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Yeast Chaperone Hsp104 Controls Gene Expression at the Posttranscriptional Level

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Abstract—Yeast chaperone Hsp104 is known as a protein responsible for dissociation of aggregates of heat-damaged proteins and prion aggregates into smaller pieces or monomers. The effects of Hsp104 on PrP-GFP and GFP were analyzed. PrP-GFP forms high-molecular-weight aggregates, whereas GFP is unable to aggregate in yeast cells. Hsp104 proved to regulate the amount of PrP-GFP and GFP in yeast cells, and the direction of chaperone action depended on the promoters controlling the production of these proteins. Overproduction of Hsp104 increased the levels of PrP-GFP and GFP when their genes were controlled by the *CUP1* promoter. In contrast, overproduction of Hsp104 decreased the levels of PrP-GFP and GFP in the case of their expression under the control of the *GPD* promoter. The effects of Hsp104 were not related to any changes in the contents of mRNAs of the genes under investigation nor to the ability of the proteins to form aggregates. Thus, the Hsp104 functions were not confined to dissociation of protein aggregates. Hsp104 was assumed to regulate gene expression at the posttranscriptional level.

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Key words: Hsp104 chaperone, expression regulation, yeast *Saccharomyces cerevisiae*, mammalian prion, promoter structure

INTRODUCTION

Historically, chaperones are referred to as heat-shock proteins, which are produced in response to elevated temperatures to assist the cell in coping with stress consequences. Yet many chaperones are produced at physiological temperatures, being essential for life support. Yeast chaperone Hsp104 is not a vital protein. Normally, Hsp104 is present in the cell in a small quantity, but its production dramatically increases under stress [1]. Under heat shock conditions, the viability of strains with deletions of *HSP104* is significantly reduced [2]. Unlike other chaperones, Hsp104 does not prevent abnormal folding or protein aggregation after stress [3]. Hsp104 is involved in the disassembly of stress-induced aggregates and thereby assists monomer reactivation [2]. The mechanism of disassembly of protein aggregates by Hsp104 is not understood as of yet. Structural analysis indicates that Hsp104 operates in the form of a hexamer, each monomer having two ATP-binding sites [4]. A hexamer in which at least one monomer has distortions in the ATP-binding motifs cannot perform the disaggregase function [5].

Hsp104 is essential for stable maintenance of yeast prions, proteins forming regular self-propagating aggregates. Sup35, Ure2, and Rnq1 can exist in prion isoforms ([*PSI*$^+$], [*URE3*], and [*PIN*$^+$]) in a sequence of generations only at a certain level of Hsp104 production [6]. It has been shown that Hsp104 utilizes free energy from ATP hydrolysis to split up large [*PSI*$^+$] polymers into small oligomers, which initiate new prion transformation cycles [7]. If *HSP104* is deleted, prion aggregates are not split, which results in their failure to be transmitted to daughter cells [7]. Overproduction of this yeast chaperone causes the elimination of [*PSI*$^+$] [8] but not [*URE3*] or [*PIN*$^+$] [10]. The mode of interactions between Hsp104 and other amyloid proteins, having no N/Q tracts, is open to discussion.

We studied the effect of Hsp104 on the murine amyloid protein PrP (prion protein) fused with the green fluorescent protein (GFP). Aggregation of PrP causes prion-related neurodegenerative diseases in mammals. PrP forms amyloid polymers, although lacking N/Q-rich tracts [12]. In the aggregated isoform, PrP forms a protease-resistant fragment, which includes region 90–231. This fragment is necessary

and sufficient for prion transformation [13, 14]. The interaction between Hsp104 and PrP has been demonstrated in vitro [15, 16]. In a cell-free system, the yeast chaperone favors PrP aggregation, but only in the presence of preexisting partly denatured PrP aggregates [16].

In this study, the sequences encoding PrP(90–231)-GFP and GFP were expressed under the control of the *GPD* and *CUP1* yeast promoters in cells with Hsp104 deletion or overproduction. We found that Hsp104 positively regulated the PrP-GFP and GFP expression controlled by the *CUP1* promoter and reduced their expression controlled by the *GPD* promoter. These effects were not related to the ability of the proteins to aggregate. A change in the level of Hsp104 production did not alter the amounts of the corresponding mRNAs. Thus, this is the first demonstration of the Hsp104 role in posttranscriptional regulation of differential gene expression.

