Coupling of cell division to cell growth by translational control of the G_1 cyclin *CLN3* in yeast

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The eukaryotic cell cycle is driven by a cascade of cyclins and kinase partners including the G_1 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 (G_1) when cells monitor their environment before replicating their DNA (Pardee 1989; Norbury and Nurse 1992). The point in G_1 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 G_1 cyclins that induce the $G_1 \rightarrow S$ transition when coupled with their cdk partners (Murray and Hunt 1993). One such G_1 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).

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Cln3p functions upstream of all other G_1 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 G_1 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 G_1 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 G_1 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 $G_1 \rightarrow S$ 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 3η (eIF3η), are particularly interesting because their mutants (*cdc33* and *cdc63*) arrest in G_1 (Hanic-Joyce et al. 1987a,b; 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 3η (*cdc63*) (Fig. 1) to evaluate it as a specific target involved in the G_1 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 G_1 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 nuclease 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

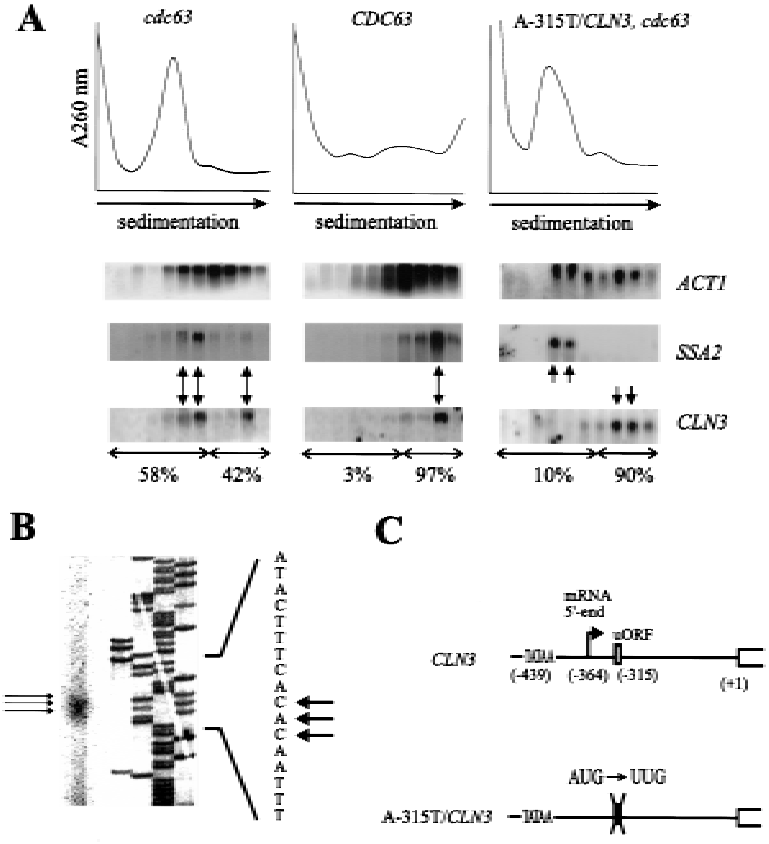

Figure 1. Inhibition of the protein synthesis machinery influences the translational efficiency of the *CLN3* message, caused by the presence of a short uORF. (*A*) Initiation of translation of particular mRNAs was evaluated by fractionating polysomes on sucrose gradients followed by RNA hybridization 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 TATAA 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-315/*CLN3* cells eliminates the uORF.

inhibit translation initiation events downstream (Geballe 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 efficiency of the *CLN3* message. When the A-315T mutation 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 comparing the ratio of budding in mutant cells with budding in cells expressing *CLN3* with wild-type mRNA leader sequences (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 completion of Start (Fig. 2A). Increased budding coincided with the point when synthesis of ribosomes decreased as the cultures approached saturation, but before entering stationary phase (Fig. 2B; Warner 1989). The DNA content of asynchronous cell populations confirmed that inactivation of the uORF in the *CLN3* mRNA leader increased the proportion of cells initiating DNA replication as nutrients 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. Consequently, the uORF in *CLN3* actually slightly stimulates 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 premature budding phenotype in nutritionally deprived media by use of glycerol as a carbon source. As predicted, inactivation of the upstream AUG shortened G_1 (with a concomitant delay in G_2/M) in synchronized cultures, obtained by centrifugal elutriation of small G_1 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 G_1 and acceler-

