Amino Acids (2011) 40:623–631
DOI 10.1007/s00726-010-0687-z

ORIGINAL ARTICLE

Mcm1p binding sites in the *ARG1* promoter positively regulate *ARG1* transcription and *S. cerevisiae* growth in the absence of arginine and Gcn4p

Suntaek Hong · Sungpil Yoon

Received: 3 May 2010 / Accepted: 30 June 2010 / Published online: 13 July 2010

Abstract In this study, we investigated the activating role of Mcm1p at *ARG1* during arginine starvation. Our results showed that two Mcm1p binding sites positively contribute to *ARG1* transcription and cell growth. Especially, we provide strong evidence that the Mcm1p binding sites play a positive role in *ARG1* transcription to overcome arginine starvation in the absence of Gcn4p. In addition, we found that the Mcm1p binding sites are not only regulated by the presence or absence of arginine but also in the presence or absence of other amino acids. These findings suggest that the *ARG1* promoter utilizes different DNA elements to control transcription, depending on which amino acids are detected in the medium.

Keywords Gcn4p · Mcm1p · *ARG1* · SD and SC media · Arginine starvation

Abbreviations

SD	Synthetic defined minimal
SC	Synthetic defined complete
ARC	Arginine control
MADS	*M*CM1, *A*GAMOUS, *D*EFICIENS, and *s*erum response factor
WT	Wild type
ARG	Arginine
URA	Uracil
Can	Canavanine

Electronic supplementary material The online version of this article (doi:10.1007/s00726-010-0687-z) contains supplementary material, which is available to authorized users.

S. Yoon (✉)
Research Institute, National Cancer Center,
809 Madu 1-dong, Ilsan-gu, Goyang-si,
Gyeonggi-do 411-764, Republic of Korea
e-mail: yoons@ncc.re.kr

S. Hong
Lee Gil Ya Cancer and Diabetes Institute,
Gachon University of Medicine and Science,
Incheon 406-840, Republic of Korea

Introduction

Gcn4p and Mcm1p are two important transcription factors in *Saccharomyces cerevisiae*. Transcription of most amino acid biosynthetic genes, including the *ARG* genes, is induced by Gcn4p in cells starved for any amino acid through increased expression of *GCN4* at the translational level (Natarajan et al. 2001). Mcm1p is an MCM1, AGAMOUS, DEFICIENS, and serum response factor (MADS) box transcription factor protein that regulates responses to environmental conditions (Messenguy and Dubois 1993). Mcm1p cooperates with diverse sequence-specific transcription factors such as α1p, α2p, Ste12p, Yox1p, Yhp1p, and Fkh2p at their promoters, either stimulating or impeding the function of transcription factors that regulate cell type specification or the cell cycle (De Rijcke et al. 1992; Messenguy and Dubois 1993, 2003). Mcm1p also mediates repression of *ARG* genes by forming the ArgR/Mcm1p repressor complex through interactions with Arg80p, Arg81p, and Arg82p (Messenguy and Dubois 1993, 2003; Yoon et al. 2004).

Transcription of four arginine biosynthesis genes (*ARG1*, *ARG3*, *ARG5,6*, and *ARG8*) is regulated by the presence or absence of arginine (Hinnebusch 1992; Messenguy and Dubois 2003; Yoon et al. 2004). The *ARG1*

gene has been widely studied as a representative model for transcriptional regulation of arginine biosynthetic genes. The *ARG1* gene is subject to dual regulation by the transcriptional activator Gcn4p and repressor Mcm1p. Genetic and biochemical analyses of the *ARG1* promoter have shown that two arginine control elements, ARC1 and ARC2, are required for efficient repression of *ARG1* expression by the ArgR/Mcm1p complex in arginine-containing medium and that two Gcn4p binding sites contribute to the efficient activation of the gene by Gcn4p. Each of the half sequences of the two ARC elements contains a MADS box, the binding site for Mcm1p (Hinnebusch 1992; Crabeel et al. 1995; Yoon et al. 2004; Yoon and Hinnebusch 2009) (Fig. 1a).

Previously, we investigated the *ARG1* promoter in Gcn4p-inducing conditions using the Chromation immunoprecipitation (ChIP) assay and mutational analysis at binding sites of the regulators (Yoon et al. 2004; Yoon and Hinnebusch 2009). We found a novel role for Mcm1p as a transcriptional activator at *ARG1*. Based on these results, we proposed that Mcm1p contributes to active transcription at the *ARG1* promoter by increasing the binding of the activator Gcn4p and by recruiting the co-activator complex SWI/SNF at *ARG1* under Gcn4p-induced conditions (Yoon and Hinnebusch 2009).

It has not been determined whether the arginine starvation condition requires Mcm1p for *ARG1* transcription and growth, as does Gcn4p-inducing conditions upon isoleucine and valine starvation (Yoon et al. 2004; Yoon and Hinnebusch 2009). It is important to determine which Gcn4p or Mcm1p binding sites require these entities under arginine starvation conditions. It is also of interest to determine whether Mcm1p still plays a positive role for *ARG1* transcription in the absence of Gcn4p. Therefore, the present study was undertaken to investigate the role of DNA elements in the *ARG1* promoter for transcription and growth under arginine starvation conditions with canavanine in the presence or absence of Gcn4p. Our results indicate that the *ARG1* promoter utilizes different DNA elements for its transcription as a function of the presence

