An early step in wobble uridine tRNA modification requires the Elongator complex

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ABSTRACT

Elongator has been reported to be a histone acetyltransferase complex involved in elongation of RNA polymerase II transcription. In *Saccharomyces cerevisiae*, mutations in any of the six Elongator protein subunit (*ELP1–ELP6*) genes or the three killer toxin insensitivity (*KTI11–KTI13*) genes cause similar pleiotropic phenotypes. By analyzing modified nucleosides in individual tRNA species, we show that the *ELP1–ELP6* and *KTI11–KTI13* genes are all required for an early step in synthesis of 5-methoxycarbonylmethyl (mcm^5) and 5-carbamoylmethyl (ncm^5) groups present on uridines at the wobble position in tRNA. Transfer RNA immunoprecipitation experiments showed that the Elp1 and Elp3 proteins specifically coprecipitate a tRNA susceptible to formation of an mcm^5 side chain, indicating a direct role of Elongator in tRNA modification. The presence of mcm^5U, ncm^5U, or derivatives thereof at the wobble position is required for accurate and efficient translation, suggesting that the phenotypes of *elp1–elp6* and *kti11–kti13* mutants could be caused by a translational defect. Accordingly, a deletion of any *ELP1–ELP6* or *KTI11–KTI13* gene prevents an ochre suppressor tRNA that normally contains mcm^5U from reading ochre stop codons.

Keywords: 5-methoxycarbonylmethyl-2-thiouridine; 5-methoxycarbonylmethyluridine; 5-carbamoylmethyluridine; Elongator complex; *KTI* genes

INTRODUCTION

In the RNA polymerase II transcription cycle, various accessory factors are needed for efficient and selective initiation and elongation of transcription on DNA templates. Some of these accessory factors, such as histone acetyltransferases, modify chromatin to make DNA accessible for transcription (Roth et al. 2001). The *Saccharomyces cerevisiae* Elongator complex, first described to include three proteins (Elp1, Elp2, and Elp3), was found to be associated with the hyperphosphorylated form of RNA polymerase II (Otero et al. 1999). Subsequently, Elp4, Elp5, and Elp6 proteins were identified to be part of the Elongator complex as a subcomplex (Krogan and Greenblatt 2001; Winkler et al. 2001). In vitro, the six-subunit Elongator complex has histone H3 and H4 acetyltransferase activity, and this activity was associated with the Elp3 protein in particular (Wittschieben et al. 1999; Winkler et al. 2002). Orthologs of Elp1–Elp4 can be found in humans as part of a six-subunit Elongator complex, which has in vitro histone H3 and H4 acetyltransferase activity (Winkler et al. 2001; Hawkes et al. 2002; Kim et al. 2002). Whether the remaining two components of human Elongator are orthologs of Elp5 and Elp6 proteins has not yet been determined (Hawkes et al. 2002).

Mutations in the human Elp1 ortholog IKAP (I kappa B kinase complex-associated protein) can result in the severe human hereditary disorder familial dysautonomia, a neurodegenerative disease (Anderson et al. 2001; Slaugenhaupt et al. 2001). Null alleles of the *S. cerevisiae* Elongator subunit genes cause pleiotropic phenotypes, including resistance to the *Kluyveromyces lactis* killer toxin (Otero et al. 1999; Frohloff et al. 2001; Jablonowski et al. 2001; Krogan and Greenblatt 2001). The three-subunit killer toxin secreted from *K. lactis* will irreversibly arrest sensitive *S. cerevisiae* strains in the G_1 phase of the cell cycle (Butler et al. 1991). In addition to the *elp1–elp6* mutants, a set of mutants has been identified to be killer toxin insensitive (*KTI*), including *kti11*, *kti12*, and *kti13* (Frohloff et al. 2001; Fichtner and Schaffrath 2002). Mutations in any of the *KTI11–KTI13* genes generate pleiotropic phenotypes similar to those caused by mutations in the *ELP1–ELP6* genes (Frohloff et al. 2001; Jablonowski et al. 2001; Fichtner and Schaffrath 2002). Furthermore, *KTI11* and *KTI12* gene products physi-

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cally interact with subunits of the Elongator (Fichtner et al. 2002, 2003). Unexpectedly, we have found that the six subunits in the Elongator complex and the Kti11–Kti13 proteins are all required for the biosynthesis of modified uridine nucleosides present at the wobble position in tRNA.

Transfer RNAs are adapter molecules, which decode mRNA into protein and thereby play a central role in gene expression. The primary tRNA transcript undergoes a series of processing and modification events in order to generate a mature tRNA (Hopper and Phizicky 2003). In this maturation process, specific subsets of the four normal nucleosides adenosine (A), guanosine (G), cytidine (C), and uridine (U) are modified. The modifications are introduced post-transcriptionally, and the formation of a modified nucleoside may require one or several enzymatic steps (Björk 1995). Of the 50 modified nucleosides so far identified in eukaryotic tRNAs, 25 are present in cytoplasmic tRNAs from *S. cerevisiae* (Sprinzl et al. 1998; Rozenski et al. 1999). In the anticodon region of tRNA, especially in positions 34 and 37, nucleosides are frequently modified. Their function seems to be primarily in the decoding process of mRNA, i.e., reading frame maintenance and/or restriction or improvement of codon–anticodon interactions (Agris 1991; Lim 1994; Björk 1995; Yokoyama and Nishimura 1995). They also act as identity elements in aminoacyl-tRNA synthetase recognition (Giege et al. 1998).

In this study, we show that nine proteins, Elp1 to Elp6 and Kti11 to Kti13, are required for the formation of 5-methoxycarbonylmethyl (mcm^5) and 5-carbamoylmethyl (ncm^5) groups on uridines at the wobble position in tRNA. Absence of these modifications influences decoding, providing a likely explanation for the pleiotropic phenotypes of *elp* and *kti* mutants.

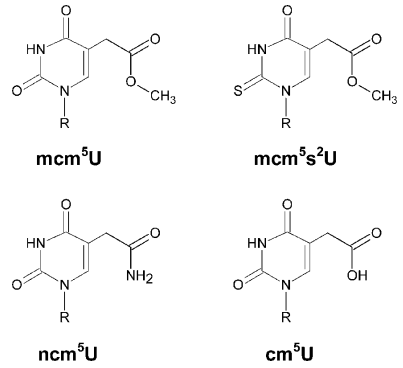

FIGURE 1. Structure of 5-methoxycarbonylmethyluridine (mcm^5U), 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U), 5-carbamoylmethyluridine (ncm^5U), and 5-carboxymethyluridine (cm^5U). R represents ribose.

RESULTS

The Elp3 protein homolog Sin3p influences modification of wobble uridines

In *Schizosaccharomyces pombe*, a *sin3-193* mutant showed reduced levels of the modified nucleoside 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) (see Fig. 1 for structure; Heyer et al. 1984; Grossenbacher et al. 1986), which is located at the wobble position (base 34) in a subset of tRNAs. The *sin3-193* mutation caused an antisuppressor phenotype, i.e., the ochre serine tRNA suppressor encoded by the *sup3-18* gene was no longer able to suppress the *ade7-413* ochre allele (Thuriaux et al. 1976). The cell volume, length, and amount of dead cells increased slightly in the *sin3-193* mutant. This was independent of the presence or absence of the ochre tRNA suppressor, suggesting that the Sin3 protein affected cell cycle regulation (Heyer et al. 1984; Grossenbacher et al. 1986).

We have identified the *sin3*$^+$ gene as the uncharacterized open reading frame (ORF) *SPAC29A4.20* and found that a null allele caused a lack of mcm^5s^2U in $tRNA^{Glu}_{mcm^5s^2UUC}$ and 5-methoxycarbonylmethyluridine (mcm^5U) (see Fig. 1 for structure) in the *sup3-18*-encoded ochre suppressor $tRNA^{Ser}$ (data not shown; see Materials and Methods). Lack of mcm^5U at the wobble position in the suppressor $tRNA^{Ser}$ correlated with an inability to suppress the *ade7-413* ochre allele (data not shown). The *sin3*$^+$ gene encodes a highly conserved protein showing 77.0% identity on the amino acid level to the *S. cerevisiae* Elp3 protein, which has been extensively studied (Wittschieben et al. 1999, 2000; Frohloff et al. 2001; Jablonowski et al. 2001). Elp3p has in vitro histone H3 and H4 acetyltransferase activity and is a subunit of the Elongator complex, implicated in elongation of RNA polymerase II transcription (Otero et al. 1999; Wittschieben et al. 1999; Krogan and Greenblatt 2001; Winkler et al. 2001).

