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Binding of Eukaryotic Translation Initiation Factor 4E (eIF4E) to eIF4G Represses Translation of Uncapped mRNA

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mRNA translation in crude extracts from the yeast *Saccharomyces cerevisiae* is stimulated by the cap structure and the poly(A) tail through the binding of the cap-binding protein eukaryotic translation initiation factor 4E (eIF4E) and the poly(A) tail-binding protein Pab1p. These proteins also bind to the translation initiation factor eIF4G and thereby link the mRNA to the general translational apparatus. In contrast, uncapped, poly(A)-deficient mRNA is translated poorly in yeast extracts, in part because of the absence of eIF4E and Pab1p binding sites on the mRNA. Here, we report that uncapped-mRNA translation is also repressed in yeast extracts due to the binding of eIF4E to eIF4G. Specifically, we find that mutations which weaken the eIF4E binding site on the yeast eIF4G proteins Tif4631p and Tif4632p lead to temperature-sensitive growth in vivo and the stimulation of uncapped-mRNA translation in vitro. A mutation in eIF4E which disturbs its ability to interact with eIF4G also leads to a stimulation of uncapped-mRNA translation in vitro. Finally, overexpression of eIF4E in vivo or the addition of excess eIF4E in vitro reverses these effects of the mutations. These data support the hypothesis that the eIF4G protein can efficiently stimulate translation of exogenous uncapped mRNA in extracts but is prevented from doing so as a result of its association with eIF4E. They also suggest that some mRNAs may be translationally regulated in vivo in response to the amount of free eIF4G in the cell.

mRNA translation in eucaryotes requires at an early step the recruitment of the ribosome to the mRNA (reviewed in reference 22). In the yeast *Saccharomyces cerevisiae*, the ribosome recruitment step is stimulated by a network of protein-protein interactions between the cap-binding protein eukaryotic translation initiation factor 4E (eIF4E), the poly(A) tail-binding protein Pab1p, and the translation initiation factor eIF4G. The eIF4G protein serves as an adapter in this step of translation initiation (15) since it also binds to the ribosome-associated eIF3 complex, which leads to the placement of the ribosome on the mRNA.

The eIF4G protein has been highly conserved throughout evolution of the eucaryotic genomes. *S. cerevisiae* contains two homologs of eIF4G that are encoded by the genes *TIF4631* and *TIF4632* (11). These two homologs will hereafter be referred to as eIF4G1 and eIF4G2, respectively. Subdomains within the yeast eIF4G proteins have been identified by both biochemical analyses and sequence homology. Located near the N termini of these proteins is a Pab1p binding site (26, 27). This is followed by an eIF4E binding site (18, 26) and, on the basis of sequence homology to mammalian eIF4G, an eIF3 binding site (17). Located within this eIF3 binding region is a possible RNA recognition motif (RRM)-like RNA binding domain that has been identified by sequence homology (11). Yeast eIF4G has been shown to be capable of binding RNA (26). In contrast to the mammalian proteins, the yeast eIF4G proteins do not have the C-terminal domain that has been suggested to bind the RNA-dependent ATPase eIF4A (17).

The yeast eIF4G protein is essential for transmitting the stimulatory signal of the poly(A) tail and the cap structure to the remainder of the translational machinery. For instance, mutagenesis of the Pab1p binding domain on eIF4G leads to the loss of both poly(A) tail-stimulated translation initiation and the synergistic interaction between the cap structure and the poly(A) tail. However, loss of the Pab1p binding site does not affect the ability of the cap structure to stimulate translation (27). It has also been shown in mammalian cell extracts that removal of the eIF4E binding region of eIF4G by proteolytic cleavage leads to the loss of cap-stimulated translation initiation on mRNA (5, 14, 20, 21). The translational consequences of mutating the eIF4E binding site on yeast eIF4G is one of the subjects of this paper.

Cap-stimulated translation initiation can also be inhibited by a family of eIF4E-binding proteins (the 4E-BPs). Association of the 4E-BPs with eIF4E inhibits the binding of eIF4G to eIF4E and therefore cap-stimulated translation (13, 18). The binding of the 4E-BPs to eIF4E and, as a consequence, the binding of eIF4E to eIF4G are regulated through the activities of 4E-BP protein kinases (reviewed in reference 24). In *S. cerevisiae*, the *CAF20* gene encodes a functional homolog of the mammalian 4E-BPs (1, 7). Its high degree of phosphorylation (29) is suggestive of a potential regulatory pathway controlling eIF4E binding to eIF4G in this organism.

Extracts from both mammalian and yeast cells can also translate uncapped mRNA. In the mammalian extracts, cleavage of the N-terminal eIF4E binding domain from eIF4G further stimulates the translation of uncapped mRNA (5, 14, 20, 21). This stimulation appears to result from an activity of the C-terminal fragment of eIF4G. It has recently been reported that the addition of excess mammalian 4E-BP1 to reticulocyte lysates inhibits uncapped-mRNA translation when eIF4G is intact but does not inhibit uncapped-mRNA translation when the eIF4E binding domain of eIF4G is removed (19, 21). These data have been interpreted to suggest that the binding of eIF4E to eIF4G stimulates eIF4G's ability to mediate uncapped-mRNA translation. Our results with yeast lysates (see below) do not agree with this interpretation. The role of eIF4E in regulating uncapped-mRNA translation has also been explored in yeast (28). Through electroporation experiments, cells containing mutant forms of eIF4E were

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found to exhibit diminished abilities to preferentially translate capped versus uncapped mRNA (28). Although each of these studies suggests that eIF4E is in some way involved in regulating the ability of eIF4G and the translational apparatus to utilize uncapped mRNA, none of them directly examined the effects of mutation of the eIF4E binding site on eIF4G on this regulation. An understanding of this putative regulatory role of eIF4E is important, since it would be predicted to impact upon the relative expression of mRNAs translated through cap-dependent and cap-independent mechanisms in vivo.

Here, we report that the translation of uncapped mRNA in yeast extracts is stimulated when the eIF4E binding site on eIF4G is partially destroyed. We also find that a mutation in eIF4E which inhibits its association with eIF4G leads to a stimulation of uncapped-mRNA translation. Each of these effects results from poor eIF4E binding to eIF4G since the addition of excess recombinant eIF4E to the mutant extracts represses their translation of uncapped mRNA. These data suggest that the binding of eIF4E to eIF4G represses the ability of eIF4G to mediate uncapped-mRNA translation in yeast. They also suggest that loss of eIF4E binding to eIF4G in vivo could both inhibit the translation of capped mRNA and stimulate the translation of other mRNAs through a cap-independent mechanism.

MATERIALS AND METHODS

Nucleic acid techniques. Each of the eIF4G genes was expressed in yeast on *TRP1*-containing vectors containing either a centromere (pUN10 [10]) or a 2μ origin (derivatives of pRS304 [23]) (Table 1). Each of these vectors contains 449 nucleotides of *TIF4632* DNA upstream of the initiation codon, 627 nucleotides of *TIF4632* DNA downstream of the translation termination codon, and intervening *Nde*I, *Bam*HI, and *Eco*RI restriction sites (26). The sequence of the first three codons of each eIF4G gene is ATG GGA TCC, which spans both an *Nde*I site and a *Bam*HI site. The termination codon is followed by the sequence GAA TTC, which contains an *Eco*RI site. Consequently, all eIF4G genes are shuffled between these vectors as *Bam*HI/*Eco*RI fragments. Furthermore, the oligonucleotide duplex which epitope tags the genes is readily introduced between the *Nde*I and *Bam*HI restriction sites.

PCR mutagenesis of each eIF4G gene was performed by the method of Barettino et al. (4) with a single mutagenic oligonucleotide and two other oligonucleotides that flanked the *Bam*HI and *Eco*RI sites within the gene. The mutagenized PCR fragments obtained from the second round of amplification were gel purified, digested with *Bam*HI or *Eco*RI, and subcloned into either the yeast or bacterial expression vectors that had also been digested with these two enzymes.

Templates encoding the various luciferase (LUC) mRNA species, as well as the methods for their use, have been previously described (16, 25). RNA concentrations in each sample were determined by measurements of optical density at 260 nm.

Yeast techniques. Yeast strains (Table 1) were propagated on standard YPD or YM medium containing either 2% glucose or 2% galactose as their carbon source and the amino acids and bases necessary for growth (12). All starting strains were nearly isogenic with each other as a result of multiple backcrosses to a W303-derived parent strain. Mutant eIF4G genes were introduced on the indicated *TRP1* plasmids into yeast strain YAS1948, which has disruptions in its genomic copies of the eIF4G1 and eIF4G2 genes *TIF4631* and *TIF4632*, by lithium acetate transformation and subsequent plating onto YM-Trp medium. Transformants which had lost their wild-type version of eIF4G2 on a *URA3CEN* plasmid were then selected for on YM-Trp medium containing 1 mg of 5-fluoroorotic acid per ml (12).

The yeast strains indicated in Fig. 2A were grown to saturation in liquid YM-TrpUra medium with galactose, plated as serial dilutions onto the same medium, grown at 26°C for 7 h to allow the resumption of growth, and then grown at the indicated temperatures for 6 days. For the experiment whose results are shown in Fig. 2B, logarithmic-phase cultures of yeast strain YAS2069 or YAS2074 in YM-Trp medium with galactose were shifted to 37°C at the beginning of the experiment. Serial dilutions of culture aliquots were plated on YPD medium at 26°C at the indicated times to determine the number of viable cells within each milliliter of the culture.

Recombinant-protein expression. Each of the recombinant glutathione *S*-transferase (GST)-eIF4G fusion proteins was expressed in bacterial strain BL21 from the pGEX2T expression vector (Pharmacia) containing the eIF4G gene inserted as a *Bam*HI/*Eco*RI fragment (26) into the expression vector *Bam*HI/*Eco*RI sites. The methods used for the induction and purification of recombinant protein were as previously described (26). Approximately 2 to 4 μg of full-length recombinant fusion protein was purified per ml of bacterial culture by use of 25 μl of glutathione resin. Bacterial strain BAS2027 produces GST-eIF4G1, BAS3140 produces GST-eIF4G1-459, BAS2030 produces GST-eIF4G2, and BAS3031 produces GST-eIF4G2-430.

