

Transcriptional Regulation of *CLN3* Expression by Glucose in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae*, the transition from the G₁ phase of the mitotic cycle into S phase is controlled by a set of G₁ cyclins that regulate the activity of the protein kinase encoded by *CDC28*. Yeast cells regulate progress through the G₁/S boundary in response to nutrients, moving quickly through G₁ in glucose medium and more slowly in poorer medium. We have examined connections between glucose and the level of the message encoding Cln3, a G₁ cyclin. We found that glucose positively regulates *CLN3* mRNA levels through a set of repeated AAGAAAA (A₂GA₅) elements within the *CLN3* promoter. Mutations in these sequences reduce both transcriptional activation and specific interaction between *CLN3* promoter elements and proteins in yeast extracts. Creation of five point mutations, replacing the G's within these repeats with T's, in the *CLN3* promoter substantially reduces *CLN3* expression in glucose medium and inhibits the ability of the cells to maintain a constant size when shifted into glucose.

Saccharomyces cerevisiae cells coordinate growth and proliferation so that cells growing under a variety of conditions are able to maintain an almost constant average size. Cell growth and division are thus in some way tethered. Were it not so, an increase in growth rate in the absence of a corresponding increase in the rate of cell division would generate larger cells. Conversely, a decrease in growth rate in the absence of a corresponding lengthening of the cell cycle would produce very small cells. Yeast cells adjust the length of the cell cycle in different media by varying the length of the G₁ phase (11, 12). In rich media containing glucose, yeast cells grow rapidly and have a short G₁ phase, whereas in a poor carbon source such as ethanol, yeast cells prolong the cell cycle by spending more time in G₁. As nutrients are depleted, G₁ lengthens until the cells eventually arrest in G₁ (25). While cell size clearly plays a role in governing the length of G₁, the effects of nutrients on cell cycle length cannot be explained solely by a model in which nutrients affect the time required to reach a threshold size for budding. This can be inferred from the fact that G₁ length is strongly influenced by nutrients in mother cells, which have already reached critical size (10b, 15, 16).

Progress through G₁ requires the association of the Cdc28 kinase with one of three G₁ cyclins, Cln1, Cln2, or Cln3. Mutation in all three G₁ cyclins or inactivation of Cdc28 leads to cell cycle arrest in late G₁ (9). Recent observations suggest that Cln3 is an upstream regulator of other G₁ cyclins (5, 23, 24). It has been shown that transcriptional manipulation of *CLN3* alters the duration of G₁: overexpression of *CLN3* shortens G₁, while deletion of *CLN3* leads to a delay in G₁ (2, 18). Recently, work in several laboratories has demonstrated nutrient regulation of Cln3/Cdc28 kinase activity. Nutrients regulate Cln3 protein levels through effects on transcription, translation, and protein stability (8, 10, 19, 20). *CLN3* mRNA levels are high in log-phase cells rapidly growing on glucose and decline as cells

enter oxidative growth in ethanol. Carbon source regulation of *CLN3* transcription involves a mechanism that is independent of growth and does not require the Ras-cyclic AMP pathway (19). Changes in *CLN3* mRNA seen in response to different carbon sources are reflected in Cln3 protein levels and Cln3/Cdc28 kinase activity (10).

Addition of glucose to post-log-phase cells can produce a 5- to 10-fold increase in *CLN3* mRNA levels within 5 min, indicating that the cells are in some way responding to a glucose signal. Several transcriptional responses to glucose have been studied, including the induction of glycolytic enzymes, repression of genes necessary for growth in nonfermentable carbon sources, and repression of genes involved in respiration (13). Thinking that regulation of *CLN3* by glucose might involve a known glucose signalling pathway, we have tested several mutations in glucose repression and induction pathways for alterations in the regulation of *CLN3*. These experiments have produced negative results: *CLN3* induction is normal in these mutants. This indicates that the mechanism of glucose regulation of *CLN3* is probably distinct from previously characterized processes. In addition, although the Mcm1 protein is thought to play a role in cell cycle-specific variations in *CLN3* transcript levels (17), we have found that temperature-sensitive *mcm1* mutants have normal glucose induction of *CLN3* at the restrictive temperature (10a). This, taken together with the finding that induction of *CLN3* by glucose is cell cycle independent (19), indicates that glucose induction of *CLN3* is also distinct from the cell cycle-dependent transcriptional regulation reported by McInerney et al. (17).

We have therefore examined the *CLN3* promoter in order to identify regions that regulate transcription in response to glucose. Here we report the presence of repeated sequences within the *CLN3* promoter that are important for transcriptional induction by glucose. Mutations in these sequences reduce both transcriptional activation and specific interactions between *CLN3* promoter elements and proteins in yeast extracts. Southwestern blots identify a 69-kDa protein that specifically binds to these repeated sequences.

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MATERIALS AND METHODS

Strains and media. The yeast strains used in this work were DS10 (*MAThis3-11,15 leu2-3,112 his1 lys2 ura3-52 trp1Δ*) and TG3 (isogenic to DS10 except for a *CLN3* deletion [3]). These strains were transformed with various plasmids as indicated. Cells were grown either in YEPD containing 1% yeast extract, 2% Bacto Peptone, and 2% glucose or in synthetic complete medium containing 6.7-g/liter yeast nitrogen base (Difco) supplemented with adenine, uracil, amino acids, and 2% glucose (21). Cell size measurements were performed with a Coulter Counter Channelizer using a 70- μ m aperture calibrated with latex beads.

RNA preparation and Northern blotting. Total yeast RNA was isolated as described previously (6). The RNA samples were separated by formaldehyde gel electrophoresis and transferred to a GeneScreen Plus membrane (New England Nuclear). To ensure uniform loading and transfer of RNA, ethidium bromide was added to the samples, and the ethidium-stained rRNA was visualized on the blots under UV illumination. Blots were also probed with a radiolabeled 0.6-kb *SacI* fragment from *U2* to confirm uniform loading. Northern blots were probed with a 1.1-kb *EcoRI* fragment from *URA3* or a 1.8-kb *BamHI* fragment from *CLN3*. All probes were radiolabeled to a specific activity of 10^9 cpm/ μ g by using [α - 32 P]dCTP (3,000 μ Ci/nmol) and random priming.