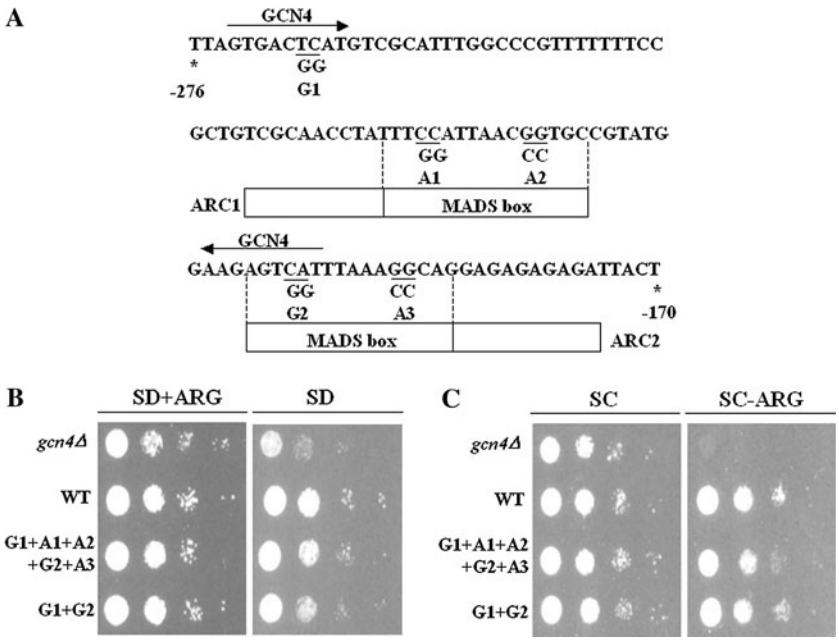

Fig. 1 Structure of the *ARG1* promoter and mutational analysis of cell growth in the absence of arginine. **a** Nucleotide sequence of the *ARG1* promoter region. The sequence is numbered (−276 to −170, indicated by *asterisks*) relative to the main transcription start site. The Gcn4p binding sites (GTGACTCAT and AAGAGTCAT) are *overlined*. The consensus sequence for Gcn4p contains nine nucleotides, ATGACTCAT. Both the 5′-most Gcn4p binding site and the second Gcn4p binding site (present in reverse orientation) contain eight consensus nucleotides. There are two ARC elements (ARC1 and ARC2) indicated by *boxes*. The putative Mcm1p binding sites are boxed within the ARC elements and are designated as MCM1, AGAMOUS, DEFICIENS, and serum response factor (MADS) *boxes*. The consensus sequence for the MADS box, called the P box, contains 16 nucleotides, TTTCCCTATTAGGTAA. The first MADS box in ARC1 includes a greater number of consensus nucleotides (nine) than the second MADS box in ARC2 (six nucleotides). Both Gcn4p binding sites and MADS boxes are in opposite orientations and designated with *arrows*. Six base pairs in the second Gcn4p site overlap the second MADS box. The site-directed mutagenized sequences are *underlined* and indicated below by G1, A1, A2, G2, and A3. Note that the GG mutation in the second Gcn4p binding site also mutated two residues in the MADS box in ARC2. **b** and **c** Spot assay of SS5 strains containing mutations as indicated (Table 1): *gcn4Δ* (SY722), WT (SY539), mutant G1 + A1 + A2 + G2 + A3 (SY747), and mutant G1 + G2 (SY749). Cells were grown to saturation in SD + URA medium at 30°C, serially diluted tenfold, and spotted onto SD + ARG, SD, SC, or SC-ARG plates supplemented with uracil. The growth phenotype following 1 day incubation at 30°C is shown

or absence of excess amino acids. We also provide strong evidence that the Mcm1p binding site plays a positive role in *ARG1* transcription to overcome arginine starvation in the absence of Gcn4p.

Materials and methods

Reagents and medium

L-Canavanine was purchased from Sigma-Aldrich (St. Louis, MO). SD medium (nitrogen base, ammonium sulfate, and glucose) or SC medium (nitrogen base, ammonium sulfate, glucose, and complete amino acids) supplemented with uracil was used in the presence or absence of arginine (Sherman 1991).

Yeast strains and plasmids

All strains used in this study are listed in Table 1 of the Supporting Information. Strain SS5 (*MATα ura3Δ argΔPH*) (Crabeel et al. 1995) was a gift from Marjolaine Crabeel. SS5 was transformed with *Eco*RI-digested 3-kb *ARG1* fragments from plasmids pSY507-pSY527 (Table 2) carrying mutations in the Gcn4p or Mcm1p binding sites, and selected for Arg^+ colonies (Yoon and Hinnebusch 2009). Deletion of *GCN4* in SS5 Arg+ transformants was achieved by transformation with SspI-digested plasmid pHQ1240 containing *gcn4Δ::hisG-URA3-hisG* and selection of Ura^+ colonies (Yoon et al. 2003). The *URA3* gene was subsequently removed by selecting for growth on medium containing 5-fluoroorotic acid.

To construct pSY507, the 3-kb *ARG1 Eco*RI fragment (from −862 bp upstream of the start codon to −356 bp from 3′ of the stop codon) from M13mp7-*ARG1* (a gift from Marjolaine Crabeel) was cloned into *Eco*RI-digested pRS425 to produce plasmid pSY507. We designed 35- to 40-mer mutagenic primers containing specific mutations in the *ARG1* sequence and used a Mutagenesis Kit (Stratagene, San Diego, CA) to introduce these mutations into pSY507. Mutagenized plasmids are described in Table 2 of the Supporting Information, and those produced in this study were verified by sequence analysis (Yoon and Hinnebusch 2009).

Spot assay

Cells were grown to saturation in SD + URA or SC + URA media containing arginine, serially diluted tenfold, and spotted onto SD + URA, SD + URA + ARG, SC + URA, and SC + URA-ARG plates in the presence or absence of canavanine. Plates were incubated at 30°C for at least 1 day. We performed more than two independent tests for all experiments.

