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Central role of Ifh1p–Fhl1p interaction in the synthesis of yeast ribosomal proteins

Dipayan Rudra, Yu Zhao and Jonathan R Warner*

Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY, USA

The 138 genes encoding the 79 ribosomal proteins (RPs) of *Saccharomyces cerevisiae* form the tightest cluster of coordinately regulated genes in nearly all transcriptome experiments. The basis for this observation remains unknown. We now provide evidence that two factors, Fhl1p and Ifh1p, are key players in the transcription of RP genes. Both are found at transcribing RP genes *in vivo*. Ifh1p, but not Fhl1p, leaves the RP genes when transcription is repressed. The occupancy of the RP genes by Ifh1p depends on its interaction with the phospho-peptide recognizing forkhead-associated domain of Fhl1p. Disruption of this interaction is severely deleterious to ribosome synthesis and cell growth. Loss of functional Fhl1p leads to cells that have only 20% the normal amount of RNA and that synthesize ribosomes at only 5–10% the normal rate. Homeostatic mechanisms within the cell respond by reducing the transcription of rRNA to match the output of RPs, and by reducing the global transcription of mRNA to match the capacity of the translational apparatus.
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Introduction

With the recent addition of Asc1p (Link *et al*, 1999), we now know that the *Saccharomyces cerevisiae* ribosome has 79 proteins, encoded by 138 ribosomal protein (RP) genes that are responsible for nearly 50% of all Pol II transcriptional initiations (Velculescu *et al*, 1997; Holstege *et al*, 1998; Warner, 1999). Their transcription is rigorously controlled as a cohort in response to both positive and negative signals (Gasch *et al*, 2000; Causton *et al*, 2001). Indeed, they represent the most prominent cluster in most transcriptome studies.

The transcriptional activating regions of most RP genes are characterized by a pair of sites that bind Rap1p and are essential for high-level transcription (Rotenberg and Woolford, 1986; Schwindinger and Warner, 1987; Nieuwint *et al*, 1989). In a few cases, the Rap1p sites are replaced by a site for Abf1p (Hamil *et al*, 1988; Herruer *et al*, 1989) or for Reb1p (Lascaris *et al*, 1999). The coordinate regulation of transcription of the RP genes appears independent of which transcriptional regulator is present.

Rap1p is a protein of many functions, as its name, repressor-activator-protein, suggests (reviewed in Morse, 2000; Pina *et al*, 2003). It is responsible for the transcription not only of the RP genes, but also of many genes encoding translation factors and enzymes of glycolysis. It binds to the TG repeats of telomeric DNA. It serves to nucleate complexes that repress transcription of genes both adjacent to the telomeres and at the silent MAT loci.

Yet alone Rap1p has only weak transcriptional activating ability (Tornow *et al*, 1993). It is reported to act by interfering with nucleosomes, thus facilitating the access of activation factors to their binding sites (Yu and Morse, 1999). Although acetylated histones have been observed throughout the UAS of RP genes (Reid *et al*, 2000), recent reports suggest that the promoter regions of actively transcribed genes, especially RP genes, are nearly devoid of nucleosomes (Bernstein *et al*, 2004; Lee *et al*, 2004).

The specificity of Rap1p in its several roles presumably lies in its recruitment of specific coactivators, such as Gcr1p at the glycolytic genes (Tornow *et al*, 1993), or corepressors, such as Sir3p, at telomeres and silent MAT loci (Moretti *et al*, 1994). Recently, a genome-wide chromatin immunoprecipitation (ChIP) analysis showed that the promoter of nearly every RP gene is occupied by the hitherto obscure potential transcription factor Fhl1p (forkhead-like) (Lee *et al*, 2002). Fhl1p was originally identified as a multicopy suppressor of a Pol III mutant, and was then shown to be important for ribosome biosynthesis (Hermann-Le Denmat *et al*, 1994). Subsequently, the same group identified *IFH1* as a multicopy suppressor of the slow growth phenotype of a Δ*FHL1* strain. Ifh1p, essential for growth, was also implicated in ribosome biosynthesis. Surprisingly, cells with deletions of both *FHL1* and *IFH1* survive (Cherel and Thuriaux, 1995).

We have now explored in more detail both the roles of Fhl1p and Ifh1p in ribosome biosynthesis and the physiological effects of their absence. We confirm that Fhl1p, as well as Ifh1p, is found at the UAS of RP genes. By co-immunoprecipitation (Co-IP) analysis, we find that Fhl1p and Ifh1p interact with each other through the 'forkhead (FH)-associated' (FHA) domain of Fhl1p (Durocher and Jackson, 2002). Mutation of the FHA domain, reducing its interaction with Ifh1p, leads to loss of Ifh1p from RP genes and to severe defects in ribosome synthesis and growth. Treatment of cells with rapamycin, which represses strongly the transcription of RP genes (Cardenas *et al*, 1999; Powers and Walter, 1999), leads to the loss of Co-IP of Fhl1p with Ifh1p and to the disappearance of Ifh1p from the RP genes. Together, these observations suggest that the Fhl1p–Ifh1p interaction is responsible for active transcription of the RP genes.

Cells lacking Fhl1p or both Fhl1p and Ifh1p grow exceedingly slowly and have less than one-quarter the normal

*Corresponding author. Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA. Tel.: +1 718 430 3022; Fax: +1 718 430 8574;
E-mail: warner@aecom.yu.edu

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amount of ribosomes, presumably because of deficient transcription of RP genes. Nevertheless, these ribosome-deprived cells utilize homeostatic mechanisms both to reduce their transcription of rRNA to match the available RPs and to balance their total mRNA population to the available ribosome complement.

Results

Both Fhl1p and Ifh1p are associated with RP genes

To confirm and extend the results reported by Lee *et al* (2002), we performed ChIP analysis on a strain carrying Fhl1p C-terminally tagged with HA_3 and Ifh1p C-terminally tagged with Myc_9. As shown in Figure 1A (lanes 4 and 6), ChIP with either anti-HA or anti-Myc enriched for DNA fragments from the promoter regions of RP genes, *RPL3*, *RPL7A*, *RPL28*, *RPL30* and *RPS6A*. No such enrichment was seen for promoters of *PGK1*, which is also driven by Rap1p (Packham *et al*, 1996), or for *ACT1*. Parallel analysis of an untagged strain showed no enrichment (lanes 3 and 5).

Quantitative PCR analysis of ChIP products (Figure 1B) showed a 10- to 20-fold enrichment of Fhl1p and a five- to eight-fold enrichment of Ifh1p at the promoters of several RP genes, with a lesser, but reproducible, enrichment at other RP genes. These results show that both Fhl1p and Ifh1p can be found at RP promoters. Their presence at RP gene promoters cannot depend on Rap1p alone as *RPL3* has a single Abf1p site rather than two Rap1p sites (Hamil *et al*, 1988).

Loss of Ifh1p from the RP genes during repression

Rapamycin leads to a rapid reduction in transcription of rRNA and RP genes (Cardenas *et al*, 1999; Powers and Walter, 1999). ChIP analysis shows that after treatment of the cells with rapamycin, Fhl1p nevertheless remains at the promoters of the RP genes (Figure 1C). On the other hand, Ifh1p does not (Figure 1D). This result suggests that the presence of Ifh1p is associated with the activation of transcription of the RP genes. Note that Rap1p is constitutively bound to RP promoters, whether transcription is occurring or not (Reid *et al*, 2000) as we have confirmed (data not shown). Thus, the repression of RP gene transcription due to rapamycin is accompanied by the loss of Ifh1p from the RP genes.

