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Testing the *faux*-UTR model for NMD: analysis of Upf1p and Pab1p competition for binding to eRF3/Sup35p

Stephanie Kervestin^a, Chunfang Li^{b,c}, Richard Buckingham^a, and Allan Jacobson^{b,*}

^aCNRS UPR9073 associated with Université Paris Diderot, Sorbonne Paris Cité, Institut de Biologie Physico-chimique (IBPC), 13 rue Pierre et Marie Curie, 75005 Paris, France

^bDepartment of Microbiology and Physiological Systems, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester MA 01655-0122, USA

Abstract

Nonsense-mediated mRNA decay (NMD) is a surveillance mechanism that accelerates the degradation of mRNAs containing premature translation termination codons. This quality control pathway depends on the NMD-specific factors, Upf1p, Upf2p/Nmd2p, and Upf3p, as well as the two release factors, eRF1 and eRF3 (respectively designated Sup45p and Sup35p in yeast). NMD activation is also enabled by the absence of the poly(A)-binding protein, Pab1p, downstream of a termination event. Since Sup35p interacts with both Upf1p and Pab1p we considered the possibility that differential binding of the latter factors to Sup35p may be a critical determinant of NMD sensitivity for an mRNA. Here we describe three approaches to assess this hypothesis. First, we tethered fragments or mutant forms of Sup35p downstream of a premature termination codon in a *mini-pgk1* nonsense-containing mRNA and showed that the inhibition of NMD by tethered Sup35p does not depend on the domain necessary for the recruitment of Pab1p. Second, we examined the Sup35p-interaction properties of Upf1p and Pab1p *in vitro* and showed that these two proteins bind differentially to Sup35p. Finally, we examined competitive binding between the three proteins and observed that Upf1p inhibits Pab1p binding to Sup35p whereas the interaction between Upf1p and Sup35p is relatively unaffected by Pab1p. These data indicate that the binding of Upf1p and Pab1p to Sup35p may be more complex than anticipated and that NMD activation could involve more than just simple competition between these factors. We conclude that activation of NMD at a premature termination codon is not solely based on the absence of Pab1p and suggest that a specific recruitment step must commit Upf1p to the process and Upf1p-associated mRNAs to NMD.

Keywords

Premature translational termination; mRNA decay; Release factors; Upf proteins; Poly(A)-binding protein

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*Corresponding author: Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester MA 01655-0122, USA, phone: 1-508-856-2442, fax: 1-508-856-5920, allan.jacobson@umassmed.edu.

^cPresent address: MedImmune, 319 North Bernardo Avenue, Mountain View, CA 94043, USA

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1. Introduction

The nonsense-mediated mRNA decay (NMD) pathway degrades mRNAs that contain premature translation termination codons (PTCs), thus protecting cells from the synthesis of potentially deleterious truncated proteins [1–5]. NMD has a minimal dependence on three factors, Upf1p, Upf2p/Nmd2p, and Upf3p, which form the core components of the highly conserved surveillance complex [1–4, 6, 7]. Upf1p has ATPase and RNA helicase activities and appears to serve as the central regulator of the NMD pathway, with Upf2p/Nmd2p and Upf3p regulating Upf1p function [8–10]. Activation of NMD also requires recognition of the premature stop codon by the translation machinery [11–14], a process whose terminal events include formation of a termination complex comprised of eRF1 (Sup45p in yeast) and eRF3 (Sup35p in yeast) and the binding of that complex to the A site of a ribosome positioned on a UAG, UAA, or UGA codon [15]. eRF1 recognizes the three stop codons and triggers release of the newly synthesized polypeptide chain. eRF3 is a GTPase and hydrolysis of GTP facilitates stop codon decoding by eRF1 [16–18]. The Upf factors have been shown to interact with eRF1 and eRF3, underscoring the connection between NMD and translation termination. Interaction of eRF3 with Upf1p has been described in yeast and human cells [19–22]. Less is known about interactions between eRF1 and Upf1p and the possibility that they are mediated through eRF3 [19, 22]. In general, interaction between the release factors and the Upf proteins has been thought to trigger NMD when termination takes place at a PTC, although the mechanism of decay activation is unknown.

A fundamental question about NMD activation is how mRNAs with PTCs are distinguished from normal mRNAs. Several lines of evidence indicate that one component of this substrate selectivity is the proximity of the poly(A)-binding protein, Pab1p, to a terminating ribosome. Experiments utilizing tethered Pab1p, and other approaches, in yeast [23], *Drosophila* [24], human [22, 25–27], *C. elegans* [28], and plant cells [29] have all demonstrated that the presence of Pab1p close to a translation termination site protects an mRNA from NMD. Consistent with this observation, mRNAs that normally have unusually long 3'-UTRs, or which garner long 3'-UTRs as a consequence of mutational or processing events, are subject to degradation by NMD [30–32] and mRNAs that would normally be substrates for NMD are stabilized by deletions that eliminate the majority of coding sequences downstream of the premature terminator [33, 34]. As suggested by the *faux*-UTR model [1, 23, 35], these data are consistent with the notion that Pab1p may play a role in orchestrating the assembly of an mRNP competent for efficient termination, a possibility consistent with experiments in yeast cell-free extracts showing that premature termination is less efficient than normal termination [23, 36].

An additional feature that appears to distinguish NMD substrates from normal mRNAs, particularly in mammalian cells, is the presence of an exon junction complex (EJC) downstream of the termination codon and 24 nt upstream of an exon-exon boundary [37]. Here, Upf2p and Upf3p, recruited by the EJC, are thought to interact with Upf1p that has been recruited by the translation termination complex, thereby activating the decay of nonsense-containing mRNA [21, 38]. In a recent model roles for the EJC and Pab1p have been integrated such that a termination event is thought to be recognized as normal or premature as a consequence of a balance between NMD-antagonizing factor(s) (such as Pab1p) and NMD stimulating factor(s) (such as the EJC) associated with the mRNA [26, 39]. NMD stimulatory factors should, however, be viewed cautiously since NMD depends on ongoing translation [13, 14, 40] and EJC factors have been shown to play important roles in determining the translatability of an mRNP [41–44].

