CLN3 expression is sufficient to restore G_1-to-S-phase progression in *Saccharomyces cerevisiae* mutants defective in translation initiation factor eIF4E

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The essential cap-binding protein (eIF4E) of *Saccharomyces cerevisiae* is encoded by the *CDC33* (wild-type) gene, originally isolated as a mutant, *cdc33-1*, which arrests growth in the G_1 phase of the cell cycle at 37 °C. We show that other *cdc33* mutants also arrest in G_1. One of the first events required for G_1-to-S-phase progression is the increased expression of cyclin 3. Constructs carrying the 5′-untranslated region of *CLN3* fused to *lacZ* exhibit weak reporter activity, which is significantly decreased in a *cdc33-1* mutant, implying that *CLN3* mRNA is an inefficiently translated mRNA that is sensitive to perturbations in the translation machinery. A *cdc33-1* strain expressing either stable Cln3p (Cln3-1p) or a hybrid *UBI4* 5′–*CLN3* mRNA, whose translation displays decreased dependence on eIF4E, arrested randomly in the cell cycle. In these cells *CLN2* mRNA levels remained high, indicating that Cln3p activity is maintained. Induction of a hybrid *UBI4* 5′–*CLN3* message in a *cdc33-1* mutant previously arrested in G_1 also caused entry into a new cell cycle. We conclude that eIF4E activity in the G_1-phase is critical in allowing sufficient Cln3p activity to enable yeast cells to enter a new cell cycle.

Key words: *cdc* mutant, cell cycle, cyclin, yeast.

INTRODUCTION

The proliferation of eukaryotic cells is tightly regulated by intra- and extra-cellular signals, which are normally monitored in the G_1-phase of the cell cycle, prior to cells undergoing DNA replication [1]. The decision to enter a new mitotic cycle for *Saccharomyces cerevisiae* is made in a time window in the late G_1-phase of the cell cycle called 'start' [2]. 'start' is defined by attainment of critical rate of protein synthesis to commit to DNA replication (S-phase) [3]. This growth-control mechanism ensures the completion of the cell cycle once a commitment has been made.

Passage through 'start' is characterized by bud formation, spindle-pole-body duplication, acquisition of pheromone resistance and onset of DNA replication (for a review, see [4]). These events are ultimately triggered by a cyclin–cyclin-dependent kinase complex composed of Cdc28p, a cyclin-dependent kinase, and cyclin 3 protein (Cln3p), an unstable protein which is present at very low levels throughout the cell cycle and which moderately accumulates in the early G_1-phase (the so-called 'early G_1 cyclin') [4–7]. This complex triggers a cascade of cyclin–CDK-dependent effects which ultimately drives entry into S-phase, after transient activation of unstable late-G_1 cyclins including Cln1p and Cln2p [4,6–8]. According to this view, the transient expression of the unstable late-G_1 cyclins (driven by Cln3p) is the key event that determines the onset of 'start' [3].

Translation activity plays an important role in the regulation of the cell cycle in *Saccharomyces cerevisiae* [9]. Translation is required for the cell mass increase during the G_1-phase and is therefore required to achieve a critical rate of protein synthesis. Regulation of translational activity may also play a more specific role by promoting the synthesis of proteins required by the cell to perform 'start'. The latter is supported by the finding that *cdc33-1*, a temperature-sensitive mutation in the gene encoding translation initiation factor eIF4E, *CDC33*, leads to G_1-phase arrest of cells at the non-permissive temperature. Translation initiation factor eIF4E [10,11] binds specifically to the 5′ m^7Gppp cap structure of mRNAs and recruits additional translation initiation factors which then act together to prepare the 5′-untranslated region (5′-UTR) of the mRNA for binding of the small ribosomal subunit during initiation of protein synthesis (for reviews, see [12,13]). *cdc33-1* cells contain eIF4E with lowered cap-binding activity [14], but since these cells still synthesize protein, albeit at a decreased rate [14], the synthesis of a specific protein may limit progression of the cell cycle. A candidate for such a protein is Cln3p, whose abundance depends critically on its relative rates of synthesis and degradation [15].

In the work presented below, we devised experiments to test the hypothesis that *CLN3* mRNA translation is limiting for G_1-to-S-phase progression in *Saccharomyces cerevisiae* cells. The results show that, owing to its 5′-UTR, *CLN3* mRNA is a poorly translated mRNA which is sensitive to mutations in eIF4E. Artificial restoration of Cln3p levels in cells with lowered protein synthetic activity due to mutations in eIF4E leads to inappropriate progression of cells from the G_1-phase into the S-phase.

EXPERIMENTAL

Strains, plasmids and media

The strains used in this study were *CDC33 (cdc33::LEU2 ura3 trp1 pCDC33, CEN, TRP1)*, *cdc33-1 (cdc33::LEU2 ura3 trp1 pcdc33-1, CEN, TRP1)* and *cdc33-42 (cdc33::LEU2 ura3 trp1 pcdc33-42, CEN, TRP1)* [16]. These strains were grown in YPD medium [1 % (w/v) yeast extract/2 % (w/v) peptone/2 %

Abbreviations used: eIF, eukaryotic initiation factor; UTR, untranslated region; (u)ORF, (upstream) open reading frame; TOR, target of rapamycin; FKBP, FK506-binding protein.

