Identification of partners of TIF34, a component of the yeast eIF3 complex, required for cell proliferation and translation initiation

Marie-Hélène Verlhac^{1,2},
Ruey-Hwa Chen^{1,2,3}, Parisa Hanachi⁴,
John W.B.Hershey⁴ and Rik Derynck^{1,2,5}

¹Departments of Growth and Development and Anatomy and

²Programs in Cell Biology and Developmental Biology, University of California at San Francisco, San Francisco, CA 94143-0640, USA,

³Institute of Molecular Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan and ⁴Department of Biological Chemistry, School of Medicine, University of California, Davis, CA 95616, USA

⁵Corresponding author

Eukaryotic initiation factor-3 (eIF3) in the yeast *Saccharomyces cerevisiae* plays a central role in initiation of translation. The eIF3 complex contains at least eight different proteins, but, as yet, little is known about the function of the individual proteins. In this study we have characterized the role of TIF34 (eIF3-p39), a recently identified WD-40 domain-containing protein of 39 kDa, in the eIF3 complex. Using temperature-sensitive mutants of TIF34 we show that this protein is required for cell cycle progression and for mating and plays an essential role in initiation of protein synthesis. By two-hybrid screening we have identified two partners that directly associate with TIF34: PRT1, a previously characterized eIF3 subunit, and a novel protein of 33 kDa (eIF3-p33) which is part of the eIF3 complex and has an RNA binding domain. TIF34 and p33 interact with each other and overexpression of p33 complements the growth defect of a *tif34-ts* mutant. Our results provide support for both physical and functional interactions between three subunits, TIF34, PRT1 and p33, in the eIF3 complex.

Keywords: eIF3/translation/WD-40 repeats/yeast

Introduction

Protein synthesis plays a major role in cell cycle regulation in eukaryotic cells in general and in yeast in particular. In the absence of protein synthesis yeast cells do not initiate DNA synthesis and mitosis. It is therefore of great biological significance to understand how protein synthesis is regulated in yeast and, in particular, how it is initiated. The process of protein synthesis initiation is complex and requires at least 10 initiation factors (for a review see Merrick and Hershey, 1996). The largest of these is eukaryotic initiation factor 3 (eIF3), a multiprotein complex of at least eight subunits (Trachsel and Staehelin, 1979; Naranda *et al.*, 1994; Hannig, 1995). Many roles have been assigned to eIF3 in the translational initiation pathway in mammalian cells. It has been implicated in dissociating 80S ribosomes into the 40S and 60S ribosomal subunits by binding to the 40S subunit (Benne and Hershey,

1978; Goumans *et al.*, 1980). It prevents dissociation of the Met-tRNA_i·eIF2-GTP ternary complex caused by addition of RNA (Gupta *et al.*, 1990) and stabilizes ternary complex binding to 40S ribosomal subunits (Benne and Hershey, 1978). eIF3 is also required for mRNA binding to 40S and 80S ribosomes (Benne and Hershey, 1978; Trachsel and Staehelin, 1979), in part by binding to the eIF4G subunit of the cap binding complex, eIF4F (Lamphear *et al.*, 1995; Mader *et al.*, 1995). Knowledge of the composition of the eIF3 complex and characterization of its different proteins and their interaction is essential for understanding how translation is initiated.

The yeast eIF3 complex comprises eight subunits of apparent molecular masses 16, 21, 29, 33, 39, 62, 90 and 135 kDa (Hershey *et al.*, 1997). Genes for four of the subunit proteins have been identified so far. The 62 kDa protein is encoded by *GCD10* and has been identified as the RNA binding subunit of eIF3 (Garcia-Barrio *et al.*, 1995). The second largest subunit, p90, is encoded by *PRT1* and is involved in Met-tRNA_i binding to the 40S ribosomal subunit (Moldave and McLaughlin, 1988). Interestingly, *PRT1* was also identified as a cell division cycle (*cdc*) mutant, *cdc63* (Hartwell and McLaughlin, 1968). Temperature-sensitive *prt1* mutants arrest in the G₁ phase of the cell cycle (Hanic-Joyce *et al.*, 1987a). The smallest subunit of eIF3, p16, is the product of *SUI1* (Naranda *et al.*, 1996). Mutant forms of *SUI1* were identified in a genetic screen for mutations that affect initiator codon recognition and allow initiation at a UUG codon (Yoon and Donahue, 1992). Finally, p39, a member of the WD-40 repeat family of proteins, has recently been identified as the product of *TIF34* (for translation initiation factor 3, subunit 4; Naranda *et al.*, 1997).

The *TIF34* gene and its corresponding protein, eIF3-p39 (TIF34), is required for cell viability and plays an important, although not necessarily direct, role in initiation of translation, possibly because it is required for assembly and maintenance of the eIF3 complex. This possible involvement in protein synthesis was suggested by analysis of yeast cells depleted of TIF34 (Naranda *et al.*, 1997). TIF34 also has considerable sequence similarity with mammalian TRIP-1 and may be a homolog (Chen *et al.*, 1995). TRIP-1 has the ability to directly interact with the cytoplasmic domain of the type II TGF- β receptor (Chen *et al.*, 1995). Its possible role in TGF- β receptor function has as yet not been reported.

To define more precisely the role of TIF34 in yeast, we generated temperature-sensitive (*ts*) alleles of the *tif34* gene. We show that TIF34 is essential for cell cycle regulation and mating ability. We also demonstrate that TIF34 is required to initiate protein synthesis. Using two-hybrid screening we have isolated two partners of TIF34, i.e. PRT1, a known component of eIF3, and a novel protein, eIF3-p33, which contains an RNA binding domain

Fig. 1. Growth of wild-type and *tif34-ts* cells at permissive or restrictive temperatures; structure of the proteins encoded by *tif34-ts6* and *tif34-ts31* alleles. **(A)** Wild-type (aW303), *tif34-ts6* and *tif34-ts31* cells were first grown at 24°C to an OD_{600} of ~0.4, then pelleted and resuspended in medium prewarmed at 24, 30 or 37°C. Samples were collected at the indicated time points and OD_{600} was measured. **(B)** The WD-40 repeats of TIF34 are schematically shown as boxes. Amino acid substitutions in both *ts* alleles are represented as lollipops; the silent mutations are not shown.

and is another subunit protein of eIF3. eIF3-p33 interacts directly with TIF34 and can functionally complement a deficiency in TIF34.

Results

TIF34 is essential for G₁/S and G₂/M progression.

To characterize the biological function of the TIF34 protein, we first disrupted the endogenous yeast gene by homologous recombination using a plasmid that carries the TIF34 coding sequence interrupted by a large deletion replaced by the *HIS3* gene. Tetrad analysis of the transformants revealed that only two of the four spores in each of the 20 asci formed colonies on rich medium and these had a *HIS*[−] phenotype. This finding indicates that the *tif34* null mutation was lethal, consistent with previous results (Naranda *et al.*, 1997). This lethal phenotype was rescued by introducing a plasmid that contained wild-type *tif34* (data not shown). To better characterize the function of TIF34, we generated temperature-sensitive alleles of *tif34* using a mutagenic polymerase chain reaction (see Materials and methods). We produced 35 temperature-

sensitive alleles that conferred no growth at 37°C yet showed a growth similar to the wild-type at 24°C. Two of these, *tif34-ts6* and *tif34-ts31*, were selected for further analyses. Both alleles conferred normal growth at 24°C, however, the *tif34-ts6* allele was unable to confer growth at 30°C, whereas *tif-ts31* cells grew at 30°C similarly to wild-type cells (Figure 1A). Both *ts* alleles were able to rescue the inability of cells with an inactivated *tif34* gene to grow at 24°C, but not at 37°C (Figure 1A).

