Volume 17 Number 15 1989 Nucleic Acids Research

Altered mRNA cap recognition activity of initiation factor 4E in the yeast cell cycle division mutant cdc33

Michael Altmann and Hans Trachsel*

Institut für Biochemie und Molekularbiologie der Universität Bern, Bühlstrasse 28, CH-3012 Bern, Switzerland

Received May 30, 1989; Revised and Accepted June 29, 1989

ABSTRACT

The mutation in the *S.cerevisiae* cell cycle division mutant cdc33 consists of a single G to A transition in the open reading frame encoding translation initiation factor 4E (eIF-4E). This leads to the substitution of glycine113 by aspartic acid close to tryptophane 115 in the protein. This mutation reduces cap binding activity of eIF-4E as measured by binding of eIF-4E to m^7GDP agarose columns and slows down overall protein synthesis at the non-permissive temperature. Comparison of the cdc33 mutation with other mutations affecting eIF-4E function supports the view that tryptophane residues and their flanking regions are involved in cap binding activity of eIF-4E.

INTRODUCTION

Translation initiation in eukaryotes is facilitated by the 5′ mRNA cap structure m^7G(5′)ppp(5′)G (for reviews, see 1−4). This structure is recognized by the initiation factor 4E (eIF-4E, cap binding protein) which, together with other translation initiation factors, mediates binding of ribosomes to mRNA (for reviews, see 5, 6). Initiation factor eIF-4E has been isolated from several species (5) including the yeast *S.cerevisiae* (7). Also, from *S.cerevisiae* the gene encoding eIF-4E has been isolated and sequenced (8).

Recently, the gene CDC33 was cloned and sequenced (9). This gene is involved in the regulation of entry of yeast cells from G_1- into S-phase (10, 11). This is illustrated by the fact that the temperature-sensitive mutant cdc33 arrests growth in the G_1- phase at the non-permissive temperature. Rather surprisingly, it turned out that the gene CDC33 encodes eIF-4E (9). At present it is unknown, how the cdc33 mutation in eIF-4E exerts its effect on cell cycle control. It was speculated, that altered translation initiation at the non-permissive temperature might be involved (9).

We have cloned and sequenced the cdc33 mutation and show here, that this mutation alters the cap recognition activity of eIF-4E. Since different mRNAs show different dependence on eIF-4E cap binding activity for translation (12) this finding supports the view that alteration of translation through modulation of eIF-4E activity may be involved in cell cycle control.

MATERIALS AND METHODS

1. *Cloning and sequencing of the cdc33 mutation*

All yeast strains used in this work are specified in Table 1. The autonomously replicating plasmid pMDA105 carrying the TRP1 gene as selectable marker and the yeast eIF-4E gene with flanking sequences on a 2176 bp Hind III fragment (8) was cut with the restriction enzyme SpeI. There are two unique SpeI-sites on pMDA105 located 19 bp upstream of the translation start codon in the 5′ untranslated region of the eIF-4E gene and 83 bp

Table 1: Characteristics of strains used

Strain	Genotype	Doubling time at 25° C	Colony formation [a]	
			30° C	37° C
T93C	a, eIF-4E::LEU2, ura3, trp1 <GAL1-eIF-4E; ARS1, CEN4, URA3>	2.0 h [b]	+++	+++
CB101 [c]	α, cdc33-1, leu1, ura3, trp1	3.8 h [d]	—	—
MDA110X	T93C transformed with pMDA105 (<eIF-4E, ARS1, TRP1>)	2.0 h [d]	+++	++
MDA105X	T93C transformed with pMDA107-1 (<cdc33 allele of eIF-4E, ARS1, TRP1>)	3.9 h [d]	+++	—

a Cells were grown for 5 days

b Cells were grown YPG (1 % yeast extract, 2 % Bacto-peptone, 2 % galactose)

c Strain CB101 was obtained form Dr. K. Matsumoto

d Cells were grown in YPD

LEU2, β-isopropylmalate; URA3, orotidine-5'-phosphate decarboxylase; TRP1 phosphoribosyl-anthranilate isomerase; ARS1, autonomously replicating sequence; CEN4, centromeric sequence.