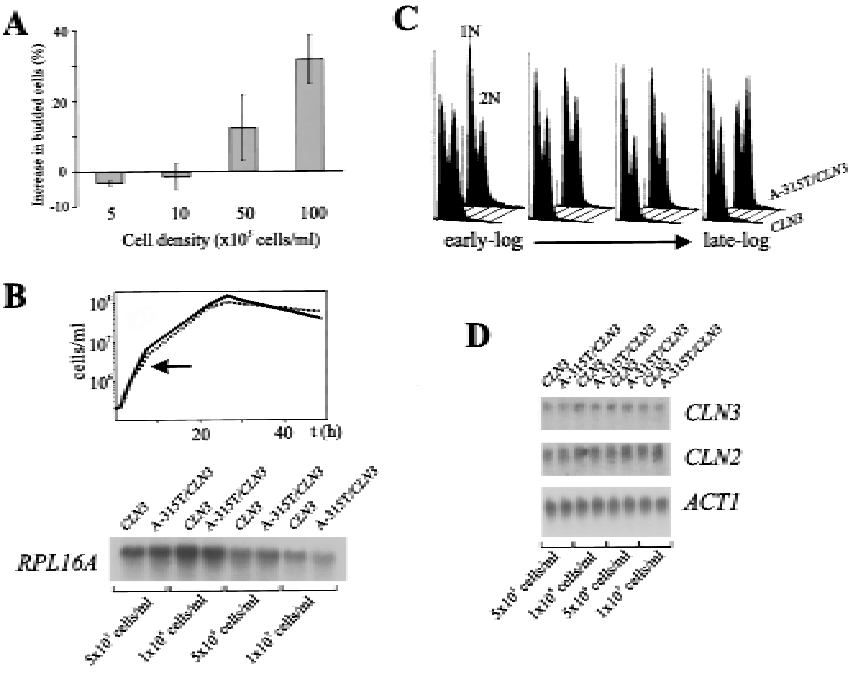

Figure 2. Inactivation of the uORF in the *CLN3* mRNA accelerates budding and DNA replication in a growth-rate dependent manner. (*A*) Percent difference of budded A-315T/*CLN3* cells compared with *CLN3* control cells during growth in rich media. Mutant and control cells were grown in YPD and budded cells were counted. The change in budded cells was calculated at four cell densities and the averages and standard deviations of three independent A-315T/*CLN3* transformants from a representative experiment are shown. (*B*) Growth curves of *CLN3* (solid line) and A-315T/*CLN3* (dotted line) cells in YPD. Arrow indicates the point where accelerated completion of Start becomes evident in A-315T/*CLN3* cells. (*Below*) Steady-state mRNA levels of ribosomal protein L16 (*RPL16A*) are shown on an RNA blot. Each lane was loaded with total RNA prepared from 3×10^7 cells, growing in YPD at the indicated cell densities. (*C*) FACS analysis of cellular DNA content during growth in rich medium at the same cell densities as in *A*. (*D*) RNA blots showing the levels of *CLN3*, *CLN2*, and *ACT1* mRNAs during growth in rich medium in the control (*CLN3*) and mutant (A-315T/*CLN3*) strain. Each lane was loaded with 10 μg of total RNA.