To characterize the structural basis of the temperature-sensitive phenotype and the much stronger *ts* phenotype of *tif34-ts6* than of *tif34-ts31*, we sequenced both alleles. The *tif34-ts6* allele encodes a protein with three mutations in different WD-40 repeats, two of which replace aspartic acid residues that are conserved from yeast to humans (Chen *et al.*, 1995). In contrast, the two mutations in *tif34-ts31* affect only sequences outside the WD-40 repeats (Figure 1B). The correlation of the stronger *ts* phenotype with mutations in WD-40 sequences suggests an important role of these domains in the function of TIF34.

Microscopic observation of yeast containing the *tif34-ts* alleles revealed that a shift of exponentially growing

Fig. 2. Flow cytometry of cells synchronized using different drugs. Wild-type (aW303) and *tif34-ts31* cells were synchronized using one of three drugs as shown and then shifted to 37°C for the times indicated (0, 40, 80, 120 or 180 min) after release from the drug. The graphs show the relative number of cells with their DNA content between 1N and 2N as determined using propidium iodide staining (PI). We only show the DNA quantitation of aW303 cells and *tif34-ts31* cells at 37°C, since *tif34-ts31* cells at 24°C behave as wild-type control cells. Cells were released from growth arrest induced using α -factor (A), hydroxyurea (B) or nocodazole (C). The top panel shows wild-type (wt) control cells and the bottom panel shows *tif34-ts31* mutant cells.

cells from 24 to 37°C for 3 h resulted in an accumulation of cells primarily in the G₁ stage of the cell cycle. Approximately 60% of the cells were arrested as unbudded cells or cells with a small bud, characteristic of G₁, in contrast to only 30% of the wild-type cells. An additional 35% of the cells arrested with larger buds, characteristic of the G₂/M stage (data not shown). To better characterize the growth arrest, we synchronized cultures of wild-type and *ts* mutant cells using α -factor, nocodazole or hydroxyurea. Following the temperature shift from 24 to 37°C the drug was removed and the DNA content of the cells was analyzed at different times using fluorescence-activated cell sorting. As shown in Figure 2A, following α -factor-induced growth arrest at Start, *tif34-ts31* cells were unable to exit G₁ at the restrictive temperature (Figure 2A). Similarly, only a small proportion of the *tif34-ts31* cells were able to exit nocodazole-induced arrest at G₂/M (Figure 2C). Finally, *tif34-ts31* cells were able to replicate their DNA at the restrictive temperature after synchronization in the early S stage using hydroxyurea (Figure 2B). Using all three treatments the results obtained with *tif34-ts6* cells were similar to those with *tif34-ts31* cells (data not shown). Taken together, these results strongly suggest that TIF34 is required for progression through the G₁/S and G₂/M stages of the cell cycle. However, once DNA replication is initiated, TIF34 is no longer required to complete DNA replication.

***tif34-ts* mutant cells are defective in mating and α -factor response at restrictive temperature**

To further characterize the function of TIF34 in yeast, we also determined the mating ability of the two *tif34-ts* mutants in comparison with wild-type cells at permissive and restrictive temperatures. Since the cells with the *ts* alleles arrest at different points of the cell cycle, we performed the mating assays, using either cells that were synchronized in G₁ with α -factor at the permissive tem-

Table I. Quantitative mating assays performed at 24 or 37°C

	24°C	37°C
α -Factor pretreatment		
aW303	7.8	1.8
atif34-ts31	7.3	0.1
atif34-ts6	0.7	0.00008
No α -factor pretreatment		
aW303	3.1	0.3
atif34-ts31	1.3	0.01
atif34-ts6	0.2	<0.000001
α tif34-ts6	0.3	0.000006
α W303	3.2	0.5

Cells were synchronized for 2 h in an α -factor (20 μ g/ml)-containing medium at 24°C prior to mating (left) or were used as an asynchronous population (right). The numbers indicate the percentage of diploids formed in the total population of cells in the mating assay. Each mating assay was performed at least three times.

perature before mating or asynchronous cell populations. As shown in Table I, the mating ability of the cells with either *ts* allele was not greatly affected by arresting the cells with α -factor prior to mating, which is consistent with our observations that the *ts* mutants tend to accumulate in G₁. At the restrictive temperature both *tif34-ts31* and *tif34-ts6* cells showed strongly decreased mating ability compared with the wild-type control cells. However, *tif34-ts6* cells showed a much more dramatic decrease in mating ability at 37°C when compared with *tif34-ts31* cells. In α -factor-arrested cells the mating efficiency of *tif34-ts6* cells was ~10 000-fold reduced in comparison with the 70-fold reduction of *tif34-ts31* cells. The decrease in mating ability did not depend on the mating type of the *tif34-ts6* cells, since it was similarly observed with MATa or MAT α *tif34-ts6* strains. Similarly to what we noticed with the growth characteristics, *tif34-ts6* cells displayed a stronger phenotype than *tif34-ts31* cells, as is apparent in

Fig. 3. *tif34-ts* mutant cells do not respond to α -factor at the restrictive temperature. Wild-type (aW303) and *tif34-ts* cells transformed with a reporter *FUS1-LacZ* gene were grown to an OD₆₀₀ of 0.3, then shifted into α -factor (20 μ g/ml)-containing medium prewarmed at 24 or 37°C and samples harvested at the time points indicated. (A) Percentage of cells with a shmoo-like morphology after 3 h treatment with α -factor at 24 or 37°C. The number in parentheses corresponds to the total number of cells examined. (B) α -Factor response as measured by *FUS1-LacZ* induction in wild-type and *tif34-ts* cells after 0, 2 and 4 h treatment with α -factor at 24 or 37°C.

the much stronger decrease in mating ability at 37°C and also the decreased mating efficiency of these cells at 24°C.

We also analyzed the ability of *tif34-ts* cells to respond to α -factor at restrictive temperatures. Cells treated with α -factor arrested in G₁, changed their morphology and made a pointed projection (a 'shmoo') towards their presumptive mating partner and induced a set of reporter genes (for a review see Herskowitz, 1989), such as *FUS1* (Trueheart *et al.*, 1987). Although both *tif34-ts6* and *tif34-ts31* cells were able to respond to α -factor at 24°C at levels similar to the wild-type control, they did not form shmoo-like projections (Figure 3A) and had much lower β -galactosidase expression from the *FUS1* promoter than wild-type cells (Figure 3B) when treated with α -factor at 37°C. After 2 or 4 h treatment with α -factor at 37°C, *tif34-ts31* cells showed a small induction of *FUS1-LacZ* (7-fold reduced compared with levels of induction at 24°C), whereas *tif34-ts6* cells showed no induction of the reporter gene. Clearly, at a restrictive temperature the response to α -factor of *tif34-ts6* cells was even weaker than *tif34-ts31* cells, which may explain why *tif34-ts6* cells mate so poorly at 37°C.

tif34-ts mutant cells are defective in translation initiation

Previous experiments have shown that repression of TIF34 synthesis from the regulated *GALI* promoter inhibited initiation of translation (Naranda *et al.*, 1997). However, TIF34 depletion required prolonged incubation in glucose

Fig. 4. [³⁵S]methionine incorporation in wild-type and *tif34-ts* mutant cells at the permissive or restrictive temperature. (A) As described in Materials and methods, wild-type (aW303), *tif34-ts6* and *tif34-ts31* cells were labeled for 15 min at 1 or 2 h after shifting to 37°C. Equal amounts of protein were loaded in each lane. Lanes 1, 2, 7 and 8, wild-type cells; lanes 3, 4, 9 and 10, *tif34-ts31* cells; lanes 5, 6, 11 and 12, *tif34-ts6* cells. Lanes 1, 3, 5, 7, 9 and 11, culture at 24°C; lanes 2, 4, 6, 8, 10 and 12, culture at 37°C. (B) *tif34-ts6* cells were grown to an OD₆₀₀ of 0.8 at 24°C, shifted for 1 h to 24 or 37°C, then pelleted and lysates analyzed by Western blot using the anti-eIF3 serum. Equal amounts of protein were loaded in both lanes. Lane 1, 1 h culture at 24°C; lane 2, 1 h culture at 37°C.

and inhibition of growth and protein synthesis was observed only after 10 h, i.e. three generations. Furthermore, TIF34 depletion was accompanied by a loss of the different proteins that constitute the entire eIF3 complex. As a result, the inhibition could not be unambiguously assigned as a direct consequence of TIF34 depletion, but rather reflects a more global effect on translation initiation. We therefore used our temperature-sensitive mutants of TIF34 at the permissive and restrictive temperatures to determine the role of TIF34 in translation initiation.

