

Distributed control for recruitment, scanning and subunit joining steps of translation initiation

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ABSTRACT

Protein synthesis utilizes a large proportion of the available free energy in the eukaryotic cell and must be precisely controlled, yet up to now there has been no systematic rate control analysis of the *in vivo* process. We now present a novel study of rate control by eukaryotic translation initiation factors (eIFs) using yeast strains in which chromosomal eIF genes have been placed under the control of the *tetO7* promoter system. The results reveal that, contrary to previously published reports, control of the initiation pathway is distributed over all of the eIFs, whereby rate control (the magnitude of their respective component control coefficients) follows the order: eIF4G > eIF1A > eIF4E > eIF5B. The apparent rate control effects of eIFs observed in standard cell-free extract experiments, on the other hand, do not accurately reflect the steady state *in vivo* data. Overall, this work establishes the first quantitative control framework for the study of *in vivo* eukaryotic translation.

INTRODUCTION

Protein synthesis is an essential activity that accounts for a large part of the ATP turnover in living cells. This process is performed by large macromolecular machines composed of proteins and rRNA molecules. These ribonucleoprotein complexes, called ribosomes, each comprise a small subunit and a large subunit that cooperate to decipher (translate) the information encoded in mRNA molecules. Recruitment of the small (40S) eukaryotic ribosomal subunit onto a cellular mRNA generally occurs via the capped 5' end. Since the AUG start codon of a gene can be located many hundreds (in some cases thousands) of nucleotides downstream of the 5' end, the 40S subunit then has to translocate (scan) along the mRNA to reach the initiation site (1). This processive, sequence-independent scanning phase involves at least 11 eukaryotic initiation factors [eIFs (2)], equivalent in *Saccharomyces*

cerevisiae to at least 21 proteins, that participate in a series of different complexes, as well as a number of (largely undefined) conformational states. The control of translation initiation is a key determinant of growth, is important to the stress response (3) and, when malfunctioning, contributes to disease states (4,5). It is therefore important to understand rate control of this process at a quantitative level. Moreover, as understanding of the molecular mechanisms underlying eIF function progresses, we will be able to fit them into a quantitative framework that can serve as a robust model for the overall process (6).

Binding of the ternary complex (comprising Met-tRNA_i^{Met}, eIF2 and GTP) to the 40S subunit is stabilized by eIF1A and eIF3 (7) (Figure 1A). Yeast eIF3 binds to eIF2, thus promoting binding of mRNA to 40S; mammalian eIF3, on the other hand, seems to be capable of binding (in an RNA-dependent manner) directly to the 40S subunit (2). eIF4F is anchored to the 5' end of the mRNA via the cap-binding protein eIF4E which, despite its small size, may play roles in a number of cellular processes (8,9). Genetic experiments in yeast have indicated that eIF1, eIF2 and eIF5 influence start codon selection (10), while *in vitro* biochemical experiments have shown that eIF1 and eIF1A play roles in scanning and formation of the 48S complex, which comprises 40S, the eIFs and mRNA (11,12). A growing body of evidence indicates that, at least in budding yeast, eIF1, eIF2, eIF3 and eIF5 may bind to the 40S subunit as a preformed multifactor complex (MFC (13); Figure 1A). Thus the MFC components, together with eIF1A, play a key role in 40S-mRNA recruitment, scanning of the 5' untranslated region, and start codon recognition (7,13–15). Recognition of the start codon in an mRNA leads to hydrolysis of eIF2-bound GTP, followed by 60S joining aided by eIF5B-GTP. Hydrolysis of the latter GTP precedes initiation of protein synthesis.

The functioning of living cells depends on the coordinated action of many molecular processes, including translation. However, thinking on the rate control of processes such as translation has been influenced by the expectation that control is usually determined by a single rate-limiting step. For example, it has frequently been suggested or assumed that eIF4E acts as a rate-limiting