Regardless of the precise mechanism of NMD activation, interactions between eRF3, Upf1p, and Pab1p are likely to influence the process. Interaction between Pab1p and eRF3 is

considered to be a major determinant of a normal termination event, whereas interaction between eRF3 and Upf1p appears to be a significant component of PTC recognition. Hence, the possibility that Upf1p and Pab1p are in competition for eRF3 binding must be considered [1, 2, 26]. Since the hUpf1p interaction domain on heRF3 has been mapped to different sites (including the eRF3 N-terminal region, possibly the same site as the Pab1p interaction domain [26], or to an eRF3 C-terminal domain, in residues 200–450 of the GTPase region [22]) it remains to be determined whether eRF3 binding to Pab1p excludes Upf1p binding or vice versa. In the experiments that follow we have evaluated the interactions between yeast Pab1p, Upf1p, and Sup35p (eRF3) using three different approaches. In the first, we utilized tethering methods to assess whether Sup35p molecules lacking specific domains or activities retained their ability to stabilize a nonsense-containing mRNA. In the second, we utilized purified recombinant proteins to determine binding properties of Pab1p and Upf1p for Sup35p. Finally, we analyzed the binding of Upf1p or Pab1p to Sup35p in the presence of the other factor. Our results indicate that simple competition between Upf1p, Pab1p, and Sup35p is an insufficient explanation for NMD activation and suggest that a specific recruitment step must commit Upf1p to NMD.

2. Materials and methods

2.1 Yeast strains and media

All strains used for tethering experiments were derived from the MBS background (*MAT α* , *ade2-1*, *his3-11,112*, *trp1-1*, *ura3-1*, *can1-100*, *leu2-3,112* [rho+] L-o, M-o) and have been described previously [23]. Strain BJ5457 (*MAT α* , *ura3-52*, *trp1- Δ 1*, *lys2-801*, *leu2-1*, *his3 Δ 200*, *pep4::HIS3*, *prb1 Δ 1.6R* *can1 GAL*) harboring FLAG-*UPF1* on a high-copy-number plasmid was used to purify Upf1p from yeast cell extracts [45]. Cells were grown in synthetic complete (SC) medium lacking the amino acids required to maintain plasmid selection [46]. Yeast cells were transformed by treatment with lithium acetate and polyethylene glycol as described [46]. Haploid strain *sup35::KAN* used for the complementation assay was obtained from heterozygote strain *sup35::KAN/SUP35* (obtained from Open Biosystems, isogenic to wild-type BY4741) after transformation with the plasmid pRS316-*SUP35* (*URA3*), sporulation, and selection for haploid strains KAN+, URA+, unable to grow on 5-Fluoroorotic Acid (5-FOA).

2.2 Plasmids

2.2.1 Yeast plasmids—The *mini-pgk1-MS2* reporter gene, carried on a pRS316 plasmid, has been described previously [23] under the name *PGK1-MS2*. Briefly, this construct expresses an mRNA containing the initial portion of the *PGK1* ORF, with a UAG after 23 codons (at position 70; AUG is position +1). MS2 binding sites are localized at position 110–227 and do not introduce any stop codons. Following the MS2 binding sites, the *mini-pgk1-MS2* ORF contains codons 283 to 309 and codons 380 to 416 of *PGK1*. The ORF downstream of the PTC is 122 codons long.

A plasmid expressing an MS2-*Sxl* fusion (*Sxl=sex lethal* protein), under the control of the *ADHI* promoter, was described previously [47] under the name pA-*sxl*. MS2-Sup35p WT, H348Q or R419G fusion proteins were expressed from YCplac111 under control of the *TPII* promoter, yielding YCplac111-MS2-*SUP35*, YCplac111-MS2-*sup35*-H348Q, and YCplac111-MS2-*sup35*-R419G). Coding sequences of *SUP35*, *sup35*-H348Q, and *sup35*-R419G were amplified by PCR from plasmids pDB663, pDB670, and pDB783, respectively [18], using the following oligonucleotides: Sup35-EcoRI-3' 5'-TTGAATTCATATATAACAGCTTTATTAAACCTTGTT-3' and XhoI-Sup35 5'-GCCCTCGAGATGTCGGATTCAAACCAAGGCAA-3'. The amplified fragments were digested by XhoI and EcoRI and then subcloned in YCplac111 using a three piece ligation

reaction with: (1) NcoI-EcoRI digested YCplac111-MS2-*PAB1* [23] that removed the *MS2-PAB* coding sequence, (2) an NcoI-XhoI fragment containing the MS2-coat protein coding sequence from YCplac111-*MS2-PAB1*, and (3) XhoI-EcoRI fragments containing *SUP35*, *sup35-H348Q*, or *sup35-R419Q* coding sequences amplified by PCR. YCplac111-derived plasmids expressing MS2-Sup35p-ΔN, MS2-Sup35p-ΔNM, or MS2-Sup35p-ΔC under control of the *TP11* promoter were obtained by recombination in yeast (yielding YCplac111-MS2-*SUP35*, YCplac111-MS2-*SUP35*-ΔC, YCplac111-MS2-*SUP35*-ΔN, and YCplac111-MS2-*SUP35*-ΔNM). All coding sequences for *SUP35* deletions were amplified by PCR. The fragment encoding domain 114–685 of *SUP35* (MS2-Sup35p-ΔN) was amplified using the following oligonucleotides: MS2-Sup35-Δ1–113: 5'-GCAGCAAACCTCCGGCATCTACGCTAGCGCCCTCGAGCAAGCTGGTTT-CCAACCACAGTCTCAAGGTATG-3' and MS2-3': 5'-CATATGGTCGACCTGCAGTCTAGAGAATTCCTCGAGCAGCTTTATTAAACCTGTGTTTTTAATATAGAA-3'. The fragment encoding domain 254–685 of *SUP35* (MS2-Sup35p-ΔNM) was amplified using the following oligonucleotides: MS2-Sup35-Δ1–254: 5'-GCAGCAAACCTCCGGCATCGCTAGCGCCCTCGAGATGTTTGGTGGTAAAGATCACGTTTCTTTAATTT-3' and MS2-3'. The fragment encoding domain 1–253 of *SUP35* (MS2-Sup35p-ΔC) was amplified using the following oligonucleotides: MS2-5-Sup35-NM: 5'-ATCGCAGCAAACCTCCGGCATCTACGCTAGCGCCCTCGAGATGTCGGATTCAAA CCAAGGCAACAATCAGCAAACCTAC-3' and MS2-3-Sup35-NM: 5'-CTTCATATGGTCGACCTGCAGTCTAGAGAATTCCTCGAGTTAATCGTTAACAACCTCGTCATCCACTTCTTCTTCCTG-3'. PCR fragments were transformed into yeast cells with YCplac111-*MS2-PAB1* partially digested by XhoI and EcoRI to remove the *PAB1* coding sequence. After selection of LEU⁺ transformants, plasmids were rescued and sequenced. Positive clones were transformed into MBS strains containing the *mini-pgk1*-MS2 reporter and URA⁺, LEU⁺ transformants were selected for further analysis. Plasmid pG-FLAG-*UPF1* encoding epitope tagged *UPF1* was described previously [45]. Plasmid pRS316-*SUP35* (*URA3*) contains a yeast genomic XhoI-XbaI fragment harboring the coding region of *SUP35* under the control of its own promoter and terminator.