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(w/v) glucose] at 25 °C unless otherwise indicated. Plasmids for β-galactosidase assays were as follows. p281 contains the *GAL1* promoter and 5′-UTR containing a synthetic polylinker fused to the reading frame of *lacZ* [17]. To make p*GAL1*promoter-*CLN3* 5′-UTR-*lacZ* (*CLN3-lacZ* construct) oligonucleotides 5′ CCAGATCTC ACA ATT TCT TTC TTG ATT TT 3′ (forward; *Bgl*II site) and 5′ GGG AAT TCA GT*C AT*C GTA CAG AAA GCG TA 3′ (reverse; *Eco*RI site; italics correspond to the start codon of *CLN3*) were used to amplify a fragment containing the entire *CLN3* 5′-UTR which was cloned into the *Bgl*II and *Eco*RI sites of plasmid p281. To make p*GAL1*promoter-*UBI4* 5′-UTR-*lacZ* (*UBI4-lacZ* construct) oligonucleotides 5′ CCAGATCTT ACG GAT AAG GAT AAG TAT AT 3′ (forward; *Bgl*II site) and 5′ GGGAATTCA GT*C AT*A ATC TAT TAG TTA AAG TAA AG 3′ (reverse; *Eco*RI site, italics correspond to the start codon of *UBI4*) were used to amplify a fragment containing the entire *UBI4* 5′-UTR which was cloned into the *Bgl*II and *Eco*RI sites of plasmid p281. Plasmid pBF30 contains the *CLN3-1* gene (PEST-sequence deleted) [18]. Plasmid p*UBI4* 5′-*CLN3* contains the entire *CLN3* gene under control of the 5′ region (promoter and untranslated leader) of the *UBI4* gene [19] and plasmid p*UBI4* 5′ contains only the 5′ region of the *UBI4* gene [19]. pGalp *UBI4* 5′-UTR *CLN3* was constructed by isolating a *UBI4* 5′-UTR *CLN3* fragment from plasmid p*UBI4* 5′-*CLN3* using oligonucleotides 5′ CCA GAT CTT ACG GAT AAG GAT AAG TAT AT 3′ (forward; *Bgl*II site) (see above; corresponds to start of *UBI4* transcript) and oligo 5′ CCA TCT CGA GAT CAT TAA TGT ATG TTA ACG T (reverse; *Xho*I site). This fragment was inserted into p281 (see above) at the unique *Bgl*II and *Xho*I sites. All transformations were performed using the lithium acetate procedure [20].

Measurement of β-galactosidase activity

Assays were performed essentially as described in [17]. β-Galactosidase activity was normalized against the protein concentration of the extracts. A representative data set is shown from three independent experiments.

Flow cytometry

Cell-culture samples were prepared as previously described [21].

Preparation of total yeast RNA and Northern blotting

Cell-culture samples (10 ml) were pelleted, washed with water, and the pellet resuspended in 600 μl of 10 mM Tris/HCl, pH 7.5, 2 mM EDTA, 150 μM LiCl, 1 % lithium dodecyl sulphate, 0.8 g of glass beads (diameter 0.5 mm) and 600 μl of phenol (equilibrated with Tris buffer at pH 8) were added; the mixture was vortex-mixed for 3 × 1 min and centrifuged at 2000 ***g*** for 5 min at 0 °C. The aqueous phase was collected, re-extracted with phenol and precipitated with 0.1 M KCl and 2.5 vol. of 100 % ethanol. The precipitate was washed with 70 % ethanol, dried, and finally dissolved in water. The RNA concentration was determined by measuring the absorption at 260 nm. RNA samples were separated by 1 % formaldehyde/agarose-gel electrophoresis (15 μg/lane) and transferred to a Zeta-Probe membrane as recommended by the manufacturer (Bio-Rad). After blotting, ethidium bromide-stained rRNA was revealed on the blots by UV illumination. The blots were probed with the 1.1 kb *Xho*I–*Hin*dIII fragment of the *CLN2* gene (a gift from Dr. S. Lanker, Department of Molecular and Medical Genetics, School of Medicine, Oregon Health Sciences University, Portland, OR, U.S.A.). Probes were radiolabelled with ^{32}P by the random-primer method and blots washed twice in 5 × SSC (20 × SSC is 3 M NaCl and 0.3 M trisodium citrate)/0.1 % SDS, twice in 2 × SSC/0.1 % SDS and once in 0.1 × SSC/0.1 % SDS for 20 min at room temperature. Filters were exposed to X-ray film (Kodak X-OMAT AR) for 16 h with an intensifying screen.