downstream of the translation stop codon. SpeI-cut pMDA105 was religated under high dilution conditions and recloned in E.coli. The resulting plasmid, pMDA106, is an E.coli-yeast shuttle vector where a 741 bp SpeI fragment carrying the whole eIF-4E open reading frame is missing but flanking sequences of the chromosomal locus of eIF-4E are still present.

pMDA106 (5 μg) was linearized with SpeI and transformed into the strain CB101 (carrying the cdc33 mutation, Table 1) by selecting for tryptophane prototrophs. Plasmid DNA from transformants was reisolated and cloned into E.coli strain MH3. Random plasmid analysis from 12 E.coli clones by restriction with SpeI showed that in 2 cases the missing 741 bp SpeI fragment had been repaired on the gapped pMDA106 plasmid at the cdc33 chromosomal locus (gap repair, 13). Both plasmids (pMDA107-1 and pMDA107-2) carrying the cdc33 mutation were transformed into strain T93C (12, Table 1). Strain T93C only grows on galactose-containing media since the unique copy of the eIF-4E gene is under the control of the GAL1-promotor on a plasmid (pGAL1-eIF-4E, Table 1) carrying the URA3 gene as a selectable marker.

All yeast transformants harbouring pMDA107−1 and pMDA107−2 showed a temperature-sensitive phenotype on glucose- but not on galactose-containing media. This is in contrast to transformants that had been transformed with the wild-type copy of the eIF-4E gene (pMDA105, control experiment). They could grow at 37°C irrespective of the carbon source in the medium.

The 741 bp SpeI fragment from plasmid pMDA107-1 was subcloned into the XbaI site in the polylinker of M13mp18 and the entire eIF-4E open reading frame was sequenced unidirectionally using appropriate complementary oligonucleotides equally distributed along the eIF-4E sequence (14).

2. *m^7GDP-agarose affinity chromatography*

One liter cultures of strains MDA110X (wild-type) and MDA105X (cdc33 mutation, Table 1) were grown at 25°C to A_{600} = 1.5−2.0 in 1% yeast extract, 2% peptone, 2% glucose (YPD). Cells were collected by centrifugation at 2000×g for 5 min., washed once in dest. H_2O and homogenized with glass beads in a Braun homogenizer in 5 ml of buffer A containing 10 mM Hepes, pH 7.0, 100 mM KCl, 0.2 mM EDTA, 14 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM ATP.

Post ribosomal supernatants were prepared and chromatographed on 1 ml m^7GDP-agarose columns according to previous protocols (7, 12, 15).

3. *Sodium dodecylsulfate polyacrylamide gel electrophoresis and Western blotting*

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE) (16) and Western blotting (17) were performed as described earlier. Gels were stained with silver according to (18). Western blots were reacted with polyclonal anti-yeast cap binding protein antibody (8) diluted 1 : 1000 in 10 mM Tris-HCl, pH 7.0, 150 mM NaCl (TBS) containing 0.5% bovine serum albumin (BSA) for 3 hours, decorated with rabbit anti-rat antibody (DAKO, Denmark), 1 : 1000 diluted in TBS, 0.5% BSA for 1 hour and subsequently with swine anti-rabbit immunoglobulin coupled to alcaline phosphatase (DAKO, Denmark), 1 : 1000 diluted in TBS, 0.5% BSA for 1 hour and finally stained with 5-bromo-4-chloro-indolyl phosphate as substrate and nitrobluetetrazolium as coupler (19).