Deletion of the *ELP3* gene influences ochre $tRNA^{Tyr}$ suppression

In *S. cerevisiae*, eight genes encode $tRNA^{Tyr}_{G\Psi A}$, all of which can exist as suppressor derivatives. The *SUP4* allele codes for a $tRNA^{Tyr}$ suppressor with a G_{34}-to-U_{34} exchange in the anticodon, resulting in a tRNA reading UAA ochre stop codons. The wild-type *sup4*$^+$ gene was replaced with the *SUP4* allele in a strain containing the two ochre alleles *ade2-1* and *can1-100*. The *ADE2* gene encodes an enzyme participating in adenine biosynthesis, and suppression of the *ade2-1* mutation allows the strain to grow on medium lacking adenine. The *CAN1* gene encodes an arginine permease, and suppression of the *can1-100* mutation results in no growth of the strain on medium lacking arginine and containing the toxic arginine analog canavanine (for suppression assay, see Materials and Methods). The *SUP4* strain was able to grow on plates lacking adenine, but not on media lacking arginine and containing canavanine, showing suppression of the *ade2-1* and *can1-100* alleles (Fig. 2). Since unmodified uridines are very rare wobble nucleo-

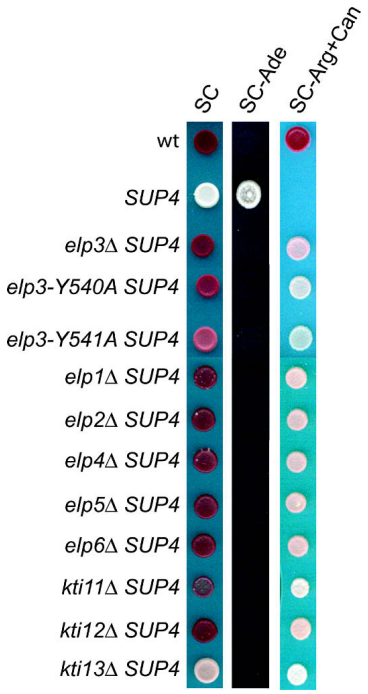

FIGURE 2. Strains with *elp* and *kti* mutations confer a loss-of-suppression phenotype. Approximately 10^4 cells of wild type (W303-1B), *SUP4* (UMY2893), *SUP4 elp1-elp6* (UMY2916, UMY3039, UMY3040, UMY2912, UMY2914, UMY2918, UMY2920, UMY2922), and *SUP4 kti11-kti13* (UMY2936, UMY2938, UMY2940) strains were spotted on SC, SC−Ade, or SC−Arg+Can plates and incubated for 3 d at 30°C.

sides in cytosolic tRNAs (Sprinzl et al. 1998), it was of interest to identify the nucleoside present in the *SUP4* encoded tRNA. We purified $tRNA^{Tyr}$ species from the wild type and *SUP4* strains using an oligonucleotide coupled to magnetic beads. As the oligonucleotide was complementary to both $tRNA^{Tyr}_{G\Psi A}$ and the suppressor derivative, only a fraction of the isolated tRNAs from the *SUP4* strain represents the suppressor tRNA. The purified tRNAs were degraded to nucleosides and the composition analyzed by HPLC. We found that mcm^5U was present in $tRNA^{Tyr}$ species from the *SUP4* strain, whereas it was not detected in $tRNA^{Tyr}$ isolated from wild type (data not shown). Thus, the *SUP4* encoded $tRNA^{Tyr}$ contains mcm^5U ($tRNA^{Tyr}_{mcm^5U\Psi A}$).

As a *sin3* mutation in *S. pombe* renders the *sup3-18* encoded ochre $tRNA^{Ser}$ suppressor deficient in mcm^5U and unable to decode ochre codons, we assumed that a deletion of the *ELP3* gene would cause a loss-of-suppression phenotype in *S. cerevisiae*. Accordingly, introduction of an *elp3::KanMX4* deletion into the *SUP4* strain abolished the formation of mcm^5U in the ochre suppressor $tRNA^{Tyr}$ and simultaneously nullified suppression of the *ade2-1* and *can1-100* ochre alleles (Fig. 2; data not shown). The *elp3* deletion strain was transformed with a plasmid carrying the wild-type *ELP3* gene, and the ability to suppress the *ade2-1* and *can1-100* alleles was restored (data not shown). Thus, lack of the mcm^5 side chain at the wobble U in the *sup3-18* and *SUP4* encoded suppressor tRNAs prevents them from reading ochre stop codons.

An *elp3*-null mutant is deficient in formation of mcm^5U, mcm^5s^2U, and ncm^5U in tRNA

Loss of suppression in the *sin3*- and *elp3*-null mutants correlates with lack of mcm^5U in the *sup3-18* and *SUP4* encoded suppressor tRNAs, respectively (data not shown). Furthermore, the *sin3* mutant lacked mcm^5s^2U in $tRNA^{Glu}_{mcm^5s^2UUC}$ (data not shown). In *S. cerevisiae*, mcm^5U_{34} is present in $tRNA^{Arg}_{mcm^5UCU}$ (Kuntzel et al. 1975), and $mcm^5s^2U_{34}$ is present in $tRNA^{Glu}_{mcm^5s^2UUC}$ and $tRNA^{Lys}_{mcm^5s^2UUU}$ (Smith et al. 1973; Kobayashi et al. 1974). Another wobble nucleoside, 5-carbamoylmethyluridine (ncm^5U_{34}), present in $tRNA^{Pro}_{ncm^5UGG}$ and $tRNA^{Val}_{ncm^5UAC}$ (Yamamoto et al. 1985; Keith et al. 1990), has a structure related to mcm^5U (Fig. 1). We reasoned that an early step(s) is common in the synthesis of these modified nucleosides. Therefore, we determined whether the *elp3* deletion affected the levels of mcm^5s^2U, mcm^5U, and ncm^5U in purified $tRNA^{Glu}_{mcm^5s^2UUC}$, $tRNA^{Arg}_{mcm^5UCU}$, and $tRNA^{Pro}_{ncm^5UGG}$, respectively. In the wild-type strain, but not in the *elp3* mutant, mcm^5s^2U, mcm^5U, and ncm^5U nucleosides were present in $tRNA^{Glu}_{mcm^5s^2UUC}$, $tRNA^{Arg}_{mcm^5UCU}$, and $tRNA^{Pro}_{ncm^5UGG}$ (Table 1; Fig. 3). Introduction of a plasmid carrying the wild-type *ELP3* gene into the *elp3* mutant restored the ability to form these nucleosides (Table 1). No intermediates of mcm^5U and ncm^5U were observed in $tRNA^{Arg}$ and $tRNA^{Pro}$ from the *elp3*-null mutant. However, in the mutant an additional compound was detected in $tRNA^{Glu}$ (Table 1; Fig. 3). This compound was identified as 2-thiouridine (s^2U), based on its spectrum, retention time, and comigration with synthetic s^2U (data not shown). Thus, the *elp3* deletion abolished the formation of the mcm^5 or ncm^5 side chains on the uridine, whereas the thiolation at position 2 was still present in $tRNA^{Glu}$ ($tRNA^{Glu}_{s^2UUC}$).