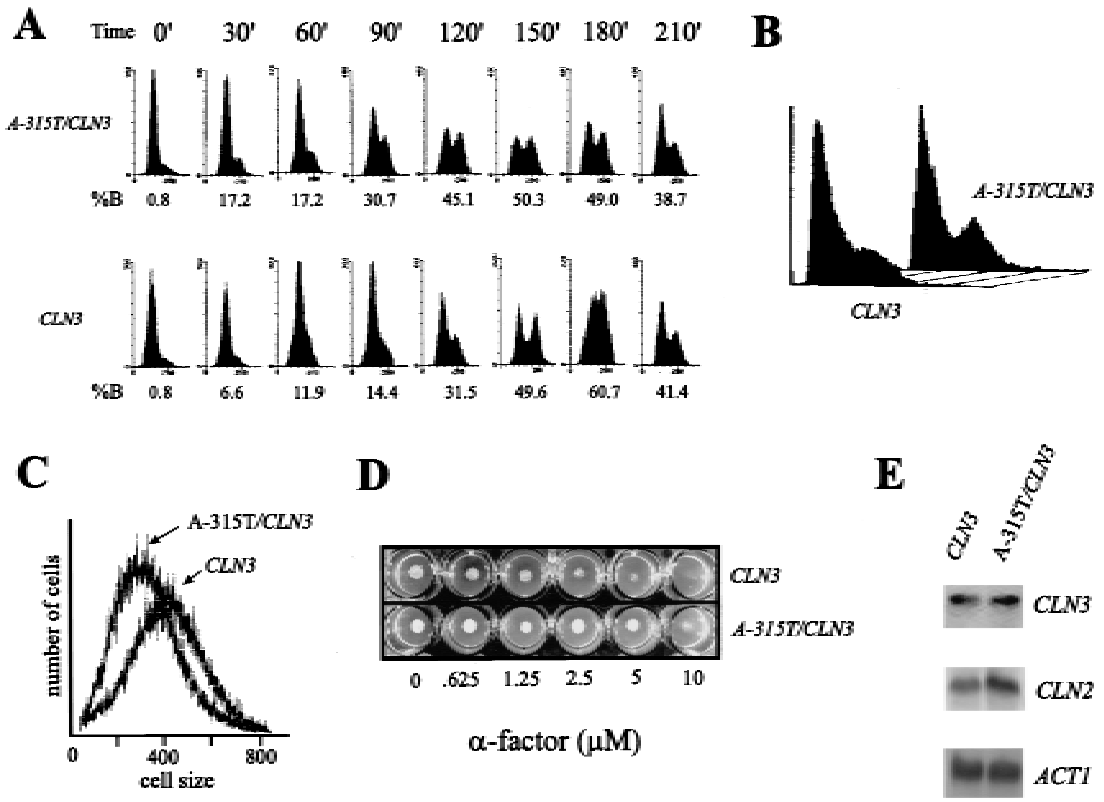

Figure 3. Inactivation of the uORF in the *CLN3* mRNA accelerates budding and DNA replication, decreases cell size at Start, and increases resistance to α-factor in poor growth conditions. (*A*) Small G_1 mutant and control cells were obtained by centrifugal elutriation from cultures growing in YPG to a cell density of 1×10^6 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 1×10^6 cells/ml. (*C*) Cell size measurements of the whole cell populations grown in YPG to a density of 1×10^6 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 1×10^6 cells/ml.

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 G_1 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 overexpressing *CLN3* are resistant to the antimitogenic effects of α-factor (Cross 1988; Nash et al. 1988), probably because they have a very short G_1, and non-G_1 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

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).

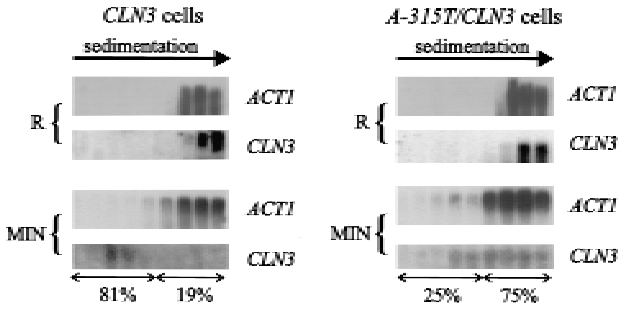

Figure 4. Polysomal profiles of wild-type (*CLN3*) and mutant (A-315T/*CLN3*) cells growing in rich (R) YPD medium, or minimal (MIN) SD medium. Polysomal extracts were fractionated as in Fig. 1, and RNA hybridization identified the distribution of *ACT1* and *CLN3* messages. The percentage of *CLN3* message found in light and heavy polysomal fractions in MIN media, determined by densitometry, is indicated at the *bottom* of each panel.