We first determined whether global protein synthesis was affected by the temperature-sensitive allele of *TIF34*. At defined time points after shift to the restrictive temperature the newly synthesized proteins were labeled for 15 min with [³⁵S]methionine and analyzed by SDS-PAGE. As shown in Figure 4A, after incubation of the cells for 1 h at 37°C new protein synthesis was reduced by ~70% in *tif34-ts31* cells (compare lanes 3 and 4) and by ~99% in *tif34-ts6* cells (compare lanes 5 and 6). Two hours after shift to the restrictive temperature [³⁵S]methionine incorporation into newly labeled proteins of *tif34-ts31* cells was decreased to 5% of the level at the permissive temperature (lanes 9 and 10), whereas no new protein

Fig. 5. Polysome profile analysis of W303 and *tif34-ts* cells. *tif34-ts31* and *tif34-ts6* cells and their isogenic wild-type parent w303 cells were grown at 24°C. Cultures were shifted to 37°C and samples were collected at 30 min and processed for polysome analysis by velocity sedimentation of whole cell extracts on sucrose gradients. Gradients were fractionated by scanning at 254 nm and the resulting absorbance profiles are shown, with sedimentation from left to right. (A) W303; (B) *tif34-ts31* cells; (C) *tif34-ts6* cells.

synthesis was detected in *tif34-ts6* cells (lanes 11 and 12). Importantly, the level of TIF34 in *tif34-ts6* cells was drastically decreased 1 h after shift to the restrictive temperature, whereas the levels of the other proteins that constitute the eIF3 complex were not significantly affected (Figure 4B), thus allowing us to assign the defect in protein synthesis specifically to TIF34. It is unclear how the *ts6* mutation confers a high instability and rapid disappearance of TIF34. Whereas it is conceivable that the absence, or decreased level, of TIF34 results in dissociation of the eIF3 complex, fractionation analyses of *tif34-ts6* cells suggest that a multiprotein eIF3 complex is formed at the restrictive as at the permissive temperature (data not shown). Taken together, these results indicate a direct requirement of functional TIF34 for new protein synthesis and again illustrate the stronger phenotype of *tif34-ts6* when compared with *tif34-ts31*.

The evidence that functional TIF34 is required for global protein synthesis suggested that the temperature-sensitive phenotype was due to a failure in translation initiation. We therefore analyzed the polysome profiles in *tif34-ts* cells at 30 min after shift to the restrictive temperature. The polysome profiles of the *ts* mutants and the wild-type cells were similar at 24°C and wild-type cells at 24 and 37°C also showed similar profiles (Figure 5). In contrast, the profiles of the *tif34-ts31* and *tif34-ts6* cells at 37°C showed a strong reduction in the polysome to monosome ratio (Figure 5B and C respectively). These data strongly suggest that the decrease in protein synthesis that results from temperature-sensitive inactivation of TIF34 is associated with a defect in initiation in translation. Thus an essential role of TIF34 in initiation of translation is supported by the pulse labeling experiments and the polysome profiles.

Table II. (A) Two-hybrid interactions between TIF34 and PRT1 and between TIF34 and p33

DNA binding domain	Activation domain fusion	LacZ expression (MU)
p33	vector	2.1 ± 0.1
p33	TIF34	46.2 ± 1.3
TIF34	vector	5.8 ± 1.0
TIF34	p33	325.5 ± 20
p33ΔC	vector	1.7 ± 0.5
p33ΔC	TIF34	73.1 ± 3.8
TIF34	p33ΔC	560.4 ± 51
PRT1	vector	4.3 ± 0.2
PRT1	TIF34	32.4 ± 0.8

The numbers correspond to the β-galactosidase activities in Miller units (MU). Standard deviations are based on three samples.

(B) Schematic representation of the polypeptide structures of eIF3-p33 and eIF3-p33ΔC protein structure

The striated box in eIF3-p33 represents the RNA-binding domain of the protein, which has been removed in eIF3-p33ΔC.

Physical interaction of TIF34 with PRT1, a subunit of eIF3, and a novel eIF3 component of 33 kDa

To better characterize the function of TIF34 in translation initiation, we screened a yeast cDNA library using the two-hybrid method to identify proteins that can directly associate with TIF34. The cDNAs that were isolated using the Gal4-based interaction system (Harper *et al.*, 1993) were then re-evaluated using the LexA-based two-hybrid system (Fields and Song, 1989; Gyuris *et al.*, 1993). Thus only cDNAs that encoded potentially interacting proteins in both two-hybrid systems were considered for further characterization. Among the 52 cDNA clones isolated in this way, 19 cDNAs encoded various C-terminal segments of PRT1, a previously identified 90 kDa component of the eIF3 complex. In addition, the full-length coding sequence of PRT1 was also able to interact with TIF34 in yeast two-hybrid assays (Table II). These data strongly suggest that TIF34 and PRT1 have direct physical interactions in the eIF3 complex. Based on the identity of the partial cDNAs that scored in the two-hybrid assays (the shortest cDNA that scored encoded for amino acid 570 to the last amino acid at 888), this association with TIF34 is mediated by sequences in the C-terminal 318 amino acids of PRT1.

From the yeast two-hybrid screens we also isolated 27 cDNAs encoding most or all coding sequence for a novel protein of 274 amino acids (Figure 6). The C-terminal segment of this protein contains a predicted RNA binding domain that belongs to the RNP-1 type present in many eukaryotic proteins that interact with single-stranded RNA (Bandziulis *et al.*, 1989). This protein has a predicted size of 33 kDa, i.e. the same size as p33 in the purified eIF3 complex, which so far had remained uncharacterized. Furthermore, as determined by direct peptide sequencing of eIF3-p33, the polypeptide sequence of p33 contained the sequences of several peptides of eIF3-p33 (P.Hanachi,