The postulated acetyl-CoA binding domain of Elp3p is critical for mcm^5U, mcm^5s^2U, and ncm^5U formation

The Elp3 protein has histone H3 and H4 acetyltransferase activity in vitro (Wittschieben et al. 1999; Winkler et al. 2002). In the C-terminal part of the Elp3 protein there is a potential acetyl-CoA binding domain consisting of motifs D, A, and B (Wittschieben et al. 2000). Two tyrosines in the B motif have been suggested to be important for the histone acetyltransferase activity (Wittschieben et al. 2000). To examine if tyrosine Y540 and Y541 are required for tRNA modification activity in vivo, we replaced the wild-type allele of *ELP3* with *elp3-Y540A* or *elp3-Y541A*. Such strains showed no suppression in the plate assay (Fig. 2). Replacement Y540A abolished the formation of mcm^5s^2U in $tRNA^{Glu}_{mcm^5s^2UUC}$, mcm^5U in $tRNA^{Arg}_{mcm^5UCU}$, and ncm^5U

TABLE 1. Content of modified nucleosides in $tRNA^{Glu}_{mcm^5s^2UUC}$, $tRNA^{Arg}_{mcm^5UCU}$, and $tRNA^{Pro}_{ncm^5UGG}$ isolated from *elp1–elp6* and *kti11–kti13* mutants

tRNA species	Modified nucleoside	*SUP4*	*elp1Δ*[a]	*elp2Δ*	*elp3Δ*	*elp3-Y540A*	*elp3-Y541A*	*elp3Δ/pELP3*	*elp4Δ*	*elp5Δ*	*elp6Δ*	*kti11Δ*	*kti12Δ*	*kti13Δ*
$tRNA^{Glu}_{mcm^5s^2UUC}$	Ψ	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	m^5C	1.00	0.89	0.94	0.71	0.79	0.92	0.67	0.92	1.01	0.89	0.79	0.82	0.83
	m^5U	1.00	0.99	1.01	0.95	0.96	1.00	0.81	1.00	1.00	0.94	0.89	0.99	0.96
	s^2U	–[c]	+[d]	+	+	+	+	–	+	+	+	+	+	+
	mcm^5s^2U	**1.00**	**<0.09**[b]	**<0.07**	**<0.02**	**<0.05**	**0.13**	**1.22**	**<0.07**	**<0.03**	**<0.04**	**<0.05**	**<0.06**	**0.18**
$tRNA^{Arg}_{mcm^5UCU}$	Ψ	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	m^1A	1.00	0.93	0.90	1.24	1.20	1.17	1.12	0.89	1.14	1.04	1.03	0.85	0.85
	m^5U	1.00	1.00	0.98	1.01	1.07	1.05	1.09	1.00	1.05	1.03	1.04	0.98	1.02
	m^1G	1.00	1.04	1.08	1.07	0.88	0.96	0.79	1.04	0.94	0.98	0.90	1.10	1.04
	m^2G	1.00	1.04	1.08	1.10	0.87	0.97	0.79	1.05	0.87	0.94	0.88	1.11	1.04
	m^2_2G	1.00	1.05	1.09	1.09	0.88	0.97	0.79	1.06	0.88	0.95	0.89	1.11	1.05
	t^6A	1.00	1.05	1.05	1.29	1.11	1.12	1.11	1.14	0.85	0.98	1.04	1.14	1.14
	mcm^5U	**1.00**	**<0.05**[b]	**<0.04**	**<0.01**	**<0.04**	**0.12**	**0.78**	**<0.05**	**<0.04**	**<0.04**	**<0.05**	**<0.05**	**0.15**
$tRNA^{Pro}_{ncm^5UGG}$	Ψ	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	m^1A+m^5C[e]	1.00	1.01	0.98	0.96	0.96	0.91	1.07	0.99	0.99	1.00	1.04	0.98	1.05
	Cm	1.00	0.93	0.95	1.03	0.83	0.87	0.67	0.91	0.94	0.93	0.77	0.99	0.95
	m^5U+m^7G[f]	1.00	1.09	1.03	1.02	1.01	0.94	1.10	1.03	1.05	1.04	1.15	1.01	1.07
	m^1G	1.00	0.94	0.92	0.90	0.92	0.89	0.96	0.93	1.01	0.98	0.89	0.96	1.00
	ncm^5U	**1.00**	**<0.03**[b]	**<0.05**	**<0.03**	**<0.04**	**<0.03**	**0.60**	**<0.04**	**<0.04**	**<0.04**	**<0.04**	**<0.04**	**0.16**

Pseudouridine (Ψ), present three times in $tRNA^{Glu}_{mcm^5s^2UUC}$, three times in $tRNA^{Arg}_{mcm^5UCU}$, and four times in $tRNA^{Pro}_{ncm^5UGG}$, was used as the internal standard. The numbers given are the ratios of the various modified nucleosides (modified nucleoside/Ψ) in respective tRNA isolated from the *SUP4, SUP4 elp,* or *SUP4 kti* mutants normalized to the ratio in the *SUP4* strain. Nucleosides s^2U, mcm^5s^2U, mcm^5U and ncm^5U are shown in bold.

[a]Deletion with a *KanMX* cassette.

[b]Detection limit.

[c]No detection of s^2U.

[d]Presence of s^2U.

[e]m^1A comigrated with m^5C.

[f]m^5U comigrated with m^7G.

Abbreviations: (Ψ) pseudouridine; (m^5C) 5-methylcytidine; (m^5U) 5-methyluridine; (s^2C) 2-thiouridine; (mcm^5s^2U) 5-methoxycarbonylmethyl-2-thiouridine; (m^1A) 1-methyladenosine; (m^1G) 1-methylguanosine; (m^2G) N^2-methylguanosine; (m^2_2G) N^2,N^2-dimethylguanosine; (t^6A) N^6-threonylcarbamoyladenosine; (mcm^5U) 5-methoxycarbonylmethyluridine; (Cm) 2′-*O*-methylcytidine; (m^7G) 7-methylguanosine; and (ncm^5U) 5-carbamoylmethyluridine.

in $tRNA^{Pro}_{ncm^5UGG}$ (Table 1). However, small amounts of mcm^5s^2U and mcm^5U were present in the *elp3-Y541A* mutant, whereas no ncm^5U could be detected in $tRNA^{Pro}_{ncm^5UGG}$ (Table 1). As in the case of the *elp3*-null allele, $tRNA^{Glu}$ isolated from the *elp3-Y540A* and *elp3-Y541A* mutants contained s^2U (Table 1). We conclude that the Y540A and Y541A amino acid substitutions generate a loss-of-suppression phenotype and affect the formation of the mcm^5 and ncm^5 groups at U_{34}.

All six components of the Elongator complex are required for the synthesis of mcm^5s^2U, mcm^5U, and ncm^5U

Because an *elp3*-null allele generated a loss-of-suppression phenotype and abolished the formation of mcm^5s^2U, mcm^5U, and ncm^5U, it seemed possible that mutations in the five other genes encoding components of the Elongator complex would cause similar phenotypes. Each of the *ELP* genes was independently deleted in the *SUP4 ade2-1 can1-100* strain. All the *elp* deletion strains showed the same phenotypes as the *elp3*-null mutant, i.e., no suppression of the *ade2-1* and *can1-100* ochre alleles, and absence of mcm^5U, mcm^5s^2U, and ncm^5U in $tRNA^{Arg}_{mcm^5UCU}$, $tRNA^{Glu}_{mcm^5s^2UUC}$, $tRNA^{Pro}_{ncm^5UGG}$, respectively (Fig. 2; Table 1). In all of these mutants, $tRNA^{Glu}$ contained s^2U (Table 1), demonstrating that these genes affect the synthesis of the side chain at position 5, but that the thiolation at position 2 is independent. Each mutant was transformed with a plasmid containing the corresponding wild-type *ELP* gene, and in all cases the suppression was restored (data not shown). We conclude that all six Elongator subunits are required for formation of the mcm^5 and ncm^5 groups at U_{34} in tRNA.

