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Mtt1 is a Upf1-like helicase that interacts with the translation termination factors and whose overexpression can modulate termination efficiency

KEVIN CZAPLINSKI,[1,4] NIMA MAJLESI,[1] TAPO BANERJEE,[2] and STUART W. PELTZ[1,2,3]

[1]Department of Molecular Genetics and Microbiology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, USA
[2]The Graduate Program in Cell and Developmental Biology, Rutgers University, Piscataway, New Jersey 08854, USA
[3]The Cancer Institute of New Jersey, Piscataway, New Jersey 08854, USA

ABSTRACT

Translation termination is the final step that completes the synthesis of a polypeptide. Premature translation termination by introduction of a nonsense mutation leads to the synthesis of a truncated protein. We report the identification and characterization of the product of the *MTT1* gene, a helicase belonging to the Upf1-like family of helicases that is involved in modulating translation termination. *MTT1* is homologous to *UPF1*, a factor previously shown to function in both mRNA turnover and translation termination. Overexpression of *MTT1* induced a nonsense suppression phenotype in a wild-type yeast strain. Nonsense suppression is apparently not due to induction of [*PSI*+], even though cooverexpression of *HSP104* alleviated the nonsense suppression phenotype observed in cells overexpressing *MTT1*, suggesting a more direct role of Hsp104p in the translation termination process. The *MTT1* gene product was shown to interact with translation termination factors and is localized to polysomes. Taken together, these results indicate that at least two members of a family of RNA helicases modulate translation termination efficiency in cells.

Keywords: helicase; nonsense-mediated mRNA decay; release factor; RNA; translation termination

INTRODUCTION

Translation machinery must be able to determine the precise sites on the mRNA where decoding should begin and where it should end. The selection of the translation start site is usually delineated by the first AUG codon encoding the amino acid methionine. After initiation of translation, the ribosome manufactures the polypeptide by progressing along the mRNA in the 5′-to-3′ direction, decoding one codon at a time. The final step in the translation process occurs when one of three termination codons occupies the A-site of the ribosome, resulting in hydrolysis of the peptide (reviewed in Stansfield & Tuite, 1994; Buckingham et al., 1997; Nakamura et al., 1996).

Reprint requests to: Stuart W. Peltz, Department of Molecular Genetics and Microbiology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, USA; e-mail: Peltz@rwja.umdnj.edu.

[4]Present address: European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117, Heidelberg, Germany.

Although translation termination normally occurs after completion of the full-length polypeptide, base substitutions and frameshift mutations in DNA often lead to the synthesis of an mRNA that contains a premature stop codon within its protein coding region. The occurrence of a premature stop codon arrests translation at the site of early termination leading to synthesis of a truncated protein and rapid degradation of the mRNA (reviewed in Ruiz-Echevarria et al., 1996; Weng et al., 1997; Czaplinski et al., 1999; Hentze & Kulozik, 1999; Hilleren & Parker, 1999). Interestingly, nonsense and frameshift mutations cause approximately 20–40% of the individual cases of over 200 different inherited diseases (reviewed in McKusick, 1994).

Although nonsense mutations often result in loss of function of a protein, it is possible to reduce the efficiency of the termination process resulting in a "nonsense suppression phenotype." Under these circumstances sufficient amounts of the full-length protein are synthesized that are capable of either reducing or eliminating the phenotype that arises as a consequence of

the nonsense mutation. Nonsense suppression results when a near cognate tRNA successfully competes with the termination factors at a nonsense codon so that amino acid incorporation into the peptide chain occurs at the site of mutation rather than premature termination of translation. Nonsense suppression can result from an amino-acyl tRNA that decodes a termination codon (suppressor tRNA) or mutations that affect the process of translation termination. Interestingly, recent reports have demonstrated that subinhibitory concentrations of certain aminoglycoside antibiotics that bind to the ribosome suppress the translation termination process and restore functional activity to genes harboring a nonsense mutation, both in cell culture and in animals (Howard et al., 1996; Bedwell et al., 1997; Barton-Davis et al., 1999). Thus, identifying and characterizing the factors that regulate the efficiency of translation termination will be important for understanding the biology of the termination process as well as in developing therapeutics for the treatment of a wide array of genetic disorders that arise as a consequence of a nonsense mutation.

Translation termination in eucaryotes is carried out by the interacting peptidyl release factors Release Factor 1 (eRF1) and Release Factor 3 (eRF3; Frolova et al., 1994; Stansfield et al., 1995; Zhouravleva et al., 1995). eRF1 catalyzes peptidyl-tRNA hydrolysis (Frolova et al., 1994; Song et al., 2000) whereas eRF3 is an EF1A-like protein that demonstrates eRF1- and ribosome-dependent GTPase activities, and enhances the peptidyl hydrolysis activity of eRF1 in a GTP-dependent manner (Kikuchi et al., 1988; Hoshino et al., 1989, 1998; Didichenko et al., 1991; Zhouravleva et al., 1995; Frolova et al., 1996). eRF1 and eRF3 from budding yeast, fission yeast, and humans have been shown to interact both in vitro and in vivo and purified human eRF1 and eRF3 are capable of forming a heterodimeric complex (Stansfield et al., 1995; Paushkin et al., 1997a, 1997b; Frolova et al., 1998; Ito et al., 1998; Eurwilaichitr et al., 1999).

Factors that modulate the efficiency of the translation termination process have been identified (Song & Liebman, 1987; All-Robyn et al., 1990; Weng et al., 1996a, 1996b; Czaplinski et al., 1998, 1999b). The Upf1 protein is an example of a factor that modulates the efficiency of translation termination. Disruption of the *UPF1* gene from yeast results in a dramatic stabilization of nonsense-containing mRNAs and promotes suppression of certain nonsense alleles (Leeds et al., 1991; Cui et al., 1995; Weng et al., 1996a, 1996b; Czaplinski et al., 1998). Recent results suggest that the Upf1p modulates the translation termination process by directly interacting with eRF1 and eRF3 (Czaplinski et al., 1998). The Upf1p contains a cysteine- and histidine-rich region near its amino terminus and all the motifs characteristic of the superfamily group I helicases (Leeds et al., 1991; Altamura et al., 1992; Koonin, 1992; Weng et al., 1996a, 1996b). The yeast Upf1p has been purified and demonstrates RNA-dependent ATPase and helicase activity (Czaplinski et al., 1995; Weng et al., 1996a, 1996b, 1998). A human homolog of the *UPF1* gene, called RENT1 or HUPF1 (Perlick et al., 1996; Applequist et al., 1997) has been identified and its conserved portions have been shown to function in yeast cells in enhancing translation termination, indicating that its role in this process is evolutionarily conserved (Czaplinski et al., 1998). Based on this example it is possible that the other factors that have been identified as modulators of translation termination efficiency may also act by modulating the activities of the peptidyl release factors eRF1 and eRF3.

The results presented here utilized a computer analysis to identify a set of superfamily group I helicases in yeast cells with significant homology to Upf1p. This analysis led to the identification of a Upf1-like family of helicases. In particular, we have characterized one gene and its protein product, which we have called *MTT1* (for Modulator of Translation Termination). Similar to *UPF1*, *MTT1* encodes a protein harboring a superfamily group I helicase domain at its carboxyl terminus and a cysteine-histidine-rich region in its amino terminus. Like Upf1p, the Mtt1p interacts with the translation termination factors and can modulate the translation termination process. These results suggest that there is a family of RNA helicases that are modulators of the translation termination process.