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 G_1 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 G_1 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-

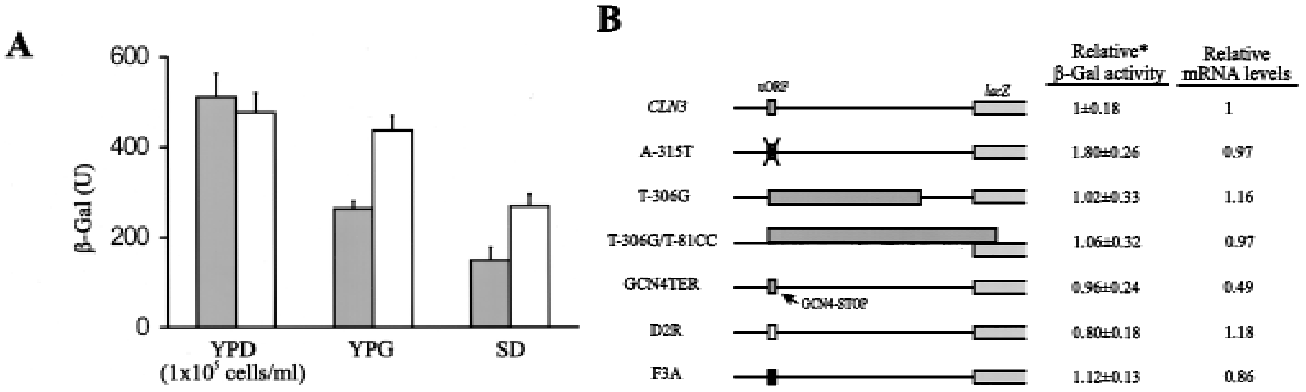

Figure 5. (*A*) β-Galactosidase reporter assays in various growth conditions. The averages and standard deviations of β-galactosidase activity from multiple independent transformants of *CLN3-lacZ* (shaded bars) and A-315T/*CLN3-lacZ* (open bars) reporter plasmids, during growth at the indicated media are shown. (*B*) Translation of *CLN3* is achieved by a leaky scanning mechanism. The averages and standard deviations of β-galactosidase activity from multiple independent transformants of each mutant plasmid are shown relative to that of the wild-type *CLN3-lacZ* plasmid, from cells growing in minimal (SD) medium. *In each case, the reported values were normalized for the steady-state levels of *lacZ* mRNA present in the cells (*right*). The *lacZ* mRNA levels for each construct were in turn normalized against actin mRNA levels (not shown).

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 *CLN3* mRNA in *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–$tRNA^{Met}$ ternary complex, with mRNA and with cap-binding factors (Hannig 1995). The Start arrest seen in the *cdc63* allele of the η subunit of eIF3 indicates that synthesis of polypeptides involved at the $G_1 \rightarrow S$ 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 *cdc63* cells influenced by eIF3, however, because overexpression of *CLN3*, or a stable dominant *CLN3* allele, does not rescue the Start arrest of *cdc63* cells (data not shown). Thus, the effects of eIF3 are pleiotropic and not limited to *CLN3*.

Growth, rapamycin treatment, and inactivation of eIF3 affected translational efficiency of *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-

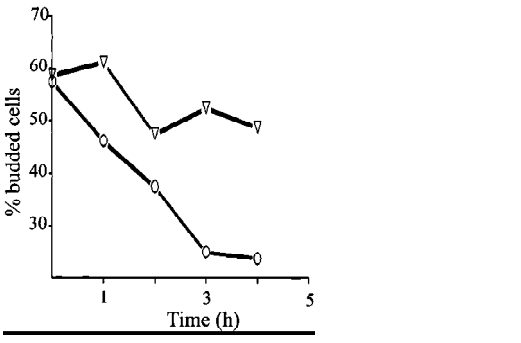

Figure 6. Eliminating the uORF in the *CLN3* mRNA allows cells to complete Start in the presence of rapamycin. Rapamycin (0.2 μg/ml) was added to YPD cultures (cell density 1×10^6 cells/ml) of control (*CLN3*) and mutant (A-315T/*CLN3*) strains, (○ and ▽, respectively). At the indicated time points, the percent of budded cells was counted.

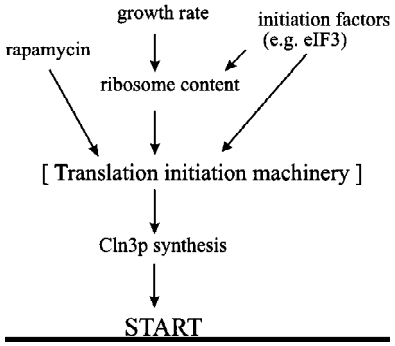

Figure 7. Cln3p synthesis, and completion of Start, are particularly responsive to changes in the concentration of the translation initiation machinery. *CLN3* expression is growth-rate dependent, because growth rate correlates with the cell's ribosome content and, therefore, with the concentration of ribosomes competent to initiate translation. eIF3 (and possibly other initiation factors) influences translation of *CLN3* because it is involved in all the steps along the formation of a preinitiation complex. Similarly, because the rapamycin-sensitive signal transduction pathway controls translation initiation (Barbet et al. 1996), it has a pronounced effect in Cln3p synthesis. Connections between these inputs are likely, because nutrients are known to affect the rapamycin-sensitive signaling pathway (Di Cono and Arndt 1996) and rapamycin also inhibits the function and synthesis of translation factors in mammalian cells (Terada et al. 1994; Beretta et al. 1996; Brown and Schreiber 1996; Brunn et al. 1997; Redpath et al. 1996).

