Cooperative Regulation of ADE3 Transcription by Gcn4p and Bas1p in Saccharomyces cerevisiae

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The one-carbon response regulon is essential for the biosynthesis of nucleic acids as well as several amino acids. The ADE3 gene is known to encode a crucial one-carbon regulon enzyme, tetrahydrofolate synthase, which is involved in the biosynthesis of purine and the amino acids methionine and glycine. Therefore, the mechanism through which ADE3 transcription is regulated appears to be critical for the cross-talk among these metabolic pathways. Even so, the direct involvement of ADE3 transcription through gene-specific transcription factors has not been shown clearly. In this study, the promoter structure of the ADE3 gene was investigated in detail, and a genuine Gcn4p responsive element (GCRE) was confirmed among three putative GCRE elements in vivo and in vitro. Through gene deletion studies of Gcn4p and Bas1p, it was established that both factors are involved in the transcriptional regulation of the ADE3 gene. Direct binding to this GCRE and the occupancy of the ADE3 promoter by these factors were also confirmed. Taking these results together, we concluded that Gcn4p is responsible for the basal and inducible expression of the ADE3 gene, while Bas1p is required for its basal expression.

One-carbon metabolism, using folate as a coenzyme, is important for methyl group biosynthesis in yeast (3, 27). This folate-dependent metabolic pathway is required for the biosynthesis of various amino acids and purines, and genes encoding the enzymes participating in this metabolism are termed the one-carbon regulon. Purine biosynthesis, one of the major metabolic pathways influenced by one-carbon metabolism, is executed by many ADE genes (4). It has been known that the expression of the one-carbon regulon and ADE genes were regulated mostly in a cooperative manner at the transcriptional level (18, 24, 33). Gcn4p and Bas1p are well-known transcription factors mediating this regulation (26, 39).

The well-studied mechanism through which yeast adapts to amino acid starvation is known as general amino acid control, a series of processes controlled by the yeast transcription factor Gcn4p. Gcn4p functions as a mammalian transcription activator protein AP-1, the Candida albicans protein CaGcn4, Aspergillus niger protein CPCA, and the Saccharomyces cerevisiae protein Gcn4p all regulate transcription by binding to the same DNA consensus sequence [ATGA (C/G)TCAT], a specific DNA sequence similar to that of GCRE. This element, called the AP-1 site, is located in the promoter region of various Gcn4p target genes in Saccharomyces cerevisiae. Gcn4p functions as a positive transcriptional regulator of many metabolic genes during UV stress and amino acid, glucose, or purine depletion (8, 12, 23, 25). Under these conditions, Gcn4p induction is regulated in a translational manner through the activity of the protein kinase Gcn2p. In amino acid-starved cells, uncharged tRNA binds to and activates this kinase, which then phosphorylates the alpha subunit of eIF2 (for eukaryotic initiation factor 2), leading to elevated Gcn4p translation (10). In yeast, the expression of genes encoding amino acid biosynthetic enzymes is upregulated by Gcn4p in response to amino acid starvation. This derepression is induced by 3-aminotriazole (3-AT), a competitive inhibitor of His3p. Gcn4p also activates the transcription of genes responsible for purine biosynthesis (43). For example, previous studies demonstrated that Gcn4p activates ADE4 (25), and microarray results using GCN4 wild-type and deletion strains showed that Gcn4p stimulates the transcription of purine and pyrimidine biosynthetic genes following 3-AT treatment (26). This result implies that Gcn4p controls the expression of genes related not only to amino acid biosynthesis but also to central metabolism.

Bas1p is a myb-related transcription factor that binds to TGACTC, a specific DNA sequence similar to that of GCRE (13, 29, 40). Bas1p is required for the activation of adenylate biosynthetic genes under conditions of adenine depletion and in an SAGA- and SWI/SNF-dependent manner (18, 33). In addition, Bas1p activates not only adenylate biosynthesis but also the one-carbon metabolic regulon under glycine-replete conditions (39).