RESULTS

Identification of a family of yeast superfamily group I helicases that are similar to the *UPF1* gene product

A sequence comparison to identify other yeast genes that are homologous to *UPF1* was undertaken and the results are shown in Figure 1. As expected, the *SEN1* gene demonstrated significant homology with *UPF1* (Fig. 1; Koonin, 1992). *SEN1* was identified in a screen for mutations that affect tRNA splicing and harbors all of the motifs to be considered a superfamily group I helicase (Winey & Culbertson, 1988; DeMarini et al., 1992; Koonin, 1992). The previously identified *DNA2* gene also demonstrated significant homology to *UPF1* (Fig. 1). DNA2 is thought to have a role in DNA replication, possibly in processing Okazaki fragments (Budd et al., 1995; Budd & Campbell 1997). Two additional yeast genes encoding superfamily group I helicases with high homology to *UPF1* were also identified and in previous studies have been named Helicase A (*HCS1*; Biswas et al., 1997a, 1997b) and Helicase B (*HCS2*; Biswas et al., 1995) or scHel1 (Bean et al., 1993). For reasons that will be described below, we have named the gene encoding scHel1 *MTT1* (for Modulator of Translation Termination). The proteins en-

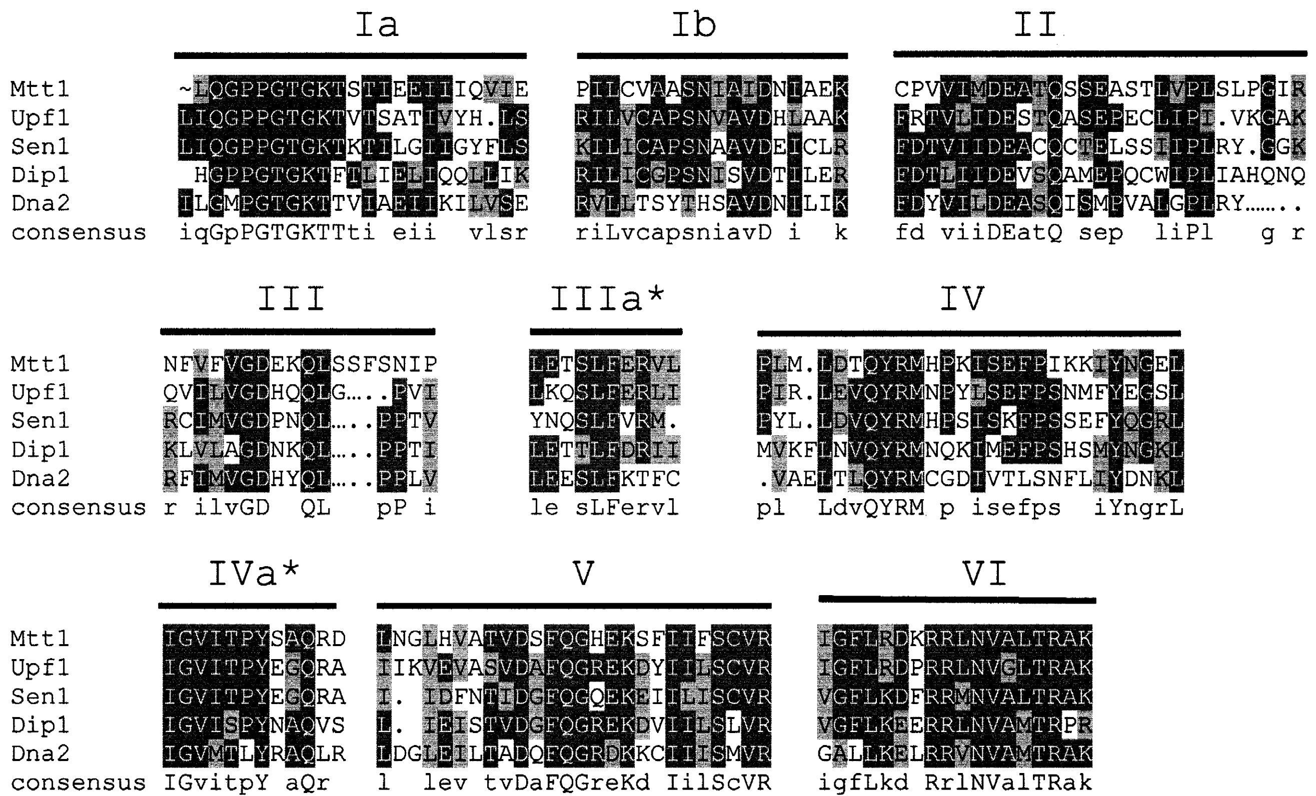

FIGURE 1. Five helicases of *Saccharomyces cerevisiae* define a subclass of superfamily group I. The amino acid sequences of the conserved helicase motifs from the *UPF1*, *MTT1*, *SEN1*, *DNA2,* and *HCSA* genes were aligned using the PILEUP program from GCG. Gap creation penalty was kept at the default (12) while the gap extension penalty was lowered to 1. The motifs Ia, Ib, and II of *SEN1* and *DNA2* were not included unless the extension penalty was lowered. Labeling of the original seven superfamily group motifs is indicated by roman numerals, and the two new motifs identified in this family, IIIa and IVa, are denoted by *.

coded by the *HCS1* and *MTT1* genes have been previously purified and demonstrated to have $5' \rightarrow 3'$ DNA-dependent helicase activity (Bean et al., 1993; Biswas et al., 1995, 1997a, 1997b). Hcs1 and Mtt1 are thought to be involved in chromosome replication (Biswas et al., 1995, 1997a, 1997b). This notion is based on two observations. First, the yeast single-stranded DNA binding protein Rpa1p enhances the DNA helicase activities to HCS1 and MTT1 in vitro (Biswas et al., 1995, 1997a, 1997b). Second, Hcs1 copurifies with DNA polymerase and displays the predicted biochemical properties of replicative helicases (Biswas et al., 1997a, 1997b).

To date there is no in vivo evidence to support the involvement of *HCS1* or *MTT1* in replication. Both *hcs1* and *mtt1* single deletion strains are viable (Bean & Matson 1997; Lussier et al., 1997; data not shown). *mtt1*Δ strains do not display significant defects in growth, sensitivity to DNA damage, or respiration defects (Bean & Matson, 1997). Transposon insertion into the promoter region of *MTT1* has been reported to cause hypersensitivity to calcofluor white, a cell wall synthesis inhibitor and hygromycin B, a drug which induces translational misreading (*ECM32*; Lussier et al., 1997). Homology of Hcs1 and Mtt1 has been noted previously (Biswas et al., 1997a). The homology among these five yeast helicases appears to be confined to their helicase domains (Fig. 1).

Seven conserved motifs are associated with all superfamily group I helicases (Gorbalenya et al., 1988; Koonin, 1992). Within these seven motifs, a limited number of residues is conserved among all superfamily group I helicases. Although these seven motifs are spaced variably from protein to protein, according to the crystal structure of two different superfamily group I helicases, these conserved residues may be in close proximity (Korolev et al., 1998; Subramanya et al., 1996; Velankar et al., 1999). A more careful analysis of the yeast genes with similarity to *UPF1* identifies these five proteins as a subclass of superfamily group I, which we will refer to as the *UPF1*-like subclass. A distinguishing feature of this subclass is a more extensive homology surrounding the conserved superfamily group I residues in motifs II, IV, V, and VI, which has been noted previously for Upf1p and Sen1p (Fig. 1; Perlick et al., 1996). Furthermore, two additional motifs within this domain are conserved among these five genes. The first is located between motifs III and IV (consensus

lexSLFervl; Fig. 1) and the second is located between motifs IV and V (consensus lgvitpYxaQxxxl; Fig. 1). We refer to them as motifs IIIa and IVa, respectively. The identification of motif IIIa is consistent with a new conserved motif indicated by the analysis of the structural data (Korolev et al., 1998). These additional motifs are also present in the human homolog of the Upf1 gene (data not shown). Two other superfamily group I helicases from yeast, Pif1 and RadH, and two well-characterized group I helicases from *Escherichia coli*, Rep and uvrD, could not be aligned to these sequences under these parameters (data not shown), indicating that the homology is not general to all superfamily group I helicases and supporting the notion for a distinct subclass.

As described above, a unique feature of the Upf1p is that it contains a cysteine-histidine-rich region near its amino terminus. Mutations in this region have been shown to reduce translation termination efficiencies at nonsense codons and enhance programmed −1 ribosomal frameshifting efficiencies (Cui et al., 1996; Weng et al., 1996b). This region has been identified as the Upf2p interaction domain (He et al., 1997; Weng et al., 1996b). Interestingly, the Mtt1p also contains a cysteine-rich region near its amino terminus. Within the first 127 amino acids, 13 cysteines and 3 histidines are present. Although the cysteine-histidine-rich regions of *UPF1* and *MTT1* contain no apparent homology at the level of primary amino acid sequence, both regions have the potential to form ring fingers (see Weng et al., 1996b; Bean & Matson, 1997). Alternatively, these regions can be aligned to multiple zinc-binding motifs (Applequist et al., 1997; data not shown). However, due to the considerable number of cysteine and histidine residues, any alignment of this type leaves several of these residues unaccounted for within the same region. Considering the many potential structural features of a region containing such an abnormally high concentration of cysteine residues, it is difficult to define the precise structure that this cysteine-histidine-rich region may fit based solely upon amino acid sequence.