2.2.2 E. coli plasmids—Plasmid pET22-*PAB1*-HIS6, used to express yeast Pab1p with a (His)₆-tag at its C-terminus in *E. coli*, was constructed as described [48]. Plasmid pGEX-GST-*SUP35*, used to express and purify the fusion protein GST-Sup35p from *E. coli*, was constructed by PCR amplification of the *SUP35* ORF using yeast genomic DNA extracted from the MBS strain and oligonucleotides EcoRI-SUP35-5': 5'-CGGAATTCTCGGATTCAAACCAAGGC-3' and XhoI-SUP35-3': 5'-GGCTCGAGTTACTCGGCAATTTTAACAAT-3'. The resulting PCR fragment was digested by EcoRI-XhoI and introduced in EcoRI-XhoI sites of pGEX-6P (GE Healthcare Life Sciences).

2.3 RNA analysis

RNA isolation and northern blotting procedures were performed as described previously [49]. Hybridization probes were prepared by random priming of double stranded DNA as described in [23] and [8]. Transcript-specific signals were quantified with a Storm PhosphorImager and normalized to levels of *SCR1* RNA, a stable RNA polymerase III transcript.

2.4 Protein expression and purification

Flag-Upf1p protein was expressed in yeast from pG-FLAG-*UPF1* in strain BJ5457 and growing in selective media at 30 °C. Flag-Upf1p was purified as described [45] except that

yeast cell extract was directly loaded onto an anti-FLAG M2 affinity chromatography column (Sigma-Aldrich) without a prior DEAE Sephadex purification. Final fractions containing Upf1p were pooled and dialysed against 20 mM Tris-HCl, pH 8, 50 mM KCl, 0.1% Triton X-100, 1 mM DTT, 20% glycerol. For Pab1p-His₆ and GST-Sup35p in *E. coli*, BL21(λDE3) transformed with plasmids pET22-*PAB1*-HIS6 or pGEX-GST-*SUP35* was grown at 37 °C in LB with 100 µg/ml ampicillin to OD₆₀₀ = 0.3. IPTG (0.5 mM) was added and cells were grown for 6 h at room temperature for Pab1p-His₆ or 3 h at 37 °C for GST-Sup35p. Pab1p was extracted and purified on a nickel-agarose column as described by the manufacturer (QIAGEN). Final fractions containing Pab1p were pooled and dialysed against storage buffer (20 mM Tris-HCl, pH 8, 100 mM KAc, 2 mM MgAc, 10% glycerol). For preparation of Sepharose-GST-Sup35p fusion complexes, Glutathione Sepharose High Performance resin (GE Healthcare) was used and binding of GST-Sup35p to the resin was performed as described [19]. Briefly, we used 150 µl of a 50% slurry of resin that was washed and resuspended in binding buffer (25 mM Tris-HCl, pH 7.4, 50 mM KCl, 10 mM MgCl₂, 0.1% Triton X-100, 2% glycerol, 100 µg/ml BSA), and incubated 2 h at 4 °C with the extract corresponding to 500 ml of culture. The bound fraction was washed 4 times with the binding buffer and resuspended to a final volume of 150 µl. Quantification of GST-Sup35p bound to the Sepharose complex was estimated using the Bradford method. Yields averaged 1 pmole/µl of resuspended resin.

2.5 Binding reactions

All binding reactions were performed in 25 mM Tris-HCl, pH 7.4, 50 mM KCl, 10 mM MgCl₂, 0.1% Triton X-100, 2% glycerol, 100 µg/ml BSA, with incubation for 20 min at 20 °C. Sepharose-GST-Sup35p slurry corresponding to 25 nM of GST-Sup35p was incubated with different amounts of purified Pab1p-His₆ and/or FLAG-Upf1p in a final reaction volume of 200 µl. After incubation, the reaction was centrifuged 1 min at 4 °C in a benchtop centrifuge to pellet the Sepharose-GST-Sup35p and associated factors. The resin was washed three times with 200 µl of the binding buffer and ultimately resuspended in 25 µl of 2X protein loading buffer. For the preincubation competition experiments, a volume of Sepharose-GST-Sup35p complex corresponding to 5 pmoles (25 nM at the end of the reaction) of GST-Sup35p was first incubated with purified Pab1p-His₆ or FLAG-Upf1p in a final volume of 100 µl for 20 min at 4 °C. The other partner was added in a final volume of 200 µl of binding buffer and incubated 20 min at 4 °C. The bound fractions were analyzed by SDS-polyacrylamide gel electrophoresis (12%) and revealed by western blotting using anti-Pab1p antibodies (commercially prepared from pure proteins overproduced in *E. coli*), anti-Upf1p (commercially prepared from pure proteins purified from yeast), anti-eRF3 antibodies (a generous gift from Tricia Serio), and anti-GST antibodies (Sigma Aldrich) as primary antibodies and anti-Rabbit peroxidase conjugated (Sigma Aldrich) as secondary antibody. Western blot signals were revealed using Amersham ECL Plus™ Western Blotting Detection Reagents (GE Healthcare) and quantified with a Molecular Dynamics personal densitometer SI on multiple exposures.

