

A fission yeast general translation factor reveals links between protein synthesis and cell cycle controls

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SUMMARY

In two independent screens we isolated fission yeast mutations with phenotypes suggesting defects in B-cyclin function or expression. These mutations define a single gene which we call *ded1*. We show that *ded1* encodes a general translation factor that is related in sequence and function to RNA helicases required for translation in other species. Levels of the B-cyclins Cig2 and Cdc13 are dramatically reduced upon inactivation of Ded1, and this reduction is independent of degradation by the anaphase promoting complex. When a *ded1* mutant is grown under semi-restrictive conditions, the translation of Cig2 (and to a lesser extent Cdc13), is impaired relative to other

proteins. We show that B-cyclin translation is specifically inhibited upon nitrogen starvation of wild-type cells, when B-cyclin/Cdc2 inactivation is a prerequisite for G₁ arrest and subsequent mating. Our data suggest that translational inhibition of B-cyclin expression represents a third mechanism, in addition to cyclin degradation and Rum1 inhibition, that contributes to Cdc2 inactivation as cells exit from the mitotic cell cycle and prepare for meiosis.

Key words: Cell cycle, B-type cyclin, *ded1*, RNA helicase, Translation

INTRODUCTION

The replication and segregation of chromosomal DNA are the two most important events of the cell cycle, and in eukaryotes the initiation of both these processes is triggered by activation of one or more cyclin-dependent kinases (or CDKs). Whilst higher eukaryotes have multiple CDKs that regulate cell cycle progression, a single CDK regulates both S-phase and mitosis in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, though other CDKs play a role in certain circumstances (Espinoza et al., 1994; Measday et al., 1994; Nishizawa et al., 1998). In both yeasts, B-type cyclins promote entry into both S-phase and mitosis. Three B-cyclins are responsible for the activation of Cdc2 during the fission yeast cell cycle. Cig2 plays the major role in promoting entry into DNA replication (Fisher and Nurse, 1996; Martin-Castellanos et al., 1996; Mondesert et al., 1996; Obara-Ishihara and Okayama, 1994) and the levels of Cig2 and its associated Cdc2 kinase activity peak around G₁/S (Martin-Castellanos et al., 1996; Mondesert et al., 1996). Cdc13 is essential for the onset of mitosis (Booher and Beach, 1988; Hayles et al., 1994; Moreno et al., 1989) and Cig1 represents a third B-cyclin whose precise role is unclear (Bueno et al., 1991) though it is

an important regulator of the stability of the Rum1 CDK inhibitor (Benito et al., 1998; Correa-Bordes et al., 1997).

Since Cdc2 can only function in association with a cyclin partner, periodicity in the levels of B-cyclins provides an important mechanism by which Cdc2 kinase activity is regulated during the cell cycle. Whilst *cdc13* mRNA levels are constant throughout the cell cycle (J. Hayles, unpublished data), Cdc13 protein accumulates from S-phase to the end of mitosis, when it is degraded by the anaphase promoting complex (or APC; Moreno et al., 1989; Yamano et al., 1996). In contrast, Cig2 protein and mRNA levels both peak around G₁/S, when the level of Cdc13 protein is low (Connolly and Beach, 1994; Martin-Castellanos et al., 1996; Mondesert et al., 1996; Obara-Ishihara and Okayama, 1994).

Although proteolysis is clearly of major importance for the regulation of cyclin periodicity, cyclin expression can also be regulated at the level of translation. The translation of *Drosophila* cyclin B mRNA is inhibited during early development (Dalby and Glover, 1993), preventing accumulation of cyclin B until just before proliferation resumes in the germ cells. In budding yeast, expression of the G₁ cyclins *CLN2* and *CLN3* is also inhibited by means of translational repression under certain conditions (Gallego et al., 1997; Hall

et al., 1998; Philpott et al., 1998; Polymenis and Schmidt, 1997), thereby linking translation to regulation of progression through the G₁ phase of the cell cycle.

Here we characterise mutations in a fission yeast general translation factor, that give rise to phenotypes suggesting defects in B-cyclin expression. The B-cyclins Cdc13 and Cig2 are especially sensitive to mutation of this factor, suggesting that translation may be relevant to the regulation of B-cyclin expression. We show that translation of both Cdc13 and Cig2 is inhibited in response to nitrogen starvation, when cells become arrested in G₁ as a prelude to mating and meiosis. Our results suggest that inhibition of B-cyclin translation may play a role in this important cell cycle transition.

MATERIALS AND METHODS

Fission yeast strains and methods

All our strains are congenic with the *Schizosaccharomyces pombe* 972h⁻ strain, and all basic growth and media conditions were as described by Moreno et al. (1991). The *cig2Δ* and *rum1Δ* strains were described by Obara-Ishihara and Okayama (1994), and Moreno and Nurse (1994). Since *rum1Δ* and *nuc2-663* strains are sterile, the *ded1-1D5 rum1Δ* and *ded1-1D5 nuc2-663* strains were made by protoplast fusion. The *cig2::HA* strain was described by Martin-Castellanos et al. (1996).

Experiments in liquid culture were performed using EMM medium, and exponential cultures were grown to a density of 2–4 × 10⁶ cells/ml at the start of each experiment.

To induce expression of the *nmt1* promoter, cells growing in the presence of 5 μg/ml thiamine were washed twice and then resuspended in fresh medium lacking thiamine, at a dilution calculated to produce a cell density of 2–4 × 10⁶ cells/ml at the time of peak expression from the promoter.

Cloning of *ded1*⁺

The *ded1*⁺ gene was cloned by complementation of the t.s and c.s defects of the *ded1-1D5* and *ded1-61* mutants, using the fission yeast genomic library described by Barbet et al. (1992), and the cDNA library generated by Bruce Edgar and Chris Norbury (Moreno and Nurse, 1994). Restriction maps of the clones isolated from the genomic library suggested the presence of the same gene in each plasmid, and the minimal complementing fragment was then sequenced on both strands. The sequence of a cDNA showed that the *ded1*⁺ gene lacks introns, and the cDNA sequence was submitted to the EMBL/GenBank/DBJ database with the accession number AJ237697. In order to confirm that we had isolated the *ded1*⁺ gene, and not a suppressor, we subcloned the *sup3-5* nonsense suppressor into a vector containing an *ded1*-rescuing genomic fragment, and transformed this plasmid into an *ded1* mutant containing the *ade6-704* allele, which is rescued by *sup3-5*. White colonies were selected, and integration by homologous recombination at the site corresponding to the *ded1*-rescuing fragment was confirmed by Southern blotting. The integrants were then back-crossed to a wild-type *ade6-704* strain, and the ability of all progeny of this cross to grow at both high and low temperatures shows that the rescuing plasmid had integrated at the *ded1*⁺ locus.

Plasmids

A plasmid expressing a myc-tagged version of Ded1 was constructed by amplifying the *ded1*⁺ cDNA with *NdeI* sites at the first ATG and at the end of the gene, before cloning into pREP41MycHis (Craven et al., 1998), such that Ded1 is expressed from the medium strength *nmt1* promoter with two copies of the c-myc epitope and six His residues at the N terminus. Expression of this fusion protein rescues the c.s phenotype of *ded1-61*, showing that the tagged Ded1 is functional.