***CLN3* promoter-reporter fusion constructs.** A series of *CLN3* promoter fragments were generated by PCR and restriction digests and then inserted into the multiple cloning site of yeast 2 μ plasmid pCA205 (a gift from Cathy Atchinson) upstream of *URA3*, which served as a reporter gene. The *URA3* coding region in pCA205 is preceded by only 24 bp of the untranslated *URA3* sequence containing one transcriptional start site and no known upstream activating sequences.

The -726 to +18 fragment was amplified by PCR using a primer (5'-TGCG ACCGTGATGATACCGA-3') upstream of the *SacI* site at -726 as an upstream primer and a primer (5'-TAATTATGGGATCCCTCAATATGGCC-3') into which a *BamHI* site (underlined) was introduced as the downstream primer. The product was then cut with *BamHI* and *SacI* and inserted into the *SacI/BamHI* sites of pCA205. A similar strategy was used to subclone the -549 to +18 fragment into pCA205 by using the same downstream primer and an upstream primer (5'-CTCACTGTAATGATCAAGTTAC-3') into which a *BclI* site was introduced. After digestion with *BclI* and *BamHI*, the PCR product was inserted into the *BamHI* site of pCA205. The -726 to -143 fragment was generated by using the primer described above upstream of the *SacI* site and a downstream primer (5'-ATTGGAGATCTGAGATTGCG-3') with an introduced *BglII* site. After digestion with *SacI* and *BglII*, the PCR product was inserted into the *SacI/BamHI* site of pCA205. The -726 to -250 fragment was amplified by using an upstream primer (5'-GAGACACTGCAGAGGCTACATTA-3') and a primer (5'-GGTTTAAGTCTGCAGAGGACTAC-3') containing an artificial *PstI* site downstream. The PCR product was digested with *Sall* (native site at -931) and *PstI* was then inserted into pBSK⁺. Digestion with *SacI* (-726) and *PstI* released the fragment from the plasmid for subcloning into *SacI*- and *PstI*-digested pCA205. The plasmid containing the -726 to -414 fragment was produced by deletion of the *XhoI/BamHI* segment from the -726 to +18 plasmid. After digestion, the ends were filled in with Klenow and ligated. The plasmid carrying the -549 to -414 segment was generated by digesting the -549 to +18 pCA205 plasmid with *XhoI* and *BamHI*. Again, the ends were ligated after Klenow filling. The -726 to -549 plasmid was made by using a primer (5'-GAGACACTGCAGAGGCTACATTA-3') upstream of the naturally occurring *SacI* (-726) site and a downstream primer (5'-GTA ACT TGA TCA TTA CAG TGA G-3') flanking the naturally occurring *BclI* site. The fragment was cut with *SacI* and *BclI* and inserted into the *SacI/BamHI* site of pCA205. The plasmid containing the -626 to -570 promoter fragment was generated by annealing two synthetic oligonucleotides (5'-AATTCAAGAAAAAAGAAAAA GAAAAAGTGAATAATATCAGGCAAGAAAAAAGAAATTAC-3' and 5'-GATCGTAATTTCTTTTCTTGGCTGATAATTTTTCACCTTTTCTTTTCTTTT TTTTCTT-3') such that the resulting four-base overhangs on the ends were compatible with *EcoRI* and *BamHI* overhangs. This double-stranded oligonucleotide was then cloned into the *EcoRI/BamHI* site of pCA205. The corresponding plasmid with the -626 to -570 fragment carrying mutations in the A₂G₅ repeats was made in the same manner, by using the following oligonucleotides (the substitutions are underlined): 5'-AATTCAAAAAAAGAAAAA AAAAAAGTGAATAATATCAGGCAAGAAAAAAGAAATTAC-3' and 5'-GATCGTAATTTCTTTTCTTGGCTGATAATTTTTCACCTTTTCTTTTCTTTT TTTTCTT-3'. Both plasmids were sequenced to ensure proper positioning of the inserts. All plasmid constructs were sequenced to ensure that no mutations were generated by PCR. Plasmid copy number varied by less than 25% (data not shown).

Gel shift assays. Yeast cells (DS10) were grown in YEPD medium (500 ml) to an A₆₀₀ of 6. The cells were resuspended in fresh medium and, after incubation at 30°C for 30 min, collected by centrifugation at 2,000 rpm for 20 min. Under these conditions, transcription of *CLN3* is high. The cells were resuspended in protein extraction buffer containing 0.2 M Tris (pH 8.0), 0.4 M ammonium sulfate, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 2 mM β -mercaptoethanol, 1- μ g/ml pepstatin A, 0.2-mg/ml phenylmethylsulfonyl fluoride, and 20- μ g/ml aprotinin. Frozen cell pellets were prepared by immediately dripping the cell suspension into liquid nitrogen. Cell disruption was performed by extensive chopping of the cell pellets in a household mini-chopper. A fine powder was obtained and allowed to thaw on ice. Cellular debris were removed by centrif-

ugation at 20,000 rpm for 20 min at 4°C. The supernatant was precipitated with 0.534 volume of 3.8 M ammonium sulfate for 30 min at 4°C. The precipitated proteins were collected by centrifugation at 20,000 rpm for 20 min. Pellets were resuspended in 500 μ l of protein buffer (20 mM HEPES [pH 8.0], 100 mM KCl, 1 mM MgCl₂, 0.2 mM dithiothreitol [DTT], 5% glycerol, 0.5 mg of phenylmethylsulfonyl fluoride) and dialyzed for 3 h against two 500-ml changes of the same buffer. Insoluble materials were removed by 5 min of centrifugation at 14,000 rpm in a microcentrifuge at 4°C. The supernatant was immediately frozen on dry ice and stored at -70°C. Protein concentration was determined by using a Bio-Rad kit.

Binding reactions were carried out in a 20- μ l reaction volume containing 20 mM HEPES (pH 8.0), 0.1% Nonidet P-40, 5% glycerol, 1 mM DTT, 50 mM KCl, 0.5 mM MgCl₂, 2 μ g of poly(dI-dC), 2 to 15 fmol of labeled DNA probe, and 4 to 8 μ g of crude yeast extract. The protein extract was first incubated on ice with a molar excess of the unlabeled competitor (when added) for 5 min and then incubated for 15 min at room temperature in the presence of the labeled probe. Samples were subjected to electrophoresis on a 4% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29:1) in 0.5 \times TBE buffer (Tris-borate [pH 8.0], 1 mM EDTA) after the gel had been prerun for 1 h at room temperature. The gel was dried on Whatman paper for 1 h at 80°C and analyzed by using a Molecular Dynamics PhosphorImager.

