Perturbation of the Activity of Replication Origin by Meiosis-specific Transcription*

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We have determined the activity of all ARSs on the Saccharomyces cerevisiae chromosome VI as chromosomal replication origins in premeiotic S-phase by neutral/neutral two-dimensional gel electrophoresis. The comparison of origin activity of each origin in mitotic and premeiotic S-phase showed that one of the most efficient origins in mitotic S-phase, ARS605, was completely inhibited in premeiotic S-phase. ARS605 is located within the open reading frame of MSH4 gene that is transcribed specifically during an early stage of meiosis. Systematic analysis of relationships between MSH4 transcription and ARS605 origin activity revealed that transcription of MSH4 inhibited the ARS605 origin activity by removing origin recognition complex from ARS605. Deletion of UME6, a transcription factor responsible for repressing MSH4 during mitotic S-phase, resulted in inactivation of ARS605 in mitosis. Our finding is the first demonstration that the transcriptional regulation on the replication origin activity is related to changes in cell physiology. These results may provide insights into changes in replication origin activity in embryonic cell cycle during early developmental stages.

Eukaryotic chromosomes consist of multiple replication units. Each origin of DNA replication is strictly controlled to fire only once in the cell cycle in the fixed sequential order (1, 2). This temporal program for origin firing is utilized for the maintenance of genome integrity through DNA replication checkpoint mechanism (3, 4). It was shown that a checkpoint effector kinase Rad53 repressed firing of late origins when replication was perturbed by hydroxy urea or methyl methane sulfonate. Furthermore, recent genome-wide studies of eukaryotic chromosome DNA replication using Saccharomyces cerevisiae have provided us with a kinetic map of progression of DNA replication along the chromosome (5, 6). However, these studies were restricted to DNA replication during mitotic cell cycle and little is known about how these multiple replication origins behave under different cell cycle, i.e. meiotic cell cycle.

The decision to enter the meiotic cell cycle is made in G1 phase and this affects the way in which the G1/S transition is controlled. In budding yeast (S. cerevisiae), poor nutrient conditions are the cue to embark on the meiotic cell cycle, which culminates in the production of spores (7). The replication of chromosome is the first detectable cytological event in meiosis. The coordinated synthesis of genomic DNA requires multiple levels of regulation and a large number of gene products. Genetic analysis in S. cerevisiae indicates that the replicative machinery used to synthesize DNA in vegetative cells is also required for the duplication of chromosome in meiosis (8–10). In S. cerevisiae, mitotic S-phase and premeiotic S-phase appear to be differently regulated. For example, premeiotic S-phase is 1.5–2 times longer than mitotic S-phase (11). However, replication kinetics as measured by neutral/neutral two-dimensional gel electrophoresis of 100-kb segment of chromosome III of S. cerevisiae in premeiotic S-phase are similar to those measured in mitotic S-phase (12). Furthermore, the same study showed that DNA replication of chromosome III initiates at the same origins in meiosis and mitosis. These two conflicting results should be resolved by studying replication of chromosomes other than chromosome III.

Several lines of evidence suggest that premeiotic DNA replication links to the initiation of meiotic recombination events. Borde et al. (13) showed that delaying the replication of a chromosome segment specifically delays the formation of the break at that segment. Cha et al. (14) showed that Spo11, which is required for interhomolog interaction, and Rec8, a meiosis-specific cohesin subunit, are negative and positive regulators for the progression of premeiotic S-phase, respectively. These results suggest that there are many other possible regulations of replication at each chromosomal level that affect the progression of S-phase in meiotic cell cycle.

The chromosome VI of S. cerevisiae is 270 kb in length and the best-characterized chromosome for replicon structure in mitotic cell cycle. It contains ten ARSs, ARS601–609, of which nine ARSs are active as replication origins that comprise five active replicons in mitotic S-phase (1, 2, 15). Thus, three origins (ARS605, 606, and 607) are used in high frequency, four (ARS601/2, 603, 603.5, and 609) are in intermediate frequency, and two (ARS604 and 608) are in low frequency, less than 5% of cell cycles. Replication of each arm of chromosome starts from one major origin (ARS605 and 607 on the left and the right arm, respectively) and extends sequentially in both directions (1, 2, 6). During mitotic growth, all of these replication origins are bound by ORC (16, 17). The aim of this study is to determine the replicon structure of the chromosome VI of S. cerevisiae in the premeiotic S-phase.
and to understand the difference from that of the S-phase replicon at molecular level. Interestingly, we found that activity of one origin ARS605, an efficient origin during mitotic cell cycle, was completely inhibited during premeiotic S-phase. As ARS605 resides within the open reading frame (ORF) of a meiosis-specific gene, MSH4, we tested the possibility that ARS605 was inhibited by transcription. We found that transcription of MSH4 by RNA polymerase II removed ORC from ARS605 during premeiotic S-phase. This is the first demonstration of origin inactivation by transcription that changes depending on the physiology of the cell.