RESULTS

Independent mutations in the gene encoding eIF4E result in G_1-arrest of *Saccharomyces cerevisiae* cells

The temperature-sensitive strain *cdc33-1* arrests growth in the G_1-phase of the cell cycle at the non-permissive temperature of 37 °C [11]. A collection of several temperature-sensitive yeast strains, mutated in the gene encoding eIF4E, which show decreased protein synthesis at 37 °C, including strain *cdc33-42* [16], was used to investigate whether these mutations also cause cells to arrest growth in the G_1-phase of the cell cycle at the non-permissive temperature. Arrest of growth in the G_1-phase is characterized by a 1n DNA content (‘1n DNA content’ refers to the DNA content of haploid cells before DNA replication). To distinguish arrest in the G_1-phase from random arrest, we determined the DNA content of cells by flow cytometry for several *cdc33* temperature-sensitive mutants. Analysis of the DNA content of *cdc33-1* and *cdc33-42* cells by flow-cytometric analysis shows that the transfer of mutant cells to 37 °C leads to the accumulation of cells with a 1n DNA content (Figure 1, top row); in the *cdc33-1* cell culture (Figure 1A), about 40 % of the cells have a 1n DNA content at 25 °C, which changes to 70 % after 2 h at 37 °C and about 80 % after 6 h at 37 °C. A similar result is obtained with a culture of strain *cdc33-42* (Figure 1B, top row), in which about 80 % of the cells have a 1n DNA content after 4 h of shift to 37 °C, with minimal further change at later time points. In the wild-type cell culture about 40 % of the cells have a 1n DNA content, and there is very little change at 37 °C (Figure 1C, top row). Furthermore, the *cdc33-44* strain (a strain carrying another temperature-sensitive allele of *CDC33*, [14]) also arrests growth in the G_1-phase at 37 °C (results not shown). We conclude from these data that different independent lesions in the *cdc33* gene cause a G_1-arrest or prolonged G_1-phase at the non-permissive temperature.

The 5′-UTR of *CLN3* mRNA prevents efficient translation

The finding that temperature-sensitive mutations in the gene encoding eIF4E lead to G_1-arrest or a prolonged G_1-phase suggested that these mutations decrease the synthesis of a protein(s) required for the G_1-to-S-phase transition. Cln3p is a candidate for such a protein because its level is critical for entry of cells into the S-phase. In addition to being turned over rapidly, Cln3p may also be synthesized at a low rate. This is suggested by the observation that *CLN3* mRNA has a rather long 5′-UTR of about 350 nucleotides carrying a short upstream open reading frame (ORF) close to the 5′ end [22,23]. We tested this prediction by fusing the *CLN3* 5′-UTR to the reporter gene *lacZ* (*CLN3-lacZ* construct) and expressing the reporter construct from the *GAL1* promoter in different yeast strains in galactose-containing medium (Figure 2). The synthesis of β-galactosidase from this construct was compared with that of constructs carrying either the 5′-UTR of *GAL1* fused to the *lacZ* gene (*GAL1-lacZ* construct; plasmid p281) or the 5′-UTR of the *UBI4* gene fused to the *lacZ* gene (*UBI4-lacZ* construct). This leader sequence was shown earlier to confer less eIF4E-dependent translation *in vivo* [11].

In the wild-type strain at 25 °C, 3 h after transcriptional induction, the *CLN3-lacZ* construct produced about 20-fold less β-galactosidase activity than the *UBI4-lacZ* construct and more

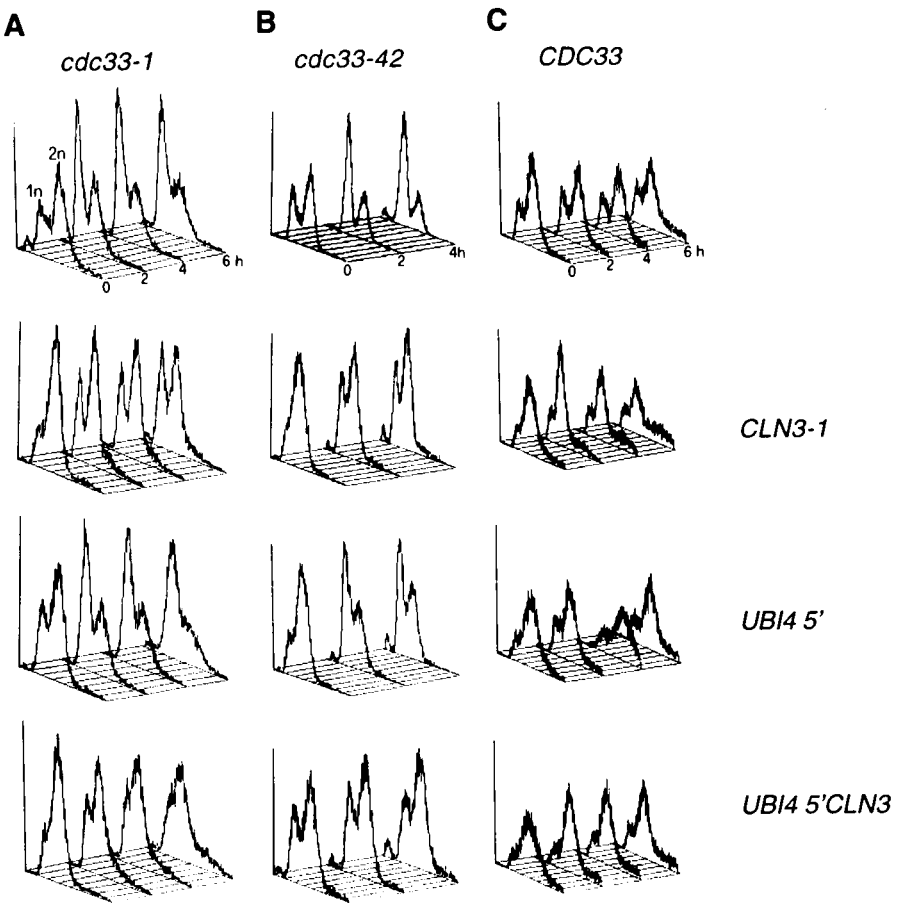

Figure 1 Artificially increased expression of Cln3p abrogates the G_1 arrest of *cdc33-1* and *cdc33-42* mutants

