The interaction of the cap-binding complex (CBC) with eIF4G is dispensable for translation in yeast

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ABSTRACT

In eukaryotes, the m7GpppN cap structure is added to all nascent RNA polymerase II transcripts, and serves important functions at multiple steps of RNA metabolism. The predominantly nuclear cap-binding complex (CBC) binds to the cap during RNA synthesis. The predominantly cytoplasmic eukaryotic initiation factor 4F (eIF4F) is thought to replace CBC after export of mature mRNA to the cytoplasm, and mediates the bulk of cellular translation. Yeast as well as mammalian CBC interacts in vitro with eIF4G, a subunit of eIF4F. In this work, we investigate a potential role of this interaction during translation in yeast. We identify a mutation (DR548/9AA) in Tif4631p, one of two isoforms of yeast eIF4G, that abolishes its binding to CBC. Cells expressing this mutant protein as the sole source of eIF4G grow at wild-type rates, and bulk cellular translation, as assessed by metabolic labeling and polysome profile analysis, is unchanged. Importantly, we find that the DR548/9AA mutation neither diminishes nor delays the translation of newly induced reporter mRNA. Finally, microarray analysis reveals marked transcriptome alterations in CBC subunit deletion strains, whereas eIF4G point mutants have essentially a wild-type transcriptome composition. Collectively, these data suggest that in yeast, the phenotypic consequences of CBC deletions are separable from its interaction with eIF4G, and that the CBC–eIF4G interaction is dispensable for a potential “pioneering round” of translation in yeast.

Keywords: Cap-binding protein; CBC; eIF4E; “pioneer round” of translation

INTRODUCTION

The m7GpppN cap structure at the 5′ end of eukaryotic mRNAs influences many aspects of their metabolism including splicing (Izaurralde et al. 1994; Lewis et al. 1996), 3′ end formation (Flaherty et al. 1997), export from the nucleus (Görlich et al. 1996; Visa et al. 1996; Shen et al. 2000), stability (Furuichi et al. 1977), and translation (Shatkin 1985). Principally, the roles of the cap structure are mediated through the binding and dynamic interchange of specific protein complexes (Lewis and Izaurralde 1997; Mitchell and Tollervey 2001; Preiss 2002). Two well-characterized complexes of this kind are the predominantly nuclear CBC and the predominantly cytoplasmic eIF4F. CBC is a heterodimer consisting of cap-binding protein (CBP) 20 (or MUD13 in yeast) and CBP80 (STO1 or GCR3 in yeast; Ohno et al. 1990; Colot et al. 1996). The smaller subunit CBP20 contains a common RNA-binding motif, the RNP domain (Izaurralde et al. 1994) and is highly conserved from yeast to human. CBP80 is far less conserved and comprises three tandem domains resembling the middle domain of eIF4G (MIF4G, see below; Aravind and Koonin 2000; Ponting 2000). CBP20 recognizes capped RNA in conjunction with eIF4F, and the RNA-helicase eIF4A (Dominguez et al. 1999; Gingras et al. 1999; Neff and Sachs 1999). CBP80 recognizes capped RNA in conjunction with eIF4F (Mazza et al. 2002). Furthermore, CBC exits the nucleus to the cytoplasm together with the mRNA (Görlich et al. 1996; Visa et al. 1996; Shen et al. 2000) where it is thought to be replaced by the eIF4F complex.

The eIF4F complex plays a central role in cap-dependent initiation of translation (for review, see Gingras et al. 1999). eIF4F consists of the cap-binding subunit eIF4E, the modulatory protein eIF4G, and the RNA-helicase eIF4A (Dominguez et al. 1999; Gingras et al. 1999; Neff and Sachs 1999). There are two isoforms of eIF4G in yeast (eIF4G1 and 2), which are encoded by the TIF4631 and TIF4632 genes (Goyer et al. 1993). Like CBP80, eIF4G is a member of the MIF4G do-
main family of proteins, which comprises several proteins involved in RNA metabolism (Aravind and Koonin 2000; Ponting 2000). In addition to eIF4E and eIF4A, eIF4G also interacts with the poly(A)-binding protein PABP (Pab1p in yeast; Tarun and Sachs 1996). Based on observations in higher eukaryotes (Lamphear et al. 1995), it is also thought to contact the small ribosomal subunit-associated multimeric factor eIF3, thus recruiting translation preinitiation complexes to the mRNA. The interaction between PABP and eIF4G facilitates the functional association of the 3′ end of an mRNA with its 5′ end to promote translation (Tarun et al. 1997; Preiss 2002), whereas the association of eIF4G with eIF4E markedly enhances the binding of the latter to the cap structure (Haghighat and Sonenberg 1997).