To make pREP1 *DED1* (*S. cerevisiae*), the budding yeast *DED1* open reading frame was amplified by PCR from genomic DNA isolated from the wild-type budding yeast strain W303-1a, using oligos that introduced *NdeI* sites at the ATG of the *DED1* ORF, and also at the 3' end of the gene. The sequence of the oligos was as follows: 5' oligo GTCTATCACATATGGCTGAAGTGAAGCAACAAGTGCAAAATT-TAAG; 3' oligo ATCTGAATCATATGTCACCACCAAGAAGAGT-TGTTTGAACCACCGCTG. The amplified product was then digested with *NdeI* and subcloned into the *NdeI* site of pREP1, so that expression of *DED1* was placed under the control of the *nmt1* promoter.

pREP3X-DBP2 was made by subcloning the *XhoI-NsiI* fragment containing a *DBP2* cDNA from pIG69 (Iggo et al., 1991), into *XhoI-SmaI* digested pREP3X. pREP3X-*vasa* was made by subcloning an *XhoI-SmaI* fragment containing the *vasa* gene from Bluescript-*vasa* (Hay et al., 1988) into *XhoI-SmaI* digested pREP3X.

The *cig2* cDNA constructs described in Fig. 7 were amplified by PCR and subcloned into vectors containing the medium strength *nmt1* promoter (Basi et al., 1993). cDNA I corresponds exactly to the *cig2* cDNA sequence described by Obara-Ishihara and Okayama (1994). cDNA II is identical except that it lacks the first 32 nucleotides of cDNA I. cDNA III represents the *cig2* cDNA without any UTRs, and was kindly provided by Hiroyuki Yamano.

Protein extracts and western blots

Protein extracts for western blotting were made by TCA extraction, as described previously (Foiani et al., 1994). For western blot analysis the following rabbit polyclonal antibodies were used: SP4 anti-Cdc13 at a dilution of 1/2000, MOC8 anti-Cig2 at a dilution of 1/500, anti-Cdc21 antibody at a dilution of 1/500. In addition, two mouse monoclonal antibodies were used: 100.4 anti-Cdc2 at a final concentration of 5 μg/ml (kindly provided by Hiroyuki Yamano), and anti-α-tubulin (Sigma T5168) at a dilution of 1/10000. The secondary antibodies were either Protein A-HRP or anti-mouse IgG-HRP conjugates, used at a dilution of 1/10000. Detection was performed using the enhanced chemiluminescence procedure (Amersham ECL kit).

Northern blot analysis

Total RNA was prepared as described (Moreno et al., 1991) and 10 μg of each sample was resolved on a formaldehyde gel. Probes were labelled using the 'Prime-a-Gene' kit (Promega). An *NdeI-NcoI* *cig2* cDNA fragment was used to detect the *cig2* mRNA, and an *HindIII* genomic fragment of the *ura4* gene was used to detect the *ura4* mRNA.

Quantitation of Cig2 protein and mRNA levels

For the experiments described in Figs 7C and 8, Cig2 and α-tubulin protein levels were quantitated using a phosphoimager after ECF detection (Amersham). For each sample, the Cig2 protein level was expressed relative to the level of α-tubulin, to provide an internal loading control. In order to avoid errors due to signal saturation the samples were serially diluted before immunoblot analysis.

To quantitate *cig2* mRNA levels by northern blot analysis, signals were quantitated directly using a phosphoimager, and expressed relative to *ura4* mRNA levels, which again served as an internal loading control.

Flow cytometry and microscopy

About 10⁷ cells were spun down for each sample and fixed in 70% ethanol before storing at 4°C. Samples were processed for flow cytometry as described (Sazer and Sherwood, 1990), and analysed with a Becton-Dickinson FACScan or FACSCalibur. In order to stain cells for fluorescence microscopy with the DNA binding dye 4',6-diamidino-2-phenylindole (DAPI), ethanol fixed cells were rehydrated in water and treated as described previously (Moreno et al., 1991).

Immunofluorescence

A wild-type strain was transformed with pREP41MycHis-Ded1, and

expression from the medium strength *nmt1* promoter induced as described above. The localization of Myc-Ded1 was determined by immunofluorescence as described previously (Maiorano et al., 1996), using the 9E10 anti-Myc monoclonal.

[³⁵S]methionine labelling and immunoprecipitation of Cig2 and Cdc13

In order to label newly synthesised proteins with [³⁵S]methionine, cells were grown in minimal medium to log phase before adding 1 mCi ProMix (Amersham) to 10⁸ cells in a volume of 10 ml. Incubation was continued for 40 minutes at 25°C or 24 minutes at 36°C. The labelled cells were then harvested and washed once with ice-cold STOP buffer (Moreno et al., 1991), before freezing in liquid nitrogen. Protein extracts were then prepared in HB buffer (Moreno et al., 1991).

For immunoprecipitation, 2 µl of the SP4 polyclonal antibody (anti-cdc13) or 12CA5 monoclonal antibody (Boehringer, anti-HA-Cig2) were bound to 20 µl Protein A-Sepharose beads (Pharmacia) for two hours at 4°C, before washing the beads with PBS. The beads were then incubated with 1 mg labelled extracts for one hour at 4°C, washed four times with HB buffer, and boiled in Laemmli buffer.

RESULTS

Novel mutants with phenotypes suggesting defects in B-cyclin function or expression

In a screen for novel fission yeast cell cycle genes (Grallert and Nurse, 1996) we isolated a temperature-sensitive (t.s.) mutant, *ded1-1D5*, with a phenotype suggesting defects in B-cyclin/Cdc2 function. Upon shifting an asynchronous culture of the *ded1-1D5* mutant to 36°C, we found that cells are unable

to enter S-phase and mitosis and so become arrested with a single interphase nucleus with 1C or 2C DNA contents (Fig. 1). Most cells arrest before mitosis, since G₂ is the predominant phase of the fission yeast cell cycle in nitrogen rich media, but if cells are synchronised in G₁ by nitrogen starvation, subsequent inactivation of Ded1 prevents G₁ progression upon return to nitrogen-rich medium (not shown). The G₁ arrest of

Table 1. Strains used in this work

Strain	Source
Wild-type L 972 h ⁻	P. Nurse
<i>ded1-1D5</i> h ⁻	This work
<i>rum1::ura4⁺ ded1-1D5 ade6-704 ura4-D18 cig2Δ</i>	This work
<i>cig2Δ</i>	H. Okayama
<i>cdc21-M68</i>	P. Nurse
<i>cdc21-M68 cig2Δ</i>	This work
<i>cdc19-P1</i>	P. Nurse
<i>cdc19-P1 cig2Δ</i>	This work
<i>cdc18-K46</i>	P. Nurse
<i>cdc18-K46 cig2Δ</i>	This work
<i>cdc22-M45</i>	P. Nurse
<i>cdc22-M45 cig2Δ</i>	This work
<i>ded1-61</i>	This work
<i>ded1-78</i>	This work
<i>cdc21-M68 ded1-78</i>	This work
<i>cdc18-K46 ded1-78</i>	This work
<i>cdc19-P1 ded1-78</i>	This work
<i>cdc19-P1 ded1-1D5</i>	This work
<i>cdc18-K46 ded1-1D5</i>	This work
<i>ded1-1D5 cdc2-33</i>	This work
<i>ded1-78 cdc2-33</i>	This work
<i>nuc2-663</i>	T. Toda
<i>nuc2-663 ded1-1D5</i>	This work
<i>cdc2-M26 h⁻</i>	P. Nurse
<i>leu1-32 h⁻</i>	P. Nurse
<i>leu1-32 h⁺</i>	P. Nurse
<i>ded1-1D5 leu1-32 h⁻</i>	This work
<i>cig2:HA h⁻</i>	Martin-Castellanos et al. (1994)
<i>ded1-1D5 cig2:HA h⁻</i>	This work
<i>cdc2-M26 cig2:HA</i>	This work
<i>cdc21-M68 ded1-78 leu1-32</i>	This work
<i>cdc18-K46 ded1-78 leu1-32</i>	This work