The yeast ADE3 gene product has been identified as the cytoplasmic trifunctional enzyme C1-tetrahydrofolate (C1-THF) synthase (35). C1-THF synthase plays a critical role in purine biosynthesis, as it is responsible for synthesizing 10-formyl-THF, the essential C-2 and C-8 purine base donor (6). ADE3 gene regulation is predominantly pretranslational; steady-state levels of the mRNA encoding C1-THF synthase are two- to threefold higher in derepressed cells than in normal
cells (1). However, there is some controversy about the transcriptional regulation of ADE3. Although adenine depletion does alter the expression of most ADE genes through the activities of Gcn4p, Bas1p, and Bas2p, it does not alter the transcription of the ADE3 gene (4). In another study with 106 yeast transcription factors, it was found that the ADE3 promoter did not bind Bas1p using chromatin immunoprecipitation (ChiP)-chip analysis (21). However, Mieczkowski and coworkers recently revealed that the ADE3 gene is a target site for Bas1p by using another set of ChiP-chip analyses (24). It also has been reported that ADE3 transcription is activated in a Bas1p-dependent manner under glycine-replete conditions (39). To understand the regulation mechanism of these closely related metabolic pathways, it is very important to understand how yeast regulates ADE3 transcription.

In this study, we examined cis and trans elements of the ADE3 gene. First, it was revealed that the ADE3 promoter region has complex and overlapping sets of potential regulatory motifs, including a TATA box and three putative Gen4p responsive elements (GCRES). We next demonstrated that one of these along with Gcn4p itself both are required for the steady-state expression of the ADE3 gene. It was found that the basal transcription levels of the BAS1 and GCN4 deletion strains were significantly decreased. Moreover, we discovered that these proteins bound to the same GCRE in vitro. Finally, the promoter occupancy of the ADE3 gene by these two factors was confirmed. Taken together, we present a simple regulatory model of ADE3 transcription that proposes that Gcn4p is responsible for the basal and inducible transcription of the gene during starvation, while Bas1p is responsible for the basal transcription only.

MATERIALS AND METHODS

Plasmids and primers. Primers used in this study are summarized in Table S1 in the supplemental material. The vector pRS80 has a 6.0-kb fragment containing an ADE3(1-333)-lacZ fusion gene with an intact ADE3 promoter (36). The multicopy plasmid p410 was constructed from p880 and YEp24, which contains the URA3 marker gene in addition to a replicating origin (the 2μ sequence). The ADE3(1-333)-lacZ fusion gene in p880 was excised with BamHI and SmaI and inserted into the BamHI and PvuII sites in YEp24, generating p410 WT (516 to 440), -d1 (236 to 440), and -d2 (516 to 236, 236 to 440). The p410-d2 strain was recovered by site-directed mutagenesis by PCR. The p410-d1 strain was recovered by site-directed mutations in the putative GCREs in the ADE3 region. We next demonstrated that one of these along with Gcn4p itself both are required for the steady-state expression of the ADE3 gene. It was found that the basal transcription levels of the BAS1 and GCN4 deletion strains were significantly decreased. Moreover, we discovered that these proteins bound to the same GCRE in vitro. Finally, the promoter occupancy of the ADE3 gene by these two factors was confirmed. Taken together, we present a simple regulatory model of ADE3 transcription that proposes that Gcn4p is responsible for the basal and inducible transcription of the gene during starvation, while Bas1p is responsible for the basal transcription only.

TABLE 1. S. cerevisiae strains used in this study

| Strain       | Relevant genotype | Source or reference |
|--------------|------------------|---------------------|
| GT48         | MATα ade3-130    | 35                  |
|              | ser1-171        |                     |
|              | ura3-52         |                     |
|              | gcn4::Δ        |                     |
| KY803        | MATa repl1::Δ   | 12                  |
|              | ura3-52         |                     |
|              | gcn4::Δ        |                     |
| JS134-7D     | MATα leu2-3.12   | 36                  |
|              | ura3-52         |                     |
|              | gcn4::Δ        |                     |
| YJK101       | JS134-7D, except | This study          |
|              | gcn4::LEU2      |                     |
|              | JS134-7D        |                     |
| YJK111       | JS134-7D, except | This study          |
|              | gcn4::GCN4-HA1  |                     |
|              | bas1::BAS1-HA3  |                     |
| BY7471       | MATα bas1::Δ    | Clontech            |
|              | leu2::Δ         |                     |
|              | mat1::Δ         |                     |
|              | ura3-52         |                     |
|              | gcn4::Δ        |                     |
| BY7471Δgcn4  | BY4741, except   | BY4741              |
|              | gcn4::KANMX     | Clontech            |
| BY7471Δbas1  | BY4741, except   | BY4741              |
|              | bas1::KANMX     | Clontech            |
| YJK309       | BY4741Δbas1, except | BY4741           |
|              | gcn4::LEU2      |                     |

For the induction of galactose, cultured cells in SC medium were transferred into YPD medium containing 0.6% yeast nitrogen base without amino acid (BY4741) and confirmed by PCR. KY803 and GT48 strains were used for the reporter assay. Wild-type and mutant promoter-reporter fusion genes were integrated into the host chromosomal DNA of these strains. Single-copy integration was confirmed in parallel with a marker test and genomic Southern blotting. The YJK309 strain was generated from BY4741Δbas1. The LEU2 deletion cassette introduced into the strains was scored as exhibiting no growth on medium containing 20 mM 3-AT and confirmed by PCR. KY803 and GT48 strains were used for the reporter assay. Wild-type and mutant promoter-reporter fusion genes were integrated into the host chromosomal DNA of these strains. Single-copy integration was confirmed in parallel with a marker test and genomic Southern blotting.

