AZF1 Is a Glucose-Dependent Positive Regulator of CLN3 Transcription in Saccharomyces cerevisiae

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Transcription of the CLN3 G1 cyclin in Saccharomyces cerevisiae is positively regulated by glucose in a process that involves a set of DNA elements with the sequence AAGAAAAA (A2GA5). To identify proteins that interact with these elements, we used a 1-hybrid approach, which yielded a nuclear zinc finger protein previously identified as Azf1. Gel shift and chromatin immunoprecipitation experiments show that Azf1 binds to the A2GA5 CLN3 regulatory sequences in vitro and in vivo, thus identifying a transcriptional regulatory protein for CLN3 and a DNA sequence target for Azf1. We show that glucose-induced expression of a reporter gene driven by the A2GA5 CLN3 regulatory sequences is dependent upon the presence of AZF1. Furthermore, deletion of AZF1 markedly reduces the transcriptional induction of CLN3 by glucose. In addition, Azf1 can induce reporter expression in a glucose-specific manner when artificially tethered to a promoter via the DNA-binding domain from Gal4. We conclude that AZF1 is a glucose-dependent transcription factor that interacts with the CLN3 A2GA5 repeats to play a positive role in the regulation of CLN3 mRNA expression by glucose.

Saccharomyces cerevisiae grows rapidly in glucose medium. With depletion of glucose, growth slows dramatically, as the cells move from glucose fermentation to oxidative metabolism of ethanol. The carbon source affects both the rate of growth in cellular mass and the rate of progression through the cell cycle. Cells rapidly increasing in mass on glucose medium proceed rapidly through the cell cycle, while cells slowly increasing in mass on nonfermentable carbon sources have a correspondingly prolonged cell cycle. This adjustment of the cell cycle length by nutrients is accomplished primarily by regulating the length of time that the yeast cells spend in G1 phase (16, 18).

The G1 cyclin encoded by CLN3 provides one of several links between growth in mass and proliferation. Changes in CLN3 expression alter G1 duration: increased expression of CLN3 shortens G1, while loss of CLN3 prolongs G1 (7, 19). CLN3 expression is in turn regulated by nutrients through several distinct pathways. Both the Tor phosphatidylinositol 3-kinase and the GPA2/RAS/cyclic AMP (cAMP) pathways are thought to regulate Cln3 translation through effects on protein synthesis rates. Translation of CLN3 is especially sensitive to decreases in the abundance of translational initiation complexes (1, 13, 14, 22). Since both of these pathways regulate pleiotropic responses to nutrients, these mechanisms can accelerate both growth in mass and proliferation in response to nutrient signals.

In addition to translational regulation, nutrients affect transcription of CLN3. CLN3 mRNA levels are induced by glucose and decrease in the presence of nonfermentable carbon sources. This involves a set of repeated sequences upstream of the CLN3 coding region. Induction of CLN3 mRNA levels by glucose does not require active growth, and it is not blocked by loss of the Ras/cAMP pathway (21) or by blocking the Tor pathway (1). Mutations in a gene such as RTG1, RTG2, or SNF3, known to affect induction of glucose transporters, do not block glucose induction of CLN3, nor is it blocked by mutations in HXK2, MIG1, REG1, SNF1, or SNF4, each of which is involved in glucose repression (26). Instead, induction of CLN3 by glucose appears to parallel the large increase in total mRNA that glucose produces (5, 6, 26).

We have identified cis elements within the CLN3 promoter that are responsible for the transcriptional induction of CLN3 in response to glucose. These repeated elements, with the sequence AAGAAAAA (A2GA5), are sufficient to drive glucose-dependent transcription of reporter genes and bind proteins from cell extracts in gel shift assays. Both the transcriptional and protein binding activities are DNA sequence specific (20).

In this work, we set out to identify proteins that interact with the A2GA5 elements from the CLN3 promoter. We now report that Azf1, a putative zinc finger transcription factor, binds to the A2GA5 repeats. The Azf1 protein functions as a glucose-dependent transcriptional activator, and deletion of Azf1 reduces the glucose induction of CLN3.