EXPERIMENTAL

Strains, media, and growth conditions. Experiments were carried out with the following *Saccharomyces cerevisiae* strains: GT113 (*MATa/MATα ade1-14/ade1-14 his3Δ/his3Δ leu2-3, 122/leu2-3, 112 lys2/lys2 ura3-52/ura3-52 trp1-289/trp1-289 SUP35/sup35Δ::HIS3* [*psi*$^-$] [*pin*$^-$]) [17], BY4742 (*MATα his3Δ-1 leu2Δ-0 lys2Δ-0 ura3Δ-0*) (Invitrogen, United States), and BY4742 (*hsp104Δ*) (*MATα his3Δ-1 leu2Δ-0 lys2Δ-0 ura3Δ-0 hsp104::KAN*^{r}) (Invitrogen, United States). Plasmid DNA was produced in *Escherichia coli* strain DH5α (*sup*E44 Δlac*U169* (φ*80lacZΔM15*) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A). The bacterial strain was grown in the liquid and solid LB media and in the liquid SOB medium [18]. Yeast strains were grown in the solid and liquid YAPD media at 30°C and in synthetic media with appropriate vitamins, microelements, and amino acids [19, 20]. For gene expression under the control of the inducible *CUP1* promoter, $CuSO_4$ was added to the media at 3–100 μM.

Plasmids. All plasmids used in the study contained the yeast and bacterial replication origins. Plasmids pLH105 and pRS316CG [21] were kindly provided by Yu.O. Chernoff. Plasmid peDNA3-1-3F4 [22], bearing the murine *PrnP* gene, was kindly provided by D. Harris. Plasmid pRS424-GPD-HSP104-KT was constructed as follows. The *GPD* promoter flanked by *Sal*I–*Bam*HI sites was excised from pLH105 and inserted into pFL39 digested with *Xho*I–*Bam*HI. Then, the 3.2-kb *Bam*HI–*Sac*I fragment bearing the mutant *HSP104-KT*-218,620 sequence (hereafter referred to as *HSP104-KT*) of pFL39-GAL-HSP104-KT was inserted into the multiple cloning site following the *GPD* promoter [23]. Plasmid pFL39-GAL-HSP104-KT contained the mutant *HSP104* gene with two amino acid substitutions of Thr for Lys at codons 218 and 620. Expression of the *HSP104-KT* sequence has a dominant negative influence on the ATPase activity of native Hsp104 [24]. To construct P_{CUP1}-GFP(URA3), the 1.2-kb *Xho*I–*Sac*I fragment, bearing *GFP* under the control of the *CUP1* promoter, was excised from pRS316CG and cloned into pFL44S [25] digested with *Xho*I and *Sac*I. The sequence encoding the murine PrP protein fragment 90–231 was PCR-amplified from pcDNA3-1-3F4. The primers were F (5'-gttcatggatcctatatgtctcaaggaggggtacccat) and R (5'-gggccgcggttatcagctggatcttctcccgtcgta).

To construct P_{CUP1}-PrP-GFP(URA3), the amplified *PrP(90–231)* fragment was cloned into P_{CUP1}-GFP(URA3) at the *Bam*HI and *Sac*II sites located downstream of the *CUP1* promoter. Plasmid P_{GPD}-PrP-GFP(URA3) was constructed by replacing the *Xho*I–*Bam*HI fragment containing the *CUP1* promoter with the *Xho*I–*Bam*HI fragment containing the *GPD* promoter from pLH105.

RNA isolation, reverse transcription, and real-time PCR. RNA was isolated using Trisol (TRI Reagent, Sigma). Samples were digested with RNase-free DNase I (Sileks M). Reverse transcription with random hexanucleotide primers was carried out with a REVERTA kit (Institute of Epidemiology, Russia) according to the manufacturer's recommendations. The 224-bp *ADH1* fragment amplified with primers ADH1-F (5'-cggtggtcacgaaggtgcc) and ADH1-R (5'-gagcggcttgaacagcgtca) was used as a reference. The course of amplification in the real-time mode was monitored with primers allowing amplification of a 246-bp *GFP* fragment: sGFP-F (5'-ccaacacttgtcactactttcacttatggt) and sGFP-R (5'-gtgcccaagaatgtttccatcttct).