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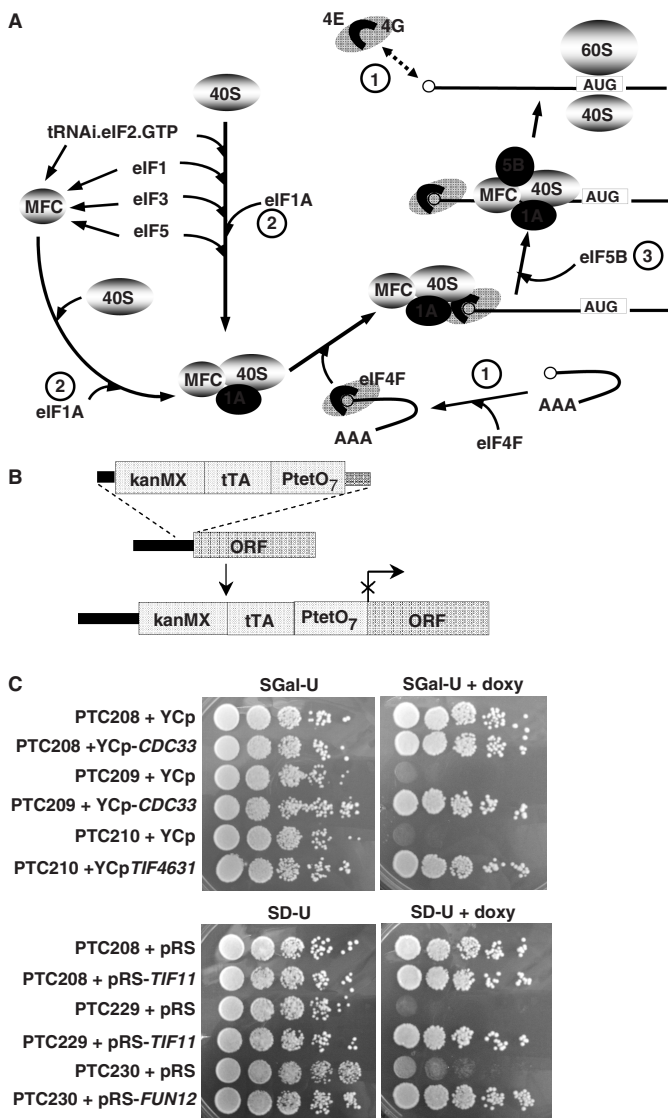


Figure 1. Construction and characterization of strains (see genotypes in Table 2) with doxycycline-regulatable eIF genes. (A) Eukaryotic translation initiation pathway, highlighting involvement of eIF4E, eIF4G, eIF1A and eIF5B in the phases of 40S recruitment [1], scanning and AUG recognition [2] and subunit joining [3]. (B) Strategy for promoter substitution upstream of eIF-encoding genes. The *tetO7* promoter (33) was inserted between 50 and 200 bp upstream of each ORF. (C) Complementation of doxycycline ($2 \mu\text{g ml}^{-1}$)-induced growth phenotypes was tested on SGal-uracil plates [YCp33Supex2-*CDC33* (YCp-*CDC33*) and YCp33Supex2-*TIF4631* (YCp-*TIF4631*)], or on SD-uracil plates [pRS316-*TIF11* (pRS-*TIF11*) and pRS316-*FUN12* (pRS-*FUN12*)].

factor in translation initiation (16–20). This approach to posttranscriptional gene expression probably derives from early models of metabolic control, widely disseminated in biochemistry teaching texts (21–23), in which it has been assumed that one enzyme-catalysed step would be responsible for rate control through each pathway. On the other hand, uncertainty about the role of eIF4E in rate control has been created by contrasting observations of the effects of artificially enhanced eIF4E synthesis in primary cell cultures as opposed to cell lines (19,20,24).

Up to now, translation has not been subjected to detailed quantitative control analysis, and therefore the mode of control in the translation initiation pathway could not be precisely elucidated. In this article, we address the issue of rate control in the translation initiation pathway using genetic titration combined with control analysis of eIF synthesis rates *in vivo*. We have chosen to perform this work in *S. cerevisiae*, the eukaryotic organism with the best-defined translation system as well as the eukaryotic organism of choice for attempts to achieve comprehensive characterization of metabolic and genetic pathways in the coming years. Studies of this relatively simple eukaryote are far more readily integrated into coherent models of rate control than are the largely qualitative results derived from the diversity of dissimilar higher eukaryotic systems under study. In our study, we find that control follows a distributed model in which all steps investigated contribute to the determination of overall rate, albeit to differing degrees. The approach taken here has broad relevance for research on gene expression.

MATERIALS AND METHODS

Construction of doxycycline-regulatable eIF strains

pCM225 (25) was employed as template for PCR amplifications of the promoter-substitution cassettes (Figure 1B). Transformants containing the promoter-substitution cassette were selected on YPD-G418 plates and further tested via analytical PCR. For construction of PTC210, *TIF4632* (encoding eIF4G2) was deleted using a PCR-based disruption method (26). Complementation of the doxycycline-inducible phenotypes of strains PTC209, 210, 229, 230 was achieved via transformation using plasmids expressing the eIF-encoding genes. The eIF4E- and eIF4G1-encoding plasmids were YCp33Supex2-*CDC33* and YCp33Supex2-*TIF4631* (27). The eIF1A- and eIF5B-encoding plasmids, pRS316-*TIF11* and pRS316-*FUN12*, were constructed via PCR amplification of *TIF11* and *FUN12* from chromosomal DNA and cloning into pRS316 (28), respectively.

