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Upf1p, a Highly Conserved Protein Required for Nonsense-Mediated mRNA Decay, Interacts with the Nuclear Pore Proteins Nup100p and Nup116p

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Abstract

Saccharomyces cerevisiae Upf1p is a 971-amino-acid protein that is required for the nonsense-mediated mRNA decay (NMD) pathway, a pathway that degrades mRNAs with premature translational termination codons. We have identified a two-hybrid interaction between Upf1p and the nuclear pore (Nup) proteins, Nup100p and Nup116p. Both nucleoporins predominantly localize to the cytoplasmic side of the nuclear pore and participate in mRNA transport. The two-hybrid interaction between Upf1p and the nuclear pore proteins, Nup100p and Nup116p, is dependent on the presence of the C-terminal 158 amino acids of Upf1p. Nup100p and Nup116p can be coimmunoprecipitated from whole-cell extracts with Upf1p, confirming in vitro the interaction identified by the two-hybrid analysis. Finally, we see a genetic interaction between *UPF1* and *NUP100*. The growth of *upf1Δ*, *can1-100* cells is inhibited by canavanine. The deletion of *NUP100* allows *upf1Δ*, *can1-100* cells to grow in the presence of canavanine. Physiologically, the interaction between Upf1p and the nuclear pore proteins, Nup100p and Nup116p, is significant because it suggests a mechanism to ensure that Upf1p

associates with newly synthesized mRNA as it is transported from the nucleus to the cytoplasm prior to the pioneer round of translation.

Keywords: mRNA surveillance, mRNA transport

Abbreviations: NMD, nonsense-mediated mRNA decay; Upf, up frameshift; Nup, nuclear pore; eRF, eukaryotic release factor; DSE, downstream sequence element; PCR, polymerase chain reaction; YEPD/YPD, yeast extract, peptone, and dextrose medium; YAPD, YPD supplemented with adenine; 3-AT, 3-aminotriazole; EDTA, ethylenediaminetetraacetic acid; NaCl, sodium chloride; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HA, influenza hemagglutininin epitope; GLFG, motif consisting of repeats of glycine, leucine, phenylalanine, and glycine interspersed with glutamine-, asparagine-, serine-, and threonine-rich spacers; DNA, deoxyribonucleic acid; tRNA, transfer ribonucleic acid; rRNA, ribosomal ribonucleic acid; NRM, nucleoporin RNA-binding motif; GLEBS, Gle2p-binding sequence; $GAL4_{BD}$, Gal4 binding domain; $GAL4_{AD}$, Gal4 activation domain; SDSPAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

1. Introduction

mRNAs containing premature translation termination codons (nonsense mRNAs) are degraded faster than their wild-type counterparts by nonsense-mediated mRNA decay (NMD; reviewed in Culbertson and Leeds, 2003). Nonsense mRNAs can arise by the transcription of genes with nonsense or frame-shift mutations and from errors in processing or transcription. The rapid decay of nonsense mRNA prevents the synthesis of potentially harmful truncated peptides. Thus, NMD ensures that only properly processed mRNAs encoding functional polypeptides persist.

NMD has been intensively studied in the yeast *Saccharomyces cerevisiae*, *Caenorabditis elegans*, and vertebrates (reviewed in Culbertson and Leeds, 2003). Yeast has three NMD-specific proteins: Upf1p, Upf2p, and Upf3p (Leeds et al., 1992; Cui et al., 1995; Lee and Culbertson, 1995). Sixteen UPF1, 11 UPF2, and 7 UPF3 orthologues have been found in a diverse array of eukaryotes (Culbertson and Leeds, 2003). These proteins act in a single pathway and, more recently, are proposed to be part of a dynamic surveillance complex that acts as a sensor for nonsense mRNAs (Atkin et al., 1997; Lykke-Andersen et al., 2001).

Upf1p is critical for NMD. It aids in the termination of translation at premature translation termination codon, by interacting with the eukaryotic translation release factors eRF1 and eRF3 (Czaplinski et al., 1998). Then, it triggers decapping by interacting with the decapping complex (Dunckley and Parker, 1999). Decapped mRNAs are then rapidly degraded by Xrn1p, a 5′→3′ exoribonuclease.

In the current view of the NMD pathway, premature termination codons are recognized through cooperative interactions between the Upf proteins and a protein complex, which is deposited either immediately upstream of exon-exon junctions on mammalian mRNAs or on the downstream sequence element (DSE) of yeast mRNAs (Gonzalez et al., 2000; Le Hir et al., 2001). First, Upf3p and, then, Upf2p join the complex (Lykke-Andersen et al., 2001). It has been proposed that Upf1p is recruited to the complex when translation terminates upstream of the complex, triggering NMD thorough interactions with Upf2p and the

translation release factors (Czaplinski et al., 1998; Le Hir et al., 2001). On the other hand, if translation terminates downstream of the mark, Upf1p is not recruited to the complex, and the mRNA escapes NMD.

mRNA degradation via NMD requires at least one round of translation, and it might even occur during the pioneer round of translation (Ishigaki et al., 2001). CBP80 and CBP20 form a complex that binds to the mRNA cap while it is still in the nucleus. The complex travels with the mRNA to the cytoplasm. CBP80/CBP20-bound mRNA is then translated at least once before eIF-4E replaces the CBP80/CBP20 complex. eIF-4E functions in subsequent translation initiation. Three lines of evidence suggest that NMD takes place on CBP80-bound mRNA: (1) CBP80- and eIF4E-bound nonsense mRNAs are similarly reduced in abundance (Ishigaki et al., 2001). (2) Consistent with the dependence of NMD on translation, a cognate suppressor transfer ribonucleic acid (tRNA) or inhibition of translation by cycloheximide stabilized the CBP-bound nonsense mRNA (Ishigaki et al., 2001). (3) Finally, hUpf2p, hUpf3p, and the components of the exon-exon junction complex copurify with CBP80-bound but not eIF-4E-bound mRNA (Ishigaki et al., 2001).

In this study, we observe an interaction between yeast Upf1p and two components of the nuclear pore (Nup) complex, Nup100p and Nup116p. Both of these nucleoporins predominantly localize to the cytoplasmic side of the nuclear pore and participate in mRNA transport (reviewed in Suntharalingam and Wente, 2003). The observation of an interaction between Upf1p and these nuclear pore proteins suggests that Upf1p may associate with newly synthesized mRNA as it is transported from the nucleus to the cytoplasm prior to the pioneer round of translation and, thus, earlier than previously proposed.

2. Materials and methods

2.1. Yeast strains, genetic methods, and culture conditions

The *S. cerevisiae* strains are described in Table 1. The yeast strains were constructed, grown, and maintained by standard procedures (Ausubel et al., 1998). SSX-leu-his plates were prepared as in Chien et al. (1991). Yeast were transformed using the lithium acetate method (Ausubel et al., 1998).

2.2. Bacterial strains and plasmid DNA construction

Escherichia coli DH5α (Life Technologies, Rockville, Maryland) was used for the preparation of plasmid deoxyribonucleic acid (DNA). The methods used for the growth, maintenance, and transformation of *E. coli* are in Ausubel et al. (1998). Plasmid DNAs were prepared from *E. coli* using a QIAprep spin plasmid miniprep kit (Qiagen, Chatsworth, California). New plasmids were constructed using standard methods (Ausubel et al., 1998). The plasmid DNAs are listed in Table 1.

pAA158 (Table 1) was used to screen yeast libraries for proteins that interact with Upf1p. A DNA fragment coding for the region of Upf1p with sequence similarity to Sen1p was amplified by polymerase chain reaction (PCR) using oligonucleotides oAA16 (5′-dCGA TGC GTC CGG CGT AGA GGA) and oAA18 (5′-dGGC GAA TTC TCT GCT TCT GAT ACA AAA CG). oAA18 was designed to create an *Eco*RI site (underlined) at nucleotides +1569 to +1574 within the *UPF1* ORF. The DNA fragment was amplified from pPL46

(Leeds et al., 1992) using *Taq* DNA polymerase (Cetus, Emeryville, California). pPL46 contains a 4.0-kb *Eco*RI-*Bam*HI fragment of DNA carrying the *UPF1* gene cloned into the *Eco*R1-*Bam*HI sites of YIp5. The PCR product was digested with *Eco*RI and *Bam*HI, cloned into the *Eco*R1-*Bam*HI sites of pUC19 and sequenced using the Sequenase 2.0 kit (United States Biochemical, Cleveland, Ohio). An *Eco*RI-*Bam*HI fragment containing wild-type *UPF1* sequence was subcloned into the *Eco*R-*Bam*HI sites of pMA424 (Chien et al., 1991), creating pAA158. pAA158 contains an in-frame fusion of the *GAL4* DNA binding domain and the 3′ half of *UPF1* under the control of the constitutive *ADH1* promoter. On its own, this plasmid did not activate the expression of the *lacZ* reporter gene in GGY1::171 (data not shown).