DNA sequences complementary to internal regions of the amplified sequences were used as TaqMan probes: ADH1-Cy5 (5'-tcgtgggtgtaaccagacaagtcagcgtga, fluorochrome Cy5, quencher RTQ-2) and sGFP-ROX (5'-tttcaagagtgccatgcccgaaggttatgt, fluorochrome ROX, quencher BHQ2). All primers and probes were manufactured by Sintol (Russia). The reaction was carried out in an ANK-16 device (Institute of Analytical Instrumentation, Russia). The reaction parameters were determined with the ANK software (Bauman Moscow State Technical University, Moscow).

Immunochemical analysis. Protein isolation and differential centrifugation were performed by the conventional method [26]. The cell lysate was separated into a pellet (insoluble) and supernatant by centrifuging at 12,000 *g* for 30 min. After SDS-PAGE in 12% gel, proteins were transferred onto a nitrocellulose membrane. Western blotting was performed with primary monoclonal antibody 3A9 against GFP (Institute of Bioorganic Chemistry, Moscow) and an ESL kit (Amersham Biosciences). Normalization of the total

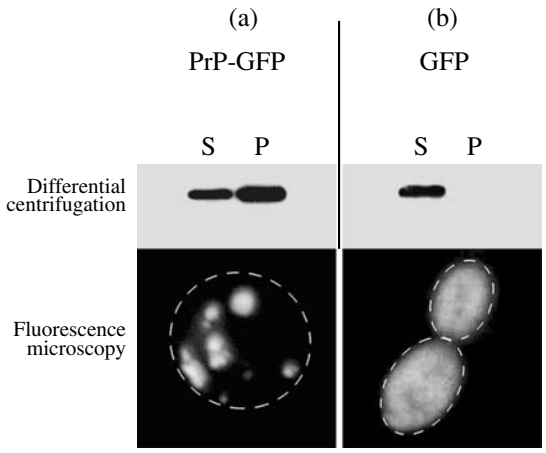

Fig. 1. Analysis of protein aggregation by differential centrifugation and fluorescence microscopy: (a) PrP-GFP, (b) GFP. S, supernatant (soluble fraction); P, pellet (insoluble fraction).

protein amount of gel stained with Coomassie Blue and measurement of the intensity of the specific band on the X-ray film were performed by densitometry, using the ImageJ 1.37a software package. Comparisons of the intensity of the specific signal were performed in triplicate with independent transformants.

Fluorescent analysis of proteins fused with GFP was performed using a Leica DM6000B microscope (Leica Microsystems, Germany) with the Leica QWin Standart V3.2.0 software package for image analysis. Fluorescence was analyzed with a GFP cube (Leica) with a 415-nm emission filter and a 480-nm excitation filter. Cells with PrP-GFP aggregates were counted on day 3 of growing transformants on a solid synthetic medium with appropriate supplements. Ten randomly chosen fields of vision (no less than 400 cells) were analyzed for each transformant. The rate of cells with cytologically detectable aggregates was counted. The significance of differences was evaluated by the nonparametric Mann–Whitney test.

RESULTS

PrP-GFP Forms High-Molecular-Weight Aggregates in Yeast Cells

To analyze PrP-GFP aggregation, GT113 [*psi*$^-$] [*pin*$^-$] cells were transformed with a plasmid containing the *PrP-GFP* sequence fused with the P_{CUP1} promoter. Transformants bearing a plasmid with P_{CUP1}-*GFP* were used as a control. To induce the *CUP1* promoter, the selective medium was supplemented with 100 μM $CuSO_4$. The protein extract obtained from the transformants by differential centrifugation was separated into the soluble and insoluble fractions. The bulk of PrP-GFP was in the aggregated isoform (Fig. 1a), whereas GFP, as expected, was detected only in the soluble fraction (Fig. 1b).

Aggregation of PrP-GFP was confirmed by fluorescence microscopy. Fluorescent grains were detected in cells producing PrP-GFP (Fig. 1a), whereas cells containing GFP were characterized by homogeneous green glowing (Fig. 1c). In the culture grown with 100 μM $CuSO_4$, cytologically detectable aggregates were present in 17–27% of the total number of cells examined (Table 1).