Quantitative analysis of eIF strains

For western blotting, standardized amounts of recombinant eIF4E and eIF1A were applied to SDS-PAGE gels next to the extracts or, alternatively, extract amounts equivalent to different numbers of cells were used to calibrate the band intensities for eIF4G and eIF5B. Antibodies against eIF4E and eIF1A were generated by immunization of rabbits with the purified factors (Abcam, Cambridge, UK). Antibodies against eIF4G1 were generated as described previously (29), while an anti-eIF5B serum was kindly provided by Tom Dever (NIH, Bethesda, MD, USA). The membranes were incubated with FITC-labelled anti-rabbit IgG (Sigma, St Louis, MO, USA), washed and then scanned using a Typhoon Imager (Molecular Dynamics, Piscataway, NJ, USA). Translation in cell-free extracts was studied as described previously (30). Each quantitation experiment was repeated at least three times.

Analysis of rate control data

We applied basic concepts derived from metabolic control analysis (MCA), which was originally developed to examine the relative control exerted by each step in a metabolic pathway (31,32), to the translation initiation pathway. Specifically, we applied the concept of the sensitivity coefficient (32), which expresses the dependence of a variable of the system (in this case the flux through the pathway, i.e. the translation initiation rate) on the rate of a certain step in the pathway. The original term referred to the effects of changes in enzyme concentration on catalytic flux through a metabolic pathway. The component control coefficient, as defined here, refers to the effect of changes in the concentration of a component engaged in the assembly of a macromolecular complex that has catalytic activity. This coefficient is defined at the steady state as $C_{eIF}^J = (\partial \ln J / \partial \ln eIF) (\cong (\% \Delta J / \% \Delta [eIF]))$, where J is the flux through the system (in this case the translation rate).

RESULTS

Imposed modulation of eIF synthesis

Our overall aim was to analyse key steps of control at the three major phases of the initiation pathway: 40S recruitment, 40S scanning leading to AUG recognition, and ribosomal subunit joining (Figure 1A). In order to do this, we placed transcription of the chromosomal genes encoding eIF4E and eIF4G (*CDC33* and *TIF4631*, respectively; 40S recruitment), eIF1A (*TIF11*; scanning and AUG recognition) and eIF5B (*FUN12*; subunit joining), under the control of the Tet-off operator system (33) (Figure 1B). We chose both eIF4E and eIF4G because there has been a long-standing debate in the field as to whether eIF4E, as opposed to any other factor acting on the initiation pathway, acts as the 'rate-determining' factor in translation (16–20).

The doxycycline-dependent slow-growth phenotypes of the strains could be suppressed by genetic complementation upon transformation using expression plasmids bearing the corresponding wild-type genes (Figure 1C). Additionally, PCR was used to confirm the structure of the chromosomal insertions (data not shown). We next tested whether the four strains constructed as described earlier would allow us to regulate synthesis continuously over a wide range of steady-state levels. Calibrated western blot analysis was used to quantitate the abundance of each eIF over a range of doxycycline concentrations (Figure 2A, B, C, D and E). These 'genetic titrations' enabled us to investigate the relationship between intracellular eIF levels and the rates of cell growth and of protein synthesis over a range that does not become excessively restrictive to cell function (see below). In control experiments, we investigated whether the levels of eIFs whose synthesis was not subject to Tet-off regulation were affected by doxycycline (data not shown). The results revealed that, in each of the four strains, only the eIF encoded by the *tetO7*-regulated gene was subject to limitation.