Overexpression of Mtt1 protein results in nonsense suppression

Based on the similarity between the Upf1 and Mtt1 proteins, we asked whether Mtt1p has a role in modulating either translation termination (as measured by nonsense suppression) or nonsense-mediated mRNA decay (NMD). Nonsense suppression occurs when a near cognate tRNA successfully competes with the termination factors at a nonsense codon, resulting in amino acid incorporation rather than premature termination of translation. Sufficient levels of nonsense suppression allow production of enough completed polypeptide protein to support growth. Translation termination is sensitive to levels of translation termination factors (Stansfield et al., 1995; Le Goff et al., 1997); thus deletion or overexpression of factors that are involved in translation termination may lead to nonsense suppression. Deletion of the *MTT1* gene does not cause nonsense suppression (data not shown). We asked whether overexpression of the Mtt1p leads to a nonsense suppression phenotype. The *MTT1* ORF was cloned into vector pG-1, a 2μ plasmid in which MTT1 transcription is driven by the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (pG1-MTT1). We transformed pG1-MTT1 into a strain harboring *leu2-2* (UGA) and *tyr7-1* (UAG) nonsense alleles and assayed for the ability of the increased dosage of *MTT1* to promote suppression of these alleles (Leeds et al., 1992). The suppression phenotype of the transformants was monitored by plating cells on medium lacking tryptophan, leucine, and tyrosine. As a control, these cells transformed with pG1 lacking MTT1 were plated on medium lacking tryptophan. The results demonstrated that cells transformed with pG-1MTT1 grew on medium lacking leucine and tyrosine, whereas cells transformed with a *TRP1* vector did not grow (Fig. 2A). An epitope-tagged version of the Mtt1 protein that harbored an amino terminal FLAG epitope was also cloned into Flag-Mtt1. Strains harboring the *FLAG-MTT1*-containing plasmid also demonstrated growth on medium lacking leucine and tyrosine (Fig. 2A). Strong overexpression of Mtt1 in both cases was confirmed using an antibody to the protein (Bean & Matson, 1997; data not shown). These results demonstrate that overexpression of *MTT1* leads to a nonsense suppression phenotype.

We next investigated whether overexpression of *MTT1* might cause nonsense suppression by induction of the [PSI^+] phenotype of yeast. In a [PSI^+] state, eRF3 forms high-molecular-weight aggregates that reduce eRF3 activity and leads to increased readthrough of translation termination codons by ribosomes (Wickner, 1994; Patino et al., 1996; Paushkin et al., 1996, 1997a; Glover et al., 1997). Overexpression of *HSP104* has been demonstrated to cure the [PSI^+] phenotype of yeast (Chernoff et al., 1995). Therefore, the effect of overexpressing Hsp104p on the nonsense suppression phenotype was monitored. The *tyr7-1 leu2-2* strain harboring the pG-1MTT1 plasmid was transformed with either a high copy *URA3* plasmid containing *HSP104* or the vector. These strains were monitored for the ability to grow on medium lacking uracil, tryptophan, leucine, and tyrosine or medium lacking uracil and tryptophan as a control. The strain harboring the pG-1MTT1 plasmid and the *URA3* vector grew on medium lacking leucine and tyrosine (Fig. 2B). The strain transformed with the plasmid overexpressing the *HSP104* gene, however, was not able to grow in the absence of leucine or tyrosine (Fig. 2B). These strains demonstrated no differences in growth on medium lacking tryptophan and uracil (Fig. 2B).

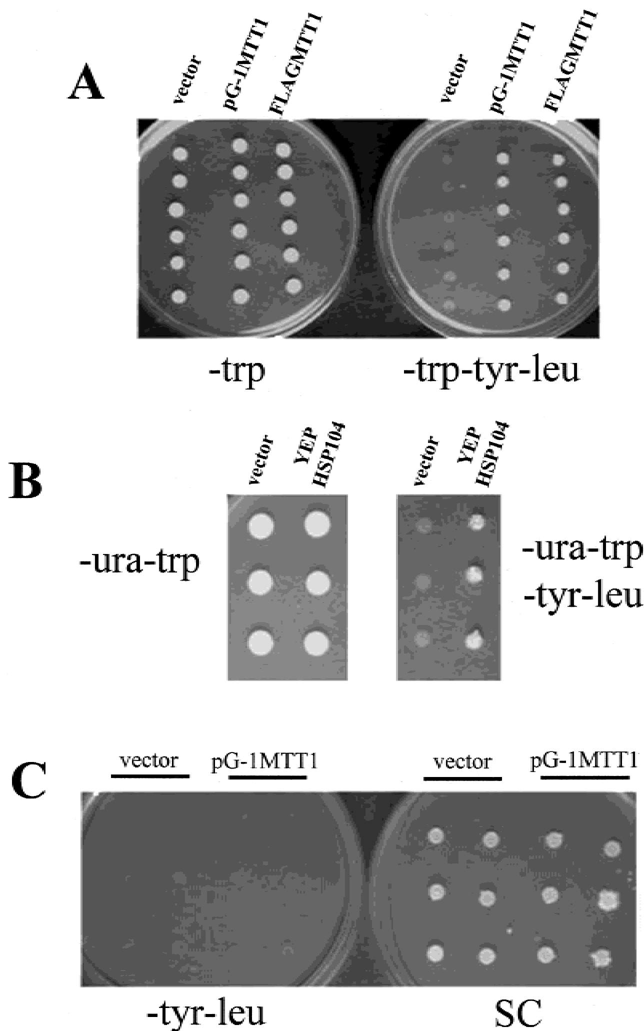

FIGURE 2. Overexpression of *MTT1* causes nonsense suppression. **A**: Overexpression of Mtt1 causes nonsense suppression. Strain KC2 was transformed with a *TRP1*-containing vector, pG-1MTT1 or pG-1FLAGMTT1, and six independent transformants were grown in −trp medium and plated on −trp and −trp−tyr−leu media. Cells were monitored for growth at 30 °C and the photograph was taken at 7 days. **B**: HSP104 suppresses the effect of overexpression of *MTT1*. Three pG-1MTT1 transformants from **A** were transformed with a high copy *URA3*-containing vector or pFL44L (a high-copy *URA3* plasmid-containing *HSP104*). Three independent transformant colonies were grown in −ura−trp medium and plated on −ura−trp or −ura−trp−tyr −leu media. Cells were monitored for growth at 30 °C and the photograph was taken at 7 days. **C**: Loss of *MTT1* overexpression abolishes the nonsense suppression phenotype. Vector and pG-1MTT1 transformants from **A** were grown in YPD and plated for single colonies. Single-colony plates were replica plated to Synthetic complete (SC) and −trp plates, and trp− colonies were selected. Six independent trp− colonies for each were then grown in SC and plated simultaneously on SC (not shown), −tyr −leu, or −trp media. Cells were monitored for growth at 30 °C and the photograph was taken at 14 days. No growth on the −trp plates was evident, confirming loss of the plasmid.

The results above are consistent with the notion that overexpression of Mtt1 may induce the [PSI^+] phenotype. An alternate hypothesis is that the Hsp104p can play a role in modulating translation termination independent of its role in propagating the [PSI^+] phenotype. A prediction of the [PSI^+] phenotype is that it becomes a stably inheritable trait that will persist in cells that lose the factor that induced the [PSI^+] state. For example, overexpressing eRF3p from a plasmid leads to the [PSI^+] state, which is maintained even when the plasmid expressing eRF3 is lost. Therefore we asked whether the nonsense suppression phenotype is maintained in cells that lose the plasmid overexpressing the *MTT1* gene. *leu2-2* and *tyr7-1* cells containing pG-1MTT1 or the vector were grown nonselectively and screened for Trp- colonies, which would indicate loss of the plasmid harboring the *MTT1* gene. These colonies were subsequently assayed for their nonsense suppression phenotype by their ability to grow on medium lacking leucine and tyrosine. The results demonstrated that neither strain grew on medium lacking these amino acids (Fig. 2C). This result indicates that the nonsense suppression phenotype is dependent on the Mtt1p being overexpressed and that the nonsense suppression phenotype observed is not due to the induction of [PSI^+] phenotype.