The Elp1 and Elp3 proteins coimmunoprecipitate $tRNA^{Glu}_{UUC}$

Two discrete subcomplexes consisting of Elp1–Elp3 and Elp4–Elp6 proteins form the Elongator (Winkler et al. 2001). To investigate whether Elongator binds tRNA, we

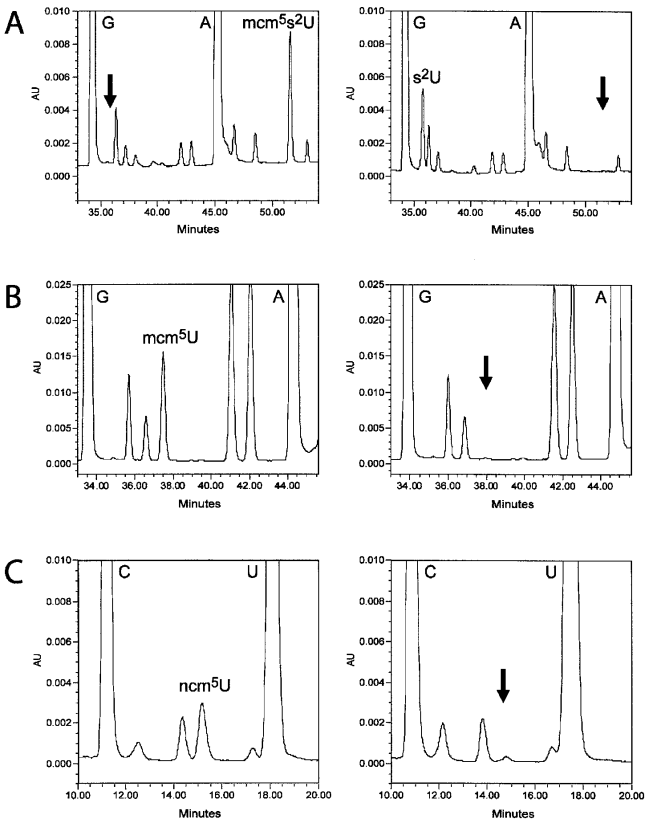

FIGURE 3. An *elp3*-null mutant is lacking mcm^5 and ncm^5 side chains at wobble uridines. HPLC analysis of modified tRNA nucleosides from *SUP4* (UMY2893, *left* panels) and *SUP4 elp3::KanMX4* (UMY2916, *right* panels) strains. (*A*) Analysis of modified nucleosides in $tRNA^{Glu}_{mcm^5s^2UUC}$. The part of the chromatogram between retention times 33 and 54 min is shown. Arrows indicate the expected retention time of s^2U (*left* panel) and mcm^5s^2U (*right* panel). (*B*) Analysis of modified nucleosides in $tRNA^{Arg}_{mcm^5UCU}$. The part of the chromatogram between retention times 33 and 45.5 min is shown. The arrow indicates the expected retention time of mcm^5U (*right* panel). (*C*) Analysis of modified nucleosides in $tRNA^{Pro}_{ncm^5UGG}$. The part of the chromatogram between retention times 10 and 20 min is shown. The arrow indicates the expected retention time of ncm^5U (*right* panel). The small peak at this position represents an unrelated compound with a spectrum different from that of ncm^5U.

constructed strains bearing genomically $(HA)_3$-tagged versions of *ELP1*, *ELP3*, and *ELP5*. Extracts from these strains and a strain with no tag were incubated with ^{32}P-labeled T7-transcribed $tRNA^{Glu}_{UUC}$, which should be a substrate for formation of the mcm^5 group at U_{34}. Following UV-cross-linking, proteins were immunoprecipitated, and the presence of coimmunoprecipitated tRNA was investigated. The analysis revealed that Elp1 and Elp3 protein coimmunoprecipitated $tRNA^{Glu}_{UUC}$, whereas the signal in the Elp5 immunoprecipitate was at background level (Fig. 4A). To assess the specificity of the interaction, we performed an experiment using a genomically tagged $(HA)_3$ version of *TIF4632* as the control. *TIF4632* encodes one of two isoforms of translation initiation factor eIF4G and is not directly involved in $tRNA^{Met}_i$ binding (Prevot et al. 2003). The signals from the Tif4632p and Elp5p immunoprecipitates were similar, i.e., background level, whereas the Elp1 and Elp3 proteins coimmunoprecipitated $tRNA^{Glu}_{UUC}$ (Fig. 4A,B). To exclude unspecific tRNA binding of the Elp1 and Elp3 proteins, we used as a control ^{32}P-labeled T7-transcribed $tRNA^{Met}_i$, which has a C at position 34. No difference in $tRNA^{Met}_i$ signal was observed in the four immunoprecipitates (Fig. 4B). These data suggest that the subcomplex consisting of Elp1–Elp3 proteins associates with tRNA that should obtain an mcm^5 group at U_{34}.

Lack of mcm^5s^2U, mcm^5U, and ncm^5U correlates with resistance to *K. lactis* killer toxin

Strains with null alleles in any of the *ELP* genes show resistance to the *K. lactis* killer toxin (Frohloff et al. 2001; Jablonowski et al. 2001). The killer toxin insensitive *kti11–kti13* mutants displayed similar pleiotropic phenotypes as strains with mutations in the *ELP* genes (Frohloff et al. 2001; Fichtner and Schaffrath 2002). We confirmed the resistance of *elp1–elp6* and *kti11–kti13* mutants using the killer toxin eclipse assay (Kishida et al. 1996; data not shown). In this assay the *elp3-Y540A* and *elp3-Y541A* strains showed the same resistance as the *elp3*-null mutant (data not shown).

To investigate whether the Kti11–Kti13 proteins affected the synthesis of mcm^5U, mcm^5s^2U, and ncm^5U, we independently deleted the genes encoding these proteins in the

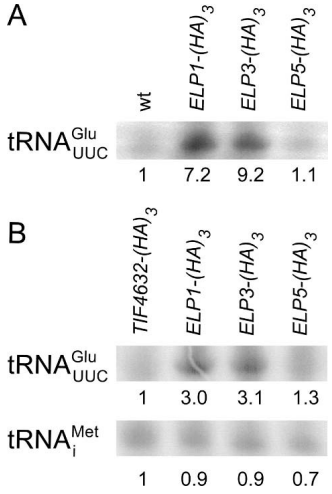

FIGURE 4. Elp1p and Elp3p coimmunoprecipitate $tRNA^{Glu}_{UUC}$. (*A*) T7-transcribed ^{32}P-labeled $tRNA^{Glu}_{UUC}$ was mixed with extracts from wild-type, *ELP1-(HA)*$_3$, *ELP3-(HA)*$_3$, or *ELP5-(HA)*$_3$ strains. After UV-cross-linking, immunoprecipitation using agarose conjugated anti-HA antibodies was performed. The immunoprecipitated material was digested with proteinase K and analyzed on a denaturing polyacrylamide gel. The signals were quantified and normalized to the background signal in the control, which was set to 1. (*B*) T7-transcribed ^{32}P-labeled $tRNA^{Glu}_{UUC}$ or $tRNA^{Met}_i$ was mixed with extracts from *TIF4632-(HA)*$_3$, *ELP1-(HA)*$_3$, *ELP3-(HA)*$_3$, or *ELP5-(HA)*$_3$ strains. The experiment and quantifications were performed as described in *A*.

SUP4 ade2-1 can1-100 strain. The *kti11* and *kti12* deletions prevented the suppression of the *ade2-1* and *can1-100* ochre alleles and the synthesis of mcm^5U in $tRNA^{Arg}_{mcm^5UCU}$, mcm^5s^2U in $tRNA^{Glu}_{mcm^5s^2UUC}$, and ncm^5U in $tRNA^{Pro}_{ncm^5UGG}$ (Fig. 2; Table 1). The *kti13* deletion strain showed no suppression in the plate assay, but ~15% of mcm^5U, mcm^5s^2U, and ncm^5U was present in $tRNA^{Arg}_{mcm^5UCU}$, $tRNA^{Glu}_{mcm^5s^2UUC}$, and $tRNA^{Pro}_{ncm^5UGG}$, respectively (Fig. 2; Table 1). In $tRNA^{Glu}$ from the *kti11–kti13* mutants, s^2U was detected (Table 1). Each mutant was transformed with a plasmid containing the corresponding wild-type *KTI* gene, which restored suppression (data not shown). We conclude that reduced levels of mcm^5U, mcm^5s^2U, and ncm^5U in tRNA correlate with resistance to *K. lactis* killer toxin.