3. Results

3.1 MS2 coat-Sup35p fusion protein complements the growth deficiency of a sup35Δ strain

Previously, we used fusions with the RNA-binding domain of bacteriophage MS2 coat protein to tether specific factors downstream of a PTC in yeast nonsense-containing mRNAs. These experiments showed that tethered MS2-Pab1p stabilizes a PTC-containing mRNA and recruits Sup35p and, reciprocally, that tethered MS2-Sup35p stabilizes an NMD substrate mRNA and recruits Pab1p [23]. *PAB1* is an essential gene and MS2-Pab1p complements a *pab1Δ* strain [47]. *SUP35* is also an essential gene and, as a prelude to analyzing mutants of tethered Sup35p, we wanted to ensure that MS2-Sup35p could provide

normal Sup35p function(s) *in vivo*. A plasmid carrying the wild-type *SUP35* gene and a *URA3* marker was introduced into yeast harboring a *sup35Δ* allele. This strain was then transformed with a second plasmid expressing the MS2-Sup35p fusion (Fig. 1A). As a negative control, we used a plasmid expressing MS2-Sup35p-ΔC in which the essential domain of Sup35p, encompassing amino acids 254 to 685, was deleted. Transformants were analyzed on medium containing 5-fluoro-orotic acid (5-FOA), which selects for loss of the *URA3* plasmid carrying wild-type *SUP35*. As expected, cells expressing the MS2-Sup35p-ΔC fusion protein did not support viability any better than the vector control. In contrast, cells expressing the full-length MS2-Sup35p fusion protein, or fusion proteins lacking up to 253 N-terminal amino acids of Sup35p, grew on 5-FOA (Fig. 1B). The ability of MS2-Sup35p to complement a *sup35Δ* strain indicates that this fusion protein retains the principal activities of wild-type Sup35p. Hence, this molecule comprises a suitable substrate for mutational analyses of its mRNA-stabilizing capabilities.

3.2 Mutations in the GTPase domain do not impede the stabilization of PTC-containing mRNA by tethered MS2-Sup35p

Tethering experiments suggest that artificially bringing factors normally present on a 3'-UTR into close proximity of a PTC can convert a translation termination event from one which is premature, or abnormal, to one that is normal [1, 23, 35, 50]. Such results led us to propose that efficient termination and mRNA stability are dependent on a properly configured 3'-UTR and that the latter was probably mediated in part by interactions between Pab1p and Sup35p [1, 23, 35]. Since Sup35p is a GTPase and GTP hydrolysis is essential for translation termination [18, 51–53] we decided to evaluate whether the GTPase activity of tethered Sup35p is required for mRNA stabilization. Accordingly, we analyzed two viable mutations, H348Q and R419G, located in the GTPase domain of Sup35p [18]. The proteins encoded by these mutations have normal GTP binding, decreased efficiencies of GTP hydrolysis, and increased levels of readthrough for some stop codons and contexts [18]. These two mutations were incorporated into tethered MS2-Sup35p fusion proteins (Fig. 1A) that were expressed in cells also harboring *mini-pgk1-MS2* mRNA, a transcript containing MS2-binding sites downstream of a PTC (see [23]). The stability of this reporter transcript was then assessed by northern blotting (Fig. 2), using MS2-Sxl tethered at the same position as a negative control. *SCR1* was used as a loading control, and the relative levels of the *CYH2* mRNA and pre-mRNA were used as assays for the activity of the NMD pathway [54]. The stability of the reporter transcript was also monitored in wild-type cells lacking MS2-fusion proteins and in cells lacking Upf1p. *Mini-pgk1-MS2* mRNA was stabilized up to 5-fold in *upf1Δ* cells (compared to wild-type cells; Fig. 2A,B), confirming that it is an NMD substrate. The validity of this conclusion is underscored by the observation that the *CYH2* pre-mRNA was also stabilized in the *upf1Δ* strain (Fig. 2A). As previously reported [23], tethering wild-type MS2-Sup35p led to a ~3.5-fold stabilization of the *mini-pgk1-MS2* mRNA (Fig. 2A,B). Tethering H348Q and R419G mutant forms of MS2-Sup35p respectively increased the stabilizing effect to ~6-fold or manifested stability comparable to that observed with wild-type MS2-Sup35p (Fig. 2A,B). GTP hydrolysis activity has been shown to be lower for Sup35p-H348Q than Sup35p-R419G and the H348Q mutation also leads to higher levels of nonsense codon readthrough than the R419G mutant [18]. Since the inhibition of GTP hydrolysis should promote accumulation of the GTP-bound form of Sup35p, and since the GTP status of Sup35p does not affect its interaction with Pab1p [55], the enhanced stabilizing effect of tethered MS2-Sup35p-H348Q is presumably not due to differential recruitment of Pab1p.

3.3 Large deletions do not reduce the ability of MS2-Sup35p to stabilize mini-pgk1-MS2 mRNA

Since tethering Pab1p or Sup35p leads to the stabilization of a PTC-containing mRNA it could be postulated that both proteins are required for mRNA stabilization and that tethering one of the two proteins is sufficient to recruit the other. Hence, we wanted to determine whether the stabilization of the *mini-pgk1-MS2* mRNA reporter by MS2-Sup35p was dependent on its ability to recruit Pab1p. A minimal interaction domain for Pab1p has been identified within residues 1–113 of Sup35p, but maximal interaction between the two factors requires the entire Sup35p NM domain (residues 1–253) [56, 57]. To evaluate the importance of these regions and the C-terminus of the molecule in mRNA stabilization, three MS2-Sup35p constructs (see Fig. 1A) were tested for their ability to stabilize the *mini-pgk1-MS2* nonsense-containing reporter mRNA: the first lacked the N-terminal domain (Δ N, deletion of residues 1–113), the second lacked domains N and M (Δ NM, deletion of residues 1–253), and the third was deleted for the entire C-terminal domain (Δ C, deletion of residues 254–685). Coimmunoprecipitation assays demonstrated that MS2-Sup35p- Δ N and MS2-Sup35p- Δ NM respectively had diminished and barely above background abilities to interact with Pab1p (Supplementary data, Fig. S1). The results of the tethering experiments (Fig. 3A,B) showed that deletion of the N and NM domains of Sup35p still allowed for stabilization of the *mini-pgk1-MS2* mRNA to an extent comparable to that observed with full length MS2-Sup35p (FL). Moreover, MS2-Sup35p- Δ C led to essentially the same level of mRNA stabilization. These data preclude assignment of the stabilization effect to a specific domain of Sup35p, or simply to an interaction with Pab1p. Since we previously reported that tethering Pab1p lacking its C-terminal Sup35p-interaction domain leads to partial stabilization of *mini-pgk1-MS2* mRNA [23] it appears likely that the stabilizing effects of tethered Pab1p or Sup35p are not simply a consequence of mutual recruitment.