1	ATGAGTGAAGTTGCACCAGAGGAGATCATAGAGAATGCAGATGGATCTAGATCTATCATT	
	M S E V A P E E I I E N A D G S R S I I	20
61	ACATACAAGATTGAAGACGGTGTCAAGTATAAGATTACTCAGAAAGTAAAAGAGGTTAAA	
	T Y K I E D G V K Y K I T Q K V K E V K	40
121	GTATTGGAAAAAGTCCATAAATCTGTTGCAGAAAGAAAAAAGTGGCATAAATATGGCTCT	
	V L E K V H K S V A E R K N W H K Y G S	60
181	GAAAAAGGGTCTCCAGCAGGTCCAAGTGTGTACAGCAAGATTGGGTGAAGAAGTAGAA	
	E K G S P A G P S A V T A R L G E E V E	80
241	TTAAGATTGAGTAGGAAGTGGAAACAAGCTGAAGAAGAAAGAAATACAAAAAGAAAGGCA	
	L R L S R N W K Q A E E E R I Q K E K A	100
301	AGTCTCACAAAGACCGGTCTACAATGTAGATTATGTGGTAACGACCATATGACCATGAAC	
	S L T K T G L Q C R L C G N D H M T M N	120
361	TGTCCATTCAAGACAATTTTAAGTGAACCTTCAGCATTAGAAGCCCAGCCACTAATGAA	
	C P F K T I L S E L S A L E D P A T N E	140
421	GGCGGTGTCGAGGCCGCCAGTGAAGAGAAGGCAGGCCAAGTTGGCGGAGCTGGTTCATC	
	G G V E A A S E E K A G Q V G G A G S I	160
481	CCAGGGCAGTATGTTCCACCTTCTCGTCGTGCTGGTGCAAGAGATCCTTCATCCGATGCT	
	P G Q Y V P P S R R A G A R D P S S D A	180
541	TACAGAGACTCTAGAGAACGTGATGATATGTGTACTTTGAAGATTATGCAAGTTAATGAA	
	Y R D S R E R D D M C T L K I M Q V N E	200
601	AATGCCGATGAAAAAGTTTGGAGAGAAGAGTTATTGTTCCCATTCGCACCCATTCCTAAGA	
	N A D <u>E N S L R E E L L F P F A P I P R</u>	220
661	GTTTCTGTCGTTAGAAACAAAGAAACAGGTAAATCAAGAGGTCTCGCCTTTGTTACCTTT	
	<u>V S V V R N K E T G K S R G L A F V T F</u>	240
721	TCGAGCGAAGAAGTTGCCGAACAAGCTTTACGTTTCTTGGATGGTAGGGTTATATGAAT	
	<u>S S E E V A E Q A L R F L D G R G Y M N</u>	260
781	TTAATTTTACGTGTGAATGGTCCAAACCTAAGGTTAAGGAATAG	
	<u>L I L</u> R V E W S K P K V K E *	274

Fig. 6. cDNA sequence and corresponding polypeptide sequence of eIF3-p33 (TIF35). Only the DNA sequence of the coding region is shown. The derived amino acid sequence for eIF3-p33 is aligned below. The RNA binding region is underlined.

H.-P.Vornlocher and J.Hershey, manuscript in preparation). As is apparent from Table II, the affinity of TIF34 for p33 greatly exceeded the affinity of TIF34 for PRT1. In addition, removal of the C-terminal 71 amino acids, which contain the RNA binding domain, in p33ΔC did not interfere with association of p33 with TIF34 and in fact somewhat increased the efficiency of association.

To assess whether TIF34 and the newly identified protein p33 were also able to interact *in vivo*, we constructed expression plasmids for epitope-tagged versions of both proteins and co-expressed them in yeast. As shown in Figure 7, immunoprecipitation of myc-tagged TIF34 resulted in co-precipitation of MRGS-tagged p33, as determined by a subsequent Western blot (Figure 7). Furthermore, deletion of the RNA binding domain in MRGS-tagged p33ΔC did not affect its association with TIF34. These results thus demonstrate that both TIF34 and the novel protein eIF3-p33 display a strong affinity for each other in both yeast two hybrid assays and *in vivo*. This ability to associate with each other is the basis for their interaction in the eIF3 complex and does not require the RNA binding domain of p33.

p33 and p33ΔC, without the RNA binding domain, can functionally rescue TIF34 deficiency

In an independent screen we also sought to identify proteins that could functionally complement an inactivated *TIF34*. Thus, a yeast genomic library constructed in the high copy number YEp13 plasmid was introduced into *tif34-ts31* mutant cells and the transfected cells were screened for their ability to grow at 35°C. Most of the plasmids recovered from the colonies that were able to grow at 35°C contained the *TIF34* gene, thus indicating that our screen was successful and well saturated. In addition, one of the plasmids contained three genes, one of which encoded eIF3-p33. Transfection of this high copy suppressor plasmid rescued the growth of *tif34-ts31* cells, but not *tif34-ts6* cells at the restrictive temperature. Considering the ability of eIF3-p33 to physically interact with TIF34, we evaluated whether expression of only eIF3-p33 was able to complement TIF34 inactivity. Thus, we introduced plasmids that allowed expression of p33 either from a *Gall* promoter or its own promoter. As shown in Figure 8, expression of p33 from either promoter was able to rescue the inability of *tif34-ts31* cells, but not

Fig. 7. Co-immunoprecipitation of TIF34 and eIF3-p33 and of TIF34 and eIF3-p33ΔC. The *tif34* deletion strain expressing TIF34Myc under a constitutive promoter and transformed with either p415 (empty vector), p415p33 (expressing MRGSp33 under a *GAL1* promoter) or p415p33ΔC (expressing MRGSp33ΔC under a *GAL1* promoter) was cultured to log phase in appropriate medium. Lysates were produced and anti-Myc immunoprecipitations were performed, followed by Western blotting using an anti-TIF34 serum or an antibody against the MRGS epitope (anti-MRGS). Lane 1, anti-Myc immunoprecipitation of the strain transformed with p415p33; lane 2, anti-Myc immunoprecipitation of the strain transformed with vector p415 alone; lane 3, crude lysate of cells transformed with p415p33 before immunoprecipitation; lane 4, anti-Myc immunoprecipitation of the strain transformed with p415p33ΔC; lane 5, crude lysate of cells transformed with p415p33ΔC before immunoprecipitation. IP, immunoprecipitation. The lower bands in lanes 1 and 2 of the anti-MRGS Western blot are non-specific bands associated with anti-Myc immunoprecipitations.

tif34-ts6 cells, to grow at the restrictive temperature, similarly to the original suppressor plasmid containing the three genes. To evaluate whether the RNA binding domain is required for functional complementation, we performed similar complementation assays using the p33ΔC mutant, which lacks the RNA binding domain. As shown in Figure 8, expression of p33ΔC was also able to rescue the growth defect of *tif34*-ts31, although the extent of rescue was always milder than with p33 (smaller colonies, which appeared more slowly than with p33 overexpression). These results demonstrate that the RNA binding domain of eIF3-p33 is not required for functional rescue.

Fig. 8. Suppressor activity of p33 and p33ΔC in *tif34*-ts31 cells. In (A) and (B) wild-type (aW303), *tif34*-ts6 and *tif34*-ts31 cells were transformed with p425 (v, empty vector) or p425p33 (p33 expressed under its own promoter), grown on plates at 24°C (A), then replica plated and grown for 2 days at 35°C (B). In (C) and (D) wild-type (aW303) and *tif34*-ts31 cells were transformed with p415 (v, empty vector) or p415p33 (MRGS-p33 expressed under a *GAL1* promoter) or p415p33ΔC (MRGS-p33ΔC expressed under a *GAL1* promoter). Cells were grown on Gal plates at 24°C (C), then replica plated and grown for 2 days at 35°C (D).

Discussion

We have studied the physiological role of TIF34, a recently identified component of eIF3 in yeast. Using two temperature-sensitive mutants we have shown a requirement for TIF34 in progression through the cell cycle, in mating and in initiation of protein synthesis. Furthermore, using the yeast two-hybrid system we show that TIF34 interacts directly with PRT1, another component of the eIF3 complex, and with a novel protein of 33 kDa in the eIF3 complex. This protein, which we named TIF35 (for translation initiation factor 3, subunit 5), has an RNA binding domain and can suppress a defect in TIF34 function.