4. *In vivo [^{35}S] methionine incorporation*

Yeast cells carrying the wild-type eIF-4E gene (MDA110X) or the temperature-sensitive cdc33 allele (strain MDA105X) were grown at 25°C in labeling medium (20). To 1 ml of exponentially growing cells 20 μCi [^{35}S] methionine (1355 mCi/mmol, NEN) was

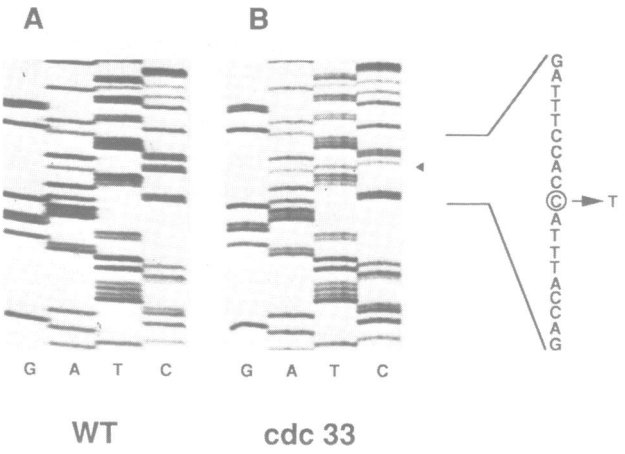


Figure1. Section of a sequencing gel. (A), wild-type, (B), cdc33.
The C → T transition (G → A in the complementary strand) in cdc33 is indicated by an arrowhead

added and the incubation continued at 25°C for 1 hour. Then 0.5 ml of the cells were transferred to 37°C. At intervals, 50 μl samples were taken and protein synthesis stopped by adding 1 ml icecold 10% trichloroacetic acid (TCA). The samples were heated for 3 min. at 80°C to deacylate charged tRNAs, cooled on ice and the precipitated protein collected on glass fiber filters (GF/C, Whatman). Filters were washed with 10 ml 5% TCA, 10 ml 70% ethanol and 1 ml acetone. Dried filters were counted in a liquid scintillation counter.

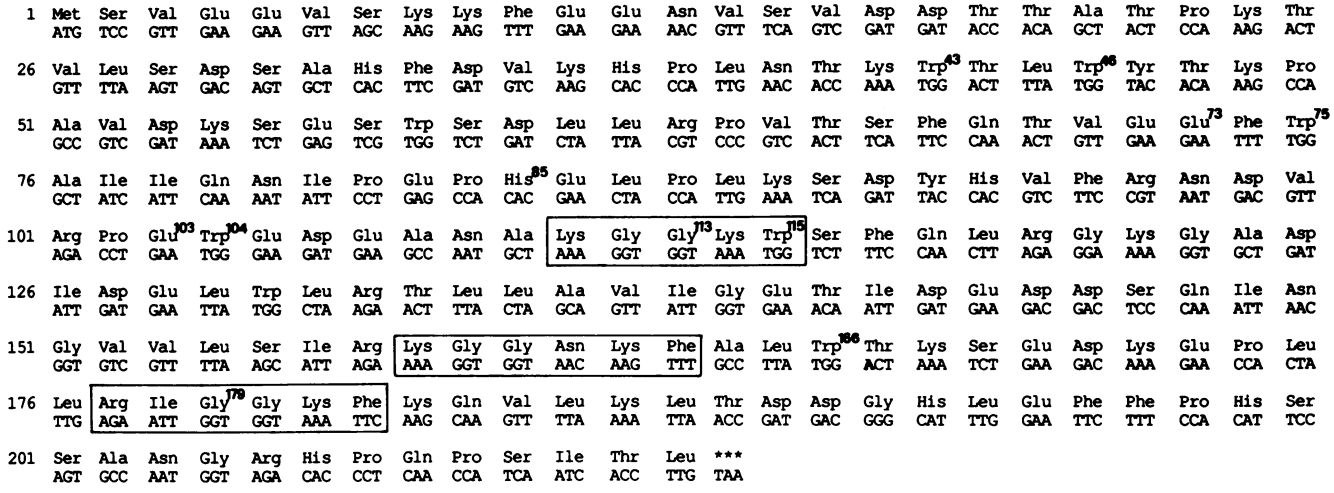
RESULTS

1. *Cloning and sequencing of the cdc33 mutation*

The mutant cdc33 allele of the eIF-4E gene was cloned from strain CB101 (Table 1, 9) by the gap mitotic repair technique (13). In short, an autonomously replicating plasmid (pMDA106, Materials and Methods) lacking the entire eIF-4E open reading frame but carrying flanking sequences of the chromosomal locus of the eIF-4E gene was used to obtain 'repaired' plasmids carrying the cdc33 allele. Two independent clones were isolated and sequenced in their entirety using oligodeoxynucleotides. A single base change, a G to A transition was found (Fig. 1). This changes the glycine residue 113 into an aspartic acid residue in the vicinity of tryptophane 115 (Fig. 2). Other features and mutations indicated in Fig. 2 will be discussed below.