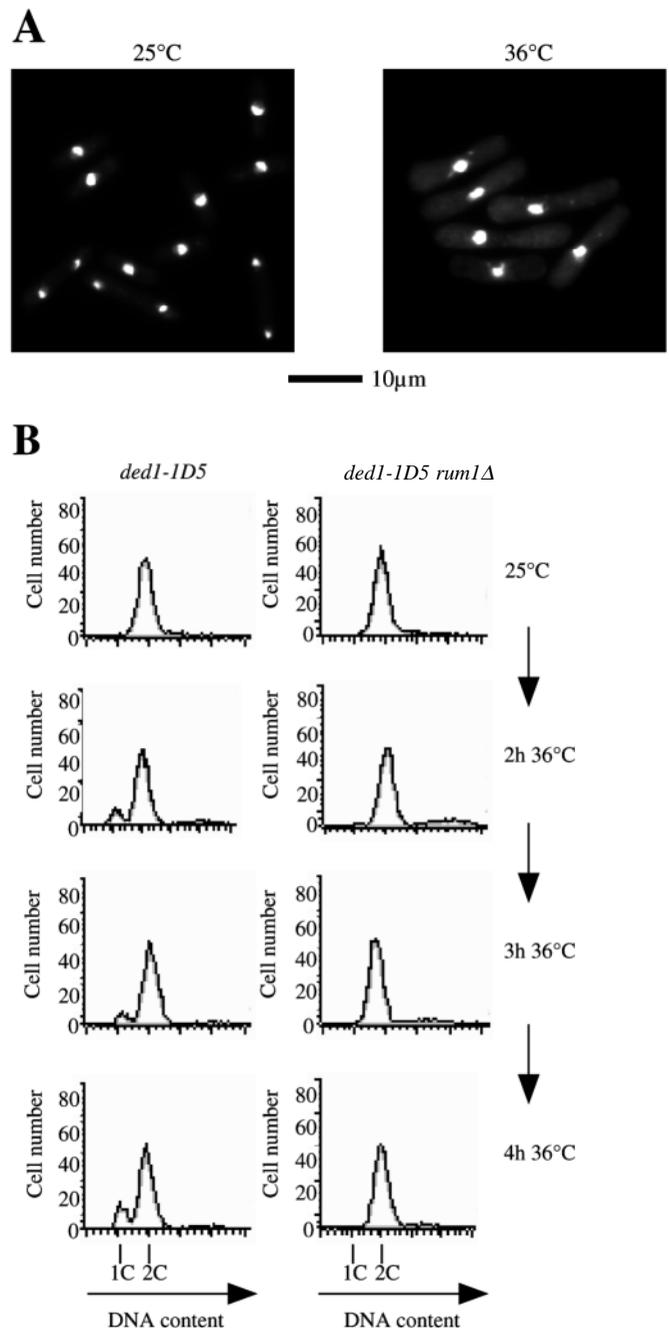


Fig. 1. A novel cell cycle mutant with phenotypes suggesting defects in B-cyclin/Cdc2 function. (A) An asynchronous culture of the *ded1-1D5* mutant was grown at 25°C and shifted to 36°C for 5 hours before cell fixation and staining with the DNA binding dye DAPI. (B) An asynchronous culture of the *ded1-1D5* mutant was grown at 25°C in parallel with an *ded1-1D5 rum1Δ* double mutant, and the two cultures were then shifted to 36°C for the times indicated, before fixing cells and processing them for flow cytometry.

Table 2. Loss of the B-type cyclin Cig2 specifically suppresses defects in Cdc18 and minichromosome maintenance proteins

Strain	25°C	35°C
Wild-type	+++	+++
<i>cig2Δ</i>	+++	+++
<i>cdc21-M68</i>	+++	cdc
<i>cdc21-M68 cig2Δ</i>	+++	+ / ++
<i>cdc19-P1</i>	+++	cdc
<i>cdc19-P1 cig2Δ</i>	+++	+ / ++
<i>cdc18-K46</i>	+++	cdc
<i>cdc18-K46 cig2Δ</i>	+++	+ / ++
<i>cdc22-M45</i>	+++	cdc
<i>cdc22-M45 cig2Δ</i>	+++	cdc

Deletion of the *cig2* gene rescues the t.s. phenotype of *cdc18-K46* as previously reported (Jallepalli et al., 1997; Lopez-Girona et al., 1998). In addition, we find that *cig2* deletion is able to suppress the t.s. defects of *cdc21-M68* and *cdc19-P1*. Cdc21/Mcm4 and Cdc19/Mcm2 are minichromosome maintenance (Mcm) proteins that are essential for DNA replication and probably have a role in origin firing. Deletion of *cig2* does not suppress *cdc22-M45*, a mutation producing a t.s. defect in the large subunit of ribonucleotide reductase. Wild-type growth is indicated by '+++', cell cycle arrest is denoted by 'cdc', whilst '+ / ++' indicates intermediate growth.

cdc2-33 (not shown). Taken together, these experiments show that mutation of *ded1* produces similar phenotypes to inactivation of cyclin B/cdc2 kinase activity.

Ded1 inactivation causes post-transcriptional inhibition of B-cyclin expression

Genetic analysis showed that the *ded1* gene is unlinked to any previously characterised regulator of B-cyclin/Cdc2 function, and we considered the possibility that mutation of Ded1 may affect the expression of B-cyclins or of Cdc2. An asynchronous culture of the *ded1-1D5* strain was grown at 25°C together with a wild-type control, and samples were taken after shifting to 36°C, in order to prepare protein extracts. As shown in Fig. 3A, immunoblot analysis revealed that although the levels of Cdc2, Cdc21/Mcm4 and α -tubulin were unchanged at 36°C, the levels of both the Cig2 and Cdc13 B-type cyclins fell dramatically. Both *cig2* and *cdc13* mRNA levels were unchanged at 36°C (not shown). Importantly, the decrease in B-cyclin levels did not require degradation by the APC, since Cdc13 and Cig2 still disappear when a *nuc2-663 ded1-1D5* double mutant is shifted to 36°C (Fig. 3B), in conditions where the APC is inactivated (Goebel and Yanagida, 1991; Hirano et al., 1988). The disappearance of Cig2, which is normally only expressed around G₁/S, is not simply a consequence of cell cycle arrest in G₁ and G₂, since a similar decrease is not seen when a *cdc2-M26* t.s. mutant is shifted to 36°C (Fig. 3C). In addition, Ded1 inactivation does not cause a dramatic reduction in the level of Cdc18 (Fig. 3A), another highly unstable protein that is expressed around G₁/S. Our results suggest that B-cyclin expression at some post-transcriptional step is particularly sensitive to inactivation of Ded1.

Ded1 is a general translation factor that is functionally homologous to RNA helicases from *Saccharomyces cerevisiae* and *Drosophila*

We cloned the *ded1*⁺ gene by complementation of both the t.s. and c.s. alleles with fission yeast cDNA and genomic libraries. Conceptual translation of the *ded1* ORF predicts a protein containing all the conserved motifs typical of RNA helicases

(Schmid and Linder, 1992). During the course of this work, the same sequence was published as *sum3*, a high-copy suppressor of specific checkpoint and stress responses (Forbes et al., 1998), *moc2*, a high-copy suppressor of sterility (Kawamukai, 1999), and the same gene was also identified in a third screen (Hsing-Yin Liu and Nancy Walworth, unpublished data).