Yeast strains and media. The yeast strains used are listed in Table 1. The YJK101 strain was derived from the JS143-7D strain (34) by replacing the chromosomal GCN4 gene with pRSFRgcn4. The retention of the gcn4::LEU2 allele was verified on synthetic complete (SC) medium lacking leucine. Mutants were scored as exhibiting no growth on medium containing 20 mM 3-AT and confirmed by PCR. KY803 and GT48 strains were used for the reporter assay. Wild-type and mutant promoter-reporter fusion genes were integrated into the host chromosomal DNA of these strains. Single-copy integration was confirmed in parallel with a marker test and genomic Southern blotting.

Assay of β-galactosidase activity. Strains to be assayed were grown to late log phase in SC medium supplemented with appropriate amino acids at 30°C, followed by a 1.10 dilution with fresh SC medium and grown at 30°C to an optical density of 0.600 nm of 1 to 2. A growth culture of 1 ml was harvested, and the activities of the harbored reporter constructs were measured using chloroform. The composition of the solutions as well as the data calculations were performed as previously described (4). Values provided were acquired from triplicate assays of three independent colonies.

Electrophoretic mobility shift assay (EMSA). The GST, GST-Gcn4, and GST-Bas1 fusion proteins were oversaturated in Escherichia coli BL21(DE3, RIL), which contains a pGEX vector carrying each gene downstream of a T7 promoter. For the construction of mutants of the ADE3 promoter were constructed in two steps. First, site-directed mutations in the ADE3 promoter were introduced using PCR. Oligonucleotides carrying specific mutations in the ADE3 promoter sequences were as follows: S-126f and S-126r for the mutation of GCREC1, S-129r and S-129r for the mutation of GCREC2, and S-130f and S-130r for the mutation of GCREC3. Point mutations were confirmed by DNA sequencing. Wild-type and mutant promoter-reporter genes integrated at the ade4 locus in the GT48 chromosome lack a replication origin, and single-copy integration was confirmed by the Southern blotting of the genomic sequence. The plasmid pRSFRgcn4 was constructed to create the Δgcn4 strain (YJK101) from the wild-type GCN4 gene (JS134-7D). The upstream 382-bp sequence and the downstream 284-bp sequence of the GCN4 open reading frame were acquired by PCR. These two fragments possessed BglII sites on one end and either HindIII or XbaI site on the other. All PCR products were digested accordingly and inserted into the pRS315 vector (NEB). The plasmid pRS305 contains an LEU2 marker lacking its own replicating origin to allow integration into a specific host chromosome locus.
The crude soluble extract was obtained by sonication. Each fusion protein tagged by glutathione S-transferase (GST) was purified with glutathione-Sepharose 4B (Amershams Biosciences) by following general methods of batch purification. Probes were prepared by annealing with two complementary single-stranded DNAs (see Table S1 in the supplemental material). These double-stranded DNA probes were end labeled with 32P by T4 polynucleotide kinase, after which 15-μl binding reaction mixtures were prepared that contained 20,000 cpm of the radiolabeled DNA probe each. Probes from the different constructs were incubated with different amounts of eluted recombinant Gcn4p in EB buffer [20 mM Tris-HCl (pH 8.0), 60 mM KCl, 0.1 mM EDTA, 20% glycerol, 0.25 mg/ml bovine serum albumin, and 0.05 μg/μl poly(dIdC)](26). Binding was allowed to proceed for 30 min at 30°C, followed by the analysis of the binding mixtures by electrophoresis on 5% Tris-borate-EDTA (TBE)–polyacrylamide gels (100 mA for 1 h in 0.5% TBE buffer). Autoradiography signals were detected with a BAS-2500 image reader (FujiFilm).

