Coupling of cell division to cell growth by translational control of the G₁ cyclin CLN3 in yeast

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The eukaryotic cell cycle is driven by a cascade of cyclins and kinase partners including the G₁ cyclin Cln3p in yeast. As the first step in this cascade, Cln3p is uniquely positioned to determine the critical growth-rate threshold for division. To analyze factors regulating CLN3 expression, we identified a short upstream open reading frame (uORF) in the 5' leader of CLN3 mRNA as a translational control element. This control element is critical for the growth-dependent regulation of Cln3p synthesis because it specifically represses CLN3 expression during conditions of diminished protein synthesis or slow growth. Inactivation of the uORF accelerates the completion of Start and entry into the cell cycle suggesting that translational regulation of CLN3 provides a mechanism coupling cell growth and division.

[Key Words: Primer extension; prt1; cyclin; CLN3; uORF; translation]

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Cell proliferation is primarily regulated during the first gap phase of the cell cycle (G₁) when cells monitor their environment before replicating their DNA (Pardee 1989; Norbury and Nurse 1992). The point in G₁ at which cells decide to pass through the cell cycle is called Start in yeast and the restriction point in animal cells. This point was first defined by critical growth rate requirements in both systems (Pardee 1974; Hartwell and Unger 1977; Johnston et al. 1977). Start was particularly defined by attainment of a critical rate of protein synthesis that was required for cells to enter S phase (Popolo et al. 1982; Moore 1988). Such a control mechanism ensures that a critical growth rate has been achieved for completion of cell division. Despite the importance of this coordination, mechanisms connecting cell division to growth are poorly understood.

Cell division requires the activity of cyclins complexed with one or more members of the cyclin-dependent kinase (cdk) family (Sherr 1996). Passage through Start depends on the presence of G₁ cyclins that induce the G₁ → S transition when coupled with their cdk partners (Murray and Hunt 1993). One such G₁ cyclin, Cln3p, is the first step in a cascade of cyclin-regulated cell cycle events in Saccharomyces cerevisiae making it a good candidate for coordination of cell growth and division at Start (Cross 1988; Nash et al. 1988; Tyers et al. 1992, 1993; Dirick et al. 1995).

Cln3p functions upstream of all other G₁ cyclins and it is necessary for the punctual execution of Start (Tyers et al. 1993; Dirick et al. 1995; Stuart and Wittenberg 1995). Expression of CLN3 is strongly associated with the size threshold for division, as CLN3 dosage is increased, cells divide at smaller than normal size, and G₁ is shortened; cells divide at larger than normal size when CLN3 is deleted (Cross 1988; Nash et al. 1988). Cells expressing stable forms of Cln3p also have a very short G₁ and divide at a smaller than normal size, because wild-type Cln3p is extremely unstable (Tyers et al. 1992; Cross and Blake 1993; Yaglom et al. 1995). Whereas mRNA levels are similar among the G₁ cyclins CLN1, CLN2, and CLN3, Cln3p levels are extremely low compared with those of Cln1p and Cln2p (Tyers et al. 1993). Furthermore, the observation that Cln3p synthesis is especially sensitive to rapamycin-mediated inhibition of translation (Barbet et al. 1996), suggests that post-transcriptional mechanisms might be important in its regulation.

Despite evidence suggesting a requirement for new protein synthesis at the G₁ → S transition, little is known about translational control of specific polypeptides at Start. Protein synthesis is regulated by specific translation initiation factors (Hershey 1991). Two translation initiation factors, eukaryotic initiation factor 4E (eIF4E) and 3₇ (eIF3₇), are particularly interesting because their mutants (cdc33 and cdc63) arrest in G₁ (Hanic-Joyce et al. 1987a; Brenner et al. 1988). Generalized protein synthesis is decreased in mutant cdc63 cells cultured at 37°C that accumulate monosomes.
cdc63 cells, however, continue to grow in size and are mating competent at the restrictive temperature suggesting that specific mRNAs involved in the regulation of Start are particularly affected by this mutation. Identification of specific mRNAs whose translation rate is affected by cdc63 should therefore reveal genes of potential importance in coupling cell division to the rate of protein synthesis and growth.