Ifh1p acts as a regulator of RP genes

The observation that the rapid transcription of RP genes is coincident with their occupancy by Ifh1p, but not Rap1p or Fhl1p, suggests that Ifh1p is an important regulator. To test this notion, we generated a strain with *IFH1* under control of the *GAL1* promoter (GAL_{UAS}-*IFH1*). Although deletion of *IFH1* is lethal (Cherel and Thuriaux, 1995), these cells grow slowly on the limiting amount of Ifh1p synthesized under glucose

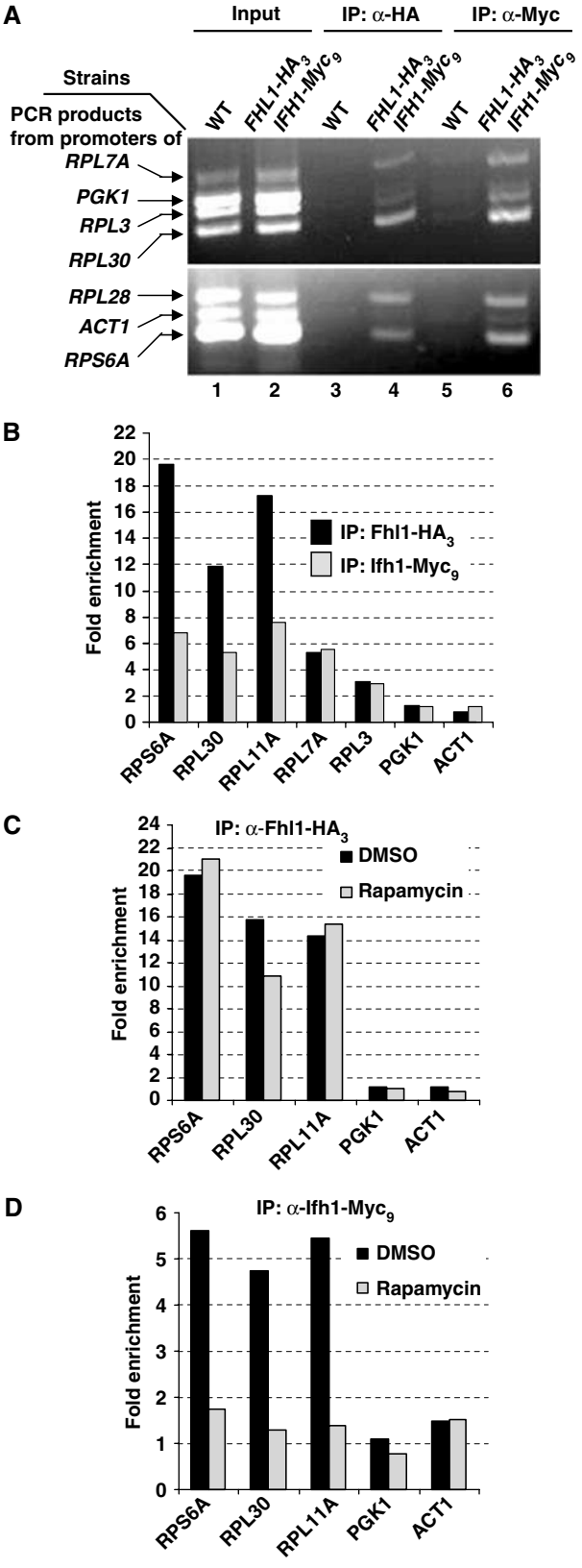


Figure 1 Fhl1p and Ifh1p are associated with RP gene promoters. (**A**) ChIP was performed using anti-HA or anti-Myc antibodies on W303a (WT) and DR36 (*FHL1-HA*$_3$, *IFH1-MYC*$_9$) double-tagged strains. Following IP, PCR was performed on total chromatin (input) and the immunoprecipitated (IP) DNA with primers specific for the promoters of the indicated RP genes. Primers specific for the promoters of non-RP genes *PGK1* and *ACT1* were used as controls. (**B**) A real-time PCR performed on the samples from strain DR36 (*FHL1-HA*$_3$, *IFH1-MYC*$_9$) in (A) using primers for the promoters of the indicated genes. Calculation of the 'fold enrichment' values is documented in Materials and methods. (**C, D**) *FHL1-HA*$_3$, *IFH1-MYC*$_9$ (strain DR47) double-tagged cells were pretreated for 30 min with rapamycin or the drug vehicle DMSO prior to formaldehyde crosslinking. This was followed by ChIP using anti-HA (C) or anti-Myc (D) antibodies followed by real-time PCR analysis.

repression, while in the presence of galactose they grow comparably to wild-type (WT) cells (Figure 2A). Although Ifh1p is initially below detection, it is rapidly synthesized after the culture is shifted from glucose to galactose (Figure 2B). The appearance of Ifh1p is accompanied by a rapid increase in transcription of RP genes, without much change to the levels of non-RP mRNAs derived from *ACT1* or *TEF1* (Figure 2C). This result suggests that limiting Ifh1p leads to limiting transcription of RP genes.

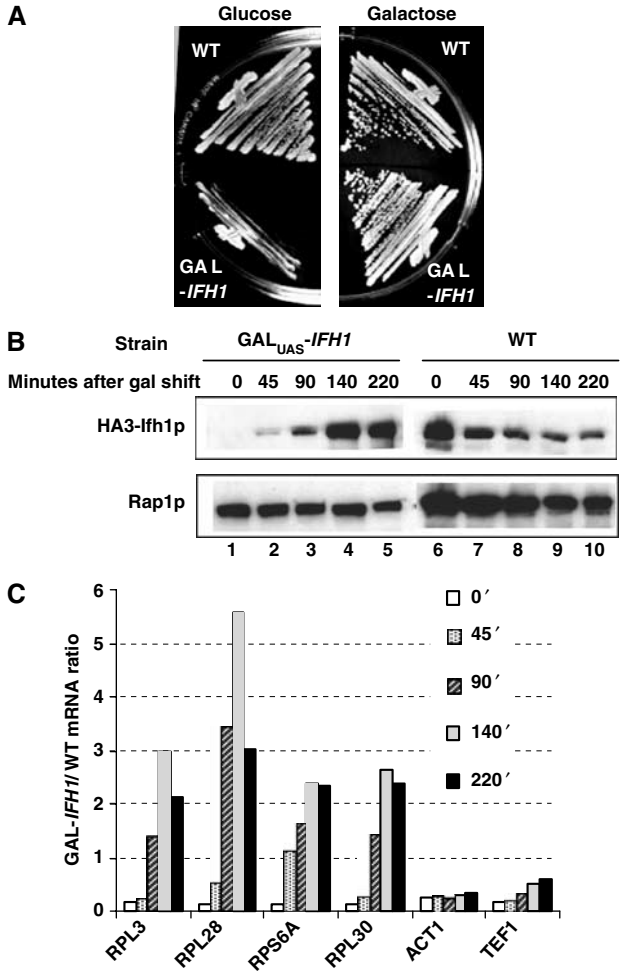


Figure 2 Ifh1p as a regulator of RP genes. (**A**) Growth of YZ146 (*IFH1-HA3* (WT)) and YZ147 (GAL$_{UAS}$-*HA3-IFH1*) in glucose (YPD) and galactose (YPGal) respectively. Cultures of WT (YZ146) and GAL$_{UAS}$-*HA3-IFH1*(YZ147) cells were grown in YPD media, and were shifted to YPGal by filtering. Cells were harvested at the indicated time points. (**B**) A portion was prepared for Western analysis using antibodies directed against the HA epitope or against Rap1p. (**C**) From the rest, RNA was prepared and Northern analysis was performed to determine the level of the indicated mRNAs by normalizing with the U3 snoRNA. A graphical representation of the ratio (GAL-*IFH1*/WT) at each time point is shown. Note that the total RNA level of these cells when grown in glucose is only 1/5 that of WT cells. As will be discussed below (Figures 7 and 8), limiting availability of Ifh1p leads to a downregulation of total RNA as well as of all mRNAs to match the availability of the translational apparatus. The levels of U3 snoRNA remain relatively constant. Thus when normalized against U3 snoRNA, at the 0 time points the ratio of both RP and non-RP mRNAs in mutant versus WT cells in glucose medium is approximately 0.2.