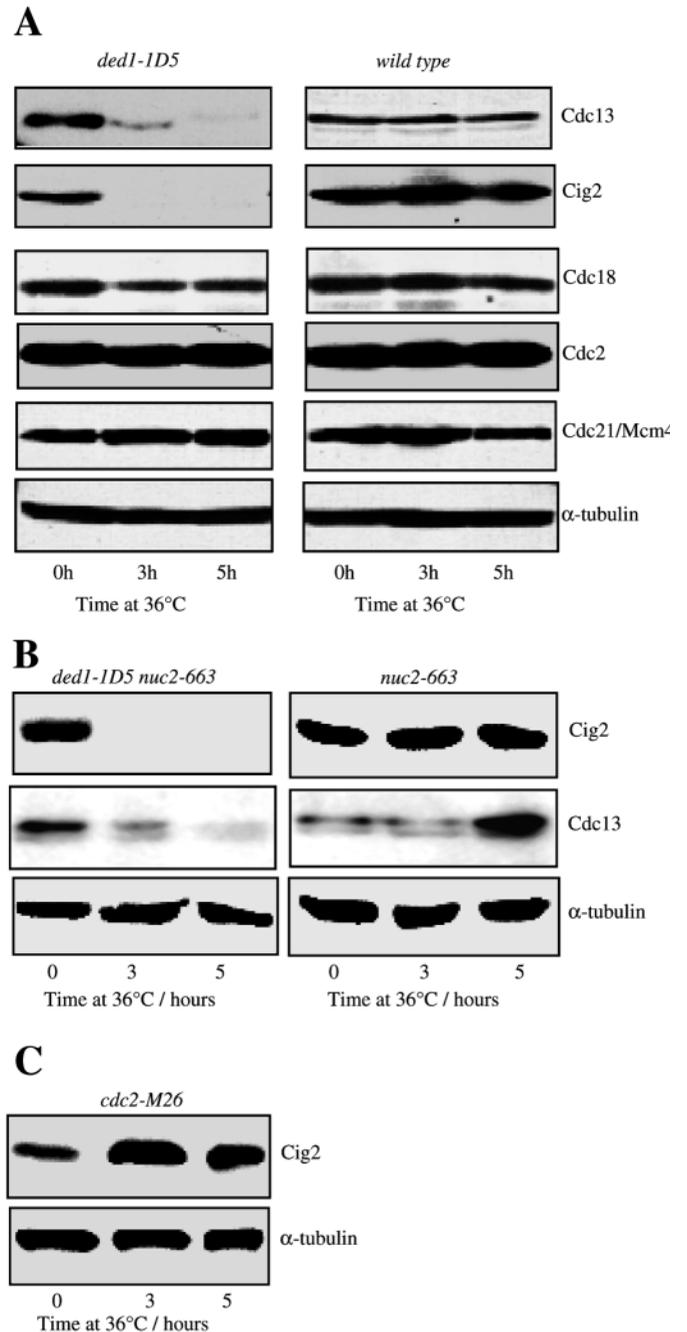


Fig. 3. APC and cell cycle arrest-independent reduction in Cdc13 and Cig2 levels upon inactivation of Ded1. (A) Protein extracts were made after shifting an asynchronous culture of the *ded1-1D5* strain to 36°C for the times shown. The levels of the indicated proteins were determined by immunoblotting. (B) A similar experiment was performed using a *ded1-1D5 nuc2-663* double mutant strain grown in parallel with a *nuc2-663* single mutant. (C) The effect of cell cycle arrest in G₁ and G₂ upon Cig2 levels was determined by shifting a *cdc2-M26* mutant from 25°C to 36°C for the indicated times.

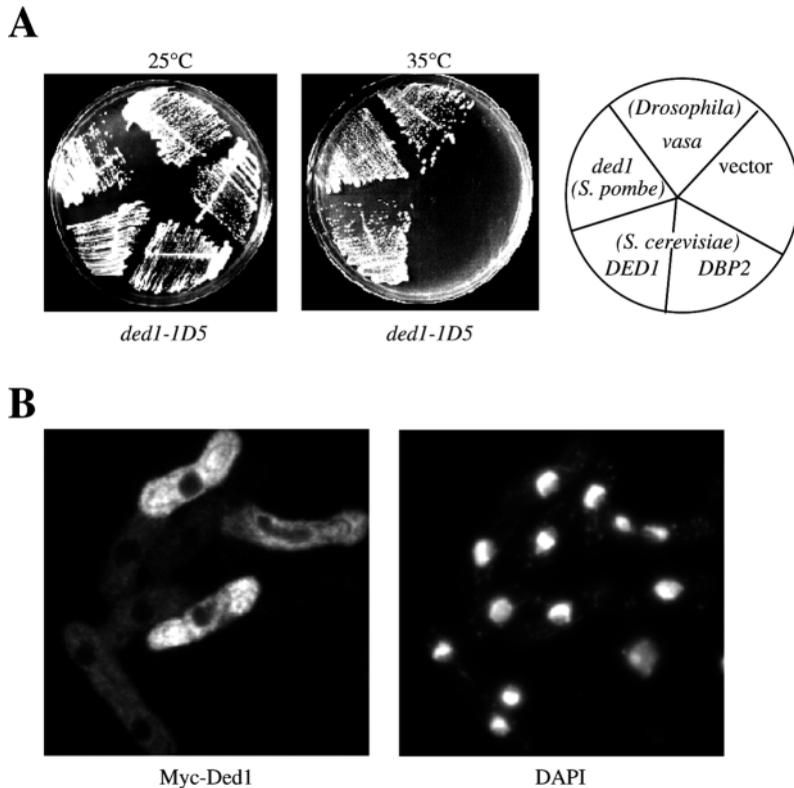


Fig. 4. Fission yeast Ded1 is functionally homologous to the budding yeast Ded1 and *Drosophila* Vasa RNA helicases. (A) Vectors expressing a variety of RNA helicases were transformed into *ded1-1D5*, and the transformants tested for growth both at the permissive temperature of 25°C and the restrictive temperature of 35°C. (B) The subcellular localization of an epitope tagged version of Ded1 was determined by immunofluorescence.

Having consulted with these groups we have agreed to adopt the name *ded1*, since the encoded protein is 60% identical to the budding yeast Ded1 protein (Forbes et al., 1998). It has recently been shown that *Saccharomyces cerevisiae* Ded1 has RNA helicase activity in vitro (Iost et al., 1999) and a general role in the initiation of translation in vivo (Chuang et al., 1997; De La Cruz et al., 1997). Budding yeast and fission yeast Ded1 proteins are also very similar to other cytoplasmic RNA helicases required for translation, such as the Vasa/PL10 subfamily of RNA helicases, essential for germ cell formation in a wide variety of animals (Gee and Conboy, 1994; Gruidl et al., 1996; Hay et al., 1988; Leroy et al., 1989).

We found that expression of either the *vasa* or budding yeast *DED1* genes can rescue the t.s. phenotype of the fission yeast *ded1-1D5* mutant, in contrast to expression of the *DBP2* gene, which encodes a budding yeast member of the p68 family of nuclear RNA helicases (Fig. 4A). This shows that *ded1*⁺, *vasa* and *DED1* encode functionally homologous proteins, and strongly suggests that fission yeast Ded1 is also an RNA helicase with a role in translation. Consistent with this view, we find that the Ded1 protein is cytoplasmic and appears to be excluded from the nucleus (Fig. 4B).

To investigate the efficiency of protein synthesis in *ded1* mutants, we examined the incorporation of [³⁵S]methionine into newly synthesised proteins during a short pulse given at different times after shifting to the restrictive temperature. General protein synthesis is profoundly inhibited when an

asynchronous culture of the cold-sensitive *ded1-61* mutant is shifted from the permissive temperature of 32°C to the restrictive temperature of 20°C (Fig. 5A). A fivefold reduction in general translation is also seen when the t.s. *ded1-1D5* mutant is shifted from 25°C to 36°C (Fig. 5B). This indicates that fission yeast Ded1 is a general translation factor analogous to Ded1 in budding yeast.