Here we show that CLN3 expression is translationally regulated through a short uORF in the 5' leader of its mRNA. To examine the role of this control element, we studied its effects in cell cycle progression. Our studies indicate that CLN3 expression and completion of Start depend on the overall availability of functional protein synthesis machinery and suggest a mechanism by which cell growth and division are coordinated.

Results

Identification of an upstream open reading frame in the 5' leader of CLN3

We examined the translation initiation rate of CLN3 mRNA with polysomal profiles of cells mutant in translation initiation factor 3h (cdc63) (Fig. 1) to evaluate it as a specific target involved in the G1 arrest of cdc63. Translation initiation of CLN3 mRNA was indeed specifically inhibited in cdc63 cells as shown by the shift of CLN3 transcripts to polysomes of lower density in nonpermissive conditions (Fig. 1A). This shift was similar to the position of HSP70 (SSA2) transcripts previously shown to be translationally repressed (Barnes et al. 1993). Translation initiation of control actin (ACT1) mRNA was unaffected in the mutant (Fig. 1A; Barnes et al. 1993). The shift of CLN3 transcripts to polysomes of lower density was specific to the cdc63 mutation and it was not observed when G1 arrest was caused by other means such as cdc28 mutants (data not shown).

Because CLN3 mRNA was poorly translated as a consequence of the translation initiation defect in cdc63 cells, we evaluated its 5' mRNA leader sequence for structural features found in mRNAs that are translationally regulated (Geballe 1996). To define the 5' mRNA leader, we first identified a TATA box at position −439 relative to the start of the coding sequence in the sequence of CLN3 (Cross 1988; Nash et al. 1988). Primer extension confirmed a site 364 nucleotides upstream of the translation initiation site as the site for initiation of transcription of CLN3 predicted by this TATA box (Fig. 1B). By use of S1 nuclelease protection assays, McInerny et al. (1997) recently verified the same 5' end of CLN3 mRNA. Identification of this initiation site revealed an upstream open reading frame (uORF) encoding the tripeptide Met-Asp-Phe at position −315 in the 5' mRNA leader of CLN3 (Fig. 1C). Because short uORFs generally

Figure 1. Inhibition of the protein synthesis machinery influences the translational efficiency of the CLN3 message, caused by the presence of a short uORF. (A) Initiation of translation of particular mRNAs was evaluated by fractionating polysomes on sucrose gradients followed by RNA hybridization to identify fractions containing specific mRNAs. RNA harvested from polysomal profiles of cdc63, CDC63, and A-315T/CLN3, cdc63 cells was monitored at 260 nm. Arrows indicate the position of CLN3 and SSA2 transcripts. The percentage of CLN3 message found in light and heavy polysomal fractions, determined by densitometry, is indicated at the bottom of each panel. (B) Primer extension analysis of the transcription initiation site of CLN3. Arrows indicate the extended products and their position in the CLN3 sequence. (C) Schematic representation of the region upstream of the CLN3 coding sequence and the A-315T/CLN3 mutant. Relative positions of nucleotide sites including the TATA box, the 5' end of the mRNA and of the predicted upstream open reading frame (uORF), are indicated and numbered with respect to the initiation codon in CLN3 coding sequences. An ATG → TTG mutation in A-315T/CLN3 cells eliminates the uORF.
inhibit translation initiation events downstream (Ge-
balle 1996; Hinnebusch 1996), we predicted that an
A → T point mutation in the ATG start codon of this
uORF (Fig. 1C) might improve the translational effi-
ciency of the CLN3 message. When the A-315T muta-
tion was introduced into the cdc63 mutant background,
CLN3 mRNA shifted to dense polysomal fractions (Fig.
1A, A-315T/CLN3, cdc63). Thus, inactivating the uORF
increased the translational efficiency of the CLN3
mRNA when availability of functional ribosomes was
limited by the cdc63 mutation.