Total RNA preparation and Northern blotting. Total yeast RNA was isolated from early-log-phase cells by the hot phenol extraction method (27). A total of 5 to 10 μg RNA was separated on a 1% formaldehyde–agarose gel with RNA running buffer (1× morpholinepropane sulfonic acid), transferred to a nylon membrane (Gene Screen Plus; NEN) using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer, and then UV fixed (UVC; 1,500 J) by a UV cross-linker. The probes were labeled with digoxigenin for ADE3, TRPS, HIS4, and ACT1, and detection was performed using the procedures recommended by the manufacturer (Boehringer Mannheim).

Western blotting. To prepare the protein lysate, PBS-washed cell pellets were resuspended in FA lysis buffer composed of 50 mM HEPES-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 25 mM β-glycerophosphate, 25 mM NaF, 1 mM PMSF, 1 μg/ml peptatin A, 5 μg/ml leupeptin, and 1 μg/ml aprotinin. Cells were broken with glass beads using a bead beater (MP), and lysate samples were generated by centrifugation at 10,000 × g for 20 min at 4°C. The protein concentrations from different samples were determined. Equal amounts of each lysate were separated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis, followed by the electrotransfer of proteins to a 0.45-μm nitrocellulose membrane (Bio-Rad). Immunoblotting was performed with anti-c-myc (Santa Cruz Biotechnology), anti-HA (Santa Cruz Biotechnology), and anti-Pgk1p (Molecular Probes).

ChIP. Chromatin DNA preparation, immunoprecipitation, and semiquantitative PCR were performed as previously described, with several modifications (29). Cell cultures (200 ml grown to an absorbance of 600 nm of 1.0) were treated with 1% formaldehyde and gently swirled for 20 min at room temperature. Glycine was added to a final concentration of 350 mM, and cells were swirled for 5 min, followed by being harvested and washed twice with FA lysis buffer. The cell pellet then was resuspended with an equal amount of FA lysis buffer. After combining the FA buffer with the acid-washed glass beads, the cells were broken by being vortexed with a bead beater, and the lysate was prepared by centrifugation. The input chromatin DNA fraction was obtained by ultracentrifugation. The crude soluble extract was obtained by sonication. Each fusion protein tagged by glutathione S-transferase (GST) was purified with glutathione-Sepharose 4B (Amershams Biosciences) by following general methods of batch purification. Probes were prepared by annealing with two complementary single-stranded DNAs (see Table S1 in the supplemental material). These double-stranded DNA probes were end labeled with 32P by T4 polynucleotide kinase, after which 15-μl binding reaction mixtures were prepared that contained 20,000 cpm of the radiolabeled DNA probe each. Probes from the different constructs were incubated with different amounts of eluted recombinant Gcn4p in EB buffer [20 mM Tris-HCl (pH 8.0), 60 mM KCl, 0.1 mM EDTA, 20% glycerol, 0.25 mg/ml bovine serum albumin, and 0.05 μg/μl poly(dIdC)](26). Binding was allowed to proceed for 30 min at 30°C, followed by the analysis of the binding mixtures by electrophoresis on 5% Tris-borate-EDTA (TBE)–polyacrylamide gels (100 mA for 1 h in 0.5% TBE buffer). Autoradiography signals were detected with a BAS-2500 image reader (FujiFilm).

RESULTS

The GCRE1 element in the ADE3 promoter is a functional, cis-acting element that promotes basal-level transcription. To identify the cis element of the ADE3 gene, we analyzed the sequence of the promoter. We found that the promoter sequence contains putative DNA binding sites for transcription factors such as TBP, Gcn4p, and Bas1p (Fig. 1A). We further compared the sequence of three putative GCREs to the consensus GCRE sequence (Fig. 1B). Among the three GCREs, GCRE1 most closely resembles the consensus sequence. Using internal deletions, we next generated a series of ADE3 promoter constructs upstream of the lacZ expression reporters (p410, p410-WT, p410-d1, p410-d2, p410-d3, and p410-d4) (Fig. 1C, left). These reporter gene constructs were cloned into multicopy plasmids and transformed into the GT48 strain, followed by the performance of the β-galactosidase assay (Fig. 1C, right). The promoter activities of all constructs containing GCRE1 (p410-WT and p410-d1) were much higher than those lacking GCRE1 (p410-d2, p410-d3, and p410-d4). We also found that the TATA box is important for adequate ADE3 transcription, which is supported by the fact that the promoter activity of the TATA-less promoter construct (p410-d4) was diminished to the same level as that of the vector control (p410). From these data, it was suggested that there are two functional cis elements in the ADE3 promoter region, GCRE1 and the TATA box.