Translation of Cig2 is highly sensitive to inactivation of Ded1

Our data suggest that synthesis of the B-type cyclins Cig2 and Cdc13 is particularly sensitive to the efficiency of the translational machinery. This may explain why partial inactivation of Ded1 produces a similar effect to deletion of the *cig2* gene. To test this idea, we compared the translation of Cig2 and Cdc13 with that of other proteins, under conditions where partial inactivation of Ded1 suppresses defects in the initiation step of DNA replication. The *ded1-1D5* mutant was grown at 25°C in parallel with a wild-type strain, and both cultures were then shifted to 34.5°C, since at this temperature the lethality of the *cdc19-P1* mutant is suppressed (Fig. 2D). At both temperatures, cells were given a short pulse of [³⁵S]methionine. Cell extracts were made, and the incorporation of radiolabel into immunoprecipitates of Cig2 and Cdc13 was compared with incorporation into total protein. Fig. 6 shows that general protein synthesis increased almost 2-fold in both the wild-type and *ded1-1D5* strains at 34.5°C. In addition, the translation of Cig2 and Cdc13 was not impaired when the wild-type strain was shifted from 25°C to 34.5°C. However, incorporation of [³⁵S]methionine into Cig2 was reduced by 60% in the *ded1-1D5* mutant at 34.5°C, while incorporation into Cdc13 was reduced by 40%. This experiment shows that translation of Cig2, and to a lesser extent Cdc13, is especially sensitive to mutation of Ded1, in comparison to the majority of other proteins.

If the ability of *ded1* mutants to suppress defects in the initiation step of DNA replication is indeed due to reduced translation of Cig2, increased expression of Cig2 should prevent *ded1* mutants from suppressing such defects. It has previously been shown, by 5' and 3' RACE PCR, that the *cig2*⁺ mRNA has large 5' and 3' UTRs, each of around 1 kb (Obara-Ishihara and Okayama, 1994). Such UTRs often contain elements that limit the efficiency of translation. Although the full sequence of the *cig2* UTRs has yet to be described, the sequence of a *cig2* cDNA containing shorter UTRs has been reported (Obara-Ishihara and Okayama, 1994). We compared this cDNA (cDNA I in Fig. 7A) with two shorter versions, either lacking a short upstream open reading frame in the 5' UTR (cDNA II in Fig. 7A), or lacking all *cig2* UTR sequences (cDNA III in Fig. 7A), and asked whether expression of these cDNAs prevented suppression of *cdc21-M68* and *cdc18-K46* by mutation of *ded1*. As shown in Fig. 7B, suppression of *cdc21-M68* at 35°C by *ded1-78* was partially blocked by expression of *cig2* cDNA I, and more completely inhibited by expression of cDNAs II and III. A similar result was obtained when we examined the ability of

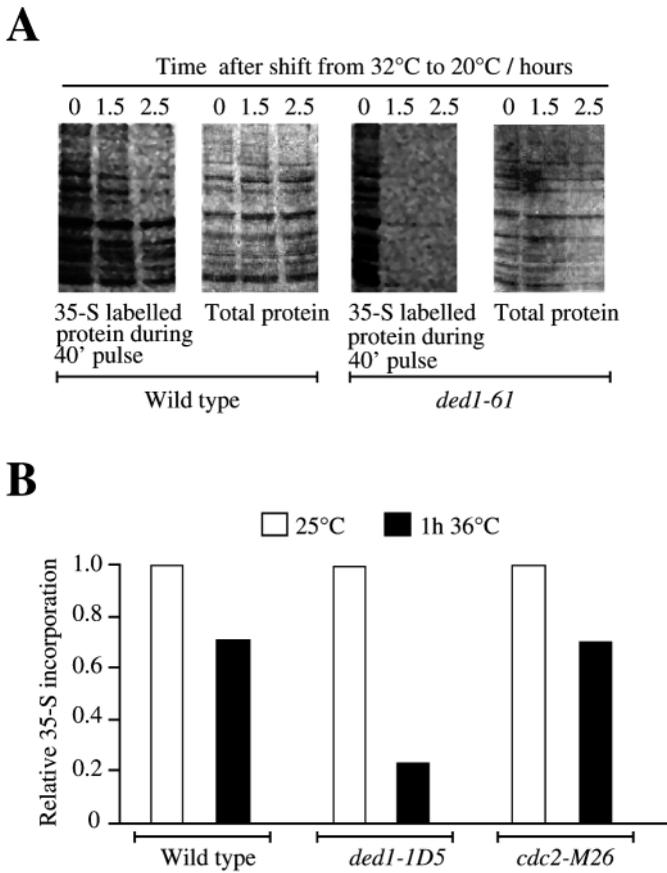


Fig. 5. Fission yeast Ded1 is a general translation factor. (A) Asynchronous cultures of wild-type and *ded1-61* strains were grown at 32°C. Protein synthesis at this temperature was compared with the rate of translation 1.5 or 2.5 hours after shifting the culture to 20°C, by exposing the cells to a short pulse of [³⁵S]methionine at each of these time points. Protein extracts were prepared and samples analysed by SDS-PAGE, followed either by detection of ³⁵S-labelled proteins using a phosphorimager, or by SyproRed staining to show total protein levels. (B) In a similar fashion, protein synthesis was compared in wild-type, *ded1-1D5* and *cdc2-M26* strains, either at 25°C, or 1 hour after shifting to 36°C. Samples were analysed as before and total incorporation quantified using a phosphorimager.

ded1 c.s. alleles to suppress *cdc18-K46* in the presence of the *cig2* cDNAs (not shown). Therefore, reduced expression of Cig2 is likely to be an important reason why defects in the initiation step of DNA replication are suppressed by partial inactivation of Ded1.

Expression of cDNA III, lacking all *cig2* UTR sequences, produces a 4-fold increase in the Cig2 protein/mRNA ratio, relative to cDNA I (Fig. 7C). This is unlikely to be due to saturation of Cig2 degradation by the APC, since the level of Cdc13 does not increase correspondingly (Fig. 7D). This suggests that the efficiency of Cig2 translation is increased by removal of the UTRs. Consistent with this view, the decrease in Cig2 protein levels upon inactivation of Ded1 at 36°C is progressively reduced as the UTR sequences are removed from the *cig2* cDNA (Fig. 8).

Translational regulation of B-cyclins as cells exit from the mitotic cell cycle

The sensitivity of Cig2 protein levels to perturbations of the translation machinery led us to consider whether Cig2 expression is regulated at the level of translation during the fission yeast life cycle. A variety of evidence suggests that translational regulation of the *cig2* mRNA might play a role under conditions of nitrogen starvation, when the fission yeast cell switches from the mitotic cell cycle to conjugation and the meiotic cycle. In contrast to many other proteins such as Cdc2, both Cig2 and Cdc13 disappear in starving cells (Wu and Russell, 1997; Yamaguchi et al., 1997; see Fig. 9A), and this is important for the cell to arrest in G₁ as a prelude to conjugation (Kominami et al., 1998; Martin-Castellanos et al., 1996; Obara-Ishihara and Okayama, 1994; Yamaguchi et al., 1997). Although Cig2 protein disappears upon nitrogen starvation of wild-type cells, expression of the *cig2*⁺ mRNA is strongly induced (Obara-Ishihara and Okayama, 1994). This raises the possibility that proteolysis may not be sufficient to explain the timely decrease in the level of Cig2 protein as cells switch from the mitotic cell cycle to conjugation and meiosis.