Recombinant yeast eIF4E was purified from bacterial extracts by cap analog affinity chromatography (9) followed by dialysis against buffer A (100 mM potassium acetate, 2 mM magnesium acetate, 30 mM HEPES [pH 7.4], 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The eIF4E expression vector (28) was kindly provided by John McCarthy (Manchester, United Kingdom).

In vitro translation assays. The preparation of yeast extracts competent for translation initiation has been previously described (16, 25). All experiments utilized non-nuclease-treated extracts which had been made 2 mM EGTA prior to the addition of the LUC mRNA. As also described previously (25), approximately 100 ng of the indicated mRNA in a 7.5-μl mixture of compounds and salts was added to 7.5 μl of the extract, and the mixture was incubated at 26°C for 30 min. The production of LUC protein in these extracts was determined by a luminescence assay that utilized between 3 and 10 μl of the cooled translation extract and 50 μl of luciferin reagent (Promega). Luminescence was measured for 15 s with a Turner TD-20e luminometer.

For the cap analog studies, the appropriate amount of ^{7m}GpppG (New England Biolabs) in 1 μl of water was added to 7.5 μl of extract prior to being mixed with 6.5 μl of the mRNA mixture. For the monoclonal antibody studies, approximately 8 μg of the yeast Pab1p monoclonal antibody 4G1 in 1 μl of buffer A was added to the protein extract, and the mixture was incubated for 10 to 15 min at 4°C prior to the addition of the mRNA-containing mixture (see reference 25 for

TABLE 1. Yeast strains and plasmids used in this study[a]

Yeast strain	Mutation/plasmid (BAS)
538[b]	None
1888[b]	*cdc33-1*
1951	p*TIF4632TRP1CEN* (2068)
1948	p*TIF4632URA3CEN* (2004)
1955	p*(HA)TIF4632TRP1CEN* (2077)
2001	p*tif4632-233TRP1CEN* (3036)
2002	p*tif4632-430TRP1CEN* (3037)
2003	p*tif4632-233,430TRP1CEN* (3038)
2008	p*(HA)tif4632-430TRP1CEN* (3044)
2069	p*TIF4631TRP1CEN* (2078)
2074	p*tif4631-459TRP1CEN* (3113)
2075	p*tif4631-213TRP1CEN* (3120)
2093	p*tif4631-213,459TRP1CEN* (3123)
2107	p*TIF4631TRP1CEN* (2078) p*URA3CEN* (550)
2108	p*TIF4631TRP1CEN* (2078) p*GPF::CDC33URA3CEN* (3163)
2109	p*tif4631-459TRP1CEN* (3113) p*URA3CEN* (550)
2110	p*tif4631-459TRP1CEN* (3113) p*GPF::CDC33URA3CEN* (3163)
2111	p*TIF4632TRP1CEN* (2068) p*URA3CEN* (550)
2112	p*TIF4632TRP1CEN* (2068) p*GPF::CDC33URA3CEN* (3163)
2113	p*tif4632-430TRP1CEN* (3037) p*URA3CEN* (550)
2114	p*tif4632-430TRP1CEN* (3037) p*GPF::CDC33URA3CEN* (3163)
2115	p*TIF4631TRP12*μ (3142) p*TIF4632URA3CEN* (2004)
2116	p*TIF4632TRP12*μ (3146) p*TIF4632URA3CEN* (2004)
2117	p*tif4631-459TRP12*μ (3147) p*TIF4632URA3CEN* (2004)
2118	p*tif4632-430TRP12*μ (3148) p*TIF4632URA3CEN* (2004)
2131[b]	p*(HA)TIF4632TRP1CEN* (2077) p*(MYC)TIF4631URA3CEN* (2081)
2132[b]	*cdc33-1* p*(HA)TIF4632TRP1CEN* (2077) p*(MYC)TIF4631 URA3CEN*(2081)
2133	*caf20::URA3* p*tif4631-459TRP1CEN* (3113)
2134	*caf20::URA3* p*tif4632-430TRP1CEN* (3037)
2136	p*(HA)TIF4631TRP1CEN* (3157)
2137	p*(HA)tif4631-459TRP1CEN* (3158)
2158[b]	p(HA)TIF4631TRP1CEN (3157)
2159[b]	*cdc33-1* p*(HA)TIF4631TRP1CEN* (3158)

[a] Numbers denote the yeast (YAS) and bacterial (BAS) strain numbers to be used when reagents are requested. The listed plasmids are found in the indicated bacterial strains.

[b] This strain has the following genotype: *MAT***a** *ade2-1 his3-11,15 leu2-3,111 trp1-1 ura3-1 pep4::HIS3*. All other strains are derived from YAS1948 and have the following genotype: *MAT***a** *ade2-1 his3-11,15 leu2-3,111 trp1-1 ura3-1 pep4::HIS3 tif4631::LEU2 tif4632::ura3.*

Wheat	(60)	HERVRYSRDQ**LL**DLRKIT
Human	(411)	EEKKRYDREF**LL**GFQFIF
Rabbit	(412)	EEKKRYDREF**LL**GFQFIF
S. C. 4G1	(449)	HVKYTYGPTF**LL**QFKDKL
S. C. 4G2	(430)	SVKYTYGPTF**LL**QFKDKL
S. C. 4G1-459	(449)	HVKYTYGPTF**AA**QFKDKL
S. C. 4G2-430	(420)	SVKYTYGPTF**AA**QFKDKL

FIG. 1. Mutagenesis of the conserved eIF4E binding domain of eIF4G. An amino acid sequence alignment of subregions of eIF4G from the indicated organisms is shown. The positions substituted in the *S. cerevisiae* (S. C.) eIF4G proteins to yield eIF4G1-459 and eIF4G2-430 (underlined and boldface) and the position number of the first residue in each sequence within the full-length protein (in parentheses) are indicated.

more details). For the eIF4E addition studies, the indicated amount of recombinant yeast eIF4E in 2 μl of buffer A was added to the yeast extract, and the mixture was incubated for 10 to 15 min at 4°C before the addition of the mRNA mixture.

All translation experiments were repeated at least twice. The data shown in the figures are representative of all of the results obtained.

Protein binding assays. Methods for studying the binding of 5 μg of pure recombinant eIF4E to 0.5 to 1 μg of glutathione agarose-bound GST-eIF4G fusion protein have been described elsewhere (26). For the experiments whose results are shown in Fig. 5, bacterial extracts containing 60 μg of eIF4E or cdc33-1p (bacterial strains overexpressing eIF4E or cdc33-1p were the kind gifts of M. Altmann, University of Bern) were incubated with the immobilized GST fusion proteins in a final volume of 100 μl or with the nitrocellulose membrane in 20 ml of blocking buffer (see below). The use of these crude extracts for similar purposes has been reported elsewhere (1). The subsequent steps were identical to those used for analysis of the pure eIF4E protein. Methods for performing the coimmunoprecipitation studies with approximately 100 μl of crude yeast mini-extract (5 to 7 mg/ml) per immunoprecipitation sample have also been described previously (26).

For far-Western analysis, approximately 0.5 μg of each full-length eIF4G protein resolved on a 0.75-mm-thick sodium dodecyl sulfate (SDS)-polyacrylamide gel was electroblotted onto a Hybond ECL membrane (Amersham) in glycine transfer buffer (26) for 45 min at 70 V in a Bio-Rad mini-gel transfer chamber. Following incubation of the membrane with blocking solution (phosphate-buffered saline plus 0.1% Triton X-100 plus 0.01% SDS plus 5% dry milk) for 1 h, the membrane was incubated for 1 h with 1 μg of recombinant eIF4E per ml or bacterial extract containing 30 μg of eIF4E in 20 ml of the blocking solution. Following three washes with 20 ml of wash buffer (phosphate-buffered saline plus 0.1% Triton X-100 plus 0.01% SDS), the eIF4E protein was detected by Western analysis by the method described below.

Immunological techniques. Rabbit antiserum to recombinant yeast eIF4E was generated through standard protocols and used at a dilution of 1:2,000 for Western analysis. Ascites fluid containing mouse monoclonal antibody 12CA5, which recognizes an influenza virus hemagglutinin (HA) protein epitope, was used at a dilution of 1:2,000 for Western analysis. For immunoprecipitation studies, 10 μl of the ascites was preabsorbed onto 100 μl of protein A-Sepharose, and 10 μl of resin was used per point as previously described (26). Western analysis was performed as described previously (26).

