The Initiation Factor eIF4A Is Involved in the Response to Lithium Stress in Saccharomyces cerevisiae*

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A gene, TIF2, was identified as corresponding to the translation initiation factor eIF4A and when overexpressed it confers lithium tolerance in galactose medium to Saccharomyces cerevisiae. Incubation of yeast with 6 mM LiCl in galactose medium leads to inhibition of [35S]methionine incorporation. By polysome analysis we show that translation is inhibited by lithium at the initiation step, accumulating 80 S monosomes. We further show by immunoblot analysis that when cells are incubated with lithium eIF4A does not sediment with ribosomal subunits. Overexpression of TIF2 overcomes inhibition of protein synthesis and restores its sedimentation with the initiation complex. In vivo, eIF4A is induced by lithium stress. We have shown previously that lithium is highly toxic to yeast when grown in galactose medium mainly due to inhibition of phosphoglucomutase, an enzyme responsible for the entry of galactose into glycolysis. We show that conditions that revert inhibition of phosphoglucomutase also revert inhibition of protein synthesis. Interestingly, glucose starvation leads to loss of polysomes but not to dissociation of eIF4A from the preinitiation complexes. Overexpression of SIT4, a protein phosphatase related to the TOR kinase pathway, reverses inhibition of protein synthesis by lithium and association of eIF4A with the initiation complex.

We have used Saccharomyces cerevisiae as a eukaryotic cell model to study the mechanisms of the action of lithium. Lithium is a drug that has been used for the last 5 decades to treat manic-depressive disorder (1). Yeast cells adapt to high osmolarity by changing the protein expression patterns (2–4). We have observed previously that lithium toxicity is dependent on the carbohydrate source used for growth (5, 6). Lithium toxicity in galactose medium is not related to osmotic stress because concentrations as low as 15 mM are lethal. Galactose is metabolized in yeast, as in humans, by the Leloir pathway during glycolysis. We have shown that one of the main targets of lithium toxicity in galactose is phosphoglucomutase, the enzyme that converts reversibly glucose-1-P to glucose-6-P (6). Because phosphoglucomutase is an essential protein for galactose metabolism, its inhibition impairs glycolysis and leads to cell death. In glucose-grown cells inhibition of phosphoglucomutase by lithium reduces the levels of UDP-glucose, and consequently, the biosynthetic pathways that use this key metabolite are impaired (6). We have recently cloned the Ser-Thr phosphatase, SIT4, that when overexpressed confers lithium tolerance in galactose medium. We have observed that SIT4 acts in a parallel pathway not involving induction of transcription of the Na+ transporter ENA1; it does not involve extrusion of lithium from the cells but alters the monovalent cation homeostasis and internal pH (5).

In this work we have cloned another gene, TIF2, that when overexpressed confers lithium tolerance in galactose medium. TIF2 encodes an ATP-dependent RNA helicase involved in the initiation of protein synthesis, homologous to mammalian eIF4A1 (7–9), and here will be referred to as eIF4A. Protein synthesis is metabolically important for cell growth and division. This process responds to the nutritional state of the cell and to stress signals (10). Several pathways transduce these signals and modify the protein synthesis machinery. Protein synthesis can be divided into the following three steps: initiation, elongation, and termination. The initiation step is specifically regulated, and the main targets are the initiation factors eIF2α and eIF4E (11). eIF2α is a subunit of the factor eIF2 that binds to Met-tRNA and GTP. GTP is hydrolyzed upon formation of the 43 S preinitiation complex and recognition of the initiation codon in the mRNA. The eIF2α-GDP complex is recycled to eIF2α-GTP by eIF2B. Phosphorylation of eIF2α by amino acid starvation leads to inhibition of protein synthesis, and GTP is not recycled (11).

eIF4 proteins are involved in unwinding of the secondary structure of mRNA and thus facilitate its binding to the 43 S preinitiation complex (12). The proteins required for this event are eIF4F, eIF4A, and eIF4B. eIF4F is composed of three subunits as follows: p220 (eIF4G), p46 (eIF4A), and p24 (eIF4E). eIF4E phosphorylation up-regulates protein synthesis (11). eIF4A is a 46-kDa protein that belongs to the DEAD/DEHX box family of helicases (12). Recently it was shown (13) that eIF4A coexists in the free form and bound to eIF4F. Activity of eIF4A is stimulated by eIF4H and by increasing mRNA binding, and eIF4B stimulates its helicase activity (14). In yeast cells two homologues of eIF4A have been cloned, TIF1 and TIF2. These two are identical at the protein level, and deletion of both is lethal to the cell (7).