Two radiolabeled DNA probes were used. The first was the -726 to -549 fragment from the corresponding pCA205 construct released with *EcoRI* and *Sall*. The second probe (-622 to -603) was a double-stranded synthetic oligonucleotide made by annealing the oligonucleotides (5'-GGGCAAGAAAAA AAAAAGAAAAAG-3' and 5'-GGGCTTTTCTTTTCTTTTCTTTG-3') corresponding to the region from -622 to -603 and producing non-*CLN3* GGG overhangs for fill-in labeling with Klenow. Both probes were radiolabeled with [α - 32 P]dCTP and Klenow fragment. Competition studies were performed with a molar excess of the unlabeled probe fragment or other double-stranded oligonucleotides, as indicated.

Southwestern blotting. The protocol described by Bassel-Duby et al. (1) was used with minor modifications. Protein extracts (50 μ g) were separated by electrophoresis on a sodium dodecyl sulfate-7.5% polyacrylamide gel and transferred to nitrocellulose. To confirm uniform loading, the blot was stained with Ponceau S. The air-dried nitrocellulose paper was immersed in binding buffer containing 25 mM HEPES (pH 8.00), 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 6 M guanidine hydrochloride and gently shaken for 10 min at 4°C. To renature the proteins, guanidine hydrochloride was gradually removed by repeating the same procedure eight times, each time with a concentration of guanidine hydrochloride twofold lower than that used in the previous wash. After the final wash without guanidine hydrochloride, the nitrocellulose was incubated for 1 h (room temperature) in 1 \times binding buffer containing 5% nonfat milk and 5- μ g/ml salmon sperm DNA. The filter was then incubated in binding buffer with 0.25% nonfat milk and 10⁶ cpm of the radiolabeled probe per ml either with or without competitor, as indicated. The probe for the Southwestern experiments was the -726 to -549 fragment described above for the gel shift experiments. Binding was carried out overnight. The filter was washed four times for 7 min each with 1 \times binding buffer at room temperature. The blot was analyzed by using a Molecular Dynamics PhosphorImager.

Site-directed mutagenesis. A PCR-based mutagenesis strategy, using *Pfu* DNA polymerase to minimize errors, was used to generate a *CLN3* promoter fragment corresponding to positions -931 to -414 in which the G's at positions -620, -608, -583, -577, and -455 were replaced with A's. CEN-based plasmid pKL001 (14), containing an epitope-tagged copy of the *CLN3* gene driven by the normal *CLN3* promoter, served as a template. First, a PCR fragment was made by using an upstream oligonucleotide flanking the *XhoI* site in plasmid pKL001 (5'-GGTACCGGGCCCCCTCGAGGTCGAC-3'; the *XhoI* site is underlined) and a downstream primer (-627 to -569) with four of the mutations (5'-GCAGGCTTGGTAATTTAATTTATGCTGATAATTTTTCACCTTTT TTTTCTTTTATTTGAAAG-3'; mutated bases are in boldface underlined). The product (positions -931 to -569) obtained with this first PCR step was then gel purified and used as the upstream primer in a second PCR with an oligonucleotide (5'-GATTAAGCTCGAGGAAAGTACAGATATACAAATTATAA ATAGGTAGGAGGAATAAAAAAG-3') in which the C at position -455 was replaced with a T (underlined) as a downstream primer. The final PCR product was digested with *XhoI* and used to replace the corresponding *XhoI* fragment in pKL001, replacing the wild-type region of the *CLN3* promoter between positions -931 and -414, with a fragment containing the mutations. The resulting plasmid, pKL001 Δ G, was sequenced to confirm the presence of the mutations and the orientation of the fragment. pKL001 Δ G and the original wild-type pKL001 plasmid were transformed into TG3 cells carrying a deletion in *CLN3* so that the only copy of *CLN3* in the cell was that which was carried on the single-copy plasmid.

Immunoprecipitation-Western blotting. Immunoprecipitation-Western blotting using epitope-tagged protein Cln3 was done as described by Tyers et al. (24). For each immunoprecipitation assay point, 5 mg of extract protein was used. Monoclonal antibody 12CA5 was used in the immunoprecipitations and as the primary antibody after blotting. An ECL detection kit (Amersham) was used to develop the Western blots.