A *mtt1Δ* strain does not affect nonsense-mediated mRNA decay

Previous results demonstrated that the Upf1p has a role in regulating both mRNA turnover and translation termination (Weng et al., 1996a, 1996b, 1998; Czaplinski et al., 1998). The effect of *mtt1Δ* on NMD was examined by monitoring the abundance of the CYH2 precursor and mature mRNA, which encodes a ribosomal protein. The inefficiently spliced CYH2 precursor, which contains an intron near the 5′ end, is a naturally occurring substrate for the nonsense-mediated mRNA decay pathway (He et al., 1993). The results demonstrated that the steady-state levels of CYH2 precursor and CYH2 mRNA were equivalent to that found in a wild-type strain (Fig. 3, compare lanes 1 and 3). The CYH2 precursor abundance increases in a *upf1Δ* strain, which is expected in this strain, as the Upf1p is required to promote NMD (Fig. 3, compare lanes 1 and 2). The abundance of the wild-type CYH2 transcript, which is not a substrate of the NMD pathway, was equivalent in all strains tested (Fig. 3). Furthermore, the abundance of the transcripts that are substrates for the NMD pathway are not affected to any greater extent in a *upf1Δ mtt1Δ* strain versus only a *upf1Δ* strain (Fig. 3, compare lanes 2 and 4). pG-1MTT1 did not alter the levels of nonsense containing transcripts, or complement deletion of *UPF1* (data not shown), demonstrating that *MTT1* plays an independent role in translation termination.

The Mtt1p interacts with the peptidyl release factor eRF3

Previous results showed that Upf1p interacts with the translation termination factors eRF1 and eRF3 to di-

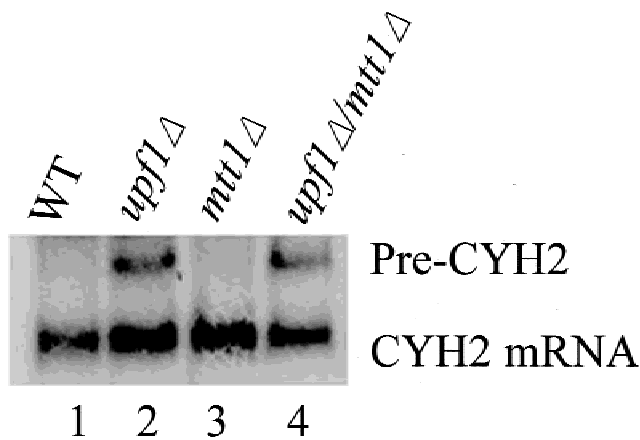

FIGURE 3. Mtt1p does not promote decay of nonsense-containing mRNAs. Total RNA was isolated from KC2 cells harboring either YCplac33 vector (wild-type), *upf1*Δ, *mtt1*Δ or *upf1*Δ, *mtt1*Δ, at OD_{600} = 0.8. Forty micrograms of RNA were separated on a formaldehyde/agarose gel, and subjected to Northern blotting analysis and probed with a CYH2 probe.

rectly affect the efficiency of translation termination (Czaplinski et al., 1998). Because overexpressing the *MTT1* gene promotes nonsense suppression of the *tyr7-1* and *leu2-2* alleles, we hypothesized that Mtt1p may also interact with the peptidyl release factors. To test this prediction, eRF1 and eRF3 were individually expressed in *E. coli* as glutathione-S-transferase (GST) fusion proteins and purified using glutathione sepharose beads. The purified GST-RF (release factor) fusion proteins associated with the glutathione sepharose beads were added to a yeast cytoplasmic extract containing Flag-Mtt1p. Following incubation, the GST-RFs and associated proteins were purified by affinity chromatography and subjected to SDS-PAGE. Immunoblotting was performed and the presence of the Flag-Mtt1p was assayed using an antibody against the FLAG epitope. The anti-FLAG antibody recognized only the 127-kDa Mtt1p in cytoplasmic extracts from cells transformed with plasmid expressing the Flag-Mtt1p (Fig. 4A, compare lanes 1 and 2). This analysis also demonstrated that the Mtt1p specifically copurified with eRF3 (Fig. 4A, compare lanes 3 and 4 to lane 5). Mtt1p did not copurify with either GST-RF1 or the GST domain (Fig. 4A, lanes 3 and 4).

The interaction of purified Mtt1p directly with either eRF1 or eRF3 was also monitored. Mtt1p was purified from yeast cells harboring the *FLAG-MTT1* gene using a FLAG antibody column exactly as described for Flag-Upf1 and Flag-Upf2 (Czaplinski et al., 1995, 1998). Purified Flag-Mtt1 contained only the 127-kDa Flag-Mtt1 protein in a Coomassie blue-stained gel (data not shown), comparable to that reported for purification of Flag-Upf1. This protein cross-reacts with the Flag antibody (Fig. 4A,B, lane 2). The purified GST-RF fusion proteins associated with the glutathione sepharose beads were added to purified Flag-Mtt1p. Following

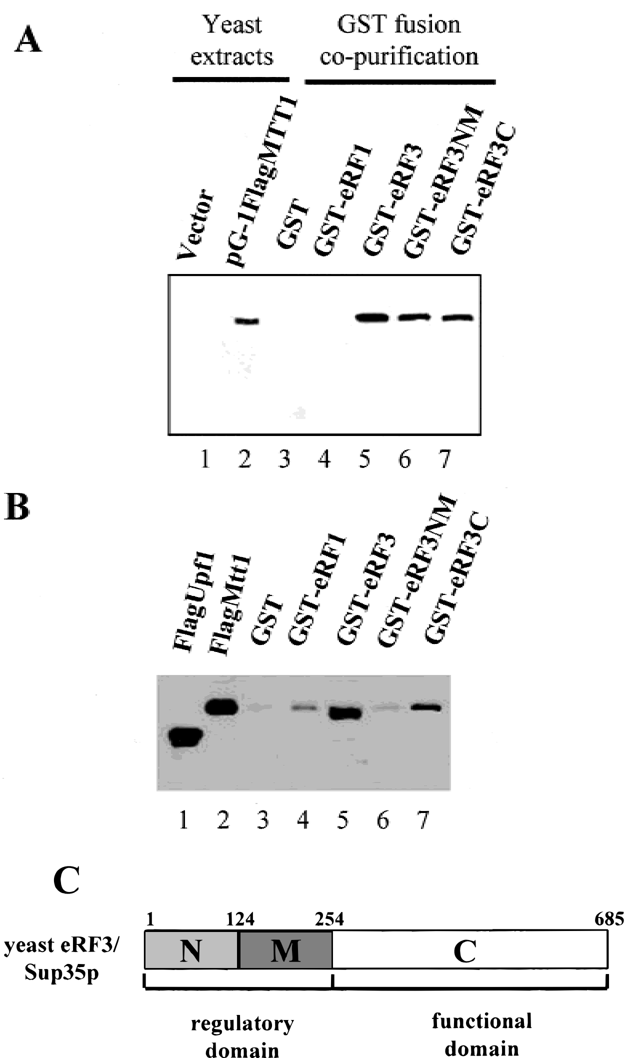

FIGURE 4. Mtt1p interacts with eRF3. **A**: GST-eRF3 fusion protein binds specifically to Mtt1p in a yeast extract. Cytoplasmic extracts from a yeast strain BJ3505 transformed with either pG-1 (vector) or pG-1FLAGMTT1 (Flag-Mtt1p) were prepared in IBTB and incubated with 10 μL GST, GST-eRF1, GST-eRF3, GST-eRF3NM, or GST-eRF3C sepharose–protein complexes. The sepharose–protein complexes were washed two times in IBTB (see Materials and methods), resuspended in SDS-PAGE loading buffer, separated on an 8% SDS-PAGE gel and immunoblotted using anti-FLAG antibody. **B**: Mtt1p interacts directly with eRF3. FlagMtt1p was purified using immunoaffinity chromatography against the N-terminally fused FLAG epitope in a manner identical to that for Upf1p (Czaplinski et al., 1998). Two hundred nanograms of Mtt1p were added to 10 μL of GST, GST-eRF3, GST-eRF3NM, or GST-eRF3C sepharose–protein complexes in a total reaction volume of 200 μL in IBTB. After 1 h at 4 °C sepharose–protein complexes were washed for 3 min with 1 mL of IBTB supplemented with KCl to the final concentration indicated above each lane. The purified sepharose–protein complexes were resuspended in SDS-PAGE loading buffer and separated on a 7.5% SDS-PAGE gel and immunoblotted as in **A**. **C**: The domain structure of the SUP35 gene of *S. cerevisiae*.