Hsp104 Posttranscriptionally Regulates the Expression of the Genes under Study

Strains producing PrP-GFP and GFP under the control of the *CUP1* promoter were transformed with pLH105 for Hsp104 overproduction. This increased the levels of the proteins under study in yeast cells (Fig. 2a). The ratio between the PrP-GFP amounts in the soluble and insoluble fractions did not significantly change. With the example of PrP-GFP, we demonstrated that the observed phenomenon stably reproduced at various levels of *CUP1* promoter activation

Table 1. Rates of cells containing PrP-GFP aggregates in Hsp104-overproducing and control cultures

Transformant	Hsp104 overproduction		Regular Hsp104 production	
	cells examined	cells with aggregates, %*	cells examined	cells with aggregates, %*
1	418	43.06 ± 2.42	725	20.97 ± 1.51
2	764	38.74 ± 1.76	699	18.31 ± 1.46
3	985	31.98 ± 1.49	655	18.67 ± 1.52
4	872	37.84 ± 1.64	793	17.78 ± 1.36
5	527	50.29 ± 2.18	580	27.59 ± 1.86
6	473	46.94 ± 2.29	538	21.93 ± 1.78
7	1134	47.88 ± 1.48	412	20.15 ± 1.98
8	1122	55.35 ± 1.48	730	23.56 ± 1.57

* Cell percentage ± standard error of the mean.