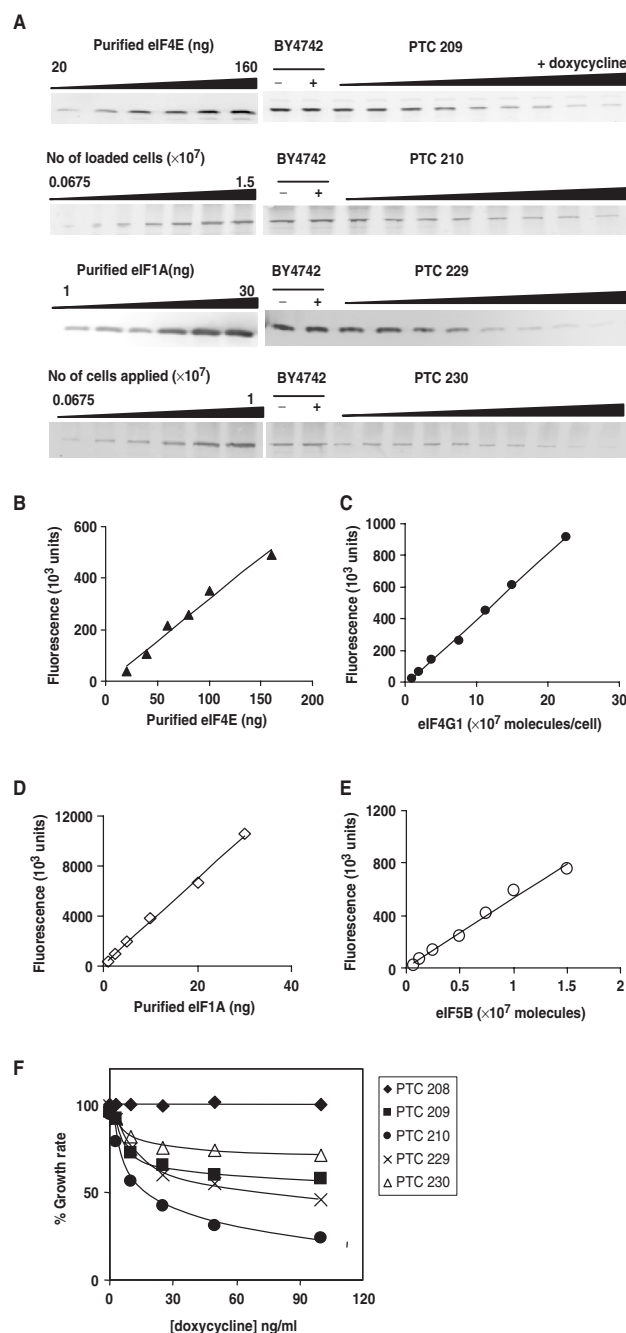


Figure 2. Quantitation of the eIF factors at different levels of doxycycline. (A) Western blots of purified recombinant eIF4E and eIF1A (left-hand panels) were used to calibrate eIF concentrations in extracts. Right-hand panels compare the levels of the eIFs in extracts derived from strains PTC209, 210, 229, 230 that had been grown in SD-Met medium containing different levels of doxycycline (1.5–100 ng ml⁻¹). For PTC210 and PTC230, calibration was performed against cell extracts containing known amounts of eIF4G1 and eIF5B (41). Control lanes show the levels of the eIFs in BY4742 extracts. Standard curves were plotted of western blot band intensity versus amount of purified recombinant eIF4E (B) and eIF1A (D) and of western blot band intensity versus known contents of eIF4G1 (C) and eIF5B (E) in different amounts of BY4742 extract. (F) Plots of relative growth rates (originally measured as OD₆₀₀) as a function of doxycycline concentrations for the strains listed in Table 2 grown in SD-Met medium. Each data point represents the average of three separate log-phase growth rate determination experiments. The absolute growth rate observed in the absence of doxycycline was set to 100%.

Growth rate and protein synthesis as functions of eIF abundance

We next determined the doubling times of each strain in logarithmic growth as a function of intracellular eIF abundance. Growth limitation by doxycycline was explored down to a growth rate of <20% of wild type, thus providing a broad range of data points for analysis (Figure 2F). Control experiments revealed no effects of doxycycline upon growth of a strain that did not have a gene subject to control by the Tet-off control system. We also followed the rate of protein synthesis *in vivo* as a function of eIF abundance in the steady state. Polysomal gradients revealed shifts in the distribution of ribosomes from the polysomal fractions into the monosomal fractions (see SupplementaryData), and indicated differences in the sensitivity of the initiation pathway to comparable changes in abundance of the four eIFs.

In order to obtain a more exact picture (Figure 3A, B, C and D), we determined the rates of ^{35}S -methionine incorporation into cells. Plotting these rates versus relative abundance of each eIF, we were then able to identify the quantitative differences between them in terms of control (Figure 3E). A remarkable feature of the plots in Figure 3 is that they all seem to approximate to a biphasic structure, with a break at 80% of maximum [eIF] or higher between two regions of distinct C^J values. One possible explanation for this behaviour is that progressively reducing the amount of intracellular eIF at some point restricts the redundancy of the macromolecular assembly routes, thus limiting eIF binding to a route that has a higher C^J value. Irrespective of the mechanistic basis for this effect, the most relevant region in terms of normal assembly of the translation initiation apparatus is the

region with the smaller C^J value that is observed at higher eIF concentrations—this tells us the contribution to control of each eIF at or near its wild-type level in the cell.