Neither Fhl1p nor Ifh1p binds to RP promoters in vitro

In an attempt to dissect the system, we carried out band-shift experiments using the intergenic region upstream of the RP gene, *RPL11A*, together with partially purified TAP derivatives (Puig *et al*, 2001) of Fhl1p and Ifh1p, alone, together and with Rap1p. While Rap1p binds tightly, no evidence of binding by Fhl1p or Ifh1p was observed, alone or in combination with the others, using several concentrations of the proteins (Figure 3). Similar results were obtained using sequences upstream of *RPL28* and *RPL30* (data not shown). By contrast, other 'FH' proteins of yeast, Fkh1p and Fkh2p, bind to multiple targets (Hollenhorst *et al*, 2001), sometimes with the assistance of other proteins (Kumar *et al*, 2000). The lack of direct binding by Fhl1p and by Ifh1p suggests that they require other factors or specific chromatin structures to associate with the RP gene promoters.

Fhl1p and Ifh1p interact with each other

We carried out Co-IP experiments to ask if the genetic interaction of Fhl1p and Ifh1p (Cherel and Thuriaux, 1995) arises from a physical interaction between the two proteins. As shown in Figure 4A, HA-tagged Fhl1p will co-immunoprecipitate Myc-tagged Ifh1p (lane 3); conversely, Myc-tagged Ifh1p will co-immunoprecipitate HA-tagged Fhl1p (Figure 4B, lane 5). No IP was observed in untagged strains (Figure 4A, lane 4; Figure 4B, lane 5). The Co-IP is not mediated through common interaction with DNA, because it is unaffected by the presence of ethidium bromide, which intercalates into DNA, thereby inhibiting normal protein–DNA interactions (Lai and Herr, 1992) (Figure 4B, lane 7). In cells pretreated with rapamycin, the interaction between Fhl1p and Ifh1p is greatly diminished (Figure 4C, compare lanes 3 and 4). Thus, rapamycin leads to the repression of RP gene transcription, the loss of Ifh1p from the RP genes and sharply reduced

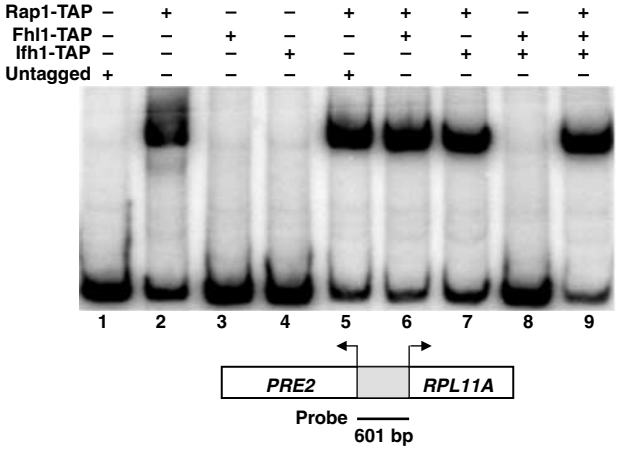


Figure 3 Failure of Fhl1p and Ifh1p to bind an RP promoter. A radiolabeled PCR-amplified fragment encompassing the intergenic region between *RPL11A* and *PRE2* (1 ng) was mixed with partially purified TAP-tagged Rap1p (5–10 ng), Fhl1p and Ifh1p (50–100 ng), or mock-purified product from an untagged strain, either separately or together as indicated in the figure, for 60 min at 0°C in 20 µl of solution containing 20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT, 5% glycerol, 5 µg poly(dI-dC), 20 µg BSA and 2 mM PMSF. Nondenaturing polyacrylamide gel electrophoresis on 8% acrylamide gels run in 25 mM Tris–borate and 0.25 mM EDTA to resolve any DNA–protein complex formed was followed by autoradiography of the dried gel.

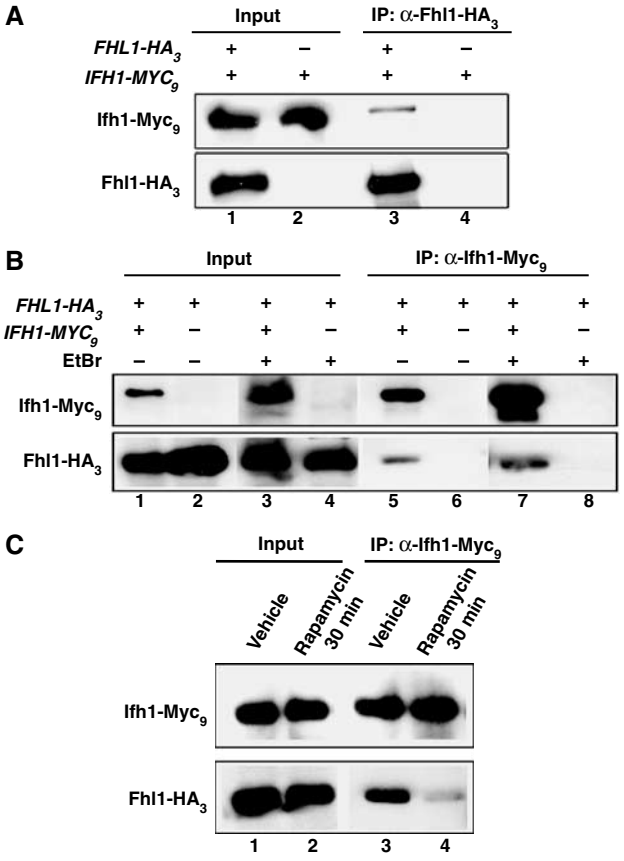
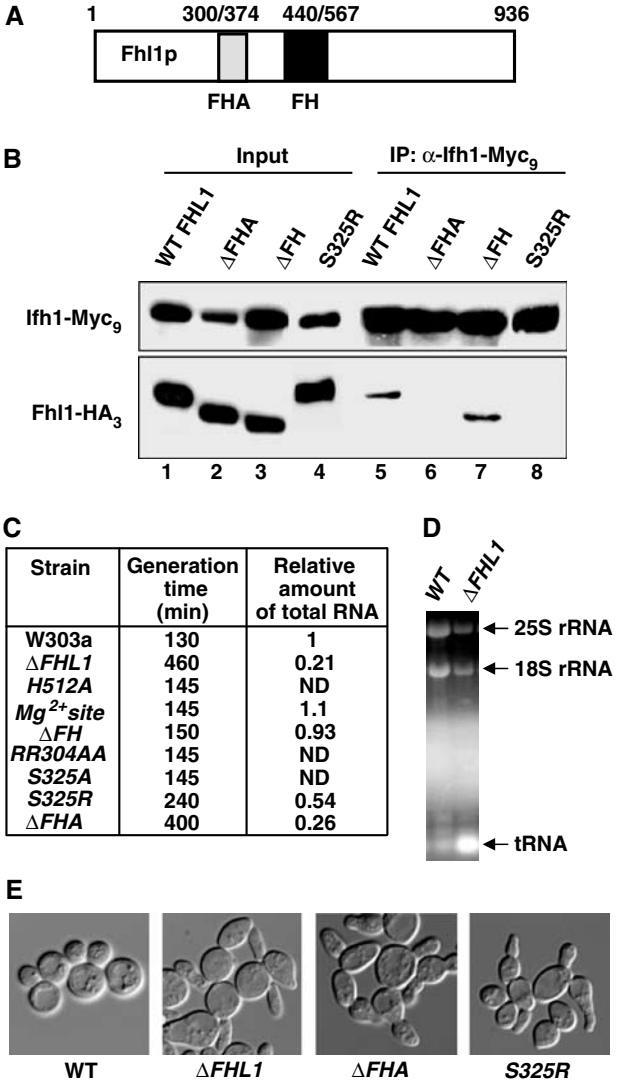