In order to test whether translation of B-cyclins is inhibited upon nitrogen starvation, we exposed cells to a short pulse of [³⁵S]methionine at different times after transfer into medium lacking a nitrogen source. General protein synthesis was compared with de novo synthesis of Cig2 and Cdc13, by SDS-

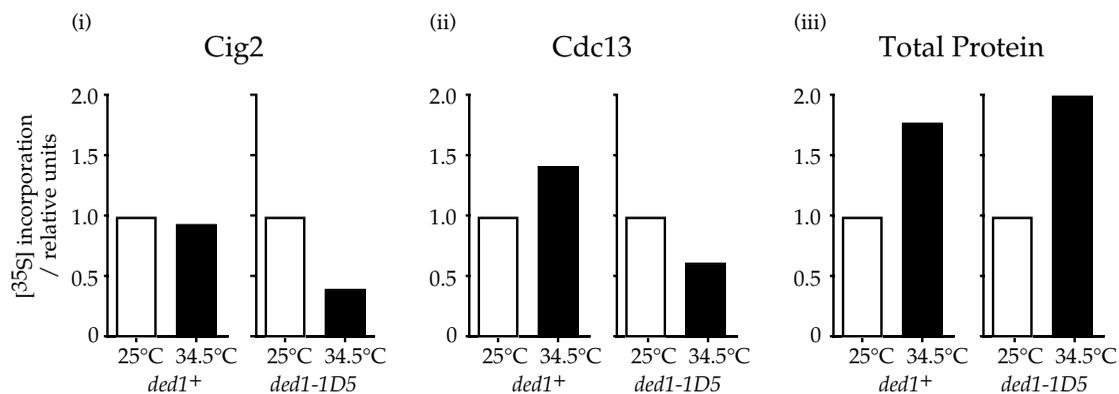
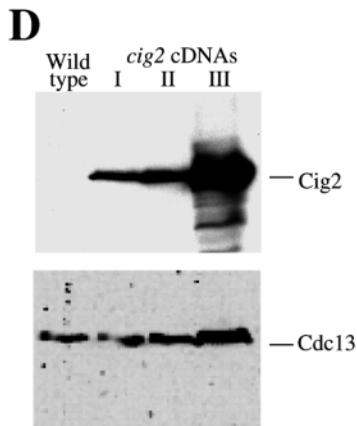
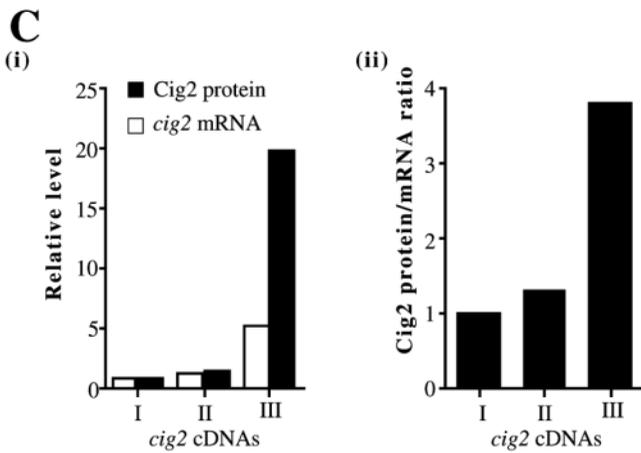
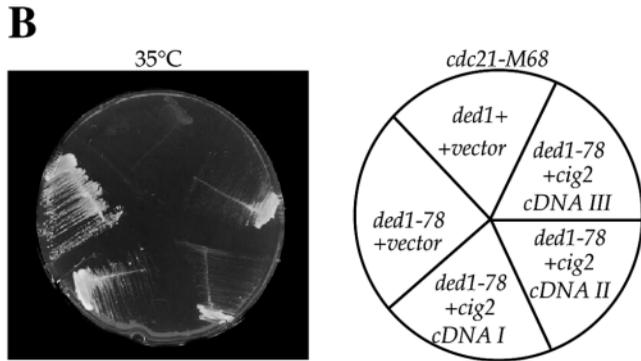
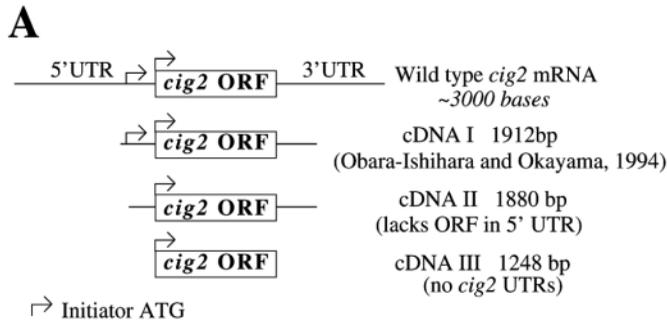


Fig. 6. Translation of Cig2 and Cdc13 is particularly sensitive to inactivation of Ded1. Wild-type and *ded1-1D5* strains were grown at 25°C and then shifted to 34.5°C for 1 hour. At both temperatures, cells were exposed to a short pulse of [³⁵S]methionine, and cell extracts were made. Cig2 and Cdc13 were then immunoprecipitated and the incorporation of [³⁵S]methionine into Cig2 (i), Cdc13 (ii) and total protein (iii) was determined as above. Values are expressed relative to the 25°C samples.



PAGE analysis of total protein samples together with immunoprecipitates of Cig2 and Cdc13, followed by phosphorimager detection of the incorporation of radiolabel

Fig. 7. Increased expression of Cig2 prevents suppression of *cdc21-M68* by *ded1-78*. (A) A variety of *cig2* cDNAs – see text for details. (B) *cdc21-M68* strains containing the indicated plasmids and alleles of *ded1* were streaked out at 35°C and incubated for 2 days. (C) Wild-type strains containing plasmids expressing the various *cig2* cDNAs from the medium strength *nmt1* promoter were grown at 25°C. Expression from the *nmt1* promoter was induced for 26 hours, and samples prepared to analyse Cig2 protein and mRNA levels by means of immuno- and northern blots. Cig2 protein and mRNA levels were quantitated as described in Materials and Methods. (i) Cig2 protein and mRNA levels are shown for cells expressing the various *cig2* cDNAs. The levels are expressed relative to cDNA I. (ii) The Cig2 protein/mRNA ratio is shown for the various *cig2* cDNAs. (D) Immunoblots showing Cig2 and Cdc13 protein levels in cells expressing the various *cig2* cDNAs.

into newly made proteins. The rate of total [³⁵S]methionine incorporation increased somewhat upon nitrogen starvation (Fig. 9B,C), probably due to a depletion of the cellular methionine pools. Nevertheless, incorporation of [³⁵S]methionine into Cig2 and Cdc13 decreases between 2 and 4 hours after shifting to nitrogen-free medium (Fig. 9D), despite the general increase seen for the majority of proteins. This shows that the translation of the Cig2 and Cdc13 B-type cyclins is indeed impaired upon nitrogen starvation. Inhibition of B-cyclin translation occurs with similar kinetics to the disappearance of Cdc13 and Cig2 proteins (Fig. 9A) and is therefore likely to contribute to their timely disappearance as cells exit the mitotic cell cycle. Moreover, the level of Cig2 protein remains high upon starvation if the *cig2* gene is expressed in the absence of the native UTRs (Fig. 10A). This is not due to saturation of APC mediated proteolysis, since Cdc13 disappears on schedule (Fig. 10A). Failure to downregulate the level of Cig2 protein is associated with a 10-

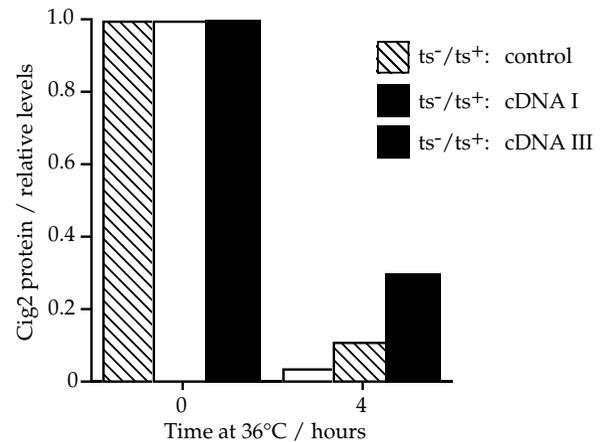


Fig. 8. Removal of the UTRs from the *cig2* mRNA reduces the sensitivity to Ded1 inactivation. Wild-type and *ded1-ID5* strains transformed with a vector control, *cig2* cDNA I, or *cig2* cDNA III, were grown at 25°C in the presence of thiamine. The cells were washed into thiamine-free medium, to induce expression of *cig2* cDNAs from the *nmt1* promoter, and incubation was continued for a further 26 hours. At this point, the cultures were shifted to 36°C for 4 hours. The ratio of Cig2 and α -tubulin was determined for each sample by immunoblotting (see Materials and Methods). The values of this ratio for the *ded1-ID5* strains are expressed relative to the corresponding values for the wild-type strains.