Table 1. The yeast strains and plasmids used in this study

Yeast strain	Genotype	Source
GGY1::171	*gal4Δ gal80Δ GAL1::LacZ his3 leu2*	Chien et al., 1991
PJ69-4α	*MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 MET2::GAL7-LacZ*	Phil James
PJ69-4a	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 MET2::GAL7-LacZ*	James et al., 1996
AAY320	*MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 upf1-Δ2 (URA3)*	Kebaara et al., 2003
AAY327	*MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 upf1-Δ6 (TRP1)*	This study
SWY67	*MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 nup100::URA3*	Susan Wente
SWY31	*MATα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 nup116::URA3*	Wente et al., 1992
W303a	*MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*	Wente and Blobel, 1993
W303α	*MATα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100*	Wente and Blobel, 1993
SWY3	*MATα ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 nup100::URA3*	Wente et al., 1992
AAY273	*MATa ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 upf1-Δ5(upf1::LEU2)*	This study
PLY146	*MATα ura3-52 trp1-1α tyr7-1 leu2-2 upf1-Δ2 (URA3)*	Leeds et al., 1992
PLY38	*MATa upf1-2 his4-38 SUF1-1 ura3-52*	Atkin et al., 1995

Table 1 continued next page

Plasmid	Relevant markers	Source
pAA158	*GAL4*$_{BD}$*UPF1*$_{525–971}$, *HIS3*, 2μ, *Amp*^{R}	This study
pEE5 (GAL4-(1-147)-SNF1)	*GAL4*$_{BD}$*SNF1*, *HIS3*, 2μ, *Amp*^{R}	Chien et al., 1991
pAA303	*GAL4*$_{BD}$*UPF1*$_{525–971}$, *URA3*, 2μ, *Amp*^{R}	This study
pAA265	*GAL4*$_{AD}$*NUP100*, *LEU2*, 2μ, *Amp*^{R}	This study
pAA378	*GAL4*$_{AD}$*NUP116*, *LEU2*, 2μ, *Amp*^{R}	This study
pAA296	*GAL4*$_{BD}$*SNF1*, *URA3*, 2μ, *Amp*^{R}	This study
pGAD-SIP1	*GAL4*$_{AD}$*SIP1*, *LEU2*, 2μ, *Amp*^{R}	Phil James
pGBDU-C1	*GAL4*$_{BD}$, *URA3*, 2μ, *Amp*^{R}	James et al., 1996
pGAD-C1	*GAL4*$_{AD}$, *LEU2*, 2μ, *Amp*^{R}	James et al., 1996
pRS316UPF1-3EP	*URA3 CEN6 ARSH4 UPF1-3EP Amp*^{R}	Atkin et al., 1997
pRS316	*URA3 CEN6 ARSH4 Amp*^{R}	Sikorski and Hieter, 1989
pRS316UPF1	*URA3 CEN6 ARSH4 UPF1 Amp*^{R}	Atkin et al., 1995
pRS316UPF1-D4	*URA3 CEN6 ARSH4 UPF1-D4 Amp*^{R}	Atkin et al., 1997
pAA78	*TRP1 CEN6 ARSH4 UPF1 Amp*^{R}	This study
pYCplac22DE572AA	*TRP1 CEN4 FLAG-DE572AA Amp*^{R}	Weng et al., 1996a
pYCplac22RR793AA	*TRP1 CEN4 FLAG-RR793AA Amp*^{R}	Weng et al., 1996a
pYCplac22TR800AA	*TRP1 CEN4 FLAG-TR800AA Amp*^{R}	Weng et al., 1996a
pAA377	*TRP1 2l upf1-D814-917 Amp*^{R}	This study
pAA301	*GAL4*$_{BD}$*RR793AA*$_{525–971}$, *URA3*, 2μ, *Amp*^{R}	This study
pAA302	*GAL4*$_{BD}$*TR800AA*$_{525–971}$, *URA3*, 2μ, *Amp*^{R}	This study
pAA304	*GAL4*$_{BD}$*UPF1-D4*$_{525–971}$, *URA3*, 2μ, *Amp*^{R}	This study
pAA318	*GAL4*$_{BD}$*DE572AA*$_{525–971}$, *URA3*, 2μ, *Amp*^{R}	This study
pAA324	*GAL4*$_{BD}$*UPF1*$_{525–813}$, *URA3*, *2l*, *Amp*^{R}	This study

GAL4$_{BD}$ encodes the Gal4p DNA binding domain.
GAL4$_{AD}$ encodes the Gal4p activation domain.

pAA303 contains an in-frame fusion of the *GAL4* DNA-binding domain and the 3′ half of UPF1. It was constructed by subcloning an *Eco*RI-*Bam*HI fragment from pAA158 into pGBDU-C1. pGBDU-C1 is an expression vector for two-hybrid analysis containing the *GAL4* DNA-binding domain under the control of the *ADH1* promoter, followed by a multiple cloning site (James et al., 1996). pAA301, pAA302, pAA304, and pAA318 were constructed by replacing the wild-type *UPF1 Hin*dIII-*Bam*HI fragment in pAA303 with a *Hin*dIII-*Bam*HI fragment from *RR793AA*, *TR800AA*, *UPF1-D4*, and *DE572AA*, respectively. *RR793AA*, *TR800AA*, and *DE572AA* are mutant *UPF1* alleles constructed by Weng et al. (1996a). *UPF1-D4* has a mutation in the helicase domain, where the arginine at codon 779 has been replaced with cysteine (Leeds et al., 1992). This mutation causes a dosage-dependent, dominant-negative inhibition of wild-type Upf1p function. pAA324 was constructed by deleting a 1363-bp *Bgl*II fragment from pAA303. This deletion removes the codons for amino acids 814 to 971 and *UPF1* terminator elements and replaces it with sequences for the amino acids methionine, asparagine, arginine, arginine, and tyrosine, and the *ADH1* terminator elements. pAA377 was constructed by subcloning a 2836-bp *Eco*RI-*Bgl*II fragment containing the *UPF1* promoter and codons 1–813 into pGBD-C1 (James et al., 1996). This results in a plasmid carrying a gene with the *UPF1* promoter, codons 1–813 from the *UPF1* ORF, followed by the codons for the amino acids methionine, asparagine, arginine,

arginine, and tyrosine, and the *ADH1* terminator elements. This plasmid also has a *TRP1* selectable marker and a 2μ origin of replication.

pAA265 was recovered in the library screen for yeast proteins that interact with Upf1p and contains an in-frame fusion of the GAL4 transcription activation domain to +49 of the *NUP100* ORF. pAA378 contains the *NUP116* ORF fused in-frame to the GAL4 activation domain (GAL4AD). The *NUP116* ORF PCR fragment was subcloned into the *Cla*I-*Bgl*II sites of pGAD-C1 (James et al., 1996). The DNA fragment with the *NUP116* ORF was amplified by PCR using oligonucleotides oAA104 (5′-dGGC *ATC GAT* ATG TTT GGA GTT AGC CGT GG) and oAA116 (5′-dGGC *AGA TCT* TCA GGT CTG CTC TGC AGC G) using *PfuTurbo* DNA polymerase (Stratagene, La Jolla, California). oAA104 creates a *Cla*I site (in italics) and oAA116 creates a *Bgl*II site (in italics) immediately upstream and downstream of the NUP116 ORF, respectively. pAA296 contains the *SNF1* ORF fused in-frame to the GAL4 DNA-binding domain. A PCR fragment containing the *SNF1* ORF subcloned into the *Eco*RI-*Bam*HI sites of pGBDU-C1 (James et al., 1996). The DNA fragment with the *SNF1* sequences was amplified by PCR using oligonucleotides oAA59 (5′-dGGG *GAA TTC* ATG AGC AGT AAC AAC AAC ACA AAC ACA GC) and oAA60 (5′-dGTC GCA GAC TCC GTT CTT G) using *Taq* DNA polymerase. OAA59 creates an *Eco*RI site (underlined) immediately upstream of *SNF1* ORF. The inserts in these plasmids were sequenced by the UNL Genomics Core Research Facility.

2.3. Two-hybrid screening

The screening of libraries for interacting yeast proteins was carried out using the two-hybrid system of Chien et al. (1991). The yeast strain GGY1::171 (Table 1) was transformed simultaneously with pAA158 (Table 1), and each of three libraries: YL1, YL2, and YL3 (Chien et al., 1991). The transformants were selected on synthetic complete medium lacking leucine and histidine. To screen for interacting proteins, the transformants were replica plated onto SSX-leu-his plates. Blue colonies were selected and plated onto synthetic complete medium lacking leucine and histidine, for single colony isolates. β-galactosidase filter assays were used to identify isolated colonies with potential interacting clones. False positives resulting from the intact *GAL4* gene in the library vector were identified by PCR using the *GAL4* specific primers oAA21 (5′-dGCC CTA TCG TGC ACT CAC CGA CGC) and oAA22 (5′-dGTG AAG GCC CTA CTG AGC CAG GAG). The remaining potential positives were streaked onto synthetic complete medium lacking leucine, and colonies were selected that had lost pAA158. The clones containing only the library plasmid were replica plated onto SSX-leu plates to identify false positives that do not require Upf1p for the activation of the reporter gene. The remaining positive library plasmid DNAs were rescued into library efficient *E. coli* DH5α competent cells (Life Technologies). Unique library plasmids were retransformed into GGY1::171, alone and with pAA158 or pEE5 (Table 1). The library plasmids requiring pAA158 for the activation of the reporter gene in GGY1::171 were sequenced by the UNL DNA Sequencing Facility using oAA68 (5′-dGAA GAT ACC CCA CCA AAC CC). This oligonucleotide is complementary to the template strand at the 3′ end of the *GAL4* activation domain. The sequences from the library plasmid inserts were used to search the Saccharomyces Genome database (http://genome-www.stanford.edu/Saccharomyces/).