Although GCRE1 is the core region for ADE3 transcriptional regulation, as shown in Fig. 1C, 5′-to-3′ serial deletion promoter constructs could not exclude the possibility that GCRE2 and GCRE3 participate in ADE3 transcription. Therefore, we generated seven promoter constructs containing one or more GCRE mutants [from ATGA(C/G)TCAT to AT GAATTCAT] capable of abolishing the direct binding of Gcn4p (see Fig. 5) (15). We next integrated these into the uua3 locus of the GT48 strain and measured the promoter activities by β-galactosidase assay (Fig. 2). All of the constructs possessing the GCRE1 mutation (p414-m1, p414-m4, p414-m5, and p414-m7) showed significantly lower levels of activity (20 to 30%) than that of the wild type (100%). However, mutations in GCRE2 and GCRE3 did not change promoter activity at all. A comparison of the activities of the WT, m2, and m3 constructs clearly revealed that GCRE2 and GCRE3 are not functional for the basal-level transcription. In addition, the results from constructs containing multiple mutations (p414-m4, p414-m5, p414-m6, and p414-m7) clearly showed that there was no cooperativity among GCREs. From these results, we concluded that only GCRE1 is the functional element for basal transcription, not GCRE2 and GCRE3.

ADE3 transcription is upregulated in a Gcn4p-dependent manner. After finding the functional cis element in the ADE3 promoter region, trans elements were examined. Since the functional GCRE was present, we monitored ADE3 mRNA levels under stress conditions such as amino acid starvation and 3-amino-1,2,4-triazole (3-AT) treatment using Northern blotting. As shown in Fig. 3A, the transcription levels of the ADE3 gene increased under derepression conditions (data not shown). We therefore speculated that ADE3 transcription is directly correlated with the expression of Gcn4p. To further verify Gcn4p-dependent transcription, a reporter assay was performed. We transformed the low-copy YA410 plasmid containing ADE3 (1-333)-lacZ into the isogenic GCN4 (IS143-7D) and Δgcn4 (YJK101) strains. The ADE3 promoter activity of the low-copy plasmid was about threefold higher in the strain containing wild-type GCN4 (Fig. 3B). To confirm that this phenomenon is true in the multicopy state, we transformed the multicopy wild-type construct (p410-WT) into the KY805 strain (gcn4 genotype) and GT48 strain (wild type). The ADE3 promoter activity in GT48 was about 13-fold higher than that
in KY803 (Fig. 3C). The results show that the level of increase (threefold) in the low-copy-number state was less robust than that in the multicopy state. Northern blotting was performed to determine the \( ADE3 \) mRNA level following exogenous Gcn4p-myc7 overexpression using a galactose-inducible promoter. The transcript levels of overexpressed \( ADE3 \) and \( TRP3 \), which is known to have a single Gcn4p response element (9, 23), were much higher than that of the vector control (Fig. 3D). From this result, we concluded that Gcn4p is responsible for the transcription of the \( ADE3 \) gene, some portion of basal transcription, and transcription under derepression conditions caused by amino acid starvation.

Both Gcn4p and Bas1p are needed for \( ADE3 \) gene expression. As shown in Fig. 3A, there was a slight increase in \( ADE3 \) transcription in the \( \Delta gc4 \) strains under amino acid starvation. Moreover, the basal \( ADE3 \) transcript level in \( \Delta gc4 \) was about half of that in the wild type. Specifically, the loss of the GCRE1 region diminished promoter activity by about 20% compared to that of the constructs containing GCRE1 (p410-d1 and p410-d2) (Fig. 1C). Accordingly, we suggested that another regulatory factor acts through GCRE1. To examine this, we searched for other factors containing the GCRE1 sequence that activate transcription. Among the many transcription factors in yeast, the well-known protein Bas1p was presented as a strong candidate, as it is bound to a sequence similar to that of GCRE, 5'-TGACTC-3', and also is known to regulate the \( ADE \) genes and the one-carbon regulon. However, unlike other \( ADE \) genes, it was reported that the \( ADE3 \) gene was not activated by Bas1p under adenine limitation (4). Why is the \( ADE3 \) gene regulated differently from other \( ADE \) genes? Although the mutation of \( ADE3 \) leads to a requirement for purines, this is not directly required for the de novo pathway of purine biosynthesis. \( ADE3 \) encodes an enzyme participating in the one-carbon metabolism. Therefore, there was a possibility that \( ADE3 \) gene transcription is regulated by Bas1p only under basal conditions, similarly to other one-carbon metabolic and histidine biosynthetic genes, and not under the derepression conditions caused by adenine limitation, unlike other \( ADE \) genes. To investigate this further, we examined the transcript levels of the wild-type, \( \Delta gc4 \), and \( \Delta bas1 \) strains (Fig. 4). Surprisingly, the basal transcription levels of both deletion strains were lower than that of the wild type. The level of basal \( ADE3 \) mRNA in \( \Delta gc4 \) and \( \Delta bas1 \) was 30 and 40% of the level of wild-type mRNA, respectively. Although the basal level was remarkably decreased in both deletion strains, the derepression patterns were quite different under amino acid starvation. Even though all of the expression levels were decreased, the pattern of gene expression in \( \Delta bas1 \) was not too different from...
that of the wild type, indicating that the basal transcription level of this strain was affected. However, for \( \Delta gc\text{en4} \), there was a dramatic change upon amino acid starvation (Fig. 4B, lane 5), as evidenced by the affected induction level. Under adenine depletion, factor-dependent transcription and derepression under starvation were not detected. Thus, we conclude that Gcn4p is required for both basal and inducible \( ADE3 \) transcription, and that Bas1p is required only for the basal-level expression of \( ADE3 \).