Metabolic control analysis (31,32,34,35) was originally developed for analysing the behaviour of enzyme systems that interconvert metabolites. Here we have developed the concept of the component control coefficient (C^J) for a macromolecular assembly pathway (see Materials and Methods section). The strength of rate control (component control coefficients) declined in the order: eIF4G > eIF1A > eIF4E > eIF5B (Figure 3E). The rationale for calculating the component control coefficients as we have done in this work is summarized in the Materials and Methods section. The largest C^J value obtained at near wild-type levels was 0.50 for eIF4G1. This means that for every change of 2% in [eIF4G1], the rate of initiation changes by 1%. Rate control imposed by eIF5B, in contrast, is more than three times weaker.

In control experiments, we examined whether the abundance of typical endogenous mRNAs in the constructed strains is affected by *tetO7*-regulated changes in eIF gene transcription rate. We quantitated the abundance of a typical long-lived mRNA (*PGK1*) and of a typical short-lived mRNA (*MAT α 1*) in the strains listed in Table 2 as a function of doxycycline addition. In all cases, doxycycline-induced inhibition of *tetO7*-regulated eIF production had no effect on mRNA abundance (data not shown). We, therefore, conclude that the changes in protein synthesis rate associated with reductions in the intracellular availability of the selected eIFs are not likely to be attributable to reduced mRNA stability. This finding is fully consistent with our earlier observations that reduced eIF activities, at least over a certain range,

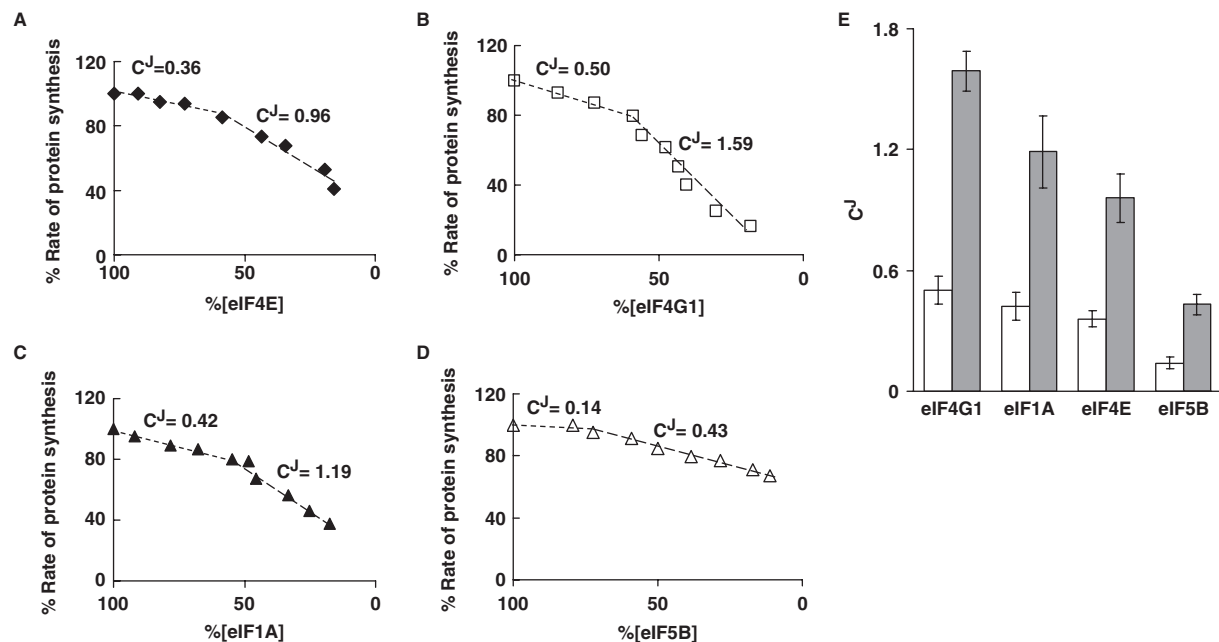


Figure 3. Dependence of protein synthesis rates on amounts of eIF4E, eIF4G1, eIF1A and eIF5B in strains PTC209, PTC210, PTC229 and PTC230 grown in SD-Met medium (A–D). The data fit well to biphasic plots; C^J values are indicated for the respective slopes. (E) Comparison of the two C^J values for each eIF. In each case, the white bar represents the C^J value for the higher [eIF] range, and the grey bar represents the value for the lower [eIF] range.

do not cause general destabilization of mRNA in yeast (36,37).