2.4. Two-hybrid interaction assay

Potential two-hybrid interactions were directly tested using the system of James et al. (1996), with the following modification. The plasmids containing the *GAL4* DNA binding domain (bait; pAA303, pGBCU-C1, pAA296, pAA301, pAA302, pAA304, pAA318, and pAA324) were transformed into PJ69-4a (Table 1), and the plasmids containing the *GAL4* activation domain (prey; pAA265, pAA378, pGAD-C1, and pGAC-SIP1) were transformed into PJ69-4α. Appropriate bait transformants were mated to prey transformants by coincubation on yeast extract, peptone, and dextrose medium (YEPD) plates for 4 h at 30°C, followed by replica plating onto synthetic complete medium lacking uracil and leucine, to select for diploids carrying the bait and prey plasmids. These diploids were tested for reporter gene activity as previously described by plating on synthetic complete medium lacking adenine, uracil, and leucine and on synthetic complete medium lacking histidine, uracil, and leucine and containing 1 mM 3-aminotriazole (3-AT; James et al., 1996). Gal4 binding domain ($GAL4_{BD}$)SNF1 (encoded on pAA296) and GAL4-ADSIP1 (encoded in pGAD-SIP1) were a positive control for two-hybrid interaction (James et al., 1996).

2.5. β-Galactosidase assay

β-Galactosidase assays were done with the Gal-Screen System (Applied Biosystems, Bedford, Massachusetts) using the Gal-Screen Buffer B as described by the manufacturer. Yeast cells were grown in synthetic complete medium lacking uracil and leucine, to an optical density of 0.4–0.6 at 600 nm, and assayed at 50,000–75,000 cells per well in 96-well, white tissue, culture-treated microtiter plates. Standard curves were generated from serial dilution series of a 1 mg/ml β-galactosidase stock solution. The stock solution was prepared from lyophilized β-galactosidase (G-8511, Sigma-Aldrich, St. Louis, Missouri) as described in the protocol for the Gal-Screen System.

2.6. Western analysis of proteins and co-immunoprecipitation assays

Whole-cell extracts from W303α, SWY67 (*nup100Δ*), and SWY31 (*nup116Δ*) were prepared as described in Atkin et al. (1995), using total protein lysis buffer [5 mM ethylenediaminetetraacetic acid (EDTA), 250 mM NaCl, 0.1% Igepal CA-630, 50 mM Tris–HCl, pH 7.4, 1 μg/ml chymostatin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml antipain, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)]. Cell lysates for the immunoprecipitations were prepared from AAY320 (*upf1Δ*) and AAY327[pR S316UPF1-3EP], as described in Atkin et al. (1995), except that cells were washed and lysed in a nondenaturing lysis buffer [50 mM Tris·Cl, pH 7.5, 0.1% Triton X-100, 0.1 M NaCl, 1 mM dithiothreitol (DTT), 10% glycerol, 5 μg/ml chymostatin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, 5 μg/ml antipain, 1 μM PMSF, 1mM benzamidine, 15 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.3 mg/ml sodium metavanadate, 2.5 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM β-glycerol phosphate; Ausubel et al., 1998].

In a co-immunoprecipitation buffer (50 mM Tris·Cl, pH 7.5, 15 mM EGTA, 100 mM NaCl, 0.1% TritonX-100, 5 μg/ ml chymostatin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, 5 μg/ml antipain, 1 mM PMSF, 1 mM benzamidine, 0.3 mg/ml sodium metavanadate, 2.5 mM sodium orthovanadate, 10 mM NaF, 10 mM β-glycerolphosphate; Ausubel et al.,

1998), 0.5–1 mg cell lysate was incubated with 1.0 μg of 12CA5 anti-HA monoclonal antibody (Berkeley Antibody, Richmond, California), at a 0.5-ml final volume, for 90 min on ice with mixing by inversion every 15 min. Nonspecific aggregates were removed by a 10-min spin at 12,000 rpm, at 4°C in a microcentrifuge. The supernatant was transferred to a Handee Spin Cup Column (Pierce Biotechnology, Rockford, Illinois), and 50 μl of protein G-sepharose slurry (Pierce Biotechnology) was added. The tubes were incubated with continuous shaking for 60 min at 4°C. Unbound proteins were removed by centrifugation for 3 min at 1000 rpm in a 4°C microcentrifuge. The unbound proteins were saved. The bound proteins were washed three times with the co-immunoprecipitation buffer and eluted with 20 μl 2 × protein sample buffer.

The proteins in whole-cell lysates, input, and bound and unbound fractions were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blotting. The Western blots were probed with 12CA5 anti-HA monoclonal antibodies and affinity-purified polyclonal anti-GLFG (WU956) antibodies as described in Atkin et al. (1995) and Bucci and Wente (1998), respectively. The primary antibodies were detected with HRP-conjugated secondary antibodies using the Super Signal ELISA Femto Maximum Sensitivity Substrate (Pierce Biotechnology) on a BioChemi System (UVP, Upland, California).

2.7. RNA preparation and Northern analysis

Yeast total RNA was extracted by the hot phenol method (Atkin et al., 1995); 15 Ag of RNA was resolved on formaldehyde gels. The RNA was transferred to GeneScreen Plus nylon membrane and was hybridized with oligolabeled DNA probes in the conditions recommended by the manufacturer (NEN Life Science Products, Boston, Massachusetts). Oligolabeled DNA probes were prepared from purified PCR products using an Oligolabeling Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey) and [α^{32}P]dCTP (NEN Life Science Products). The amount of probe hybridized to the Northern blots was quantified using a Storm (Amersham Pharmacia Biotech).

2.8. Nonsense suppression assay

The *can1-100* suppressible marker was used to measure nonsense suppression in liquid assays and drop tests. For liquid assays, the strains were grown overnight to saturation in YPD supplemented with adenine (YAPD). The next morning, the cultures were diluted to an OD_{600} of 0.1 in YAPD or synthetic complete medium lacking arginine and containing 100 μg/ml canavanine. The OD600 for each culture was monitored. The drop tests were done on YPD and YPD containing 25, 100, and 300 μg/ml canavanine, as described in Atkin et al. (1995). Sensitivity to canavanine is indicative of suppression of *can1-100*.

3. Results and discussion

3.1. Isolation of proteins that interact with Upf1p

The two-hybrid system of Chien et al. (1991) was used to identify and clone genes for proteins that interact with Upf1p. The C-terminal half of Upf1p (amino acids 525–971) was fused to the *GAL4* DNA binding domain (Fig. 1A; pAA158) and used to screen for Upf1p-

interacting activation domain hybrids (see Materials and methods). The region of Upf1p encompassing codons 525–971 was selected because it shares similarity with a region in Sen1p, a transfer ribonucleic acid (tRNA) and ribosomal ribonucleic acid (rRNA) processing protein (Leeds et al., 1992). Our goal was to identify the protein(s) that interact with this conserved region. Eleven unique Upf1p-interacting clones were recovered. Nine clones were in-frame fusions to the short regions of the noncoding DNA and were not of further interest. However, the remaining two clones were in-frame fusions, the first of which was to position +49 of the *NUP100* ORF and the second of which was to the entire *GSY1* ORF. *NUP100* encodes a nuclear pore complex protein that is localized predominantly to the cytoplasmic side of the central transporter region. It participates in mRNA transport (Strawn et al., 2001). These proteins could interact because Upf1p is predominantly localized in the cytoplasm (Atkin et al., 1995). In contrast, *GSY1* encodes a glycogen synthetase (Farkas et al., 1990). Because it is not clear why Upf1p might interact with this protein, we have not characterized this interaction further.