The uORF in the CLN3 mRNA regulates the timing of
cell division during slow growth

Inactivation of the uORF in mutant A-315T/CLN3 cells
should increase CLN3 levels and accelerate completion
of Start. Because bud appearance marks completion of
Start (Pringle and Hartwell 1981), we tested the growth-
rate dependence of this potential phenotype by compar-
ing the ratio of budding in mutant cells with budding in
cells expressing CLN3 with wild-type mRNA leader se-
quencies (Tyers et al. 1992) at several stages of growth in
rich media. Interestingly, mutant cells budded slightly
less frequently relative to the control strain during the
early-log phase of growth when nutrients were abundant
(Fig. 2A). In late-log phase, budding increased markedly
in A-315T/CLN3 cells, indicating accelerated comple-
tion of Start (Fig. 2A). Increased budding coincided with
the point when synthesis of ribosomes decreased as the
cultures approached saturation, but before entering sta-
tionary phase (Fig. 2B; Warner 1989). The DNA content
of asynchronous cell populations confirmed that inactiv-
ation of the uORF in the CLN3 mRNA leader increased
the proportion of cells initiating DNA replication as nu-
trients were depleted (Fig. 2C). These differences did not
result from increased steady-state CLN3 mRNA levels
(Fig. 2D). The effect of the A-315T/CLN3 mutation,
therefore, varied with growth conditions because it
mildly inhibited Start in rich conditions, but accelerated
Start in poor conditions. The function of the wild-type
uORF is the opposite of the effect of the mutation. Con-
sequently, the uORF in CLN3 actually slightly stimu-
lates cell division in rich media and inhibits division as
growth slows—a mechanism coupling cell division to
growth conditions.

We confirmed the growth-dependence of this prema-
ture budding phenotype in nutritionally deprived media
by use of glycerol as a carbon source. As predicted, inac-
tivation of the upstream AUG shortened G1 (with a con-
comitant delay in G2/M) in synchronized cultures, ob-
tained by centrifugal elutriation of small G1 cells (Fig.
3A), and again increased the proportion of cells with 2N
DNA content (Fig. 3B) ~30% in asynchronously growing
cells. Further, growth rate-dependent acceleration of
Start in our mutant strain is nutrient-mediated but not
nutrient-specific, because this phenotype was observed
when growth was limited by carbon source, nitrogen
source, or both (data not shown). Thus, inactivation of
the uORF in the CLN3 mRNA shortens G1 and acceler-
ates completion of Start during slow growth. Similar to what has been observed in strains over-expressing CLN3 (Nash et al. 1988), however, our mutant strain does not divide faster under these conditions, because the shortened G1 is compensated by a delay in subsequent phases of the cell cycle.

The role of the uORF in sensitivity to α-factor and the critical size threshold for division

Overexpression of CLN3 reduces cell size at budding (Cross 1988; Nash et al. 1988). Furthermore, cells over-expressing CLN3 are resistant to the antimitogenic effects of α-factor (Cross 1988; Nash et al. 1988), probably because they have a very short G2, and non-G2 cells are refractory to α-factor-induced arrest. Consistent with these changes, cell size decreased by 20% to 30% (Fig. 3C) and α-factor-resistance increased (two- to fivefold) in our mutant A-315T/CLN3 strain in glycerol-containing media (Fig. 3D). Moreover, steady-state CLN3 mRNA levels did not increase under these conditions (Fig. 3E). Thus, inactivation of the uORF in the CLN3 mRNA decreases the critical size threshold for division and α-factor sensitivity during slow growth. As observed for dominant CLN3 mutants (Nash et al. 1988), however, inactivation of the uORF does not prevent normal arrest and viability upon entering stationary phase (not shown).

The translational efficiency of CLN3 mRNA in poor growth conditions is enhanced in cells lacking the uORF

The accelerated passage through Start that we observed in A-315T/CLN3 cells suggested that Cln3p levels were increased in our mutant. In vivo levels of Cln3p protein are extremely difficult to detect (Cross and Blake 1993; Tyers et al. 1992, 1993). Because association of mRNAs with heavy polysomal fractions directly reflects their efficiency in initiating translation, however, we analyzed polysomal profiles of cells lacking the uORF in different growth conditions to obtain direct biochemical evidence.