RESULTS

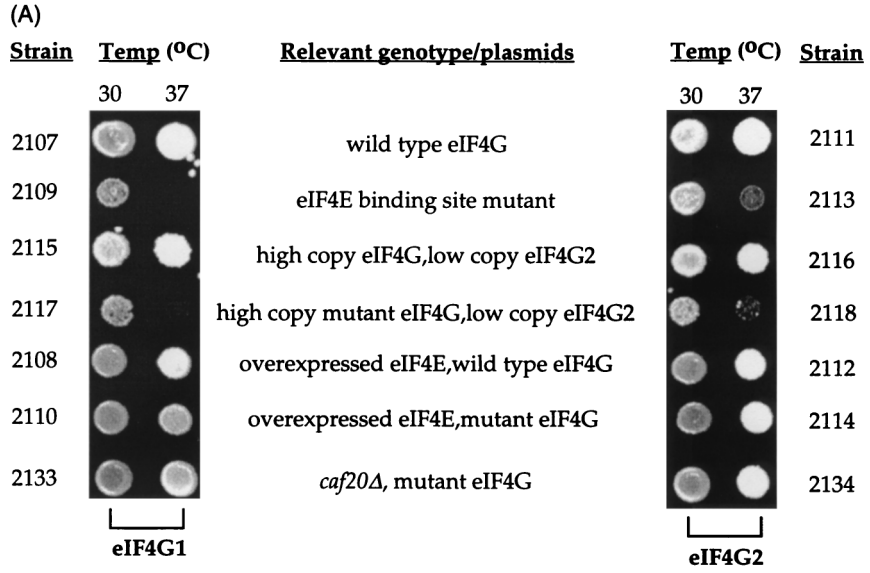
Mutagenesis of yeast eIF4G. Eucaryotic translation initiation factor eIF4G contains highly conserved regions throughout much of its primary structure. The region of eIF4G responsible for binding to eIF4E has been previously defined by deletion and substitution mapping in mammalian cells and in yeast (18, 26). Furthermore, the 4E-BPs have been shown to contain a similar region (18). For human eIF4G, replacement of either a highly conserved tyrosine or two highly conserved leucines with alanine destroys the ability of the protein to interact with eIF4E in vitro (18) (Fig. 1). For each of the yeast eIF4G homologs, replacement of the highly conserved tyrosine within this region with alanine results in cell lethality, while replacement of the two highly conserved leucine residues in eIF4G1 with alanines (eIF4G1-459) results in a temperature-sensitive growth phenotype (27). We describe below a more extensive analysis of the in vitro and in vivo consequences of mutation of these highly conserved leucine residues to alanine in both eIF4G1 (eIF4G1-459) and eIF4G2 (eIF4G2-430) (Fig. 1).

The temperature-sensitive growth phenotypes of eIF4G1-459 and eIF4G2-430 mutants are suppressed by increased eIF4E levels. Several different experiments were performed in order to characterize the in vivo consequences of these mutations within the eIF4G proteins. First, the effects of mutation of the eIF4E binding regions within eIF4G1 and eIF4G2 on yeast cell viability were assayed. Both mutations resulted in temperature-sensitive growth phenotypes of various degrees. A yeast strain containing eIF4G2-430 (YAS2113) was able to form microcolonies only on galactose medium at 37°C (Fig. 2A). A yeast strain containing eIF4G1-459 (YAS2109) showed marked temperature-sensitive growth on galactose medium at 34°C and on glucose medium at 35°C (data not shown). Due to the enhanced aberrant growth phenotypes on galactose versus glucose medium, all further growth studies were performed with galactose medium. We imagine that galactose may be a more restrictive carbon source for the eIF4E mutants as a result of the added stress to the cells growing in this suboptimal sugar. The eIF4G1-459 cells arrested at 37°C in galactose medium without going through a cell division (Fig. 2). Cells harboring eIF4G1-459 at 37°C also lost viability at a higher rate than their wild-type counterparts (Fig. 2B), suggesting that this mutation is a temperature-sensitive lethal mutation. The weaker phenotypes associated with the eIF4G2 mutations may in part be due to a residual ability of this protein to bind to eIF4E in vivo (i.e., see Fig. 3A).

Overexpression of either eIF4G1-459 (YAS2117) or eIF4G2-430 (YAS2118) in a yeast strain containing a wild-type copy of eIF4G2 led to a marked temperature-sensitive growth phenotype of the strain (Fig. 2A). This dominant negative phenotype suggests that the mutant eIF4G proteins are being made within the yeast cell and are capable of competing with the wild-type eIF4G2 for its biological targets. They also suggest that the temperature sensitivity of the strains does not result from an overall lowering of the levels of eIF4G at the restrictive temperature, since eIF4G2 is being normally expressed. In accordance with the hypothesis that the mutant proteins are not being underexpressed, Western analysis of extracts containing either wild-type or mutant eIF4G proteins expressed from the same low-copy-number plasmid revealed that their levels of eIF4G were essentially equal (data not shown). In summary, these studies of yeast cells overexpressing eIF4G1-459 or eIF4G2-430 show that these proteins are partially functional and stable at the restrictive temperature.

In order to address whether the temperature-sensitive growth phenotypes were due to a loss of eIF4G's ability to bind eIF4E, two different approaches were used to increase the amounts of functional eIF4E in the mutant strains. In the first experiment, the eIF4E gene *CDC33* was overexpressed by utilizing an expression plasmid that linked it to the active, galactose-inducible GPF promoter (28). This plasmid overexpresses eIF4E approximately eightfold relative to its normal level (28). As shown in Fig. 2A, the growth deficiencies of the eIF4G1-459 and eIF4G2-430 mutants were nearly completely restored at 37°C when eIF4E was overexpressed (YAS2110 and YAS2114, respectively). This suggests that the temperature sensitivities of these strains result from the inability of their eIF4G proteins to bind to eIF4E.

The levels of functional eIF4E in each of the mutant strains were also increased by deleting their *CAF20* gene. Caf20p has been reported to be a negative regulator of eIF4E that functions in a manner similar to the 4E-BPs in mammalian cells (1). As a result, we anticipated that a deletion of this gene would lead to greater levels of functional eIF4E within the mutants and therefore partial suppression of their temperature-sensitive phenotypes. As shown in Fig. 2A, deletion of *CAF20* led to



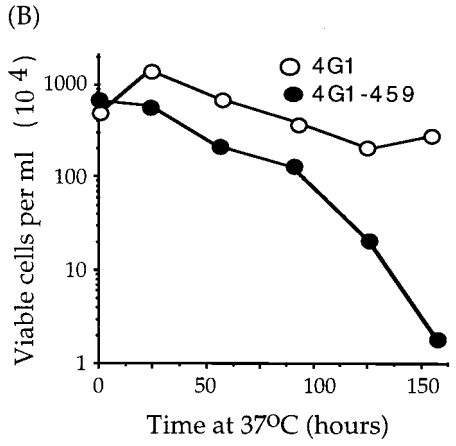


FIG. 2. Phenotypic characterization of the eIF4G1-459 and eIF4G2-430 mutants. (A) The indicated yeast strains with the listed relevant genotypes and plasmids were grown on YM-galactose plates at either 30 or 37°C for 5 days. See Table 1 for more details about the strains. Note that these strains contain chromosomal disruptions of their eIF4G1 and eIF4G2 genes and carry the indicated eIF4G gene on a plasmid. (b) Yeast eIF4G1-459 mutants exhibit increased rates of killing at 37°C. Mid-log-phase cultures of yeast strains in YM-galactose medium containing either eIF4G1 (YAS2069) or eIF4G1-459 (YAS2074) were shifted to 37°C and grown for the indicated times. Cell viability was determined by plating aliquots of the cultures at 26°C.

substantial suppression of the temperature sensitivities of the eIF4G1-459 and eIF4G2-430 strains (YAS2133 and YAS2134, respectively). In conjunction with the above suppression data, these results support the conclusion that the substitution of the highly conserved leucine residues within the eIF4E binding domain of yeast eIF4G leads to temperature-sensitive growth as a consequence of reduced eIF4E binding.

The eIF4G1-459 and eIF4G2-430 proteins exhibit decreased affinities for eIF4E in vitro. Each of the mutant yeast eIF4G proteins, as well as their wild-type counterparts, was fused to GST and immobilized on glutathione resin (26). The association of eIF4E with each of these fusion proteins was then assayed for by incubating recombinant eIF4E with the resin and then measuring the relative amount of eIF4E retained on the resin by Western analysis (26). As shown in Fig. 3A, eIF4G1-459 exhibited no detectable binding to eIF4E. In contrast, eIF4G2-430 exhibited a two- to threefold loss of eIF4E binding. Because nearly equal amounts of the mutant and wild-type recombinant eIF4G proteins were immobilized on each of these resins (Fig. 3A), these data provide a relative measure of the effects of the alanine-for-leucine substitutions within the yeast eIF4G proteins on their ability to associate with eIF4E in solution.

As an alternative means to measure the association of eIF4E with eIF4G in vitro, a far-Western analysis was employed (Fig. 3B). The amount of eIF4E bound to the immobilized eIF4G was easily quantified by Western analysis using antibodies to eIF4E. As shown in Fig. 3B, both eIF4G1 and eIF4G2 exhibited high levels of eIF4E binding in this assay, and neither eIF4G1-459 nor eIF4G2-430 exhibited significant eIF4E binding. These data suggest that the mutated leucines within these two proteins are essential determinants for eIF4E binding in this assay method. However, given that eIF4G2-430 still associated with eIF4E in solution (Fig. 3A), the data also suggest that other determinants of eIF4E binding to eIF4G are not detected with a far-Western assay.

The relative amounts of binding of each of the four eIF4G proteins to eIF4E within crude yeast extracts were also determined. Extracts from yeast expressing influenza virus HA-tagged wild-type or mutant eIF4G proteins were subjected to immunoprecipitation with monoclonal antibody 12CA5. The immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the amounts of either eIF4G or eIF4E within them were determined by Western analysis. As shown in Fig. 3C, only trace amounts of eIF4E coimmunoprecipitated with either eIF4G1-459 or eIF4G2-430. In contrast, each of the wild-type eIF4G proteins immunoprecipitated substantial amounts of eIF4E. One possible explanation for why eIF4G2-430 bound to eIF4E in the experiments using recombinant proteins (Fig. 3A) but not in crude extracts is the differences in eIF4E and eIF4G concentrations in the two assays. In combination with the other in vitro experiments, these immunoprecipitation data indicate that substitution of the two highly conserved leucine residues within the eIF4E binding region of the yeast eIF4G proteins can lead to significant decreases in the ability of the proteins to associate with eIF4E in vitro.