One additional possibility that warranted attention was the PSI state of the cells that were subjected to analysis of mRNA levels. Sup35p demonstrates prion properties and results in the [PSI⁺] phenotype when aggregated. The [PSI⁺] state causes readthrough at stop codons and can be induced by overexpression of either full-length Sup35p or just its NM domain [58]. To rule out the possibility that stabilization of *mini-pgk1-MS2* mRNA by full-length MS2-Sup35p or its truncated forms is indirect and due to [PSI⁺]-mediated stop codon readthrough, we tested nonsense suppression in strains expressing either form of MS2-Sup35p, using the *can1-100* allele as a reporter [8] (Supplementary data, Fig. S2). Although the assay manifests the expected nonsense suppression [59] upon deletion of *UPF1* or *UPF2*, the results of this experiment demonstrate that overexpression of either full-length or truncated MS2-Sup35p does not induce nonsense suppression.

3.4 Binding of Pab1p to Sup35p saturates at five-fold lower concentration than binding of Upf1p to Sup35p

Tethering experiments with Pab1p and Sup35p have led to a model of NMD which postulates that the binding of Upf1p and Pab1p to Sup35p may be mutually exclusive [1, 2, 23]. We tested this hypothesis *in vitro*, using purified recombinant proteins, to determine whether Pab1p and Upf1p could compete for binding to Sup35p. As a first step, we evaluated the individual binding activities of Upf1p or Pab1p to Sup35p (Fig. 4A,B). A fixed amount of GST-tagged Sup35p corresponding to a final concentration of 25 nM, purified from *E. coli*, was incubated with increasing amounts of FLAG-tagged Upf1p purified from yeast or His₆-tagged Pab1p purified from *E. coli*. After several washes, the relative amounts of FLAG-Upf1p or Pab1p-His₆ bound to GST-Sup35p were analyzed by western blotting and quantified using densitometry and multiple exposures. Control experiments showed that: a) FLAG-Upf1p and Pab1p-His₆ do not bind GST alone (Supplementary data, Fig. S3A), b) as expected, purified Sup45p binds GST-Sup35p

(Supplementary data, Fig. S3B), and c) FLAG-Upf1p and Pab1p-His₆ both showed similar binding properties at 4 °C and 20 °C (Supplementary data, Fig. S4). The latter results led us to perform all experiments at 20 °C with an incubation time of 20 min. To normalize the different binding curves, we defined 100% binding as the fraction bound when 200 pmoles (corresponding to a final concentration of 1 μM) of Pab1p or Upf1p were incubated with GST-Sup35p. Using these approaches, the binding of Pab1p-His₆ and FLAG-Upf1p to GST-Sup35p differed significantly (Fig. 4B and S4). For FLAG-Upf1p, 50% of maximal binding was reached at approximately 125 nM whereas comparable binding of Pab1p-His₆ was reached at approximately 25 nM. Although these results suggest that Pab1p-His₆ binds GST-Sup35p with a higher affinity than FLAG-Upf1p, the experimental system was not suitable for determination of the respective K_d values. However, the lower relative efficiency of FLAG-Upf1p/GST-Sup35p binding was also observed in analyses using surface plasmon resonance to monitor interaction (Supplementary Data, Fig. S5) and has also been reported for the homologous human factors [26]. These data imply that if these factors were to compete directly for binding to Sup35p, Pab1p would efficiently outcompete Upf1p unless the latter was present at much higher concentrations. The available data suggest that just the opposite is true in yeast cytoplasm, i.e., Pab1p may be as much as 100 times more abundant than Upf1p (there are approximately 198000 ± 48500 molecules of Pab1p (<http://yeastgfp.yeastgenome.org/>) and 1,600 molecules of Upf1p [8] per cell).

3.5 Sequential interaction of Pab1p and Upf1p with Sup35p provides only limited support for the direct competition hypothesis

To test whether the binding of Pab1p to Sup35p could affect the binding of Upf1p to Sup35p, or vice versa, a fixed amount of GST-Sup35p (25 nM) was incubated with a saturating amount of one binding partner (Pab1p-His₆ to a final concentration of 375 nM; FLAG-Upf1p to a final concentration of 500 nM) and binding was assessed in the presence of increasing amounts of the other factor. Binding was quantified and normalized as in Fig. 4. The data in Fig. 5A demonstrate that the binding of FLAG-Upf1p to GST-Sup35p is not affected by the preincubation of GST-Sup35p with excess Pab1p-His₆. In either the presence or absence of Pab1p-His₆, 50% of binding was reached at approximately 70 nM Upf1p (Fig. 5A). The difference between this value and the 125 nM value for 50% binding in the experiments of Fig. 4 may reflect the fact that the experiments of Fig. 5A include a 20 minute preincubation of GST-Sup35p with or without Pab1p-His₆ prior to the addition of FLAG-Upf1p. Further evidence that the binding of Pab1p to Sup35p does not exclude subsequent Upf1p-Sup35p interaction is the lack of displacement of Pab1p-His₆ when FLAG-Upf1p interacts with GST-Sup35p (Fig. 5A, lanes 1 to 6). These experiments indicate that the binding of Pab1p-His₆ and FLAG-Upf1p to Sup35p is not mutually exclusive and thus may take place on independent binding sites.

Although preincubation of Pab1p-His₆ with GST-Sup35p did not affect the subsequent binding of FLAG-Upf1p to GST-Sup35p, the binding of Pab1p-His₆ to GST-Sup35p was affected by the initial presence of FLAG-Upf1p. Whereas Pab1p-His₆ alone bound GST-Sup35p with high efficiency, reaching 50% binding at 25 nM (Fig. 4B), preincubation of GST-Sup35p with a saturating concentration of FLAG-Upf1p led to a reduction in the binding efficiency of Pab1p-His₆ (Fig. 5B). The presence of FLAG-Upf1p inhibited binding by low amounts of Pab1p-His₆ and displaced the concentration required for 50% of binding to approximately 280 nM Pab1p-His₆ (Fig. 5B). In spite of this displacement high concentrations of Pab1p-His₆ (> 500 nM) bound to Sup35p to equivalent extents in the presence or absence of FLAG-Upf1p (compare Fig. 4 and 5B).