**Figure 3.** Inactivation of the uORF in the CLN3 mRNA accelerates budding and DNA replication, decreases cell size at Start, and increases resistance to α-factor in poor growth conditions. (A) Small G1 mutant and control cells were obtained by centrifugal elutriation from cultures growing in YPG to a cell density of 1 x 10^6 cells/ml, and resuspended at the same cell density in the original clarified medium. At the indicated time points after resuspension, the percent of budded cells (%B) and cellular DNA content was determined. (B) The cellular DNA content of mutant and control cells was determined from cultures growing asynchronously in YPG to a density of 1 x 10^6 cells/ml. (C) Cell size measurements of the whole cell populations grown in YPG to a density of 1 x 10^6 cells/ml was determined by FACS. Cell numbers are plotted on the y-axis and the x-axis represents the forward angle scattering. Cell size measurements are relative and not absolute. (D) Sensitivity to α-factor of cells growing on YPG solid medium was tested at the indicated concentrations of α-factor. (E) RNA blots of CLN3, CLN2, and ACT1 mRNAs, as in Fig. 2D from cells grown in YPG to a density of 1 x 10^6 cells/ml.
for the role of the uORF in translational control of Cln3p synthesis. During growth in rich (R) medium in both the wild-type (CLN3) and mutant (A-315T/CLN3) cells, the CLN3 mRNA is found associated with heavy polysomes, indicating that it is translated efficiently in both cases (Fig. 4). When shifted to minimal (MIN) medium, however, it is evident that the CLN3 mRNA in cells lacking the uORF is found mostly in heavy polysomal fractions, whereas in wild-type cells, little of the CLN3 message is found in heavy polysomal fractions (Fig. 4). Thus, the uORF in the CLN3 mRNA represses its translational efficiency in a growth-dependent manner.

Cln3p synthesis is achieved via a leaky scanning mechanism from ribosomes that bypass the uORF

We further tested the role of the uORF in regulating Cln3p levels with a fusion gene containing the CLN3 mRNA leader and promoter directing expression of the β-galactosidase gene in various growth conditions (Guarente and Ptashne 1981). Interestingly, the lacZ construct containing the A-315T mutation again resulted in a 5%–10% decrease in β-galactosidase activity during the early-log phase of growth in rich media when nutrients were abundant (Fig. 5A). In poor growth conditions, β-galactosidase activity increased markedly in the A-315T mutant compared with the reporter construct with the wild-type CLN3 5′ leader. This growth rate dependence matched the effect of the same mutation on CLN3 function as reflected in the cell cycle profile in Figure 2.

We evaluated additional mutants in the 5′ mRNA leader of CLN3 to understand mechanisms by which the uORF regulates Cln3p levels. uORFs affect translation initiation through potential mechanisms including control of reinitiation in sequences downstream of the uORF, by expression of specific inhibitory peptides in the uORF, or by control of leaky scanning past the uORF (Geballe 1996). We tested these mechanisms with appropriate mutations in our β-galactosidase reporter constructs (Fig. 5B). Because alterations in yeast 5′ mRNA leader sequences can lead to severe message instability complicating interpretations related to translational control, care was taken so that the introduced mutations only minimally altered the primary structure of the CLN3 5′ mRNA leader. Furthermore, the reported values were normalized for mRNA levels (Fig. 5B).