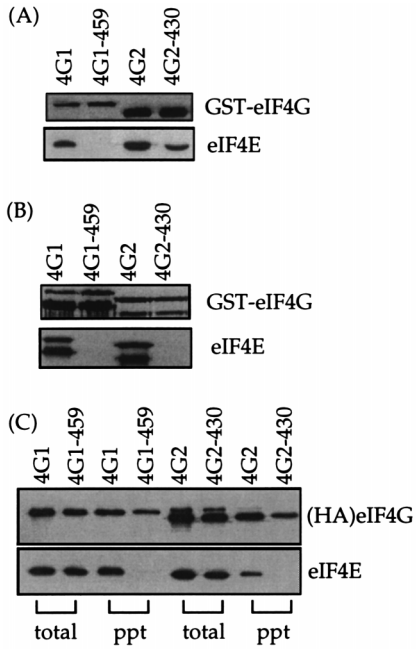


FIG. 3. The eIF4G mutant proteins associate poorly with eIF4E in vitro. (A) Recombinant eIF4E associates poorly with recombinant eIF4G1-459 and eIF4G2-430. A 5-μg sample of pure yeast eIF4E was incubated with 0.5 to 1 μg of the indicated glutathione resin-immobilized GST-eIF4G fusion protein. Following washing of the resin, bound proteins were eluted in SDS, resolved by SDS–10% PAGE, and then visualized by either Coomassie brilliant blue staining (GST-eIF4G) or Western analysis (eIF4E). (B) Yeast eIF4E associates poorly with the eIF4G mutants in a far-Western analysis. Approximately 0.5 μg of GST-eIF4G fusion proteins was resolved by SDS-PAGE, electroblotted onto a nitrocellulose filter, and then incubated with a solution containing 1 μg of pure eIF4E per ml. Following washing of the filter, the eIF4E bound to the GST-eIF4G protein was visualized by Western analysis (bottom panel). The top panel shows a Coomassie brilliant blue-stained SDS-polyacrylamide gel containing the GST-eIF4G proteins electroblotted onto the nitrocellulose membrane. The bands below the full-length eIF4G fusion proteins are N-terminal proteolytic fragments of the protein. (C) Yeast eIF4E associates poorly with the eIF4G mutants in crude yeast extracts. Extracts containing the indicated influenza virus HA epitope-tagged eIF4G proteins were subjected to immunoprecipitation using the 12CA5 monoclonal antibody. Samples were resolved by SDS–10% PAGE, and either eIF4G (top panel) or the associated eIF4E (bottom panel) was detected by Western analysis using the appropriate antibody. Three percent of the extract (total) and 30% of the immunoprecipitate (ppt) was loaded onto the gels. The yeast strains used in this study were, from left to right, YAS2136, -2137, -1955, and -2008.

Enhanced translation of uncapped mRNA in extracts containing eIF4G1-459 or eIF4G2-430. Having established that the two yeast eIF4G homologs exhibited decreased affinities for eIF4E both in vitro and in vivo, we next analyzed the effects of these mutations on the in vitro translation properties of extracts containing each of them. The translation of LUC mRNA in these extracts is dependent on the presence of a cap structure (capLUC) or a poly(A) tail (LUCpA) (16, 25). In the presence of both of these elements (i.e., capLUCpA), a synergistic stimulation of translation is observed (16, 25). Synergy is defined as the fold difference in expression of capLUCpA mRNA versus the sum of expression of the capLUC and LUCpA mRNAs. In the absence of these elements, the LUC mRNA is very poorly translated. In this paper, uncapped mRNA refers to both LUC and LUCpA mRNAs. The cap-dependent translation in these extracts has been previously shown to require eIF4E (25). The poly(A) tail-dependent translation has been shown to require Pab1p and a region of eIF4G residing just N terminal to this protein's eIF4E binding site (26, 27). The synergistic stimulation of translation requires eIF4E, Pab1p, and at least the Pab1p binding site on eIF4G (27). Measures of the production of the LUC protein from in vitro-synthesized LUC mRNA have to date accurately reflected the translational properties of the extracts when they are programmed with other mRNAs (16, 25, 27). Because the measure of LUC protein production is quantitative over a 10,000-fold range, we chose to analyze the translational properties of mutant eIF4G extracts with this method. We also chose to use yeast extracts that had not been treated with micrococcal nuclease prior to the addition of the LUC mRNA since, as a result of the presence of normal levels of endogenous mRNAs, such untreated extracts are most representative of the in vivo situation.

Non-nuclease-treated extracts prepared from yeast strains harboring eIF4G1 exhibited the previously reported stimulation by the cap and the poly(A) tail structures, the synergistic stimulation by both of these structures, and the absence of significant translation in the absence of these structures (Fig. 4A) (see also reference 27). In contrast to these results, extracts containing the eIF4G1-459 protein exhibited very different properties. First, the expression of the LUC mRNA lacking a cap and a poly(A) tail went from being nearly undetectable to being nearly equal to that of capLUC mRNA. Second, the expression from the LUCpA mRNA was increased over 400-fold compared to that seen in the wild-type extract. Extracts containing eIF4G2 exhibited characteristics similar to those containing eIF4G1, with the notable exceptions that the level of translation of the LUC mRNA lacking a cap and a tail was higher than in the eIF4G1 extracts and that the translation of LUCpA mRNA was significantly greater than in the eIF4G1 extracts (Fig. 4A) (27). Results qualitatively similar to those for the eIF4G1-459 extracts were obtained with eIF4G2-430 extracts, although the absolute magnitudes of the differences were not as large. We also note that, as expected, the synergistic stimulation of translation induced by the presence of the cap and the poly(A) tail on mRNA, as measured by comparing the expression of capLUCpA mRNA to the sum of expression of capLUC and LUCpA mRNAs (Fig. 4A), is lost when the eIF4E binding site mutations are introduced into the eIF4G proteins.

The large translational enhancement of LUCpA mRNA in each of the two mutant eIF4G extracts was shown to be dependent on the presence of Pab1p in two different ways. First, we previously reported that point mutations within the Pab1p binding domains of either eIF4G mutant protein (eIF4G1-213 or eIF4G2-233) inhibit the expression of LUCpA mRNA by approximately 10-fold (Fig. 4B and C) (27). We found that these mutations, when combined with the eIF4E binding site mutations to yield eIF4G1-213,459 or eIF4G2-233,430, also inhibited the stimulated expression of LUCpA mRNA resulting from the eIF4E binding site mutations to a similar degree (Fig. 4B and C). Second, we previously reported that immunoneutralization of Pab1p in yeast extracts inhibits poly(A) tail-dependent translation by greater than 95% (Fig. 4B and C) (25). We found that the antibody used also inhibited the stimulation of LUCpA mRNA translation induced by the eIF4E binding site mutations to a similar degree without affecting the translation of capLUC mRNA (Fig. 4B and C) or LUC mRNA (data not shown). These data show that the enhanced translation of LUCpA mRNA in extracts containing either eIF4G1-459 or eIF4G2-430 does not arise from a novel mechanism of translational enhancement by the poly(A) tail, but instead results from an enhanced ability of the poly(A) tail to stimulate translation through its normal, Pab1p-dependent mechanism.