EXPERIMENTAL PROCEDURES

Strains—All strains used in this study (Table 1) are isogenic to NKY730 or NKY278. Orc1 protein was tagged by 3×HA epitope tag cassette at its C terminus using the plasmid p306ORC1-HA/C (18). An Δume6 mutant is a deletion mutant that is constructed by insertion of the KanMX cassette (19).

Induction of Sporulation—Cells were grown and sporulated as described (20). In short, a single colony from the YPG (3% glycerol, 2% Bacto peptone, 1% yeast extract) plate was inoculated into 5 ml of SPS presporulation medium (0.5% yeast extract, 1% Bacto peptone, 0.17% yeast nitrogen base without ammonium sulfate and amino acids, 0.05 M potassium phthalate, 1% potassium acetate, 0.5% ammonium sulfate, pH 5.0), and cells were cultured at 30 °C overnight. Small amounts of the preculture suspension were then inoculated into 0.5 liter of SPY, and cells were cultured at 30 °C to a density of 4×10^7 cells/ml. Cells were grown to 1×10^7 cells/ml at 23 °C in 100 ml of YPD and then fixed with 1% formaldehyde at room temperature for 30 min. Synchronous cultures induced for meiosis were withdrawn every 1 h at 0–5 h and fixed in the same way. Orc1-tagged strains undergo meiosis normally.

Chromatin Immunoprecipitation (ChIP)—Mitotic cells were grown to 1×10^7 cells/ml at 23 °C in 100 ml of YPD and then fixed with 1% formaldehyde at room temperature for 30 min. Synchronous cultures induced for meiosis were withdrawn every 1 h at 0–5 h and fixed in the same way. Orc1-tagged strains undergo meiosis normally.

Reverse Transcriptase (RT)-PCR—Total RNA was extracted from yeast cells using the RNeasy kit (Qiagen). RT-PCR was performed using the TaKaRa RNA PCR kit (AMV) version 3.0 (TaKaRa) by following the manufacturer's instructions. Regions of MSH4 and ACT1, cDNAs were amplified using the following primer sets: MSH4 5’TGTTTTGGGTTTGGAATCTG and 5’TTCCTTTCAATGAC and 5’CGCTCAGAGGTTTGAAC (323 bp); ARS1 5’TGGTGTTGATGTAAGCGGAG and 5’AAAGTCAACCCCCTCGGATG (270 bp); non-ARS 5’TTGGCAGTAACTGAC and 5’CCGTCAGAGGTTTGAAC (323 bp); ARS1 5’TGGTGTTGATGTAAGCGGAG and 5’AAAGTCAACCCCCTCGGATG (270 bp); non-ARS 5’TGGTGTTGATGTAAGCGGAG and 5’AAAGTCAACCCCCTCGGATG (270 bp); non-ARS 5’TGGTGTTGATGTAAGCGGAG and 5’AAAGTCAACCCCCTCGGATG (270 bp); non-ARS 5’TGGTGTTGATGTAAGCGGAG and 5’AAAGTCAACCCCCTCGGATG (270 bp); non-ARS 5’TGGTGTTGATGTAAGCGGAG and 5’AAAGTCAACCCCCTCGGATG (270 bp); non-ARS 5’TGGTGTTGATGTAAGCGGAG and 5’AAAGTCAACCCCCTCGGATG (270 bp).

RESULTS

Origin Activities of ARSs on Chromosome VI in Premeiotic S-phase—Fig. 1, A and B show S. cerevisiae cell growth during the transfer from mitotic to meiotic cell cycle. In response to nitrogen starvation, mitotic cells enter the meiotic cell cycle synchronously at the point in G1 phase called “Start” to undergo premeiotic DNA synthesis, which is followed by recombination, twice of chromosome division and finally spore formation. To determine the period of the premeiotic S-phase precisely, we assessed the progression of meiotic cell cycle by FACs and by microscopic observation of nuclear division. We used the SK1 strain as a wild type because of its efficient sporulation properties compared with other strains (24).