Because the process of reinitiation depends on the distance between the termination codon of the uORF and the downstream AUG (Hinnebusch 1996), we first extended the uORF by inactivating its termination codon (T-306G mutation). We also tested reinitiation by a double mutation causing the uORF to overlap the downstream ORF (T-306G/T-81CC mutation), and by replacing the termination codon and ten nucleotides downstream of the uORF with the highly efficient termination signal from the fourth uORF of GCN4 (GCN4TER mutation) (Hinnebusch 1996). None of these mutants had any significant effect on reporter gene expression, arguing strongly against a reinitiation mechanism. Mutations changing the amino acid at position 2 (D2R mutation) or 3 (F3A mutation) of the uORF also did not significantly alter expression from the downstream ORF (Fig. 5B), arguing against a role for the tripeptide encoded by the uORF. Taken together, these results showed that the uORF represses expression of CLN3, and translation from the downstream initiation codon is achieved by ribosomes that bypass the uORF through leaky scanning.

The uORF is the control element that makes Cln3p synthesis sensitive to the TOR-mediated signal transduction pathway

What signals could impinge on the translational control of CLN3? Translational mechanisms regulating growth control have been found in response to GCN2 and in the rapamycin-sensitive TOR pathway. The effects of the uORF on CLN3 expression particularly presented analogies to the uORF-mediated translational control of GCN4 by GCN2 (Hinnebusch 1996). The absence of a reinitiation mechanism for translation regulation, however, makes GCN2 regulation of the CLN3 uORF unlikely. We confirmed this view because neither addition of the amino acid analogue 3-aminotriazole nor amino acid starvation derepressed CLN3 expression in our reporter constructs (data not shown). Rapamycin arrests cells in G1 by globally inhibiting translation initiation and decreasing Cln3p synthesis (Barbet et al. 1996). In contrast to control (CLN3) cells, a significant fraction of mutant (A-315T/CLN3) cells arrested outside the G1 phase of the cell cycle in the presence of rapamycin (Fig. 6). This result indicates that the uORF makes CLN3 expression, and consequently completion of Start, particularly sensitive to limitations of protein synthesis mediated by rapamycin.

**Discussion**

The identification of a uORF in the CLN3 mRNA serving as a translational control element shows the poten-
tial to directly link protein biosynthesis and initiation of cell division. Because inactivation of this uORF alters the timing of Start during slow growth, our results provide a molecular mechanism for the coordination of growth and division in the life cycle of proliferating cells.

Inefficient translation initiation of $\text{CLN3}$ mRNA in $\text{cdc63}$ cells is not surprising given the important roles of eIF3 in the formation of the 43S translation preinitiation complex. eIF3 regulates the supply of 40S ribosomal subunits, and their association with the eIF–2–GTP–tRNAMet ternary complex, with mRNA and with cap-binding factors (Hannig 1995). The Start arrest seen in the $\text{cdc63}$ allele of the $\text{h}$ subunit of eIF3 indicates that synthesis of polypeptides involved at the $\text{G}_1\rightarrow\text{S}$ transition is particularly sensitive to eIF3 and protein synthesis in general. We provided evidence for a role of eIF3 in Cln3p synthesis. Cln3p cannot be the only polypeptide involved at Start in $\text{cdc63}$ cells influenced by eIF3, however, because overexpression of $\text{CLN3}$, or a stable domin-ant $\text{CLN3}$ allele, does not rescue the Start arrest of $\text{cdc63}$ cells (data not shown). Thus, the effects of eIF3 are pleiotropic and not limited to $\text{CLN3}$.

Growth, rapamycin treatment, and inactivation of eIF3 affected translational efficiency of $\text{CLN3}$ mRNA (Fig. 7). How might these diverse signals alter Cln3p levels? Classic experiments with translation of globin-mRNAs as a model showed that any treatment that de-
increases the concentration of functional translation initiation complexes disproportionately and specifically affects mRNAs which, like CLN3, are not efficiently translated (Lodish 1974). Thus, identification of a leaky scanning mechanism suggests that the uORF in CLN3 mRNA could repress CLN3 expression simply by decreasing the numbers of scanning ribosomes reaching the downstream AUG, thereby decreasing its intrinsic translation initiation rate (Geballe 1996; Hinnebusch 1996). The growth rate-dependence in CLN3 expression would then be readily explained by changes in the cellular ribosome content (Figs. 2B and 7) that correlate with growth rate in yeast (Warner 1989). This model further predicts that changes in rate-limiting components of the cellular protein synthesis machinery mediated by alternative pathways should also affect translation of these same mRNAs as we observed with CLN3 in response to rapamycin treatment and inactivation of eIF3.