A truly rate-limiting factor would have a C^J value of 1, and as we can see from Figure 3, all of the eIF C^J values determined here fall far short of 1 (in the near-wild-type concentration range). This unpredicted result therefore tells us that the eIFs share rate control of the overall pathway, and that no single eIF exercises complete rate control (Table 1). A further key issue relates to the summation rule (35), which states that the sum of all control coefficients should equal 1. More recent results

have indicated that this sum can in fact be >1 (approaching 2) for metabolic pathways if there is macromolecular crowding (38) or where the enzymes on a pathway exchange substrate groups (39). Our data now show that rate control summation in the translation initiation pathway, and therefore probably in other macromolecular assembly pathways, follows a different rule to that applicable to most metabolic pathways.

The objective of the straightforward control analysis performed in this paper is to relate flux through the overall system (the translation initiation pathway) to local activities of the eIFs. However, we need to bear in mind that as protein synthesis is attenuated, this affects cell growth and physiology that in turn could, at least theoretically, feed back on the rate control relationships of the respective factors. A useful indication of the global state of the cell as a function of changes in the rate of protein synthesis is provided by the plots shown in Figure 4, which derive from the data presented in Figure 3 and in the Supplementary Data section. In all four cases,

Table 1. Values for C^J (near maximum J) and C^{J^*} (lower J values)

Initiation factor	C^J	C^{J^*}
eIF4E	0.36	0.96
eIF4G1	0.50	1.59
eIF1A	0.42	1.19
eIF5B	0.14	0.43

Table 2. Yeast strains used in this study

Name	Genotype	Origin or comments
PTC208 (BY4742)	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	EUROSCARF, Frankfurt
PTC209	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 <i>CDC33(-198,-1)::KanMX4-tTA-tetO7</i></i>	This study
PTC210	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tif4632Δ <i>TIF4631(-306,-1)::KanMX4-tTA-tetO7</i></i>	This study
PTC229	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 <i>TIF11(-309,-1)::KanMX4-tTA-tetO7</i></i>	This study
PTC230	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 <i>FUN12(-121,-1)::KanMX4-tTA-tetO7</i></i>	This study

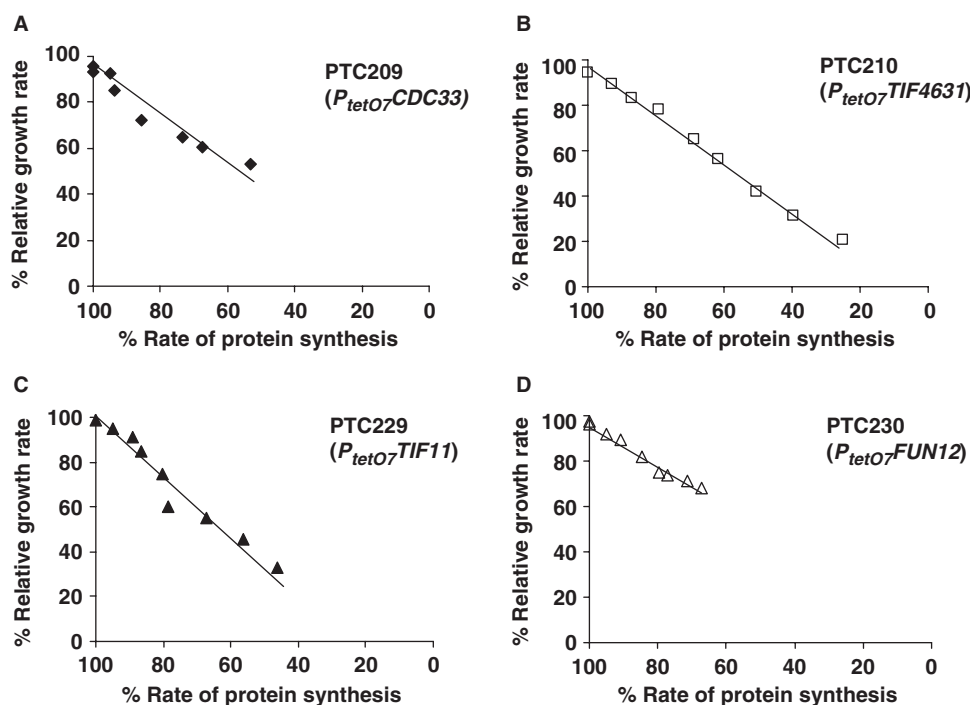


Figure 4. Protein synthesis rate closely follows growth rate in strains PTC209 (eIF4E), PTC210 (eIF4G), PTC229 (eIF1A) and PTC230 (eIF5B).