2. *Cap binding activity of eIF-4E carrying the cdc33 mutation*

Cells carrying the wild-type eIF-4E gene (MDA110X, Table 1) and cells carrying the eIF-4E cdc33 allele on an autonomously replicating plasmid (MDA105X, Table 1) were grown at 25°C in rich medium (YPD) and ribosomal salt wash fractions prepared. To test whether the cdc33 mutation affects cap binding activity of eIF-4E the ribosomal salt wash fractions were passed over m^7GDP-agarose affinity columns (Fig. 3). After the application of protein (lanes 1, 9 and 5, 11) the columns were washed extensively with buffer A (Materials and Method) (flow through fractions, lanes 2 and 6), with buffer A containing 100 μM GDP (GDP eluates, lanes 3 and 7) and finally with buffer A containing 80 μM m^7GDP (m^7GDP eluates, lanes 4 and 8). Bound eIF-4E (and associated components) was eluted



cdc 33 Gly ^{113} → Asp
(GGT) (GAT)

ts 4-0 Gly ^{179} → Asp
ts 4-2 Gly ^{179} → Asp, Glu ^{73} → Lys
ts 4-3 Gly ^{179} → Asp, Glu^{103} → Lys
ts 4-4 Gly ^{179} → Asp, His ^{85} → Tyr

Figure 2. DNA sequence of eIF-4E gene and mutations. A conserved amino acid motif in the protein is boxed. ts, temperature-sensitive

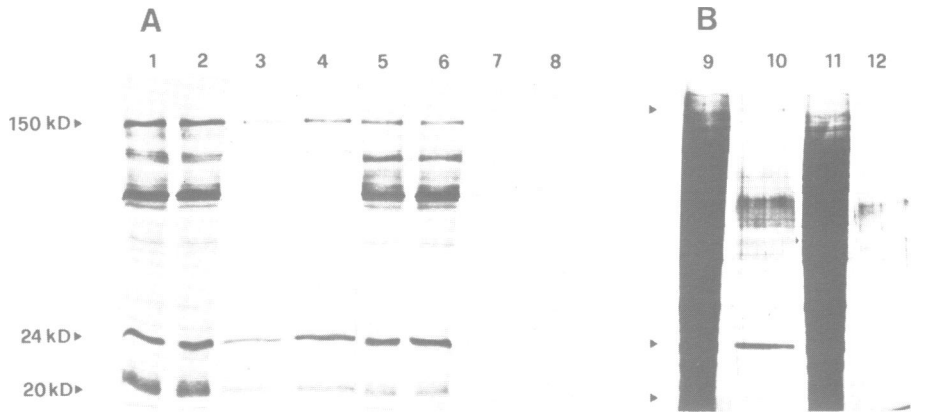


Figure 3. Binding of eIF-4E to m^7GDP-agarose affinity columns. Ribosomal salt wash fractions (10 mg protein) of strain MDA105X (wild-type) and strain MDA110X (cdc33) were applied to 1 ml m^7GDP-agarose columns. The columns were washed successively with buffer A (flow through fractions), 100 μl GDP in buffer A (GDP eluates) and 80 μM m^7GDP in buffer A (m^7GDP-eluates, cap binding proteins). Aliquots of fractions were fractionated by SDS PAGE followed by Western blotting (A) or silver staining (B). The Western blot was reacted with polyclonal rat anti-yeast cap binding protein antibody (8). The arrowheads point to the 150, 24 (eIF-4E) and 20 KD proteins.
(A) Lane 1, 15 μg protein of ribosomal salt wash fraction of wild-type, lane 2, 15 μg protein of flow through fraction, lane 3, 20 μl GDP eluate (from a total of 200 μl), lane 4, 20 μl m^7GDP eluate (from a total of 500 μl), lane 5, 15 μg protein of ribosomal salt wash fraction of strain cdc33, lane 6, 15 μg protein of flow through fraction, lane 7, 20 μl GDP eluate (from a total of 200 μl), lane 8, 20 μl m^7GDP eluate (from a total of 500 μl).
(B) Lane 9, see lane 1, lane 10, see lane 4, lane 11, see lane 5, lane 12, see lane 8.