DISCUSSION

The Elongator complex in yeast, consisting of Elp proteins 1–6, was suggested to participate in elongation of transcription (Otero et al. 1999; Krogan and Greenblatt 2001; Winkler et al. 2001). Mutations in any of the *KTI11–KTI13* genes generate pleiotropic phenotypes similar to those caused by mutations in the *ELP1–ELP6* genes, and Kti11 and Kti12 proteins interact with subunits of the Elongator complex (Frohloff et al. 2001; Jablonowski et al. 2001; Fichtner et al. 2002, 2003; Fichtner and Schaffrath 2002). Unexpectedly, we have found that *ELP1–ELP6* and *KTI11–KTI13* genes are required for the formation of the modified nucleosides mcm^5U, mcm^5s^2U, and ncm^5U (see Fig. 1 for structures). These modified nucleosides are located at position 34 (the wobble position) in a subset of tRNA species (Sprinzl et al. 1998). In all the *elp*- and *kti*-null mutants studied here, except *kti13*, formation of mcm or ncm side chains at position 5 of the uridine was abolished (Table 1; Fig. 3). A *kti13*-null allele significantly reduced the levels of mcm^5U, mcm^5s^2U, and ncm^5U, suggesting a regulatory role of Kti13p in formation of these nucleosides. In $tRNA^{Glu}_{mcm^5s^2UUC}$, s^2U is still present in the *elp1–elp6* and *kti11–kti13* mutants (Table 1; Fig. 3), suggesting that the thiolation at position 2 is unaffected and that the gene products affect the synthesis of either the first step or of an unstable intermediate(s) of the mcm^5 and ncm^5 side chains. Consistently, no intermediates of mcm^5U and ncm^5U were observed in $tRNA^{Arg}_{mcm^5UCU}$ and $tRNA^{Pro}_{ncm^5UGG}$ from the *elp1–elp6* and *kti11–kti13* mutants (Fig. 3; data not shown).

Biosynthesis of mcm^5 and ncm^5 side chains at the wobble position

Elongator consists of two subcomplexes, Elp1p–Elp3p and Elp4p–Elp6p (Winkler et al. 2001). We have shown that Elp1p and Elp3p coimmunoprecipitated in vitro transcribed $tRNA^{Glu}_{UUC}$, which should be a substrate in the formation of the mcm^5 group at U_{34}. In contrast, there was no apparent coimmunoprecipitation of $tRNA^{Met}_i$ that is not a substrate for formation of mcm^5U or ncm^5U derivatives (Fig. 4). These data indicate specificity of the Elp1–Elp3 subcomplex in tRNA binding and a direct involvement of Elongator in wobble uridine modification. Differential labeling experiments showed that both carbons of acetate were utilized with equal efficiency to form the 5-carboxymethyl (cm^5) part of mcm^5U (Tumaitis and Lane 1970). Based on structural similarities, ncm^5U is likely to be a derivative of 5-carboxymethyluridine (cm^5U) (Fig. 1). Mutations in the putative acetyl-CoA binding site of Elp3 (Y540A or Y541A) result in a reduced histone acetyltransferase activity in vitro (Wittschieben et al. 2000). These mutations abolished (Y540A) or severely reduced (Y541A) synthesis of the mcm^5U, mcm^5s^2U, and ncm^5U nucleosides in tRNA (Table 1), implying that acetyl-CoA could be a donor in the tRNA modification reaction. However, the labeling experiment showed that acetate or acetyl-CoA is not an immediate donor in formation of the cm^5 group of mcm^5U. Acetate had to be metabolized before the carbons are incorporated into the cm^5 side chain and the actual donor is not known (Tumaitis and Lane 1970). In the tRNA modification reaction, Elongator components could be involved in generating the donor. In addition to the acetyl-CoA binding domain, the Elp3 protein shows homology to the catalytic domain of S-adenosyl-methionine (SAM) radical enzymes (Chinenov 2002). Radical SAM enzymes contain a labile iron–sulfur cluster and require SAM to generate 5′-deoxyadenosyl radicals that can initiate an enzyme reaction by abstracting a hydrogen atom from the substrate (Layer et al. 2004). Thus, a radical mechanism could be involved in the formation of cm^5U. To date, two iron–sulfur cluster containing RNA-modifying enzymes are known, MiaBp and RumAp (Agarwalla et al. 2002; Pierrel et al. 2002). Of these, the MiaB protein is a bifunctional radical SAM enzyme involved in formation of 2-methylthio-N^6-isopentenyladenosine ($ms^2i^6A_{37}$) in tRNA by introducing the methylthio group at position 2 of the adenosine (Pierrel et al. 2004). In formation of cm^5U in tRNA, the donor is not known and we have so far not been able to establish an in vitro assay.

The esterified methyl constituent of mcm^5 originates from SAM and is transferred by the mcm^5U/mcm^5s^2U tRNA carboxyl methyltransferase, Trm9p (Tumaitis and Lane 1970; Kalhor and Clarke 2003). No candidate gene product(s) has been identified for the final step(s) in formation of ncm^5U. One of the Elp1–Elp6 or Kti11–Kti12 proteins could potentially catalyze this step. If so, the integrity of the Elongator complex and the associated Kti11–Kti12 proteins must be important as no nucleoside intermediates are observed in any mutant.

Dual function of the Elongator complex?

The Elongator complex was found to cofractionate with the hyperphosphorylated form of RNA polymerase II, sug-

gested to be the elongating form of the polymerase (Otero et al. 1999). Elongator has histone acetyltransferase activity in vitro and was proposed to assist RNA polymerase II during transcription through chromatin (Wittschieben et al. 1999). Human Elongator showed a weak stimulation of transcription by human RNA polymerase II in vitro and human Elp1 and Elp3 associated with the pS2 gene promoter in chromatin immunoprecipitation (ChIP) experiments (Kim et al. 2002; Metivier et al. 2003). In contrast, the yeast Elongator did not stimulate elongation by RNA polymerase II in vitro and was not occupying open reading frames in ChIP experiments analyzed for specific genes or in a genome-wide manner (Krogan et al. 2002; Pokholok et al. 2002; Gilbert et al. 2004). The association of Elongator with RNA polymerase II has been questioned as no RNA polymerase II copurified with tagged Elp1p, Elp2p, Elp3p, or Elp5p (Krogan et al. 2002). However, the hyperphosphorylated form of RNA polymerase II has been observed to coimmunoprecipitate with tagged Elp2p and Elp5p (Frohloff et al. 2003). Localization studies showed that Elongator subunits are primarily cytosolic, both in yeast and HeLa cells (Kim et al. 2002; Pokholok et al. 2002; Huh et al. 2003). This localization is consistent with a role in wobble base tRNA modification as modifications in the anticodon region normally take place in the cytosol (Hopper and Phizicky 2003). Recently, Elp1 and Elp3 proteins were found to coimmunoprecipitate unspliced and spliced mRNA (Gilbert et al. 2004; Petrakis et al. 2004), implying that a small fraction of Elongator localizes to the nucleus and participates in RNA metabolism. In summary, Elongator has been extensively studied, but the experimental data are not conclusive.

The lack of mcm^5 and ncm^5 side chains in *elp1–elp6* mutants could formally be explained by a defect in transcription of a gene(s) encoding the tRNA-modifying enzyme(s). As the *elp1–elp6* mutants show a complete lack of mcm^5 and ncm^5 side chains at wobble uridines, it would indicate that the tRNA-modification gene is not at all expressed, which we find unlikely to be a consequence of a defect in elongation of transcription. In fact, only a small set of genes show reduced mRNA levels in Elongator mutants (Krogan and Greenblatt 2001). However, it cannot be excluded that Elongator has a dual function, as some tRNA-modifying enzymes have other substrates than tRNA. It is known that the pseudouridine synthases Pus1p and Pus7p in addition to tRNA have U2 snRNA as a substrate (Massenet et al. 1999; Ma et al. 2003). Recently, the mammalian Pus1p (mPus1p) was identified as a coactivator for retinoic acid receptor mRARγ-dependent RNA polymerase II transcription (Zhao et al. 2004). In this process, mPus1p is a component of the coactivator complex and pseudouridylation of the RNA component, Steroid Receptor RNA Activator, is required for transcriptional activation (Zhao et al. 2004). Thus, Elongator might participate both in transcription and tRNA modification.