Exponentially growing cells containing no construct (top row), *CLN3-1* construct (second row), the *UBI4* 5′ construct (third row) or the *UBI4* 5′-*CLN3* construct (bottom row) were shifted from 25 to 37 °C for the times indicated (0, 2, 4 and 6 h after temperature shift) and samples analysed by flow cytometry. (**A**) *cdc33-1* cells; (**B**) *cdc33-42* cells; (**C**) *CDC33* (wild-type) cells. Event count (*x*-axis) versus DNA content (*y*-axis) are shown.

than 25-fold less than the *GAL1-lacZ* construct (Figure 2A). In the mutant strain *cdc33-1*, the differences between the *CLN3-lacZ* construct and the others were more pronounced. At 25 °C the *CLN3-lacZ* construct produced 90- and 25-fold less activity than the *UBI4-lacZ* construct and the *GAL1-lacZ* construct respectively, as compared with the wild-type strain at the same temperature. Thus, in the *cdc33-1* mutant, at the permissive temperature for growth, the *CLN3* 5′-UTR allows lower levels of translation than the other two leaders.

We then examined the effects of a shift to the non-permissive temperature using the same three leader constructs in the two strains. In the wild-type strain after 3 h of galactose induction coupled with a shift to 37 °C 1 h prior to induction, the *CLN3-lacZ* construct produced approx. 250-fold less β-galactosidase activity than both the *UBI4-lacZ* construct and the *GAL1-lacZ* construct (Figure 2B, *CDC33* columns). The same constructs were then analysed under the same conditions but in the *cdc33-1* strain. The *CLN3-lacZ* construct produced β-galactosidase activity that was more than 600-fold less than that produced by the *UBI4-lacZ* construct, and 90-fold less than that produced by the *GAL1-lacZ* construct (Figure 2B, *cdc33* columns). Importantly, in the *cdc33-1* strain at 37 °C, the actual levels of translated β-galactosidase produced using the *CLN3-lacZ* construct are the lowest of any observed in this experiment. The mRNA levels produced from the different *lacZ* constructs were similar as shown by Northern-blotting experiments, except that the *CLN3-lacZ* message was routinely 2–3-fold less abundant in both *CDC33* and *cdc33* strains (results not shown). Even taking these data into account, it is clear that the *CLN3* 5′-UTR acts as a poor leader, the poor translation of which is exacerbated in a strain carrying defects in an mRNA-recognition component of the translation-initiation machinery. This suggests that *CLN3* mRNA is a weakly translated mRNA and that this is due at least in part to its 5′-UTR.

Stable Cln3p suppresses the G_1-arrest of mutant eIF4E strains

The above analysis of the *CLN3* 5′-UTR suggests that the G_1 arrest of *cdc33* mutants may be due primarily to the insufficient synthesis of Cln3p. To elucidate whether this is a valid hypothesis, we expressed a stable form of Cln3p [24] in *cdc33* mutant cells and assayed whether this would be sufficient to allow cells to proceed into the S-phase. Exponentially growing *cdc33-1* and *cdc33-42* yeast strains containing the *CLN3-1* gene (encoding a C-terminally truncated stable form of Cln3p [24]), on a low-copy plasmid, were shifted from 25 to 37 °C. At 2 h intervals samples were collected and analysed by flow cytometry. At the permissive temperature, strains carrying the *CLN3-1* plasmid have fewer

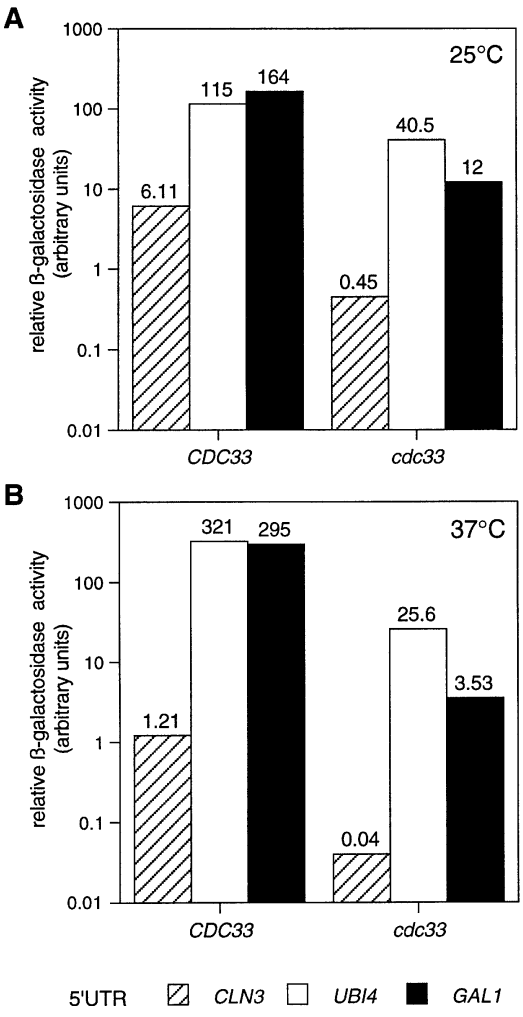

Figure 2 The 5′-UTR region of *CLN3* mRNA causes inefficient translation

Wild-type (*CDC33*) or *cdc33-1* mutant (*cdc33*) strains carrying a *GAL1*promoter driven *lacZ* gene fused to the 5′-UTR of either *CLN3, UBI4* or the p281 derived *GAL1* leader were grown in minimal medium containing raffinose to early exponential phase at 25 °C. Half of each culture was left at 25 °C (**A**) while the other half was shifted to 37 °C for 1 h (**B**). After this time galactose was added to both sets of cultures for a further 3 h before harvesting by centrifugation. Standard β-galactosidase assays were performed as described in [17]. Results shown are a representative set from three independently performed experiments.