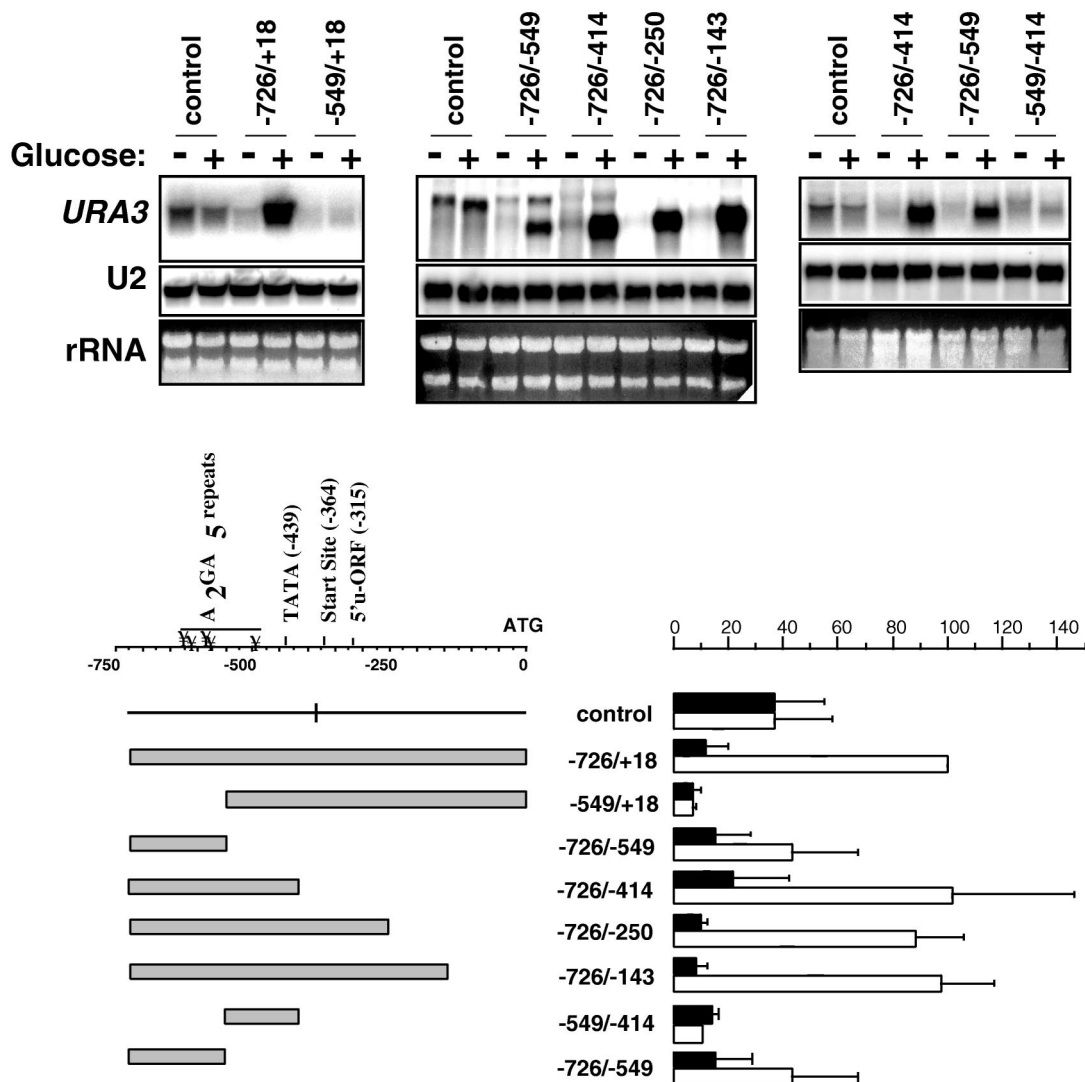


FIG. 1. Glucose stimulation of *CLN3* promoter-*URA3* reporter constructs. DS10 cells were transformed with a set of plasmids carrying *CLN3* promoter fragments of various lengths, as indicated, and grown in synthetic complete medium to post-log phase (optical density at 2 to 2.5). Cells were collected before and 20 min after transfer to fresh medium for RNA preparation. RNA (7.5 μ g/lane in the left and center panels and 15 μ g/lane in the right panel) was run on an agarose gel for Northern blotting with a *URA3* probe. The bars represent the averages of PhosphorImager data for the *URA3* reporter from at least three separate experiments for each construct, normalized to the -726 to +18 signal in fresh medium. Dark bars represent post-log-phase cells, and light bars indicate cells in fresh medium. Error bars represent standard deviations. The *CLN3* transcriptional start site, the TATA box, the A₂GA₅ regulatory elements, and the upstream open reading frame (u-ORF) identified by Polymenis and Schmidt (20) are designated on the map.

RESULTS

To identify elements within the *CLN3* promoter that are involved in glucose regulation of transcription, we tested the ability of *CLN3* promoter fragments to drive the expression of *URA3* as a reporter gene. Fragments of various lengths from the region of the *CLN3* gene 5' of the open reading frame, as indicated in Fig. 1, were inserted into the polylinker of 2 μ vector pCA205, a gift from Cathy Atchinson. This places the sequences to be tested upstream of a *URA3* gene that contains the *URA3* coding region with only a minimal portion of the promoter, 24 bp in length. To test the constructs, yeast cells carrying the plasmids were grown to post-log phase in selective media. Glucose was added back to the cells, and the ability of the promoter fragments to enhance transcription of *URA3* was assessed with Northern blots.

As shown in Fig. 1, the vector alone with no *CLN3* insert

expresses some of the *URA3* message, which is driven by a cryptic promoter within 2 μ sequences upstream of the polylinker (data not shown). It is important to note that this basal level of transcription is glucose independent. Insertion of a fragment (-726 to +18) corresponding to the 726 bp immediately upstream of the *CLN3* coding sequence caused *URA3* transcription to become strongly glucose dependent. In this construct, transcription was initiated from the normal *CLN3* transcriptional start site, as evidenced by primer extension mapping (data not shown) and the fact that the message decreases in size with 3' deletion of the insert (Fig. 1). In the control plasmid, transcriptional initiation takes place within 2 μ sequences approximately 350 bp upstream of the *URA3* ATG. The addition of the full-length *CLN3* promoter places the *CLN3* initiation site (at -364) approximately the same distance from the ATG. For this reason, the control message and that produced

by the -726 to +18 construct are approximately the same size. As 3' deletions are made from the *CLN3* promoter segment, the *CLN3* initiation site moves closer to the ATG, leading to progressively smaller messages.

While the region extending 726 bp upstream from the *CLN3* translational start produced strong glucose induction of the reporter gene, 5' deletion of 177 bp from this fragment, leaving the region from -549 to +18 driving the promoter, led to loss of the glucose response. In contrast, truncation of the promoter from the 3' end produced relatively little effect on the *URA3* transcript level, such that the -726 to -549 element alone remained strongly glucose inducible. However, while the -726 to -549 fragment confers glucose-dependent transcription, there is a decrease in the *URA3* message when driven by the -726 to -549 fragment compared to the -726 to -414 fragment. This may suggest that removal of the region between -549 to -414 caused loss of some glucose-responsive elements. On the other hand, the difference in transcriptional activity between the two fragments could also be due to the fact that the -726 to -549 element lacks the *CLN3* TATA box. Further analysis of the -549 to -414 element shows that although this region may play a role in the strong glucose response manifested by the -726 to -414 fragment, it cannot enhance transcription on its own (Fig. 1).