incubation, the GST-RFs and associated proteins were purified by affinity chromatography and subjected to SDS-PAGE. Immunoblotting was performed and the presence of the Mtt1p was assayed using an antibody against the FLAG epitope. The anti-FLAG antibody rec-

ognized only the 127-kDa Mtt1p (Fig.4B, compare lane 2 to lane 1). This analysis demonstrated that the Mtt1p strongly copurified with full-length eRF3 (Fig. 4B, compare lanes 3 and 5) and demonstrated a weak but specific interaction with eRF1 (Fig. 4B, compare lanes 3 and 4). Mtt1p did not copurify with the GST domain (Fig. 4B, lane 3).

The translation termination factor eRF3 in budding yeast is encoded by the *SUP35* gene and consists of three distinct domains, the amino terminal (N), middle (M), and carboxyl terminal (C) domains, (Fig. 4C; Ter-Avanesyan et al., 1993; Paushkin et al., 1997b). The N and M domains (abbreviated as NM) are dispensable for cell viability (Ter-Avanesyan et al., 1993) but the N domain is required for formation and maintenance of the eRF3 aggregates responsible for the [PSI^+] phenotype (Doel et al., 1994; Paushkin et al., 1996, 1997a; Glover et al., 1997). The C domain includes the conserved region of homology to EF1A, and is essential for cell viability (Fig. 4C). We asked if the individual domains of eRF3 are capable of interacting with Mtt1p. To accomplish this, GST fusion proteins of the NM or C domains of eRF3 were individually expressed and purified as described above for full-length eRF3 (Paushkin et al., 1997b). The interaction of Mtt1p with each of the domains was determined by incubating the purified FLAG-Mtt1p with the GST-RF3 fusion proteins and the interactions of these proteins were monitored as described above. The results demonstrated that the purified FLAG-Mtt1p interacts most strongly with the C domain of eRF3 (Fig. 4B, compare lanes 3 and 7). A weaker interaction was also detected with the NM domain of eRF3 (Fig. 4B, lane 6). The results from the experiments of Figure 4 indicate that Mtt1p strongly interacts with eRF3.

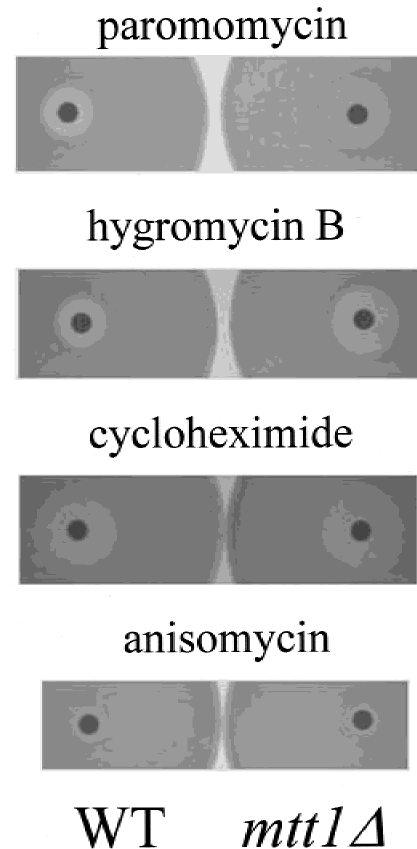

FIGURE 5. *mtt1Δ* strains are sensitive to translational inhibitors. Wild-type and isogenic *mtt1Δ* strains were spread out on YPD plates and a sterile disk containing 750 mg hygromycin B, 2,500 mg paromomycin, 7.5 mg cycloheximide, or 75 mg anisomycin was placed in the center and growth was monitored at 30 °C.

A *mtt1Δ* strain is sensitive to translation inhibitors

Strains harboring mutations that diminish translational fidelity are hypersensitive to antibiotics such as hygromycin B and paromomycin, which cause an increased frequency of misreading in yeast (Palmer et al., 1979; Singh et al., 1979). A *mtt1Δ* strain was previously reported to be hypersensitive to hygromycin B (Lussier et al., 1997). We determined whether a *mtt1Δ* strain also demonstrates hypersensitivity to paromomycin. As a control we monitored sensitivity to cycloheximide and anisomycin, two other antibiotics that target the translation machinery. Hygromycin B, paromomycin, cycloheximide, and anisomycin sensitivities were monitored in isogenic wild-type and *mtt1Δ* strains by determining the zone of growth inhibition around a disc containing each of the drugs placed onto a lawn of cells. The results demonstrated that a *mtt1Δ* strain was clearly hypersensitive to paromomycin and hygromycin B (Fig. 5). The *mtt1Δ* strain was hypersensitive to cycloheximide to a lesser extent, and no significant sensitivity to anisomycin was detected (Fig. 5).

The Mtt1p is polysome-associated

Based on the phenotypes of a *mtt1Δ* strain, we hypothesized that the Mtt1p is associated with ribosomes. The absorbance profiles of polysome extracts from isogenic wild-type and *mtt1Δ* strains demonstrated no significant differences (data not shown). To determine whether the Mtt1p is ribosome associated, postmitochondrial extracts were prepared from cells harboring the Flag-*Mtt1* gene and the polysome fractions were separated by centrifugation through sucrose gradients. The various fractions were collected and the presence of the Flag-Mtt1p protein in the gradient fractions was determined by probing the blots with an antibody directed against the Flag epitope. The results from these experiments indicated that the Flag-Mtt1p is polysome and monosome associated, whereas the upper fractions contained no detectable full-length Flag-Mtt1p (Fig. 6A). Treatment of the polysome extracts with

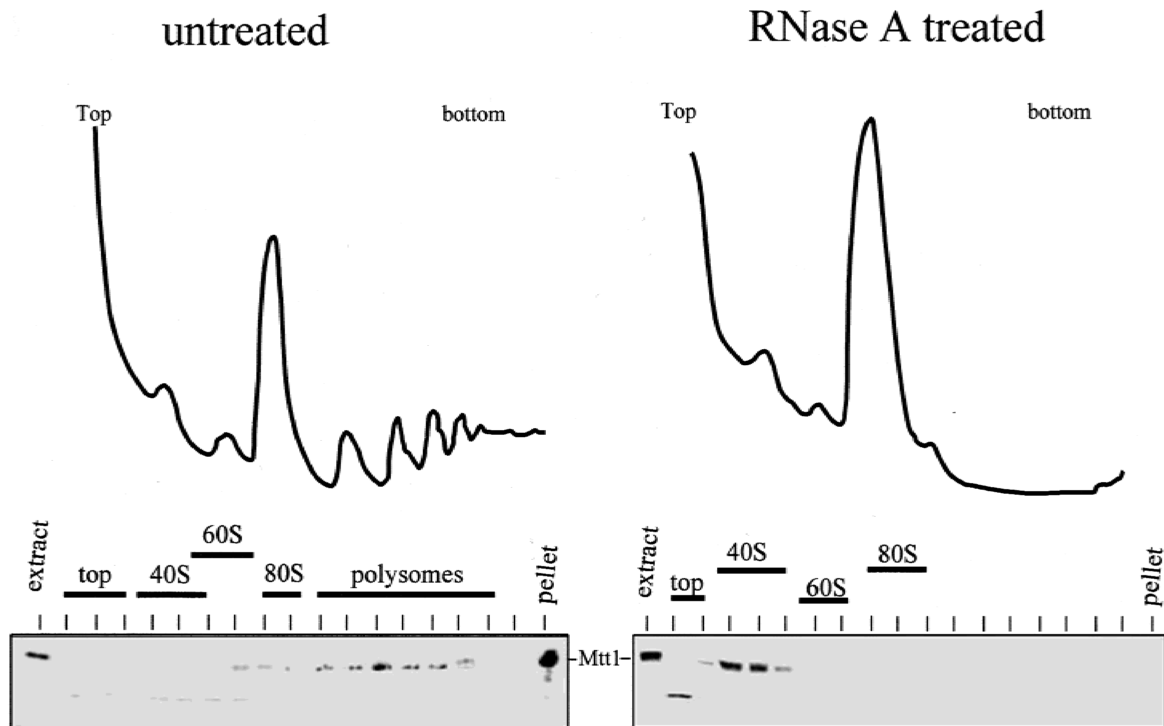

FIGURE 6. Mtt1p is associated with polyribosomes. Polysome extracts of cells transformed with pG-1FLAG*MTT1* were prepared and centrifuged through a 7–47% linear sucrose gradient. An identical sample was treated with RNAse A and centrifuged in an identical manner. A_{254} was monitored as gradient fractions were collected. The absorbance profiles are located at the top of each panel. Total protein in each fraction was TCA precipitated and analyzed by SDS-PAGE followed by western blotting using anti-FLAG M2 monoclonal antibody as described previously (Czaplinski et al., 1995).