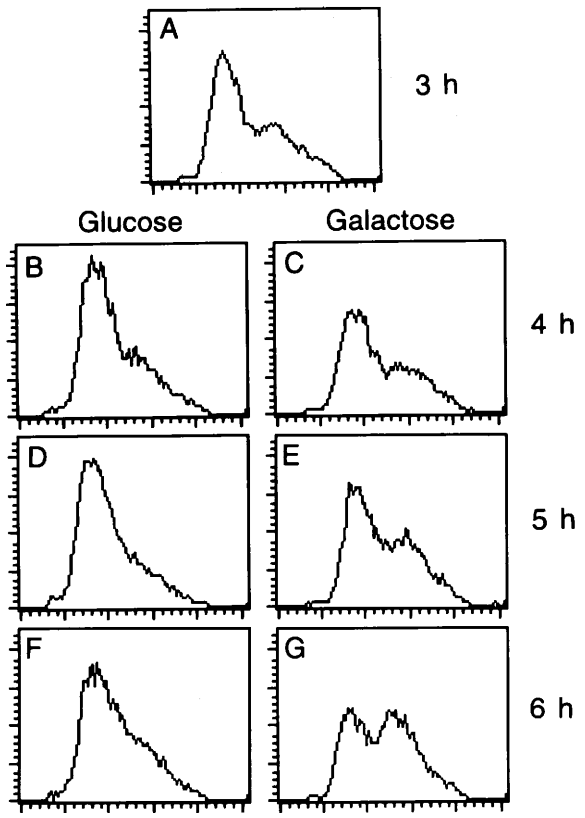

Figure 3 *UBI4* 5′-UTR-*CLN3* expression drives previously arrested *cdc33-1* cells into S-phase

A *cdc33-1* strain carrying a GAL1-promoter-driven *UBI4* 5′-UTR-*CLN3* construct was grown to mid-exponential phase in YPD medium, and shifted to 37 °C for 3 h, washed once in water and resuspended in either YPD or YPGal medium (the latter is like YPD, but with 2% galactose instead of glucose) and incubated for a further 3 h at 37 °C. The Figure shows the resulting flow cytometry. (**A**), (**B**), (**D**) and (**F**) show glucose-grown cells at 3, 4, 5 and 6 h respectively subsequent to temperature shift, and (**C**), (**E**) and (**G**) show cells resuspended in galactose at 4, 5 and 6 h respectively subsequent to the temperature shift.

cells in the G_1 phase, a phenotype expected when stable Cln3p is being expressed [24]. Comparison of the *cdc33* strains containing no plasmid with those expressing Cln3-1p clearly indicates that continuous expression of this stabilized Cln3 protein prevents these mutants arresting in G_1 after shift to the non-permissive temperature (compare the first and second rows in Figures 1A and 1B). Thus the loss of translation in these mutants appears to critically affect the rate of translation of Cln3p.

Expression of a *CLN3* mRNA with decreased dependence on eIF4E restores G_1-to-S-phase progression in an eIF4E-deficient strain

If mutations in *CDC33* decrease the expression of the *CLN3* gene sufficiently to cause a G_1 arrest, then eIF4E-independent translation of *CLN3* mRNA may allow G_1-to-S-phase progression in *cdc33* mutants. We and others have demonstrated that translation of *UBI4* mRNA, like that of other starvation- or stress-induced proteins, is less dependent on eIF4E than that of bulk mRNA (see Figures 2A and 2B and [11,19,25]). Importantly, the constructs used above demonstrate that, at 37 °C, whereas *CLN3* 5′-UTR-dependent translation is decreased 30-fold in a *cdc33-1* mutant as compared with the wild-type, *UBI4* 5′-UTR-dependent translation is affected less than 3-fold (Figure 2B). Therefore we used a construct in which the *CLN3* ORF is fused to the 5′ region (promoter and untranslated leader) of the *UBI4* gene, p*UBI4* 5′-*CLN3* [19], to see whether it could suppress the G_1-arrest of *cdc33-1* and *cdc33-42* cells at 37 °C. Exponentially growing *cdc33-1* and *cdc33-42* cells containing the *UBI4* 5′-*CLN3* construct on a low-copy plasmid were shifted from 25 to 37 °C, and at 2 h intervals samples were collected to determine the ratio of 1n to 2n DNA in the cells. As a control we used *cdc33-1* and *cdc33-42* cells that contained only the *UBI4* 5′ construct (without the CLN3 insert [19]). Flow cytometry shows that *cdc33* cells containing the *UBI4* 5′-*CLN3* construct (Figures 1A and 1B, bottom row) display a predominantly 2n DNA content at 25 °C, which remains so after a shift to 37 °C for 2–6 h, whereas the control cells (*UBI4* 5′) alter their distribution to an almost exclusively 1n DNA content at 37 °C (Figures 1A and 1B, third row). This suggests that a significant fraction of the *cdc33* cells containing *UBI4* 5′-*CLN3* are able to pass through 'start' into S-