MATERIALS AND METHODS

Yeast strains and growth conditions. The media used were YEP (1% yeast extract, 2% peptone) and S (0.67% yeast nitrogen base) with the indicated carbon source at 2%. Strains are listed in Table 1. TNL22 was made by Gal80 deletion in DS10 by using an XbaI fragment from pBM290 as described previously (12). AZF1 was deleted in TNL80 and TNL81 by PCR amplification of the region spanning the AZF1 deletion in strain 2369 and by using this fragment to delete AZF1 in DM16 and DS10, respectively. All disruptions and gene replacements were confirmed with PCR. JRY-675 and the isogenic azf1Δ were obtained from Thomas Lisowsky. BY4741 and 2369 were obtained from Research Genetics.

Plasmid constructs and 1-hybrid screen. HIS3 reporter constructs for integration at LYS2 were based on the plasmid pBM1499 (12). To make pLN3, the GAL1 upstream activator sequence was removed by digestion with EcoRI and BamHI and replaced with a double-stranded oligonucleotide corresponding to 58 bp from CLN3 between positions −626 and −569 with the sequence 5′-gatc TTTCAGAGAAGAAAAAGAAAATGGAAGAAAATTATCACGGAAG AAAAAAGAATTACgat-3′. The lower-case letters at the ends indicate 5′-single-stranded overhangs for cloning into the restriction sites. To make the control plasmid pLN4, pBM1499 was digested with EcoRI to release the GAL1 upstream activator sequence and religated. The identities of both pLN3 and
The plasmids were integrated at LYS2, and the URA3 marker was removed as described previously (12). The plasmid pYES-AZF1 expressing a V5 epitope-tagged Azf1 protein was made by PCR amplification of AZF1 by using primers with the sequences 5'-TAG GAA CAG ACC ACC ATG CCT CCT CCA ACT GCA CAG-3' and 5'-GCT TTT GTA ATT AAT GTP CTT AAA TCT C3'. The PCR product was TA cloned into pYES2.1/V5-His-TOPO according to the manufacturer's instructions (Promega) and confirmed by sequencing. Production of AZF1 mRNA and protein was confirmed by Northern and Western blotting. The control plasmid with lacZ inserted instead of AZF1 was obtained from Marijane Russell, Invitrogen.

TLN25 cells were transformed using the EZ transformation kit (ZYMO Research) with pGAD-C libraries (17) (obtained from Phil James) in all three reading frames. Transformants were selected and replica plated onto SD-His plus 40 mM 3-aminotriazole (AT). The positive candidate plasmids were isolated from surviving colonies and retransformed into TLN25 and TLN26. Those plasmids that rescued TLN25 but not TLN26 on SD-His plus AT were sequenced.

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**TABLE 1. Saccharomyces cerevisiae strains**

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<th>Strain</th>
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<td>MATa his3-11,15 leu2-3,112 lys1 lys2 ura3-52 trp1Δ</td>
<td>This study</td>
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<tr>
<td>DM16</td>
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<td>This study</td>
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<td>TH146</td>
<td>Strain JRY-675 carrying pYES-AZF1::URA3</td>
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FIG. 1. Interaction of Azf1 with CLN3 regulatory sequences in vitro. Cell extracts from the wild-type (BY4741) and azf1Δ (2369) strains or the azf1Δ strain carrying either pYES-AZF1 (TLN58) or pYES-lacZ (TLN59) as a control were prepared for gel shift experiments as described in Materials and Methods. The extracts were incubated with a 32P-labeled double-stranded oligonucleotide corresponding to the indicated CLN3 regions containing the A2GA5 repeats (bold type), and the DNA protein complexes were loaded onto a nondenaturing polyacrylamide gel and visualized by phosphorimager. WT, wild type. (A and B) Unlabeled oligonucleotides were added as competitors at concentrations 25- and 100-fold greater than that of the labeled probe as indicated. The arrows indicate a specific set of bands that disappear in the azf1Δ lanes. The five-rayed star indicates an AZF1-independent complex. (C) Cell extracts from either the wild-type strain or the strain (TH146) carrying the pYES-AZF1 plasmid expressing a V5 epitope-tagged Azf1 grown in galactose were incubated with labeled oligonucleotides as in panel A. An antibody against the V5 epitope was added to the indicated samples prior to loading as described in Materials and Methods. The lower arrow indicates a specific pair of bands; the upper arrow indicates a supershifted band.
FIG. 2. Specificity of Azf1-DNA interactions. Cell extracts from an azf1Δ strain carrying the pYES-AZF1 plasmid (TLN18), grown in galactose, were incubated with the 32P-labeled CLN3 oligonucleotide as described for Fig. 1. The indicated unlabeled double-stranded oligonucleotide competitors were added at concentrations 25- and 100-fold greater than that of the labeled probe. The 2x and 3x designations for the competitor oligonucleotides indicate tandem repeats of the sequence in 2 and 3 copies, respectively. A2G5 repeats are underlined.
RESULTS