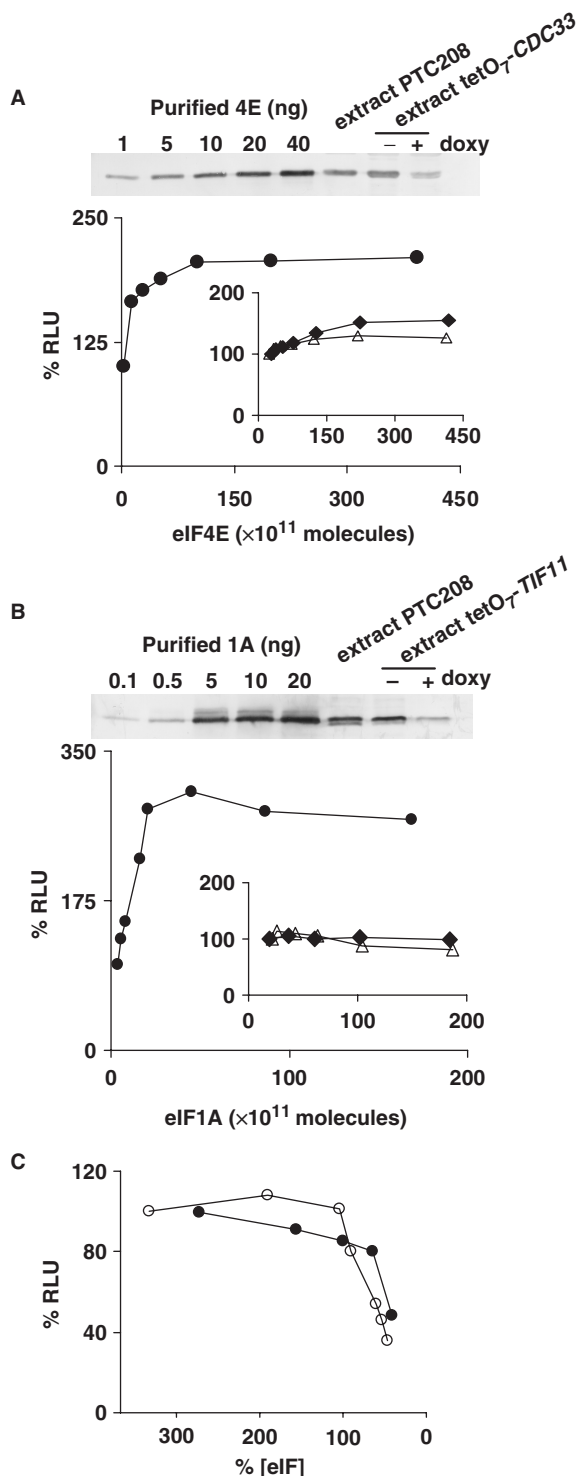


Figure 5. *In vitro* translation by cell-free extracts from PTC209 and PTC229. (A) Dependence of luciferase activity encoded by capped *LUC* mRNA on the concentration of eIF4E (supplemented using purified recombinant eIF4E; see Figure 2). PTC 209 was grown in SD-met medium containing doxycycline (doxy; to a final concentration of 10 ng ml⁻¹). The main graph plots luciferase activity (as% relative light units) against amount of eIF4E in the extract from PTC209 grown in the presence of doxycycline (filled circle). The inset shows control data obtained with the extract obtained from PTC209 grown without doxycycline (filled diamond) and with the extract from PTC208 (BY4742) (open triangle); these plots start at a higher level of

growth is tightly linked to protein synthesis rate, with some variation in the slope. This suggests that there is a common fundamental relationship between growth and protein synthesis, with only a small dependence on which eIF is mediating limitation, thus simplifying the analysis and interpretation of the rate control data. However, it should be noted that we have not characterized the influence of changes in the cell physiological state on the C^J values estimated here, which would be necessary for hierarchical control analysis (40) of this system. Thus the control relationships we describe can only be assumed to apply to the log phase growth conditions specified.