### Table 1

| Strain          | Genotype                                      | Ref./Source |
|-----------------|-----------------------------------------------|-------------|
| NKY278          | MATα ho::LYS2/ ura3/ lys2/                   | Ref. 14     |
| NKY274          | MAT α ho::LYS2 ura3 lys2                    | Ref. 14     |
| NKY730          | MATα ho::LYS2/ hsp306ORC1-HA/C               |            |
| SKY10201        | MATα ho::LYS2/ orc1::ORC1-3HA-URA3/         |            |
| SKY10009        | MATα ho::LYS2/ orc1::ORC1-3HA-URA3/         |            |
| SKY10201        | MATα ho::LYS2/ orc1::ORC1-3HA-URA3/         |            |
| SKY10205        | MATα ho::LYS2/ orc1::ORC1-3HA-URA3/         |            |
| RY260 (W303)    | MATα ha::LYS2/ rplb1-1                      | Ref. 34     |
| SKY10010 (50% SK1) | MATα ho::LYS2/ rplb1-1/(RY260 × NKY274)   |            |
| SKY10011 (75% SK1) | MATα ho::LYS2/ rplb1-1/ ure6::KanMX/     |            |

2 The abbreviations used are: ORF, open reading frame; HA, hemagglutinin; FACS, fluorescence-activated cell sorter; SPM, sporulation medium; N/N two-dimensional gel electrophoresis, neutral/neutral two-dimensional agarose gel electrophoresis; RT, reverse transcriptase; ChIP, chromatin immunoprecipitation; WCE, whole cell extract; ARS, autonomously replicating sequence; ORC, origin recognition complex.

![Image 357x26 to 384x38]
This result indicated that ARS605 was inactivated in the pre-meiotic S-phase except for ARS605 (Fig. 1A). The activity of origins was similar between the mitotic and pre-meiotic S-phase. To determine the activity of 10 ARSs on chromosome VI as replication origins, we pooled cells every 15 min from 1–4 h after the induction of sporulation and performed the N/N two-dimensional gel electrophoresis (Fig. 1B, upper row). The activity of origins was examined from the FACS profile and kinetics of meiotic divisions (Fig. 1A). The spore formation rate reached 99% (Fig. 1B) 10 h after induction, suggesting that DNA synthesis observed by FACS indeed occurred in the pre-meiotic S-phase.

We determined that 90% of cells entered premeiotic S-phase between 1–2 h and finished around 4–5 h after induction, judging from the FACS profile and kinetics of meiotic divisions (Fig. 1A). The spore formation rate reached 99% (Fig. 1B) 10 h after induction, suggesting that DNA synthesis observed by FACS indeed occurred in the premeiotic S-phase.

To determine the activity of 10 ARSs on chromosome VI as replication origins, we pooled cells every 15 min from 1–4 h after the induction of sporulation and performed the N/N two-dimensional gel electrophoresis (Fig. 1C). Because the SK1 strain is genetically distant from the S288C strain, the genomic dimension of which has been determined (24); we newly measured the activity of all ARSs during mitotic growth in the SK1 strain. This result indicated that ARS605 was inactivated in the premeiotic S-phase. ARS605 is located within the ORF of MSH4 (a MsiS homolog) and transcribed in early meiosis (25, 26). We assumed that the loss of ARS605 activity was because of the MSH4 transcription because it is known that transcription from a strong galactose inducible promoter interferes with origin functions on plasmid (27).

**Replicon Structure in Meiosis**

Transcription of MSH4 Interferes with Origin Activity of ARS605 in Meiosis—To confirm our hypothesis, we examined the relationships between MSH4 transcription and ARS605 origin activity in the premeiotic S-phase (Fig. 2). The transcription of MSH4 was induced at between one and two hours and reached the level of 10-fold of time 0 at three hours after the induction of meiosis, which is in good agreement with the previous data (24). The level of MSH4 transcripts at 3 h was maintained at the same level up to 5 h (data not shown), suggesting that MSH4 was transcribed actively during the premeiotic S-phase.