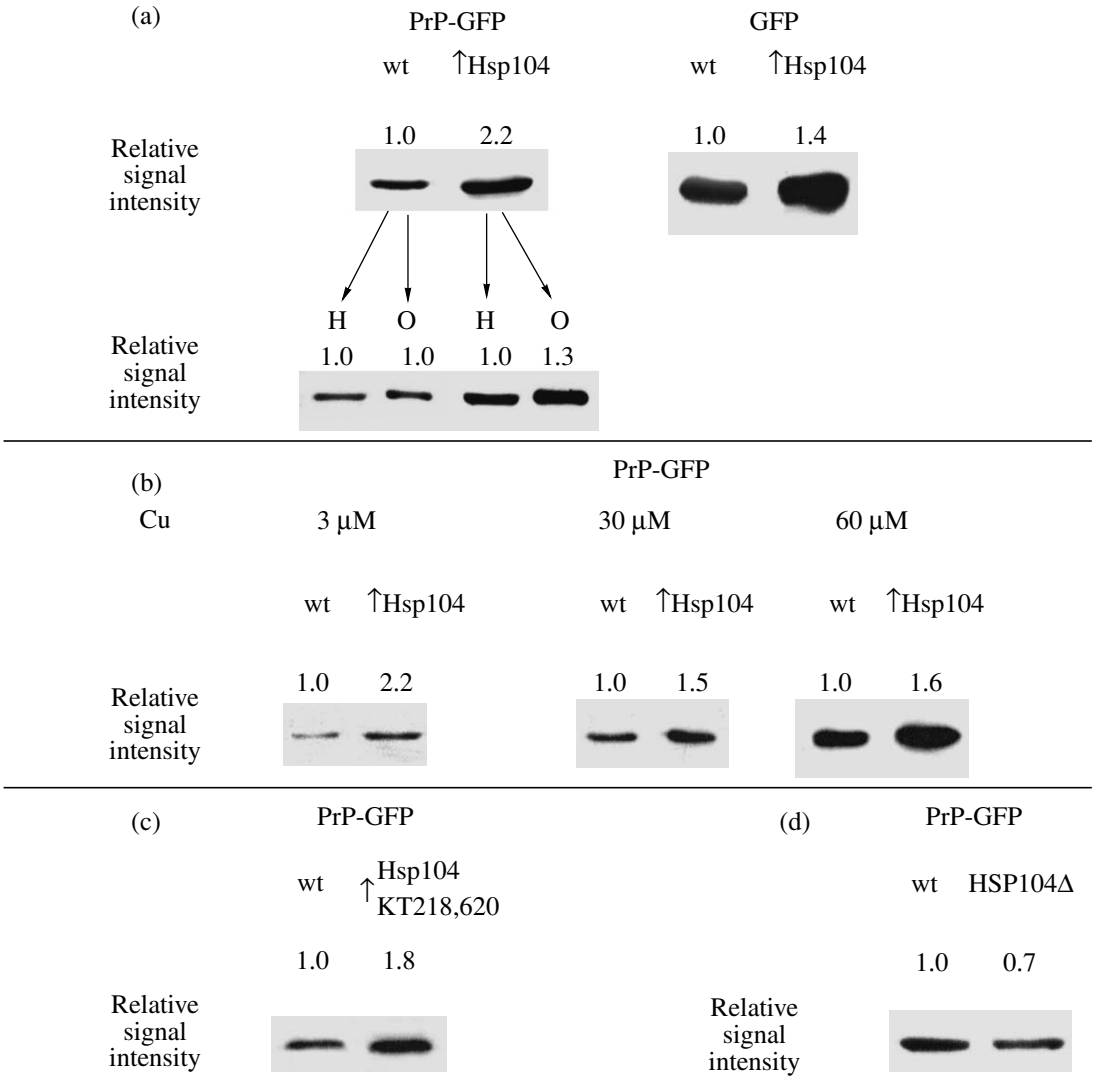

Fig. 2. Effect of Hsp104 on the expression of the *PrP-GFP* and *GFP* sequences controlled by the *CUP1* promoter. In all experiments except Fig. 3b, the promoter was activated by adding 100 μM $CuSO_4$ to the medium. (a) Effect of Hsp104 overproduction on the amounts of PrP-GFP and GFP in yeast cells. (b) Effect of Hsp104 overproduction at different levels of *PrP-GFP* expression controlled by the *CUP1* promoter. (c) Effect of the overproduction of the mutant chaperone variant Hsp104KT218,620 with inactivated ATPase activity on the concentration of PrP-GFP. (d) Effect of *HSP104* deletion on the amount of PrP-GFP: wt, normal Hsp104 production; ↑Hsp104 and ↑Hsp104KT218,620, overproduction of Hsp104 and its mutant variant Hsp104KT218,620, respectively; Hsp104Δ, deletion of the *HSP104*.

(Fig. 2b). This result was confirmed by fluorescence microscopy. Upon Hsp104 overproduction, the rate of cells with cytologically visible PrP-GFP aggregates increased approximately twofold (Table 1). The significance of differences was evaluated by the Mann–Whitney test, whose value equaled 3.36 (P = 0.00078). As Hsp104 did not affect PrP-GFP aggregation, the increase in the rate of aggregate-containing cells could be related to an increase in the amount of the protein studied.

In addition, we analyzed the effect of inhibition of the ATPase activity of Hsp104 and deletion of its gene on the PrP-GFP production. The strain bearing P_{CUP1}-PrP-GFP was transformed with pRS424-GPD-HSP104-KT, containing a mutant *HSP104*, which had a dominant negative effect on the ATPase activity of native Hsp104 [24]. An increase in the expression of the gene for the mutant chaperone variant, as well as the overexpression of intact *HSP104*, increased the amount of PrP-GFP (Fig. 2c). The signal increased by factors of 1.5–2 in three experimental replications. Thus, the positive expression regulation mediated by Hsp104 did not depend on the ATPase activity of the chaperone. Deletion of *HSP104* had opposite consequences. Strain BY4742 and isogenic strain BY4742(*hsp104*Δ), bearing a deletion of the chromosomal *HSP104* copy, were transformed with P_{CUP1}-PrP-GFP. Upon the *HSP104* deletion, the amount of