RNase A shifted polysome-associated Mtt1p exclusively to fractions that contain free 40S ribosomal subunits (Fig. 6B). This observation is consistent with the notion that the Mtt1p in Figure 6A is localized to polysomes, and not found in very large protein complexes, as has been observed for eRF3 aggregates in [*PSI*$^+$] strains (Paushkin et al., 1996). We do not know whether the shift in Mtt1 after RNAse treatment reflects association with 40S ribosomal subunits or that it is an RNP complex that comigrates with the 40S subunit.

DISCUSSION

The results presented here describe a potential cellular role for the Mtt1p, a previously characterized helicase with significant homology to the Upf1p, a factor involved in regulating both translation termination and NMD (Czaplinski et al., 1995, 1998; Weng et al., 1996a, 1996b, 1998). Evidence is presented indicating that Mtt1p is a second member of the Upf1-like family of helicases and has a role in modulating translation termination. Interestingly, comparison of the *MTT1* gene with other superfamily group I helicases identified unique features that tag this as a specific subfamily of superfamily group I helicases, possibly being involved in either RNA-dependent or RNA/DNA-dependent processes (Fig. 1). As will be discussed below, these results suggest that a subset of the superfamily group I (Upf1p family) of RNA helicases are involved in modulating the efficiency of the translation termination process.

The Mtt1p is a second yeast helicase that interacts with the translation termination release factors

Previous results demonstrated that the Hcs2/Hel1p/Mtt1p exhibited $5' \rightarrow 3'$ DNA helicase activity (Biswas et al., 1995; Bean & Matson, 1997). We have observed RNA-dependent ATPase activity in vitro, supporting a role for Mtt1p in RNA-dependent processes (data not shown), demonstrating that, like the Upf1 protein from this family, Mtt1 has both DNA- and RNA-dependent ATPase activity in vitro (Czaplinski et al., 1995, Weng et al., 1996a, 1996b). A comparison of the *MTT1* and *UPF1* genes identified several regions of similarity. Both proteins contain a cysteine-histidine-rich region near the amino terminal end of the protein and harbor all of the motifs characteristic of a superfamily group I helicase (Fig. 1). The primary sequences of the cysteine-histidine-rich regions of *UPF1* and *MTT1* are not very homologous; however, it is conceivable that these cysteine-histidine-rich regions form an unidentified type of cysteine-histidine-rich protein structure. Interestingly, mutations in the cysteine-histidine-rich region of

UPF1 have been shown previously to increase programmed −1 frameshifting efficiencies and promote nonsense suppression (Cui et al., 1996; Weng et al., 1996b).

The results of the experiments described here demonstrated that: (1) high levels of the Mtt1p result in a nonsense suppression phenotype (Fig. 2A); (2) Mtt1p directly interacts with the peptidyl release factors (Fig. 4A,B); (3) a *mtt1*Δ strain demonstrates sensitivity to drugs that affect translation fidelity (Fig. 5); and (4) Mtt1p was found associated with polyribosomes. These results are all consistent with a role for the Mtt1p in translation and the ability to interact with eRF3.

eRF3 in *Saccharomyces cerevisiae* demonstrates prion-like properties and in an aggregated state results in a cytoplasmically inherited suppressor element known as [*PSI*$^+$] (Patino et al., 1996; Paushkin et al., 1996). The product of the *HSP104* gene is required for formation and maintenance of [*PSI*$^+$] aggregates of eRF3, and when overexpressed can cure the suppression observed in a [*PSI*$^+$] strain (Chernoff et al., 1995). Overexpression of HSP104 can disrupt eRF3 protein aggregates that could lead to the [*PSI*$^+$] state. Several lines of evidence, however, indicate that the Mtt1p does not induce the [*PSI*$^+$] phenotype. For example, the experiments observed in Figure 6 demonstrated that the Mtt1p was located in large complexes as part of polysomes. Consistent with this view is the observation that the Mtt1p migrates as much smaller particles if the polysomes are treated with RNAse. Previous results have demonstrated that eRF3 aggregates [*PSI*$^+$] strains are resistant to RNase treatment (Paushkin et al., 1996). Furthermore, the results of overexpressing Mtt1p are not consistent with the notion that overexpression of Mtt1p induces the [PSI+] phenotype. Loss of the *MTT1* plasmid resulted in loss of the nonsense suppression phenotype. This result is not characteristic of a [*PSI*$^+$] phenotype. Once the [*PSI*$^+$] inheritable phenotype is induced, for example, by overexpression of eRF3, the plasmid harboring the *SUP35* gene is dispensable and the [*PSI*$^+$] phenotype can be perpetuated in the absence of the inducer plasmid. The fact that nonsense suppression does not occur when the excess Mttp1p is lost indicates that Mtt1p did not induce the [*PSI*$^+$] phenotype.

Because *HSP104* acts to dissolve protein aggregates (Glover & Lindquist, 1998), it is possible that overexpression of *HSP104* may be dissolving Mtt1p aggregates that are induced by Mtt1 overexpression. In this scenario, the protein aggregates specifically lead to suppression, because Mtt1 expression would still be elevated in the presence of excess HSP104. This hypothesis suggests that putative Mtt1 aggregates can sequester eRF3 by virtue of direct protein–protein interaction and/or inhibit proper eRF3 function.

An alternative explanation for these phenotypes is that the Mtt1p may be involved with *HSP104* to affect translation termination. This model suggests that *HSP104* and *MTT1* are involved in a common step of translation termination and that dosage of these two factors plays a role in their ability to influence termination. *HSP104* has been proposed to make specific interactions with eRF3 (Patino et al., 1996), and interactions of this type may also indicate another role of *HSP104* in translation termination other than acting to disassociate [*PSI*$^+$] aggregates. Although *HSP104* is involved in regulation of eRF3 aggregates, it is surprising that there has been no report on the ability of *HSP104* to regulate the [*URE3*] phenotype of yeast. [*URE3*] has been demonstrated to result from a prion-like aggregation of the Ure2 protein (Wickner 1994; Masison et al., 1997). A [*PSI*$^+$]-specific effect for involvement of *HSP104* in aggregate regulation would support a specific interaction of this protein with the translation termination machinery. Interestingly, a tobacco homolog of *HSP104* is required for translational regulation of TMV, in part by a direct interaction with the TMV mRNA (Wells et al., 1998).

The Upf1-like family of helicases may be involved in RNA-dependent processes

Sequence comparisons of superfamily group I helicases initially identified *SEN1* and the mouse MOV-10 genes as having strong regions of homology to the *UPF1* helicase region (Koonin, 1992). The *MTT1* gene also demonstrates homology to *UPF1* and to other members of the *UPF1*-like subfamily (Figs. 2 and 7). In particular, another member of the *UPF1* family of helicases in yeast is the recently isolated *HCS1* gene, Helicase A (Biswas et al., 1997a, 1997b). This helicase was initially isolated as part of the multienzyme polymerase complex (Biswas et al., 1993a, 1993b, 1995), although deletion of this gene is not lethal (K. Czaplinski, unpubl. experiments). Functional evidence for the extended homology is exemplified in the *upf1-D4* mutation. This mutation is an arginine-to-cysteine mutation in position 779 (R779C) and it confers dominant negative inhibition of NMD in yeast (Leeds et al., 1992). The equivalent mutation in the human homolog (R844C) demonstrates a similar dominant negative effect in mammalian cells (Sun et al., 1998). This mutation lies within the extended sequence of motif V from this subfamily and is conserved in all five family members in yeast (Fig. 1A, the last residue in this motif).