It was reported previously that the origin function of an ARS1 was inactivated by strong transcription that run through it (27). However, it is not yet clear how the origin function is actually perturbed by transcription at the molecular level. One possible mechanism of inactivation of ARS605 is that transcription disturbs the assembly of prereplication complex, which is the binding of ORC and mini-chromosome maintenance complexes to DNA sequence of the ARS.

We therefore examined whether the ORC binding was affected by transcription using the conventional ChIP method (18). Three primer sets that cover ARS605, ARS607, and a region 4 kb away from ARS1 (non-ARS) were designed. We examined the binding of Orc1, the largest subunit of ORC, during the premeiotic S-phase. Fig. 2B shows that binding of Orc1 to ARS605 was comparable to ARS607 until 1.5 h after the induction but decreased gradually starting from 2 h and reached one tenth of the binding level to ARS607 by 4 h. We further confirmed the decreased binding of Orc1 to ARS605 using ARS1 as a positive control (Fig. 2C) because ARS1 was an active origin in the premeiotic S-phase (28). The decrease in Orc1 binding to ARS605 paralleled negatively with the increase in the transcription of MSH4, suggesting strongly that the removal of Orc1 by transcription had disrupted the origin function of ARS605.

Transcription of MSH4 Interferes with Origin Activity of ARS605 in Mitosis—Induction of the early meiosis-specific gene family including MSH4 is dependent on the transcription factors Ime1 and Ume6 (29). In an ume6 deletion mutant, early meiosis-specific genes are derepressed during vegetative growth (24, 29). Therefore, we further confirmed the relationships between MSH4 transcription and ARS605 origin activity by introducing a deletion mutation of ume6 to the cell. We...
Replicon Structure in Meiosis

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

FIGURE 2. Regulation of MSH4 transcription and Orc1 binding to ARS605 in meiosis. A, abundance of MSH4 mRNA measured by quantitative RT-PCR. The amount of the MSH4 mRNA in the wild-type cell was measured by quantitative PCR method. The mRNA of a housekeeping gene, \( \beta \)-actin (ACT1), was used as an internal standard to normalize technical variations. The amplified products were separated by agarose gel electrophoresis, and their densities were determined by an Image Gauge software (Fujifilm). The number of cycle used was MSH4 20 cycles and ACT1 20 cycles. \( t_0 \) corresponds to the time of resuspension of cells in SPM. Under the condition, target cDNA fragments were amplified linearly. Bars indicate standard errors in two measurements. B, the physical association of Orc1 with ARS605 and ARS607. The chromatin immunoprecipitation assay was used to measure the amount of Orc1 bound to ARS605. The HA-tagged Orc1 was immunoprecipitated with anti-HA monoclonal antibody from cells in SPM at indicated times or from asynchronously growing mitotic cells (M). \( t_0 \) corresponds to the time of resuspension of cells in SPM. The DNA from whole cell extract (WCE) and DNA co-immunoprecipitated (IP) with HA antibody were amplified by using primer sets corresponding to sequences around ARS607, ARS605, and non-ARS sequences (located at 4 kbp away from ARS1). The expected PCR products for ARS605 (323 bp), ARS607 (279 bp), and non-ARS (228 bp) are shown on the WCE column. To ensure the linearity of the PCR signal, appropriate dilutions of WCE and IP samples were used in PCR amplification. The amplified products were examined by agarose gel electrophoresis. To quantify the result, the intensities of the bands of PCR products were measured by an Image Gauge software (Fujifilm), and the relative values were presented in the bottom graph. Values were normalized against WCE. Bars indicate standard errors in two measurements. C, the physical association of Orc1 with ARS605 and ARS1. The DNA from WCE and DNA co-immunoprecipitated with HA antibody (IP) were amplified by using primer sets corresponding to sequences around ARS1, ARS605, and non-ARS sequence. The expected PCR products for ARS605 (323 bp), ARS1 (270 bp), and non-ARS (228 bp) are shown on the WCE column. Levels of Orc1 binding were measured and analyzed as in shown in B. Bars in the bottom graph are standard errors of two experiments.