**Gen4 and Bas1 bind directly to GCRE1 in vitro.** We next investigated the direct binding of Gcn4p and Bas1p to the GCRE1 of \( ADE3 \). Accordingly, EMSA was performed with 32P-end-labeled probes harboring \( ADE3 \) promoter regions that contained the wild-type sequence and the mutated GCRE1 sequences GST-Gcn4 and GST-Bas1 (Fig. 5A). Both proteins were incapable of binding to the mutant probe (\( A3\text{Gm} \)) despite strong interaction with the wild-type promoter (\( A3\text{GW} \)). The extent of DNA-protein complex formation was dependent upon the concentration of GST-Gcn4 and GST-Bas1 in the binding mixture. To examine whether both proteins bind simultaneously to the GCRE1 element, EMSA was performed with a fixed concentration of GST-Gcn4 and an increased concentration of GST-Bas1 (Fig. 5B, left) or vice versa (Fig. 5B, right). Interestingly, we found that increasing the amount of one recombinant protein competed with the binding of the other. These results suggest that Gcn4 and Bas1 bind directly to the same GCRE1 as trans-activators; however, both proteins could not bind to the element at the same time.

**Gcn4p and Bas1p are associated with the \( ADE3 \) promoter region in vivo.** To confirm promoter occupancy, strains containing Gcn4-HA3 (YJK101) and Bas1-HA3 (YJK111) were generated from the JS143-7D strain and subjected to ChIP analysis. Yeast strains were verified by PCR, and normal functionalities of these tagging proteins were confirmed by the Northern blotting of transcriptional target genes of each factor. ChIP assays for Gcn4p and Bas1p binding were performed after 2 h of 3-AT treatment. Since previous studies have shown the association of Gcn4p with the \( HIS3 \) promoter (16, 17), we used primers specific for the \( HIS3 \) promoter region as a positive control and a specific primer set for the \( POL1 \) CDS as a negative control, similarly to a previous study (28). Compared to the input and communoprecipitated signals, it was clear that Gcn4p and Bas1p weakly bind to the \( ADE3 \) promoter under basal conditions. However, only Gcn4p increased binding to this region under amino acid starvation (Fig. 6). These results confirm that Gcn4p and Bas1p associate with the \( ADE3 \) promoter region in vivo, activating the basal transcription of \( ADE3 \). In addition, Gcn4p, but not Bas1p, binds increasingly for the bulk of \( ADE3 \) gene induction during amino acid starvation.

**Gcn4p and Bas1p are needed to fully induce \( ADE3 \) transcription.** According to previous reports, the maximal expression of several genes, including \( ADE2, ADE4, HIS4 \), and \( HIS7 \), appears to require the transcriptional activators Gcn4p and Bas1p (7, 25, 37, 38). Our data also supported this for \( ADE3 \) gene transcription. To prove this, we generated a \( GCN4 \) and \( BAS1 \) double deletion strain and then compared mRNA levels of \( ADE3, TRP3, HIS4 \), and \( ACT1 \). As shown in Fig. 7A, the level of \( ADE3 \) transcript in the steady state of the double deletion strain was significantly lower than that of the wild type or that of each of the single-deletion strains, as is the case for \( HIS4 \). Furthermore, the \( TRP3 \) mRNA level was not affected at all in the \( BAS1 \) deletion strain (Fig. 7B). As mentioned previously, the \( TRP3 \) promoter has a single Gcn4p response element, while the \( HIS4 \) gene is regulated by two transcription factors, Gcn4p and Bas1p. Therefore, we concluded that Gcn4p and Bas1p are responsible for basal-level transcription through the same response element, namely GCRE1, which is located in the promoter region. Moreover, these two proteins activate transcription independently from each other under conditions tested.