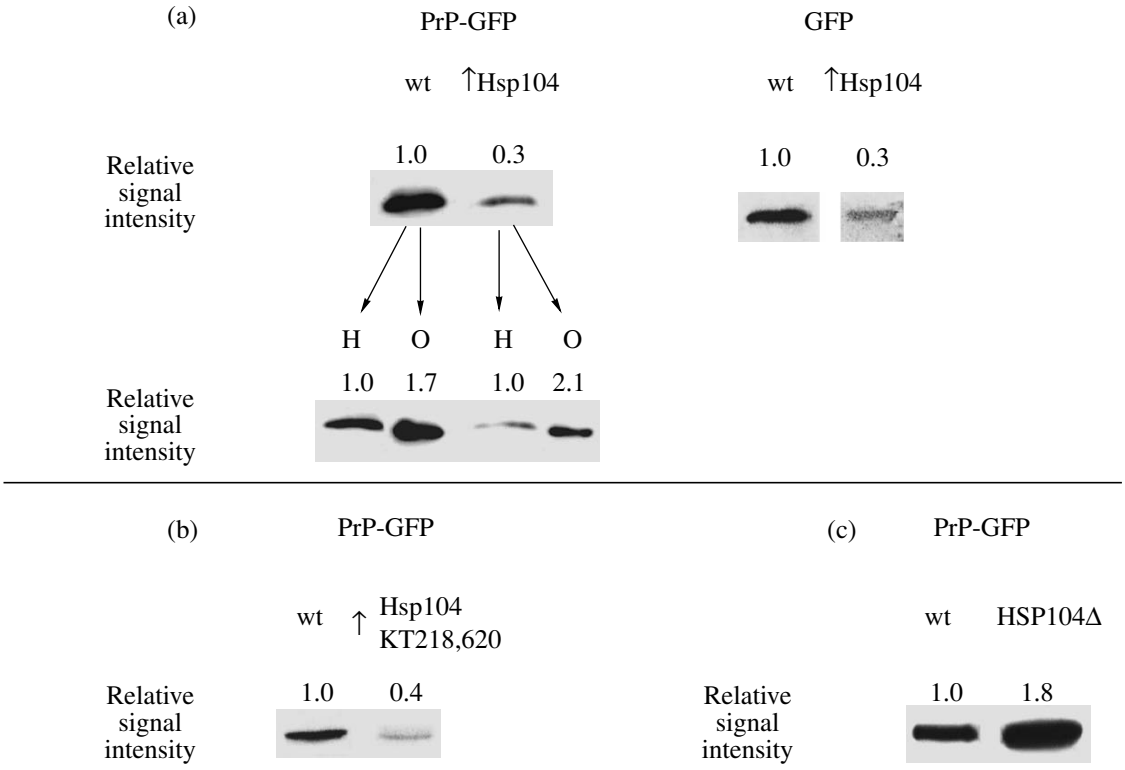

Fig. 3. Effect of Hsp104 on the expression of the *PrP-GFP* and *GFP* sequences controlled by the *GPD* promoter. (a) Effect of Hsp104 overproduction on the amounts of PrP-GFP and GFP in yeast cells. (b) Effect of the overproduction of the mutant chaperone variant Hsp104KT218,620 with inactivated ATPase activity on the concentration of PrP-GFP. (c) Effect of *HSP104* deletion on the amount of PrP-GFP: wt, normal Hsp104 production; ↑Hsp104 overproduction of Hsp104 or its mutant variant Hsp104KT218,620, respectively; Hsp104Δ, deletion of *HSP104*.

PrP-GFP insignificantly decreased (Fig. 2d), but these differences were reproduced throughout the series of independent experiments. Based on the results shown in Fig. 2, we suggest that Hsp104 positively regulates the PrP-GFP and GFP production under the control of the *CUP1* promoter.

The next step of the study concerned the influence of Hsp104 on the expression of the same sequences under the control of another yeast promoter, *GPD*. Strains having the P_{GPD}-*PrP-GFP* or P_{GPD}-*GFP* sequences were transformed with pLH105, overproducing Hsp104, or the control pRS315 vector. Immunochemical analysis showed that, when the sequences under study were controlled by P_{GPD}, Hsp104 overproduction reduced the amounts of the proteins (Fig. 3a). Overexpression of the mutant copy of the gene, *HSP104-KT*, with inactivated ATPase activity had the same effect on the P_{GPD}-*PrP-GFP* expression (Fig. 3b). This result was not altered in the presence of 100 or 150 μM $CuSO_4$. Thus, the direction of the chaperone effect did not depend on copper ions in the growth medium. The *HSP104* deletion increased the amount of PrP-GFP when the hybrid sequence was expressed under the control of the *GPD* promoter (Fig. 3c). These results indicate that Hsp104 regulates the amounts of the proteins in yeast cells and that the direction of the chaperone effect depends solely on the promoter controlling the corresponding genes.

It was reasonable to suggest that Hsp104 indirectly regulates transcription of sequences controlled by the *CUP1* and *GPD* promoters. To verify this suggestion, we compared the amounts of the *PrP-GFP* and *GFP* mRNAs in experiments with regular Hsp104 production and its overproduction. As seen from Table 2, the mRNA copy numbers did not differ in experiments with regular Hsp104 production and overproduction. Thus, Hsp104 overproduction did not affect the levels of the *PrP-GFP* and *GFP* mRNAs synthesized under the control of the *CUP1* and *GPD* promoters. From these results, we conclude that Hsp104 regulates the expression of the sequences under study at the translational or posttranslational level.