In this work we show a novel regulation of protein synthesis by induction of expression eIF4A. We show that lithium inhibi-

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1 The abbreviation used is: eIF4A, eukaryotic initiation factor 4A.
its protein synthesis and that overexpression of eIF4A reverts this inhibition. This effect is related to phosphoglucomutase inhibition and to Ser-Thr phosphatase SIT4.

**EXPERIMENTAL PROCEDURES**

**Strains—**Escherichia coli strain XLI-Blue was used for plasmid construction. S. cerevisiae strains R757 (MATa, his-4-5, leu3-52, lys 9, hOl), and FY833 (MATa, his3Δ200, ura3-52, leu2Δ1, lys2Δ200, trp1Δ63, GAL2Δ1) that were kindly provided by Dr. M. Ghislain were used. Strains JF291-PGM2 (Mata, leu2Δ3-112, his3-1, ura3-52, PGM1, PGM2, PGIt2, and FY333-SIT4) (MATα, his3Δ200, ura3-52, leu2Δ1, lys2Δ200, trp1Δ63, GAL2Δ1, pGAL-PGM2) were described previously (5, 6). FY833-TIF2 (MATα, his3Δ200, ura3-52, leu2Δ1, lys2Δ200, trp1Δ63, GAL2Δ1, pGAL-TIF2) was constructed by transforming strain FY833 with plasmid pRN93-TIF2 as described below.

**Isolation of Genes That Confer Lithium Resistance—**Wild type strain R757 was transformed with an expression cDNA library under control of a galactose-inducible promoter (15). Transformed cells were plated in minimal medium (YNB-gal) containing the following: YNB 6.7 g/liter, galactose 2%, his, ura, lys, and methionine 0.003%, and 30 ml LiSO4. Only six recombinants expressing a protein conferring lithium resistance were able to overcome the lithium stress. The plasmid from the selected colonies was isolated, and the DNA insert was subcloned in pBlueScript SK− and sequenced using AutoRead Sequencing Kit and ALF DNA Sequencer (Amersham Biosciences). The sequence was compared with the known open reading frames from the S. cerevisiae genome database.

**Characterization of Clones Resistant to Lithium—**The DNA insert of the selected clone was subcloned in the expression vector pRN93 (kindly donated by Dr. C. W. Slattery from Yale University), which contains a GAL1 promoter and the URA3 gene marker. This plasmid was named pRN93-TIF2. This plasmid was used to transform strain FY833 by the lithium acetate method (16).

**eIF4A Incorporation—**Yeast cells were grown to mid-logarithmic phase (Nmax = 1.0) on YP-galactose medium and incubated with 6 mM LiCl. An aliquot of 0.1 ml was withdrawn, and 0.1 mCi of [35S]methionine (200 Ci/mmol) was added. Incorporation was stopped with 1 N NaOH and incubated at 30 °C for 10 min. Trichloroacetic acid to a final concentration of 10% was added together with 2% bovine serum albumin. The precipitate was collected by vacuum filtration using Whatman GF/A glass fiber filters. For determination of incorporation, the filter was counted on a Packard 1600CA counter using scintillation mixture.

**[35S]Methionine Uptake—**Yeast cells were grown to mid-logarithmic phase (Nmax = 1.0) on YP-galactose medium and incubated with 6 mM LiCl. An aliquot of 3 ml was collected and washed twice with 2% galactose. Cells were resuspended in 700 ml of 2% galactose plus 20 μl [35S]methionine (6000 cpm/μmol) at 30 °C. Aliquots were withdrawn after 60, 120, and 600 s of incubation and diluted in 1 ml of 20 mM icc cold methionine. Cells were collected by vacuum filtration using 0.45 μm Millipore filters. For determination of uptake, the filter was counted on a Packard 1600CA counter using scintillation mixture.