A 1-hybrid selection identifies AZF1 as a positive regulator of CLN3. Induction of CLN3 by glucose is mediated by sequences between positions −626 and −570 containing A2GA5 repeats. We used a 1-hybrid selection to identify proteins that interact with the A2GA5 repeats. We constructed a plasmid in which the −626 to −570 region from CLN3 was inserted upstream of HIS3. This construct was integrated into a his3Δ strain, and the resulting strain was used to screen a library of yeast genomic sequences fused with the Gal4 transcriptional activation domain. Library fusion proteins that direct the Gal4 transcriptional activation domain to the HIS3 reporter gene produce cells able to grow in the absence of histidine in the presence of 3-aminotriazole. Plasmids from AT-resistant colonies were isolated and retransformed into the starting strain to confirm their activity as well as into a control strain carrying the HIS3 reporter without the CLN3 promoter sequences to confirm specificity.

This screen yielded a positive clone that increased transcription of both the HIS3 reporter and the normal chromosomal CLN3 gene. This plasmid did not confer increased expression of the control HIS3 reporter without A2GA5 sequences. Sequencing of this clone revealed that the fusion contains the zinc finger domain of the protein encoded by AZF1 (asparagine-rich zinc finger 1). AZF1 was originally isolated as a high-copy suppressor of a mutation in the mitochondrial RNA polymerase gene RPO41 and was identified as a putative transcription factor based on its zinc finger domain and nuclear localization (4, 24). However, Azf1 has not previously been shown to regulate transcription, and no DNA sequences have been identified that interact with Azf1.

Azf1 binds the A2GA5 repeats in vitro. We used the 57-bp region from CLN3 between positions −626 and −570 as a probe in gel shift assays to determine whether AZF1 plays a role in forming the complex that binds the A2GA5 repeats. Cell extracts from wild-type cells produced a set of prominent bands indicated by the arrows in Fig. 1A. These bands were absent in the lanes containing extracts prepared from an AZF1 deletion strain, indicating that Azf1 is necessary for the formation of the complexes. The pYES-AZF1 plasmid, encoding a V5 epitope-tagged Azf1 protein, restored the specific bands, but the control plasmid did not. The AZF1-dependent bands were competed by oligonucleotides containing the A2GA5 repeats but were not competed by other oligonucleotides in which the repeats were altered or absent (Fig. 1B). Antibodies against the epitope tag decreased the mobility of the prominent gel shift band in extracts containing the tagged Azf1 (Fig. 1C). These did not affect the mobility of the bands in lanes with wild-type extracts in which Azf1 was not tagged. These results support the idea that the Azf1 protein is a part of the protein-DNA complex.

An additional gel shift band is also observed in Fig. 1A. This band is not abolished by AZF1 deletion and showed faster mobility on the gel than the AZF1-dependent bands. This band is most prominent in the azf1Δ extracts and may represent another regulatory protein that can bind to this region of the CLN3 promoter.