Northern blot analysis

Northern blot analysis was carried out as described previously (Qiu et al. 2004; Yoon et al. 2004) with the following modifications. Briefly, cells were grown overnight at 30°C in YPD. Cells were inoculated in SD + URA + ARG, SD + canavanine, or SC + URA-ARG + canavanine media and incubated at 30°C for 6 h. RNA were prepared from these cultures and subjected to Northern blot analysis. The *SCR1* gene is transcribed to produce a non-coding RNA. The blots were probed for *ARG1* and *scR1* (as internal controls). The primers used to amplify the *ARG1* open reading frame (ORF) to generate 523 bp polymerase chain reaction (PCR) products were 5′ TCTGGTG GTTTAGATACCTC 3′ (sense) and 5′ CTTGGTTTTCG TCAGTAGAC 3′ (antisense). The primers used to amplify *SCR1* ORF to generate 486 bp PCR products were 5′ ATACGTTGAGAATTCTGGCC 3′ (sense) and 5′ TGGT TCAGGACACACTCCAT 3′ (antisense). The PCR products were resolved by agarose gel electrophoresis and purified from the agarose matrix. The PCR products were used for probe synthesis, and the radiolabeled PCR probes were used for membrane hybridization after gel-filtration column purification. ULTRAhyb ultrasensitive hybridization buffer (Ambion, Austin, TX) was used for prehybridization and hybridization as described by the vendor except for the washing conditions. After hybridization, the membranes were washed once with 2× SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature, once with the same buffer at 55°C, and once with 1× SSC, 0.1% SDS buffer at 55°C for 15 min each; then they were washed twice with 0.1× SSC, 0.1% SDS at 55°C for 20 min each. The washed membranes were subjected to phosphorimaging analysis for autoradiography. More than two independent tests were performed in all experiments.

Results and discussion

The Mcm1p binding site of *ARG1* positively contributes to growth during arginine starvation with canavanine

The *ARG1* promoter includes two ARC elements and two Gcn4p binding sites that are designated in Fig. 1a. We introduced point mutations into the chromosomal copy of *ARG1* for the predicted Mcm1p binding sites (MADS boxes) in the ARC elements and Gcn4p binding sites to determine whether Mcm1p and Gcn4p play roles in growth and *ARG1* transcription during arginine starvation (Table 1 in Supporting Information) (Yoon and Hinnebusch 2009). We designated the mutants G1, A1, A2, G2, and A3 (Fig. 1a). To eliminate Mcm1p binding at each ARC

element, we replaced the conserved CC residues in each predicted half-site with GG residues in each of the two ARC elements (Tan and Richmond 1998; Yoon and Hinnebusch 2009). We also made GG mutations in one of the half-sites in each Gcn4p binding site to determine the effect of mutation of Gcn4p binding sites on growth and transcription (Fig. 1a). The GG mutation in the second Gcn4p binding site also mutated two residues at the Mcm1p binding site in ARC2. Even though the mutation G2 altered the Gcn4p binding site and the ARC2 element, we assumed that the mutation plays a major role in the Gcn4p binding site, but not for the Mcm1p binding site, in the presence of Gcn4p. We also generated multiple mutations to observe redundant functions of each mutant G1, A1, A2, G2, and A3. In addition, multiple mutations were introduced into the chromosomal copy of *ARG1* (Tables 1 and 2 in Supporting Information).

Next, we tested which mutants showed growth defects with arginine-dependence on SD or SC plates. In the *gcn4* deletion strain, we observed more severe growth defects on SC than on SD plates in the absence of arginine, suggesting that an excess of amino acids might suppress *ARG1* expression (Fig. 1b, c); thus, Gcn4p is more necessary on the SC plate during arginine starvation. We assumed that excess of other amino acids negatively regulated *ARG1* transcription. When we observed growth of the mutants, strains mutated at all five sites (mutant G1 + A1 + A2 + G2 + A3) or at the two Gcn4p binding sites (mutant G1 + G2) did not exhibit growth defects in the absence of arginine (Fig. 1b, c). These results indicate that mutants of Gcn4p binding sites could maintain sufficient *ARG1* transcription to preserve normal growth in the absence of arginine.

Next, we tested their growth with addition of canavanine, which renders arginine-deficient mutant cells unable to grow (Penn et al. 1983). Due to its structural similarity to arginine, canavanine behaves as a competitive inhibitor of arginine, interfering with arginine-utilizing enzymes. We hypothesized that the addition of canavanine increases the requirement for *ARG1* transcription, allowing the investigation of DNA elements in the *ARG1* promoter that are important for growth and transcription. Mutant G1 exhibited a slight growth defect on SD plates containing a high concentration of canavanine, while no growth defects were evident for the other single mutants A1, A2, G2, and A3 (Fig. 2a, b). These results suggest that the first Gcn4p binding site is more important for growth during arginine starvation than other single mutants. When we examined the phenotypes of mutants with multiple binding site mutations, we observed a growth defect in cells carrying mutations G1 and G2 (Fig. 2b). This suggested that the two Gcn4p binding sites in *ARG1* have redundant functions for growth on SD plates. At low concentrations of canavanine (0.05 μg/ml), mutant G1 + G2 had the same phenotype as mutant G1 + A1 + A2 + G2 + A3 (Fig. 2b), suggesting that the MADS boxes were not required for growth. However, comparing the growth of mutant G1 + G2 with mutant G1 + A2 + G2 or mutant G1 + A1 + A2 + G2 + A3 at a high concentration of canavanine (0.1 μg/ml), we found that the growth defect of mutant G1 + G2 was more pronounced when combined with mutation A2 (Fig. 2b), indicating that the first Mcm1p binding site positively influences cell growth under these conditions. We did not detect any growth defect in mutants A2 + G2, G1 + A2, or A1 + A2 + G2 + A3, suggesting that the first Mcm1p binding site positively influences growth only when both Gcn4p binding sites are mutated. The results support the conclusion that the first Mcm1p binding site has a positive role for growth during arginine starvation and when the two Gcn4p binding sites are mutated.