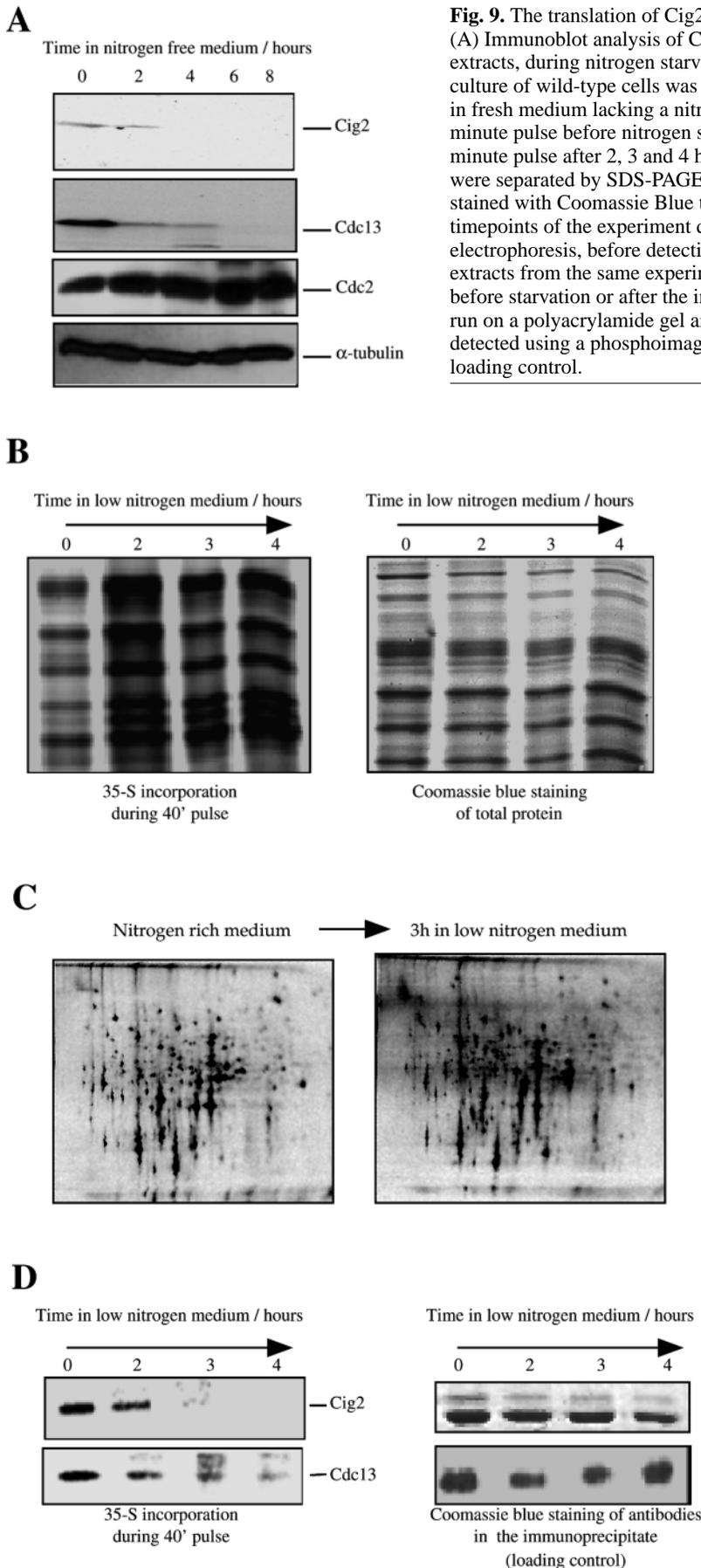


Fig. 9. The translation of Cig2 and Cdc13 is inhibited upon nitrogen starvation.

(A) Immunoblot analysis of Cig2, Cdc13, Cdc2 and α -tubulin protein levels in total cell extracts, during nitrogen starvation of a wild-type strain at 25°C. (B) An asynchronous culture of wild-type cells was grown at 25°C and cells were then washed and resuspended in fresh medium lacking a nitrogen source. Incorporation of [³⁵S]methionine during a 40 minute pulse before nitrogen starvation was compared with incorporation during a 40 minute pulse after 2, 3 and 4 hours of growth in low nitrogen medium. Labelled proteins were separated by SDS-PAGE, and detected with a phosphoimager. Parallel samples were stained with Coomassie Blue to show total protein levels. (C) Samples from 0 and 3 hour timepoints of the experiment described in (B) were subjected to 2-dimensional gel electrophoresis, before detection of labelled proteins with a phosphoimager. (D) Protein extracts from the same experiment were used to immunoprecipitate Cig2 and Cdc13, either before starvation or after the indicated times in low nitrogen medium. The samples were run on a polyacrylamide gel and incorporation into newly synthesised proteins was detected using a phosphoimager. Parallel samples were stained with coomassie blue as a loading control.

fold inhibition of conjugation and meiosis (Fig. 10B; Obara-Ishihara and Okayama (1994) also reported a three fold reduction in conjugation efficiency upon increased Cig2 expression), emphasising the importance of the inhibition of Cig2 function for sexual differentiation in fission yeast.

DISCUSSION

We have identified a functional homologue of the budding yeast Ded1 RNA helicase, which is required for general translation together with the eIF4A RNA helicase (Chuang et al., 1997; De La Cruz et al., 1997). Fission yeast Ded1 is also required for protein synthesis and our *ded1* alleles are likely to provide useful experimental tools, since fission yeast strains allowing rapid inactivation of the translation machinery have not previously been isolated. Although the precise role of Ded1 in translation remains to be determined, it is likely to be involved in the initiation step, since budding yeast *ded1* mutants show reduced levels of polysomes (Chuang et al., 1997; De La Cruz et al., 1997) and overexpression of the *DED1* gene rescues the lethality associated with defects in either the cap-binding protein Cdc33, or in the RNA helicase Tif1/eIF4A (De La Cruz et al., 1997); for general reviews of translational initiation see (Gray and Wickens, 1998; McCarthy, 1998; Sachs et al., 1997). Defects in fission yeast Ded1 function can also be rescued by expression of the *vasa* gene, encoding a highly related RNA helicase from *Drosophila* (Hay et al., 1988; Liang et al., 1994). *Vasa* is required in vivo to overcome translational repression of genes such as *nanos* (Gavis et al., 1996). This inhibition is mediated by a motif in the 3'UTR of the *nanos* mRNA, that is predicted to have a complex secondary structure (Gavis et al., 1996). It is possible that Ded1 homologues in budding and fission yeasts may also be required to overcome the inhibitory effects of such motifs upon translation.