The 57-bp region of the CLN3 promoter from −626 to −570 contains two sets of A2GA5 repeats (see Fig. 2). We used a gel shift assay to determine if the shifted complex containing the Azf1 protein could bind both of these regions. Extracts from galactose-grown cells carrying the pYES-AZF1 plasmid were incubated with the entire 57-bp CLN3 probe. Smaller unlabeled sections from within the 57-bp region were added to determine whether they could compete with the labeled probe for binding (Fig. 2). Oligonucleotides containing either upstream or downstream repeats effectively competed for binding, indicating that both sets can compete with the entire 57-bp region. Two competitors with mutations in the core A2GA5 repeats, with changes of the G to a T or a C (oligonucleotides D and F in Fig. 2), did not compete, indicating sequence-specific interaction.

The Azf1 protein is present at the CLN3 promoter in vivo. To determine if Azf1 is associated with the CLN3 promoter in vivo, we used chromatin immunoprecipitation assays. In these experiments, protein-DNA complexes are cross-linked in vivo and isolated by immunoprecipitation of the DNA-binding protein. The presence of specific DNA regions can then be measured in the immunoprecipitated samples by quantitative PCR amplification. Cell lysates were prepared from azf1Δ cells car-
AZF1 is a regulator of transcription in S. cerevisiae

Deletion of AZF1 reduces glucose induction of CLN3. Deletion of AZF1 decreased CLN3 mRNA levels in cells grown in glucose but had little effect on CLN3 message levels in cells growing in ethanol (Fig. 4). Thus, the induction of CLN3 by glucose was reduced in the azf1Δ mutant. This CLN3 transcriptional defect is rescued in the azf1Δ strain by pYES-AZF1, which overexpresses AZF1. In our hands, while this plasmid could be induced by galactose, it produced a significant level of expression in glucose medium as well. Overexpression of AZF1 increased CLN3 expression in both carbon sources. However, the effect of AZF1 overexpression on CLN3 levels was more prominent in glucose medium, despite the fact that pYES-AZF1 produces higher levels of Azf1 in nonfermentable medium than in glucose. The average band intensities from three different experiments are shown in Fig. 4B. Deletion of AZF1 had little effect on CLN3 mRNA expressed from the CUP1 promoter (Fig. 4C), nor did it affect mRNA levels for CDC28 or BCK2 (Fig. 4D). Because CLN3 regulates progression through Start, we might expect loss of Azf1 to indirectly produce a change in Start-specific transcripts. We observed a decrease in CLN2 message levels in the azf1Δ strain but no effect on CLN1 mRNA levels.

We found that AZF1 is required for the glucose induction of a reporter construct driven by the CLN3 A2GA5 repeats. Wild-type cells carrying a HIS3 reporter driven by CLN3 A2GA5 repeats show a glucose-dependent increase in HIS3 expression. This allowed growth on glucose in the absence of histidine but not with glycerol-lactate as the carbon source (Fig. 5A). This was more apparent when 2 mM AT was added to the medium to inhibit the function of the HIS3 gene product. This glucose-dependent expression of HIS3 was specific to the A2GA5 reporter and was not observed in cells carrying the identical HIS3 reporter in which the A2GA5 elements were not present. This response also requires AZF1; deletion of AZF1 blocked the increase in HIS3 expression from the CLN3 A2GA5 repeats. These results support a role for Azf1 in glucose transcriptional induction of CLN3 via the A2GA5 repeats. The requirement for Azf1 and the A2GA5 sequences in glucose induction of the HIS3 reporter is also evident in the Northern blot shown in Fig. 5B.
Glucose-dependent transcriptional activation by Azf1.

One possible mechanism for regulating CLN3 in response to glucose would be to alter Azf1 binding to DNA target sites. However, we were unable to identify any differences in gel shift patterns between protein extracts from yeast grown in glucose or glycerol-lactate (Fig. 6). Minor differences in band intensities were observed between extracts but were not reproducibly associated with a carbon source. In addition, the Azf1-Gal4 activation domain fusion isolated in the 1-hybrid selection increased reporter and CLN3 mRNA levels in either glucose or glycerol-lactate (data not shown). This indicates that the zinc finger portion of Azf1 was able to recruit the Gal4 activation domain to the CLN3 sequences in either carbon source.