Interestingly, eIF4G interacts with CBC in yeast as well as in human cells (Fortes et al. 2000; McKendrick et al. 2001). In yeast, the interaction site for CBC on eIF4G is located between the eIF4E-binding motif and the MIF4G domain (Fortes et al. 2000). eIF4E and CBC can simultaneously bind to eIF4G, but adversely affect each others affinity for eIF4G. Moreover, CBC addition to yeast in vitro translation reactions inhibited cap-dependent translation in wild-type extracts but stimulated the expression of capped mRNA in extracts containing a mutant form of eIF4G that is deficient for eIF4E and Pab1 binding (Fortes et al. 2000). These findings are consistent with a role for the CBC-eIF4G interaction in the exchange of CBC for eIF4F and/or a direct recruitment of nascent mRNA for translation. Based on conventional thinking, these events might be expected to take place once the mRNA reaches the cytoplasm or concomitantly with its nucleocytoplasmic transport. However, because CBC ( Görlich et al. 1996) and many components of the translation machinery (Iborra et al. 2001; Dahlberg et al. 2003; Nathanson et al. 2003) are present in both cellular compartments, it cannot be excluded that the eIF4G-CBC interaction functions in the nucleus. Indeed, the interaction between the mammalian factors was revealed in nuclear preparations (McKendrick et al. 2001). Further support for a direct role of CBC in translation comes from recent work in human cell lines suggesting that both nuclear and cytoplasmic nonsense mediated decay (NMD) occurs on CBC-associated rather than eIF4E-associated mRNA (Ishigaki et al. 2001; Lejeune et al. 2002). NMD is a surveillance mechanism comprising the recognition and subsequent degradation of mRNAs bearing a premature termination codon and, importantly, is translation dependent (Hentze and Kulozik 1999; Maquat and Carmichael 2001; Schell et al. 2002; Wilkinson and Shyu 2002). Thus, it has been proposed that NMD takes place during a “pioneering round” of translation that is stimulated by CBC—perhaps through its interaction with eIF4G.

In this study, we investigate a potential role of CBC in translation in the yeast Saccharomyces cerevisiae. For this purpose, we examine the CBC-binding site of eIF4G1, identify a point mutation (DR548/9AA) that abolishes its interaction with CBC, and study the phenotypic consequences of this mutation in vivo. In contrast to deletion of either CBC subunit in vivo, we find that cells expressing eIF4G1 with the (DR548/9AA) substitution as the sole source of eIF4G (termed eIF4G1–548 protein and 4G1–548 strain in the following) show no detectable phenotype at the level of cell growth or transcriptome composition. Unlike eIF4G1–459, a mutant protein with reduced binding to eIF4E, eIF4G1–548 shows no defect in bulk cellular translation, and we find no detectable delay in the translation of newly made mRNA molecules. Taken together, these data suggest that the eIF4G-CBC interaction is dispensable in yeast. Moreover, the indistinguishable kinetics of translation of an induced reporter mRNA in eIF4G1–548 and wild-type cells reveal that the first round of mRNA translation does not require the wild-type eIF4G–CBC interaction.

RESULTS

A point mutant of eIF4G1 defective in CBC binding

The minimal CBC-interacting region of the yeast eIF4G1 protein was previously shown to reside between amino acid residues 490 and 592, with a further contribution by amino acids 593–656 (Fortes et al. 2000). To isolate point mutants with reduced CBC binding for further functional analyses, we assayed the binding of a suitable wild-type eIF4G1 fragment (eIF4G1486–656) as well as a series of amino acid substitution mutants to CBC in vitro (Fig. 1). Antibody-immobilized CBC was incubated with in vitro translated, [35S]-labeled eIF4G1486–656 proteins, as previously described (Fortes et al. 2000). Quantitative analysis of labeled proteins bound to CBC after extensive washes revealed strongly reduced binding with mutants in the region from amino acids 527 to 549 (lanes 9–16). The mutant protein eIF4G1–548, corresponding to alanine substitutions of an aspartate and an arginine residue in positions 548 and 549, lacked any detectable CBC binding (lanes 15–16).