with m^7GDP. Aliquots of eluted fractions were analysed by Western blotting (Fig. 3A) and silver staining (Fig. 3B). The polyclonal antibody used for immunodecoration of blots was obtained by injecting rats with the m^7GDP-eluate from a m^7GDP-agarose affinity column through which yeast ribosomal salt wash fraction had been passed. Besides eIF-4E the antibody recognizes at least two further proteins (see below). From comparison of lane 4 with lane 8 (Western blot) and lane 10 with lane 12 (silver stain) it is evident that eIF-4E carrying the cdc33 mutation could not be bound to the affinity column and eluted with m^7GDP. This is in contrast to wild-type eIF-4E, where approx. 50% of eIF-4E was retained on the column and eluted with m^7GDP from the resin. After repeated passage of ribosomal salt wash fraction over fresh m^7GDP-agarose wild-type eIF-4E could be completely retained while eIF-4E cdc33 was not bound (not shown). About 10% of wild-type eIF-4E elutes already in the GDP eluate (lane 3). Note that at least two further proteins which react with polyclonal anti-yeast cap binding protein antibody (150 KD and 20 KD) are eluted together with wild-type eIF-4E. They are probably components of the cap binding complex eIF-4F. These proteins do not bind to the affinity resin in the presence of eIF-4E cdc33 (lane 8), indicating that they are attached to the column through eIF-4E.

These data clearly indicate that the cap binding activity of eIF-4E carrying the cdc33 mutation is strongly reduced compared to wild-type eIF-4E. We do not know, however, whether the altered cap binding activity of cdc33 is responsible for the temperature-sensitive phenotype of cells carrying this mutation.

3. *Growth characteristics and protein synthesis*

The expression of the eIF-4E cdc33 allele from an autonomously replicating plasmid (yeast

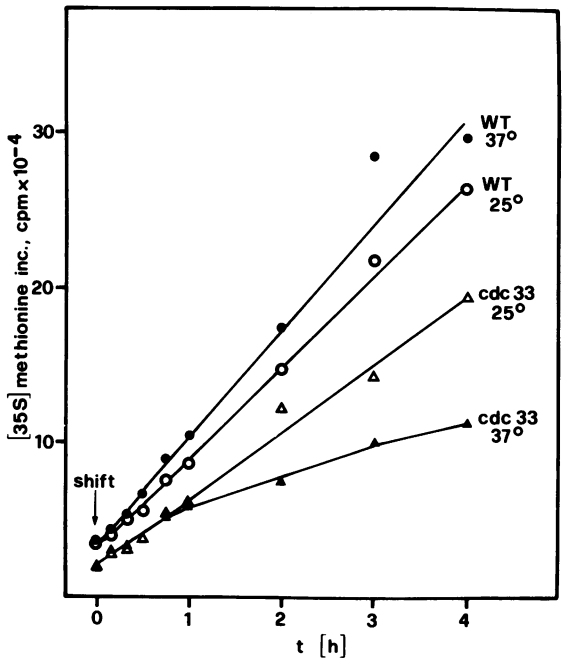


Figure 4. *In vivo* [^{35}S] methionine incorporation. [^{35}S] methionine incorporation was measured as described in Materials and Methods. WT, wild-type yeast strain MDA105X; cdc33, mutant eIF-4E yeast strain MDA110X. The arrow indicates the time when cells were shifted to 37°C.