DISCUSSION

Our results indicate that PrP-GFP forms high-molecular-weight aggregates in the yeast cytoplasm (Fig. 1a), whereas GFP does not aggregate even when overproduced under the control of the *CUP1* promoter (Fig. 1b). When *PrP-GFP* and *GFP* are expressed

Table 2. Comparison of *PrP-GFP* and *GFP* mRNA quantities in experiments with different levels of Hsp104 production

Promoter	Gene	ΔCt*		ΔΔCt**	$2^{-\Delta\Delta Ct}$***
		Hsp104 overproduction	Regular Hsp104 production		
CUP1	*PrP-GFP*	0.18 ± 0.141	0.25 ± 0.069	0.07	1.050
	GFP	0.34 ± 0.134	0.30 ± 0.035	0.03	1.021
GPD	*PrP-GFP*	1.23 ± 0.796	1.28 ± 0.835	0.05	1.035

Notes: * ΔCt is the difference between the means of the threshold mRNA cycles of the reference and examined genes.
** ΔΔCt is the difference between the ΔCt means observed upon regular production and overproduction of Hsp104.
*** $2^{-\Delta\Delta Ct}$ is the ratio between the copy numbers of the mRNAs under study in the control and test cultures.

under the control of the *CUP1* promoter, Hsp104 overproduction increases the amounts of the corresponding proteins (Fig. 2a). The same effect is observed at an elevated production of the mutant Hsp104KT, whose ATPase activity is entirely inhibited (Fig. 2c). In contrast, the deletion of the chaperone gene reduces the PrP-GFP level in yeast cells (Fig. 2d). These results indicate that the role of Hsp104 is not limited to disassembly of protein aggregates. Hsp104 regulates the expression of proteins independently of their ability to aggregate, and this regulation is not related to the ATPase activity of the chaperone.

The opposite effect of Hsp104 is observed when the expression of *PrP-GFP* and *GFP* is controlled by the *GPD* promoter (Fig. 3). This inverted action is not related to any differences in the rate of production of the proteins under the control of the *CUP1* and *GPD* promoters, because Hsp104 overproduction increases the level of PrP-GFP at any rate of transcription of the corresponding sequence under the control of the *CUP1* promoter (Fig. 2b). Neither is this phenomenon related to the presence of copper ions in the selective medium: their addition did not affect the result with the *GPD* promoter. It may be concluded that the direction of the chaperone effect on the expression of the same sequences is determined by the promoter controlling their transcription. The real-time PCR data convincingly demonstrate that the change in *Hsp104* expression does not affect the amounts of the *PrP-GFP* or *GFP* mRNAs. Thus, the expression is regulated at the posttranscriptional level.

The following working hypothesis can be proposed to explain this phenomenon. It is likely that Hsp104 activates degradation of alien proteins by interacting with other chaperones or proteins of the ubiquitin complex. In addition, according to our results, Hsp104 positively regulates the expression of sequences controlled by the *CUP1* promoter. Therefore, Hsp104 must possess an additional activity. The mRNA molecules produced from the genes under study under the control of the *CUP1* and *GPD* promoters differ only in the sequence of their 5'-untranslated regions (UTRs), because the transcription initiation sites are located several tens of nucleotides upstream from the start codon. The UTRs of many viral genes and some genes of higher eukaryotes contain sequences positively regulating the rate of translation of corresponding mRNAs [27]. The same sequences have been recently found in several yeast genes [28]. Some UTRs bind protein factors controlling translation. For example, the Zuotin yeast chaperone binds the UTR of the *TFIID* mRNA and positively regulates its translation [29].

The results obtained for the Hsp101 chaperone, a plant ortholog of Hsp104, are of special interest [24]. It has been found that Hsp101 binds to the degenerate tandem repeat $(CAA)_n$ in the UTR of the tobacco mosaic virus mRNA and positively regulates its translation [30]. According to our analysis, the start codon of the *CUP1* promoter is also preceded by four CAAT tandem repeats. This fact brings us to the suggestion that Hsp104 directly interacts with the UTR of the mRNA synthesized from the *CUP1* promoter and increases the efficiency of mRNA binding with translation initiation factors. To understand the molecular mechanisms of the Hsp104-mediated expression regulation, we intend to analyze the interaction of Hsp104 with modified UTRs of various promoters and translation initiation factors by biochemical and genetic methods.

To summarize, we showed that the yeast Hsp104 chaperone controls gene expression at the posttranscriptional level. Thus, we have revealed a new function of Hsp104, independent of its ATPase activity.

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