To study the functional consequences of the eIF4G interaction with CBC in vivo, we chose to employ a series of yeast strains expressing the eIF4G1 protein from a centromeric plasmid as a sole source of eIF4G (Table 1). Wild-type eIF4G1 protein was used as a reference (strain 4G1). Additionally, we introduced versions of eIF4G1 carrying the (DR548/9AA) substitution to affect binding to CBC (strain 4G1–548) or the previously described (LL459/60AA) substitution affecting the interaction of eIF4G with eIF4E (strain 4G1–459; Tarun and Sachs 1997), alone or in combination (strain 4G1–459,548; Fig. 2A). To ascertain the efficacy of the (DR548/9AA) substitution in vivo, we performed immunoprecipitation experiments (Fig. 2B) using antibodies raised against CBP80 protein (Görlich et al. 1996). Because the level of complex formation between CBC and wild-type eIF4G1 is below detection limits in this type of approach (Fortes et al. 2000), we chose to compare
strains 4G1–459 and 4G1–459,548. Through an elimination of negative cooperative effects between eIF4E and CBC, the –459 mutation increases the amount of CBC-eIF4G complex present at steady state to detectable levels (see Fig. 2B, lane 5, and Fortes et al. 2000). In this context, the –548 mutation strongly reduces the eIF4G1 signal in the CBC-bound fraction (lane 6). The residual level of CBC with strain 4G1–548 represents nonspecific background binding to the antibody resin, as shown by performing a control experiment with a /H9004 cbp80 yeast strain. Even in this context, a small amount of eIF4G1 signal is still detectable in the bead-bound material (lane 4).

These results show that the –548 mutation also impairs the eIF4G-CBC interaction in vivo.

In agreement with earlier reports (Taran and Sachs 1997), the strain 4G1–459 has a marked growth phenotype in complete medium at 30°C when compared to the reference strain 4G1 (145 versus 90 min doubling time, respectively, in liquid culture; Fig. 2C). By contrast, the strain 4G1–548 grows normally (90 min doubling time), and the combination of both mutations in strain 4G1–459,548 causes no additional growth retardation (145 min doubling time). Analysis of the strains on galactose-based medium and various temperatures (16°C, 23°C, 30°C, and 37°C) revealed no contribution to the growth phenotype by the –548 mutation (data not shown). These results argue that the elf4G-CBC interaction is dispensable in vivo, even when the binding between elf4G and elf4E is compromised.

### TABLE 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Systematic name</th>
<th>Name used in this study</th>
<th>Disrupted interaction</th>
<th>Plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td>YAS 2632 (a)</td>
<td>4G1</td>
<td>none</td>
<td>TRP1-CEN-tlf4631</td>
</tr>
<tr>
<td>YAS 2757</td>
<td>4G1–548</td>
<td>CBC</td>
<td>TRP1-CEN-tlf4631-548</td>
</tr>
<tr>
<td>YAS 2074 (b)</td>
<td>elf4E</td>
<td>none</td>
<td>TRP1-CEN-tlf4631-459</td>
</tr>
<tr>
<td>YAS 2758 (a)</td>
<td>elf4E, CBC</td>
<td>none</td>
<td>TRP1-CEN-tlf4631-549,548</td>
</tr>
<tr>
<td>YBJ 220 (b)</td>
<td>CBC</td>
<td>none</td>
<td>TRP1-CEN-tlf4631-URA-CEN-pGal-Luc</td>
</tr>
</tbody>
</table>

**Name Genotype**

| Δebp80          | MATa ade2 ade3 his3 leu2-3,112 trp1 ura3 ycbp80/gcr3::TRP1 |
| Δebp20          | MATa ade2 ade3 his3 leu2-3,112 trp1 ura3 ycbp20/mud13::HIS3 |

α: These strains have the following genotype: MATa ade2-1 his3-11,15 leu2-3,111 trp1-1 ura3-1 pep4::HIS3 tlf4631::leu2 trl4632::ura3.

β: This strain is described by Taran and Sachs (1997); it has the following genotype: MATa ade2-1 his3-11,15 leu2-3,111 trp1-1 ura3-1 pep4::HIS3 tlf4631::LEU2.