Protein synthesis *in vitro* as a function of eIF abundance

While the cellular system was the primary focus of this quantitative study, it is also evident that many studies of translation initiation have utilized cell-free extracts. We accordingly decided to make a comparative study *in vitro* of the rate control behaviour of two of the eIFs, namely eIF4E and eIF1A. Extracts were prepared from strains PTC209 and PTC229 (Table 2) that had been growing in the presence of doxycycline. Purified eIFs were then added back to reconstitute progressively the respective extracts. The translation competence of the extracts was followed using a capped and polyadenylated luciferase-encoding reporter mRNA (Figure 5). Analysis of translation rate as a function of total eIF present in each extract revealed that the apparent control effects exercised by eIF4E and eIF1A in the lower concentration ranges were much greater than in the comparable *in vivo* states. The relationship between translation rate and eIF1A abundance in the extract from strain PTC229 grown in the presence of doxycycline (Figure 5B, and compare Figure 5C) did not approximate to the type of biphasic form seen *in vivo*, showing instead a region of very strong dependence on factor concentration leading to a (non-responsive) plateau. On the other hand, the dependence of translation rate on eIF4E concentration in Figure 5A includes a region of intermediate factor dependence. In control experiments, it was found that extracts prepared from the same strains grown in the absence of doxycycline showed relatively minimal responses to the addition of eIF4E or eIF1A (insets in Figure 5A and B). This minimal response was slightly higher in the case of PTC209. This was most likely because the chromosomal *tet-off* construction in this strain directed a somewhat reduced maximal (non-suppressed) rate of *CDC33* transcription, and thus of eIF4E synthesis, compared to the level directed by the wild-type promoter (data not shown).

endogenous eIF4E than the main plot. (B) Equivalent plots for PTC229 showing data for the extract from PTC229 grown in the presence of doxycycline (main plot, filled circle) and control data [inset; extract from PTC229 grown without doxycycline (filled diamond) and BY4742 extract (open triangle)]. (C) Direct comparison of the main data sets from panels A and B plotted as percentages in the same orientation as in Figure 3 [eIF4E (filled); eIF1A (open circle)]. The 100% point on the x-axis is equivalent to the level of each eIF in the corresponding extract as derived from each strain grown in the absence of doxycycline. Relative luciferase activity (y-axis) is equivalent to the rate of protein synthesis (averaged over 1 h).

Replotting the data in Figure 5A and B together allows more direct comparison between the eIF4E and eIF1A experiments (Figure 5C) and also between the *in vitro* data as a whole and the *in vivo* results of Figure 3. Overall, an important feature of these data is that they emphasize the contribution of the crowded and compartmentalized environment of the living cell to rate control in translation.

DISCUSSION

This study has provided the first set of component control coefficients for eukaryotic translation initiation, and thus the first quantitative framework for defining how the components of this pathway collectively contribute to its overall control. The procedure utilized here could be applied to all of the macromolecular assembly pathways based on intermolecular interactions and different conformational states, thus building up an increasingly accurate picture of control across the network of intracellular interactions that are involved in gene expression. Establishing this common quantitative framework for representation and modelling of the control and regulation of gene expression should be generally useful.

Our study illustrates how quantitative control data provide important insight into the workings of a complex biological system. The data show that rate control is distributed over different steps (and different eIFs) in the initiation pathway. It was noted previously that distributed rate control could be an inevitable consequence of the action of evolutionary 'forces' on a pathway of this type (6). An additional outcome is that there is no simple relationship between intracellular eIF concentration and a factor's contribution to control of the system. Thus, eIF4E is significantly more abundant in yeast cells than are eIF1A and eIF4G (41), yet its C^J value is far from proportionately smaller than those of these other two factors. In other words, it is unwise to base any judgments of likely control strength of a component of such a pathway on intracellular abundance alone. This consideration is also relevant to any models of disease caused by mutationally induced deficiencies in eIF function (16–18), since the quantitative data presented here show that we need to abandon the assumption that any particular eIF commands full control over the rate of the overall pathway under any particular set of conditions.

It is of course essential to remember in a study of this type that at least eIF1A and eIF4G may act at more than one site on the initiation pathway, and thus that each C^J value for these proteins represents the sum of multi-site action. This does not detract from the usefulness of these values as quantitative indicators of factor-centric control as manifested by the system under the defined conditions, but does mean that any mechanistic interpretation must take the multi-site functions into account. In this context, for example, it is remarkable that despite being involved in several steps on the initiation pathway, eIF1A manifests a C^J value that is only 16% greater than that of eIF4E, a factor that is thought to have a single site of action on the pathway.

In relation to potential targets for regulation of translation initiation, it is notable that the comparable C^J values of three of the eIFs studied in this work make them all potentially effective sites for targeting regulation. Thus, the observation of distributed control in the translation initiation pathway also tells us not to assume that regulation is likely only to be effective if targeted to one or two of the characterized eIFs. Finally, the current study sets the stage for comprehensive rate control analysis of this, and other, eukaryotic gene expression pathways. This will ultimately lead to the discovery of new general control principles that guide such systems.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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