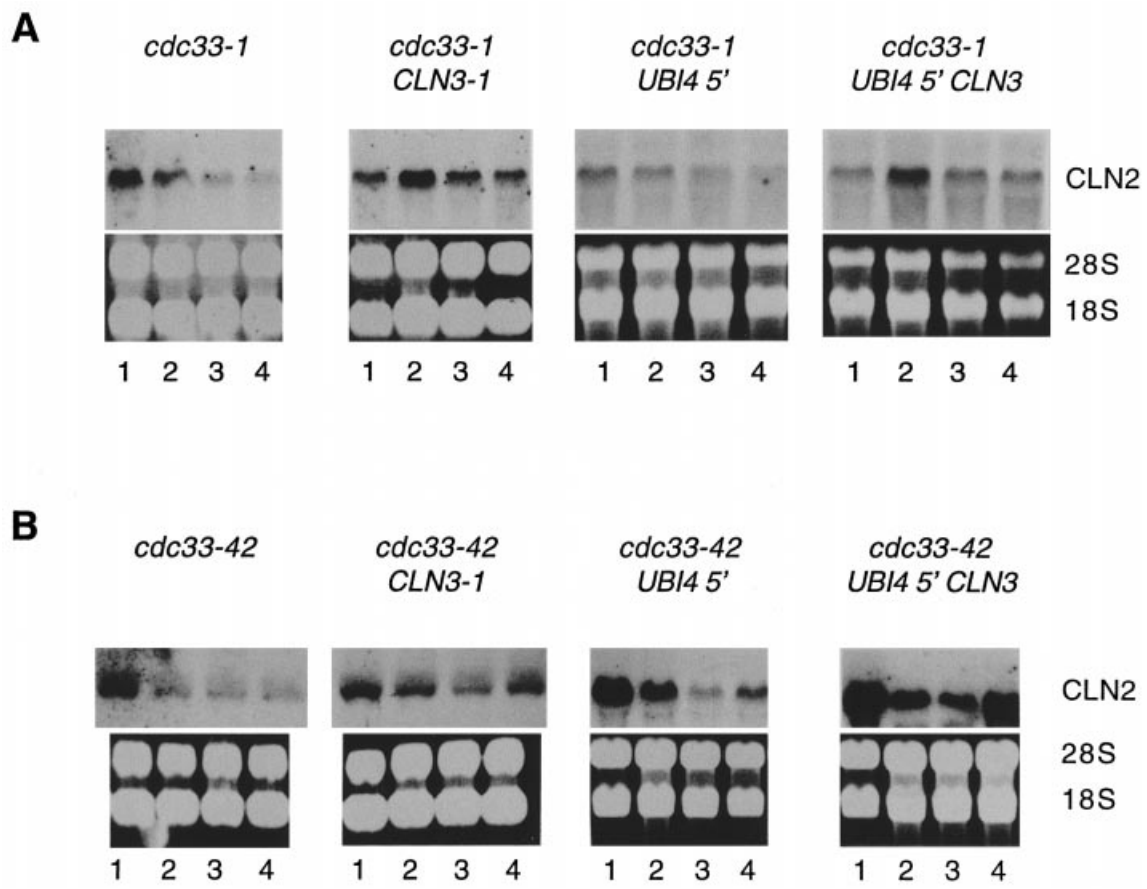

Figure 4 CLN2 expression is maintained in randomly arresting *cdc33* mutants expressing *CLN3-1* or *UBI4* 5′-*CLN3*

(**A**) The levels of *CLN2* transcript in strain *cdc33-1*, *cdc33-1* containing *CLN3-1*, *cdc33-1* containing the *UBI4* 5′, and *cdc33-1* containing the *UBI4* 5′-*CLN3* construct are shown. (**B**) The levels of *CLN2* transcript in strain *cdc33-42*, *cdc33-42* containing *CLN3-1*, *cdc33-42* containing the *UBI4* 5′, and *cdc33-42* containing the *UBI4* 5′-*CLN3* construct are shown. Total RNA was isolated from exponentially growing cells incubated at 25 °C for 6 h (lane 1 in each panel) and from cells shifted to 37 °C for 2, 4, and 6 h (lanes 2, 3 and 4) in Figure 3(**A**) and for 0, 2 and 4 h (lanes 2, 3 and 4) in Figure 3(**B**). As a control for RNA loading and blotting the ethidium bromide-stained rRNA is presented below the autoradiogram. 28 S and 18 S indicate the migration of rRNA.

phase. These data show that eIF4E-independent translation of *CLN3* mRNA mediated by *UBI4* 5′-UTR suppresses the G_1-arrest in a significant fraction of the *cdc33* mutant cells. One interpretation of these results is that artificial enhancement of the Cln3p levels, caused by either a stable Cln3p, or a *CLN3* gene with decreased eIF4E-dependence, prevents a notable proportion of temperature-sensitive *cdc33* cells from arresting in G_1 as they normally do because there is sufficient Cln3p to trigger a new cell cycle.