Several of the inserts appeared to block basal expression of the *URA3* message, compared to the control without an insert. This may be due to inhibitory sequences or transcriptional termination of this message somewhere within the insert. Our interest is in sequences that are able to alter transcription in response to glucose; therefore, we have not investigated this further. Additionally, some of the inserts appeared to allow continued expression of the basal transcript. For example, the -726 to -549 fragment produced a prominent glucose-inducible band with a transcriptional start site mapping to within the short *URA3* untranslated sequence (not shown), as well as a less prominent band that seems to correspond to the message produced in the absence of an insert. For our purposes, we have concentrated on the more prominent lower bands that are clearly glucose inducible and have not included the less prominent upper bands in the quantitation.

A closer examination of the -726 to -414 element shows the presence of four repeats of the eight-base sequence AAGAAAA (A_2GA_5), three in the forward direction at positions -622, -610, and -585 and one inverted in the antiparallel direction at position -460. In addition, there is a similar sequence, AAGAAATT, at position -579. To investigate the significance of these repeated sequences for the transcriptional activity of the -726 to -414 fragment, smaller constructs containing these sequences were tested for the ability to enhance transcription upon addition of glucose. We found that a 57-bp oligonucleotide corresponding to the region of the *CLN3* promoter from -626 to -570 is sufficient to produce a substantial glucose response (Fig. 2). This fragment contains three complete sets of the repeated sequence A_2GA_5 and AAGAAATT. Interestingly, when the G's in these four repeated sequences are mutated to A's, this fragment loses its transcriptional activity, as shown for two independent yeast transformants carrying this construct in the last four lanes of Fig. 2. This indicates that the repeated sequences play an important role in driving glucose-dependent transcription.

Looking for factors that mediate the glucose response, we used gel shift assays to determine whether yeast nuclear extracts contain a protein that will bind specifically to the DNA elements that drive glucose-dependent transcription. For this experiment, we used a DNA fragment corresponding to the sequence from -726 to -549 as a labeled probe. We found

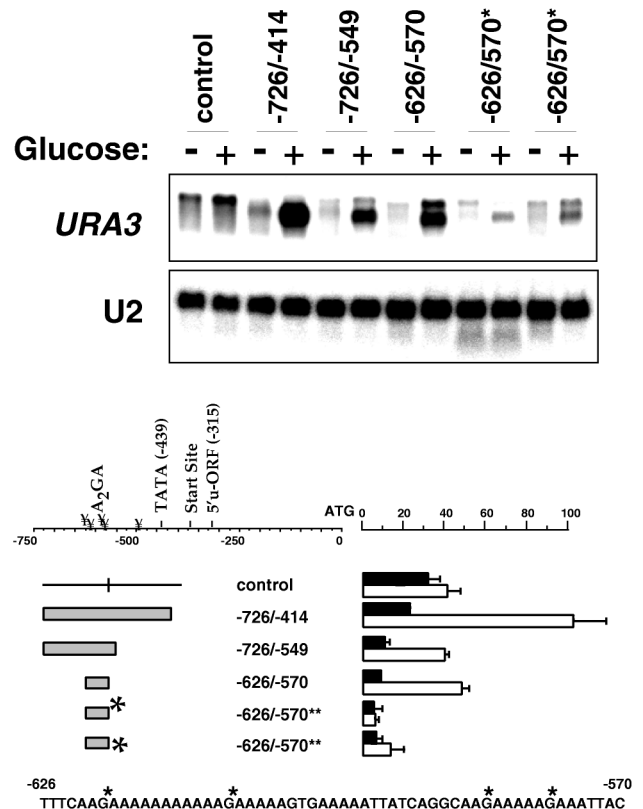


FIG. 2. A set of AAGAAAA repeats plays a role in glucose-dependent transcription. DS10 cells carrying a set of promoter-reporter fusions were treated as described in the legend to Fig. 1. Cells were collected before and 20 min after transfer to fresh medium for RNA preparation and Northern blotting (7.5 μ g/lane) with a *URA3* probe. The bars represent the averages of PhosphorImager data for the *URA3* reporter from at least three separate experiments for each construct, normalized to the -726 to +18 signal in fresh medium. Dark bars represent post-log-phase cells, and light bars indicate cells in fresh medium. Error bars represent standard deviations. Asterisks indicate the replacement of the indicated G's in the AAGAAAA repeats with A's. The results for two independent transformants are shown for the construct carrying the substitution mutations. The sequence from -626 to -570 is shown with the positions of the substitutions marked with asterisks. u-ORF, upstream open reading frame.

that this labeled fragment can form complexes with proteins in the yeast extract that can be specifically competed with a molar excess of the same fragment (Fig. 3). Shorter double-stranded oligonucleotides corresponding to positions -626 to -570 and -591 to -551, which contain four and two sets of the repeated sequence A_2GA_5 , respectively, also competed, but less effectively. We cannot tell from these experiments whether the less complete competition with the shorter oligonucleotides represents decreased affinity due to the absence of interactions with sites that are present on the longer probe or the presence of multiple proteins, some of which bind to sites on the probe that are not represented in the shorter fragments and therefore not competed. However, it is clear that competition with the shorter fragments was dependent on the A_2GA_5 repeats. Again, mutation of G's in the repeated sequences to A's diminished the ability of these fragments to compete with the labeled fragment (competing oligonucleotides containing the mutations are indicated by asterisks).

We found that a double-stranded oligonucleotide corresponding to the region between positions -622 and -603 with the sequence CCCAAGAAAAAAAAAAAAGAAAAAGGG, containing two of the A_2GA_5 repeats (underlined), was also