The data of Fig. 5 provide only partial support for the notion of simple direct competition between Pab1p and Upf1p for Sup35p binding. Although the experiments of Fig. 5A imply that there are independent FLAG-Upf1p and Pab1p-His₆ binding sites on GST-Sup35p, the

observation that pre-incubation with Upf1p inhibits Pab1p-Sup35p interaction (Fig. 5B) indicates that the two binding events are not entirely independent. The latter possibility was further supported by experiments testing simultaneous binding of Pab1p and Upf1p to Sup35p (see below).

3.6 Assays monitoring simultaneous interaction are more consistent with competition between Pab1p and Upf1p for binding to Sup35p

As an alternative approach to understanding the binding relationships between Pab1p, Upf1p, and Sup35p, we evaluated the abilities of different mixtures of FLAG-Upf1p and Pab1p-His₆ to interact with a fixed amount of GST-Sup35p (Fig. 6A,B). In binding reactions that included all three proteins at the outset the presence of FLAG-Upf1p inhibited the binding of Pab1p-His₆ to GST-Sup35p, a result consistent with that shown in Fig. 5B. More specifically, with Pab1p-His₆ at 12.5 nM, the fraction of that protein bound to GST-Sup35p decreased markedly as the amount of FLAG-Upf1p increased (compare the Pab1p signal in Fig. 6A, lanes 1, 4, 7, and 10, or in Fig. 6B, lanes 4, 5, and 6). A similar albeit less pronounced phenomenon was observed when initial Pab1p-His₆ concentrations were 10- (125 nM) or 40-fold (500 nM) higher (see Fig. 6A, lanes 2, 5, 8, and 11, as well as lanes 3, 6, 9, 12; also Fig. 6B, lanes 7, 8, 9). Fig. 6A also shows that, at any concentration of Pab1p-His₆, increasing the amount of FLAG-Upf1p leads to higher Upf1p binding to GST-Sup35p (compare lanes 4, 7, and 10, or 5, 8, and 11, or 6, 9, and 12). Notably, the extent of the increase in FLAG-Upf1p binding is lower at 500 nM Pab1p-His₆ than at 125 nM Pab1p-His₆ (compare Upf1p bands in Fig. 6A lanes 5, 8, and 11 to lanes 6, 9, and 12).

While FLAG-Upf1p has the ability to compete effectively with Pab1p-His₆, this competition can be overcome by higher levels of Pab1p-His₆ (e.g., see Pab1p bands in Fig. 6A, lanes 4, 5, 6, or 7, 8, 9, or 10, 11, 12), an observation that could be interpreted as simple competition. However, at low amounts of FLAG-Upf1p (12.5 nM) a 40-fold increase in the level of Pab1p-His₆ does not affect the interaction between FLAG-Upf1p and GST-Sup35p (Fig. 6A, compare FLAG-Upf1p signal in lanes 4 to 6). At higher amounts of FLAG-Upf1p (125 nM and 500 nM), the highest level of Pab1p-His₆ (500 nM) reduces the interaction between FLAG-Upf1p and GST-Sup35p (Fig. 6A, compare Upf1p signals in lanes 7, 8, and 9, as well as 10, 11 and 12; also, in Fig. 6B, compare Upf1p signal in lanes 6, 9, and 12).

Collectively, the data of Fig. 5 and 6 indicate that, under certain circumstances, Pab1p and Upf1p can compete for binding to Sup35p. However, instead of exhibiting binding properties characteristic of overlapping binding sites and mutually exclusive binding, Pab1p and Upf1p competition for Sup35p appears to be more complex and may reflect the consequences of indirect competition caused by binding-induced protein conformational changes. These possibilities are discussed below.

4. Discussion

Premature translation termination generally promotes NMD whereas normal termination does not. Since NMD depends on recruitment of the Upf proteins the mechanism by which normal termination excludes such recruitment is of considerable interest [2, 5]. Here we have evaluated a model suggesting that Pab1p and Upf1p compete for interaction with eRF3 (Sup35p in yeast), with normal termination depending on the ability of 3'-UTR-localized Pab1p to interact with the release factor, Sup35p [1, 23, 35, 50]. As envisaged by this “*faux-UTR*” model, the lack of proximal Pab1p at a PTC allows for Upf1p-Sup35p interaction, thus promoting NMD. Two predictions of this model are that: a) Pab1p:Sup35p interaction should be critical for normal termination and mRNA stability and b) Pab1p should effectively compete with Upf1p for binding to Sup35p. We took three general approaches to addressing these predictions. In the first we tested whether specific domains or activities of

tethered Sup35p were required for the stabilization of a nonsense-containing mRNA. In the second, we purified Upf1p, Pab1p, and Sup35p and utilized these purified factors to analyze the relative affinity of Upf1p or Pab1p for Sup35p. Finally, we used the three factors to test directly for competitive interactions.

In the tethering experiments we found that MS2-Sup35p deleted for its interaction domains with Pab1p (Sup35p- Δ N and Sup35p- Δ NM) could still stabilize a nonsense-containing mRNA when tethered downstream of a PTC (Fig. 3). Previously we showed that tethering Pab1p deleted for its interaction domain with Sup35p could still stabilize a nonsense-containing mRNA, albeit to a lower extent than full-length Pab1p [23]. Therefore, the stabilization of a PTC-containing mRNA by tethered Pab1p or Sup35p is unlikely to be due solely to interaction between these two factors. One possibility is that tethered Sup35p- Δ N and Sup35p- Δ NM might inhibit NMD by promoting efficient translation termination at the PTC, possibly by recruiting Sup45p (eRF1). However, this, too, may be an oversimplification since Sup45p tethered in place of Sup35p or Pab1p does not stabilize *mini-pgk1-MS2* mRNA [23]. An alternative possibility is that the GTPase activity of the residual C-terminal domain of Sup35p somehow plays a role in mRNA stabilization. This conclusion is consistent with the results of the tethering experiments that utilized Sup35p GTPase mutants (Fig. 2). Two mutants were tested, and the one with the larger defect in GTP hydrolysis (H348Q) was more efficient at nonsense mRNA stabilization than wild-type Sup35p (Fig. 2). Since the GTPase activity of Sup35p is required for efficient stop codon recognition by Sup45p [18, 60], and NMD is dependent on translation termination at a PTC [4, 19], tethered MS2-Sup35p-H348Q might enhance the stabilization of a PTC-containing mRNA by interfering with the termination process. Indeed, another mutation that reduces Sup35p's affinity for GTP has been shown to inhibit the NMD pathway [55]. Collectively, the tethering experiments of Fig. 1–3 indicate that multiple and redundant functions of tethered Pab1p and Sup35p are implicated in stabilizing an otherwise unstable mRNA. MS2-Sup35p could stabilize a nonsense reporter mRNA by recruiting Pab1p via its NM domain, or by altering the efficiency of translation termination with its C-terminal domain.