Figure 4 Fhl1p and Ifh1p interact with each other. (**A**) Co-IP was carried out using anti-HA antibody on extracts prepared from DR36 (*FHL1-HA*$_3$, *IFH1-MYC*$_9$ double-tagged), with DR37 (*IFH1-MYC*$_9$) as a negative control. The immunoprecipitated protein complex was resuspended in SDS loading buffer, boiled and analyzed by SDS–PAGE followed by Western blotting using anti-HA or anti-Myc antibodies. A 5 μl portion of the original cell extracts was analyzed in separate lanes as loading controls (input). (**B**) A converse Co-IP experiment to Figure 2A. In this case, the IP was carried out using anti-Myc antibody on extracts prepared from DR36 (*FHL1-HA*$_3$, *IFH1-MYC*$_9$), with DR13 (*FHL1-HA*$_3$) as a negative control. Samples in the lanes indicated were treated with 200 μg/ml of ethidium bromide for 30 min on ice before the IP (see Materials and methods). (**C**) Extracts of DR36 (*FHL1-HA*$_3$, *IFH1-MYC*$_9$) cells that had been treated with 0.2 μg/ml rapamycin or with drug vehicle (DMSO) for 30 min were subjected to Co-IP using anti-Myc antibody.



Strain	Generation time (min)	Relative amount of total RNA
W303a	130	1
Δ*FHL1*	460	0.21
H512A	145	ND
Mg^{2+}*site*	145	1.1
Δ*FH*	150	0.93
RR304AA	145	ND
S325A	145	ND
S325R	240	0.54
Δ*FHA*	400	0.26

Figure 5 The FHA domain of Fhl1p is required for its interaction with Ifh1p. (**A**) Schematic of the Fhl1p protein with the FHA domain (amino acids 300–374) and the FH domain (amino acids 440–567) indicated. (**B**) Co-IP using anti-Myc antibody on extracts prepared from equal numbers of cells of strains DR47 (*FHL1-HA*$_3$), DR48 (Δ*FH-HA*$_3$) or DR49 (Δ*FHA-HA*$_3$), carried on a CEN plasmid, covering the deleted *FHL1*; Ifh1p is tagged C-terminally with Myc$_9$. (**C**) Cultures were grown in synthetic media at 30°C with gentle shaking and the growth rate determined over several generations by light scattering at 600 nm. The mutations in *FHL1* are indicated. The Mg^{2+} site mutant consisted of the following changes: L514A, S515A, N517A and F520A. Deletion of the FH domain includes amino acids 440–567 and that of the FHA domain includes amino acids 300–374. The total RNA isolated from 1 ml of a culture of W303a at $OD_{600} \sim 1.0$ is arbitrarily defined as 1 unit. The relative amount of RNA from the indicated strains at a similar optical density is tabulated. ND: not done. (**D**) A 7.5 μg portion of RNA isolated from the indicated strains (requiring 5× as many mutant as WT cells) was mixed with ethidium bromide and analyzed on a denaturing agarose gel and photographed under UV illumination. (**E**) WT and *FHL1* mutant strains viewed at 100× magnification with Nomarski optics.

interaction between Fhl1p and Ifh1p. It seems likely that the interaction between Fhl1p and Ifh1p is a cause of the high level of transcription of RP genes.

Basis of the interaction of Fhl1p with Ifh1p

Examination of the sequence of Fhl1p reveals two conserved domains (Figure 5A). FH has been identified as a DNA-binding domain, unusual in that it requires Mg ions for binding (Clark *et al*, 1993). The FHA domain is widespread in nature, originally identified in FH proteins, but now observed in many others. The FHA domain binds to distinct phospho-peptide ligands, usually those containing a phospho-threonine (reviewed in Durocher and Jackson, 2002). By contrast, the 1085-amino-acid sequence of Ifh1p has no easily recognizable domains.

We generated mutant versions of Fhl1p in order to determine the sites of interaction with Ifh1p. Several mutations within the FH domain or deletion of the entire FH domain has no effect on the interaction (Figure 5B, lane 7). On the other hand, deletion of the FHA domain of Fhl1p, or even mutation

of a single amino acid within the core of the FHA domain, S_{325} to R, leads to loss of interaction with Ifh1p (Figure 5B, lanes 6 and 8). Since the FHA domain is known to interact with a phospho-peptide, this result predicts that transcription of RP genes is related to the phosphorylation of a site on Ifh1p that leads to its interaction with the FHA domain of Fhl1p.

Interaction of Fhl1p and Ifh1p is important for cell growth

Deletion of *FHL1*, while not lethal, reduces substantially the growth rate of a cell (Hermann-Le Denmat *et al*, 1994) (Figure 5C). Furthermore, the RNA content of such cells is markedly decreased (Figure 5C). As is evident from Figure 5D, which employed RNA from five times as many mutant as WT cells, this decrease is largely in rRNA. Quantitation of Figure 5D indicates that deletion of *FHL1* reduces the mass ratio of rRNA to tRNA from 4.8 to 0.6. Stated another way, mutant cells have only 10% the number of ribosomes but 70% the number of tRNAs of WT cells, substantiating the conclusion that Fhl1p is important for ribosome synthesis.

However, neither mutation of key residues within the FH domain nor deletion of the entire domain had much effect either on cell growth or on RNA content (Figure 5C). By contrast, deletion of the FHA domain caused nearly as slow growth, and nearly as reduced RNA, as did deletion of the entire gene. The mutation S_{325}R of *FHL1*, shown above to reduce the interaction of Fhl1p with Ifh1p, has an intermediate effect on growth and on ribosome content (Figure 5C). Note that the mutant forms of Fhl1p are present at the same level as the WT protein (Figure 5B, inputs).

The morphology of the cells is shown in Figure 5E. Although the appearance of unnatural protrusions suggests problems in cell division, the key observation is that in each strain, the cells are roughly the same size as WT cells. This contrasts with the unusually small size of cells deficient in Sfp1p, another factor implicated in ribosome synthesis (Jorgensen *et al*, 2004).