6: YBJ 220 and YBJ 221 derived respectively from the strains YAS 2632 and YAS 2757.
In the context of the –459 mutation (compare strain 4G1–459 with 4G1–459,548). These findings are substantiated by polysome profile analyses of extracts from strains expressing the different eIF4G mutants on linear sucrose density gradients (Fig. 3B). The 4G1–459 strain displays a drastic decline in polysomal complexes and a concomitant increase in monosome complexes (Fig. 3B, compare the absorbance profiles at 254 nm from strain 4G1–459, right panel, and the 4G1 strain, left panel), typical of a defect in translation initiation. By contrast, the –548 mutation induces no appreciable profile shifts in either context (compare profiles within each panel). These results demonstrate that the –548 mutation has no significant effect on bulk translation in exponentially growing cells.

The eIF4G-CBC interaction is dispensable for efficient translation of newly made mRNAs

In mammalian cells, the first (“pioneer”) round of translation has been suggested to be mediated by CBC rather than eIF4E (Lejeune et al. 2002). In this context, CBC might require its interaction with eIF4G (Ishigaki et al. 2001; Wilkinson and Shyu 2002). Because only a small proportion of total cellular protein synthesis would derive from the first round of translation at steady state, we devised an experiment to measure the accumulation of protein product from newly made mRNA molecules in cells harboring different forms of eIF4G1 protein. For this purpose, we constructed the yeast strains YBJ 220 and YBJ 221 (Table 1), which are derivatives of the strains 4G1 and 4G1–548, respectively, and bear an additional plasmid encoding a luciferase reporter mRNA under the control of a strong, galactose-inducible promoter. Both strains were shifted to galactose-based media and aliquots of cells collected for luciferase activity assays at multiple time points ranging from 0 to 90 min (Fig. 4). Given the strong and rapid induction of luciferase gene transcription, it is a reasonable assumption that the accumulation of luciferase activity over time directly reflects the initial recruitment of newly made luciferase mRNA for translation. We measured steep increases of luciferase activity over time (~50-fold within 60 min) that were, however, completely indistinguishable between the two yeast strains analyzed,
even at the earliest time points measured (that is, 10 min). These results argue against the eIF4G-CBC interaction as a requirement for a pioneering round of translation in yeast.

FIGURE 3. Effects of eIF4G mutants on general translation activity. (A) The amount of newly synthesized proteins in different strains was measured by pulse labeling with \(^{35}\)S-methionine. The data are expressed as a percentage of methionine incorporation measured with each mutant strain relative to the 4G1 wild-type control. Shown are averaged results from 10 to 16 measurements corresponding to 3 to 5 independent yeast cultures with standard deviations. (B) Polysome profile analysis. Yeast extracts from the indicated strains were analyzed by sucrose density gradient centrifugation. Absorbance profiles at 254 nm were recorded after centrifugation. The profiles are representative of several biological repeat experiments.

Effects of disrupting CBC function on the composition of the cellular transcriptome

Analysis of transcriptome changes using DNA microarrays is an alternative way to uncover the cellular consequences of compromised CBC function. Given the tight coupling between multiple steps in mRNA metabolism, including translation and mRNA decay, it is conceivable that a selective impairment of a given step for specific mRNAs might ultimately be reflected in an altered steady-state level of that mRNA. Thus, we chose to perform transcriptome profiling experiments for a selected panel of yeast strains, including 4G1–459, 4G1–548, and the wild-type reference strain 4G1, as well as strains lacking either subunit of CBC (strains \(\Delta cbp80\) and \(\Delta cbp20\)) and a suitable wild-type control. Total RNA was isolated and subjected to microarray analysis using cDNA microarrays comprising approximately 95% of all annotated ORFs in the yeast genome as detailed in Materials and Methods. Two separate array experiments were performed for each mutant to wild-type comparison with fluorescent dye swap (Fig. 5). Using a two-fold threshold for changes in expression level, both CBP deletion strains revealed relevant transcriptome alterations (Fig. 5D,E). Strain \(\Delta cbp20\) displayed increased levels for 18 and decreases for 34 different mRNAs. In strain \(\Delta cbp80\), 33 different mRNAs scored as increased and 41 as decreased in levels relative to wild-type cells. A proportion of these mRNAs showed consistent changes in both deletion strains: 15 mRNAs had a decreased abundance, and 5 genes an increased abundance (see Table 2 for a list of these mRNAs). By contrast, the analysis of the strains 4G1–548 and 4G1–459 reveal no significant specific alterations in transcriptome composition (Fig. 5B,C). Calculating a coefficient of pairwise correlation (as described in the legend of Fig. 5) as a measure to compare the array data sets with a self-to-self control experiment (Fig. 5A) confirmed this notion: al-