**Antibody Preparation—**Polyclonal antiserum was raised against GST-eIF4A, a fusion of glutathione S-transferase protein and the first 40 amino acids encoded by the TIF2 gene. These first 40 amino acids are the most variable among yeast RNA helicases but are identical to TIF1 gene. To obtain the 5′ end of the TIF2, genomic DNA was amplified using the following primers. The upstream primer was 5′-cgg tag gcc tgt cat act gat g 3′, which introduces a BamHI site shown in italics, and the downstream primer 5′-cgg cta gcc gta acc gaa acc act taa 3′, which introduces an Xhol site shown in italics and a termination codon. The PCR product was subcloned in pBluescript II SK− (Stratagene, La Jolla) using the AutoRead sequencing kit and sequenced using an ALF DNA sequencer (Amersham Biosciences). After confirming the sequence, the PCR product was subcloned into plasmid pGEX 4T-3 (Amersham Biosciences) into the BamHI and Xhol sites, and the E. coli strain XLI-Blue was transformed. Expression of the protein was induced with isopropyl-thio-β-D-galactopyranoside and purified in a glutathione-agarose column as indicated by the manufacturer. A first subset of 40 μl of a diluted culture was used after 2 h of incubation, 200 μl of purified protein was given to New Zealand rabbit, accompanied by complete Freund’s adjuvant. Other boosts at 3-week intervals were given with an equal amount of GST-eIF4A protein and incomplete Freund’s adjuvant. The rabbit was sacrificed, and the serum was tested in immunoblot using a dilution of 1:10,000. The serum recognizes a unique band of ~45 kDa in a total yeast extract as expected for eIF4A. As a control, pre-immune serum was used and did not recognize this band.

**RESULTS**

**TIF2 Confers Lithium Resistance—**Lithium is toxic to S. cerevisiae at concentrations as low as 10 mM when grown in galactose but not to yeast grown in other carbon sources such as glucose, sucrose, ethanol, or glycerol (6). In a previous work (5) we have screened a cDNA expression library under the control of a galactose-inducible promoter for clones that conferred lithium resistance in YNB galactose medium. We have reported the isolation of the gene SIT4 and partially characterized its involvement in lithium resistance. In this work we show the characterization of a second clone isolated from the same screen that after sequencing was identified as TIF2. This gene encodes the translation initiation factor known in mammals as eIF4A. As shown in Fig. 1A, overexpression of TIF2 conferred resistance to low lithium concentrations.

**Inhibition of Protein Synthesis by Lithium—**The involvement of a translation initiation factor on lithium stress led us to study the effect of lithium on protein synthesis. In Fig. 1B we show that incorporation of [35S]methionine into acid-precipitable protein was inhibited by addition of 6 mM lithium in a time-dependent manner. After 2 h of lithium incubation, [35S]methionine incorporation is 90% inhibited. Overexpres-
sion of TIF2 partially reverts this inhibition to 65%. In contrast to galactose, [35S]methionine incorporation in glucose-grown cells is not inhibited by addition of 50 mM LiCl (Fig. 1B). It is important to point out that inhibition of [35S]methionine incorporation could also be due to a reduction of the total methionine pool. We measured [35S]methionine uptake, and our results show that incubation of cells for 2 h in 6 mM lithium leads to a reduction of [35S]methionine uptake from an initial rate of 0.613 ± 0.094 to 0.323 ± 0.071 nmol/min/mg dry weight (n = 3). Although [35S]methionine uptake is inhibited by lithium (50% inhibition), incorporation is much more affected (90% inhibition) under the same experimental condition (Fig. 1B) indicating that lithium inhibits protein synthesis at another step.

We studied by polysome analysis the step at which translation is inhibited by lithium. When the wild type strain was grown in galactose and incubated for 4 h in 30 mM lithium, there was a marked increase in the level of 80 S ribosomes and a decline in the level of translating polysomes indicating that it is inhibited at the initiation step (Fig. 2). This effect is not seen even by the addition of a lethal dose of LiCl (250 mM) to glucose-grown cells (Fig. 2) confirming the result of methionine incorporation.