Phenotypes and translational decoding in *elp1–elp6* and *kti11–kti13 mutants*

Null mutations in Elongator subunit genes cause delayed transcriptional activation of *PHO5*, *GAL1*, *GAL10*, and *ENA1* upon a shift to conditions that should activate transcription of these genes (Otero et al. 1999; Wittschieben et al. 1999). It was also shown that *elp* mutants are slow growing and slow to adapt to various growth conditions (Otero et al. 1999; Frohloff et al. 2001; Jablonowski et al. 2001). Furthermore, *elp1–elp6* and *kti11–kti13* mutants are resistant to *K. lactis* killer toxin, temperature-sensitive, sensitive to caffeine and calcofluor white, and mildly sensitive to 6-azauracil (Otero et al. 1999; Frohloff et al. 2001; Jablonowski et al. 2001; Fichtner and Schaffrath 2002). In addition, *elp1–elp3*, *elp5*, and *kti12* mutants show a significant delay in G_1-to-S transition (Frohloff et al. 2001). Thus, mutations in the *ELP1–ELP6* and *KTI11–KTI13* genes cause pleiotropic phenotypes.

In *S. cerevisiae* 13 of the 42 cytoplasmic tRNA species have a U at the wobble position (Percudani et al. 1997). Of these 13 tRNAs, the identity of the nucleoside at position 34 is known in eight species, and of these, six are mcm^5U or ncm^5U derivatives (Smith et al. 1973; Kobayashi et al. 1974; Kuntzel et al. 1975; Yamamoto et al. 1985; Keith et al. 1990; Glasser et al. 1992). The two others have an unmodified U ($tRNA^{Leu}_{UAG}$) and a pseudouridine (Ψ, $tRNA^{Ile}_{ΨAΨ}$) at position 34 (Randerath et al. 1979; Szweykowska-Kulinska et al. 1994). The nature of the U_{34} nucleoside in the five remaining tRNAs had not been established. We investigated those and found that all contained mcm^5U, ncm^5U, or derivatives thereof (data not shown). In the *elp3* deletion mutant the formation of the mcm^5 or ncm^5 group at U_{34} was abolished in the 11 tRNA species having such derivatives (Table 1; data not shown).

What function would mcm^5 and ncm^5 groups have in decoding? According to the original wobble hypothesis a U at position 34 will read A and G in the decoding process (Crick 1966). Since then the wobble hypothesis has been revised and states that an unmodified U_{34} recognizes U and C in addition to A and G, whereas a modified uridine will restrict wobble recognition (Agris 1991; Lim 1994; Yokoyama and Nishimura 1995). The presence of mcm^5 or ncm^5 groups at U_{34} limits pairing to A- and G-ending codons (Lim 1994; Yokoyama and Nishimura 1995). Lack of these groups at U_{34} in tRNAs decoding split codon boxes, with codons for more than one amino acid, could result in mistranslation. Additionally, lack of mcm^5 or ncm^5 groups at U_{34} is likely to cause a general reduction in the efficiency of decoding A- and G-ending codons (Lim 1994; Yokoyama and Nishimura 1995). Consistent with this, the *SUP4* and *sup3-18* encoded ochre tRNA suppressors require the mcm^5 group to efficiently decode UAA ochre codons (Fig. 2; data not shown). By using a reporter construct, the ability of the *SUP4* encoded suppressor tRNA to decode UAA was three-

fold decreased in an *elp3* mutant (data not shown). In addition to affecting the decoding properties, modifications at U_{34} are known to act as tRNA identity elements and influence the efficiency of the aminoacylation reaction (Giege et al. 1998). Although the pleiotropic phenotypes of *elp1–elp6* mutants have been attributed to a transcriptional defect, our results provide an alternative explanation. We suggest that the phenotypes of the *elp1–elp6* and *kti11–kti13* mutants could equally well be a consequence of less efficient translation and/or mistranslation, caused by lack of mcm^5 and ncm^5 groups at the wobble position in 11 out of 42 tRNA species in yeast. In fact, lack of the modified nucleosides queuosine (Q_{34}) or $ms^2i^6A_{37}$ in *Shigella flexneri* cause a reduced posttranscriptional expression of the transcriptional activator VirFp (Durand et al. 1994, 1997). This reduction of VirF protein renders a decreased transcription of target genes, showing that translational defects caused by hypomodified tRNAs can affect transcription. Moreover, lack of modified nucleosides in the anticodon region has been shown to cause pleiotropic phenotypes in *Salmonella enterica* (Ericson and Björk 1986; Björk and Nilsson 2003).

Potential target of *K. lactis* killer toxin

Strains with null mutations in any of the *ELP1–ELP6* or *KTI11–KTI13* genes show resistance to *K. lactis* killer toxin (Frohloff et al. 2001; Jablonowski et al. 2001; Fichtner and Schaffrath 2002). Interestingly, high dosage of a gene encoding $tRNA^{Glu}_{mcm^5s^2UUC}$ suppresses the killer toxin sensitivity of a wild-type strain (Butler et al. 1994), suggesting that $tRNA^{Glu}_{mcm^5s^2UUC}$ could be the target of killer toxin. The overexpressed $tRNA^{Glu}_{mcm^5s^2UUC}$ might be hypomodified, if the modification enzyme(s) is not able to cope with increased amounts of substrate. Thus, if the target of the toxin is $tRNA^{Glu}_{mcm^5s^2UUC}$, a hypomodified derivative may be resistant. Alternatively, increased amounts of fully modified $tRNA^{Glu}_{mcm^5s^2UUC}$ might sequester the killer toxin present in the cell, leaving a pool of $tRNA^{Glu}_{mcm^5s^2UUC}$ functional in translation.

Conclusions

We have shown that the Elp1–Elp6 and Kti11–Kti13 proteins are all required for formation of mcm^5 and ncm^5 side chains at U_{34} in tRNA. It is remarkable that an early step(s) in the formation of mcm^5 and ncm^5 side chains is dependent on at least nine proteins. So far no more than two gene products have been implicated in the synthesis of a modified nucleoside in yeast (Gerber and Keller 1999; Anderson et al. 2000; Alexandrov et al. 2002). The Kti13 protein seems to have a regulatory role because a null mutant still contains ~15% of these nucleosides. The Kti11 and Kti12 proteins have also been suggested to act as regulators, since loss of the Kti11p enhances the proteolytic processing of the Elp1 protein, and Kti12p is important for the phosphorylation status of Elp1p (Fichtner et al. 2003; Jablonowski et al. 2004). We find it likely that the Kti11–Kti13 proteins regulate Elongator in the wobble uridine modification process and that the activity of Elongator modulate translational efficiency.

MATERIALS AND METHODS

Yeast strains, media, and genetic procedure

The yeast strains used are listed in Table 2. *S. cerevisiae* and *S. pombe* transformation, media, and genetic procedures have been described (Moreno et al. 1991; Burke et al. 2000).

The *sup3-18* and *ade7-413* alleles in the *S. pombe* strain h^+ *ade7-413 sup3-18 sin3-193* were amplified by PCR and sequenced. The *sup3-18* gene was confirmed to encode a $tRNA^{Ser}_{UUA}$ ochre-suppressing tRNA (Amstutz et al. 1985). The mutation in *ade7-413* creates an ochre stop codon after the 74th codon. To generate strain UMY2821, the *ura4*$^+$ gene in the *ade7-413 sup3-18 sin3-193* strain was replaced with an *ura4-D18* DNA fragment PCR-amplified from strain BP231. To delete the *sin3*$^+$ gene, a two-round PCR procedure was used (Krawchuk and Wahls 1999), generating a *sin3*::*KanMX6* fragment. Strain UMY3041 was constructed by transforming the *sin3*::*KanMX6* fragment into strain h^+ *ade7-413 sup3-18* and selecting for G418 resistance. The deletion was confirmed by PCR.