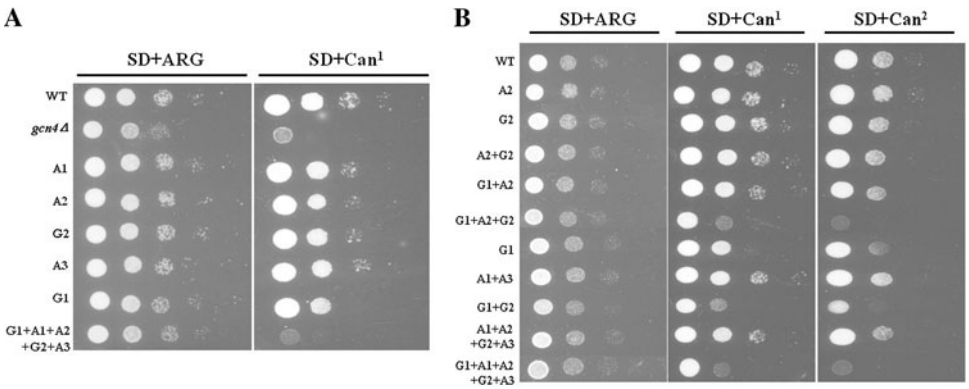

Fig. 2 The Mcm1p binding site in the *ARG1* promoter positively influences growth on the SD plate during arginine starvation. **a** and **b** SS5 strains carrying chromosomal *ARG1* mutant alleles are indicated on the *left* (see Table 1 for description of strains). Cells were grown to saturation in SD + URA medium at 30°C, serially diluted tenfold, and spotted onto SD + ARG, SD + Can^1 (Canavanine 0.05 μg/ml), or SD + Can^2 (Canavanine 0.1 μg/ml) supplemented with uracil. The growth phenotype following 1 day of incubation for SD + ARG and 2 days for Canavanine plates at 30°C is shown

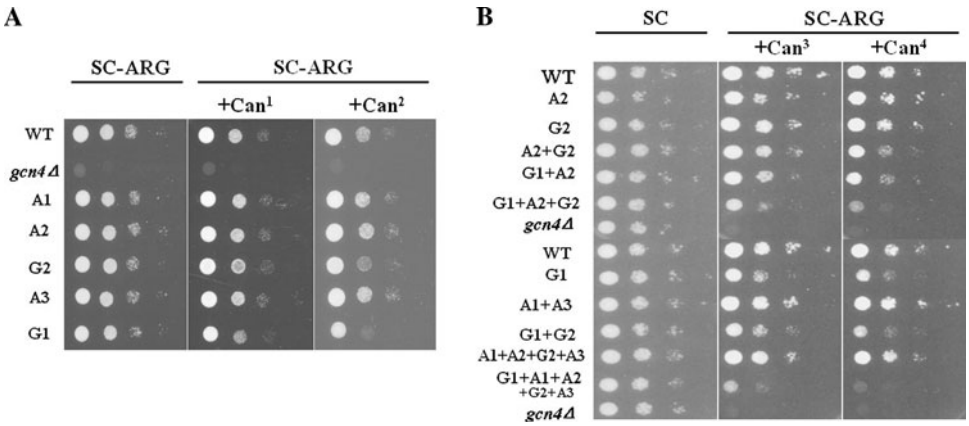

Fig. 3 The Mcm1p binding site in the *ARG1* promoter positively influences growth on the SC plate during arginine starvation. **a** and **b** SS5 strains carrying chromosomal *ARG1* mutant alleles are indicated on the *left* (see Table 1 for description of strains). Cells were grown to saturation in SC + URA medium at 30°C, serially diluted tenfold, and spotted onto SC-ARG or SC. They were also spotted onto SC-ARG + Can^1 (Canavanine 0.25 μg/ml), Can^2 (Canavanine 1.5 μg/ml), Can^3 (Canavanine 0.5 μg/ml), or Can^4 (Canavanine 1.0 μg/ml) supplemented with uracil. The growth phenotype following 1 day of incubation for SC and SC-ARG and 2 days for Canavanine plates at 30°C is shown

Studying growth phenotypes required an approximately tenfold higher canavanine concentration on SC plates than on SD plates. The growth phenotypes of mutants in SC medium were somewhat different from those observed in SD, suggesting that regulation of the *ARG1* promoter depends on an excess of amino acids. We found that the sequences mutated in single mutant G1 were required for growth on SC plates with canavanine (Fig. 3a). The mutant G1 defect seemed to be more substantial on the SC plate than the SD plates. The first Gcn4p binding site appeared to be more important than the second Gcn4p binding site in SC medium during arginine starvation, because mutant G1 had a similar phenotype to the double mutant G1 + G2 (Fig. 3b). As observed in SD medium (Fig. 2b), mutant G1 + G2 displayed a greater growth defect in SC medium when combined with mutation A2 in the Mcm1p binding site (Fig. 3b). At high canavanine concentrations, we also detected a greater growth defect in the double mutant A2 + G2 than in the single mutant G2.

Based on the mutational analysis of growth on SD and SC plates under conditions of arginine starvation, we concluded that the Mcm1p binding site of *ARG1* plays a positive role in cell growth when the Gcn4p binding sites are mutated. In addition, it is interesting that the requirement of Gcn4p binding sites was stronger on the SC plate than the SD plate (Figs. 2a, b, 3a, b), as demonstrated by an increased requirement of Gcn4p on the SC plate (Fig. 1b, c).

The Mcm1p binding site of *ARG1* positively contributes to its transcription during arginine starvation

We performed Northern blot analysis to observe whether Mcm1p binding sites also contributed to *ARG1* mRNA levels. We found that the reduced transcription of mutant G1 + G2 was more pronounced when combined with mutation A2 in the SD medium (Fig. 4a). The double mutant G1 + A2 showed a greater reduction of *ARG1* transcription levels than the G1 or A2 single mutants in the SC medium (Fig. 4b). These results suggest that the first Mcm1p binding site plays a positive role in *ARG1* mRNA induction in SC and SD media during arginine starvation.