strain MDA105X, Table 1) does not affect the slow growth phenotype of cells when compared to cells expressing this mutant gene integrated in the chromosome (strain CB101, Table 1). Both cell types have a doubling time of about 4 hours. Nevertheless, some differences can be noted. Strain MDA105X still forms colonies at 30°C, whereas strain CB101 does not. Furthermore, the chromosomally integrated mutation confers a significant lag-phase for growth to the cell when plated onto fresh medium at 25°C (3−4 days). This lag-phase is not observed in the strain that carries the cdc33 mutation on the plasmid (not shown). When both cell lines are transferred from 25°C to the non-permissive temperature of 37°C, MDA105X still undergoes 3−4 divisions, while CB101 arrests growth after approx. 1−2 divisions. These observations indicate that overexpression of the cdc33 allele of eIF-4E from an autonomously replicating plasmid compensates at least partially for the growth defect caused by this mutation.

Since eIF-4E is a translation initiation factor it was pertinent to investigate the change in methionine incorporation in the mutant cells at the non-permissive temperature. The strain carrying eIF-4E cdc33 on a plasmid (strain MDA105X) shows a decreased rate of protein synthesis when compared to the wild-type strain (MDA110X) after shifting the cells to 37°C (Fig. 4). As expected from the slow shut off of growth of this mutant after shift to 37°C (3−4 divisions) we do not observe a dramatic inhibition of protein synthesis even after 4 hours at 37°C. Similar results were obtained with the chromosomally integrated eIF-4E cdc33 (not shown), even though these cells grow only for 1−2 generations at 37°C. Pulse-labeling experiments after shifting cells to 37°C reveal that a similar pattern of proteins

is synthesized in mutant cells like in wild-type cells although at a reduced rate of approx. 20% (not shown).

DISCUSSION

The data presented in this report show that the substitution of glycine113 by aspartic acid in yeast eIF-4E results in reduced cap binding activity of eIF-4E and reduced overall *in vivo* [^{35}S] methionine incorporation into protein in cells carrying this mutation. Despite the severe effects of this single amino acid exchange in the essential yeast eIF-4E gene, protein synthesis in cells carrying this mutation can proceed for several hours after the shift to the non-permissive temperature.

We have obtained a whole collection of temperature-sensitive yeast strains modified in the eIF-4E gene (12). A common feature of all the mutants—even those that provoke a stringent conditionally lethal phenotype—is that they do not completely shut off protein synthesis after shift to the non-permissive temperature. Like cdc33 they continue protein synthesis at a more or less reduced level (down to 15%) for several hours. We do not know whether this effect is due to the ability of the modified initiation factor to maintain its function in protein synthesis at a reduced rate despite its altered cap binding activity or whether protein synthesis can be maintained in the cell for a limited time without the function of eIF-4E (cap binding protein-independent translation).

The cdc33 mutation is responsible for the arrest of growth of cells by blocking entry of these cells from G_1- into S-phase during the cell cycle (11). Comparison of the cdc33 mutation with other mutations which we have created in eIF-4E (12) reveals some interesting similarities. The mutation is located in a conserved motif ($^{K}_{R}$XGGXK$^{F}_{W}$, boxed in Fig. 2) that appears 3 times in the carboxy-terminal part of eIF-4E. Another temperature-sensitive mutant, strain 4-0 (Gly179 → Asp, weak temperature-sensitive phenotype), also shows a substitution of glycine by aspartic acid in the most carboxy-proximal of these repeated motifs. We don't know the function of this sequence which to our knowledge has not been found in other nucleic acid binding proteins.