Glucose appears to increase the transcriptional activity of the Azf1 protein. Fusion of the Gal4 DNA-binding domain (DBD) and Azf1 to direct Azf1 to an artificial promoter produces glucose-dependent growth on medium containing glucose and lacking histidine (glucose His− medium) in the presence of 2 mM AT. In glycerol-lactate medium, the fusion was inactive, producing no increase in growth compared to the control strain. This response required the Azf1 component of the fusion and was not observed with the control plasmid that expresses the Gal4 DBD alone (Fig. 7A). Glucose-dependent expression of the HIS3 reporter is also evident in the Northern blot shown in Fig. 7B. It can be seen in Fig. 7C that the levels of expression of mRNA encoding the Azf1-Gal4 DBD fusion are similar under the different growth conditions. This provides strong evidence that Azf1 is a glucose-dependent transcriptional activator.

**DISCUSSION**

Azf1 as a regulator of CLN3. Previous work demonstrated that CLN3 is positively regulated by glucose in a process that involves a set of A2GA5 repeats. We identified Azf1, a nuclear zinc finger protein thought to be a transcription factor (2, 4, 24), as a part of a complex that binds to the A2GA5 sequences. Taken together, our results indicate that Azf1 binds to the A2GA5 sequences in the CLN3 promoter and plays a role in the glucose induction of CLN3 transcription. These experiments confirm that Azf1 is a DNA-binding protein and identify a DNA sequence that interacts with Azf1. Chromatin immunoprecipitation experiments show that Azf1 binds to the CLN3 promoter in vivo, identifying a gene target for Azf1.

While Azf1 appears to play a role in regulating CLN3 transcription, several lines of evidence indicate that there are other proteins involved in this process as well. First, deletion of AZF1 reduces but does not abolish glucose induction of CLN3. This suggests that some other protein(s) also functions to regulate CLN3 expression in response to glucose. Second, we observed an additional gel shift band with the CLN3 DNA

**FIG. 5.** Azf1 and glucose are required for CLN3 reporter expression. (A) Wild-type (WT) and azf1Δ strains carrying the CLN3 A2GA5-driven HIS3 reporter (TLN25 and TLN60, respectively), along with isogenic strains carrying a control reporter lacking the CLN3 promoter sequences (control-HIS3) (TLN26 and TLN61, respectively), were serially diluted and plated in 10−2 drops containing the indicated number of cells. The media used were as indicated, and cells were incubated at 30°C. The DS10 background strain carrying a HIS-marked plasmid was used as a His− control. (B) Northern blots using RNA from the strains presented in panel A.

**FIG. 6.** Nutrient conditions do not alter the AZF1-dependent gel shift pattern. Cell extracts from wild-type (BY4741) and azf1Δ (2369) strains grown in either YEPD or YEP-glycerol-lactate, as indicated, were prepared for gel shift experiments as described for Fig. 1. Unlabeled oligonucleotides were added as competitors at concentrations 25- and 100-fold greater than that of the labeled probe as indicated. The arrows indicate a specific set of bands that disappear in the azf1Δ lanes. The five-rayed star indicates an AZF1-independent complex.
consistent with the lack of phenotypes reported for AZF1 deletion, more than one DNA-binding protein. One explanation for these results is that Azf1 is part of a redundant system with higher-order complexes when Azf1 is present. Another possibility is that Azf1 competes with another protein for DNA binding or that it is shifted to a different conformation in the absence of Azf1, suggesting that it represents a protein that is involved in a different mechanism.

Several lines of evidence suggest that Azf1 is not regulated by mechanisms that limit its ability to bind to DNA target sequences. First, access to the nucleus does not appear to be regulated in Azf1 deletion mutants. Despite the reduced levels of Azf1 abundance is regulated by carbon sources, this is not the mechanism by which glucose regulates Azf1 function. While Azf1 expression is regulated by carbon sources, with Azf1 protein levels increased in poor carbon sources and decreased in glucose (24). However, the carbon source regulation of Azf1 expression is opposite to the pattern we would expect for a transcriptional activator of CLN3. These results indicate that while Azf1 abundance is regulated by carbon sources, this is not the mechanism by which glucose regulates CLN3.