Less eIF4E-dependent *CLN3* drives previously arrested G_1 *cdc33-1* cells into S-phase

The experiments described above are open to an alternative interpretation because they were performed using asynchronous cultures. Thus the random arrest of the *cdc33* mutants seen with the *CLN3-1* plasmid and the *UBI4* 5′-*CLN3* plasmid may occur because, upon temperature shift, a certain population of the cells may be held up in the G_2/M-phase of the previous cell cycle. We devised a situation in which we could induce the transcription of a *UBI4* 5′-UTR-*CLN3* mRNA, after a *cdc33-1* mutant had been arrested in G_1. We cloned the 5′-UTR of *UBI4* fused to the *CLN3* reading frame behind the same galactose promoter used for the *lacZ* assays in Figure 2, so that we could induce this hybrid message by addition of galactose to the growth medium. A *cdc33-1* strain carrying this plasmid was grown to early exponential phase in complete medium containing raffinose at 25 °C, and shifted to 37 °C for 3 h, at which time a significant G_1 arrest was in evidence as judged by flow cytometry (Figure 3A). The culture was split and received either glucose or galactose and was incubated at 37 °C for a further 3 h, with hourly samples taken for flow cytometry. Control cells which received glucose remained in G_1 as expected (Figures 3B, 3D and 3F), whereas approx. 50% of cells in which the hybrid *UBI4* 5′-UTR-*CLN3* message was induced by galactose addition are able to enter S-phase, as judged by an increase in the 2n peak over the course of the next 3 h (Figures 3C, 3E and 3G). Sampling over longer periods demonstrated no further change in the distribution shown in of Figure 3G (results not shown). These data, coupled with those above, demonstrate that altered translational control of the *CLN3* message is sufficient to drive a new cell cycle in mutants that would otherwise arrest in G_1 because they lack a functional essential translation intiation factor.

CLN2 mRNA levels are maintained in randomly arrested cells

The data presented so far suggest that mutations in the eIF4E gene cause a G_1-arrest at 37 °C because *CLN3* mRNA cannot be

sufficiently translated. This is predicted to lead to low levels of Cln1p and Cln2p, since the Cln3p-Cdc28p kinase induces transcription of *CLN1* and *CLN2* genes. To test this prediction, we isolated total RNA from *cdc33-1* cells, *cdc33-1* cells carrying either the *CLN3-1*, *UBI4* 5′ or the *UBI4* 5′-*CLN3* constructs and performed Northern-blot experiments. The data show that, in both a *cdc33-1* strain and a *cdc33-1* strain carrying the *UBI4* 5′ control construct, *CLN2* mRNA levels decline upon shifting to the non-permissive temperature (Figure 4A, first and third panels). In contrast, *cdc33-1* cells transformed with the *CLN3-1* or *UBI4* 5′-*CLN3* construct show no decline in *CLN2* mRNA after a shift from 25 °C to 37 °C (Figure 4A, second and fourth panels). We obtained similar results with the *cdc33-42* cells (Figure 4B). We conclude that *cdc33* mutants containing stable Cln3p or *CLN3* mRNA which can be translated with decreased dependence on eIF4E are able to maintain their levels of *CLN2* mRNA, as opposed to the same mutant cells lacking these constructs, indicating that Cln3p-Cdc28p activity has been maintained as judged by *CLN2* transcription.

DISCUSSION

In the present work we have shown that two temperature-sensitive mutations in the *CDC33* gene encoding translation initiation factor eIF4E (*cdc33-1* and *cdc33-42*) result in arrest of growth of *Saccharomyces cerevisiae* cells in the G_1-phase of the cell cycle at the non-permissive temperature of 37 °C. This arrest can be suppressed by artificial enhancement of cyclin 3 protein (Cln3p), achieved either by the expression of stable Cln3p or by expression of an mRNA encoding Cln3p whose translation has a decreased dependence on eIF4E. Cln3p is difficult to quantify directly with accuracy, therefore (i) the levels of Cln3p translation were assessed using the *lacZ* reporter, and (ii) the presence of active Cln3p was verified by its stimulation of the transcription of the *CLN2* gene. Since enhanced expression of Cln3p in mutant cells suffices to induce entry into S-phase in a considerable proportion of the cells, we conclude that a reduction of eIF4E activity causes a reduction of Cln3p levels sufficient to cause G_1 arrest. This demonstrates that *CLN3* mRNA translation is crucial for G_1-to-S-phase progression in yeast cells. This dependence of *CLN3* gene expression on eIF4E is apparently due to the instability of Cln3p and to its very low synthesis rate. The long 5′-UTR, which contains a short ORF [22,23] (see below), strongly inhibits *CLN3* mRNA translation, thus contributing to the low synthesis rate (Figure 2). The level of Cln3p is thus kept low by low levels of transcription of *CLN3* mRNA [7], by low translation of *CLN3* mRNA and by the instability of Cln3p [7,15]. Therefore, a decrease in the translational activity in cells is expected to lower Cln3p levels below a critical threshold concentration, leading to arrest of growth in the G_1-phase of the cell cycle.