We also observed special features from our Northern blot analysis. We found that the single mutants G1 and G2 showed reduced *ARG1* transcription by arginine starvation (Fig. 4a, b), suggesting the importance of both Gcn4p binding sites for normal transcription of *ARG1*. In addition, the double mutant G1 + G2 showed an additive effect, such that it reduced transcription more than that of either single mutant G1 or G2 (Fig. 4a, b). These results seem to be distinct from the growth phenotypes (Figs. 2a, b, 3a, b). We assumed that quantification of *ARG1* mRNA levels by Northern blot analysis is more sensitive for detecting the differences among mutants. Considering that the single mutant A2 and double mutant G1 + A2 showed much lower *ARG1* transcription in SC medium than in SD medium, we assumed that the Gcn4p or Mcm1p binding sites are more necessary in SC medium. Although the results showed a slight difference between growth phenotype and *ARG1* transcription levels in the mutants, it could be concluded that the Mcm1p binding site of *ARG1* plays a positive role in *ARG1* transcription in both SC and SD media in arginine starvation.

The *GCN4* deletion strain requires Mcm1p binding sites for survival in the absence of arginine

The results of the aforementioned mutational analysis indicated that *ARG1* transcription could be activated

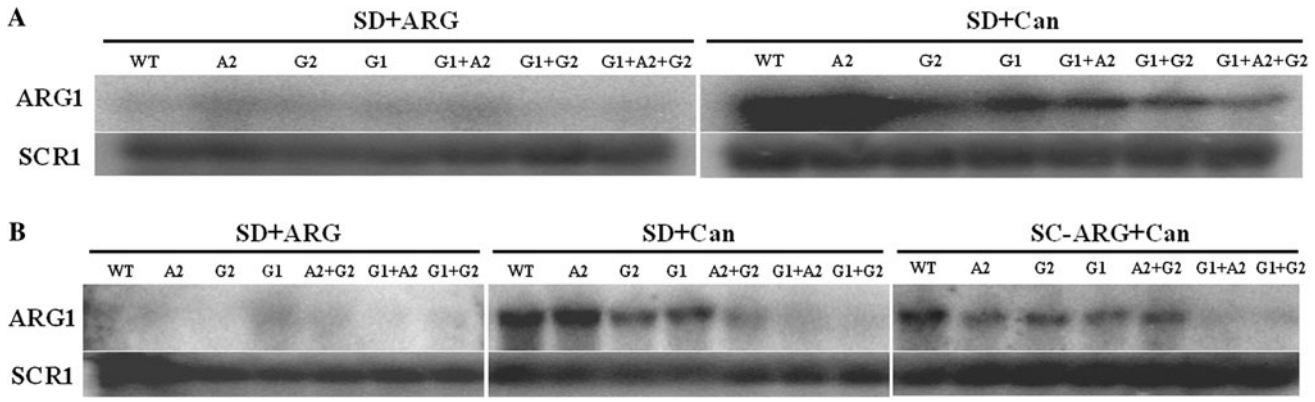

Fig. 4 The Mcm1p binding site in the *ARG1* promoter requires for *ARG1* transcription on both SD and SC plates during arginine starvation. **a** and **b** SS5 strains carrying chromosomal *ARG1* mutant alleles are indicated on the *top* (see Table 1 for description of strains). Cells were grown to saturation in SD + URA medium at 30°C and diluted 50-fold into the following culture media. The culture media were divided into three parts: SD + ARG, SD + Can (Canavanine 0.1 μg/ml), or SC-ARG + Can (Canavanine 0.5 μg/ml) supplemented with uracil. They were incubated for an additional 6-h for growth. Total RNA were prepared from these cultures and subjected to Northern blot analysis. The blots were probed for *ARG1* and *scR1* (as internal control) as described in "Materials and methods". The membranes were subjected to autoradiography

through the Mcm1p binding site independently of Gcn4p. However, it was also possible that mutation of the Mcm1p binding site could disrupt binding of Gcn4p, thus decreasing *ARG1* transcription. To distinguish between these possibilities, we examined the growth phenotypes of Gcn4p-deleted cells carrying mutations G1, A1, A2, G2, and A3. We assumed that mutation G2 plays a major role in the Mcm1p binding site in the absence of Gcn4p. The G1 mutation did not confer any growth defect, suggesting that the first Gcn4p binding site did not contribute to growth in Gcn4p-deleted cells (Fig. 5a, b). In arginine-starvation SD plates, mutations A2 and G2 resulted in growth defects; the defect was more severe in mutant G2 than in mutant A2, and the double mutant A2 + G2 exhibited a much more severe phenotype than mutant G2. Thus, both Mcm1p binding sites were required for growth in SD medium under conditions of arginine starvation in the absence of Gcn4p. The growth phenotypes of Gcn4p-deleted cells on SC plates without arginine were quite different from those observed on SD plates. Only sequence A2 was required for growth on SC (Fig. 5b); however, both mutants A2 and G2 exhibited growth defects on SD (Fig. 5a). The results supported the suggestion that sequence A2 in the *ARG1* promoter is generally required for activating *ARG1* transcription, but that sequence G2 is regulated differently in the presence or absence of excess amino acids, and is required for growth only in SD medium. It is possible that sequence G2 is a sensor for excess amino acids and activates *ARG1* transcription in the absence of excess amino acids, whereas it does not have an activating role when excess amino acids exist. For example, the absence of amino acids may induce an activating factor of *ARG1* transcription at sequence G2, or the presence of amino acids may induce inhibition of *ARG1* transcription at sequence G2. In addition, we assumed that G2 had a different role between the SD and SC media, since the growth severity varied with the presence and absence of excess amino acids. These findings suggest that the *ARG1* promoter is not only regulated by the presence or absence of arginine but also in the presence or absence of excess amino acids.