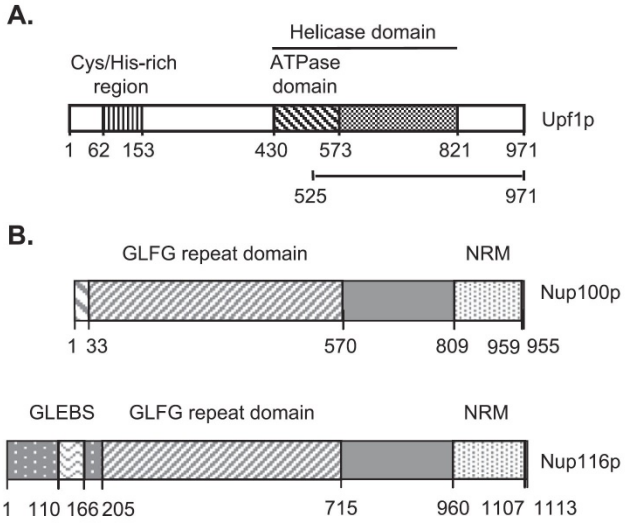


Figure 1. Schematic diagrams of Upf1p, Nup100p, and Nup116p. (A) Upf1p is a 971-amino-acid protein containing a cys/his-rich region at its N-terminus and a helicase domain with an ATPase domain at its C-terminus. The line from 525 to 971 under the schematic of Upf1p shows the region of Upf1p that was fused to the *GAL4* DNA binding domain and used as bait in a two-hybrid screen. (B) Nup100p and Nup116p are similar throughout their GLFG repeat domains and C-termini containing a nucleoporin RNA-binding motif (NRM). The GLFG repeats consist of repeats of glycine, leucine, phenylalanine, and glycine, interspersed with glutamine-, asparagine-, serine-, and threonine-rich spacers. Only amino acids 1–33 from Nup100p and 1–205 from Nup116p are unique. Nup116p amino acids 110–166 are a Gle2p-binding sequence (GLEBS).

The two-hybrid interaction between Upf1p and Nup100p is specific because reporter genes were only active in cells when both $Gal4_{BD}Upf1_{525–971}$ (expressed from pAA303) and $Gal4_{AD}Nup100$ (expressed from pAA265) were present (Table 2; Fig. 2A). $Gal4_{BD}Upf1_{525–971}$

could not activate the reporter genes with Gal4AD (expressed from pGAD-C1) on its own or with Gal4ADSip1 (expressed from pGAD-SIP1). Gal4ADNup100 also did not activate the reporter genes with Gal4BD on its own or with Gal4BDSnf1 (expressed from pAA296).

Gal4ADSip1 and Gal4BDSnf1 were positive two-hybrid interaction controls. Snf1p protein kinase is a central component of the yeast signaling pathway for glucose repression (reviewed in Jiang and Carlson, 1997). It is part of a complex that includes Sip1p. Jiang and Carlson (1997) showed that Snf1p and Sip1p have a two-hybrid interaction.

We also attempted to test whether we could observe interactions using Nup100p as a bait and Upf1p as a prey. We found that an in-frame fusion of Upf1 (originally used as the bait) and the activation domain autoactivate reporter gene expression (data not shown). This result is perhaps not surprising because Upf1p is a helicase and has been shown to bind both DNA and RNA in vitro, but this autoactivation meant that the reciprocal experiment was not possible.

Table 2. Upf1p interacts specifically with Nup100p and Nup116p in a two-hybrid interaction assay (James et al., 1996)

DNA binding domain hybrid	Activation domain hybrid	Mean β-galactosidase activity (ng/7.5 × 10^6 cells)
Gal4BDUpf1525–971	Gal4ADNup100	0.2 (±0.04, n = 8)
Gal4BD	Gal4ADNup100	0.1 (±0.03, n = 8)
Gal4BDUpf1525–971	Gal4ADNup116	0.4 (±0.11, n = 8)
Gal4BD	Gal4ADNup116	0.1 (±0.06, n = 8)
Gal4BDSnf1	Gal4ADSip1	4.4 (±2.57, n = 8)

PJ69-4a (Table 1) transformed with plasmids containing the *GAL4* DNA binding domain constructs (pAA303 [Gal4BDUpf1525–971], pGBCU-C1 [Gal4BD], and pAA296 [Gal4BDSnf1]; Table 1) were mated with PJ69-4α transformed with plasmids containing the *GAL4* activation domain (pAA265 [Gal4ADNup100], pAA378 [Gal4AD-Nup116], and pGAD-SIP1 [Gal4ADSip1]; Table 1). β-galactosidase, encoded by the *lacZ* reporter gene, activity levels were measured by the Gal-Screen System, a chemiluminescent reporter gene assay system. The standard deviation and number of replicates for the mean β-galactosidase activity are shown in parentheses. The amount of β-galactosidase per 7.5 × 10^6 cells was determined by comparison with a standard curve prepared with known amounts of purified β-galactosidase.

The two-hybrid interaction between Gal4BDUpf1525–971 and Gal4ADNup100 is weaker than the two-hybrid interaction between Gal4ADSip1 and Gal4BDSnf1. Yeast cells containing Gal4BDUpf1525–971 and Gal4ADNup100 grew slower on synthetic complete minimal medium lacking uracil, leucine, and adenine and on synthetic complete minimal medium lacking uracil, leucine, and histidine and containing 1mM 3-aminotriazole (Fig. 2A) and had lower β-galactosidase activity levels (Table 2) than did yeast cells containing Gal4ADSip1 and Gal4BDSnf1. Although the β-galactosidase activity levels were lower for Gal4BDUpf1525–971 and Gal4ADNup100 than for the Gal4ADSip1 and Gal4BDSnf1 interaction, it was still twofold above background. This difference is small but significant because the standard deviation for these experiments was only ±0.04. It is important to note that the strength of the two-hybrid interaction between Upf1p and Nup100p relative to the two-hybrid interaction between Snf1p and Sip1p may not reflect the relative strengths of their in vivo interactions because two-hybrid protein interactions are not assayed in their natural context.

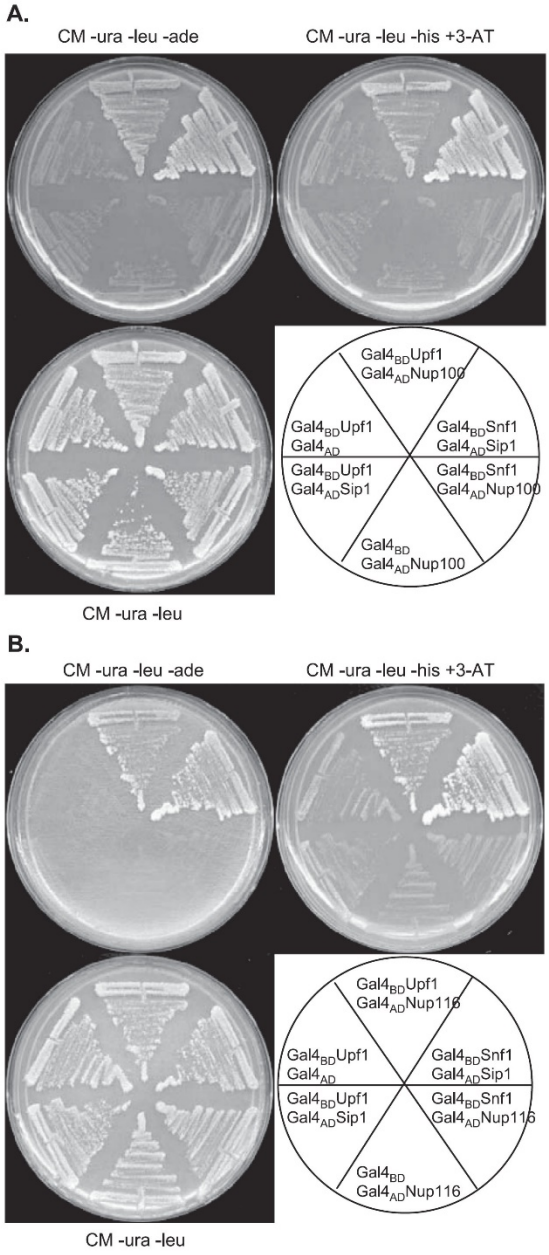


Figure 2. Upf1p interacts with Nup100p and Nup116 in a two-hybrid interaction assay. Yeast strains were prepared by mating PJ69-4a (Table 1) transformed with plasmids containing the *GAL4* DNA binding domain constructs (pAA303 [Gal4BDUpf1525–971], pGBCU-C1 [Gal4BD], pAA296 [Gal4BDSnf1]; Table 1), and were mated with PJ69-4α transformed with plasmids containing the *GAL4* activation domain (pAA265 [Gal4ADNup100], pAA378 [Gal4ADNup116], pGAD-C1 [Gal4AD], pGAD-SIP1 [Gal4ADSip1]; Table 1). *ADE2* and *HIS3* reporter gene activity was assayed by growth on synthetic complete medium lacking uracil, leucine, and adenine (CM-ura-leu-ade) medium, and synthetic complete medium lacking

uracil, leucine and histidine and containing 1mM 3-aminotriazole (CM-ura-leu-his, +3-AT) medium, respectively. Reporter gene activation is not required for growth on the synthetic complete medium lacking uracil and leucine (CM-ura-leu). The binding and activation domain hybrids are indicated for each sector. The Gal4$_{DB}$Upf1 and Gal4$_{AD}$Nup100p (A) and the Gal4$_{DB}$Upf1p and Gal4$_{AD}$Nup116p (B) interactions both activate the expression of the *GAL2-ADE2* and *GAL1-HIS3* reporter genes, which allows for growth on synthetic complete medium lacking uracil, leucine, and adenine and on synthetic complete medium lacking uracil and leucine and histinine and containing 1 mM 3-aminotriazole, respectively.