Role of TIF34 in cell cycle regulation

We have shown that progression through the cell cycle requires functional TIF34. Coordinating cell division and growth occurs in the G₁ phase of the cell cycle at a regulatory step called Start and initiation of the cell cycle occurs under favorable growth conditions, which include sufficient protein synthesis. We now show that cells that lack a functional *TIF34* gene do not proliferate. When shifted to the restrictive temperature *tif34*-ts mutant cells tend to accumulate as a G₁ population. This accumulation could be explained by an inability of *tif34*-ts mutant cells to exit G₁. Furthermore, only few cells can pass the G₂/M transition at the restrictive temperature. We therefore conclude that TIF34 is essential for passage through G₁.

and G₂/M, but is not required in S phase once DNA replication is initiated.

The requirement for TIF34 to proceed through the cell cycle is presumably associated with its function in initiation of protein synthesis. Previous studies using inhibitors of protein synthesis, such as cycloheximide, have shown that the Start point in G₁ and initiation of mitosis are very sensitive to a slight reduction in protein synthesis, whereas DNA replication, once initiated, can occur without protein synthesis (Shilo *et al.*, 1979; Burke and Church, 1991). Our results using the *tif34-ts* mutants demonstrate more directly the need for protein synthesis in the passage through Start in G₁ and in progression through the G₂/M transition. The requirement for TIF34 for progression through the cell cycle is also consistent with previous findings that a temperature-sensitive mutant of PRT1 confers cell cycle arrest in G₁ at the restrictive temperature (Hanic-Joyce *et al.*, 1987a,b). In fact, *prt1* was initially identified as *cdc6* (Dumas *et al.*, 1982), since yeast cells with the mutant gene were unable to proceed through the cell cycle. Taken together, both TIF34 and PRT1, two proteins that physically interact in the eIF3 complex, are required for cell cycle regulation, thus suggesting an important role for the entire eIF3 complex and protein synthesis in this process.

It has also been indirectly shown that the response to α -factor, and mating in general, require general protein synthesis (Throm and Duntze, 1970; Lipke *et al.*, 1976). We now show that the *tif34-ts* mutants are defective in α -factor response and mating at restrictive temperature. Our results therefore establish a link between the requirement for initiation of translation and the ability of yeast cells to respond to α -factor and to mate.

Role of TIF34 in initiation of protein synthesis

Using our temperature-sensitive mutants we have shown that inactivation of TIF34 blocks initiation of protein synthesis. This block in initiation of translation is rapid, i.e. within ~30 min, as assessed from the polysome profile. It is important to note that the other proteins of the eIF3 complex are present at that time, thus allowing us to assign an essential role in initiation of translation specifically to TIF34. Previous results have suggested a requirement for TIF34 in protein translation, but the absence of TIF34 was accompanied by loss of the other eIF3 components as well (Naranda *et al.*, 1997).

The exact role of TIF34 in initiation of protein synthesis and in the eIF3 complex is not as yet established and awaits identification of the other eIF3 components. However, our results provide some information on its role. The presence of five WD-40 repeats in TIF34 suggests an involvement in interaction with some other components of the eIF3 complex. In agreement with a proposed role of these loosely defined domains, crystallographic analysis of G β has shown that the multiple WD-40 repeats result in a propeller-like configuration which can form the structural basis for interactions with neighboring proteins (Pitcher, 1992; Clapham and Neer, 1993; Lambright *et al.*, 1996). Depletion of TIF34 results in degradation of all subunits of the eIF3 complex at a rate much faster than normal turnover of these proteins, which is consistent with a role of TIF34 in assembly and maintenance of the eIF3 complex (Naranda *et al.*, 1997). This hypothesis is substantiated

by the associations detected in the yeast two-hybrid assays. Indeed, TIF34 is able to interact directly with PRT1 and the novel protein p33, two other components of the eIF3 complex. Further support for a role in assembly of the eIF3 complex is provided by a comparison of the behavior and nature of the TIF34 mutations associated with the *tif34-ts* phenotypes. Although both *tif34-ts* mutants rapidly inhibit initiation of translation at 37°C, the *tif34-ts6* allele results in a much stronger phenotype than the *tif34-ts31* allele, as assessed by its behavior and temperature sensitivity in assays for cell proliferation assays, general rate of protein synthesis, response to α -factor and mating ability. Furthermore, only the *tif34-ts31* and not the *tif34-ts6* defective phenotype is rescued by overexpression of p33 at 35°C. The amino acid substitutions in the *ts6* mutant protein are all located in WD-40 repeats and two of the three point mutants result in replacement of an aspartic acid that is conserved from yeast to humans (Chen *et al.*, 1995). It is therefore likely that the conformational changes in the protein at the restrictive temperature disturb the proposed three-dimensional propeller-like structure and in this way drastically disturb the protein associations. Consequently, the strong *tif34-ts6* phenotype may result from an inability of the protein to be recruited into the eIF3 complex at 37°C. In contrast, the weak *tif34-ts31* phenotype arises from mutations in sequences that do not affect the WD-40 repeats and are likely not to grossly perturb the propeller-like structure of the WD-40 repeats; thus, the mutant protein may still be able to participate in the complex at 37°C. Our analyses of the temperature-sensitive mutants therefore support a role of the WD-40 repeats in the function of TIF34.

Identification of p33, a novel subunit of eIF3 that directly interacts with TIF34

Our two hybrid screening for proteins that directly interact with TIF34 resulted in the identification not only of PRT1 as an interacting protein, but also of a novel protein of 274 amino acids. This protein, p33, is a previously uncharacterized component of the eIF3 complex and is now named TIF35 (for translation initiation factor 3, subunit 5; P.Hanachi, H.-P.Vornlocher and J.Hershey, manuscript in preparation). TIF35 has no extensive sequence homology to previously identified proteins with the exception of its C-terminal segment, which corresponds to an RNA binding domain. The physical association of TIF35 with TIF34 is supported not only by the interaction scored in yeast two-hybrid assays but also by co-immunoprecipitation analyses in yeast.

The function of TIF35 remains to be characterized. One possible role is that it is involved in structural organization and in functional integrity of the eIF3 complex. This is supported by the direct physical association of TIF35 with TIF34. Importantly, this association does not require the putative RNA binding domain of TIF35, as determined by yeast two-hybrid assays and co-immunoprecipitation analyses. Another function of TIF35 is presumably associated with its C-terminal RNA binding domain. The eIF3-p62 subunit encoded by *GCD10* has previously been identified as the major RNA binding subunit of eIF3 (Garcia-Barrio *et al.*, 1995) and PRT1 also contains an RNA binding motif (Evans *et al.*, 1995). The nature of the RNA binding and the RNA targets of these proteins

have not yet been characterized. TIF35 may now be a third protein in the eIF3 complex that can interact with RNA. One function of the eIF3 complex is to promote binding of mRNA to the 40S ribosome (Benne and Hershey, 1978; Trachsel and Staehelin, 1979). It remains to be determined whether TIF35 interacts with mRNA, rRNA or Met-tRNA. However, the function of the RNA binding domain of TIF35 does not appear to be essential, as suggested by our functional complementation experiments. In these assays the RNA binding segment is not required to functionally complement the *tif34-ts31* defective phenotype. How exactly TIF35 complements the TIF34-ts31 inactivity is unclear, but this complementation strongly suggests a functional cooperation between the two proteins that does not require a functional RNA binding segment. Further studies will be required to characterize the role of TIF34 and TIF35 in initiation of protein synthesis and in control of translation.