The short upstream ORF (uORF) in the CLN3 leader has been demonstrated to be important for the translational control of Cln3p [23]. Reporter constructs comprising the entire 5′-UTR of *CLN3* bearing a mutation within the upstream AUG fused to *lacZ* allowed up to only a 1.8-fold increase in expression as compared with the wild-type 5′-UTR construct [23]. However, in our assays, the minimum differences in the values achieved when using *CLN3* 5′-UTR reporters in *CDC33* and *cdc33-1* strains are significantly greater than 1.8-fold. This indicates that, whereas the uORF does have a role to play in regulating the translation of CLN3, the uORF is not the only feature of the *CLN3* 5′-UTR responsible for making the CLN3 mRNA such a poorly translated mRNA.

Recently it was demonstrated that Cln3p is down-regulated at the translational level 8-fold under nitrogen-starvation conditions [26], providing evidence that nutrient deprivation impinges upon the synthesis of the early G_1 cyclin. Those authors stated that the 5′-UTR of Cln3p appeared to be required for this nitrogen-loss effect. This correlates well with our experiments using reporters containing only the 5′-UTR of *CLN3*, because this 5′-UTR clearly functions very inefficiently with respect to translation (Figure 2). Those authors also state that no particular region of the 5′-UTR is specifically required for this down-regulation, thus demonstrating that the uORF is not a critical control element with respect to Cln3p translation upon nutrient depletion. Interestingly, incubation of our wild-type strain containing the *UBI4* 5′-*CLN3* construct in medium lacking nitrogen prevented a G_1 arrest after 6 h, a time-point when the wild-type alone has a $> 90\,\%$ 1n DNA content (S. B. Helliwell and H. Trachsel, unpublished work), strengthening the hypothesis that the translational control of Cln3p synthesis is critical for the decision to enter the next cell cycle.

Thus translational activity and perhaps eIF4E activity is an important parameter in determining whether yeast cells can enter a new mitotic cycle. It has been well known for quite some time that yeast cells have to grow to a critical size [2,27–29] and reach a critical rate of protein synthesis [3,30] to become committed to enter a new cycle of cell division. Cln3p levels are sensitive to the growth rate [18,31,32], and artificially enhanced Cln3p levels lead to commitment to cell division at a lower critical mass [33,34]. This is consistent with the hypothesis that Cln3p levels may (i) be the (indirect) measure of the mass of the cell and (ii) reflect the activity of the cap recognition step in initiation of translation, e.g. the activity of eIF4E or one of its associated factors.

The activity of eIF4E may be subject to regulation in response to intra- and extra-cellular signals. Indeed, there is evidence that a signal-transduction pathway including target of rapamycin (TOR) [19,35] in yeast cells and mTOR/FRAP/RAFT [36,37] in mammalian cells influences translation initiation at the level of eIF4E activity [38–40] [FRAP is FK506-binding protein (FKBP)/rapamycin-associated protein; RAFT is rapamycin and FKBP target]. Blocking this signal-transduction pathway in yeast with the drug rapamycin leads to inhibition of initiation of translation and to the acquisition of a starvation-like phenotype of the cells. Most importantly, the same *UBI4* 5′-*CLN3* construct that abrogates the G_1 arrest of *cdc33* mutants has been shown to prevent the G_1 arrest of rapamycin-treated cells [19]. Recently, it has been demonstrated that amino acid depletion in mammalian cells also affects the phosphorylation and activity of two proteins controlled by mTOR, which are directly involved with translational control. Re-addition of amino acids to amino-acid-depleted CHO-IR (Chinese-hamster ovary/insulin receptor) cells restores the phosphorylation and insulin-responsiveness of p70 S6 kinase and eIF-4E binding protein 1 (‘4E-BP1’), and a rapamycin-resistant p70 S6 kinase mutant is also resistant to the effects of amino acid withdrawal [41]. This suggests that at least one response to amino acid sufficiency is normally controlled via mTOR, p70 S6 kinase and eIF-4E binding protein 1. These data suggest that, in yeast, nutrients signal through the TOR pathway to eIF4E and/or factors associated with eIF4E to enhance protein synthesis, including Cln3p synthesis [19,40].

In yeast, a second signal-transduction pathway may regulate eIF4E activity: *BCY1* encodes the negative regulatory subunit of cAMP-dependent protein kinase, and *bcy1* mutations suppress the G_1 arrest of a *cdc33-1* mutant, presumably due to high constitutive protein kinase A activity [11]. Together with data described by Tokiwa et al. [34] and Baroni et al. [33], which show that nutrients signal through the cAMP signal-transduction pathway to enhance the expression of the *CLN3* gene in the early G_1-phase and to modulate the expression of *CLN1* and *CLN2* at

'start', this indicates that the cAMP signal-transduction pathway contributes directly or indirectly to the regulation of translation at the level of eIF4E activity. Signalling through two signal-transduction pathways may therefore increase the synthesis of Cln3p which, as a Cln3p–Cdc28p complex, activates the transcription of the *CLN1* and *CLN2* genes and initiates the passage of the cells through 'start' and the entry into S-phase.

The regulation of the eIF4F complex is the logical choice for cells wishing to maintain translational control of individual mRNAs, since eIF4E and eIF4G (the two essential components of the yeast eIF4F) are presumed to be the first initiation factors which make contact with cytoplasmic mRNAs. It remains to be elucidated whether elements of the TOR and/or RAS pathways impinge in some way upon eIF4E directly, or on eIF4G, the other subunit of eIF4F.

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