Occupancy of the RP promoter by Ifh1p requires its interaction with Fhl1p

The consistency of the observations *in vivo* and *in vitro* leads to the hypothesis that a key step in driving the transcription of RP genes is the binding of Ifh1p to Fhl1p, rather than the binding of Fhl1p to DNA (or to chromatin). We carried out a ChIP experiment to determine the occupancy of Fhl1p and Ifh1p at RP promoters under conditions in which they do not interact with each other. Neither deletion of the FHA domain nor the mutation S_{325}R affects the association of Fhl1p with RP genes (Figure 6A). Strikingly, however, deletion of the FHA domain abolishes the association of Ifh1p with the RP genes, and mutation S_{325}R nearly does so (Figure 6B). Taken together, these results indicate that it is the interaction between Fhl1p and Ifh1p that is responsible for bringing Ifh1p to the RP promoters, and suggest that while the presence of Fhl1p seems to be the unique characteristic of RP genes (Lee *et al*, 2002), it is the presence of Ifh1p that leads to their active transcription.

Physiology of ribosome-deprived cells

In spite of the critical role that Fhl1p and Ifh1p play in ribosome synthesis, cells with the genotype Δ*FHL1* or

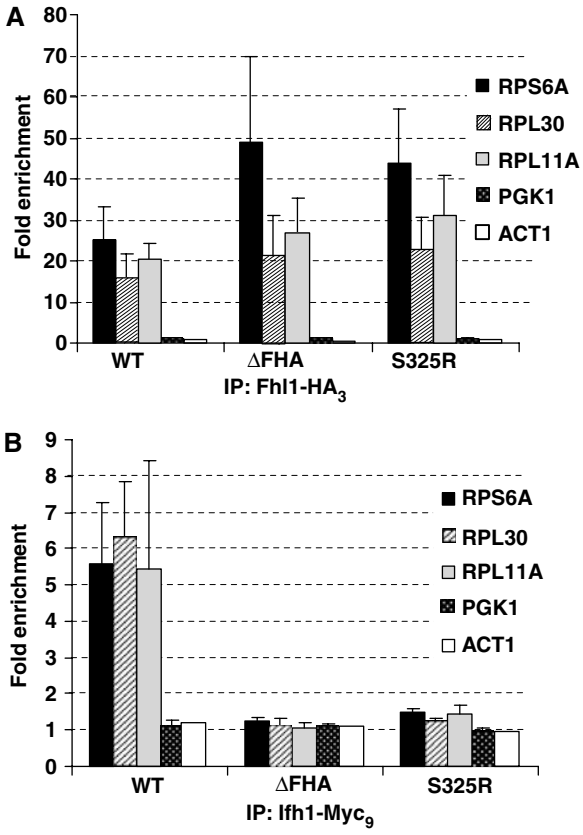


Figure 6 Interaction of Fhl1p and Ifh1p is necessary to bring Ifh1p to the RP genes. A ChIP experiment followed by real-time PCR was performed using anti-HA (**A**) or anti-Myc (**B**) antibodies on strains harboring HA_3-tagged full-length (WT), FHA domain deleted (ΔFHA) or the S_{325}R mutant version of Fhl1p (strains DR47, DR49 and DR65, respectively). The endogenous copy of *FHL1* in these strains is deleted, and Ifh1p is tagged C-terminally with Myc_9.

Δ*FHL1* Δ*IFH1* are viable, if very slow growing (Cherel and Thuriaux, 1995). While the slow growth is presumably due to the reduced content of ribosomes, how do the cells manage their transcription and translation under such conditions? We determined the level of rRNA transcription and the efficiency of rRNA processing by pulse–chase labeling with [C^3H_3]-methionine, with which it is possible to look specifically at the rRNA species (Figure 7). Two results are apparent. First, the level of incorporation of CH_3 during the 2.5 min pulse is greatly reduced in each mutant strain. Second, the processing of the pre-rRNA appears to proceed slowly but relatively efficiently in each mutant strain. This result is more apparent in the right-hand panels, which show a longer pulse and a longer chase for the mutant strains. By far, the larger part of the precursor RNAs appears to be processed normally, although there is a suggestion of some degradation. Since the mutant cells have a doubling time 3–4 × greater than the WT cells, and only 1/5 the content of RNA (Figure 5C), we calculate that they are making ribosomes at only 5–10% the rate of WT cells, a value consistent with the results of Figure 7. The important conclusion from this experiment is that cells are able to adjust their transcription of rRNA in response to the insult of reduced production of RPs.

mRNAs of ribosome-deprived cells

Because Fhl1p is found almost exclusively at RP genes (Lee *et al*, 2002) and similar results have been reported for Ifh1p (Schawalder *et al*, 2004), we expected to find that in Δ*FHL1* strains, the level of RP mRNA would be greatly reduced compared to that derived from other genes. However, Figure 8A shows that in comparison with *ACT1*, RP mRNAs are reduced marginally if at all. On the other hand, using U3 snoRNA as a loading control, the levels of both RP and non-RP mRNAs appear greatly reduced in mutant cells. To investigate more thoroughly, we examined the entire spectrum of mRNAs using an Affymetrix array (Figure 8B, D and F). The results are striking! Essentially all mRNAs are reduced similarly in the mutant cells. Note that equal amounts of total RNA were used from the WT and the mutant strains to make biotin-labeled cRNA, to be hybridized to the arrays (see Materials and methods). Since the total RNA level in Δ*FHL1* and Δ*FHL1* Δ*IFH1* cells is about a fifth of that in WT cells (Figure 5C), RNA from the mutant cultures represents five times as many cells as WT. Thus, on a per cell basis, all the genes lying on the $x = 0$ axis in the genome-wide gene expression patterns (Figure 8D and F) are approximately five times under-represented in the mutant strains. Of the ~5000 authentic genes analyzed, less than 300 showed signals that were preferentially increased or decreased more than two-fold in the mutant strains compared to the WT. Although there was substantial consistency between the two mutant strains, no discernable pattern appeared. For example, few of the more than 100 genes involved in ribosome biogenesis fell into this category.

Considering that Fhl1p binds almost exclusively to RP genes, the interesting result is that we observed only marginal deficits of the RP gene transcripts (Figure 8C, E and G). Of the 115 RP genes examined, only 11 from the Δ*FHL1* strain and 16 from the Δ*FHL1* Δ*IFH1* strain have mRNA at less than half their normal level compared to the bulk mRNA. For the most part, these were the same genes.

The raw data from the array hybridization suggest that the levels of mRNA in the mutant strains are reduced to approximately the same extent as the levels of total RNA. This result demonstrates that the mutant cells have a remarkable capacity to detect a deficiency of ribosomes and to respond by reducing the amounts of all mRNAs to ensure that the mRNA/ribosome ratio is maintained within narrow limits, presumably, although not necessarily, through reduced Pol II transcription. Such ability of the translation system to provide global feedback to the transcription system, in the interests of maintaining homeostasis, is yet another example of cellular control mechanisms of which we remain profoundly ignorant.