Two lines of evidence suggest that Nup100p is structurally and functionally similar to another nuclear pore protein, Nup116p: (1) Nup100p and Nup116p are a homologous yeast gene pair that likely resulted from a gene duplication event (Wente et al., 1992; Suntharalingam and Wente, 2003; Fig. 1), and (2) mutants with a deletion of only *NUP100* or *NUP116* are viable, while the deletion of both *NUP100* and *NUP116* is lethal (Murphy et al., 1996). The sequence and functional similarity between Nup100p and Nup116p suggests that Upf1p might also interact with Nup116p. We tested this idea directly using a two-hybrid interaction assay (James et al., 1996). An in-frame fusion between the *GAL4* activation domain and the entire *NUP116* open reading frame was constructed (pAA378). Reporter gene activation was observed in cells containing Gal4$_{BD}$Upf1$_{525–971}$ and Gal4$_{AD}$Nup116 (Fig. 2B). This interaction was specific because Gal4$_{AD}$Nup116 was not able to activate the *ADE2* and *HIS3* reporter genes on its own or in combination with Gal4$_{BD}$ or Gal4$_{BD}$Snf1. This interaction is also weaker than the two-hybrid interaction between Gal4$_{AD}$Sip1 and Gal4$_{BD}$Snf1 because yeast cells containing Gal4$_{BD}$Upf1$_{525–971}$ and Gal4$_{AD}$Nup116 grew more slowly on the synthetic complete minimal medium lacking uracil, leucine, and adenine and on the synthetic complete minimal medium lacking uracil, leucine, and histidine and containing 1 mM 3-aminotriazole than did yeast cells containing Gal4$_{AD}$Sip1 and Gal4$_{BD}$Snf1 (Fig. 2). Moreover, cells containing Gal4$_{BD}$Upf1$_{525–971}$ and Gal4$_{AD}$Nup116 had lower β-galactosidase activity levels than did yeast cells containing Gal4$_{AD}$Sip1 and Gal4$_{BD}$Snf1 (Table 2).

3.2. Upf1p interacts with Nup100p and Nup116p in cell extracts

The ability of Upf1p to bind to Nup100p and Nup116p was further tested by coimmunoprecipitation of protein complexes from whole-cell extracts. Cell lysates were prepared with nondenaturing lysis buffer from AAY320[pRS316] (*upf1Δ*) and AAY327[pRS316UPF1-3EP], which carries *UPF1-3EP*, a functional HA-epitope tagged allele of *UPF1* encoding Upf1-3HA (Atkin et al., 1995), and were incubated with the anti-HA monoclonal antibody, 12CA5. Antibody-bound Upf1p and interacting proteins were isolated with protein G-sepharose. The unbound and bound fractions were separated by SDS-PAGE and analyzed by immunoblotting (Fig. 3). We have previously shown that Upf1-3HA can be specifically detected by immunoblotting with anti-HA antibody (Atkin et al., 1995). Here, we show that Upf1-3HA can be immunoprecipitated by the anti-HA antibodies because a band of the expected size for Upf1-3HAwas observed in the bound fraction (Fig. 3B, lower panel). Furthermore, the immunoprecipitation depleted a significant fraction of the Upf1-3HA (Fig. 3B, lower panel). No bands of the expected size for Upf1-3HA were seen in the whole-cell lysates, unbound and bound fractions from AAY320 that lacks Upf1-3HA (data not shown).

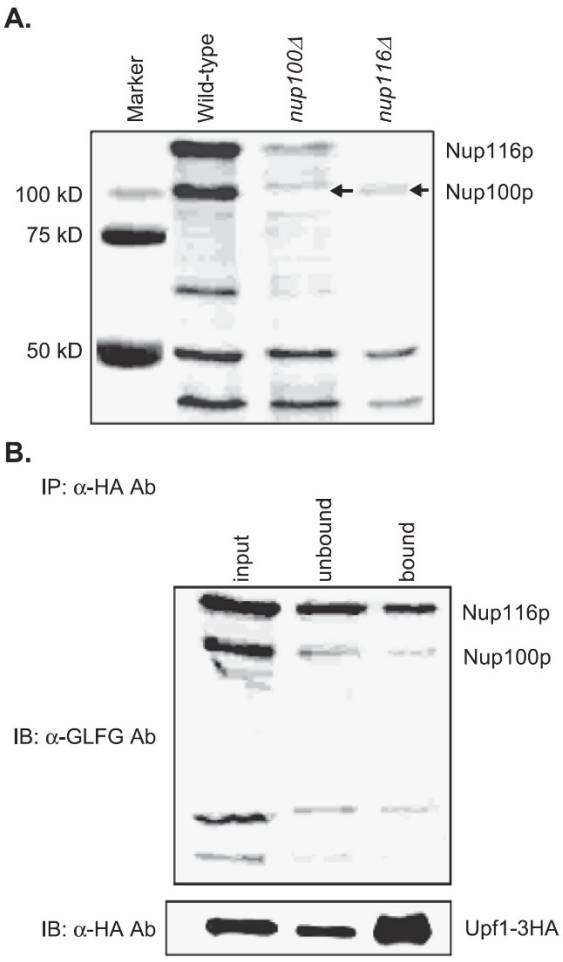


Figure 3. Upf1p interacts with Nup100p and Nup116p in cell extracts. (A) Control immunoblot for detection of Nup116p and Nup100p by anti-GLFG antibodies. Equivalent amounts of whole-cell extracts prepared with total protein lysis buffer from W303α (wild-type), SWY67 (*nup100Δ*), and SWY31 (*nup116Δ*) yeast cells were separated on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose, and analyzed by immunoblotting with affinity-purified polyclonal anti-GLFG antibodies (WU956; Bucci and Wente, 1998). The anti-GLFG antibodies were raised against the GLFG region of Nup116p and react with other yeast GLFG proteins. The Nup116p band recognized by anti-GLFG antibodies is indicated. Anti-GLFG antibodies recognize two bands of the expected size for Nup100p in whole-cell extracts from wild-type cells. The lower band is specific for Nup100p because this band is absent in whole-cell extracts from *nup100Δ* cells (closed arrow). (B) Upf1-3HA coimmunoprecipitates Nup100p and Nup116p from yeast cell lysates. Whole-cell lysates prepared with nondenaturing lysis buffer AAY327[pRS316UPF1-3EP], which expresses Upf1-3HA (input), were immunoprecipitated with 12CA5 anti-HA monoclonal antibody. The proteins in the input (20 μg) and equal amounts of unbound and bound fractions were resolved by SDS-PAGE and immunoblotted with anti-GLFG (top) or anti-HA antibodies (bottom).

To determine whether Nup100p or Nup116p copurify with Upf1-3HA, the samples were immunoblotted with an affinity-purified anti-GLFG polyclonal antibody raised against the region from Nup116p containing a GLFG motif (Fig. 1; Bucci and Wente, 1998). Yeast have five proteins that contain a GLFG motif: Nup100p, Nup116p, Nup145Np, Nup49p, and Nup57p (reviewed in Suntharalingam and Wente, 2003). This antibody recognizes all five members of the GLFG family (Strawn et al., 2001). We selected this antibody because, currently, there are no monospecific antibodies for Nup100p available. As a control, we showed that this antibody recognizes specific Nup116p and Nup100p bands by comparing the bands detected in the cell lysates extracted with total protein lysis buffer from W303α (wild-type), SWY67 (*nup100Δ*), and SWY31 (*nup116Δ*; Fig. 3A). A 116 kD band was detected in whole-cell extracts from wild-type cells that are missing in the cell extracts from the *nup116Δ* deletion mutant. Two bands of the expected size for Nup100p (100 kD) were detected in cell extracts from wild-type cells and a *nup116Δ* deletion mutant. The lower band is missing in the *nup100Δ* deletion mutant, indicating that it is specific for Nup100p. This antibody recognizes additional bands that may correspond to other members of the GLFG family. One of these bands, a ~60 kD band, is reduced in *nup100Δ* and *nup116Δ* cells relative to wild-type cells. This band is the expected size for Nup145N (60 kD), a GLFG family member, but it has not been specifically identified. The reduced abundance of this band in both *nup100Δ* and *nup116Δ* cells suggests that the expression of at least a subset of the GLFG family members may be coregulated. We did not characterize this band further because it is not extracted by the nondenaturing lysis buffer used to prepare the cell lysates for the coimmunoprecipitation.

Nup100p and Nup116p copurify with Upf1-3HA because both Nup100p and Nup116p are detected in the bound fraction (Fig. 3B, upper panel). Not all the Nup100p or Nup116p copurified with Upf1p because Nup100p and Nup116p were still detected in the unbound fraction. This is consistent with these proteins being a binding site for multiple dynamic factors (Strawn et al., 2001). Furthermore, a third protein, recognized by the anti-GLFG polyclonal antibodies, copurifies with Upf1p. This suggests that Upf1p may interact with additional members of the GLFG family. The third protein has not been characterized further.