Implicit in the conclusions of the tethering experiments is that NMD activation does not rely solely on Pab1p and Upf1p competition for interaction with Sup35p. Nevertheless, the role of mutually exclusive binding of Upf1p or Pab1p to Sup35p remains an important open question and we, therefore, used recombinant proteins and *in vitro* binding assays to address it. When tested separately for Sup35p interaction, FLAG-Upf1p and Pab1p-His₆ bind differentially to Sup35p, with Pab1p associating much more efficiently with Sup35p than Upf1p (Fig. 4 and Supplementary Data, Fig. S4). Based solely on these results, and the relative cytoplasmic abundance of Upf1p and Pab1p (<http://yeastgfp.yeastgenome.org/>; [8]), it would be expected that the higher affinity and considerably higher cellular levels of Pab1p would allow it to outcompete Upf1p, provided that: a) both proteins were freely available, b) the Sup35p binding site for both proteins was the same, and c) the binding of one of the two factors to Sup35p did not alter the Sup35p binding site for the other. However, point *a* is unlikely, given the well documented association of Pab1p with the mRNA poly(A) tail [61], and the results of the competitive binding assays of Fig. 5 and 6 suggest that points *b* and *c* also cannot be correct.

Direct competition was first addressed by assessing the consequences for subsequent binding when Sup35p was preincubated with excess Pab1p or Upf1p (Fig. 5). These experiments showed that Upf1p binding to Sup35p was not affected by prior Pab1p binding and that the association of pre-bound Pab1p to Sup35p was not significantly diminished by increased Upf1p binding. These results ruled out the possibility of identical binding sites for the two proteins on Sup35p and the possibility that binding of Pab1p to Sup35p (e.g., at a normal terminator) could simply exclude Upf1p binding to Sup35p. When the order of

factor addition was reversed the preincubation of Sup35p with excess Upf1p was found to inhibit subsequent binding of Pab1p, but any Pab1p binding did not appear to diminish the levels of Upf1p binding (Fig. 5). One explanation for the inhibition of Pab1p:Sup35p binding by Upf1p could be that Upf1p has two binding sites on Sup35p, one with high affinity and independent of Pab1p, and another with low affinity that is shared by Pab1p. However, if this were true then the maximum amount of Upf1p binding to Sup35p should be different in the presence or absence of Pab1p. Such a difference was not observed, even with the raw data before normalization (data not shown). Another possibility could be that the binding of Upf1p induces a Sup35p conformational change that inhibits the subsequent binding of Pab1p.

To gain insight into this complex relationship, Upf1p and Pab1p were added simultaneously to Sup35p and binding was monitored at different Upf1p:Pab1p ratios (Fig. 6). These experiments showed that the simultaneous presence of Upf1p was sufficient to inhibit the binding of Pab1p to Sup35p, i.e., that prior preincubation of Upf1p and Sup35p was not required for the manifestation of competition. Further, unlike the preincubation assay of Fig. 5, the experiments of Fig. 6 demonstrated that competition could be mutual, i.e., at certain concentrations, Upf1p and Pab1p could each inhibit the binding of the other protein to Sup35p. For example, although the inhibition of Pab1p binding by Upf1p is evident in both Fig. 5 and 6, it is also clear that the binding of Upf1p to Sup35p can be affected by high concentrations of Pab1p (125 nM and 500 nM) but not by low concentrations (12.5 nM) (see Fig. 6B). Possibly, the influence of Pab1p on Upf1p binding is less evident in Fig. 5 because the experiments were not performed at equilibrium. Of particular interest in Fig. 6 are the binding data from incubations where the ratios of Pab1p to Upf1p approach the disproportionate levels seen *in vivo*. Thus, in Fig. 6A, lane 6, and in Fig. 6B, lane 10, Upf1p still binds relatively well to Sup35p in spite of a 40-fold excess of Pab1p.

Collectively, the data of Fig. 5 and 6 indicate that Pab1p and Upf1p can compete with each other for binding to Sup35p, but this competition is more complex than anticipated. Pab1p and Upf1p may share at least part of a common interaction site on Sup35p or the binding of Upf1p or Pab1p to Sup35p might induce a conformational change that inhibits the subsequent interaction. Unfortunately, the localization and stoichiometry of Upf1p binding to Sup35p is still unknown so we cannot rule out the possibility of multiple binding sites. In yeast, Upf1p:Sup35p interaction has been mapped by GST-pull down experiments. One study assigned interaction to the GTPase domain of Sup35p (residues 254–465) [20], whereas another found that deletion of the Sup35p NM domain (residues 1–253) or deletion of domain C (residues 254–685) both resulted in factors that failed to interact with Upf1p [55]. In human cells, mutations in the N-terminal domain of heRF3 that abolish interaction with PABP1C also decrease interaction with hUpf1, suggesting that hUPF1 and PABPC1 interact with a similar, although not necessarily overlapping region of heRF3 [26]. Additionally, Ivanov et al. have reported that hUpf1 binds to the GTPase domain of heRF3 [22].

An alternative possibility is that the Upf proteins are selectively recruited to a PTC by virtue of the aberrant nature of premature termination. Ribosomes appear to pause at a PTC [23], and it has been suggested that this may reflect a defect in ribosome release or recycling after peptide release [23, 36]. Upf1p may thus be recruited to a ribosome having a stop codon in its A site, but lacking some other component characteristic of normal termination, e.g., a nascent peptide.