In addition, we found interesting results on the SD plate in the absence of Gcn4p. Although we did not observe any growth defects for single mutants A1 or A3, these mutations appeared to suppress the growth defects of mutants A2 and G2, since the multiple mutants A1 + G2, A2 + A3, A1 + G2 + A3, and A1 + A2 + A3 displayed reduced defects as compared with the A2 or G2 single mutant (Supplementary Fig. 1A, B). Interestingly, the suppressive functions of mutations A1 and A3 were restricted to interactions with mutations G2 and A2, respectively, since we did not detect the suppressive effect in the double mutants A1 + A2 or G2 + A3. Moreover, this suppressive effect was not observed if mutations A1 and A3 were combined with mutations of both A2 and G2, since mutant A1 + A2 + G2 + A3 displayed a growth defect (Supplementary Fig. 1A). These results suggest that the two Mcm1p binding sites might somehow interact with each other. We did not observe a suppressive effect for mutant A2 on SC plates (Supplementary Fig. 1A, B), since mutation A3 did not recover the growth defect of mutant A2 on SC plates.

We confirmed the requirements for *ARG*1 transcription of mutants A2 and G2 in Gcn4p-deleted cells using Northern blot analysis. As shown in Fig. 5c, mutants A2 and G2 showed reduced *ARG1* transcription during

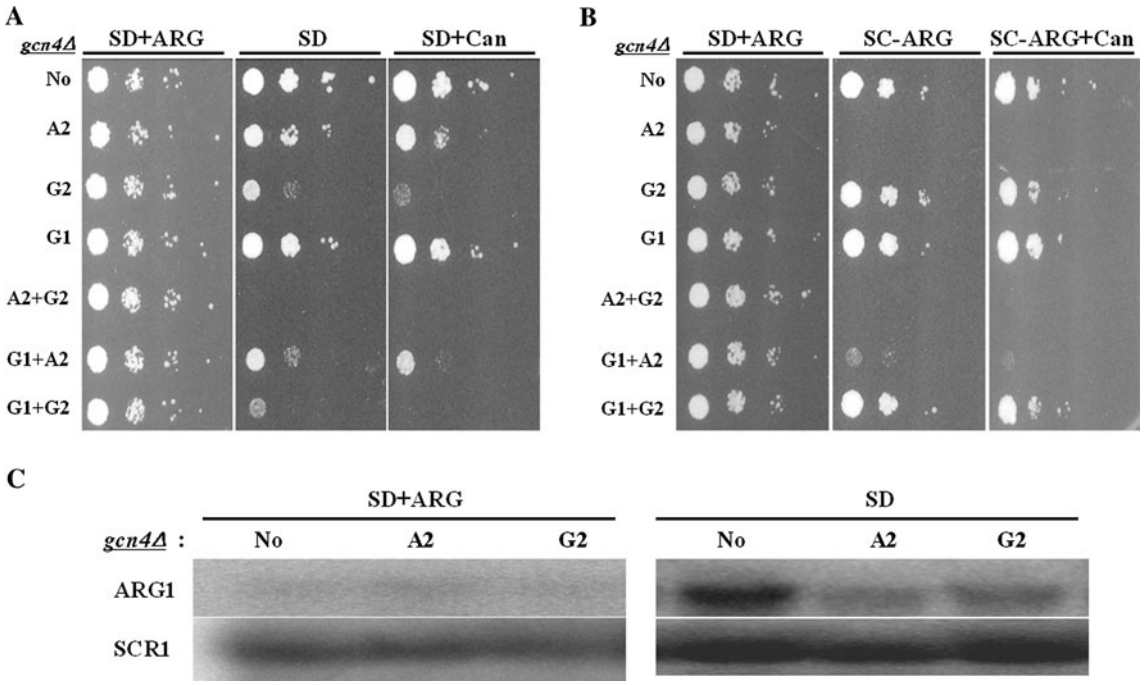

Fig. 5 The *GCN4* deletion strain requires the Mcm1p binding site for survival during arginine starvation. **a** and **b** *gcn4Δ* SS5 strains containing mutated *ARG1* alleles are indicated on the *left* (see Table 1 for description of strains). *No* indicates WT *ARG1* promoter in *gcn4Δ* SS5 strains. Cells were grown to saturation in SD + URA medium at 30°C, serially diluted tenfold, and spotted onto SD + ARG, SD, SD + Can (Canavanine 0.05 μg/ml), SC-ARG, or SC-ARG + Can (Canavanine 0.1 μg/ml) supplemented with uracil. The growth phenotype following more than 2 days of incubation at 30°C is shown. **c** *gcn4Δ* SS5 strains containing mutated *ARG1* alleles are indicated on the *top* (see Table 1 for description of strains). *No* indicates WT *ARG1* promoter in *gcn4Δ* SS5 strains. Cells were grown to saturation in SD + URA medium at 30°C and diluted 50-fold into following culture media. The culture media were divided into two parts: SD + ARG and SD supplemented with uracil. They were incubated for an additional 6 h for growth. Total RNA were prepared from these cultures and subjected to Northern blot analysis. The blots were probed for *ARG1* and *scR1* (as internal controls) as described in "Materials and methods". The membranes were subjected to autoradiography

arginine starvation. We found that expression level of mutant A2 was more reduced than mutant G2, although the growth phenotype showed more reduction in mutant G2. We assumed that different culture conditions somehow caused these differences. Nevertheless, our results clearly showed that Mcm1p binding sites play a positive role in *ARG1* transcription in the absence of Gcn4p, suggesting that Gcn4p is not the only activator involved in *ARG1* transcription.

Addition of several amino acids increases growth of mutant G2 on SD plates

Mutant G2 exhibited different growth phenotypes on SD and SC plates in Gcn4p-deleted cells (Fig. 6a), suggesting that the absence of amino acids other than arginine could positively regulate *ARG1* transcription through the second Mcm1p binding site (sequence G2) in the absence of arginine. To identify which amino acids were responsible for the growth defect of mutant G2 on SD plates, we added each single amino acid to SD plates and observed the growth phenotypes.