An interaction between Upf1p and these nuclear pore proteins is logical because both Nup100p and Nup116p are located predominantly on the cytoplasmic face of the central transporter region in the nuclear pore (reviewed in Suntharalingam and Wente, 2003, and Upf1p is predominantly located in the cytoplasm (Atkin et al., 1995). Thus, Upf1p could interact with Nup100p and/or Nup116p in vivo. Furthermore, both Nup100p and Nup116p are involved in the transport of mRNAs through the nuclear pore. The interaction between Upf1p and the nuclear pore proteins has important implications, if it is biologically significant. For example, an interaction with Nup100p and Nup116 suggests a mechanism that would ensure that Upf1p is assembled into the surveillance complex during mRNA transport and prior to or during the pioneer round of translation. To test the biological significance of the interaction, we determined if (1) there is a genetic interaction between *NUP100* and *UPF1*, and if (2) the deletion of *NUP100* or *NUP116* causes the accumulation of *CYH2* pre-mRNA, an NMD substrate, or suppression the *can1-100* nonsense allele.

3.3. A nup100 deletion is epistatic to an upf1 deletion for suppression of the can1-100 nonsense allele but not CYH2 pre-mRNA accumulation

NMD mutants have two phenotypes: They accumulate mRNAs with premature termination codons and suppress a variety of nonsense and frameshift mutations (Leeds et al., 1992). To find out if *UPF1* interacts with *NUP100* in vivo, we asked if cells with *upf1-Δ5* and *nup100::URA3* show a novel phenotype. We crossed AAY273 (*upf1-Δ5*, Table 1) with SWY3 (*nup100::URA3*, Table 1). Thirteen complete tetrads were recovered: 1 parental ditype, 2 nonparental ditypes, and 10 tetratypes. *upf1-Δ5* and *nup100Δ* spores were viable.

The mRNA accumulation phenotype can be determined by looking at the accumulation of *CYH2* pre-mRNA, a substrate of the NMD pathway (He et al., 1993). *CYH2*, which encodes ribosomal protein L29, contains an intron that is inefficiently spliced from the pre-mRNA, and consequently, a significant amount of *CYH2* pre-mRNA is transported to the cytoplasm. An in-frame premature termination codon within the *CYH2* intron targets the cytoplasmic-bound *CYH2* pre-mRNA for NMD. The spores from two tetratype tetrads were analyzed for *CYH2* premRNA accumulation (Fig. 4A). The wild-type and *nup100Δ* yeast controls and spores accumulated very low levels of *CYH2* pre-mRNA. In contrast, the *upf1-Δ5* and *upf1-Δ5 nup100Δ* cells accumulated high levels of *CYH2* pre-mRNA. The *CYH2* pre-mRNA/mRNA accumulation ratios of the two *upf1-Δ5 nup100Δ* spore clones are indistinguishable from the ratios seen in the *upf1-Δ5* cells. Thus, the presence of the *nup100Δ* had no effect on the *CYH2* pre-mRNA accumulation phenotype of *upf1-Δ5*.

can1-100 is a nonsense allele that confers resistance to canavanine and is suppressed in *upf1* mutants (Maderazo et al., 2000). *CAN1* encodes a high-affinity permease that is responsible for arginine transport into cells. The *can1-100* allele contains a single A-to-T mutation that results in the substitution of a lysine codon at position 47 for a UAA codon in the *CAN1* coding region (Maderazo et al., 2000). Canavanine is a toxic analogue of arginine that is also transported into cells via Can1p. Thus, *CAN1* cells are sensitive to canavanine, while *can1* mutants tend to be resistant to canavanine. Suppression of *can1-100* results in canavanine sensitivity. The suppression of the *can1-100* allele by the spores from one tetratype tetrad was evaluated (Fig. 4B). The optical density of liquid cultures grown in either the presence or absence of canavanine was monitored. All spores grew equally well in the absence of canavanine (Fig. 4B, top). Thus, the deletion of *UPF1*, *NUP100*, or of both has no effect on the viability of yeast strains grown in liquid YAPD cultures; *upf1-Δ5* spores were unable to grow in the presence of canavanine (Fig. 4B, bottom). Wild-type, *nup100Δ*, and *upf1-Δ5 nup100Δ* all grew equally well in the presence of canavanine. Thus suppression of *can1-100* by *upf1-Δ5* is inhibited by the deletion of *NUP100* in the *upf1-Δ5 nup100Δ* spore.

This effect is not due to a slightly reduced export of *can1-100* mRNA in the *upf1-Δ5 nup100Δ* strain for two reasons: (1) An mRNA transport defect has not been seen in *nup100Δ* strains (Iovine and Wente, 1997), and (2) *nup116Δ* strains have an mRNA transport defect (Wente and Blobel, 1993; Fig. 5), but the *upf1-Δ5 nup116Δ* strain is not more sensitive to canavanine (data not shown). Thus, the effect is not the result of a general mRNA transport defect but is specific to the combination of *upf1-Δ5* and *nup100Δ* alleles (Fig. 4B). Furthermore, the genetic interaction between *UPF1* and *NUP100* suggests that the interactions seen between Upf1p and Nup100p are biologically significant.

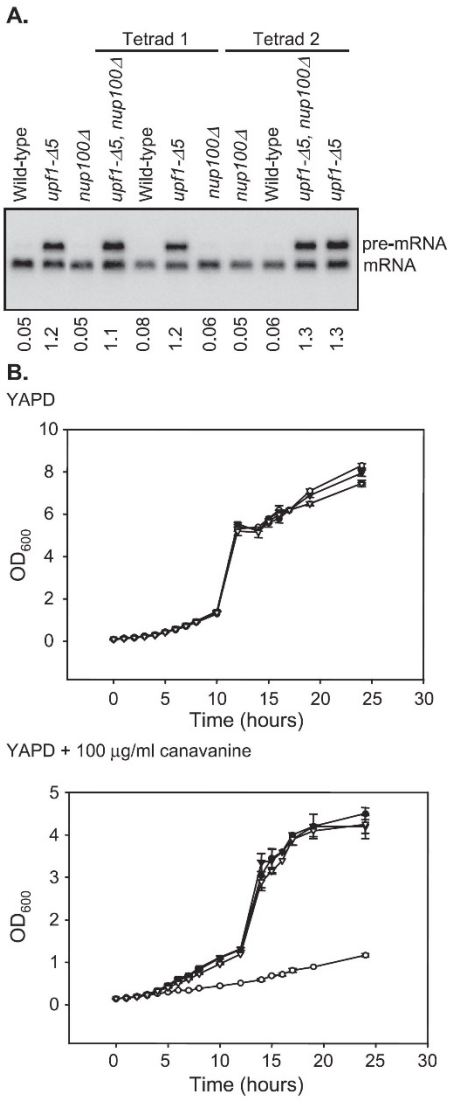


Figure 4. mRNA accumulation and nonsense suppression phenotype of representative tetratype tetrads recovered from a cross between AAY273 (*upf1Δ-5*) and SWY3 (*nup100Δ*) yeast strains. (A) Northern blot of total RNA extracted from W303a (wild-type), AAY273 (*upf1Δ-5*), and SWY3 (*nup100Δ*) yeast strains and the spore clones from two tetratype tetrads probed with labeled *CYH2* DNA. The locations of the *CYH2* pre-mRNA and mRNA bands are indicated to the right of the Northern blot. The *CYH2* pre-mRNA/mRNA ratios are under the Northern blot. *CYH2* pre-mRNA is a substrate for NMD because the intron contains an in-frame stop codon. *CYH2* mRNA is not a substrate for NMD. (B) The *can1-100* nonsense suppression phenotype of the tetratype tetrad spore clones. The OD_{600} was monitored for 25 h for cultures grown at 30°C in YAPD and 100 μg/ml canavanine. ●:Wild type; ○: *upf1-Δ5*; ▼: *nup100Δ*; and ▽: *upf1-Δ5*, *nup100Δ* cells.

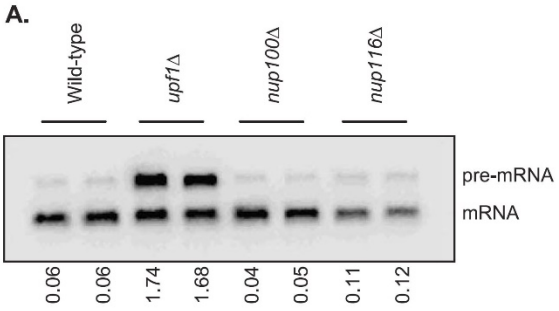
This effect is not due to a slightly reduced export of *can1-100* mRNA in the *upf1-Δ5 nup100Δ* strain for two reasons: (1) An mRNA transport defect has not been seen in *nup100Δ* strains (Iovine and Wente, 1997), and (2) *nup116Δ* strains have an mRNA transport defect (Wente and Blobel, 1993; Fig. 5), but the *upf1-Δ5 nup116Δ* strain is not more sensitive to canavanine (data not shown). Thus, the effect is not the result of a general mRNA transport defect but is specific to the combination of *upf1-Δ5* and *nup100Δ* alleles (Fig. 4B). Furthermore, the genetic interaction between *UPF1* and *NUP100* suggests that the interactions seen between Upf1p and Nup100p are biologically significant.