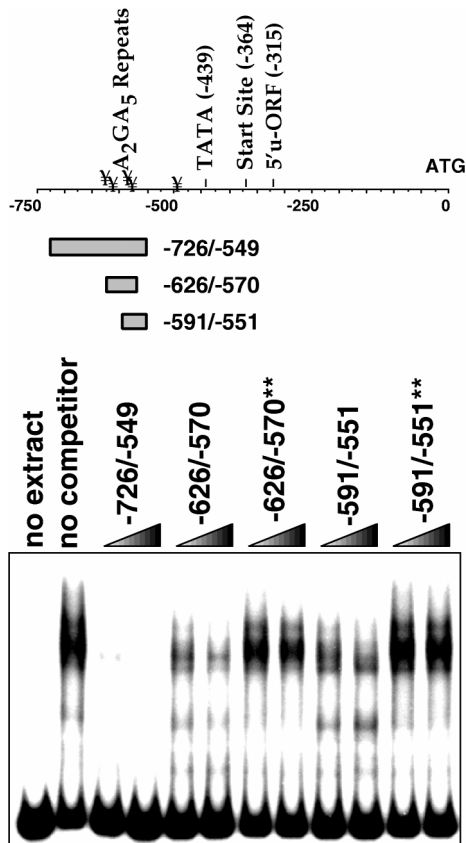


FIG. 3. A set of AAGAAAA repeats plays a role in protein binding to sequences that drive glucose-dependent transcription. A double-stranded DNA fragment corresponding to the sequence between -726 and -549 was labeled, incubated with protein extract, and run on a native polyacrylamide gel as described in Materials and Methods. Lanes: 1, probe (2 fmol) with no extract; 2 to 10, probe and extract; 2, no competitor; 3, 50 \times unlabeled -726 to -549 fragment; 4, 100 \times unlabeled -726 to -549 fragment; 5, 50 \times unlabeled -626 to -570 fragment; 6, 100 \times unlabeled -626 to -570 fragment; 7, 50 \times unlabeled -626 to -570 fragment with A's substituted for the G's in the A_2GA_5 repeats; 8, 100 \times unlabeled -626 to -570 fragment with A's substituted for the G's in the A_2GA_5 repeats; 9, 50 \times unlabeled -591 to -551 fragment; 10, 100 \times unlabeled -591 to -551 fragment; 11, 50 \times unlabeled -591 to -551 fragment with A's substituted for the G's in the A_2GA_5 repeats; 12, 100 \times unlabeled -591 to -551 fragment with A's substituted for the G's in the A_2GA_5 repeats. u-ORF, upstream open reading frame.

able to produce a DNA-protein complex (Fig. 4). In this experiment, two bands are evident, the lower labeled complex can be competed away with a molar excess of the unlabeled fragment, but this competition is abolished if T's are substituted for the G's in the two A_2GA_5 repeats. The upper band is less prominent and appears to be less specific in that it is competed by both oligonucleotides. These results indicate that the repeated sequences that are necessary for glucose induction are also important for the formation of specific DNA-protein complexes.

Because addition of glucose to the starved cells produced a large increase in the *CLN3* message, we searched for differences between DNA-protein complexes formed with extracts from post-log-phase cells and complexes formed with extracts from log-phase cells. We have been unable to identify any consistent difference in gel shift patterns between log-phase and post-log-phase extracts by using a variety of labeled probes (data not shown).

We used Southwestern blotting with a labeled double-stranded oligonucleotide probe corresponding to the region

between -626 and -570 to estimate the sizes of proteins that bind to the A_2GA_5 repeats. This probe identified two bands that were competed away by excess unlabeled probe. One of these bands, with an apparent molecular mass of 69 kDa was competed with a 15-fold excess of the short oligonucleotide, corresponding to the region between positions -622 and -603 (CCCAAGAAAAAAAAAAGAAAAAGGG), used in Fig. 4, containing just the two A_2GA_5 repeats (underlined). Competition was diminished when the unlabeled oligonucleotide was mutated to replace the G's in the A_2GA_5 sequences with T's (Fig. 5). In other experiments using a shorter double-stranded oligonucleotide as a probe, corresponding to the region between positions -591 and -551 , the upper 69-kDa band was labeled, but not the lower one, suggesting that the lower band requires sequences that are in the larger probe but are not found in the smaller for binding (Fig. 5B).

To confirm the importance of the A_2GA_5 repeats in the normal transcriptional regulation of *CLN3* by glucose, we mutated five repeated A_2GA_5 sequences in the *CLN3* promoter, replacing the central G's in the A_2GA_5 repeats with T's at positions -620 , -608 , -583 , -577 , and -455 . A restriction fragment containing the mutated promoter sequences was then used to replace the corresponding fragment from the normal *CLN3* promoter in plasmid pKL001. pKL001 is a CEN-based plasmid that contains an epitope-tagged *CLN3* coding sequence driven by the *CLN3* promoter (14). This plasmid was transformed into a *cln3* Δ strain to provide the only copy of *CLN3* in the cell. We then compared the expression of *CLN3* in cells carrying the mutant promoter (TKL1) with that in cells carrying the parent plasmid containing the wild-type promoter (TKL2). We found that mutations of the repeated sequences produced a substantial reduction in the ability of glucose to induce *CLN3* mRNA levels (Fig. 6A). This decreased *CLN3*

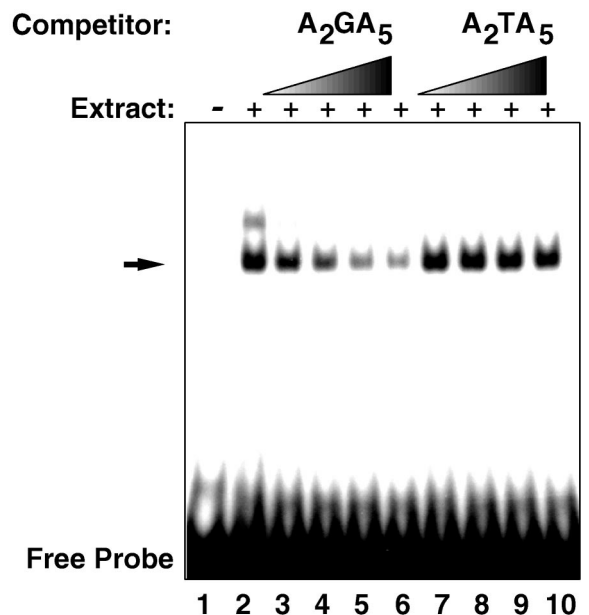


FIG. 4. Protein binding to a minimal AAGAAAA sequence. The gel shift experiment was done as described in the legend to Fig. 3, except that a double-stranded oligonucleotide with the sequence CCCAAGAAAAAAAAAAGAAAAAAGGG was used as the labeled probe. Lanes 1, probe with no extract; 2 to 10, probe and extract; 2, no competitor; 3 to 6 respectively, competition with 5-, 10-, 15-, and 20-fold excesses of unlabeled fragment; 7 to 10 respectively, competition with 5-, 10-, 15-, and 20-fold excesses of unlabeled fragment with T's substituted for the G's in the A_2GA_5 repeats. The arrow indicates the lower, specific band.