Overexpression of TIF2—Yeast was incubated with a sublethal dose of lithium (6 mM), and the effect of eIF4A overexpression and its localization were studied (Fig. 3). Analysis of the polysome profile of eIF4A-overexpressing cells shows that increasing the amount of eIF4A reversibly partially the accumulation of monosomes and increases the amount of polysomes (Fig. 3C) in comparison with the wild type cells treated with lithium (Fig. 3B). To determine the association of eIF4A to preinitiation complex, the fractions of the sucrose gradient were analyzed by immunoblot using an antibody raised against yeast eIF4A. Interestingly, we show that eIF4A in wild type yeast growing in the absence of lithium sedimented at the same position as ribosomal subunits (fractions 1–10, Fig. 3A) indicating association with the preinitiation complex. However, upon incubation with lithium it is only present in the top of the gradient (fractions 1–4, Fig. 3B). These results suggest that lithium induces eIF4A dissociation from the preinitiation complex. In Fig. 3C we show that upon overexpression of eIF4A this effect is reverted. Even in the presence of lithium, eIF4A is present on the first 10 fractions as shown for the wild type cells grown in the absence of lithium and in higher amounts. These results suggest that the increase in the amount of eIF4A is an important factor in regulation of protein synthesis.

Induction of Expression of eIF4A by Lithium Stress—Induction of transcripts involved in protein synthesis is a response of yeast to high salinity (4). We determined by Western blot whether expression of eIF4A is regulated by lithium stress (Fig. 4). eIF4A was induced by sublethal concentrations of lithium (6 and 15 mM). The induction of expression of eIF4A is time- and dose-dependent (Fig. 4B). These results suggest that overexpression of eIF4A might be a physiological response to lithium stress in galactose medium.

Overexpression of Phosphoglucomutase Reverts Inhibition of Protein Synthesis—Inhibition of protein synthesis at the initiation step has been shown to occur after depletion of glucose or fructose (20, 21). In a previous report (6) we have shown that lithium inhibits with high affinity phosphoglucomutase, an essential enzyme for galactose metabolism. We tested whether inhibition of protein synthesis by lithium is linked to inhibition of phosphoglucomutase and, thus, to reduced glycolytic flux. In vivo inhibition of phosphoglucomutase by lithium is reverted by overexpression of PGM2 or addition of high concentrations of Mg2+ to the medium (6). We treated wild type yeast cells on the presence of 100 mM Mg2+ or yeast overexpressing PGM2 with 6 mM lithium. After different time intervals cells were pulse-labeled with [35S]methionine, and incorporation into acid-precipitable material was measured. Results show that inhibition of protein synthesis was reverted in these conditions (Fig. 5A). Polysome analysis (Fig. 5B) shows that the polysome profile is normal in cells treated with lithium in the presence of 100 mM MgCl2. These results clearly show that inhibition of protein synthesis is linked to inhibition of phosphoglucomutase activity by lithium.

As a control, we tested whether the effect of overexpression of TIF2 on protein synthesis is due to protection of glycolysis (Table I). In TIF2-overexpressing and in wild type cells the production of ethanol was inhibited ~65%. These results indicate that overexpression of TIF2 interferes with the effect of lithium only on the rate of protein synthesis but not on fermentation.

A Link between SIT4 and TIF2?—SIT4, a type 1/type 2A-related protein phosphatase, is involved in the response to nitrogen starvation (22–24) and salt stress (25). Overexpression of SIT4 confers lithium resistance in galactose medium (5).
We tested whether this lithium resistance is linked to protection of the inhibition of protein synthesis. Overexpression of \textit{SIT4} partially protects protein synthesis from lithium inhibition. This is shown by measuring incorporation of $[^{35}\text{S}]$methionine into acid-precipitable material (Fig. 6A) or by polysome analysis (Fig. 6C). Furthermore, Western blot analysis of the polysome fractions shows that eIF4A sediments with ribosomal subunit fractions even in the presence of lithium in \textit{SIT4} overexpressing strain. Our results show that overexpression of \textit{SIT4} stimulates sedimentation of eIF4A with preinitiation complex. \textit{SIT4} could enhance protein synthesis by overcoming inhibition of the glycolytic flux by lithium. However, data in Table I show that this is not the case because the glycolytic flux of cells overexpressing \textit{SIT4} is also inhibited 65% as shown above for the wild type strain. These results indicate that overexpression of \textit{SIT4} does not revert inhibition of glycolysis by lithium but is probably involved on the signaling pathway controlling protein synthesis.