**FIG. 2. GCRE1 is a critical cis element in the \( ADE3 \) promoter.** Seven constructs, each containing combinations of mutant GCRE elements, were generated. Point mutant elements are indicated by filled arrowheads. To check the activity of each mutant promoter, a general \( \beta \)-galactosidase assay was performed. The reported values are averages calculated from the results of duplicate assays from at least three independent transformants. The error bars represent standard deviations. (GCRE1 point mutant, ATGA\text{CTCCT}→ATGA\text{ATTTCCT}; GCRE2 point mutant, ATGA\text{CTGAC}→ATGA\text{ATTGAC}; GCRE2 point mutant, TTGA\text{ATTTG}→TTGA\text{ATTG}.)
FIG. 3. Gcn4p upregulates the expression of ADE3 mRNA under basal and inducible conditions. (A) Exponentially growing wild-type (JS143-7D) and Δgcn4 (YJK101) strains were transferred into fresh SC-his medium alone (normal), SC-his medium lacking TRP (amino acid starvation), and SC-his medium containing 80 mM 3-AT (3-AT). After 1 h of medium transfer, total RNA was precipitated and Northern blotting was performed with probes specific for ADE3 and ACT1. mRNA band intensities were expressed numerically by a phosphorimager, and each ADE3/ACT1 mRNA ratio was calculated and normalized to levels for the WT normal sample. (B and C) Specific activities of ADE3(1-333)-lacZ fusion proteins expressed from a single-copy plasmid (B) or multicopy plasmids (C) were measured by reporter assay. (B) The single-copy plasmid carrying the ADE3(1-333)-lacZ fusion gene (YA410-WT) was transformed into GCN4 wild-type (JS143-7D) and Δgcn4 (YJK101) strains. (C) p410-WT, a multicopy plasmid bearing the ADE3(1-333)-lacZ construct, was transformed into the KY803 (GCN-negative) and GT48 (GCN-positive) strains. All indicated values for the β-galactosidase assays were averages from three independent experiments. Error bars represent standard deviations. (D) BY4741 Δgcn4 strain containing pGALGcn4-myc or pGAL-myc (32) was grown to early log phase and transferred to SRG medium. After 12 h of galactose induction, cells were harvested for the preparation of protein and RNA sampling. Western blotting was performed with antibodies specific against c-myc and Pgk1p. Northern blot analysis was performed with probe specific against ADE3, TRP3, and ACT1. IB, immunoblot.
both normal and derepressed conditions induced by amino acid starvation.

**DISCUSSION**

The folate-dependent pathway termed one-carbon metabolism is crucial for the biosynthesis of purines and several amino acids, like glycine and methionine, and it encompasses the majority of methyl group biogenesis in yeast. Gcn4p, Bas1p, and Bas2p regulate most of the genes that compose the one-carbon pathway; for example, most of the purine biosynthetic genes, such as *ADE1*, *ADE2*, *ADE5*, and *ADE8* but excluding *ADE3*, are activated in an adenine-dependent manner by Bas1p (4, 5). Previous reports indicated that 3-AT treatment induced the expression of *ADE1*, *ADE2*, *ADE3*, *ADE4*, *ADE8*, *ADE12*, and *ADE17*, and a microarray study revealed that this induction depends upon Gcn4p in several cases (*ADE1*, *ADE3*, *ADE8*, *ADE12*, and *ADE17*) (26). Even with these extensive data, the details of the transcriptional mechanism mediated by Gcn4p and Bas1p on the *ADE3* gene during purine biosynthesis have not yet been clearly investigated.

In this report, we focused on *ADE3* transcription and revealed clear evidence of its dependence upon Gcn4p and Bas1p. First, we investigated the promoter activities of the three putative GCREs upstream of the *ADE3* gene. It was confirmed that only GCRE1 is a functional cis-acting promoter...
element. When GCRE1 was mutated or deleted, the basal gene expression levels were reduced to 33% of that produced by the wild-type promoter (Fig. 2). Moreover, reduced expression levels of *ADE3* transcript were observed in the *GCN4* deletion strain compared to levels for the wild-type strain. These results confirm the role of Gcn4p as a positive regulator of *ADE3* gene expression. However, we also found several lines of evidence that another factor also is involved in *ADE3* transcription.