DISCUSSION

Through the chromosome-wide investigation of replicon dynamics during the mitotic and premeiotic S-phase, we found that the activity of an efficient origin in the mitotic S-phase, ARS605, was completely lost in the premeiotic S-phase (Fig. 1C). The inhibition was correlated with the induction of transcription of a meiosis-specific gene MSH4 after the onset of presporulation cell growth (Fig. 2). We have also demonstrated that Orc1 binding to ARS605 origin was reduced specifically in parallel with the increase in MSH4 transcription. Because ARS605 is located within the ORF of MSH4, we tested the possibility that the origin activity is inversely correlated with the transcription that runs through the origin region and removes ORC complex that is essential for the origin activity. As expected, the activity of ARS605 was completely dependent on the UME6 gene function that regulated MSH4 negatively during mitotic cell and positively during meiotic cell cycle (Fig. 3). Furthermore, we showed that RNA polymerase was required for the origin inactivation (Fig. 3). A similar result has been reported by an artificial system, where the gal promoter fused to ARS1 does abolish origin activity when transcription is induced assumed that derepression of MSH4 transcription by the \( \text{ume6} \) mutation would inhibit ARS605 activity even in the mitotic S-phase. Fig. 3A shows that MSH4 was transcribed at the level comparable to that in the meiosis in the absence of the \( \text{ume6} \) function. As expected, N/N two-dimensional gel electrophoresis showed that activity of ARS605 as indicated by bubble arc disappeared completely in the \( \text{ume6} \) deletion mutant during mitotic cell cycle (Fig. 3B). The binding of Orc1 to ARS605 was reduced and hardly detectable by ChIP analysis (Fig. 3C). These results confirm that MSH4 transcription interferes with ARS605 activity by removing ORC from its binding site.

Mutation in RNA Polymerase II Can Restore Origin Activity—To investigate whether or not the removal of ORC from the origin, ARS605, was because of the actual elongation of transcription through it, we have examined the effect of a temperature sensitive mutation of RNA polymerase II, \( rpb1-1 \) (30), on the derepression of MSH4 transcription by \( \text{ume6} \) deletion mutation. Surprisingly, the MSH4 transcription in \( \Delta \text{ume6} \) mutant cells in the mitotic phase was reduced by the introduction of \( rpb1-1 \) even at permissive temperatures (Fig. 3A). Therefore we analyzed the ARS605 activity in a \( \Delta \text{ume6}/rpb1-1 \) double mutant at permissive temperatures and found that the origin activity of ARS605 was recovered (Fig. 3B). The Orc1 binding to ARS605 was also restored under the same condition (Fig. 3C). These results indicated that the inactivation of the replication origin was caused by the removal of ORC from its binding site, possibly by collision with RNA polymerase II.
by galactose, although no molecular mechanism is indicated (27). Here, we demonstrated clearly that replication origin 
activity was negatively regulated through transcription, which 
interfered the binding of ORC complex to the replication 
origin either directly by running of RNA polymerase or indi-
rectly by transcription-associated events like changes in 
DNA topology or chromatin structure. Based on these 
results, we propose a model for the inactivation of ARS activ-
itity by transcription (Fig. 4).

Previous studies have shown that the length of S-phase is 
different between the mitotic and meiotic cell cycle, whereas 
eLONGATION rate of replication as well as initiation activity of all 
ARS on the chromosome III is invariable between the two cell 
cyCLES. Present finding of the loss of one of the three active 
origins on the chromosome VI may reduce the rate of replica-
Tion of the chromosome specifically in meiotic cycle. If inverse 
relationships between transcription of genes and initiation of 
replication are general features of the replication origins of the 
eukaryotic cells, origins located within the transcription units 
should affect the S-phase progression, dependent on physiolog-
ic conditions, that affects transcription of those particular 
genes. It is therefore interesting to determine replication or-
gins that are located within the transcription units. In particu-
ar, transcriptions specific for the meiotic cell cycle should be 
examined carefully for the presence of ORC binding sites, ACS, 
as possible origins of replication that affect S-phase progression 
during meiotic cell growth.

Chromosomes in the pre-blastula Xenopus embryonic cells 
initiate replication at many evenly spaced sites distributed at 
random throughout the chromosomes (31). And it was shown 
that reduction in number and specifications of origins are first
observed at the time of the mid-blastula transition (32, 33). The mechanisms behind the origin specification remain poorly understood. However, it is well known that mid-blastula is accompanied by the increases in transcription of zygotic genes. Similar inverse correlation between origin activation and induction of transcription proposed in Fig. 4 may explain the origin specification during early development of *Xenopus* embryonic cells.

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