In conclusion, the poor ability of Pab1p to out-compete Upf1p that is bound to Sup35p suggests that, *in vivo*, whatever recruitment event allows association of Upf1p with a prematurely terminating ribosome represents a high efficiency commitment to targeting an

mRNA for decay by the NMD pathway. The data do not, however, explain why the binding of Pab1p to Sup35p might inhibit the activation of NMD. Obviously, in addition to a better definition of the nature of the respective binding sites additional parameters must be taken into account. These include the role of ATP (Upf1p is an ATP-binding protein), GTP, the influence of RNA and other factors implicated in translation termination and NMD (especially Upf2p/Nmd2p and Upf3p) and the mechanistic differences imposed when the interactions in question are taking place on the ribosome in the course of protein synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

The Pab1p interaction domain is not necessary for stabilization of an NMD substrate by tethered eRF3/Sup35p

Mutations in the GTPase domain do not impede the stabilization of PTC-containing mRNA by tethered eRF3/Sup35p

Pab1p binds eRF3/Sup35p with a higher affinity than does Upf1p

Upf1p inhibits Pab1p binding to eRF3/Sup35p whereas the interaction between Upf1p and eRF3/Sup35p is relatively unaffected by Pab1p

The determinants of preferential eRF3/Sup35p binding to Upf1p or Pab1p are more complex than direct competition

Fig. 1.

MS2-Sup35p fusion proteins maintain cell viability. **A.** MS2 fusion constructs. DNA encoding the MS2 coat protein was fused, as indicated, to that encoding full-length *SUP35* (WT), to DNA encoding *sup35* mutations H348Q or R419G, or to *SUP35* DNA lacking the N (1–113), NM (1–253), or C (254–685) domains. Interaction domains with Pab1p and Sup45p, and the positions of mutations H348Q and R419G are indicated. Boxes with stripes represent the GTPase domains. **B.** Growth of cells expressing MS2-SUP35 tethering constructs. MS2-SUP35 fusion constructs were introduced into a *sup35Δ* strain containing *SUP35* on a *URA3* plasmid to maintain cell viability. Transformants were spotted in steps of 10-fold dilution onto either YPD or YPD containing 0.2% FOA. The presence of FOA counterselects the *SUP35 URA3* plasmid and cell growth indicates MS2-Sup35p fusion proteins able to maintain cell viability.

Fig. 2.

Tethered GTPase mutants of Sup35p still stabilize *mini-pgk1-MS2* mRNA. **A.** Total RNA isolated from wild-type (WT) or *upf1Δ* cells expressing *mini-pgk1-MS2* mRNA was analyzed by northern blotting with DNA probes detecting *mini-pgk1-MS2*, *SCR1*, and *CYH2* transcripts. Yeast strains used for the WT and *upf1Δ* contained no fusion protein, whereas the WT cells of the remaining lanes contained the indicated MS2-Sup35p fusion proteins. **B.** Quantification of the northern blotting signal. The fold-stabilization, relative to the WT sample, was determined by radioactivity measurements using a phosphorImager. Error bars indicate the standard error to the mean (s.e.m.)

Fig. 3.

Tethering of different Sup35p domains still stabilizes *mini-pgk1-MS2* mRNA. **A.** Total RNA isolated from wild-type (WT) or *upf1Δ* cells expressing *mini-pgk1-MS2* mRNA was analyzed by northern blotting with DNA probes detecting *mini-pgk1-MS2*, *SCR1*, and *CYH2* transcripts. Yeast strains used for the WT and *upf1Δ* contained no fusion protein, whereas the WT cells of the remaining lanes contained the indicated MS2-Sup35p fusion proteins. **B.** Quantification of the northern blotting signal. The fold-stabilization, relative to the WT sample, was determined by radioactivity measurements using a phosphorImager. Error bars indicate the standard error to the mean (s.e.m.)

Fig. 4. Individual binding of Pab1p-His₆ and FLAG-Upf1p to GST-Sup35p. **A.** Binding assays. Increasing amounts (quantity indicated) of purified Pab1p-His₆ (right panel) or purified FLAG-Upf1p (left panel) were incubated with GST-Sup35p (25 nM) bound to glutathione resin. After washes, the bound sample was eluted in loading buffer, separated on SDS-PAGE 12%, and analyzed by western blotting using anti-Upf1p, anti-Pab1p, and anti-GST antibodies to reveal GST-Sup35p. **B.** Quantification of different binding experiments using multiple exposures and densitometry. The intensity level of the bound fraction for 1 μ M was set at 100% to compare different sets of experiments. Error bars indicate the standard error to the mean (s.e.m.)

Fig. 5. Preincubation test for competition between Pab1p-His₆ and FLAG-Upf1p for binding to GST-Sup35p. **A.** Preincubation with Pab1p-His₆. GST-Sup35p (25nM) was preincubated for 20 min at 20 °C with Pab1p-His₆ (375 nM) (left panel) or no Pab1p (right panel), then incubated with increasing amounts of FLAG-Upf1p. The bound fractions were analyzed by SDS-PAGE electrophoresis followed by western blotting with specific antibodies. Lower panel: quantification of the western blot signal. **B.** Preincubation with FLAG-Upf1p. The same experiment as in **A**, except that GST-Sup35p was preincubated with FLAG-Upf1p (500 nM) and increasing amounts of Pab1-His₆ were then added. Error bars indicate the standard error to the mean (s.e.m.)

Fig. 6.

Simultaneous incubation of Pab1p-His₆ and FLAG-Upf1p with GST-Sup35. GST-Sup35p (25 nM) was incubated with different amounts of Pab1p-His₆ and FLAG-UPF1p. The bound fractions were analyzed by SDS-PAGE electrophoresis followed by western blotting with specific antibodies. **A.** Binding of increasing amounts of Pab1p-His₆ in the absence of FLAG-Upf1p (left) or with increasing amounts of FLAG-Upf1p, up to 500 nM (from left to right). **B** Binding of increasing amounts of FLAG-Upf1p in the absence Pab1p-His₆ (left) or increasing amounts of Pab1p-His₆, up to 500 nM (from left to right).