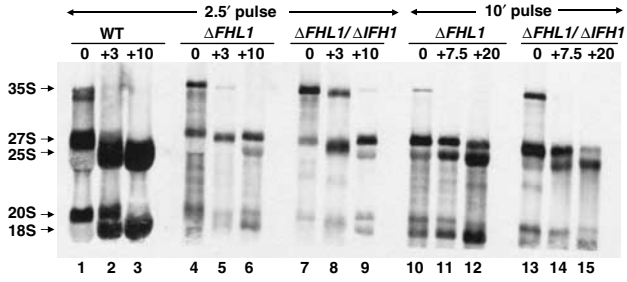
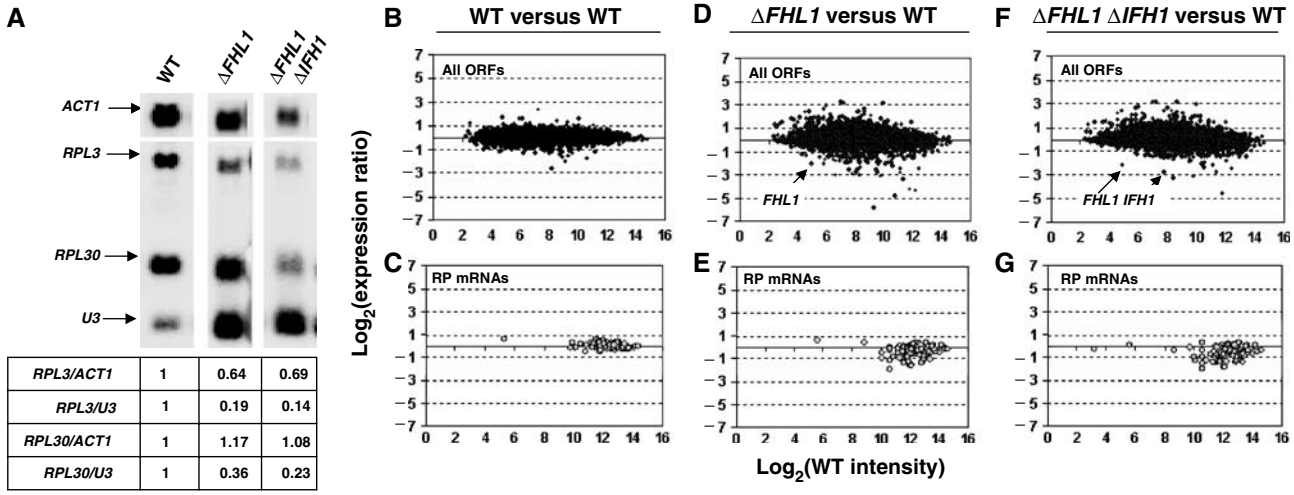


Figure 7 Slow transcription and processing of rRNA in mutant cells. Cultures of YNN281(WT), SHY35 (Δ*FHL1*) and D-105 (Δ*FHL1* Δ*IFH1*), growing in methionine drop-out medium, were pulsed with [C^3H_3]-methionine (Perkin-Elmer NET061-X) at 60 μCi/ml for 2.5 min (**A–C**) or 10 min (**D, E**). Cold methionine was added to 100 μg/ml and samples were taken at the indicated times. RNA was prepared and analyzed on a denaturing gel, transferred to nylon and treated with En^{3}Hance (Perkin-Elmer) and subjected to autoradiography for 7 days at −80. (Note that the WT lanes were loaded with RNA from half as many cells as the others.)



RPL3/ACT1	1	0.64	0.69
RPL3/U3	1	0.19	0.14
RPL30/ACT1	1	1.17	1.08
RPL30/U3	1	0.36	0.23

Figure 8 mRNA levels of Δ*FHL1* and Δ*FHL1*, Δ*IFH1* mutant strains. (**A**) Northern analysis showing the levels of RP mRNAs when normalized by U3 snoRNA or by *ACT1*. Total RNA (7.5 μg) was analyzed on denaturing agarose gels, transferred to nylon membrane and analyzed using labeled oligonucleotide probes directed against the indicated RNA species as previously described (Nierras and Warner, 1999). (**B–G**) Graphical representation of differential gene expression comparing DR36 (WT) with itself (B), with DR34 (Δ*FHL1*) (D) and with DR35 (Δ*FHL1* Δ*IFH1*) (F). The differential expressions of only the RP genes for the above samples are shown in (C, E and G), respectively. RNA from DR36, DR34 and DR35 strains was analyzed in duplicate using individual Affymetrix S98 arrays. The robust multiarray average (RMA) algorithm was used to normalize all six arrays and to compute average gene expression values for each strain. The original data are available in Supplementary Table I.

Layers of control

It is evident that a certain level of transcription of RP genes can occur in the absence of the transcription factors Fhl1p and Ifh1p (Figure 8). Is this residual transcription subject to the same controls as the high levels of transcription that occur in growing WT cells? Indeed, rapamycin can repress even the residual transcription of RP genes that occurs in the absence of Fhl1p and Ifh1p (Figure 9). Although the results presented in Figures 1D and 4C suggest that rapamycin acts by inhibiting the interaction of Ifh1p with Fhl1p, this result suggests that there is an additional layer of control of RP gene transcription beyond the interaction of Ifh1p with Fhl1p.

Discussion

New factors in the transcription of RP genes

The identification of Fhl1p and Ifh1p at the promoters of RP genes (Figure 1; Lee *et al*, 2002; Jorgensen *et al*, 2004; Schawalder *et al*, 2004) provides a new dimension for considering the regulation of this cohort of genes. If, as the bulk of the evidence suggests, these proteins are present exclusively at RP genes, they are likely to be key factors that recruit the transcriptional apparatus. This hypothesis is supported by the finding that when transcription of RP genes is repressed, Ifh1p is no longer found at the RP promoters. In contrast, Fhl1p, like Rap1p, seems to be present at the RP promoters even when transcription is repressed. Thus, the most economical hypothesis is that recruitment of Ifh1p to the promoter activates transcription of RP genes (Figure 1); depletion of Ifh1p reduces transcription of RP genes (Figure 2); deletion of *IFH1* is lethal (Cherel and Thuriaux, 1995).

Role of the FHA domain of Fhl1p

The importance of the FHA domain of Fhl1p, both for cell growth (Figure 5C) and for the presence of Ifh1p at RP genes (Figure 6B), implies that Ifh1p associates with the RP genes through its interaction with the FHA domain, which is therefore critical for RP gene transcription. The nature of the FHA domain (Durocher and Jackson, 2002) further implies that a phosphorylated residue of Ifh1p is the interacting partner. A simple model is that while Rap1p and Fhl1p (through its interaction with Rap1p?) are constitutively bound to RP genes, transcriptional activity is based on the phosphorylation of Ifh1p, permitting it to bind Fhl1p and consequently to associate with RP genes to drive transcription. Regulation, then, would depend on the balance between phosphorylation and dephosphorylation of Ifh1p.

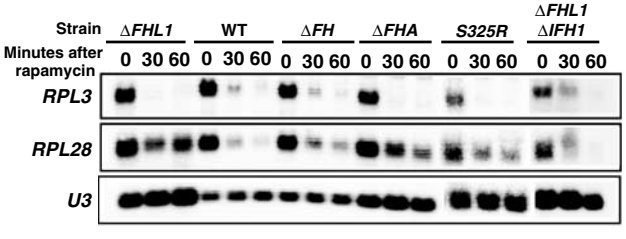


Figure 9 Rapamycin causes repression of RP genes in cells lacking Fhl1p and Ifh1p. Strains DR34, DR47, DR48, DR49, DR65 and DR35 were treated with rapamycin (0.2 μg/ml) and harvested at indicated time points. Total RNA was isolated, and 7.5 μg of RNA was analyzed by Northern blotting as described for Figure 8. Note the consistent high levels of U3 RNA from strains deficient in functional Fhl1p.