The wild-type *sup4*$^+$ locus was replaced with the *SUP4* allele in strain W303-1B using the two-step method (Scherer and Davis 1979). To target the *SUP4* allele to the correct chromosomal location, plasmid pRS306-*SUP4* was linearized with MunI and transformants selected on synthetic complete (SC) medium lacking uracil. Colonies able to grow on SC plates lacking adenine (SC-Ade) and unable to grow on SC medium lacking arginine and containing 60 μg/mL canavanine (SC−Arg+Can), were streaked on 5-fluoro-orotic acid (5-FOA)-containing medium. The *SUP4* locus from 5-FOA^R, Ade$^+$, and Can^S colonies was amplified by PCR and sequenced, confirming the gene replacement. Strains UMY2893 and UMY2894 were obtained from a tetrad in a cross between one of the generated *SUP4* strains and W303-1A.

To construct the *elp5*::*KanMX6* and *kti11*::*KanMX6* deletions, oligonucleotides containing 45-nt homology to the flanking sequences of the *ELP5* or *KTI11* genes were used to amplify the *KanMX6* cassette (Longtine et al. 1998). To delete *ELP1*, *ELP2*, *ELP3*, *ELP4*, *ELP6*, and the *KTI12*, *KTI13* genes, chromosomal DNA from the corresponding null mutants in the yeast deletion collection (Research genetics) was used as templates. Appropriate primers were used to amplify DNA fragments containing the *KanMX4* cassette and 300–400 nt of flanking sequences. Each PCR product was transformed into strain UMY2893 and transformants selected on YEPD plates containing 200 μg/mL of G418. Deletions of the *ELP* or *KTI* genes were confirmed by PCR followed by a backcross, to verify 2:2 segregation of the *KanMX4*/*KanMX6* allele and cosegregation of the G418^R and loss-of-suppression phenotypes. The strains generated were UMY2912 (*elp1*::*KanMX4*), UMY2914 (*elp2*::*KanMX4*), UMY2916 (*elp3*::*KanMX4*), UMY2918 (*elp4*::*KanMX4*), UMY2920 (*elp5*::*KanMX6*), UMY2922 (*elp6*::*KanMX4*), UMY2936 (*kti11*::*KanMX6*), UMY2938 (*kti12*::*KanMX4*), and UMY2940 (*kti13*::*KanMX4*).

The wild-type *ELP3* gene was replaced by mutant *elp3* alleles in

TABLE 2. Strains used in this study

Yeast strain	Genotype	Source
S. pombe		
BP231	*h⁺ ura4-D18*	J. Keeney, pers. comm.
	h⁺ ade7-413 sup3-18	J. Kohli, pers. comm.
	h⁺ ade7-413	J. Kohli, pers. comm.
	h⁺ ade7-413 sup3-18 sin3-193	J. Kohli, pers. comm.
UMY3041	*h⁺ ade7-413 sup3-18 sin3::KanMX6*	This study
UMY2821	*h⁺ ade7-413 sup3-18 sin3-193 ura4-D18*	This study
S. cerevisiae		
354	*MATα SUP4 trp5-38 his5-2 lys1-1 ade2-1 ura3 can1 met*	F. Sherman, pers. comm.
W303-1A	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Fiorentini et al. 1997
W303-1B	*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Fiorentini et al. 1997
UMY2893	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	This study
UMY2894	*MATa SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	This study
UMY2912	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 elp1::KanMX4*	This study
UMY2914	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 elp2::KanMX4*	This study
UMY2916	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 elp3::KanMX4*	This study
UMY2918	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 elp4::KanMX4*	This study
UMY2920	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 elp5::KanMX6*	This study
UMY2922	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 elp6::KanMX4*	This study
UMY2936	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 kti11::KanMX6*	This study
UMY2938	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 kti12::KanMX4*	This study
UMY2940	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 kti13::KanMX4*	This study
UMY3039	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 elp3-Y540A*	This study
UMY3040	*MATα SUP4 leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 elp3-Y541A*	This study
UMY3114	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 ELP1-(HA)$_3$::KanMX6*	This study
UMY3116	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 ELP3-(HA)$_3$::KanMX6*	This study
UMY3118	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 ELP5-(HA)$_3$::KanMX6*	This study
UMY3094	*MATα leu2-3,112 trp1-1 can1-100 ura3-1 his3-11,15 TIF4632-(HA)$_3$::KanMX6*	This lab

strain UMY2893 using the two-step method (Scherer and Davis 1979). To target the *elp3* alleles to the correct chromosomal location, plasmids pRS306-*elp3-Y540A* or pRS306-*elp3-Y541A* were linearized with BclI and transformants selected on SC medium lacking uracil. After selection for 5-FOA^R segregants, PCR amplification, and DNA sequencing confirmed that red colonies contained the *elp3* mutations. The generated strains, UMY2939 (*elp3-Y540A*) and UMY2946 (*elp3-Y541A*), were transformed with pRS315-*ELP3*, which restored suppression. Strains containing C-terminal $(HA)_3$-tagged Elp1, Elp3, and Elp5 proteins, UMY3114, UMY3116, and UMY3118, were derived from W303-1A and constructed by the PCR-mediated strategy using plasmid pFA6a-3HA-kanMX6 as the template (Longtine et al. 1998). The strains were confirmed by PCR and by Western blot analysis using anti-HA antibodies. Strains with tagged Elp proteins were killer toxin sensitive, confirming the functionality of the tagged proteins.

Identification of the *sin3*⁺ gene and phenotypes of *sin3* mutants

To clone the wild-type *sin3*⁺ gene, a *URA3*-based *S. pombe* genomic library was introduced into the *ade7-413 sup3-18 sin3-193 ura4-D18*strain (UMY2821) and two plasmids were identified that restored the suppression of the *ade7-413* allele (data not shown). DNA sequencing revealed that the two plasmids had the uncharacterized open reading frame *SPAC29A4.20* in common. In order to find the *sin3-193* mutation, we PCR-amplified and sequenced the *SPAC29A4.20* open reading frame from *sin3*⁺ and *sin3-193* strains. The *sin3-193* mutation generated an arginine-to-histidine substitution at position 220 (R220H) of the Sin3p (data not shown).

Introduction of a *sin3::KanMX6* allele into an *ade7-413 sup3-18* strain abolished the suppression of the *ade7-413* allele (data not shown). Analysis of tRNA revealed that $tRNA^{Glu}_{mcm^5s^2UUC}$ (Wong et al. 1979) from the *sin3*⁺ strain contained mcm^5s^2U, whereas it was reduced in the *sin3-193* (5% of wild type) and not detectable in the *sin3::KanMX6* derivative (data not shown). Furthermore, analysis of the ochre suppressor $tRNA^{Ser}$ revealed the presence of mcm^5U in the *ade7-413 sup3-18* strain, but not in the *sin3-193* and *sin3::KanMX6* derivatives or the wild-type *ade7-413* strain (data not shown).

Suppression assay

To investigate the effect of the *elp1-6*- and *kti11-13*-null alleles on tRNA suppression, we used the *ade2 can1* system (Hopper et al. 1980). Strains W303-1A and W303-1B contain the two ochre alleles, *ade2-1* (UAA) and *can1-100* (UAA). *ADE2* encodes an enzyme participating in adenine biosynthesis. Strains with an *ade2* mutation have a requirement for adenine and accumulate a red pigment. Mutations in the *CAN1* gene that codes for an arginine permease generate resistance to canavanine, an arginine analog toxic to yeast cells. The efficiency of an ochre suppressor tRNA to decode the *ade2-1* (UAA) and *can1-100* (UAA) codons can be

investigated on plates. If the suppressor tRNA is functional, the strains with the *ade2-1* (UAA) and *can1-100* (UAA) alleles will grow on SC−Ade, but not on SC−Arg+Can plates. If the suppressor tRNA is nonfunctional, the strains will grow on SC−Arg+Can, but not on SC−Ade medium.

