number of the plasmids, we wonder whether single-copy plasmid containing Cms1 gene could also complement the growth arrest of WJ-1 cells at non-permissive temperature. Single-copy plasmid YCp50 (ARS/CEN) or high-copy plasmid YEp351 (2m) and their constructs containing Cms1 gene were used for yeast transformation experiments (Materials and Methods). The results showed that WJ-1 cells trans-

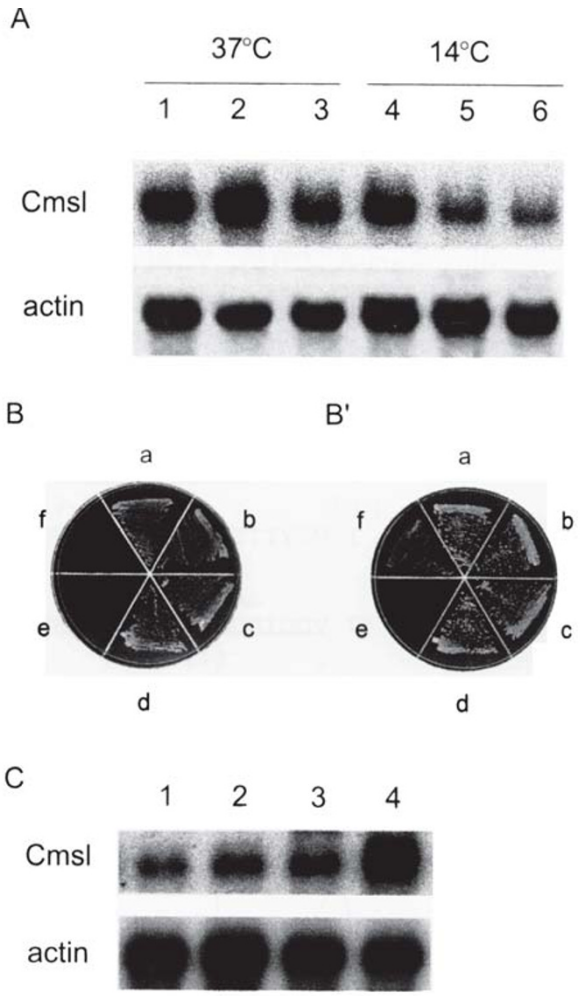

Fig 4. Rescue of the growth arrest of WJ-1 strain by overexpression of Cms1 gene (A) Northern blot analysis of Cms1 mRNA transcription. 8534-8C, mcm10-2C and WJ-1 strains were grown to log phase at 30°C. The cells were diluted to 3×10^6/ml, and then shifted to 37°C and 14°C, respectively. After incubation at 37°C for 4 h and 14°C for 8 h, total RNA was isolated and subjected to Northern blotting. Actin mRNA was used as an internal control. (1) and (4): 8534-8C; (2) and (5): mcm10-2C; (3) and (6): WJ-1. 8534-8C, mcm10-2C and WJ-1 strains were transformed with (B) low-copy plasmids: (a) 8534-8C [YCp50], (b) 8534-8C [YCpCms1], (c) mcm10-2C [YCp50], (d) mcm10-2C [YCpCms1], (e) WJ-1 [YCp50], (f) WJ-1 [YCpCms1]; (B') high-copy plasmids: (a) 8534-8C [YEp351], (b) 8534-8C [YEpCms1], (c) mcm10-2C [YEp351], (d) mcm10-2C [YEpCms1], (e) WJ-1 [YEp351], (f) WJ-1 [YEpCms1]; and incubated at 14oC for 10 d. (C) Northern blot analysis of Cms1 mRNA transcription. WJ-1 cells transformed with different plasmids grew to log phase in SC-Ura or SC-Leu medium, and then were shifted to 14°C. After incubation at 14°C for 8 h, total RNA was isolated and subjected to Northern blotting. Actin mRNA was used as an internal control. (1) WJ-1 [YCp50]; (2) WJ-1 [YEp351]; (3) WJ-1 [YCpCms1]; (4) WJ-1 [YEpCms1].

formed with single-copy plasmid containing Cms1 gene did not grow at 14°C, whereas these cells transformed with high-copy plasmid containing Cms1 gene could grow at 14°C (Compare Fig 4B "f" with 4B' "f"). To further confirm the overexpression of Cms1 gene in transformants with high-copy plasmid, the level of Cms1 mRNA was analyzed with Northern blotting assay. The results showed that the amount of Cms1 mRNA in WJ-1 cells with YEpCms1 was significantly higher than those in WJ-1 cells with YCpCms1 or empty plasmids (Fig 4C). These data suggest that the overexpression of Cms1 gene is required for complementation of the cold-sensitive phenotype of WJ-1 at 14°C.

To investigate whether Cms1 gene was mutated in WJ-1 cells, we amplified Cms1 gene from WJ-1 genome using PCR technique, and the PCR product was directly subject to sequencing. The sequencing result showed that Cms1 gene in WJ-1 genome was not mutated (data not shown), suggesting that the suppressor gene of mcm10-1 was not Cms1. Therefore, the conclusion from the sequencing result combined with the data of the yeast transformation experiment is that the single copy of Cms1 does not play a role in suppression of mcm10-1 mutant and the complementation of WJ-1 at non-permissive temperature, whereas the overexpression of Cms1 gene can rescue the growth arrest of WJ-1 cells at non-permissive temperature and CMS1protein might interact with MCM10 protein.

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