FIGURE 4. Translation of newly made mRNA is independent of the eIF4G-CBC interaction. The yeast strains 4G1 and 4G1–548 were transformed with a plasmid expressing the luciferase reporter mRNA under the control of a galactose-inducible promoter. The resulting strains YBJ 220 and YBJ 221, respectively, were used to monitor the accumulation of luciferase activity over time after galactose induction as described in Materials and Methods. The luciferase activity was normalized against the number of cells per sample. The graph represents the average of three independent experiments.
though both CBC deletion strains scored as significantly different from the self-to-self control (0.637 for the strain Δcbp20 and 0.593 for the Δcbp80), both elf4G point mutants are virtually indistinguishable from the wild-type control (0.071 for 4G1–548 and 0.056 for 4G1–459). Remarkably, we do not register any significant transcriptome alterations in strain 4G1–459, despite its marked translational and growth phenotype. Note that the microarray data normalization would “mask” any uniform changes in the amount of all mRNA present in cells to focus on mRNAs that deviate from any general trend (the normalization strategy is described in Materials and Methods). These data indicate that alterations of the transcriptome composition due to deletions of either CBC subunit are not primarily due to a disruption of the CBC–elf4G complex.

DISCUSSION

The study presented here extends our previous investigations on the interaction between CBC and elf4G in yeast. Using a series of amino acid substitution mutants in elf4G1 protein, we identify critical amino acids for CBC binding that lie approximately in the middle of the region that was previously mapped for CBC binding (Fortes et al. 2000). In particular, we identify one mutation (DR548/9AA) that abolishes binding to CBC in vitro and in vivo. Surprisingly, our experiments reveal no detectable phenotypic consequences of the disrupted CBC–elf4G complex formation at various levels of analysis. Cells expressing the elf4G1–548 protein grow normally under laboratory conditions, and display wild-type levels of bulk protein synthesis. Translation of a newly made reporter mRNA is unaffected and shows no kinetic delay associated with a lack of interaction between CBC and elf4G. Furthermore, we find no phenotypic differences between a strain carrying a doubly mutated elf4G protein with disrupted binding to both elf4E and CBC (4G1–459,548) and a strain just compromised in elf4E-binding (4G1–459). This may suggest that the previously observed synthetic lethality between the elf4G1–459 protein and a frameshift mutation in CBP80 (Fortes et al. 1999, 2000) is not simply a consequence of a disrupted binding between the two proteins, but implies a further impairment of CBP80 function.

Alternatively, disruption of the interaction between CBC and elf4G may affect the translation of a smaller subset of mRNAs that do not contribute appreciably to bulk cellular translation. The levels of the translation products of these mRNAs may not be sufficiently altered to affect cellular growth under the conditions we tested here. Although we
have not exhaustively investigated this scenario, our preliminary analysis of changes in polysome distribution of mRNAs using microarray-based methods did not reveal such a subset of mRNAs (J. Baron-Benhamou, data not shown). On balance, our observations argue against a direct role of the CBC-eIF4G in translation initiation and favor a nonessential contribution, if any, to the remodeling of mRNPs from a nuclear to a cytoplasmic configuration in yeast.

Deletion of either subunit of CBC alone, or in combination, is not lethal, but it incurs a growth impairment (Colot et al. 1996; Fortes et al. 1999; Das et al. 2000). We present evidence here that deletions of the CBP20 or CBP80 genes selectively affect the steady-state levels of a small subset of mRNAs. These transcriptome alterations do not persist in the 4G1–548 strain, suggesting that CBC’s effect on target mRNAs is not mediated through its interaction with eIF4G. CBC is known to affect multiple steps in mRNA metabolism, including a recently identified nuclear pathway for mRNA degradation (Das et al. 2000). Further experiments are required to delineate the step(s) in target mRNA metabolism that is affected by the absence of CBC subunits.