Furthermore, the cdc33 mutation is located closely to tryptophane 115. Initiation factor eIF-4E contains eight tryptophane residues and they are conserved in number and position between yeast and mouse (14). Tryptophanes 43, 46 and 166 (Fig. 2) were shown to be important for cap binding activity of yeast eIF-4E *in vitro* (14). Two further temperature-sensitive mutants (derived from strain 4-0) also have amino acid substitutions in the vicinity of tryptophane residues (Fig. 2): 4-2 (Glu^{73} → Lys; Glu^{179} → Asp) close to tryptophane 75 and 4-3 (Glu^{103} → Lys; Gly^{179} → Asp) close to tryptophane 104. This indicates that in addition to tryptophane residues also flanking amino acids are important for eIF-4E activity. Note that the mutations in a further derivative of strain 4-0, the temperature-sensitive mutant 4-4 (His ^{85} → Tyr; Gly^{179} → Asp), are not located in the vicinity of tryptophane residues.

Despite the similarities between the cdc33 mutation and other mutations affecting eIF-4E activity, the cdc33 mutation is unique in that it leads to cell cycle arrest at 37°C. This cell cycle arrest is suppressed by the bcy1 mutation (9), a mutation which leads to constitutive activation of cAMP-dependent protein kinase (21). This points to a possible direct or indirect role of eIF-4E in the cAMP signalling system or eIF-4E beeing directly or indirectly a substrate of cAMP-dependent protein kinase.

Elucidation of eIF-4E function and regulation should ultimately lead to the understanding at the molecular level of the link between translation initiation and cell cycle control in yeast.

ACKNOWLEDGEMENT

We thank Dr. K. Matsumoto for sending us strain CB101 and M. Berger for typing the manuscript. This work was supported by the grant Nr. 3.117.81 from the Swiss National Science Foundation to H.T.

*To whom correspondence should be addressed

REFERENCES

1. Shatkin, A.J. (1976) Cell *9*, 645–653
2. Filipowicz, W. (1978) FEBS Lett. *96*, 1–11
3. Banerjee, A.K. (1980) Microbiol. Rev. *44*, 175–205
4. Shatkin, A.J. (1985) Cell *40*, 223–224
5. Edery, I., Pelletier, J. and Sonenberg, N. (1987) in Translational Regulation of Gene Expression (ed. J. Ilan), 335–366, Plenum Press, N.Y.
6. Rhoads, R.E. (1988) Trends Biochem. Sci. *13*, 52–56
7. Altmann, M., Edery, I., Sonenberg, N. and Trachsel, H. (1985) Biochemistry *24*, 6085–6089
8. Altmann, M., Handschin, C. and Trachsel, H. (1987) Mol. Cell. Biol. *7*, 998–1003
9. Brenner, C., Nakayama, N., Goebl, M., Tanaka, K., Toh-E, A. and Matsumoto, K. (1988) Mol. Cell. Biol. *8*, 3556–3559
10. Pringle, J.R. and Hartwell, L.H. (1981) in The Molecular Biology of the Yeast Saccharomyces cerevisiae: life cycle and inheritance (eds. J.N. Strathern, E.W. Jones and J.R. Broach), 97–142, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. Reed, S.I. (1980) Genetics *95*, 561–577
12. Altmann, M., Sonenberg, N. and Trachsel, H., submitted
13. Orr-Weaver, T.L., Szoslak, J.W. and Rothstein, R.J. (1983) Meth. in Enzymol. *101*, 228–245
14. Altmann, M., Edery, I., Trachsel, H. and Sonenberg, N. (1988) J. Biol. Chem. *263*, 17229–19232
15. Edery, I., Altmann, M. and Sonenberg N. (1988) Gene *74*, 517–525
16. Anderson, C.W., Baum, P.R. and Gesteland, R.F. (1973) J. Virol. *12*, 241–252
17. Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA *76*, 4350–4354
18. Chamberlain, J.P. (1979) Anal. Biochem. *98*, 132–135
19. Blake, M.S., Johnston, K.H., Rusell-Jones, G.J. and Gotschlich, E.C. (1984) Anal. Biochem. *136*, 175–179
20. Yaffe, M.P. and Schatz, G. (1984) Proc. Natl. Acad. Sci. USA *81*, 4819–4823
21. Matsumoto, K., Uno, I., Oshima, Y. and Ishikawa, T. (1982) Proc. Natl. Acad. Sci. USA *79*, 2355–2359

This article, submitted on disc, has been automatically converted into this typeset format by the publisher.