Plasmid constructions

DNA manipulations, plasmid preparations, and bacterial transformations were performed according to standard protocols. Genes were PCR-amplified using *Pwo* DNA polymerase or the Expand Long Template PCR system (Roche Applied Science). An integrative vector carrying the *SUP4* locus was constructed by cloning a BamHI/SacI fragment PCR-amplified from strain 354 into the corresponding sites of pRS306 (Sikorski and Hieter 1989), generating plasmid pRS306-*SUP4*. DNA sequencing confirmed that the anticodon was TTA. The oligonucleotides used for the PCR were 5′-TTTTGGATCCGTCCAGATGCCTTTACGAGT-3′ and 5′-AT ATGAGCTCGTTTCGGCTCTAATCCACTG-3′.

A low-copy *LEU2* plasmid carrying *ELP3* (pRS315-*ELP3*) was constructed by cloning a BamHI/XhoI fragment, PCR-amplified from strain W303-1B into the corresponding sites of pRS315 (Sikorski and Hieter 1989). The oligonucleotides used were 5′-TT GTGGATCCTTGGCTTCAGGTGTCATTC-3′ and 5′-ATCTCTC GAGTATCTGGGGCCAATTGT-3′.

Plasmid pRS306-*ELP3* was constructed by cloning a BamHI/ XhoI *ELP3* fragment from pRS315-*ELP3* into the corresponding sites of plasmid pRS306. Mutant alleles of *ELP3* were obtained using the QuickChange protocol (Stratagene), generating pRS306-*elp3-Y540A* and pRS306-*elp3-Y541A*.

Plasmid pRS315-*ELP5* was constructed by cloning a SalI/SpeI fragment, PCR-amplified from strain W303-1B into pRS315. The oligonucleotides used were 5′-ATTTGTCGACCACTTGGAGTTC ACGATGTTA-3′ and 5′-GAGAACTAGTGGGGATCGACACCTA AATTCA-3′. PCR fragments containing *ELP1*, *ELP2*, *ELP4*, or *ELP6* amplified from strain W303-1B were cloned into the pGEM^{R}-T Easy Vector (Promega) following addition of an A overhang by *Taq* DNA polymerase (Roche Applied Science). The oligonucleotides used were 5′-TTCGACGTTTTCATGGACCA-3′ and 5′-CGCCAACAAACTCTAGCTCAT-3′ (*ELP1*), 5′-AATGA TGGGGAGAGTGACGTA-3′ and 5′-GTTTCGCTTTACGAGAAA AGG-3′ (*ELP2*), 5′-AAATATCGCATCGAATGGAA-3′ and 5′-TGAGTTTGAAGCTGAACCGT-3′ (*ELP4*), or 5′-AGCAAGGTG TCGAATCAAGTT-3′ and 5′-ATCACCCATAAAGGCAGGAA-3′ (*ELP6*).

Plasmids pRS315-*ELP1*, pRS315-*ELP2*, pRS315-*ELP4*, and pRS315-*ELP6* were constructed by cloning SacI/SacII (*ELP1*), ApaI/SacI (*ELP2* and *ELP4*), and SacII/SalI (*ELP6*) fragments from the respective pGEM^{R}-T Easy vector into plasmid pRS315. Plasmids pRS315-*KTI11*, pRS315-*KTI12*, and pRS315-*KTI13* were constructed by cloning BamHI/XhoI fragments, PCR-amplified from strain W303-1B into the corresponding sites of pRS315. The oligonucleotides used were 5′-CTTAGGATCCGCACATACTTTT TGTCCTGGTG-3′ and 5′-CTTTCTCGAGCATATACACCTACC CAGTATCC-3′ (*KTI11*), 5′-GAGAGGATCCGTGGACGTGGAT GTAGAATCG-3′ and 5′-CTTTCTCGAGCTGCCTCTCTTGGTA CGACA-3′ (*KTI12*) or 5′-TGTTGGATCCGCTGCTGTAGAGCG TTTGGTG-3′ and 5′-GTTTCTCGAGGTATGAGCTCCTTCGCT GGG-3′ (*KTI13*). A $tRNA^{Glu}_{UUC}$ gene under the T7 promoter gene was constructed by ligating three pairs of oligonucleotides (Sampson and Uhlenbeck 1988) into the EcoRI/BamHI sites of pUC18 (Roche Applied Science), generating p1537. The $tRNA^{Glu}_{UUC}$ gene carried two mutations (G1-C72) to improve the efficiency of T7 transcription.

tRNA methods and HPLC analysis

Cells were grown at 30°C in 2-L YEPD or appropriate selective media. The cells were harvested at OD_{600} = 1.0 and single tRNA species prepared as previously described (Björk et al. 2001). The purified tRNAs were digested to nucleosides and analyzed by HPLC using a Develosil C-30 reverse-phase column (Gehrke et al. 1982; Gehrke and Kuo 1990). The presence of only negligible amounts of other nucleosides than expected demonstrated the purity of the tRNAs analyzed. Dihydrouridine (D) was not detected at 254 nm and is not included in Table 1. Nucleosides mcm^5s^2U, mcm^5U, ncm^5U, and s^2U were identified by their spectrum, retention time, and by comigration with corresponding synthetic nucleosides. The ncm^5U nucleoside was obtained by conversion of mcm^5U as described previously (Fissekis and Sweet 1970). The T7-transcribed radiolabeled $tRNA^{Met}_{i}$ and $tRNA^{Glu}_{UUC}$ were prepared by using MvaI-linearized p2142 (Åström and Byström 1994) or p1537, 5′-[α-^{32}P]CTP (400 Ci/mmol; Amersham Biosciences), and the Riboprobe in vitro transcription system (Promega). The radiolabeled transcripts were purified as previously described (Johansson and Byström 2004).

UV cross-linking and immunoprecipitation

Cells were grown in 50 mL of YEPD at 30°C to an OD_{600} between 0.6 and 0.8. The cells were harvested, washed with cold breaking buffer (10 mM HEPES at pH 7.3, 50 mM KCl, 10 mM MgAc, 5 mM DTT, 5% glycerol), resuspended in 300 µL of cold breaking buffer supplemented with protease inhibitor mixture (Otero et al. 1999), and crude extracts were prepared by vortexing in the presence of glass beads. The glass beads were extracted twice with 300 µL of cold breaking buffer, and the extract was cleared by two centrifugations in a microfuge. Radiolabeled tRNA transcripts (1 ng, ~100,000 dpm) and 20 U of RNasin were added to 300 µL of the extracts. Fractions (20 µL) were transferred to a 96-well microtitre plate, which was incubated at room temperature for 10 min. The microtitre plate was exposed five times to 120 mJ/cm^2 of UV using a Stratalinker (Stratagene), before the fractions were pooled and diluted to 600 µL with cold breaking buffer. Immunoprecipitation was performed for at least 2 h at 8°C in a rotating chamber by using 50 µL of anti-HA agarose conjugate (Sigma). After five washes with 1 mL of cold wash buffer (10 mM HEPES at pH 7.3, 50 mM KCl, 10 mM MgAc, 0.1% Triton X-100), proteins were eluted by addition of 100 µL of TES (50 mM Tris-HCl at pH 8.0, 10 mM EDTA, 1% SDS) and a 20-min incubation at 65°C. The beads were washed once with 50 µL of TES. To confirm the presence of the immunoprecipitated protein, a fraction of the pooled eluate was analyzed by Western blot analysis, using anti-HA antibodies. Based on this analysis, the immunoprecipitations were equally efficient in all protein extracts. The remaining eluate was treated with Proteinase K, precipitated, and separated on an 8% polyacrylamide, 8 M urea gel. The gel was dried and the signals were visualized and quantified by PhosphorImager analysis.

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