Collectively, our data send a cautionary message with regard to current models of a pioneering round of translation and the molecular mechanism that mediates it (Wilkinson and Shyu 2002). It is noticeable that, unlike CBP20, CBP80 is evolutionarily not a highly conserved protein. Thus, it is possible that our findings in yeast are reflective of an evolutionary divergence between the yeast and human proteins. More detailed analyses will be required to understand the putative role of CBC in an mRNP remodeling process that prepares nascent mRNA for the initial recognition by the translation machinery.

MATERIALS AND METHODS

Plasmids constructs and yeast strains

PCR mutagenesis was used to introduce the alanine codons into the recombinant eIF4G1 gene on a TRP1CEN plasmid as previously described (Hershey et al. 1999). The mutated plasmids were used to produce templates for coupled in vitro transcription–translation reaction to express [35S]-labeled eIF4G1 fragments. The centromeric plasmid URA-CEN-pGal-Luc was obtained by insertion of a HindIII–Xhol fragment from pGEM-Luc (Promega) into the HindIII–Xhol sites of p416Gal1 kindly provided by Michael Knop (EMBL, Heidelberg, Germany). The strains of yeast S. cerevisiae used in this study are described in Table 1. Standard yeast growth conditions and manipulations were used (Sherman 2002).

CBC column preparation and in vitro binding assays

CBC column preparation and in vitro binding assays were performed as previously described (Fortes et al. 1999).

Immunoprecipitation and Western blot analysis

Binding buffer (10× concentrated: 100 mM Tris-HCl at pH 8.0, 1 M NaCl, 1% NP40) was added to equal amounts (650 µL) of yeast

| TABLE 2. mRNAs whose expression is affected in both, Δcbp20 and Δcbp80, strains |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ORF name        | Expression ratio | Δcbp20          | Δcbp80          | Description                                                                 |
| YNL338W         | 2.151           | 2.409           | MIG2 (protein containing zinc fingers very similar to zinc fingers in Mig1p) |
| YGL209W         | 2.082           | 2.257           | GPM2 (phosphoglycerate mutase, involved in glycolysis)                       |
| YDL021W         | 2.118           | 2.235           | GND1 (phosphogluconate dehydrogenase, decarboxylating)                       |
| YHR183W         | 2.503           | 2.010           | SMX2 (snRNP G protein, homolog of Human Sm-G)                                 |
| YHL050C         | 2.108           | 2.184           | ARG3 (ornithine carbamoyltransferase)                                        |
| YMR162C         | 0.274           | 0.271           | PFY1 (profiling, actin-binding protein)                                      |
| YLR012C         | 0.462           | 0.429           | BUD28                                                                       |
| YBL044W         | 0.445           | 0.428           | ADE17 (5-aminoimidazole-4-carboxamide ribonucleotide transformylase)          |
| YFL017W-A       | 0.487           | 0.416           | GPM1 (phosphoglycerate mutase)                                               |
| YL088W          | 0.394           | 0.354           | ENO1 (enolase)                                                               |
| YFR007W         | 0.446           | 0.372           | ENO2 (enolase)                                                               |
| YLR446W         | 0.366           | 0.341           | SMX2 (snRNP G protein, homolog of Human Sm-G)                                 |
| YLR181C         | 0.440           | 0.407           | ARG3 (ornithine carbamoyltransferase)                                        |
| YOR122C         | 0.440           | 0.398           | BUD28                                                                       |
| YLR062C         | 0.411           | 0.409           | ADE17 (5-aminoimidazole-4-carboxamide ribonucleotide transformylase)          |
| YMR120C         | 0.226           | 0.434           | GPM1 (phosphoglycerate mutase)                                               |
| YKL152C         | 0.209           | 0.286           | GPM1 (phosphoglycerate mutase)                                               |
| YKL153W         | 0.387           | 0.454           | ENO1 (enolase)                                                               |
| YGR254W         | 0.223           | 0.317           | ENO2 (enolase)                                                               |
| YHR174W         | 0.423           | 0.358           | SMX2 (snRNP G protein, homolog of Human Sm-G)                                 |