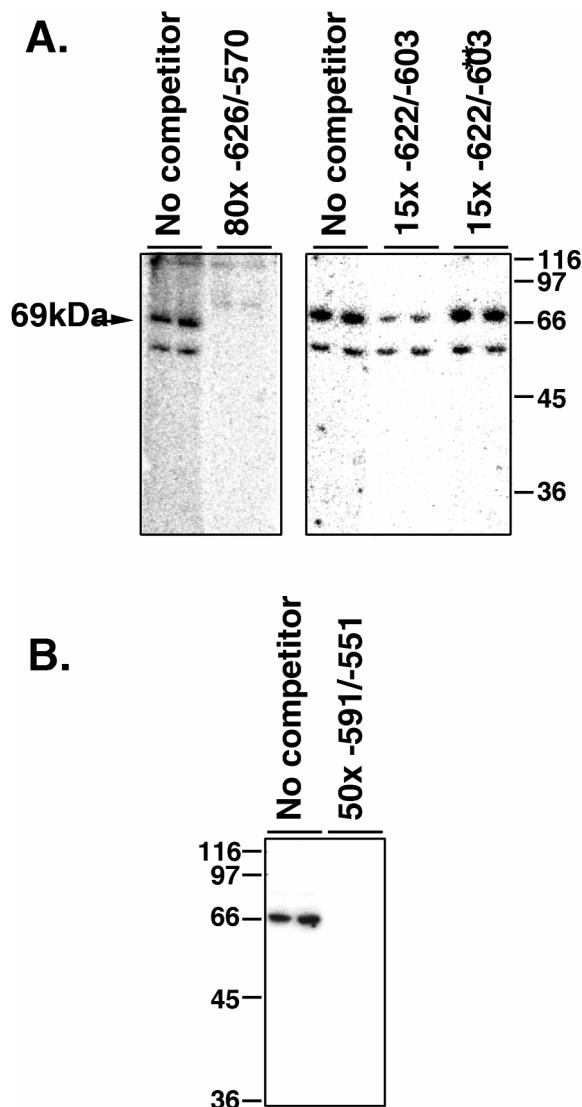


FIG. 5. Southwestern blots identify a 69-kDa protein that binds to a DNA sequence containing the A₂GA₅ repeats. Extracts were run on sodium dodecyl sulfate-containing gels and blotted as described in Materials and Methods. Blots were renatured, and strips containing duplicate lanes were incubated with the indicated labeled double-stranded DNA fragment as described in Materials and Methods. (A) Blots probed with double-stranded DNA corresponding to the sequence between positions -726 and -549. The left blot shows competition with an 80-fold molar excess of the unlabeled fragment. The right blot shows competition with a 15-fold molar excess of either an unlabeled double-stranded oligonucleotide (AAGAAAAAAAAAAGAAAAA [-622/-603]) or the corresponding fragment with T's substituted for the G's in the A₂GA₅ repeats (-622/-603**). (B) Blots were probed with double-stranded DNA corresponding to the sequence between -591 and -551 and unlabeled competitor as indicated. The values beside the lanes are molecular sizes in kilodaltons.

mRNA was also reflected in lower Cln3 protein levels, as shown by the immunoprecipitation-Western blot in Fig. 6A. Cln3 protein levels are difficult to measure in cells that are not overexpressing the protein. While it is clear that the mutant promoter produces less Cln3 protein in vivo, it is difficult to estimate the magnitude of this difference because Cln3 protein levels were close to the limit of detection.

The induction of *CLN3* mRNA by glucose suggests that cells increase *CLN3* expression in order to accelerate progress through the cell cycle. This would allow the cells to keep up

with the increase in cellular growth rate that glucose produces and maintain a relatively constant cell size. We found that while the mutations in the *CLN3* promoter produced little noticeable effect on post-log-phase cells, in glucose, the mutant cells were approximately twice as large as those expressing *CLN3* at normal levels from the wild-type promoter (Fig. 6B). This is consistent with the lower Cln3 levels and a decreased