The growth of mutant G2 was increased by addition of Valine, Tryptophan, Asparagine, Glutamine, Alanine, Isoleucine, Leucine, Methionine, Phenylalanine, Threonine, or Tyrosine to SD plates (Fig. 6b–d). Mutant G2 had a greater growth defect than mutant A2 on the SD plate (Fig. 6a); however, the individual addition of the above amino acids resulted in better growth for mutant G2 than mutant A2 (Fig. 6b–d). In particular, Isoleucine, Phenylalanine, and Threonine resulted in much better growth recovery than the other amino acids. When we increased the amount of Phenylalanine, Threonine, or Isoleucine on SD plates, we found that growth of mutant G2 was enhanced to a level similar to that of non-mutant cells (Fig. 7a). When we combined the amino acids Phenylalanine, Threonine, and Isoleucine, we also observed that growth of mutant G2 was enhanced to a level similar to that of non-mutant cells. The results suggest that the exact amino acid supplied to the cells is as important as the amount of amino acid for sequence G2 regulation. In contrast, we observed that Cysteine, Glycine, Proline, and Lysine did not increase growth of mutant G2, even at higher concentrations (Fig. 7b). This suggests that the amount of the amino acid

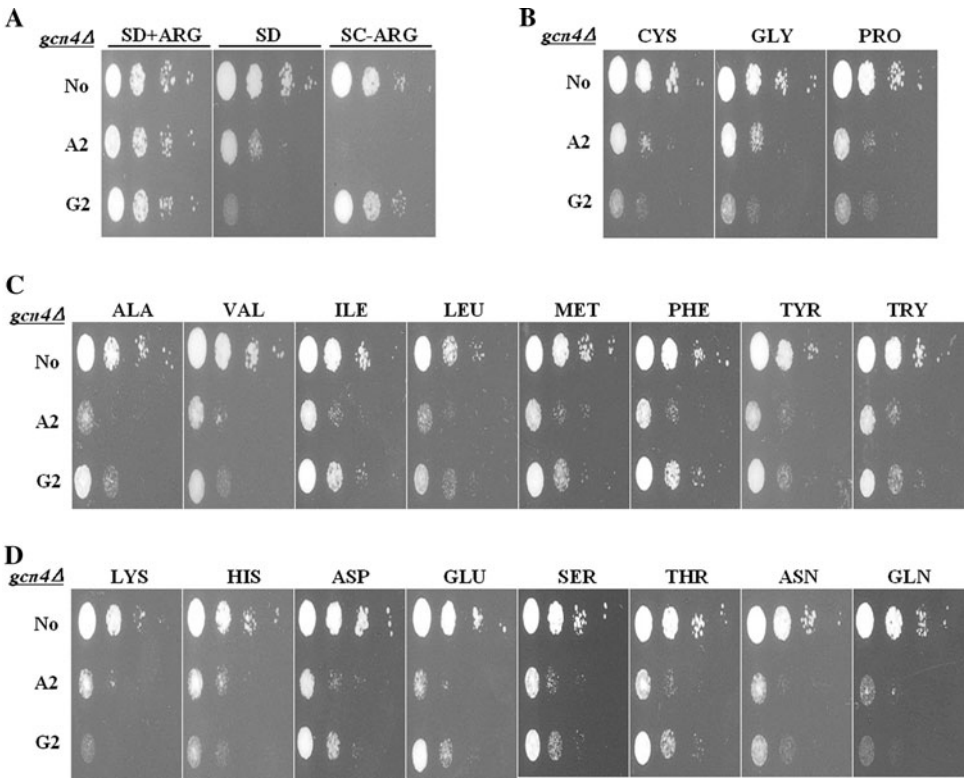

Fig. 6 Addition of several amino acids increases growth of mutant G2 on SD plates. **a** The *gcn4Δ* SS5 strains containing mutation A2 or G2 in the *ARG1* alleles are indicated at the *left* of the figure. *No* indicates WT *ARG1* promoter in *gcn4Δ* SS5 strains. The strains were grown to saturation in SD + URA medium at 30°C. The cells were serially diluted tenfold, and spotted on SD + ARG, SD, or SC-ARG, which were supplemented with uracil. They were incubated for 3 days at 30°C, and the growth phenotypes were observed. **b–d** Each amino acid was added to the SD + URA plates as indicated. The amino acids were dissolved at 1 g/100 ml, except Tyrosine (0.2 g/100 ml), and 200 µl were added to each SD + URA plate. The cells were serially diluted tenfold and spotted on the indicated plates. They were incubated for 3 days at 30°C, and the growth phenotypes were observed