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Binding buffer (10× concentrated: 100 mM Tris-HCl at pH 8.0, 1 M NaCl, 1% NP40) was added to equal amounts (650 µL) of yeast
extracts prepared as for gradient separation (see below). Polyclonal antibodies raised against the yeast CBP80 protein (Görlich et al. 1996) were chemically cross-linked to protein A sepharose beads (Amersham) as described by Harlow and Lane (1988). The extracts were then incubated with 15 µL of bead-cross-linked antibodies for 1 h 30 min at 4°C under agitation. The unbound fraction was collected and the beads were extensively washed (five times with binding buffer at 4°C). The bound fraction was eluted from the beads using 40 µL elution buffer (10 mM Tris-HCl at pH 8.0, 1 M NaCl, 0.2% SDS). One percent of the input extracts and 100% of the bound fractions were loaded on a SDS-PAGE gel. Western blot was performed as described by De Gregorio et al. (2001). Rabbit polyclonal antiserum against the N-terminal region of the yeast eIF4G1 protein was raised against the synthetic peptide SEAENTKRLFLEQVRLLKAMERKKNG (Genosys) coupled to maleimide-activated keyhole limpet hemocyanin (KLH; Pierce). α-eIF4G antibodies (dilution 1/1000) or α-CBP80 antibodies (dilution 1/1000; Görlich et al. 1996) were used for Western analysis, followed by anti-rabbit IgG coupled to horseradish peroxidase (dilution 1/5000; Amersham).

Pulse labeling

Yeast cells were grown exponentially at 30°C in minimal media lacking methionine. An equal amount of cells (0.2 OD600 nm units) was collected for each strain and incubated at 30°C with 3.5 µL of a mix containing 0.44 µCi/µL [35S]-methionine (Amersham) and 6 ng/µL cold methionine. After 5 min, the reaction was stopped by addition of an equal volume of 20% trichloroacetic acid. Samples were boiled for 20 min at 90°C and applied to glass microfibre Whatman filters (Ø = 25 mm). The amount of radioactivity, retained on the filter after extensive washes, was measured using a scintillation counter.

Yeast extract and sucrose gradient separation

Yeast cells were grown exponentially at 30°C in complete media (YPD), and 50 µg/mL cycloheximide was added to the cultures 1 min before harvesting the cells by centrifugation. Cells were washed once with breaking buffer (20 mM Tris-HCl at pH 7.4, 100 mM KCl, 2 mM MgCl2, 100 µg/mL heparin, 100 µg/mL cycloheximide, 2 mM DTT, 0.5 mM PMSF) and repelleted. One and a half times the wet cell weight of breaking buffer and five times wet cell weight of glass beads (425–600 µm) were added, and cells were broken by five times vortexing for 1 min with intermittent cooling on ice. After two consecutive centrifugations at 12,000g, supernatants were collected and the equivalent of 25 OD260 nm units was then layered onto linear 17.5 to 50% sucrose density gradients in gradient buffer (50 mM Tris-HCl at pH 7.4, 50 mM NH4Cl, 4 mM MgCl2, 2 mM DTT). Extracts were then separated by ultracentrifugation at 202,000g for 2.5 h. The gradients were then fractionated starting from the bottom and the absorbance profiles at 254 nm were recorded.

Total RNA extraction and microarray analysis

Yeast extracts prepared as for gradient separation were used to prepare total RNA by phenol-chloroform-isooamyl alcohol extraction. Twenty micrograms of total RNA were used to prepare Cy 3- and Cy5-labeled cDNA for hybridization to glass DNA microarrays comprising approximately 95% of all annotated ORF sequences in the S. cerevisiae genome as described by T. Preiss, J. Baron-Benhamou, W. Ansorge, and M.W. Hentze (in prep.). Microarray data were analyzed using Gene Pix 4.0 and Gene Spring 5.0 (Silicon Genetics) software. Only spots classified as “present” and satisfying a minimal intensity requirement above background were included in the analysis (representing approximately 90% of all yeast ORFs). Intensity ratios were normalized to the 50th percentile for each chip.

Luciferase assay

Yeast strains were grown exponentially at 30°C in selective minimal medium complemented with 2% raffinose. At time 0, galactose was added to a final concentration of 2%. At each time point 1-ml aliquots of each culture were collected, cells were washed with H2O and resuspended in 100 µL lysis buffer (50 mM Tris-HCl at pH 7.4, 100 mM NaCl, 1 mM PMSF). One hundred microliters of glass beads (425–600 µm) were added and cells were lysed by vortexing. Sixty percent of the supernatant was used to measure luciferase activity using a luciferase assay system (Promega), following the manufacturer’s instructions.

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