It has now become clear that the ability to manipulate nucleic acid duplexes by helicases is critical for almost every biological process in which DNA and RNA are involved. A large number of helicases have been shown to be involved in posttranscriptional control mechanisms. Examples include tRNA processing (Winey & Culbertson, 1988; DeMarini et al., 1992), ribosomal biogenesis (reviewed in Venema & Tollervey, 1995; Venema et al., 1997), RNA splicing (reviewed in Staley &

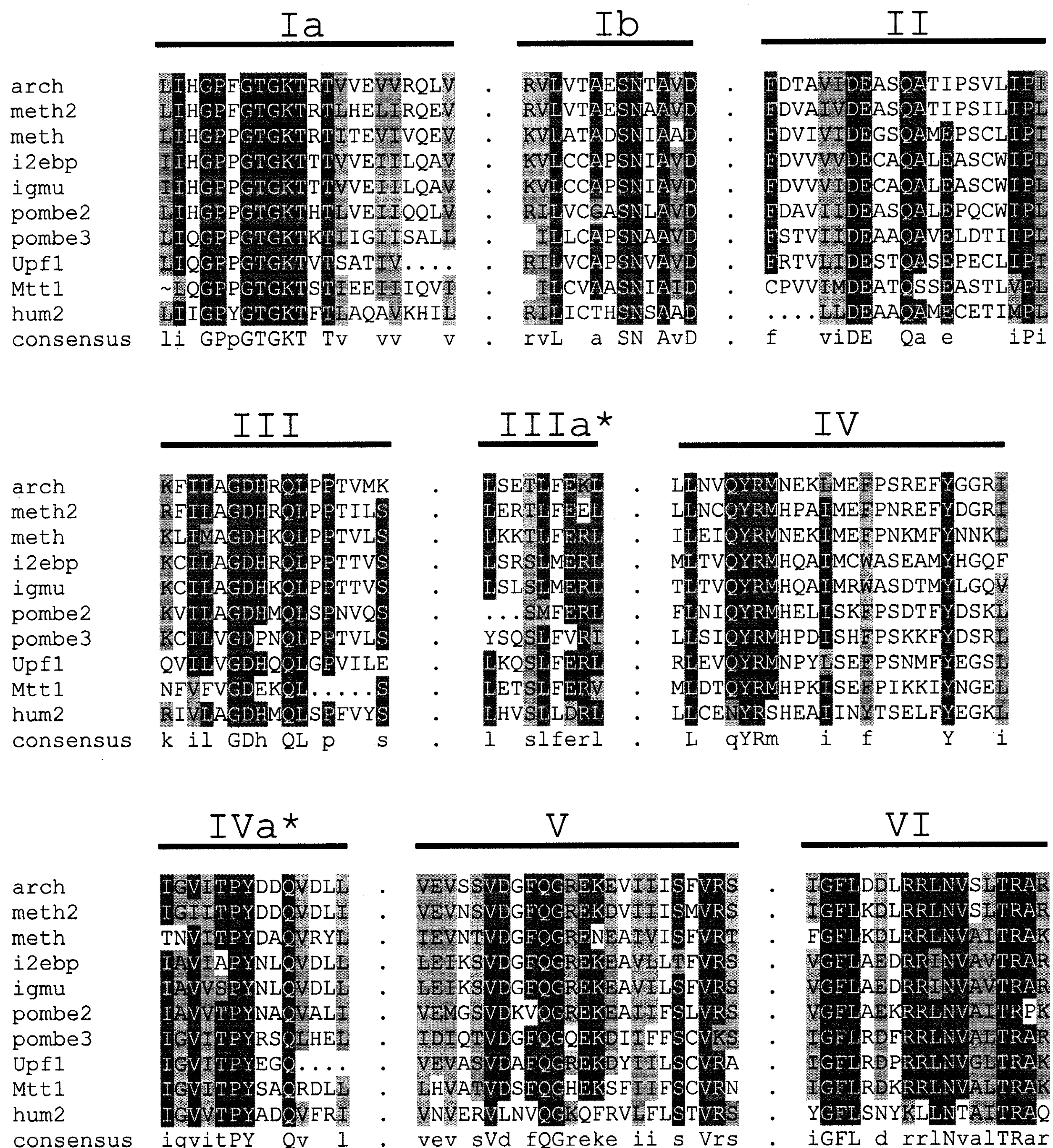

FIGURE 7. The Upf1-like helicase superfamily is conserved throughout evolution. The amino acid sequences of the conserved helicase motifs from the *UPF1*, *MTT1,* and eight members of this family from other species were aligned using the PILEUP program from GCG as in Figure 2. Labeling of the original seven superfamily group I motifs is indicated by roman numerals, and the two new motifs identified in this family, IIIa and IVa, are denoted by *. The Mov-10 gene of mice was identified as homologous to Upf1 (Koonin, 1992) and contains the newly identified motifs but has been omitted. The abbreviations are as follows: arch: *Archaeoglobus fulgidus*, accession #C69423; meth: *Methanococcus jannaschii*, accession #H64312; meth2: *Methanobacterium thermoautotrophicum*, accession #AE000922; i2ebp: insulin II enhancer binding protein, *Mus musculus,* accession #S35633; igmu: Ig mu chain switch region binding protein 2, *Homo sapiens*, accession #A47500; pombe2: *Schizosaccharomyces pombe*, accession #Z98951; pombe3: *Schizosaccharomyces pombe*, accession #Q92355; hum2: cDNA for KIAA0054, *Homo sapiens*, accession #D29677.

Guthrie, 1998), RNA transport (Snay-Hodge et al., 1998; Tseng et al., 1998), translation (Rozen et al., 1990), and mRNA turnover (Weng et al., 1996b; reviewed in Jacobs Anderson & Parker, 1996). These helicases fall into at least two families, the most prominent superfamily being the DExD/H box helicases or superfamily group II. Several helicases have demonstrated the ability to displace both DNA and RNA duplexes in vitro

(Scheffner et al., 1989; Zhang & Grosse 1994; Czaplinski et al., 1995).

It is interesting that there are at least two helicases that can interact with the translation termination machinery in yeast. A search of the available databases revealed several proteins that harbor the Upf1-like helicase domain that we have described here, suggesting that this subfamily of helicases is not limited to yeast. Several proteins previously identified as homologous (Applequist et al., 1997) were found to contain the newly identified motifs as well (Fig. 7). The mutated arginine residue of *upf1-D4* is also highly conserved within motif V of these members (Fig. 7, SxVR) with only one member harboring a conservative arginine-to-lysine substitution. The preceding valine is invariant among all of the members identified here. Given the available functional data for the members of this superfamily, it is clear that these Upf1-like helicases will not all be involved in the same biological processes, however the homology may indicate some conserved aspect of their activities. Given that the Upf1, Sen1, and Mtt1 are all likely to be involved in RNA-dependent processes, and that the role of the DNA2 protein in replication may be in processing of Okazaki fragments that are primed by RNA, we hypothesize that this superfamily may function in RNA-dependent processes and this subclass may constitute a new family of RNA helicases.

The roles of RNA helicases in translation termination

One potential role for the Mtt1p builds upon the function of Upf1 in mRNA turnover. Upf1 ATPase activity is required for NMD, and its activities are modulated by the release factors (Czaplinski et al., 1998). An *MTT1* deletion does not affect NMD (Fig. 3); however, it is conceivable that an unidentified subset of mRNAs could be stabilized in the absence of Mtt1, and thus it may act analogously to Upf1 in linking translation termination to an unidentified mRNA turnover pathway. It is also conceivable that Mtt1p may link translation termination to some unidentified cellular process, or it may play a more vital role under different growth conditions. No other proteins harboring homology to Mtt1p outside of the helicase domain have been found in database searches.