Materials and methods

Strains and genetic manipulations

All experiments were performed with W303 strains of *Saccharomyces cerevisiae* (can 1-100 leu 2-3,112 his 3-11-15 trp1-1 ura 3-1). To generate a TIF34 knock-out strain, a 2.5 kbp DNA fragment, containing the full-size coding region of TIF34 as well as its 1.4 kbp DNA flanking sequences was generated by PCR using 5'-GGCCCGGATCCTACACCCAAGAAGGAGAATG and 5'-GGCCCGGATCCTACGATTCTTGCTGCCAT as primers and *S.cerevisiae* DNA as template. The *Bam*HI fragment was then inserted into pBSIIS/K (Gibco) to generate pBSTIF34. An *Eco*RI–*Sall* fragment containing most of the coding region of TIF34 was then removed from pBSTIF34 and replaced by the *HIS3* gene. The resulting plasmid was linearized and used for transformation of diploid W303 yeast cells. The transformants were used for tetrad analysis to generate TIF34 knock-out mutants.

Mating assays and α -factor response

Quantitative mating assays were performed as described (Chenevert *et al.*, 1994). To determine the ability of *tif34-ts* mutant strains to respond to α -factor at the restrictive temperature, cultures of wild-type and *ts* mutants, transformed with CY442 (an integrating *LEU2* plasmid carrying *FUS1-lacZ*; from I.Herskowitz), were grown to an OD₆₀₀ of 0.3 at 24°C, resuspended into prewarmed 24 or 37°C medium supplemented with α -factor (20 μ g/ml). At different times cells were either fixed in formaldehyde for microscopic observation or treated for β -galactosidase activity measurements (see two-hybrid section of Materials and methods) to determine the level of induction of the *FUS1-lacZ* gene.

Plasmid construction

pRD53pTIF34Myc is a *CEN(4) URA3* plasmid which expresses TIF34-Myc under a *GAL1* promoter. This plasmid was constructed by inserting a PCR-generated *Bam*HI–*Pst*I fragment encoding a C-terminal Myc tag in-frame with the TIF34 coding sequence (*Bam*HI–*Bam*HI fragment) into the *Bam*HI–*Pst*I sites of pRD53 (a kind gift of J.Li).

The following plasmids were used in yeast two-hybrid assays. pEG-TIF34 was constructed by ligating a 1.1 kbp *Bam*HI–*Bam*HI fragment containing the TIF34 coding sequence from pBSp39 into pEG202 (Bennetzen and Hall, 1982; from R.Brent). Plasmids pJGTIF34, pJGeIF3-p33 and pJGeIF3-p33AC were constructed by PCR amplification of the sequences coding for TIF34, p33 and p33AC respectively using primers that incorporate 5'-*Eco*RI and 3'-*Xho*I sites into pJG4-5 (Gyuris *et al.*, 1993; from R.Brent). Whereas the full-size coding sequences were incorporated into pJGTIF34 and pJGeIF3-p33, the sequence for amino acids 1–203 followed by a stop codon was inserted into pJGeIF3-p33AC. pEGPRT1 was constructed by PCR amplification of the 2.7 kbp fragment encoding PRT1 using primers that incorporate 5'-*Nco*I and 3'-*Xho*I sites into pEG202 (Bennetzen and Hall, 1982; from R.Brent). Finally, plasmids pJGeIF3-p33 and pJGeIF3-p33AC were constructed by PCR amplification of the sequences coding for full-size p33 or amino acids 1–203 (p33AC) using primers that incorporate 5'-*Eco*RI and 3'-*Xho*I sites into pEG202 (Bennetzen and Hall, 1982; from R.Brent).

p425p33 is a 2 μ , *LEU2* plasmid which allows expression of p33 under its own promoter. Thus, the *p33* gene (including 643 bp of the 5'-UTR, the *p33* coding sequence and 710 bp of the 3'-UTR) was cloned as a *Bam*HI–*Bam*HI fragment into the *Bam*HI site of pRS425 (Sikorski and Hieter, 1989; from I.Herskowitz). p415p33 is a 2 μ , *LEU2* plasmid which allows expression of MRGS-tagged p33 under a *GAL1* promoter (MRGS-tag from Qiagen). To construct this plasmid, the *p33* coding sequence was PCR-amplified using 5'-CCCGGATCCGCCATGGGTAGAGGT-TCTCACCATCACCATCACCATATGAGTGAAGTGAAGTTGCACCA and 5'-CCCGTCGACCTCGAGCATATTCTGTGCATCTA as primers and then cloned as a *Bam*HI–*Sall* fragment into p415 (Sikorski and Hieter, 1989). p415p33 Δ C is a plasmid similar to p415p33 except that the encoded p33 lacks the C-terminal 71 amino acids. Thus, the coding sequence of p33 was PCR amplified from p415p33 using 5'-GATCGGATCCGCCATGGGTAGAGGTCTCACCAT and 5'-TAG-CCTCGAGTAAATCGGCATTTCATTAACCTGCA primers, thereby introducing a stop codon after the codon for amino acid 103, and ligating the *Bam*HI/*Xho*I-cut fragment into the *Bam*HI and *Xho*I sites of pRS415 (Sikorski and Hieter, 1989).

To raise anti-TIF34 antiserum, plasmid pTrc(His)₆-TIF34 was constructed by cutting the *Bam*HI fragment containing the TIF34 coding sequence from pBSTIF34 and ligating it into the *Bam*HI site of pTrcHis (Invitrogen). This plasmid allowed expression in *Escherichia coli* of His₆-tagged TIF34, which was subsequently purified by Co³⁺-agarose chromatography.

Generation of temperature-sensitive alleles of TIF34 using mutagenic PCR

A 1.1 kbp fragment containing the TIF34 coding region was PCR amplified under mutagenic conditions: 40 ng pBSTIF34 was resuspended in 10 mM Tris, pH 8.9, 50 mM KCl, 1.5 mM MgCl₂, 0.5 mM MnCl₂, 1 mM each dGTP, dTTP and dCTP, 0.2 mM dATP, 0.2 ng/ml primers (5'-GGCCCGGATCCAGACAAGGAATATGAAGGC and 5'-GGCCCGGATCCTATACATCAACATCAGCAT) and 5 U *Taq* DNA polymerase. The PCR product was purified, digested with *Bam*HI and cloned into pTCA to generate a *tif34* mutant library. The plasmid library was used to transform the haploid *tif34* Δ strain. The transformants were first grown on –ura, trp, Gal plates and then replica plated onto –ura, trp, Glu plates. Colonies that were able to grow at 24°C but not at 37°C were picked and plated onto –trp, 5FOA plates to allow isolation of *tif34-ts* mutants. The *tif34-ts* alleles were integrated into the *tif34* Δ strain using standard yeast genetic manipulation (Rose *et al.*, 1990).

Cell synchronization experiments

Cells were synchronized in different phases of the cell cycle using various drugs: α -factor (20 μ g/ml) was used for G₁ synchronization; hydroxyurea (0.2 M) for S phase synchronization; nocodazole (15 μ g/ml) for G₂/M synchronization. Early exponential wild-type or *tif34-ts* mutant cultures were synchronized for 2 h in the drug at 24°C and then shifted to 37°C for 30 min in the presence of the drug. The cells were subsequently filtered, washed with drug-free medium prewarmed at 37°C and resuspended in prewarmed, drug-free medium. Samples were harvested at different time points and processed as described for flow cytometric DNA quantitation (Epstein and Cross, 1992). The quantitation was performed using a one laser Becton-Dickinson FACScan.