\textbf{Lithium Versus Glucose Starvation}—Lithium treatment in galactose-grown cells leads to inhibition of protein synthesis as has been shown for glucose starvation (20, 21). In order to test if these two treatments lead to the dissociation of eIF4A from the preinitiation complexes, we have imposed glucose starvation in the absence of lithium by shifting cells to 0.05% glucose (Fig. 7). Interestingly, glucose starvation leads to a loss of polysomes but not to dissociation of eIF4A from the preinitiation complexes. At this condition eIF4A is accumulated on the monosome (80 S) peak (Fig. 7B). This result shows that the translational response to lithium treatment in galactose-grown cells is different from the re-
sponse to glucose starvation. Furthermore we show that neither overexpression of SIT4 (Fig. 7C) nor elf4A (Fig. 7D) reverts inhibition of translation under glucose starvation.

**DISCUSSION**

Besides the beneficial therapeutic effects of lithium, toxic effects have also been studied. In fact, the first studies on lithium action were on phenotypic effects on development. LiCl induces vegetalization in sea urchin embryos (26) and inhibits the formation of the dorsal-ventral axis in *Xenopus laevis* embryos (27) and the cell fate determination in the slime mold *Dictyostelium discoideum*. Wolcott (28) demonstrated that vegetalization was induced only to impaired protein synthesis and not to mRNA degradation. Developmental defects have also been related to the inhibition of GSK-3β, a highly conserved serine/threonine protein kinase implicated in cell fate determination and hormonal signaling (29). In this work we have shown that lithium inhibits translation initiation in cells grown in galactose and that overexpression of elf4A partially reverts this effect and confers lithium resistance (Figs. 1 and 2). Inhibition of translation is not observed in glucose-growing cells even at a lethal dose of lithium (Fig. 2). These results suggest that lithium is not inhibiting directly an enzyme of the protein synthesis machinery. Inhibition must be due to an indirect effect of lithium. We have shown that lithium inhibits phosphoglucomutase activity and the glycolytic flux when galactose is used as the carbon source (see Ref. 6 and this work).

We have shown that eIF4A is normally distributed along the polysomes and on top of the gradient where no ribosomal RNA is present. Upon lithium addition, it is only found in the free form and associated to elf4F (12), and it has been shown that it interacts directly with elf4F (30). Two different modes of regulation of elf4F complex have been described in yeast. The first one involves the yeast elf4E-binding protein p20 (4E-BP) that binds to elf4E, displaces elf4G, and therefore dissociates the elf4F factor (31). The second involves proteolysis of elf4G that depends on the TOR pathway (32). Our results show that elf4A is normally distributed along the sucrose gradient on fractions that contain the 40 S and 60 S ribosomal subunits and on top of the gradient where no ribosomal RNA is present. Upon lithium addition, it is only found on the top of the gradient (Fig. 3, A and B). This result suggests that the fraction of elf4A associated to preinitiation complexes disappears. Upon induction of elf4A expression the dissociation is reverted, and inhibition of protein synthesis is partially restored. Therefore, another factor that might regulate initiation of protein synthesis is the induction of expression of elf4A. In mouse two elf4A proteins are expressed, elf4AAI and elf4AII. The ratio of mRNA levels that encode for these two proteins varies among tissues, and it has been postulated that its concentration could influence the initiation process (33). RNA helicase activity is also induced by binding of elf4A to elf4F (14, 34), elf4B, and elf4H (14). Our results show that cells physiologically respond to lithium stress up-regulating expression of elf4A. This might be important for protein synthesis regulation because *TIF2/elf4A* overexpression confers lithium tolerance (Fig. 4). However, the induc-

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<tr>
<th>Strain</th>
<th>Fermentation rate Control</th>
<th>Fermentation rate + 6 mM LiCl</th>
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<tbody>
<tr>
<td>FY833</td>
<td>1.65 ± 0.40</td>
<td>0.52 ± 0.23</td>
</tr>
<tr>
<td>FY833-TIF2</td>
<td>1.29 ± 0.40</td>
<td>0.44 ± 0.07</td>
</tr>
<tr>
<td>FY833-SIT4</td>
<td>1.42 ± 0.42</td>
<td>0.52 ± 0.19</td>
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**Table I**

Lithium inhibits fermentation in galactose grown cells

The fermentation rate from yeast cells grown in YPGal medium with or without 6 mM LiCl were assayed as described under “Experimental Procedures.” Results are represented as mean ± S.D. of three independent experiments.