3.4. NUP100 and NUP116 deletion mutants do not accumulate nonsense mRNAs or suppress nonsense mutations

If the interaction between Upf1p and the two nuclear pore proteins, Nup100p and Nup116p, is important for NMD, we might expect *NUP100* and *NUP116* null mutants to accumulate mRNAs with premature termination codons and/or suppress nonsense mutations. *NUP100* null mutants do not accumulate *CYH2* pre-mRNA and/or suppress the *can1-100* nonsense allele (Fig. 4). We tested whether *NUP116* null mutants accumulate *CYH2* pre-mRNA and/ or suppress the *can1-100* nonsense allele.

The *CYH2* pre-mRNA accumulation phenotype of W303a (wild-type), AAY273 (*upf1-Δ5*), SWY3 (*nup100Δ*), and SWY31 (*nup116Δ*) yeast strains were determined by quantitative Northern blotting (Fig. 5A). As previously shown, *nup100Δ* yeast strains do not accumulate *CYH2* pre-mRNA. *nup116Δ* yeast strains consistently accumulate twofold more *CYH2* pre-mRNA than wild-type or *nup100Δ* yeast strains do.

nup116Δ yeast strains could accumulate *CYH2* pre-mRNA either because of a problem with mRNA transport or a defect in NMD. Defects in NMD, such as the loss of Upf1p function, result in increased stability and polyribosome association of *CYH2*, *RP51B*, and *MER2* pre-mRNAs (He et al., 1993). However, because *nup116Δ* yeast strains have a previously characterized defect in mRNA transport (Wente and Blobel, 1993), we suspected the that *CYH2* pre-mRNA accumulation was due to a defect in mRNA transport. We tested this possibility by examining the ratio of *CYH2* pre-mRNA to mRNA associated with polyribosomes in isogenic wild-type and *nup116Δ* yeast strains (Fig. 5B). If *CYH2* pre-mRNA accumulates because of a defect in mRNA transport, we expect similar polyribosome-associated *CYH2* pre-mRNA/mRNA ratios in isogenic wild-type and *nup116Δ* yeast strains. On the other hand, if *CYH2* pre-mRNA accumulates because of a defect in NMD, we expect higher polyribosome-associated *CYH2* pre-mRNA/mRNA ratios in a *nup116Δ* strain than in an isogenic wild-type strain. The polyribosome-associated *CYH2* pre-mRNA/mRNA ratios were 0.09 (±0.03, $n = 3$) and 0.08 (±0.04, $n = 4$) in isogenic wild-type and *nup116Δ* yeast strains, respectively (Fig. 5B). In contrast, the *CYH2* pre-mRNA/mRNA ratios on polyribosomes isolated from an isogenic *upf1Δ* mutant was 0.43 (±0.08, $n = 2$, data not shown). Thus, we conclude that *CYH2* pre-mRNA accumulates in *nup116Δ* yeast strains due to a defect in mRNA transport and not NMD.



C.

Strain	Canavanine concentration		
	25 μg/ml	100 μg/ml	300 μg/ml
Wild type	+	+	+
upf1-Δ5	+	+/-	-
nup100Δ	+	+	+
Nup116Δ	+	+	+

Figure 5. mRNA accumulation and nonsense suppression phenotype of *nup100Δ* and *nup116Δ* cells. (A) Northern blot of total RNA extracted from W303a (wild-type), AAY273 (*upf1Δ-5*), SWY3 (*nup100Δ*), and SWY31 (*nup116Δ*) cells probed with *CYH2* radiolabeled DNA. The locations of the *CYH2* pre-mRNA and mRNA bands are indicated to the right of the Northern as pre-mRNA and mRNA. The *CYH2* pre-mRNA/mRNA ratio is under the Northern blot for each sample. (B) Distribution of *CYH2* pre-mRNA and mRNA after sucrose density gradient sedimentation. Cell extracts from wild-type (upper PhosphorImage) and *nup116Δ* (lower PhosphorImage) cells were resolved on sucrose gradients. RNA was recovered and used for Northern blots. The Northern blots were probed with *CYH2* radiolabeled DNA. The *CYH2* pre-mRNA and mRNA bands are indicated to the right of the blots. (C) Nonsense suppression phenotypes of W303a (wild-type), AAY273 (*upf1Δ-5*), SWY3 (*nup100Δ*), and SWY31 (*nup116Δ*) yeast cells carrying the *can1-100* allele. The phenotype was determined by growth on YPD containing 25, 100, or 300 μg/ml canavanine. Sensitivity to canavanine is indicative of suppression. (+) Growth, (–) no growth, (+/–) weak growth.

The nonsense suppression phenotype of *nup116Δ* yeast cells was examined using the *can1-100* allele. We compared the growth of SWY31 (*nup116Δ*) yeast cells to W303a (wild-type), AAY273 (*upf1Δ*), and SWY3 (*nup100Δ*) yeast cells carrying the *can1-100* mutation on

plates containing canavanine (Fig. 5C). The *UPF1 NUP100 can1-100* cells are resistant to canavanine. As expected, they grew on medium containing up to 300 μg/ml canavanine. The *can1-100* allele is suppressed in *upf1Δ* yeast cells. These yeast cells cannot grow on medium containing greater than 100 μg/ml canavanine. Like the isogenic wild-type strain, the *nup100Δ* and *nup116Δ* yeast strains were able to grow on medium containing up to 300 μg/ml canavanine. Thus *can1-100* is also not suppressed in a *nup116Δ* yeast strain.

3.5. The C-terminal 158 amino acids of Upf1p are required for the interaction of Upf1p with Nup100p and Nup116p

To determine what part of Upf1p is important for the interaction with Nup100p and Nup116p, we tested whether amino acid substitutions in the Upf1p helicase domain or the deletion of the Upf1p C-terminal 158 amino acids affected the interaction of Upf1p with Nup100p and Nup116p. We replaced the wild-type *UPF1* sequence in pAA303, which encodes *UPF1-GAL4*$_{BD}$, with the sequence from four missense *upf1* alleles, *UPF1-D4*, *DE572AA*, *RR793AA*, and *TR800AA*, that have mutations in the helicase domain (Leeds et al., 1992; Weng et al., 1996a) to create pAA304, pAA318, pAA301, and pAA302, respectively. We also constructed a deletion allele of *UPF1* that is missing the C-terminal 158 amino acids (*upf1-Δ814-971*; pAA324 and pAA377). The *CYH2* pre-mRNA accumulation and suppression phenotypes of yeast strains carrying the *UPF1* alleles are summarized in Table 3. Our results generally agree with previously published works (Weng et al., 1996a; Atkin et al., 1997). All of these mutant *UPF1* alleles accumulate *CYH2* pre-mRNA and suppress nonsense and/or frameshift alleles. Yeast strains carrying *UPF1-D4*, *DE572AA*, or *RR793AA* have intermediate relative *CYH2* pre-mRNA/mRNA accumulation ratios. Yeast strains carrying *TR800AA* or an *upf1* null allele accumulate similar amounts of *CYH2* pre-mRNA. We also observed one minor difference from a previously published work. Specifically, Weng et al. (1996a) did not detect nonsense suppression in yeast strains with *RR793AA*, whereas we observed weak suppression in *RR793AA* yeast strains. This difference might be due to differences in the way nonsense suppression was assayed.

The *GAL4*$_{BD}$ fusions, containing the mutant *upf1* sequences, were transformed into PJ69-4a and mated to PJ69-4α containing the GAL4$_{AD}$fusion hybrids to create diploids with all possible combinations of DNA binding and activation domain hybrids. *ADE2* and *HIS3* reporter gene activity in the diploids was assayed by growth on (1) synthetic complete medium lacking uracil, leucine, and adenine medium, and (2) synthetic complete medium lacking uracil and leucine and histidine and containing 1 mM 3-aminotriazole medium, respectively (Fig. 6). The *UPF1-D4*, *DE572AA*, *RR793AA*, and *TR800AA GAL4*$_{BD}$ fusions all resulted in reporter gene activation with Gal4$_{AD}$Nup100 and Gal4$_{AD}$Nup116 but not Gal4$_{AD}$, on its own, or Gal4$_{AD}$Sip1. The deletion of the Upf1p C-terminal 158 amino acids abolished reporter gene activation with Gal4$_{AD}$Nup100 and Gal4$_{AD}$Nup116. Thus, we conclude that the C-terminal 158 amino acids of Upf1p are important for the interaction of Upf1p with Nup100p and Nup116p.