Two-hybrid screening

Screening for potential proteins that interact with TIF34 was done in one two-hybrid system (Harper *et al.*, 1993), whereas the interactions were subsequently verified in another system (Fields and Song, 1989; Gyuris *et al.*, 1993). The former two-hybrid system to screen for potential interacting proteins uses a yeast strain containing *lacZ* and *HIS3* reporter genes under the control of Gal4 binding sites. The bait protein is fused with a GAL4 DNA binding domain and is expressed from pAS2-1. The prey protein is expressed as a fusion with the GAL4 activation domain in pACT2. To screen for interacting proteins, we generated the bait vector pASTIF34 by PCR amplification of the TIF34 coding sequence using the same primers used to generate the *tif34-ts* alleles. The PCR product was digested with *Bam*HI and cloned into pAS2-1. Two yeast cDNA libraries (kindly provided by S.Elledge), in which the cDNA fragments were cloned into pACT or pACTII, were used for screening. The two-hybrid screen was performed essentially as described (Harper *et al.*, 1993). We isolated 52 potential positive clones from 3 $\times$ 10⁵ transformants. Sequence analysis revealed that 19 and 27 of them contained coding sequences of PRT1 and p33 respectively.

We then reconfirmed the interaction between TIF34 and PRT1 and TIF34 and p33 using the other two-hybrid system (Gyuris *et al.*, 1993).

This system uses a reporter plasmid (pSH18-34) containing binding sites for LexA located upstream of a reporter gene (*LacZ* or *HIS3*). The bait protein was fused with the LexA sequence and expressed from a pEG202 plasmid. The prey protein was expressed as a fusion to an SV40 nuclear localization sequence in a pJG4-5 plasmid. β -Galactosidase activity was assayed as described (Miller, 1972). Thus, cells were cultured overnight in 2% raffinose medium, then regrown for 5 h in 2% raffinose, 2% galactose medium. Aliquots from these log phase cultures were then collected by centrifugation and resuspended into Z buffer. To a 1 ml sample of cells were added 2.7 μ l β -mercaptoethanol, 20 μ l 0.1% SDS and 50 μ l chloroform, the suspension was then vortexed and 700 μ l 1 mg/ml *o*-nitrophenyl- β -D-galactoside in Z buffer were added. After incubating the mixture for 30 min at 30°C, the β -galactosidase reaction was stopped by adding 0.5 ml 1 M Na₂CO₃. Units of activity were calculated as $1000 \times \text{OD}_{420} / \text{incubation time (min)} \times \text{volume culture (ml)} \times \text{OD}_{600}$.

Suppressor screen

To screen for a multicopy suppressor of *tif34-ts* alleles, a YEpl3-based yeast genomic library (kindly provided by M.Peter) was introduced into *tif34-ts31* cells and the transformants grown at 24°C overnight, then transferred to 35°C. Forty-one colonies that could grow at 35°C were obtained from 4×10^4 transformants. Colony hybridization revealed that 36 out of 41 suppressors contained the *TIF34* gene. Plasmid DNAs from the five remaining clones were isolated and used to retransform *tif34-ts31* cells. Only one could confer the suppressor activity and was therefore sequenced.

Polysome profile analysis

To analyze the polysome profiles (Figure 5), strains W303, *tif34-ts31* and *tif34-ts6* were cultured overnight in YPD at 24°C to an OD₆₀₀ of ~0.8. Samples of 100 ml were removed and an equal volume of prewarmed YPD was added to each flask, after which the cultures were incubated at 37°C for 30 min. To the cultures was added 100 μ g/ml cycloheximide, followed by quick cooling by swirling the cultures on ice. The cells were collected by centrifugation and resuspended in ice-cold buffer A (10 mM Tris-HCl, 100 mM KCl, 10 mM MgCl₂, 1 mM DTT) containing 100 μ g/ml cycloheximide. The cells were disrupted using glass beads and the cell lysates were clarified by centrifugation at 4000 g for 3 min followed by 3000 g for 10 min at 4°C. Samples of 40 A₂₆₀ units of whole cell extracts were fractionated on linear 15–45% sucrose gradients for 2 h at 38 000 r.p.m. Gradients were fractionated by upward displacement while scanning at 254 nm with an ISCO UA-5 detector.

Antibodies

Rabbit antiserum (HRP Inc., Denver, CO) was raised against the purified (His)₆-p39 recombinant protein produced in *E.coli* from pTrc(His)₆-p39 and was subsequently affinity purified on antigen columns (Gerber *et al.*, 1995). Monoclonal antibody (9E10) against the Myc epitope was produced in our laboratory from a hybridoma provided by J.M.Bishop. Monoclonal antibody against the MRGS(His)₄ epitope was purchased from Qiagen.

Preparation of cell extracts and immunoprecipitation

For [³⁵S]methionine labeling cells were cultured at 24°C in 50 ml YPD to an OD₆₀₀ of ~0.5, washed in methionine-free medium prewarmed to 24 or 37°C, then resuspended into 1 ml methionine-free medium prewarmed to 24 or 37°C containing 100 μ Ci [³⁵S]methionine. Cells were labeled for 15 min at 24 or 37°C, centrifuged, washed with phosphate-buffered saline and the pellet frozen on dry ice/ethanol and kept at –80°C. Lysates were prepared as described (Gerber *et al.*, 1995). To load equal amounts of protein, the protein concentration in each lysate was determined using a BioRad protein assay. Samples for SDS–page were diluted in 2× sample buffer (Laemmli, 1970).

For anti-Myc immunoprecipitations, 50 ml logarithmic phase cells were pelleted, frozen on dry ice/ethanol and kept at –80°C. Lysates, prepared as described (Gerber *et al.*, 1995), were precleared with protein A–Sepharose beads (preincubated with rabbit anti-mouse IgG; Jackson Immunoresearch Inc.) at 4°C for 30 min. The cleared lysates were incubated with anti-Myc antibody (diluted 1:100) for 1 h at 4°C, followed by a 1 h incubation at 4°C with protein A–Sepharose beads that had been preincubated with rabbit anti-mouse serum. Beads were collected by centrifugation and washed five times with lysis buffer (Gerber *et al.*, 1995), then resuspended into a bead volume of 2× sample buffer (Laemmli, 1970).

Acknowledgements

We thank Ira Herskowitz for advice and Maarten van Lohuizen and Hans-Peter Vornlocher for help in the initial phase of this research. We also thank Matthias Peter, Nicole Valtz and all the members of the Herskowitz mating club for their precious advice, support and interesting discussions regarding this work. This research was supported by grants CA63101 to R.D. and GM22135 to J.W.B.H. M.-H.V. was supported by the CNRS and R.-H.C. was supported by a Leukemia Society of America Special Fellow Award.