The nearly equal yields of LUC enzyme activity from the

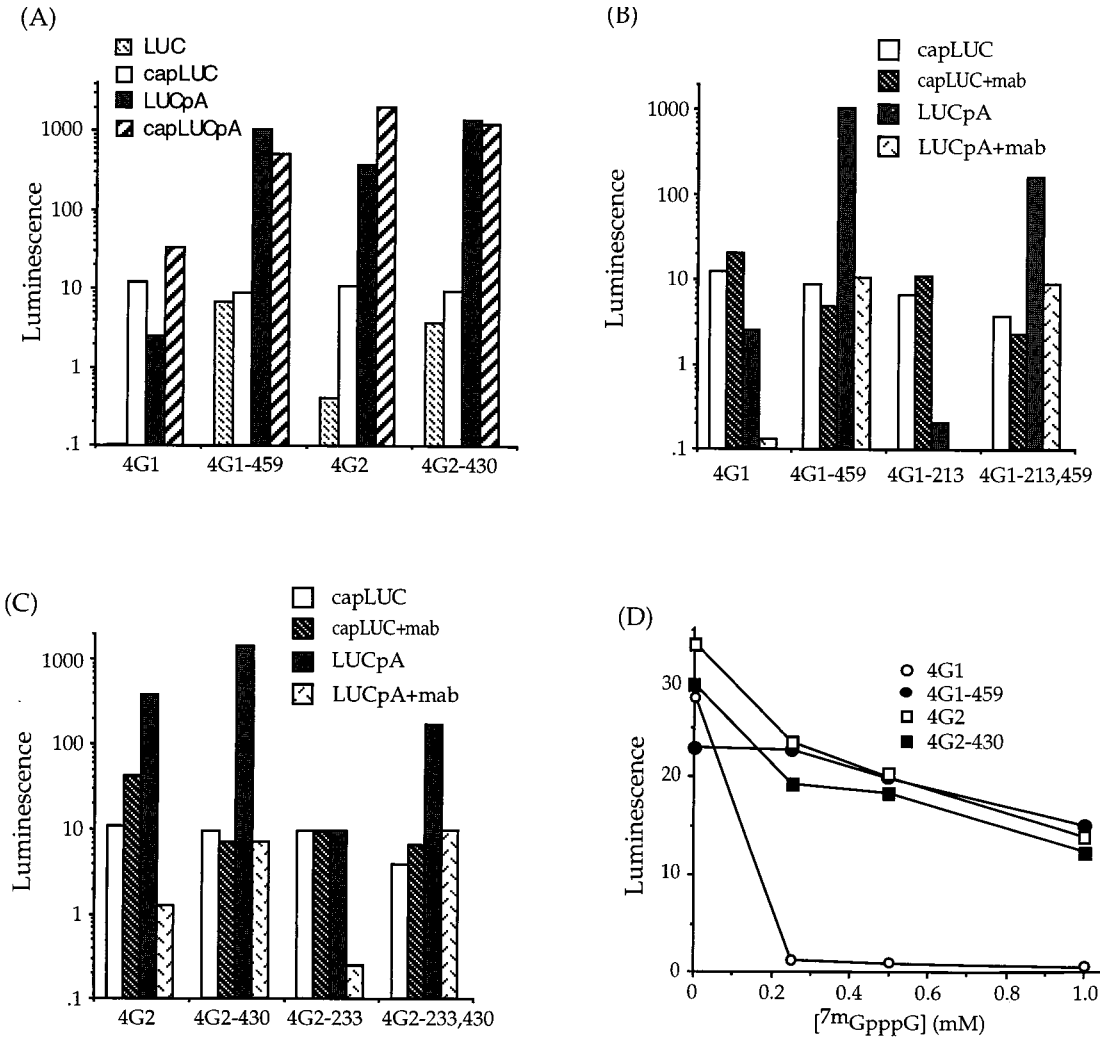


FIG. 4. Extracts from yeast eIF4G mutants exhibit enhanced translation of uncapped mRNA. (A) Translation of uncapped mRNA is enhanced in the eIF4G mutant extracts. Yeast translation extracts programmed with equal amounts of LUC mRNA containing a cap (capLUC), a poly(A) tail (LUCpA), both (capLUCpA), or neither (LUC) were analyzed for LUC protein production by using a luminescence assay (25). eIF4G1-459 and eIF4G2-430 extracts contain eIF4G proteins with point mutations in their eIF4E binding region. (B) The enhanced translation of LUCpA mRNA in eIF4G1-459 extracts occurs through a Pab1p-dependent mechanism. The indicated yeast extracts were preincubated with the Pab1p monoclonal antibody (mab) 1G1 (3) prior to the addition of mRNA. eIF4G1-213 extracts contain eIF4G1 with a series of point mutations in its Pab1p binding region, while eIF4G1-213,459 contains mutations in both the Pab1p and the eIF4E binding regions. (C) The enhanced translation of LUCpA mRNA in eIF4G2-430 extracts occurs through a Pab1p-dependent mechanism. Details are as given for panel B. eIF4G2-233 extracts contain eIF4G2 with a series of point mutations in its Pab1p binding region, while eIF4G2-233,428 contains mutations in both the Pab1p and the eIF4E binding regions. (D) The cap analog ^{7m}GpppG inhibits the translation of capLUC mRNA in eIF4G1 extracts but not in eIF4G1-459 or eIF4G2 extracts. The cap analog was added to the extract, and this was then added to the capLUC mRNA mixture. The final concentrations of the analog in the translation mixture are shown. All translation data are representative of at least two independent experiments. The yeast strains used to prepare the extracts were as follows: eIF4G1, YAS2069; eIF4G1-459, YAS2074; eIF4G1-213, YAS2075; eIF4G1-213,459, YAS2093; eIF4G2, YAS1951; eIF4G2-430, YAS2002; eIF4G2-233, YAS2001; and eIF4G2-233,430, YAS2003.

LUC and capLUC mRNAs in the eIF4G1-459 and eIF4G2-430 extracts (Fig. 4A) suggested that the cap structure was no longer enhancing translation in the mutant extracts. In order to directly test whether the cap structure contributed to the translation of capLUC mRNAs in the various extracts, inhibition studies with the cap analog ^{7m}GpppG were undertaken. This analog has previously been shown to inhibit the translation of capLUC mRNA in nuclease-treated yeast extracts (16, 25). We confirmed that this analog inhibited the translation of capLUC mRNA in the non-nuclease-treated extract containing only eIF4G1 (Fig. 4D). We then found that extracts containing eIF4G1-459 were resistant to the addition of the cap analog. The resistance of these extracts suggests that the mutations in eIF4G1-459 which disrupt eIF4E binding to it also disrupt the ability of the cap structure to stimulate in vitro translation of capped mRNA. Such a result is consistent with the hypothesis that eIF4E recruits eIF4G to the 5′ end of the mRNA via its association with the cap structure (reviewed in reference 22).

Surprisingly, the translation of capLUC mRNA in the wild-type eIF4G2 extract was resistant to the cap analog (Fig. 4D), even though the cap structure on the mRNA stimulated its expression (i.e., compare LUC to capLUC in Fig. 4A). In contrast, the eIF4G2 extracts were inhibited by the cap analog once they were treated with nuclease (data not shown). By comparison, wild-type yeast extracts, which contain both eIF4G1 and eIF4G2, were inhibited up to 90% by the cap analog (see Fig. 6C). This could suggest that eIF4G2 contributes only 10% to the translation of capped mRNA in these extracts. Although

an explanation for these data on the eIF4G-containing extracts must await further experiments, we imagine that such resistance reflects occupancy of eIF4G2 on mRNA near the cap structure. This would result in such a high local concentration of eIF4E near the cap structure that the cap analog could not effectively compete. The nuclease sensitivity of this cap analog effect could occur if fragments of mRNA remained bound to eIF4G2 after nuclease treatment and prevented eIF4G2 from binding to new mRNA binding sites near the cap structure on the capLUC mRNA.

Note that in all of our experiments, the promoter, 5′ leader, and 3′ untranslated region of the eIF4G2 gene *TIF4632* are used to express either the eIF4G1 or the eIF4G2 open reading frames. As a result, some of the observed differences between strains and extracts containing eIF4G1 and eIF4G2 may be due to unnatural expression of eIF4G1. Future work will address more directly the implications of the differences between eIF4G1 and eIF4G2 discussed here and previously (26, 27).

The mutant eIF4E protein cdc33-1p associates poorly with eIF4G. The above in vivo and in vitro data suggested that the loss of eIF4E binding to eIF4G was primarily responsible for the observed effects in the translation experiments. These results predicted that mutations within eIF4E which diminish its binding to eIF4G would exhibit similar effects. In order to test this hypothesis, we first analyzed the ability of the recombinant eIF4E mutant protein cdc33-1p (2) to bind to equal amounts of full-length GST-eIF4G1 or GST-eIF4G2 immobilized on a glutathione column (Fig. 5A, lower panel). cdc33-1p causes temperature-sensitive growth in vivo and exhibits very poor binding to cap analog columns in vitro (2). Because cdc33-1p cannot be purified by ^{7m}GDP-chromatography, we chose to incubate bacterial extracts containing either overproduced wild-type or mutant eIF4E with the resin-associated eIF4G. An identical approach has recently been successfully used by Altmann and coworkers for a similar reason (1). As expected, wild-type eIF4E in the bacterial extract bound to eIF4G1 and eIF4G2, two- to threefold less well to eIF4G2-430, and not at all to eIF4G1-459 (Fig. 5A). The similarity of these data to those shown in Fig. 3A confirms the specificity of the eIF4E interaction in this cruder assay system. In contrast to the data for wild-type eIF4E, the mutant cdc33-1p exhibited much weaker binding to both eIF4G1 and eIF4G2 (Fig. 5A). These data suggest that cdc33-1p is deficient for binding to both eIF4G proteins in vitro.

A far-Western analysis probing the various immobilized GST-eIF4G proteins with cdc33-1p was also performed. In these experiments, crude bacterial lysates containing either the wild-type or *cdc33-1* mutant eIF4E proteins were incubated with the nitrocellulose-bound GST-eIF4G proteins. Then, as described for Fig. 3B, the eIF4E which was bound to eIF4G was detected by Western analysis (Fig. 5B). As a control for specificity, the GST-eIF4G proteins containing mutated eIF4E binding sites were also included in the assay, and they were found to be unable to bind to the wild-type eIF4E. The results of these experiments show that cdc33-1p associates poorly with both eIF4G1 and eIF4G2. These data are in accord with those shown in Fig. 5A.

Coimmunoprecipitation studies were also performed in order to provide an alternative measure of the association of cdc33-1p with eIF4G1 or eIF4G2 in yeast extracts. Either wild-type or *cdc33-1* yeast strains were transformed with epitope-tagged eIF4G1 and eIF4G2 genes (26). Following the preparation of extracts from these strains, each of the eIF4G proteins was immunoprecipitated with monoclonal antibodies to the epitope tag. The immunoprecipitates were then resolved by SDS-PAGE, and either eIF4E or the eIF4G proteins were visualized by Western analysis. As seen in Fig. 5C, the cdc33-1p associated very poorly with either eIF4G1 or eIF4G2 in comparison to the association of wild-type eIF4E with these proteins. Together with the experiments whose results are shown in Fig. 5A and B, the results from these immunoprecipitation studies support the hypothesis that cdc33-1p exhibits a decreased binding affinity for eIF4G1 and eIF4G2.

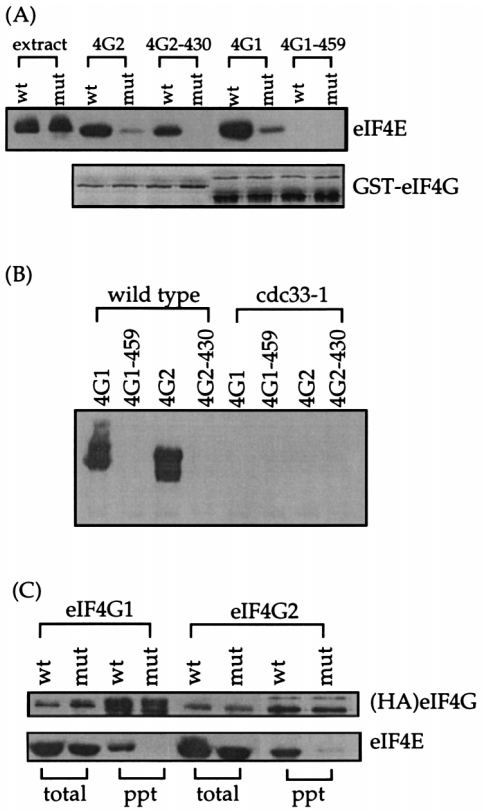
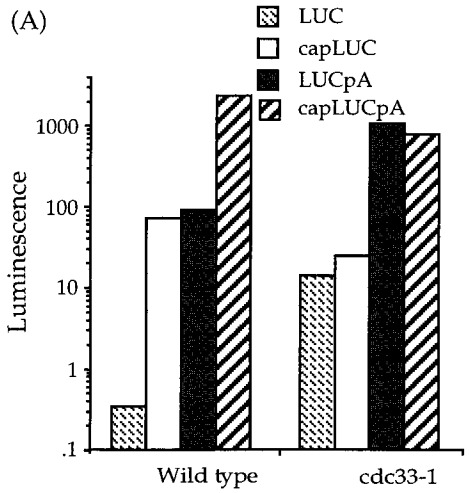
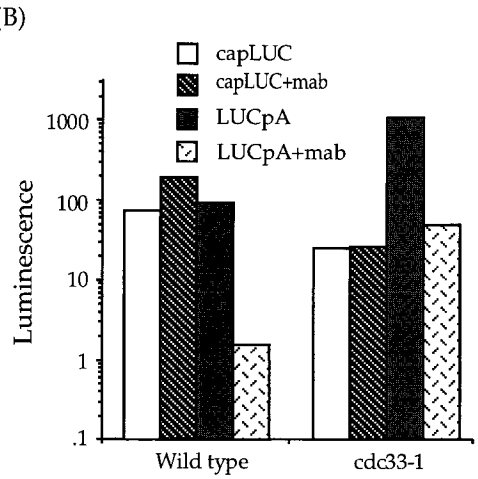