There are at least three, nonexclusive, candidates for Ifh1p kinase. The effect of rapamycin in reducing the Co-IP of Fhl1p and Ifh1p in parallel with the reduction of RP transcription (Figure 4C) implicates the TOR kinase pathway in RP gene transcription. The PKA pathway has been shown to participate in RP gene transcription (Klein and Struhl, 1994; Neuman-Silberberg *et al*, 1995; Jones *et al*, 2003) and, when constitutively activated, to overcome repression induced by rapamycin (Schmelzle *et al*, 2004). Finally, the identification of CK II in a complex with Ifh1p (Krogan *et al*, 2004; our unpublished data) is suggestive. Any or all of these kinases could maintain the phosphorylation of Ifh1p against a constitutively acting protein phosphatase, as yet unidentified.

Recent experiments have implicated Sfp1p as another factor that can be found at many RP genes (Jorgensen *et al*, 2004; Marion *et al*, 2004), but without the same degree of specificity as shown by Fhl1p (Lee *et al*, 2002) or Ifh1p (Schawalder *et al*, 2004). Sfp1p is interesting because it appears to migrate between nucleus and cytoplasm, the former when RP gene transcription is active, the latter when it is inactive (Jorgensen *et al*, 2004; Marion *et al*, 2004). The basis for this migration is unknown, as is the way in which it interfaces with Fhl1p and Ifh1p.

Jorgensen *et al* (2004) have stressed the importance of ribosome synthesis in controlling cell size, at least partly through Sfp1p, whose deletion leads to unusually small cells. Surprisingly, Δ*FHL1* cells, with greatly reduced ribosome biosynthesis, are of normal size (Figure 5E; Jorgensen *et al*, 2004). This lack of effect suggests that it is not simply the rate of ribosome synthesis itself that controls cell size, but rather some more complex interaction between the ribosome synthetic machinery and the START mechanism (Jorgensen *et al*, 2004; Rudra and Warner, 2004).

Role of Rap1p

Rap1p has long been an intriguing protein because of the number and variety of its roles in the cell, from coating telomeres to silencing the silent MAT loci to activating both glycolytic and RP genes, perhaps 50% of the Pol II transcripts of the cell. There is ample evidence that Rap1p acts by clearing nucleosomes from a region of chromatin, thereby permitting access to positive or negative transcription factors (Yu and Morse, 1999; Yarragudi *et al*, 2004). What is unclear is the basis for the specificity of these secondary factors, for example, for Sir3p at telomeres, for Gcr1p at glycolytic genes and for Fhl1p at RP genes (Figure 1). Does binding at different sites induce a specificity in Rap1p that ensures the binding of the correct factor? Or is Rap1p simply opening up the chromatin with the specificity supplied entirely by the context of surrounding sequences? Indeed, using a LexA-Rap1p fusion, we have found that the presence of Fhl1p at an RP gene requires not just the presence of Rap1p but also the direct interaction of Rap1p with its binding sites (Zhao *et al*, in preparation), suggesting that alteration of the structure of the DNA by Rap1p (Gilson *et al*, 1993; Konig *et al*, 1996) is a prerequisite for recruiting Fhl1p and Ifh1p.

Although a recent bioinformatics study has implicated two motifs as definitive characteristics of genes regulated as RP genes are (Beer and Tavazoie, 2004), the motifs are rather degenerate and seem not to be universal among the RP genes.

Indeed, we find that quite minor changes in the sequence context of the Rap1p sites can lead to drastic changes both in the occupancy by Ifh1p as measured by ChIP and in the level of transcription of the adjacent gene (Zhao *et al*, in preparation).

How is the transcription of the several RP genes that have an Abf1p site, but no Rap1p sites, so tightly coordinated with the others? While Abf1p can in some circumstances substitute for Rap1p to clear nucleosomes from chromatin (Yarragudi *et al*, 2004), the specificity issue remains since Abf1p is also found at many loci (Lee *et al*, 2002). On one gene driven by Abf1p (Hamil *et al*, 1988), *RPL3*, both Fhl1p and Ifh1p are found but at a substantially lower level than for *RPL30* (Figure 1B). Yet the transcription of *RPL3* and *RPL30* must be nearly identical, since the level (Holstege *et al*, 1998) and the $T_{1/2}$ (Kim and Warner, 1983) of their two mRNAs are almost the same. Are Fhl1p and Ifh1p more effective at *RPL3*, or are they simply associated with the gene in a way that is less effectively chromatin immunoprecipitated?

Other factors

Ultimately, we would like to know how the presence of Fhl1p and Ifh1p leads to such active transcription and how they interact with other factors that have been implicated in RP gene transcription, such as Sfp1p (Jorgensen *et al*, 2004; Marion *et al*, 2004), the TAFs (Mencia *et al*, 2002), the RSC chromatin remodeling complex (Angus-Hill *et al*, 2001) and the protein acetylase Esa1p (Reid *et al*, 2000). Interestingly, neither H4 nor H2A, the favored substrates for Esa1p, is highly acetylated at RP genes, which seem to have in common the acetylation of K18 of histone H3 (Kurdistani *et al*, 2004). Perhaps Esa1p acetylates one of the non-histone factors. Indeed, recent data indicate that the regulatory regions of RP genes are devoid of nucleosomes during active transcription (Bernstein *et al*, 2004; Lee *et al*, 2004), suggesting that these chromatin remodeling factors are operative only during periods of activation or repression of the RP genes.

Caveats

Although the data presented above are consistent with the simple model of Rap1p recruiting Fhl1p whose FHA domain recruits phosphorylated Ifh1p, which drives transcription of RP genes, a number of cautions need be considered. That cells grow, albeit slowly, without Fhl1p demonstrates an independent basal system of transcription. Furthermore, this basal level of RP mRNA is still repressed rapidly and efficiently by rapamycin (Figure 9). Thus, if Ifh1p is a target of the TOR pathway, it is not the only target. Furthermore, we find by Co-IP that Rap1p interacts with Ifh1p, even under conditions where Ifh1p does not interact with Fhl1p, for example, one that lacks the FHA domain (data not shown). This could explain why the overexpression of Ifh1p partially suppresses a Δ*FHL1* mutant, but not why the deletion of *FHL1* partially suppresses the lack of Ifh1p (Cherel and Thuriaux, 1995). Finally, at least one RP gene that is regulated coordinately with the others, *RPL18B*, appears to have no Fhl1p (Lee *et al*, 2002) or Ifh1p (confirmed by us, data not shown).

Resourcefulness of cells

A useful insight into biological homeostasis is revealed in Figures 7 and 8, which demonstrate that cells of *S. cerevisiae* have mechanisms to cope with the loss of more than 90% of their capacity to synthesize ribosomes. This is manifest through the extreme downregulation of rRNA transcription so that it roughly matches the available RPs. There is surprisingly little information about the way that rRNA and RP synthesis is coordinated in eukaryotic cells. Although Figure 7 suggests that rRNA transcription is repressed in response to a deficiency of RPs, undoubtedly multiple mechanisms couple the transcription of RP and rRNA genes, which together are responsible for such a great proportion of the total transcription of the cell. Such mechanisms remain to be identified.

Another manifestation of the cell's adjustment to deficient ribosome synthesis is the downregulation of the production of all mRNAs. A rapidly growing WT cell is estimated to have 200 000 ribosomes and 15 000 mRNAs (Warner, 1999). Were the Δ*FHL1* cell, with only 20 000 ribosomes (Figure 5D), to maintain its normal production of mRNA, there would be nearly one mRNA/ribosome, which would lead at least to inefficient if not to frankly aberrant translation. However, the array analysis described in Figure 8 shows that although the total RNA level in Δ*FHL1* cells is only 20% of WT, the ratio of mRNA to total RNA is essentially the same in both strains. This result suggests that global transcription by RNA polymerase II is somehow controlled by the capacity of the translational apparatus. Although the regulation of transcription of individual genes has been studied in great detail, little attention has been paid to the factors that limit global mRNA transcription, and how that limitation could be modulated in response to physiological insults.