Although these results do not conclusively rule out regulation of DNA binding in vivo, they are more consistent with a model in which glucose does not regulate Azf1 function by altering nuclear localization or DNA-binding activity. This leaves us with a model in which Azf1 is normally bound to the ACGA sites in both glucose and glycerol-lactate sequences. In this model, the activity of Azf1 at the CLN3 promoter is somehow increased when the cells are in glucose medium. The strongest evidence for such a model comes from the finding that Azf1 can produce glucose-regulated transcription when tethered to Gal4 binding sites. In these experiments, we expected that the ability of the fusion protein to bind to the reporter would be unaffected by the carbon source. This fusion protein is expressed from the ADH promoter, with similar mRNA levels in both glucose- and glycerol-lactate-grown cells. The Gal4 DNA-binding domain subunit recognizes and binds to Gal4 binding sites in the presence of either carbon source (15).

It is also possible that another protein is regulated by glucose in a manner that alters its interactions with Azf1. In this model, Azf1 marks the gene to be regulated and serves as a binding site for the protein that receives the glucose signal. Discovering the proteins that interact with Azf1, and the effect of the carbon source on these interactions, will be helpful in understanding regulation of Azf1 activity.

FIG. 7. Azf1 has glucose-dependent transcriptional activity. Strain Pj69-4A carrying the HIS3 gene driven by Gal4 binding sites was transformed with a plasmid carrying either the Gal4 DBD (pOB2D) or an Azf1/Gal4 DBD fusion (pBDAZF1) as indicated. (A) Cells were grown for serial dilution on the indicated medium as described for Fig. 5. (B) Cells were grown in the indicated media and harvested for RNA preparation and Northern blotting with a HIS3 probe. U2 RNA was probed as a loading control. (C) Northern blot showing expression of the Azf1/Gal4 DBD fusion in glucose and glycerol lactate.

probe that remains in the absence of Azf1 (Fig. 1). This band is apparently specific in that it is competed away by an excess of unlabeled probe. The band becomes more prominent in the absence of Azf1, suggesting that it represents a protein that competes with Azf1 for DNA binding or that it is shifted to a higher-order complex when Azf1 is present. One explanation for these results is that Azf1 is part of a redundant system with more than one DNA-binding protein.

A model in which Azf1 is part of a redundant system is also consistent with the lack of phenotypes reported for AZF1 deletion mutants. Despite the reduced levels of CLN3 mRNA in azf1Δ cells growing in glucose, we observed no growth defect in glucose-grown cells. Although the mutant cells tended to be somewhat larger than wild-type cells, we saw no obvious delay in G1 when using flow cytometry (not shown). This is perhaps not surprising, given the incomplete block in CLN3 induction produced by AZF1 deletion, along with the well-known redundancy in the G1 cyclin pathway. In addition to regulation at the level of transcription, CLN3 expression is also regulated at the level of translation and protein stability (8, 13, 22, 27). Beyond this, it is thought that Cln1 and Cln2 can serve some of the functions of Cln3 (23). In addition, BCK2 appears to play a role in G1 progression that is parallel to that of CLN3 (9, 11, 25). The presence of these alternate pathways may mask the effects of AZF1 deletion.

Glucose regulation via Azf1. Several lines of evidence suggest that Azf1 is not regulated by mechanisms that limit its ability to bind to DNA target sequences. First, access to the nucleus does not appear to be regulated in Azf1 deletion mutants. Despite the reduced levels of Azf1 abundance is regulated by carbon sources, this is not the mechanism by which glucose regulates CLN3. In addition, BCK2 appears to play a role in G1 progression that is parallel to that of CLN3 (9, 11, 25). The presence of these alternate pathways may mask the effects of AZF1 deletion.

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