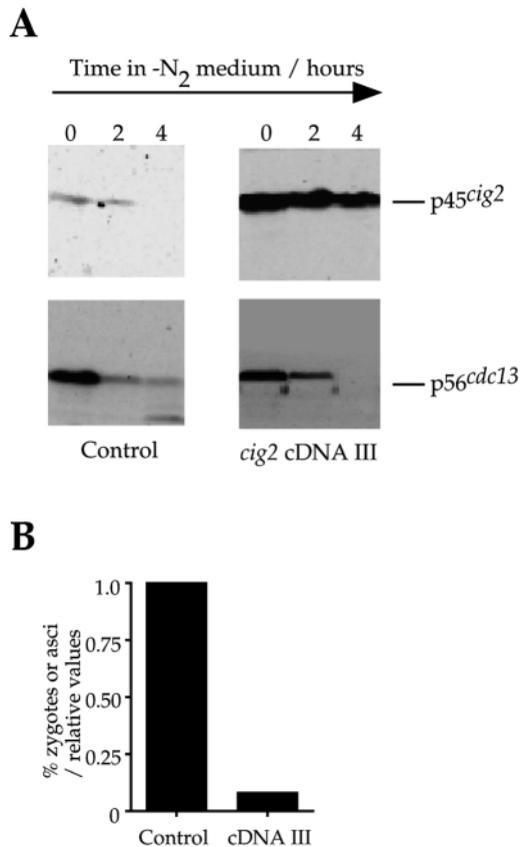


Fig. 10. Expression of *cig2* lacking UTRs prevents the decrease in Cig2 protein levels upon starvation and blocks conjugation. (A) Wild-type strains transformed with a vector control or *cig2* cDNA III, were grown at 25°C in medium containing thiamine. Cells were then washed into fresh medium lacking thiamine, and incubated for 20 hours at 25°C to induce expression of *cig2* from the *nmt1* promoter. The cultures were then washed into fresh medium lacking a nitrogen source, and samples were taken at the indicated times to analyse Cig2 and Cdc13 protein levels by immunoblotting. (B) Wild-type strains of both mating types were transformed with with a vector control or *cig2* cDNA III. The cultures were grown at 25°C and expression from the *nmt1* promoter was induced for 20 hours as before. Equal numbers of cells of the opposite mating type, containing either the vector or *cig2* cDNA III, were then mixed and vigorously shaken in malt extract medium. After 2 days incubation at 25°C, the percentage of zygotes and asci was determined microscopically. The value for cells expressing the *cig2* cDNA III is expressed relative to that of the control.

The *ded1*⁺ gene was previously identified as a multicopy suppressor of a checkpoint defect (Forbes et al., 1998), or of sterility (Kawamukai, 1999), though a role for Ded1 in translation was not demonstrated in these studies. Overexpression of *ded1*⁺ (called *sum3*⁺ in Forbes et al., 1998) was found to prevent cells with high levels of the Cdc25 tyrosine phosphatase from entering mitosis before the completion of S-phase. Elevated expression of *ded1*⁺ also prevented the advancement of mitosis that normally results from osmotic shock, without affecting transcription of genes induced by the stress-specific MAP kinase pathway (Forbes et al., 1998). Overexpression of a second putative translation factor produces the same effect (Humphrey and Enoch, 1998).

Although it is not clear how these effects are produced, it will be interesting to see whether translation is regulated by the checkpoints that link cell cycle progression to genomic integrity.

We have shown that levels of the B-cyclins Cdc13 and Cig2 are greatly reduced upon inactivation of Ded1, even if the APC is inactivated at the same time. Another unstable protein, Cdc18, is not affected to the same degree. While Ded1 appears to be a general translation factor, our data indicate that the synthesis of Cig2 and Cdc13 is particularly sensitive to a reduction in the general efficiency of translation. For Cig2, our data suggest that elements in the UTRs of the *cig2* mRNA may limit the efficiency of Cig2 translation, and it is tempting to speculate that Ded1 may be required to overcome the inhibitory effects of such sequences. We are currently characterising the UTRs of the *cig2* mRNA in more detail, in order to define which elements have a role in regulating Cig2 translation. Regarding Cdc13, we note that a recent study has shown that the 5' UTR of the *cdc13* mRNA is predicted to have a complex structure, analogous to that of the 5' UTR of the *cdc25* mRNA, which may again serve to limit the efficiency of translation (Daga and Jimenez, 1999). This study also showed that the levels of both Cdc25 and Cdc13 are sensitive to a modest reduction in the efficiency of translation initiation activity.

There is no evidence that the rate of translation of Cig2 and Cdc13 changes through the cell cycle in proliferating cells. Indeed, it has previously been shown that the rate of Cdc13 translation is similar in G₁ and G₂ phases (Correa-Bordes et al., 1997). Cig2 protein and mRNA levels peak around G₁/S (Connolly and Beach, 1994; Martin-Castellanos et al., 1996; Mondesert et al., 1996; Obara-Ishihara and Okayama, 1994), and regulated transcription and proteolysis may be sufficient to explain these changes in cycling cells. However, the translation of both Cdc13 and Cig2 is inhibited upon nitrogen starvation (Fig. 9), when the disappearance of B-cyclins is crucial for exit from the mitotic cell cycle and entry into conjugation and meiosis. This identifies a third mechanism by which Cdc2/cyclin B kinase activity is regulated during this important cell cycle transition, in addition to cyclin B proteolysis (Kominami et al., 1998; Kumada et al., 1995; Stern and Nurse, 1998), and CDK-inhibition by Rum1 (Correa-Bordes and Nurse, 1995; Kominami et al., 1998; Moreno and Nurse, 1994; Stern and Nurse, 1998). The mechanism by which B-cyclin translation is inhibited upon nitrogen starvation remains to be determined, although we find that removal of the *cig2* UTRs prevents the reduction in Cig2 protein levels upon starvation (Fig. 10). We do not know whether regulation of Ded1 plays any role in this process, and we find that the translation of most other proteins is less affected in starving cells. However, it is interesting to note that the level of Ded1 is reduced upon nitrogen starvation (H.-Y. Liu and N. Walworth, personal communication), and this may impair the translation of mRNAs that are particularly sensitive to Ded1 function. It is also possible that translational regulation of Cig2 expression may involve multiple mechanisms, since two *cig2* mRNAs are induced upon nitrogen starvation, and one of these lacks a poly(A) tail (Obara-Ishihara and Okayama, 1994). Poly(A)⁻ mRNAs are poorly translated, and subsequent polyadenylation at some point during meiosis, or in the germinating spore, could provide a means of rapidly making new Cig2 protein, as

an efficient trigger to the onset of S-phase. This would then be analogous to translational regulation of certain mRNAs by polyadenylation during *Xenopus* and *Drosophila* early development (Salles et al., 1994; Wickens, 1990).

Translational regulation of B-type cyclins has been shown to be important in higher eukaryotes for both cell cycle control and development. Cyclin B mRNA is concentrated in the posterior pole of the *Drosophila* oocyte, and becomes incorporated into the pole cells that are the progenitors of the germ-line, but is not translated until around the time that mitosis resumes in the developing gonad (Dalby and Glover, 1992). In many animals, translation of B-type cyclins is stimulated after germinal vesicle breakdown during oocyte maturation, and it has been shown in starfish that this is important for the repression of DNA replication between the two meiotic divisions (Picard et al., 1996). Translational regulation of D-type cyclins may also be important for cell cycle control, when mammalian cells exit G₀ and resume proliferation. Overexpression of the cap-binding protein eIF4E can lead to oncogenic transformation of mammalian fibroblasts (Lazaris-Karatzas et al., 1990); reviewed by Hentze (1995), and this may be related to increased translation of specific mRNAs such as cyclin D1 (Koromilas et al., 1992; Rosenwald et al., 1993). It will be of considerable interest to establish the extent to which translational control provides a general linkage between the regulation of cell growth and division.

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