FIG. 5. The eIF4E mutant protein cdc33-1p associates poorly with eIF4G in vitro. (A) cdc33-1p associates poorly with recombinant eIF4G. Bacterial lysates containing the indicated recombinant eIF4E protein (wt, wild type; mut, cdc33-1p) were incubated with the indicated glutathione resin-associated GST-eIF4G fusion protein. Following washing of the resin, bound proteins were eluted in SDS, resolved by SDS–10% PAGE, and visualized by either Coomassie brilliant blue staining (GST-eIF4G) or Western analysis (eIF4E). Equal amounts of eIF4E within the lysate (extract) were incubated with each recombinant eIF4G protein. (B) cdc33-1p associates poorly with eIF4G as determined by far-Western analysis. Bacterial lysates containing equivalent amounts of the indicated recombinant eIF4E protein (wild type or cdc33-1p) were incubated with the indicated nitrocellulose-immobilized GST-eIF4G proteins as described for Fig. 3B. The eIF4E proteins bound to the GST-eIF4G were visualized by Western analysis. A Coomassie brilliant blue-stained SDS–10% polyacrylamide gel containing the amounts of GST-eIF4G proteins electroblotted onto the nitrocellulose membrane is shown in panel A. (C) cdc33-1p associates poorly with eIF4G in crude yeast extracts. Extracts containing the indicated influenza virus epitope-tagged eIF4G proteins were subjected to immunoprecipitation using the 12CA5 monoclonal antibody. Samples were resolved by SDS–10% PAGE, and either eIF4G (top panel) or its associated eIF4E (bottom panel) was detected by Western analysis using the appropriate antibody. One percent of the extract (total) or 30% of the immunoprecipitate (ppt) was loaded onto the gel. The yeast strains used in this study were, from left to right, YAS2158, -2159, -2131, and -2132.

Enhanced translation of uncapped mRNA in extracts containing the mutant eIF4E protein cdc33-1p. Having established that cdc33-1p bound less well to eIF4G1 and eIF4G2 in a recombinant system and in crude yeast extracts, we next examined the properties of a translation extract containing it. As shown in Fig. 6, the *cdc33-1* extract exhibited many of the features found in the eIF4G mutant extracts described above.





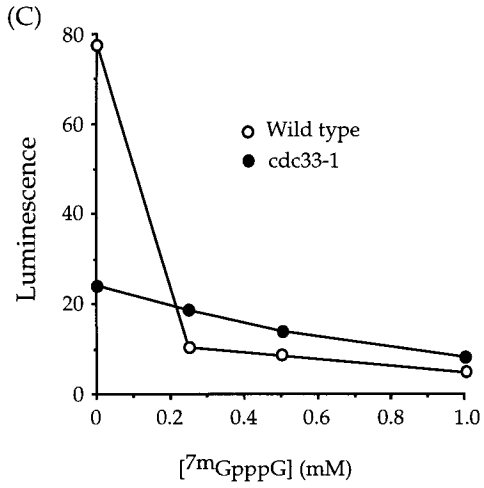


FIG. 6. Extracts from yeast *cdc33-1* mutants exhibit enhanced translation of uncapped mRNA. (A) Translation of uncapped mRNA is enhanced in the *cdc33-1* extract. Yeast translation extracts programmed with equal amounts of the indicated LUC mRNAs were analyzed for LUC protein production by luminescence assay (25). (B) The enhanced translation of LUCpA mRNA in the *cdc33-1* extracts occurs through a Pab1p-dependent mechanism. The indicated yeast extracts were preincubated with the Pab1p monoclonal antibody (mab) and analyzed as described for Fig. 4B. (C) The cap analog ^{7m}GpppG does not inhibit the translation of capLUC mRNA in *cdc33-1* extracts. The cap analog was added to the extract, and this was then added to the capLUC mRNA mixture. The final concentrations of the analog in the translation mixture are shown. All data are representative of at least two independent experiments. The yeast strains used to prepare the extracts are YAS538 (wild type) and YAS1888 (*cdc33-1*). Yeast strains were grown at 26°C prior to extract preparation.

Notably, the translation of LUC and LUCpA mRNAs was stimulated nearly 10-fold over that found in the wild-type extract, and the expression of LUC mRNA was nearly equal to that of capLUC mRNA (Fig. 6A). Similarly, the stimulation of poly(A) tail-dependent translation was found to require Pab1p, since immunoneutralization of Pab1p inhibited this effect (Fig. 6B). Furthermore, in contrast to wild-type extracts, translation of capLUC mRNA was not inhibited by the cap analog in the *cdc33-1* extract (Fig. 6C). The results from these studies of the translation properties of extracts containing an eIF4E protein which associates poorly with eIF4G are consistent with the above conclusion that loss of eIF4E binding to eIF4G leads to the stimulation of translation of uncapped mRNA in yeast.

Recombinant eIF4E reverses the alterations in translation observed in extracts containing mutant eIF4G or mutant eIF4E. The translational properties of extracts containing the mutant eIF4G homologs or mutant eIF4E could result from either a loss of association between eIF4E and eIF4G or an indirect effect of these mutations on the levels of other translation factors within the extract. They did not result from large differences in the level of expression of the mutant versus wild-type eIF4G or eIF4E proteins, since Western analysis of extracts from all of the strains used in this study revealed that the mutant proteins were expressed within 50% of the levels of their wild-type counterparts (data not shown). Because overexpression of eIF4E in vivo suppressed the temperature-sensitive growth phenotypes of the eIF4G mutants (Fig. 2A) and the eIF4E mutant (6), it seemed likely that the addition of excess recombinant eIF4E to the in vitro extracts would also suppress their abnormalities. This prediction was based partially on the inference that the ability to suppress the in vivo phenotypes of the eIF4G mutants by overexpression of eIF4E indicated that the eIF4G proteins could be bound by eIF4E at sufficiently high concentrations. Such a hypothesis is consistent with the differences observed for eIF4E binding to eIF4G2-430 in the different assays (Fig. 3).

Consequently, recombinant eIF4E was added at various concentrations to each of the six extracts described above. In these experiments, the suppression of translation of LUCpA mRNA by exogenous eIF4E would be expected to be greater for the mutant extracts than for their wild-type counterparts. As shown in Fig. 7, the recombinant eIF4E protein suppressed the differences observed between each of the wild-type and mutant extracts in LUCpA mRNA expression. Importantly, the addition of eIF4E to the wild-type extracts had very little effect on their ability to translate LUCpA mRNA. Also, the ability of the mutant extracts to translate LUC mRNA was inhibited by the addition of eIF4E to a degree similar to that of LUCpA mRNA, while the translation of this mRNA in wild-type extracts was not decreased (data not shown). We note that the *cdc33-1* extract's translation of capLUC mRNA was also stimulated by the recombinant eIF4E (Fig. 7C), as would be expected for an extract lacking functional eIF4E. On the basis of