creases 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 $p27^{Kip1}$ (Hengst and Reed 1996; Millard et al. 1997) contributes to G_1 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; Chu et al. 1997; Larminie et al. 1997) implying that inhibition of protein synthesis may play a role in inhibiting oncogenic transformation. This is in agreement with several studies showing that translation factors can function as oncogenes in transfected cells (Sonenberg 1993). In particular, the eIF4E cap-binding protein transforms NIH-3T3 cells and other fibroblasts to a malignant phenotype (Lazaris-Karatzas et al. 1990), possibly by virtue of translational enhancement of specific mRNAs (Koromilas et al. 1992). It is interesting that increased expression of eIF4E results in increased protein levels of cyclin D1 (Rosenwald et al. 1993a), and eIF4E itself is regulated by c-myc (Rosenwald et al. 1993b; Jones et al. 1996).

Our data provide important insights into the significance of translational mechanisms in the genetic coordination of growth and cell division. The presence of the uORF in the *CLN3* message ensures that Cln3p levels sufficient to pass Start will not accumulate when overall growth conditions are not optimal. Because this inhibition is relieved at fast growth rates, however, the cell is able to take advantage of rich growth conditions and initiate 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 the auxotrophic supplements. The *cdc63* strain (TC3-26-3: *MAT***a,** *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 (*MAT***a,** *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 *Eco*RI site (position +1159), with plasmid pMT10 as a template. The PCR product was cut with *Bst*XI and *Eco*RI and subcloned into the same sites of pMT10. For one-step gene replacements (Kaiser et al. 1994) the plasmids were cut with *Pvu*II and used to transform strains TC3-26-3 and W303a to obtain strains SCMSP3 (A-315T/*CLN3, cdc63*) and SCMSP4 (A-315T/*CLN3*), respectively. The strains were verified to contain only a single *CLN3* copy by Southern analysis of genomic DNA, utilizing a unique *Bam*HI 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 G_1 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 *Bst*XI–*Eco*RI (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 −236 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 1×10^6 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 1×10^6 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 *Sal*I (5′-ACGCGTCGACGTGCTGCGGTGCATGG-3′) and *Bam*HI (5′-CGGGATCCGGCCATCGTACAGAAAGCG-3′) restriction sites at their ends and plasmid pMT10, or A-315T/*CLN3*-pMT10, as templates. The PCR products were digested with *Sal*I and *Bam*HI and subcloned into the same sites of the β-galactosidase reporter plasmid pLG669-Z (Guarente and Ptashne 1981), resulting in the *CLN3–lacZ* and A-315T/*CLN3–lacZ* plasmids. The indicated mutations were introduced by the PCR-based overlap extension technique (Ho et al. 1989) as modified by Datta (1995), with appropriate mutagenic oligonucleotides. These were: 5′-GGGTATAGTCCTCTTTCCTCCGAAATCCATTTGACTGGCAG-3′ for the T-306G mutation; 5′-CTCCTCTGCATTTCTTTTCCCGACCCATAGCATTTCTTAC-3′ for the T-81CC mutation; 5′-GTCCTCTTTCCTCAGAATCTCATTTGACTGGCAGACTCAG-3′ for the D2R mutation; 5′-GGGTATAGTCCTCTTTCCTCAAGCATCCATTTGACTGGCAGAC-3′ for the F3A mutation; and 5′-CGTTTCCTAATGGGTATAGAAGGTAACCGTTAGAAATCCATTTGACTGGC-3′ for the GCN4TER mutation. The PCR products were then digested with *Xho*I and *Bam*HI and subcloned into the same sites of the *CLN3–lacZ* plasmid. The corresponding plasmids were transformed into strain W303a 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 10^2 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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