References

- Bandziulis, R.J., Swanson, M.S. and Dreyfuss, G. (1989) RNA-binding proteins as developmental regulators. *Genes Dev.*, **3**, 431–437.
- Benne, R. and Hershey, J.W.B. (1978) The mechanism of action of protein synthesis initiation factors from rabbit reticulocytes. *J. Biol. Chem.*, **253**, 3078–3087.
- Bennetzen, J.L. and Hall, B.D. (1982) The primary structure of the *Saccharomyces cerevisiae* gene for alcohol dehydrogenase. *J. Biol. Chem.*, **257**, 3018–3025.
- Burke, D.J. and Church, D. (1991) Protein synthesis requirements for nuclear division, cytokinesis and cell separation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **11**, 3691–3698.
- Chen, R.-H., Miettinen, P.J., Maruoka, E.M., Choy, L. and Derynck, R. (1995) A WD-domain protein that is associated with and phosphorylated by the type II TGF- β receptor. *Nature*, **377**, 548–552.
- Chenvert, J., Valtz, N. and Herskowitz, I. (1994) Identification of genes required for normal pheromone-induced cell polarization in *Saccharomyces cerevisiae*. *Genetics*, **136**, 1287–1297.
- Clapham, D.E. and Neer, E.J. (1993) New roles for G-protein $\beta\gamma$ -dimers in transmembrane signalling. *Nature*, **365**, 493–506.
- Dumas, L.B., Lussky, J.P., McFarland, E.J. and Shampay, J. (1982) New temperature-sensitive mutants of *Saccharomyces cerevisiae* affecting DNA synthesis. *Mol. Gen. Genet.*, **187**, 42–46.
- Epstein, C.B. and Cross, F. (1992) CLB5: a novel B cyclin from budding yeast with a role in S-phase. *Genes Dev.*, **6**, 1695–1706.
- Evans, D.R., Rasmussen, C., Hanic-Joyce, P.J., Johnston, G.C., Singer, R.A. and Barnes, C.A. (1995) Mutational analysis of the Prt1 protein subunit of yeast translation initiation factor 3. *Mol. Cell. Biol.*, **15**, 4525–4535.
- Fields, S. and Song, O. (1989) A novel genetic system to detect protein–protein interaction. *Nature*, **340**, 245–246.
- Garcia-Barrio, M.T., Naranda, T., Vazquez de Aldana, C.R., Cuesta, R., Hinnesbusch, A.G., Hershey, J.W.B. and Tamame, M. (1995) GCD10, a translational repressor of GCN4, is the RNA-binding subunit of eukaryotic translation initiation factor-3. *Genes Dev.*, **9**, 1781–1796.
- Gerber, M.R., Farrell, A., Deshaies, R.J., Herskowitz, I. and Morgan, D.O. (1995) Cdc37 is required for association of the protein kinase Cdc28 with G1 and mitotic cyclins. *Proc. Natl Acad. Sci. USA*, **92**, 4651–4655.
- Goumans, H.A., Thomas, A., Verhoeven, H.O., Voorma, H.O. and Benne, R. (1980) The role of eIF-4C in protein synthesis initiation complex formation. *Biochim. Biophys. Acta*, **608**, 39–46.
- Gupta, N.K. *et al.* (1990) New insights into an old problem: ternary complex (Met-tRNA^f-eIF2-GTP) formation in animal cells. In McCarthy, J.E.G. and Tuite, M.S. (eds), *Post-Transcriptional Control of Gene Expression*. Springer Verlag, Berlin, pp. 521–526.
- Gyuris, J., Golemis, E., Chertkov, H. and Brent, R. (1993) Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell*, **75**, 791–803.
- Hanic-Joyce, P.J., Johnston, G.C. and Singer, R.A. (1987a) Regulated arrest of cell proliferation mediated by yeast *prt1* mutations. *Exp. Cell Res.*, **172**, 134–145.
- Hanic-Joyce, P.J., Singer, R. and Johnston, G.C. (1987b) Molecular characterization of the yeast PRT1 gene in which mutations affect translation initiation and regulation of cell proliferation. *J. Biol. Chem.*, **262**, 2845–2851.
- Hannig, E.M. (1995) Protein synthesis in eukaryotic organisms: new insights into the function of translation initiation factor eIF-3. *BioEssays*, **17**, 915–919.
- Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S. (1993) The p21 Cdk-interacting protein cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805–816.
- Hartwell, L.H. and McLaughlin, C.S. (1968) A mutation of yeast apparently defective in the initiation of protein synthesis. *Proc. Natl Acad. Sci. USA*, **62**, 468–474.

- Hershey,J.W.B., Asano,K., Naranda,T., Vornlocher,H.-P., Hanachi,P. and Merrick,W.C. (1997) Conservation and diversity of translation initiation factor eIF3. *Biochimie*, **78**, 903–907.
- Herskowitz,I. (1989) A regulatory hierarchy for cell specialization in yeast. *Nature*, **342**, 749–757.
- Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Lambright,D.G., Sondek,J., Bohm,A., Skiba,N.P., Hamm,H.E. and Sigler,P.B. (1996) The 2.0 Å crystal structure of a heterotrimeric G protein. *Nature*, **379**, 311–319.
- Lamphear,B.J., Kirchweiger,R., Skern,T. and Rhoads,R.E. (1995) Mapping of functional domains in eIF4G with picornaviral proteases. Implication for cap-dependent and cap-independent translational initiation. *J. Biol. Chem.*, **270**, 21975–21983.
- Lipke,P.N., Taylor,A. and Ballou,C.E. (1976) Morphogenic effects of α -factor on *Saccharomyces cerevisiae* a-cells. *J. Bacteriol.*, **127**, 610–618.
- Mader,S., Lee,H., Pause,A. and Sonenberg,N. (1995) The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 γ and the translational repressors, 4E-binding proteins. *Mol. Cell. Biol.*, **15**, 4990–4997.
- Merrick,W.C. and Hershey,J.W.B. (1996) The pathway and mechanism of eukaryotic protein synthesis. In Hershey,J.W.B., Mathews,M.B. and Sonenberg,N. (eds), *Translational Control*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 31–69.
- Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 352–355.
- Moldave,K. and McLaughlin,C.S. (1988) The analysis of temperature-sensitive mutants of *Saccharomyces cerevisiae* altered in components required for protein synthesis. In Boltin,M. and Fukihara,Y. (eds), *Genetics of Translation*. Springer Verlag, London, pp. 271–281.
- Naranda,T., MacMillan,S.E. and Hershey,J.W.B. (1994) Purified yeast translation factor eIF3 is an RNA-binding protein complex that contains the PRT1 protein. *J. Biol. Chem.*, **269**, 32286–32292.
- Naranda,T., MacMillan,S.E., Donahue,T.F. and Hershey,J.W. (1996) SUI1/p16 subunit is required for the activity of translational initiation factor eIF3 in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **16**, 2307–2313.
- Naranda,T., Kainuma,M., MacMillan,S.E. and Hershey,J.W.B. (1997) The 39-kilodalton subunit of eukaryotic translation initiation factor 3 is essential for the complex's integrity and for cell viability in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **17**, 145–153.
- Pitcher,J.A. (1992) Role of beta-gamma subunits of G proteins in targeting the beta-adrenergic receptor kinase to membrane-bound receptors. *Science*, **257**, 1264–1267.
- Rose,M.D., Novick,P., Thomas,J.H., Botstein,D. and Fink,G.R. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shilo,B., Riddle,V.G.H. and Pardee,A.B. (1979) Protein turn-over and cell-cycle initiation in yeast. *Exp. Cell Res.*, **123**, 221–227.
- Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *S. cerevisiae*. *Genetics*, **122**, 19–27.
- Throm,E. and Duntze,W. (1970) Mating-type dependent inhibition of DNA synthesis in *Saccharomyces cerevisiae*. *J. Bacteriol.*, **104**, 1388–1390.
- Trachsel,H. and Staehelin,T. (1979) Initiation of mammalian protein synthesis: the multiple functions of the initiation factor eIF-3. *Biochim. Biophys. Acta*, **565**, 305–314.
- Trueheart,J., Boeke,J.D. and Fink,G.R. (1987) Two genes required for cell fusion during yeast conjugation: evidence for a pheromone-induced surface protein. *Mol. Cell. Biol.*, **7**, 2316–2328.
- Yoon,H. and Donahue,T.F. (1992) The *suil* suppressor locus in *Saccharomyces cerevisiae* encodes a translation factor that functions during tRNA_i^{Met} recognition of the start codon. *Mol. Cell. Biol.*, **12**, 248–260.

Received on July 3, 1997; revised on August 29, 1997