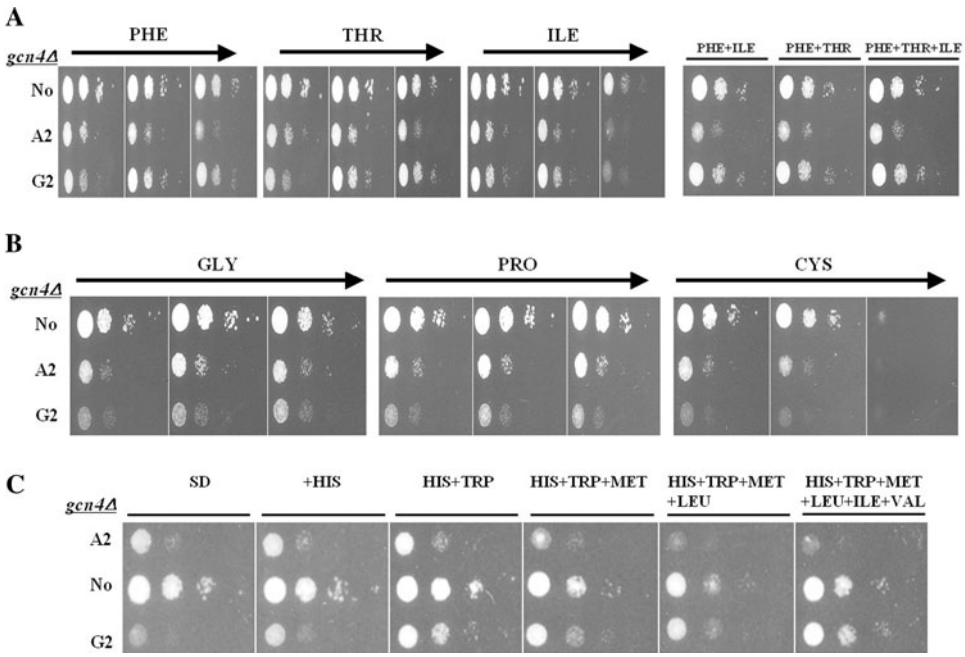

Fig. 7 Increased amounts of amino acids positively contribute to the growth rate of mutant G2. **a–c** The *gcn4Δ* SS5 strains containing mutation A2 or G2 in the *ARG1* alleles are indicated at the *left* of the figure. *No* indicates WT *ARG1* promoter in *gcn4Δ* SS5 strains. The strains were grown to saturation in SD + URA medium at 30°C. The cells were serially diluted tenfold and spotted on SD + URA plates supplemented with the indicated amino acids (1 g/100 ml). The *arrow* indicates increased concentrations of amino acids (*left* 20 µl, *middle* 100 µl, *right* 1,000 µl of amino acids in each plate). The cells were incubated for 3 days at 30°C, and the growth phenotypes were observed

is irrelevant for those amino acids that do not contribute to the regulation of mutant G2. We also found that the growth of mutant G2 was similar to that of non-mutant cells when single amino acids were added one-by-one (Fig. 7c). Although we did not find a specific amino acid that recovered the growth defect of mutant G2, the results suggest that the mutant G2 position could play a role in sensing the amount of amino acids in the medium. Our results indicate that transcription of *ARG1* is positively controlled by the Mcm1p binding site (mutant G2) in the absence of excess amino acids during arginine starvation in Gcn4p-deleted cells. Further experiments are required to identify the factors involved in this differential regulation of the *ARG1* promoter.

The collective data are consistent with the proposal that Mcm1p plays a positive role in *ARG1* transcription during arginine starvation, similar to its role as a transcriptional activator in several other promoters (Gavin et al. 2000; Darieva et al. 2003; Carr et al. 2004). Our results also indicate that transcription of *ARG1* is controlled by different mechanisms in the presence or absence of complete amino acids under conditions of arginine starvation. In addition, considering that SWI/SNF interacts with Mcm1p and plays a positive role in *ARG1* expression in the presence of Gcn4p (Yoon et al. 2004; Yoon and Hinnebusch 2009), it is also possible that SWI/SNF positively contributes to the growth in the absence of Gcn4p.

Acknowledgments We thank Marjolaine Crabeel for the SS5 strain and M13mp7-ARG1. This work was supported by Basic Science Program through the National Research Foundation of Korea Grant funded by the Korean Government (NRF-2009-0070791).

References

Carr EA, Mead J, Vershon AK (2004) Alpha1-induced DNA bending is required for transcriptional activation by the Mcm1-alpha1 complex. Nucleic Acids Res 32:2298–2305

Crabeel M, de Rijcke M, Seneca S, Heimberg H, Pfeiffer I, Matisova A (1995) Further definition of the sequence and position requirements of the arginine control element that mediates repression and induction by arginine in *Saccharomyces cerevisiae*. Yeast 11:1367–1380

Darieva Z, Pic-Taylor A, Boros J, Spanos A, Geymonat M, Reece RJ, Sedgwick SG, Sharrocks AD, Morgan BA (2003) Cell cycle-regulated transcription through the FHA domain of Fkh2p and the coactivator Ndd1p. Curr Biol 13:1740–1745

De Rijcke M, Seneca S, Punyammalee B, Glansdorff N, Crabeel M (1992) Characterization of the DNA target site for the yeast ARGR regulatory complex, a sequence able to mediate repression or induction by arginine. Mol Cell Biol 12:68–81

Gavin IM, Kladde MP, Simpson RT (2000) Tup1p represses Mcm1p transcriptional activation and chromatin remodeling of an a-cell-specific gene. EMBO J 19:5875–5883

Hinnebusch AG (1992) The molecular and cellular biology of the yeast saccharomyces: gene expression. In: Broach JR, Jones EW, Pringle JR (eds) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 319–414

Messenguy F, Dubois E (1993) Genetic evidence for a role for MCM1 in the regulation of arginine metabolism in *Saccharomyces cerevisiae*. Mol Cell Biol 13:2586–2592

Messenguy F, Dubois E (2003) Role of MADS box proteins and their cofactors in combinatorial control of gene expression and cell development. Gene 316:1–21

Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG, Marton MJ (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. Mol Cell Biol 21:4347–4368

Penn MD, Galgoci B, Greer H (1983) Identification of AAS genes and their regulatory role in general control of amino acids biosynthesis in yeast. Proc Natl Acad Sci USA 80:2704–2708

Qiu H, Hu C, Yoon S, Natarajan K, Swanson MJ, Hinnebusch AG (2004) An array of coactivators is required for optimal recruitment of TATA binding protein and RNA polymerase II by promoter-bound Gcn4p. Mol Cell Biol 24:4104–4117

Sherman F (1991) Getting started with yeast. Methods Enzymol 194:3–21

Tan S, Richmond TJ (1998) Crystal structure of the yeast MATalpha2/MCM1/DNA ternary complex. Nature 391:660–666

Yoon S, Hinnebusch AG (2009) Mcm1p binding sites in ARG1 positively regulate Gcn4p binding and SWI/SNF recruitment. Biochem Biophys Res Commun 381(1):123–128

Yoon S, Qiu H, Swanson MJ, Hinnebusch AG (2003) Recruitment of SWI/SNF by Gcn4p does not require Snf2p or Gcn5p but depends strongly on SWI/SNF integrity, SRB mediator, and SAGA. Mol Cell Biol 23:8829–8845

Yoon S, Govind CK, Qiu H, Kim SJ, Dong J, Hinnebusch AG (2004) Recruitment of the ArgR/Mcm1p repressor is stimulated by the activator Gcn4p: a self-checking activation mechanism. Proc Natl Acad Sci USA 91:11713–11718