Materials and methods

Strains and plasmid constructs

The strains used in this study are listed in Table I. Replacement of the *IFH1* promoter by the *GAL1* promoter and the epitope tagging of the proteins of interest were carried out by PCR-based gene targeting (Longtine *et al*, 1998) and TAP tagging according to Puig *et al* (2001). For the N-terminal TAP tagging of *RAP1* (strain YZ73), pBS1761 (Puig *et al*, 2001) was used as the template where the *GAL1* promoter is replaced by the *RAP1* promoter. All the tagged proteins supported normal growth.

Plasmid constructs carrying the WT and the mutated versions of *FHL1-HA*$_3$ were generated by conventional methods using the QuikChange XL site-directed mutagenesis kit (Stratagene). All were sequenced to confirm mutation. To generate strains DR47, DR48, DR49 and DR65, we transformed the respective plasmid constructs into the diploid DR57, followed by sporulation and tetrad dissection.

Preparation of yeast cell lysates, immunoprecipitation and Western blotting

A 50 ml culture was grown to an OD_{600} ~1.0. Cells were harvested, washed with IP150 buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$ and 0.1% NP-40) and lysed using glass beads in 300 μl ice-cold IP150 buffer supplemented with 'Complete Mini' protease inhibitor cocktail tablet (Roche) and 1 mM PMSF. Lysates were centrifuged at 13 000 *g* for 1 min at 4°C to remove debris. In some cases, the extract was incubated with 200 μg/ml of ethidium bromide on ice for 30 min before the centrifugation. These extracts were incubated at 4°C with anti-Myc mouse monoclonal antibody (9E10) coupled to Protein A-agarose beads (Pierce). For the IP performed with HA antibody, anti-HA affinity matrix (Roche) was used. Following incubation, beads were washed three times with IP150 buffer. The washed beads containing bound proteins were suspended in 50 μl of 1% SDS gel loading buffer and heated at 95°C for 5 min. The released polypeptides in 20 μl of heated sample were resolved in 0.1% SDS–5% polyacrylamide gels. The separated polypeptides were transferred onto a PVDF membrane, and

Table I Strains used in this work

Strain	Genotype	Reference or source
W303a and α	*ade2-1 leu2-3,112 ura3-1 his3-11,15 trp1-1 can1-100 ssd1-1*	Thomas and Rothstein (1989)
YZ73	W303α *TAP-RAP1*::*TRP1*	This study
YZ146	W303α *IFH1-HA3*::*G418r*	This study
YZ147	W303α GAL_{UAS}*IFH1*::*HIS3*	This study
DR12	W303a *FHL1-TAP*::*G418r*	This study
DR13	W303a *FHL1-*HA_3::*G418r*	This study
DR14	W303α *IFH1-TAP*::*TRP1*	This study
DR34	W303α *FHL1*Δ::*HIS3*	This study
DR35	W303α *FHL1*Δ::*HIS3, IFH1*Δ::*G418r*	This study
DR36	W303a *IFH1-*MYC_9::*TRP1, FHL1-*HA_3::*G418r*	This study
DR37	W303α *IFH1-*MYC_9::*TRP1*	This study
DR47	W303 *FHL1*Δ::*HIS3, IFH1-*MYC_9::*TRP1,* (pRS316: *CEN, URA3 FHL1-*HA_3)	This study
DR48	W303 *FHL1*Δ::*HIS3, IFH1-*MYC_9::*TRP1,* (pRS316: *CEN, URA3* Δ*FH-*HA_3)	This study
DR49	W303 *FHL1*Δ::*HIS3, IFH1-*MYC_9::*TRP1,* (pRS316: *CEN, URA3* Δ*FHA-*HA_3)	This study
DR57	W303*a*/α *FHL1*Δ::*HIS3/FHL1 IFH1-*MYC_9::*TRP1/IFH1*	This study
DR65	W303 *FHL1*Δ::*HIS3, IFH1-*MYC_9::*TRP1,* (pRS316: *CEN, URA3 FHL1(*S_{325}*R-*HA_3*)*)	This study
YNN281	*MATa ura3-52, his3-*Δ*200, trp1-1, lys2-801a, ade2-101*	Cherel and Thuriaux (1995)
SHY35	*MATa ade2-1, ura3-52, trp1-*Δ*1, lys2-801, his3-*Δ*200, fhl1-*Δ*1*::*HIS3*	Cherel and Thuriaux (1995)
D105	*MATa ura3-52, his3-*Δ*200, trp1-*Δ*1, lys2-801a, ade2-101 fhl1-*Δ*1*::*HIS3 ifh1-1*::*URA3*	Cherel and Thuriaux (1995)

analyzed by Western blotting using anti-HA (3F10) peroxidase or anti-c-Myc (9E10) peroxidase (Roche) wherever applicable.

Chromatin immunoprecipitation

A 200 ml portion of culture ($\sim 1 \times 10^7$ cells/ml) was treated with formaldehyde at a final concentration of 1% at room temperature for 30 min with occasional swirling. Glycine was added to a final concentration of 360 mM. ChIP was then carried out as described (Kuras and Struhl, 1999). To immunoprecipitate Fhl1-HA_3 or Ifh1-Myc_9, 20 μl of anti-HA mouse monoclonal antibody (12CA5) or 20 μl anti-c-Myc mouse monoclonal (9E10) antibody was added to the chromatin preparation with 20 μl Protein A-agarose beads and incubated at 4°C for 3 h. Quantitative real-time PCR analyses were performed on an Applied Biosystems 7700 sequence detector. To calculate the fold enrichment of Fhl1-HA_3 and Ifh1-Myc_9 occupancy at an individual promoter, we determined the apparent crosslinking efficiency by dividing the amount of PCR product from the immunoprecipitated sample by the amount of PCR product in the input sample prior to IP and subtracting the apparent crosslinking efficiency of a control promoter, *CYC1*, that is not occupied by Fhl1p (Lee *et al*, 2002).

Microarray hybridization

Total RNA was isolated from strains DR34, DR35 and DR36. Following the Affymetrix protocol, 5 μg of each RNA was used to prepare cDNA using reverse transcriptase (GIBCO-BRL SuperScript) that was subsequently used as a template to make biotin-labeled cRNA using an *in vitro* transcription reaction (Enzo). Each cRNA was hybridized with an individual Affymetrix Yeast Genome S98 oligonucleotide array that was subsequently processed and scanned according to the manufacturer's instructions. Biotin-labeled cRNA preparation and array hybridization was performed in duplicate. Data were saved as raw image files and converted into probe set data (as '*.cel' files) using Microarray Suite (MAS 5.0).

Analysis of microarray data

Robust multiarray average (RMA) (Irizarry *et al*, 2003) was used to analyze Affymetrix probe set data. There are four stages of RMA. First, probe set data ('*.cel' files) from all arrays are simultaneously normalized using quantile normalization, which eliminates systematic differences between GeneChips, without significantly altering the relative intensity of probes within a GeneChip. Second, mean optical background level for each array is estimated, and the intensity for each probe is adjusted to remove this. Third, the normalized, background-corrected data are transformed to the $\log_2$ scale. Finally, multiple probes are combined into a single measure of expression for each gene on each array by using a median-polish procedure. The microarray data can be accessed at GEO with the accession number GSE2096.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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