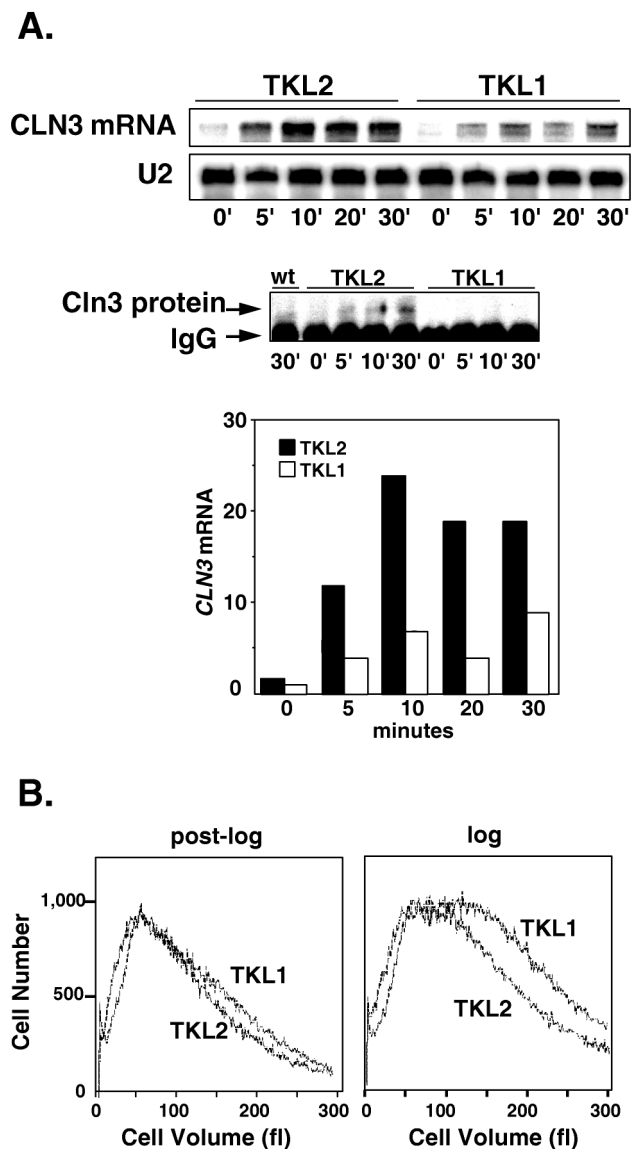


FIG. 6. Mutations in five A₂GA₅ elements in the *CLN3* promoter decrease *CLN3* expression and increase cell size in glucose. (A) Cells carrying a deletion of the chromosomal copy of *CLN3* and carrying CEN plasmids expressing *CLN3* from either the wild-type (wt) promoter (TKL2) or a promoter carrying five point mutations in which T's were substituted for the G's in the A₂GA₅ repeats (TKL1) were grown in synthetic medium to post-log phase (optical density of 2). Fresh glucose was added to 2% at time zero, and samples were collected at the indicated times for Northern blotting (top) and immunoprecipitation-Western blotting (bottom) as described in Materials and Methods. IgG (immunoglobulin G) refers to the band produced by the 12CA5 antibodies used in the immunoprecipitations. The bar graph represents PhosphorImager data for the *CLN3* mRNA for each construct, normalized to the U2 signal. The scale is arbitrary. (B) Post-log-phase cells (optical density at 660 nm of 2) and cells growing in log phase (optical density at 660 nm of 0.5) in synthetic medium were collected for size measurements with a Coulter Counter Channelizer. DS10 was used as an untagged control in the immunoprecipitation-Western blotting experiment.

ability to accelerate movement through the cell cycle in response to glucose.

DISCUSSION

We have previously demonstrated that *CLN3* mRNA levels are regulated by carbon source and that glucose induction of *CLN3* transcription does not depend on either cellular growth or progression through the cell cycle (19). By using *CLN3* promoter-reporter fusion constructs, we have identified a region between positions -762 and -414 in the *CLN3* promoter that can confer glucose regulation on a heterologous reporter gene. This region within the *CLN3* promoter contains five copies, four exact and one partial, of the sequence AAGAAA AA. Small double-stranded oligonucleotides containing these repeated sequences are able to drive glucose-dependent expression of the reporter gene. Mutations in these elements substantially reduce transcriptional induction of both a *URA3* reporter and the *CLN3* gene by glucose. Mobility shift assays demonstrate specific binding of proteins from yeast extracts to DNA fragments containing the repeats. Protein binding to the labeled oligonucleotide can be competed with a molar excess of unlabeled oligonucleotides containing the above repeated sequences, but mutation of the central G's within the repeats decreases the ability of the unlabeled DNA to compete. These results indicate the importance of the repeated elements for both induction of transcription and DNA-protein interactions. Southwestern blotting revealed a protein with an apparent size of 69 kDa that binds specifically to these sequences in a manner that depends on the intact repeats, as well as a smaller protein that binds in a manner that is not affected by changes in the repeats.

We have found no evidence to indicate that glucose regulates the binding of proteins to elements of the *CLN3* promoter that confer glucose responsiveness. This suggests that glucose regulation of *CLN3* involves a process that is more complex than the simple regulated binding of a transactivator, or repressor protein, to the *CLN3* promoter. One important step in learning how glucose affects *CLN3* expression would be to identify the protein(s) that binds to the glucose-responsive elements in the *CLN3* promoter. It would be interesting to know how these proteins are affected by glucose. Another important question is whether these factors are specific to *CLN3* expression or are part of a common pathway that coordinates the expression of a large group of genes in response to glucose. In this regard, a database search yielded only a few yeast promoters carrying these repeated elements. These genes did not fall into any obvious group in terms of function, nor do they all appear to be transcriptionally regulated by glucose. Therefore, we cannot say whether the features that regulate transcription of *CLN3* are important in regulating other genes.

Mutation of only five bases, confined to A₂GA₅ repeats within the *CLN3* promoter, produces cells that do not properly increase Cln3 levels in response to glucose. This decrease response clearly has functional significance, because in glucose medium, these cells become approximately twice as large as the controls that express *CLN3* from the wild-type promoter. We were unable to detect an appreciable amount of Cln3 protein in the TKL1 cells; however, we believe that Cln3 protein is being expressed in these cells, because they appear smaller than cells with *CLN3* completely deleted. The single-copy plasmid produced Cln3 protein levels that were, in our study, close to the limit of detection. We propose that TKL1 cells have some level of functional Cln3 protein that is too low to give a signal in our experiments. While it is clear that these elements contribute to the response to glucose, it is

equally clear that these elements alone do not, by themselves, account for the cell cycle shortening that glucose produces. First of all, while mutation of the five A₂GA₅ repeats decreased the ability of glucose to increase *CLN3* mRNA levels, this did not completely abolish the response. This may be because other elements or mechanisms that do not involve these repeated sequences remain able to increase *CLN3* transcript levels in response to glucose. It is also possible that the remaining response stems from the presence of additional imperfect copies of the repeated sequence. While we have focused on the 8-bp A₂GA₅ sequences, there are a total of 13 copies of the shorter 6-bp sequence AAGAAA within the 1,000 bases upstream of the *CLN3* coding sequence. It is possible that these also contribute to the glucose response. Secondly, Cln3 protein levels are regulated on a variety of levels in addition to transcription in response to nutrient signals. These mechanisms include posttranscriptional regulation by rich medium (20), the Ras-cyclic AMP pathway (10), protein synthesis rates (10), and nitrogen limitation (8). Finally, the *CLN3* gene is believed to act in a redundant pathway, in that deletion of *CLN3* is only lethal in combination with loss of other genes, most notably, *BCK2* (4, 7). It seems likely that nutrients will regulate other cell cycle components in addition to *CLN3*.

The closest mammalian homologs to Cln3 appear to be the D-type cyclins. Both D-type cyclins and *CLN3* have similarities in expression, being less affected by cell cycle position than other cyclins, and both types of cyclin appear to function in the process of ending G₁. The D cyclins serve to link mitogenic signals from the cellular environment to the process of exiting G₁ (22). As with the D cyclins, Cln3 levels appear to be regulated by environmental signals, in this case originating from nutrient resources. The transcriptional regulation of *CLN3* that we describe appears to be but one of the multiple layers of control that allow yeast cells to modulate G₁ length.

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