At present, we do not understand how Upf1p and Mtt1p modulate the translation termination process. We envision that the efficiency of translation termination can be affected by altering (1) the association rates of the eRFs with the ribosome, (2) the efficiency of the eRFs in promoting peptidyl hydrolysis, or (3) the rate of disassociation of the eRFs from the ribosome after translation termination has been completed. We are currently developing assays to monitor these steps in the translation termination process to begin to understand at what step these proteins function. The observation that purified Mtt1p interacts strongest with eRF3 suggests that Mtt1p overexpression may exert its effect primarily through an interaction with this component of the termination complex. Furthermore, the NM domain of eRF3 demonstrated a significantly stronger interaction with Mtt1p in cytoplasmic extracts than purified Mtt1p. This may suggest that interaction of Mtt1 with the NM domain of eRF3 may be enhanced or bridged by other factors present in the extracts. Identification of such Mtt1-eRF3 bridging factors would lead to a more detailed understanding of the biological role of this protein.

MATERIALS AND METHODS

General yeast methods

Yeast media, transformations, RNA isolation, blotting, and hybridization were as described (Scheistl & Geitz 1989; Rose et al., 1990; Hagan et al., 1995; Weng et al., 1996a).

Strains constructed for this study

MTT1 was disrupted from PLY22 (*a his4-38 SUF1-1 ura3-52 met14*) and PLY36 (**a** his4-38 SUF1-1 ura3-52 met14 upf1-2) to assay for frameshift suppression. To assay nonsense suppression of *leu2-2* and *tyr7-1*, strain KC2 was created by crossing PLY146 *(a ura3-52 leu2-2(UGA) tyr7-1(UAG) trp1 upf1::URA3;* Leeds et al., 1991, 1992) with PLY22, selecting diploids on −ura−trp media, sporulating, and assaying random spores for auxotrophy on −ura, −trp, −leu, and −tyr media independently. Subsequent analysis of strain KC2 (*a ura3-52 leu2-2(UGA) tyr7-1(UAG) trp1 his4-38 met14*) demonstrated that it also retained the *met14* and *his4-38* markers, but not *SUF1-1.* Deletion of *UPF1* from KC2 was as described (pKOM; Cui et al., 1995). To disrupt *MTT1 (mtt1::hisGURA3hisG)*, pKOMTT1 was digested with *Pvu*II and transformed into yeast. To confirm the knockout of *MTT1,* genomic DNA was digested with *Xho*I and a 2.2-kb *Xho*I-*Xba*I probe fragment was excised from pUC19*MTT1*. Wild-type *MTT1* gives a 20-kb genomic fragment, whereas *mtt1*::hisGURA3hisG gives an ~6.2-kb fragment. After selection on 5-FOA to remove hisGURA3 (mtt1::hisG), the probe yields an ~3.5-kb fragment.

Plasmids

YCplac22*MTT1* and YEplac112*MTT1* were created by amplifying the *MTT1* gene by PCR (Elongase, BRL Inc.) with a forward primer complementary to the region upstream of a *Kpn*I site (5′-gagaacatactctggcgggctt-3′) and a reverse primer complimentary to a region downstream of the MTT1 termination codon, adding a *Sal*I site (5′-ggggggtcgactctagcatc gccactagaaaattca-3′), and inserting the 5-kb *Kpn*I/*Sal*I-digested PCR product into *Kpn*I/*Sal*I-digested YCplac22 or Yeplac112. pG-1FLAG was created by insertion of a polylinker sequence in the unique *Bam*HI-*Sal*I sites of pG-1 (Schena & Yamamoto, 1988), which directs translation of the FLAG epitope, which is followed by several unique restriction

sites, one of which is *Nco*I. The ATG of this *Nco*I site is in frame with the FLAG peptide. pG-1FLAGMTT1 was created by PCR (Elongase) using a 5′ primer complementary to the 5′ end of the coding region, adding an *Nco*I site in the 5′ end in which the ATG of *Nco*I is the translation start site (5′-ccccccatggattttcagtgcagaacgtg-3′) and the same 3′ primer as above. The PCR product was ligated to an *Nco*I-*Sal*I digested pG-1FLAG. pG-1MTT1 was created by removing the *Nco*I-*Sal*I fragment from pG-1FLAGMTT1 and ligating it to the 7.4-kB *Nco*I-*Sal*I vector fragment. In this construct, the FLAG peptide sequence is removed. pUC19MTT1 was created by inserting the *Kpn*I-*Sal*I PCR fragment described above into *Kpn*I-*Sal*I-digested pUC19. The *Eco*47III-*Xba*I fragment from pUC19MTT1 was replaced with *Pvu*II-*Xba*I-digested pAS135 (Alani et al., 1987) to create pKOMTT1.

Preparation of glutathione sepharose-RF fusion complexes and of purified RF-fusion proteins

The glutathione sepharose-RF fusion complexes were prepared as described previously (Czaplinski et al., 1998). One microliter of GST-RF complexes typically contained 0.9 μg GST-eRF1 or 1.5 μg GST-eRF3, while GST complexes typically contained 4.5 μg GST per μL of resin.

Preparation of cytoplasmic extracts

BJ3505 (MAT *pep4::HIS3 prb-Δ1.6R HIS3 lys2-208 trp1-Δ10 ura3-52 gal2 can1*) cells transformed with pG-1FLAGMTT1 were grown to an OD_{600} = 1.0 and washed in cold Buffer IB (25 mM HEPES, pH 7.4 at 4 °C, 50 mM KCl, 10 mM $MgCl_2$, and 2% glycerol) with 0.5 mM PMSF. Cells were repelleted and suspended in an equal volume of cold IB with 0.5 mM PMSF and protease inhibitors (PI; 1 μg/mL each Leupeptin, Aprotinin, and pepstatin A). An equal volume of glass beads was added and lysis was achieved by vortexing six times for 20 s, with 1 min cooling on ice in between vortexing. The lysate was removed, and the beads washed two times with an equal volume of IB with 0.5 mM PMSF and PI. The washes were combined with the lysate and the cell debris was removed by centrifugation at 30,000 × *g* for 20 min.

Drug hypersensitivity assay

0.3 mL of a 1:1 mix of water and $O.D._{600}$ = 1.4 cell culture as indicated was added to 4 mL of melted top agar at 42 °C, and this mixture was plated onto a YPD plate prewarmed to 30 °C and allowed to solidify. Indicated amounts of antibiotic were applied to a sterile disk and the dried disks applied to the center of the YPD plates and growth was monitored at 30 °C. Hypersensitivity is indicated by larger zones of growth inhibition surrounding the antibiotic-containing disk.

Polysome analysis

A 250-mL culture of strain BJ3505 transformed with pG-1*FLAGMTT1* was grown to $O.D._{600}$ = 0.8 in −trp media and brought to 100 mg/mL with cycloheximide, then chilled quickly by pouring the culture into centrifuge bottles containing 50 mL of frozen media with 100 μg/mL cycloheximide and chilling on ice until the frozen media has melted. Cells were collected, washed once with water containing 100 μg/mL cycloheximide. The cell pellet was resuspended in an equal volume of Extract Buffer (10 mM Tris, pH 7.5, 100 M NaCl, 5 mM $MgCl_2$, 200 mg/mL heparin, 2 mM DTT, 0.5 mM PMSF and 1× PI) and an equal volume of glass beads was added. Lysis was performed by vortexing eight times for 20 s with 1 min on ice in between vortexing. The lysate was removed from the beads to a microcentrifuge tube and centrifuged twice for 10 min at maximum speed, removing the supernatant after each centrifugation. Thirty $O.D._{260}$ units were layered on top of an 11-mL 7–47% linear sucrose gradient in gradient buffer (50 mM Tris-Acetate, pH 7.0, 50 mM NH_4Cl, 12 mM $MgCl_2$, 2 mM DTT, 0.5 mM PMSF) and centrifuged at 39,000 rpm for 2.5 h with a Beckman Ti-45 rotor. RNAse A treatment was performed by adding RNAse A to 200 μg/mL and incubating on ice for 10 min prior to loading onto the gradient. A_{254} was monitored as gradients were harvested from the top using a Isco gradient fractionator. Total protein in fractions was precipitated by TCA and separated on an 8% SDS-PAGE gel and subjected to western blotting against the FLAG epitope as described (Czaplinski et al., 1995).

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