The de novo syntheses of histidine and purines are interconnected due to the sharing of common substrates and intermediates between these pathways. This connection is reflected in the cross-pathway transcripational regulation of certain *HIS* and *ADE* genes by transcription factors such as Gcn4p and Bas1p/Bas2p (25, 29). Since the *ADE3* gene product, C1-THF synthase, is involved in several important amino acid and purine biosynthetic pathways, it is very possible that the *ADE3* gene is highly expressed in vivo, unlike other *ADE* genes. In fact, a previous study showed that the promoter of *ADE3* was much more active than those of *ADE1* and *ADE5,7* under normal conditions (4). Cells require a unique regulatory mechanism to meet the cellular requirement for a high basal pool of C1-synthase enzyme to overcome nutrient starvation. These features may explain why Bas1p-dependent transcription occurs only under basal conditions, as well as the unremarkable down-regulation of the *ADE3* gene during adenine-limiting conditions. Moreover, we could detect more than two different lengths of *ADE3* transcripts (Fig. 4A and 7B). After checking the transcriptional start site by primer extension, we found that mRNA of *ADE3* was started at four sites, 27 bp, 30 bp, 38 bp, and 42 bp, which are consistent with the results reported by the National Center for Biological Information (NCBI). We also found that there are slightly different regulation patterns of these various types of transcripts. Therefore, it was suggested that the deletion of *gcn4* or *bas1* led to a change of the start site of *ADE3* transcription, which remains to be examine by further studies for the transcriptional regulation.

We hypothesized that both the Gcn4p and Bas1p transcription factors were responsible for the abundant transcript levels under basal conditions, as well as the robust transcriptional activation of *ADE3* in response to amino acid starvation. We therefore evaluated Bas1p-dependent transcriptional regulation using BY4741 deletion variants. The Northern blot experiments in Fig. 4 and 7 clearly confirmed that Bas1p significantly

**FIG. 5.** Gcn4 and Bas1 directly bind to the GCRE1 sequence from the *ADE3* promoter. (A) 32P-labeled DNA probes containing wild-type (A3GW; JK06101/JK06102) and mutated (A3Gm; JK06103/JK06104) GCRE1 (0.025 pmol) were mixed with the indicated amount of GST-Gcn4 or GST-Bas1. Following binding for 30 min at 30°C, the mixtures were analyzed on a 5% TBE–acylamide gel, followed by being dried for detection with a BAS-2500 image reader. The mutation site of the A3Gm probe is identical to that of the GCRE1 mutant in Fig. 2. (B) EMSA was performed with 32P-labeled A3GW probe, GST-Gcn4 (200 μg), and an increased concentration of GST-Bas1 (100, 200, 400, and 800 μg; left) or GST-Bas1 (200 μg), as well as an increased concentration of GST-Gcn4 (100, 200, 400, and 800 μg; left). DNA-protein complex and unbound, free probe are indicated with an arrowhead.
influenced basal ADE3 expression. Interestingly, Gcn4p and Bas1p bind to the same DNA sequence. Our data suggest that Gcn4p and Bas1p are functional homologs in the case of ADE3 transcription by acting through the same cis element. However, although it was predicted that derepressed Gcn4p stimulates BAS1 expression under amino acid starvation (26), the expression of Bas1p as well as the promoter occupancy of ADE3 by Bas1p did not change after 3-AT treatment in our experiment (Fig. 6A). Therefore, we suggest that Gcn4p and Bas1p maintain ADE3 gene expression at the basal level, while derepressed Gcn4p stimulates transcription that is capable of overcoming the stress condition by activating amino acid biosynthetic genes such as ADE3 only through GCRE1.

Other reports showed that Bas2p is required for the regulated expression of ADE genes under adenine-limiting conditions in a Bas1-dependent manner (22). However, the expression of Bas1p and Bas2p is not regulated by adenine (40, 44), indicating that adenine repression occurs by downregulating activator functions. Recently, a model was presented for the regulation of ADE genes (except ADE3) by Bas1p and Bas2p in an adenine-dependent manner (33). In the report, adenine-dependent activation was achieved by protein-protein interaction between Bas1p and Bas2p. Furthermore, it also was revealed that transcription occurred in a SAGA- and SWI/SNF-dependent manner (18, 33). The absence of this activation mechanism on the ADE3 promoter may lead to the adenine-independent regulation of transcription.

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