In addition, this model predicts that increasing concentrations of 43S preinitiation complexes will result in proportionally greater enhancement of translation of mRNAs with low intrinsic initiation rates (Lodish 1974). In fact, in conditions of high abundance of preinitiation complexes, these same mRNAs will be translated more efficiently than mRNAs that associate with a large number of 43S complexes. mRNAs associated with increased numbers of 43S complexes are crowded with ribosomes, and scanning along the mRNA is hindered. This prediction may explain the slight inhibitory effect in the completion of Start observed during early-log growth in rich media when the uORF in the CLN3 mRNA is absent (Fig. 2).

Translational control of gene expression is increasingly recognized among cell cycle regulatory molecules. Translational control of cdk4 (Ewen et al. 1995) and the cdk inhibitor p27Kip1 (Hengst and Reed 1996; Millard et al. 1997) contributes to G1 arrest. Importantly, the retinoblastoma tumor-suppressor protein (Rb) has been implicated in ribosomal biogenesis, because it represses RNA polymerase III transcription (White et al. 1996; Nakamura et al. 1997) implying that inactivation of growth and cell division. The presence of the uORF in the CLN3 message ensures that Cln3p levels are not efficiently inhibited cell division faster when the opportunity arises. This control of CLN3 at the level of initiation of translation is consistent with the extremely low half-life of Cln3p, because even small changes in the rate of Cln3p synthesis will then have a pronounced effect in overall Cln3p abundance and completion of Start. Although this may not be the only control of CLN3 (Hubler et al. 1993; Baroni et al. 1994; Mitsuzawa 1994; McInerny et al. 1997), its growth rate dependence makes it particularly interesting. Identification of additional signals interacting with the translational control element that we identified, has the potential to provide new insights into mechanisms coupling cell growth to division.

Materials and methods

Yeast strains and plasmids

Standard methods (Kaiser et al. 1994) of yeast manipulation were used, unless otherwise stated. The media used were YPD (1% yeast extract, 2% peptone, 2% glucose). YPG (1% yeast extract, 2% peptone, 3% glycerol), and SD (0.67% YNB (Difco), 2% glucose) with auxotrophic supplements. The cdc63 strain (T3-26-3: MATa, cdc63-1, leu2, ura3) and its CDC63 isogenic counterpart (GR2) were a gift from Dr. G. Johnston (Hanic-joyce et al. 1987a).

The wild-type (CLN3) strain used as a control in this study was GT108, an isogenic derivative of W303a (MATa, ade2, leu2, his3, trp1, ura3, ssd1-d), and was a gift from Dr. B. Futcher (Tyers et al. 1992). This strain contains a single HA-tagged CLN3 copy shown previously to have the same properties and stability as an untagged Cln3p (Tyers et al. 1992). The pMt10 plasmid carrying a genomic copy of CLN3 with a triple tandem HA-epitope tag at the carboxyl terminus was also a gift from Dr. B. Futcher (Tyers et al. 1992). To eliminate the uORF in the CLN3 mRNA we introduced the A→T mutation at position -315 by PCR with a sense primer encoding the introduced mutation and an antisense primer complementary to CLN3 sequences downstream of the EcoRI site (position +1159), with plasmid pMt10 as a template. The PCR product was cut with BstXI and EcoRI and subcloned into the same sites of pMt10. For one-step gene replacements (Kaiser et al. 1994) the plasmids were cut with PvuII and used to transform strains TC3-26-3 and W303a to obtain strains SCMS3 (A-315T/CLN3, cdc63) and SCMS4 (A-315T/CLN3), respectively. The strains were verified to contain only a single CLN3 copy by Southern analysis of genomic DNA, utilizing a unique BamHI restriction site present in the HA-epitope tag (Tyers et al. 1992, 1993).