**Fig. 6. Overexpression of SIT4 overcomes inhibition of protein synthesis induced by lithium stress.** A, yeast strains FY833 (○) and FY833-SIT4 (●) were grown in YPGal medium to mid-log phase in galactose medium and treated with LiCl 6 mM. An aliquot of 0.1 ml was withdrawn at the indicated times after lithium addition, and 0.1 mCi of [35S]methionine (200 Ci/mmol) was added. Incorporation of [35S]methionine was measured. B and C, the polysome profile of strain FY833 (B) or FY833-SIT4 (C) treated with 6 mM LiCl was analyzed. An aliquot of 0.2 ml of each fraction were analyzed by immunoblotting using an antibody against elf4A, P, polysomes.
Involvement of TIF2 and SIT4 in Lithium Stress

Here we present evidence that SIT4, a component of the TOR pathway, is involved in the regulation of protein synthesis in response to lithium stress. SIT4 overexpression confers lithium resistance in galactose medium (5) and protects translation initiation from inhibition (Fig. 6). Sit4p associates with several regulatory subunits. Association with Tap42p (22, 35) regulates initiation of protein synthesis signaled by the TOR kinase pathway. Sit4p-Tap42p complex is dissociated when cells are deprived of amino acids or when treated with the drug rapamycin, leading to inhibition of translation (24). Overexpression of SIT4 or mutation of TAP42 (tap42-11) confer resistance to rapamycin by maintaining its association (24). The regulation of protein synthesis downstream of Sit4p-Tap42p is not well understood, but it was shown that rapamycin treatment leads to degradation of the initiation factor eIF4G (32). Here we show that localization of eIF4A is modulated by SIT4. Overexpression of SIT4 leads to eIF4A association to preinitiation complexes even in the presence of lithium (Fig. 6). These results suggest that the dissociation of eIF4A from preinitiation complex induced by lithium might involve eIF4G degradation.

In S. cerevisiae different signals have been reported to inhibit protein synthesis such as amino acid deprivation, depletion of purine (36, 37), and depletion of glucose (20, 21). Here we show that protein synthesis is also inhibited when glycolysis is halted at the step catalyzed by phosphoglucomutase in galactose-growing cells. We have tested whether glucose starvation leads to dissociation of eIF4A from the preinitiation complexes as shown for lithium. Surprisingly, glucose starvation leads to polysome loss but not dissociation of eIF4A (Fig. 7). In fact eIF4A accumulates in the monosome fraction during glucose starvation. This result suggests that initiation complex is not dissociated during glucose starvation, whereas lithium dissociates eIF4A. Glucose sensing involves several well characterized pathways; however, it is difficult to differentiate between the mechanisms exerted by extracellular glucose or by intermediate metabolites of glycolysis (reviewed in Refs. 38 and 39). Ashe et al. (21) has related three signaling pathways to inhibition of protein synthesis derived from glucose depletion, cAMP, SNF1, and SNF/RGT2, because mutations within these pathways overcome inhibition of protein synthesis. They also show that the TOR pathway is not involved in the response to glucose starvation. They have used the tap42-11 mutant (resistant to rapamycin) and showed that this mutant does not protect from loss of polyribosomes upon glucose removal. Our results contrast with those published by Ashe et al. (21) because overexpression of SIT4 protects from loss of polyribosomes when glycolysis is blocked by lithium. Our data present growing evidence that there is a cross-talk between lithium stress, deprivation of nutrients, and initiation of protein synthesis. We identified TIF2 as a new gene involved in this mechanism.

**Fig. 7.** Glucose starvation leads to accumulation of eIF4A in the monosome fraction. Yeast strains FY833 (A and B), FY833-SIT4 (C), and FY833-TIF2 (D) were grown in YPD medium to mid-log phase in galactose medium and centrifuged at 3,000 x g for 10 min. Afterward they were resuspended in 50 ml of YPD (A) or YP plus 0.05% glucose (B-D) and further incubated for 25 min. Cycloheximide was added to a final concentration of 60 µg/ml before harvesting, and the polysome profile was analyzed in a 15–45% sucrose gradient. An aliquot of 0.2 ml of every two fractions was analyzed by immunoblotting using an antibody against eIF4A. P, polysomes.
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