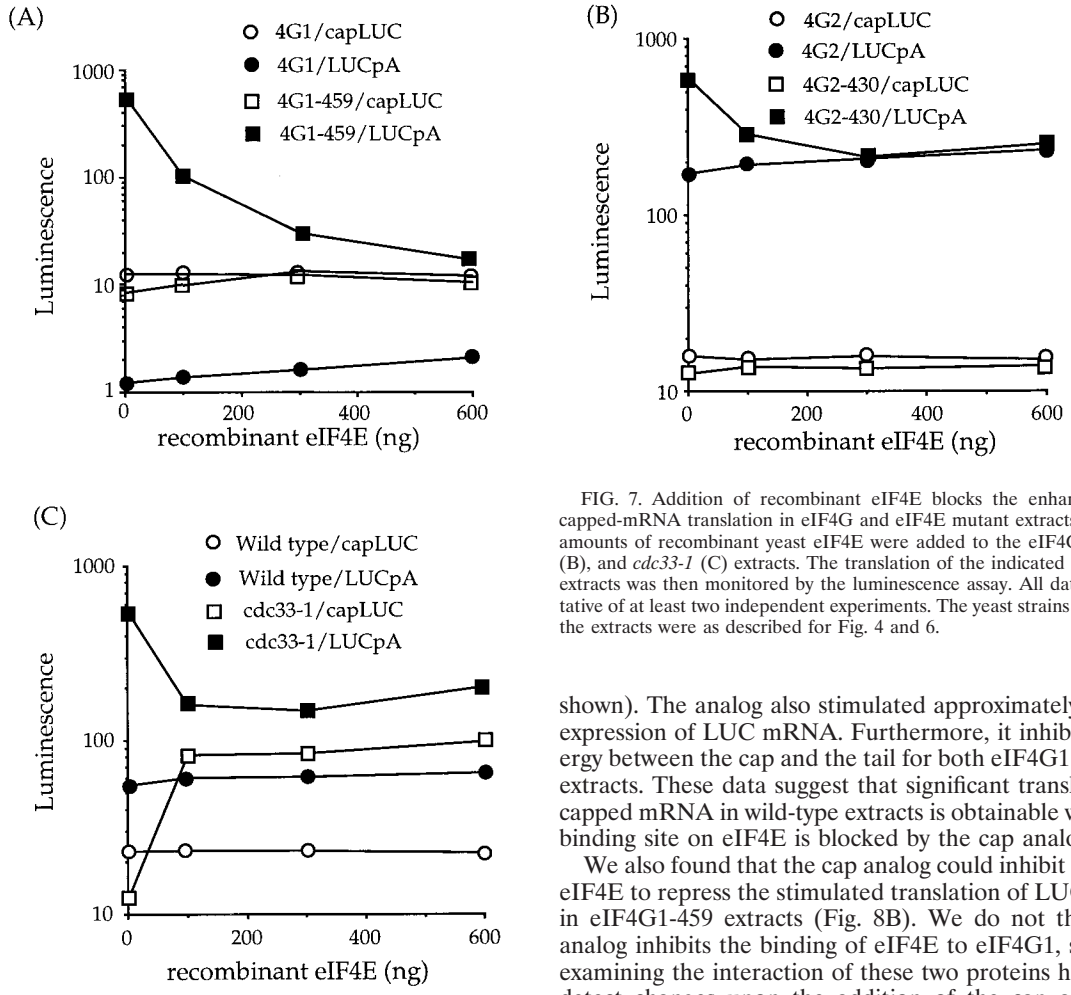


FIG. 7. Addition of recombinant eIF4E blocks the enhancement of uncapped-mRNA translation in eIF4G and eIF4E mutant extracts. The indicated amounts of recombinant yeast eIF4E were added to the eIF4G1 (A), eIF4G2 (B), and *cdc33-1* (C) extracts. The translation of the indicated mRNA in these extracts was then monitored by the luminescence assay. All data are representative of at least two independent experiments. The yeast strains used to prepare the extracts were as described for Fig. 4 and 6.

quantitative estimates from previous work (2), we estimated that in these experiments 5 to 10 times more exogenous eIF4E (i.e., 600 ng) than was present in the extracts was added. We suspect that capLUC mRNA expression was not enhanced after the addition of eIF4E to the eIF4G mutant extracts because of the offsetting effects of decreasing uncapped-mRNA translation and increasing capped-mRNA translation. The results from these experiments provide strong support for the conclusion that the lack of eIF4E binding to eIF4G in the mutant yeast extracts is responsible for the enhanced translation of uncapped mRNA, and they rule out an alternative hypothesis that the observed effects are an indirect consequence of the mutations on other proteins within the extracts.

Addition of the cap analog to translation extracts. We decided to further explore the effects of addition of the cap analog to various extracts in order to more fully understand the effects of its binding to eIF4E on translation of uncapped mRNA. First, as has previously been reported for nuclease-treated translation extracts (16, 25), the addition of the cap analog to non-nuclease-treated extracts containing wild-type eIF4G1 or eIF4G2 was found to stimulate the translation of LUCpA mRNA (Fig. 8A). The extent of stimulation was much greater for the non-nuclease-treated extracts (10- to 20-fold) than for nuclease-treated extracts (2- to 3-fold) (25), mostly because translation of exogenous mRNA in the absence of the analog is much greater in nuclease-treated extracts (data not shown). The analog also stimulated approximately 10-fold the expression of LUC mRNA. Furthermore, it inhibited the synergy between the cap and the tail for both eIF4G1 and eIF4G2 extracts. These data suggest that significant translation of uncapped mRNA in wild-type extracts is obtainable when the cap binding site on eIF4E is blocked by the cap analog.

We also found that the cap analog could inhibit the ability of eIF4E to repress the stimulated translation of LUCpA mRNA in eIF4G1-459 extracts (Fig. 8B). We do not think the cap analog inhibits the binding of eIF4E to eIF4G1, since studies examining the interaction of these two proteins have failed to detect changes upon the addition of the cap analog (28a). These data are consistent with the conclusion that blockage of the cap binding site on eIF4E can neutralize the repressing effect of eIF4E on uncapped-mRNA translation.

DISCUSSION

The data presented in this paper indicate that the substitution of alanines for two highly conserved leucine residues within each of the two yeast eIF4G homologs has several dramatic in vitro and in vivo consequences. These substitutions almost completely block the association of eIF4E with eIF4G1, and they significantly inhibit the association of eIF4E with eIF4G2 in vitro. In addition, these mutant forms of eIF4G appear to be deficient in eIF4E binding in vivo, since the temperature-sensitive phenotype of strains harboring them is suppressed by either increasing the expression of eIF4E or removing the eIF4E negative regulator Caf20p. Extracts prepared from these eIF4G mutant strains exhibit an enhanced ability to translate uncapped mRNA. Extracts containing the mutant eIF4E protein cdc33-1p, which poorly associates with eIF4G, also exhibit an enhanced ability to translate uncapped mRNA. Because the addition of recombinant eIF4E to each of the mutant extracts nearly completely reversed this enhancement, we conclude that these effects are due to loss of eIF4E binding to eIF4G. Taken together, these data support the hypothesis that yeast eIF4E represses the ability of eIF4G to stimulate the translation of uncapped mRNA in vitro and possibly in vivo.

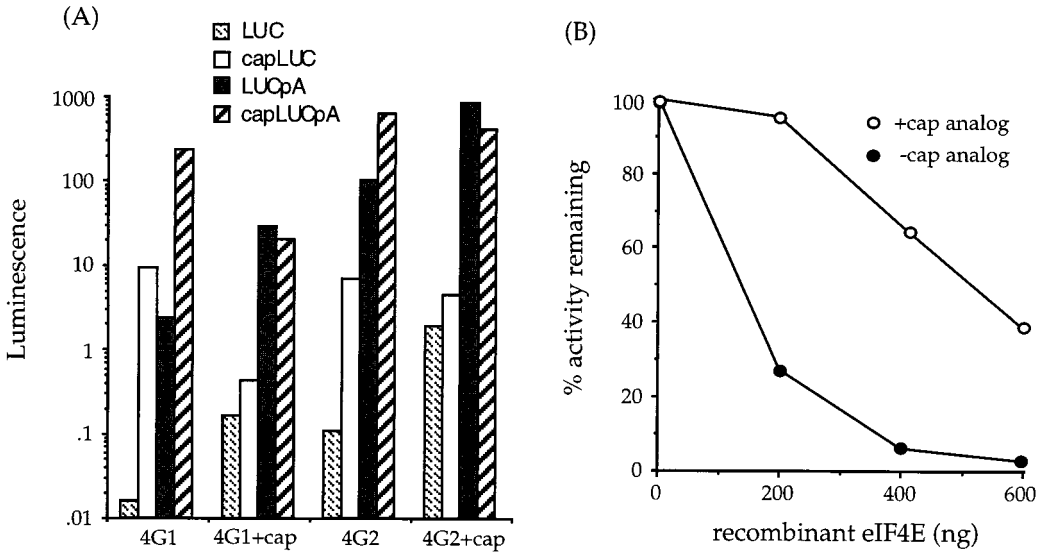


FIG. 8. The cap analog stimulates translation in wild-type extracts and prevents the inhibition of translation by eIF4E in eIF4G1-459 extracts. (A) Addition of ^{7m}GpppG stimulates translation in non-nuclease-treated translation extracts. The amounts of translation of the indicated LUC mRNAs within the indicated extracts in the presence (+cap) or absence of 0.5 mM ^{7m}GpppG are shown. (B) Addition of ^{7m}GpppG prevents the repression of LUCpA mRNA expression by eIF4E in eIF4G1-459 extracts. The amounts of translation of LUCpA mRNA within the eIF4G1-459 extract in the presence or absence of 0.5 mM ^{7m}GpppG and the indicated amounts of recombinant yeast eIF4E are shown. The *y* axis indicates the percentage of LUCpA mRNA translation under each condition relative to the 100% value recorded for no eIF4E addition.

Does eIF4E repress uncapped-mRNA translation solely by sequestering eIF4G onto the endogenous mRNA in the extract? Such sequestration should be sensitive to the presence of the cap analog. In fact, our observation that LUC and LUCpA mRNA expression is stimulated approximately 10-fold by the addition of the cap analog (Fig. 8A) supports the notion that sequestration plays a role. The addition of the cap analog to the non-nuclease-treated extracts, however, did not entirely mimic the effects of the eIF4G or eIF4E mutations on uncapped-mRNA translation. For instance, extracts containing eIF4G1-459 exhibited nearly equal translation of capped and uncapped LUC mRNA, and these levels were similar to that found for capLUC mRNA in eIF4G1 extracts (Fig. 4). In contrast, the addition of the cap analog to eIF4G1 extracts resulted in the inhibition of capLUC mRNA expression and the activation of LUCmRNA expression to 10% of the level found in eIF4G1-459 extracts (Fig. 4A and 8A). Furthermore, eIF4G2 extracts were stimulated by the cap analog to a lesser degree than was found in the eIF4G2-430 extracts. Finally, the addition of eIF4E to the mutant eIF4G proteins repressed LUCpA mRNA expression but did not stimulate capLUC mRNA expression. Such a differential effect would not be anticipated if sequestration were to explain all of the mutations' effects. As a result of these various quantitative observations, the simple hypothesis that release of eIF4G from endogenous mRNA accounts for the enhancement of uncapped-mRNA translation in the mutant extracts is not sufficient.

Instead, our data best support the alternative hypothesis that both sequestration and a direct repressing effect of eIF4E on eIF4G result in the inability of yeast extracts to efficiently translate uncapped mRNA. We imagine that eIF4E represses an activity of eIF4G as a result of protein-protein interactions, and as a result, the repressing activity is completely relieved when eIF4E dissociates from eIF4G. We suspect that it is eIF4G's ability to bind to RNA that is the target of the proposed eIF4E repression. A direct test of this model using purified recombinant eIF4G and eIF4E is in progress. We are also testing the possibility that binding of the cap analog to eIF4E inhibits its repression of eIF4G by inducing conformational changes. Unfortunately, a direct test of the sequestration model is more difficult since, by definition, it requires working with complex extracts that mimic the cellular environment.