For fluorescence-activated cell sorting (FACS) analysis, the samples were prepared as described (Lew et al. 1992), with the modifications of Heichman and Roberts (1996). Centrifugal elutriation was performed as described (Stuart and Wittenberg 1995), except that small cells were collected at a rotor speed of 2400 rpm with a pump flow of 22 ml/min (Tyers et al. 1993). In all cases, the elutriated cell population was within a very narrow range of cell sizes, composed >99% of small G1 cells. α-Factor resistance was tested as described previously (Tyers et al. 1993), on YPG solid medium.

DNA methods

Unless otherwise stated, standard methodology (Sambrook et al. 1989) was used in all procedures. PCR was performed with
the Vent enzyme (New England Biolabs, MA). The sequences of PCR-generated DNA fragments and introduced mutations were verified by DNA sequencing by use of Sequenase (U.S. Biochemical). Oligonucleotides were synthesized at the core facility of the Massachusetts General Hospital or at GIBCO BRL. The Escherichia coli strain DH5α (GIBCO BRL) was used for bacterial propagation of plasmids.

RNA methods

Yeast total RNA was prepared by standard methods (Kaiser et al. 1994). For RNA blots, we used Hybond-N membranes (Amersham, UK), and Rapid-Hyb hybridization solution (Amersham, UK). Probes were prepared by PCR from yeast genomic DNA with specific primers that corresponded to coding sequence positions +100 to +781 for ACT1, +104 to +607 for SSA2, +98 to +516 for CLN2, and the entire coding region of RPL16. For CLN3, the BstXI–EcoRI (1.5 kb) fragment from plasmid pMT10 was used as a probe.

For primer extensions, Poly(A)^+ RNA was prepared from strain W303a with the Oligotex mRNA isolation kit (Qiagen, CA) according to the manufacturer’s instructions. We performed primer extensions with the Superscript reverse transcriptase (GIBCO BRL) and an antisense primer complementary to positions −256 to −257 of the CLN3 sequence. A sequencing reaction by use of the same primer together with the pMT10 plasmid as template, which contains a genomic copy of CLN3, was run in parallel as a size marker.

For polysomal fractionation in the cdc63 experiments (Fig. 1), cells were grown in YPD at 23°C until they reached a density of 1 × 10⁶ cells/ml, and then shifted to 37°C for 4 hr. For the polysomal fractionations presented in Figure 4, cells were grown in YPD at 30°C until they reached a density of 1 × 10⁶ cells/ml. At that point, half the culture was used to prepare polysomal extracts, whereas the remaining cells were concentrated, resuspended in SD medium at the same cell density, and cultured for an additional 10 min (Tzamarias et al. 1989). Cell extract preparation, fractionation of polysomes on sucrose gradients, and RNA preparations from 1.2-ml fractions collected from the top of the gradient were performed as described (Cigan et al. 1991).

β-Galactosidase reporter assays

CLN3 sequences (positions −934 to +6) were PCR-amplified with primers containing SalI (5′-AGCGCTGACGTGCCTGC-GGTGATGGTTTTTAACTCC-3′) and BamH1 (5′-CCGGATCCGGCCATCG-TCGAGAAAGCG-3′) restriction sites at their ends and plasmid pMT10 as template, which contains a genomic copy of CLN3, was run in parallel as a size marker.

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and production of β-galactosidase was assayed (Kaiser et al. 1994) from cells grown at 30°C in SD medium to mid-log phase. The reported β-galactosidase values are normalized for the steady-state lacZ mRNA levels, to accurately account for differences in the synthesis of β-galactosidase. For the assays in Figure 5A, cells harboring the plasmids were grown in YPD, YPG, and SD media. Because during growth in YPD medium the effects of the uORF depend on the growth phase, and because β-galactosidase is extremely stable, the cultures were started at a density of 10^6 cells/ml and harvested at 10^5 cells/ml. During this period, growth was at a steady-state, and the obtained values reflected more accurately β-galactosidase synthesis.

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References


Cross, F.R. 1988. DAF1, a mutant gene affecting size control,


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**References**

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