The experiments presented here extend the observations of several different laboratories studying the roles of eIF4G and eIF4E in the translation of uncapped mRNA (5, 14, 19–21, 28). Our results are in complete agreement with those of researchers studying the effects of cleavage of the eIF4E binding site of eIF4G on the translation of uncapped mRNA. Those studies conclude that a fragment of the eIF4G protein has the ability to stimulate uncapped-mRNA translation. We further conclude that full-length yeast eIF4G, when unbound to eIF4E, is also capable of this stimulation. Furthermore, we suggest that loss of eIF4E binding to eIF4G leads to these effects as a result of release of eIF4G from the endogenous mRNA and release of eIF4G from a direct inhibition by eIF4E.

Our data do not, however, agree with those of Ohlmann et al. (19, 21), which indicated that eIF4E must bind to mammalian eIF4G in order for eIF4G to stimulate uncapped-mRNA translation. Specifically, it was reported that the titration of eIF4E from eIF4G by the addition of the eIF4E-binding protein 4E-BP1 led to the inhibition of uncapped-mRNA translation in reticulocyte lysates. Our data show that mutant eIF4G proteins which associate poorly with eIF4E, or mutant eIF4E proteins which associate poorly with eIF4G, stimulated uncapped-mRNA translation in yeast extracts. Possible reasons for this discrepancy are the differences between the yeast and the mammalian eIF4G proteins, the differences in the mechanisms by which the lysates perform uncapped-mRNA translation, the differences between working with nucleased- and non-nuclease-treated extracts, and the possibility that the 4E-BP1 titration experiments titrated out more than eIF4E in the extracts. Evidence that the addition of eIF4E to the 4E-BP1-treated extracts restored translation of the uncapped mRNA would clarify this last point.

Our data introduce the concept that eIF4E plays a negative regulatory role in cap-independent translation of mRNA in yeast. This function of eIF4E would work in concert with its positive role of recruiting eIF4G to the 5′ end of the mRNA. It is also possible that subsequent to binding to the cap structure during cap-dependent translation, the activation of eIF4G via changes in its interactions with eIF4E may also have functional consequences. The repression of eIF4G by eIF4E may be functionally relevant for the control of translation of those mRNAs which rely on eIF4G binding within their 5′ untranslated leader for their expression. For these mRNAs, it could be imagined that small increases in the amount of free eIF4G could lead to large changes in their expression. For instance, the translation of heat shock mRNAs, which are known to be translated in a cap-independent manner (8), after thermal shock may rely on the dissociation of eIF4E from eIF4G as a means to stimulate eIF4G binding to them.

In conclusion, the data presented in this paper suggest a new role for yeast eIF4E as a negative regulator of uncapped-mRNA translation through its binding to eIF4G. Questions arising from these data include how modifications of eIF4G and eIF4E could affect this aspect of their interaction, how the binding of other proteins to eIF4G could modulate its ability to stimulate uncapped-mRNA translation, and how binding of the cap structure to eIF4E alters its putative repressive interaction with eIF4G. Future studies in this area of translation initiation in both yeast and higher eucaryotes will almost certainly lead to experimental testing of each of these possibilities.

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REFERENCES

1. **Altmann, M., N. Schmitz, C. Berset, and H. Trachsel.** 1997. A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E. EMBO J. **16:**1114–1121.
2. **Altmann, M., and H. Trachsel.** 1989. Altered mRNA cap recognition activity of initiation factor 4E in the yeast cell cycle division mutant *cdc33*. Nucleic Acids Res. **17:**5923–5931.
3. **Anderson, J., M. Paddy, and M. Swanson.** 1993. PUB1 is a major nuclear and cytoplasmic polyadenylated RNA-binding protein in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**102–112.
4. **Barettino, D., M. Feigenbutz, R. Valcarcel, and H. G. Stunnenberg.** 1993. Improved method for PCR-mediated site-directed mutagenesis. Nucleic Acids Res. **22:**541–542.
5. **Borman, A., R. Kirchweger, E. Ziegler, R. E. Rhoads, T. Skern, and K. M. Kean.** 1997. eIF4G and its proteolytic cleavage products: effect on initiation of protein synthesis from capped, uncapped, and IRES-containing mRNAs. RNA **3:**186–196.
6. **Brenner, C., N. Nakayama, M. Goebl, K. Tanaka, A. Toh-e, and K. Matsumoto.** 1988. *CDC33* encodes mRNA cap-binding protein eIF-4E of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8:**3556–3559.
7. **Cruz, J., I. Iost, D. Kressler, and P. Linder.** 1997. The p20 and Ded1 proteins have antagonistic roles in eIF4E-dependent translation in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **94:**5201–5206.
8. **Duncan, R. F.** 1996. Translational control during heat shock, p. 271–293. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control, vol. 30. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
9. **Edery, I., M. Altmann, and N. Sonenberg.** 1988. High-level synthesis in *Escherichia coli* of functional cap-binding eukaryotic initiation factor eIF-4E and affinity purification using a simplified cap-analog resin. Gene **74:**517–525.
10. **Elledge, S. J., and R. W. Davis.** 1988. A family of versatile centromeric vectors designed for use in the sectoring-shuffle mutagenesis assay in *Saccharomyces cerevisiae*. Gene **70:**303–312.
11. **Goyer, C., M. Altmann, H. S. Lee, A. Blanc, M. Deshmukh, J. L. Woolford, H. Trachsel, and N. Sonenberg.** 1993. *TIF4631* and *TIF4632*: two genes encoding the high-molecular-weight subunits of the cap-binding protein complex (eukaryotic initiation factor 4F) contain an RNA recognition motif sequence and carry out an essential function. Mol. Cell. Biol. **13:**4860–4874.
12. **Guthrie, C., and G. Fink (ed.).** 1991. Methods in enzymology, vol. 194. Guide to yeast genetics and molecular biology. Academic Press, Inc., San Diego, Calif.
13. **Haghighat, A., S. Mader, A. Pause, and N. Sonenberg.** 1995. Repression of cap-dependent translation by 4E-binding protein 1: competition with p200 for binding to eukaryotic initiation factor-4E. EMBO J. **14:**5701–5709.
14. **Haghighat, A., Y. Svitkin, I. Novoa, E. Kuechler, T. Skern, and N. Sonenberg.** 1996. The eIF4G-eIF4E complex is the target for direct cleavage by the rhinovirus 2A proteinase. J. Virol. **70:**8444–8450.
15. **Hentze, M. W.** 1997. eIF4G: a multipurpose ribosome adaptor? Science **275:**500–501.
16. **Iizuka, N., L. Najita, A. Franzusoff, and P. Sarnow.** 1994. Cap-dependent and cap-independent translation by internal initiation of mRNAs in cell extracts prepared from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **14:**7322–7330.
17. **Lamphear, B. J., R. Kirchweger, T. Skern, and R. E. Rhoads.** 1995. Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. J. Biol. Chem. **270:**21975–21983.
18. **Mader, S., H. Lee, A. Pause, and N. Sonenberg.** 1995. The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4γ and the translational repressors 4E-binding proteins. Mol. Cell. Biol. **15:**4990–4997.
19. **Ohlmann, T., V. M. Pain, W. Wood, M. Rau, and S. J. Morley.** 1997. The proteolytic cleavage of eucaryotic initiation factor (eIF) 4G is prevented by eIF4E binding protein (PHAS-I; 4E-BP1) in the reticulocyte lysate. EMBO J. **16:**844–855.
20. **Ohlmann, T., M. Rau, S. J. Morley, and V. M. Pain.** 1995. Proteolytic cleavage of initiation factor eIF-4G in the reticulocyte lysate inhibits translation of capped mRNAs but enhances that of uncapped mRNAs. Nucleic Acids Res. **23:**334–340.
21. **Ohlmann, T., M. Rau, V. M. Pain, and S. J. Morley.** 1996. The C-terminal domain of eukaryotic protein synthesis initiation factor (eIF) 4G is sufficient to support cap-independent translation in the absence of eIF4E. EMBO J. **15:**1371–1382.
22. **Sachs, A. B., P. Sarnow, and M. W. Hentze.** 1997. Starting at the beginning, middle, and end: translation initiation in eucaryotes. Cell **89:**831–838.
23. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
24. **Sonenberg, N.** 1996. mRNA 5′ cap-binding protein eIF4E and control of cell growth, p. 245–269. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control, vol. 30. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Tarun, S., and A. B. Sachs.** 1995. A common function for mRNA 5′ and 3′ ends in translation initiation in yeast. Genes Dev. **9:**2997–3007.
26. **Tarun, S. Z., and A. B. Sachs.** 1996. Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. EMBO J. **15:**7168–7177.
27. **Tarun, S. Z., S. E. Wells, J. A. Deardorff, and A. B. Sachs.** 1997. Translation initiation factor eIF-4G mediates in vitro poly(A) tail-dependent translation. Proc. Natl. Acad. Sci. USA **94:**9046–9051.
28. **Vasilescu, S., M. Ptushkina, B. Linz, P. P. Muller, and J. E. G. McCarthy.** 1996. Mutants of eukaryotic initiation factor eIF-4E with altered mRNA cap binding specificity reprogram mRNA selection by ribosomes in *Saccharomyces cerevisiae*. J. Biol. Chem. **271:**7030–7037.

28a. **Wells, S., and A. Sachs.** Unpublished results.

29. **Zanchin, N. I. T., and J. E. G. McCarthy.** 1995. Characterization of the in vitro phosphorylation sites of the mRNA-cap binding complex proteins eukaryotic initiation factor 4E and p20 in *Saccharomyces cerevisiae*. J. Biol. Chem. **270:**26505–26510.