Table 3. Effect of mutations in the *UPF1* helicase domain on *CYH2* pre-mRNA accumulation and suppression

UPF1 allele	Relative *CYH2* pre-mRNA/mRNA accumulation ratio	Nonsense suppression phenotype		Allosuppression phenotype	Source
		leu2-2	*tyr7-1*		
UPF1-D4	3.5[a]	N.D.	N.D.	+	Atkin et al., 1997
DE572AA	3.7[b]	+	+/–	N.D.	This study
RR793AA	2.1[b]	+/–	+/–	N.D.	This study
TR800AA	5.3[b]	+	+/–	N.D.	This study
upf1-Δ814–971	7.2[b]	+	+	N.D.	This study
UPF1	1.0[c]	—	—	—	This study; Atkin et al., 1997
upf1	7.5[a], 6.3[b]	+	+	+	This study; Atkin et al., 1997

The *CYH2* pre-mRNA accumulation and nonsense suppression phenotypes of yeast with wild-type and mutant *UPF1* alleles were assessed in PLY146 (*leu2-2 tyr7-1 upf1-Δ2*, Table 1) transformed with pRS314, pRS314UPF1, pYCplac22DE572AA, pYCplac22RR793AA, pYCplac22TR800AA, and pAA377 (*upf1-Δ814-917*; Table 1). The relative accumulation ratios were calculated by dividing the *CYH2* pre-mRNA/mRNA accumulation ratio for each strain by the ratio established for the strain carrying wild-type *UPF1*. The *CYH2* pre-mRNA/mRNA accumulation ratio was determined by quantitative Northern blotting. The nonsense suppression phenotype was determined by growth on synthetic complete medium lacking tryptophan and leucine and on the synthetic complete medium lacking tryptophan and tyrosine. Growth in the absence of leucine or tyrosine is indicative of suppression; (+) growth, (–) no growth, (+/–) weak growth.

Previously, the allosuppression phenotypes of *UPF1*, *upf1-2*, and *UPF1-D4* mutants were assessed in PLY38 (*his4-38*, *SUF1-1*; Table 1, Atkin et al., 1997) transformed with pRS316UPF1, pRS316, and pRS316UPF1-D4 (Table 1). The allosuppression phenotype was determined by growth on synthetic complete medium lacking uracil and histidine. Growth in the absence of histidine is indicative of allosuppression; (+) growth, (–) no growth, N.D. not determined.

a. *CYH2* pre-mRNA and mRNA accumulation was measured on Northerns prepared with total RNA extracted from PLY38 (Atkin et al., 1997).
b. *CYH2* pre-mRNA and mRNA accumulation was measured on Northerns prepared with total RNA extracted from AAY320.
c. The relative *CYH2* pre-mRNA/mRNA accumulation ratios determined using total RNA from PLY38 and AAY320 were the same.

3.6. Conclusions

- Upf1p interacts with the nuclear pore proteins, Nup100p and Nup116p. We observed these interactions by two-hybrid assays (Table 2, Fig. 2) and coimmunoprecipitation (Fig. 3). These interactions are dependent on the presence of the C-terminal 158 amino acids of Upf1p (Fig. 6). Our results do not distinguish between a direct interaction or an interaction that is mediated by other molecules. These other molecules could be proteins or RNAs because Nup100p, Nup116p, and Upf1p all contain RNA binding motifs and both Nup116p and Upf1p bind RNA in vitro (Fabre et al., 1994;Weng et al., 1996a).

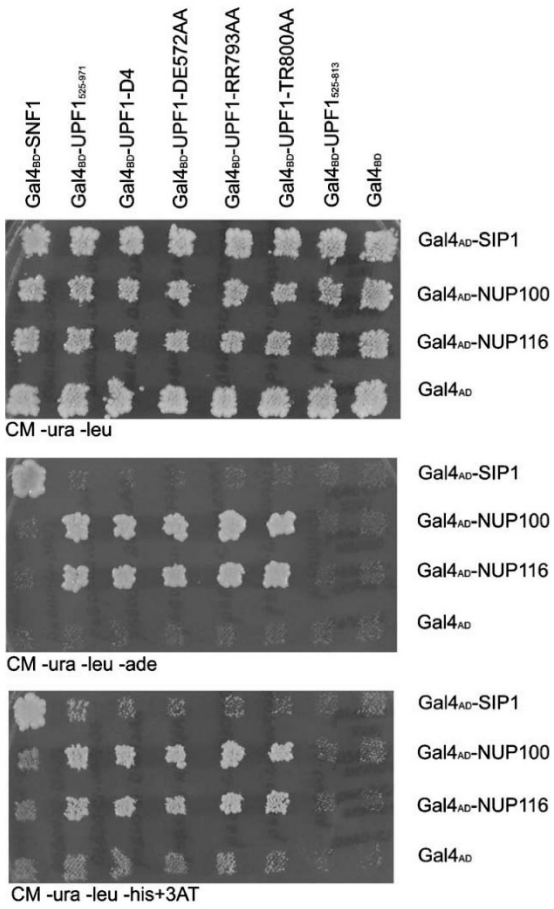


Figure 6. The C-terminal 158 amino acids of Upf1p are required for the interaction of Upf1p with Nup100p and Nup116p. Yeast strains were prepared for the two-hybrid interaction assay by mating PJ69-4a (Table 1) transformed with plasmids containing the *GAL4* DNA binding domain constructs (pAA296 [Gal4BDSNF1], pAA303 [Gal4BDUPF1525–971], pAA304 [Gal4BDUPF1-D4], pAA318 [Gal4BDUPF1-DE572AA], pAA301 [Gal4BDUPF1-RR793AA], pAA302 [Gal4BDUPF1-TR800AA], pAA324 [Gal4BDUPF1525–813], and pGBCU-C1 [Gal4BD]; Table 1), with PJ69-4α transformed with plasmids containing the *GAL4* activation domain (pGAD-SIP1 [Gal4ADSIP1], pAA265 [Gal4ADNUP100], pAA378 [Gal4AD-NUP116], pGAD-C1 [Gal4AD]; Table 1). *ADE2* and *HIS3* reporter gene activity was assayed by growth on synthetic complete medium lacking uracil, leucine, and adenine (CM-ura-leu-ade) medium and on synthetic complete medium lacking uracil, leucine, and histinine and containing 1 mM 3-aminotriazole (CM-ura-leu-his, +3-AT) medium, respectively. Reporter gene activation is not required for growth on synthetic complete medium lacking uracil and leucine (CM-ura-leu). The Gal4 DNA binding (top) and activation (right) domain hybrids are indicated. The DNA binding domain hybrids containing sequences from *upf1* missense alleles (*UPF1-D4*, DE572AA, RR793AA, and TR800AA), in conjunction with both the Nup100p and Nup116p activation domain hybrids, still activate the expression of the *GAL2-ADE2* and *GAL1-HIS3* reporter genes. However, no reporter gene activation is observed when the C-terminal 158 amino acids of Upf1p are absent.

- The biological significance of the interactions between Upf1p and the nuclear pore proteins, Nup100p and Nup116p, is supported by three lines of evidence: (1) a genetic interaction between *NUP100* and *UPF1*, (2) a common cellular location for these proteins, and (3) previously identified interactions with an overlapping set of proteins involved in mRNA transport. *NUP100* and *UPF1* interact genetically because a *nup100* deletion is epistatic to an *upf1* deletion for the suppression of the *can1-100* nonsense allele (Fig. 4).We have not yet seen a direct genetic interaction between *NUP116* and *UPF1*. Upf1p, Nup100p, and Nup116p are all predominantly located in the cytoplasmic cellular compartment. Upf1p is distributed throughout the cytoplasm (Atkin et al., 1995); Nup100p and Nup116p are located on the cytoplasmic face of the central transporter region in the nuclear pore (Suntharalingam and Wente, 2003). Thus, Upf1p could interact with Nup100p and Nup116p in vivo. All three proteins interact with mRNA transport components. Nup100p and Nup116p interact with Mex67p and Kap104p (Aitchison et al., 1996; Strawn et al., 2001). Mex67p is a homologue of the mammalian export factor TAP, which, along with p15, forms a stable complex with Y14 and REF/Aly, components of the exon junction complex required for nonsense mRNA recognition in mammals (Kim et al., 2001). Kap104 binds Hrp1p, the DSE-binding protein needed to trigger NMD through an interaction with Upf1p (Aitchison et al., 1996; Gonzalez et al., 2000). Thus, we conclude that the interaction between Upf1p and Nup100p is biologically significant.
- The role of Upf1p in nonsense suppression is separable from its role in nonsense mRNA accumulation. We showed that the deletion of *NUP100* prevents suppression of the *can1-100* nonsense allele, but not the accumulation of *CYH2* pre-mRNA in an *upf1* deletion mutant (Fig. 4). Our results are consistent with previous work by Weng et al. (1996a,b) who proposed that Upf1p is a multifunctional protein involved in modulating mRNA decay and translation termination at nonsense codons. They showed that DE572AA, RR793AA, and TR800AA mutations in the helicase region of Upf1p inactivated its mRNA decay function but prevented the cosuppression of *leu2-2* and *tyr7-1*, two nonsense alleles (Weng et al., 1996a). Furthermore, they described mutations (*upf1Δ1*, C65S, C84S, and C148S) in the amino-terminal cysteine- and histidine-rich region of Upf1p that have normal nonsense-mediated mRNA decay activities but are able to cosuppress *leu2-2* and *tyr7-1* nonsense alleles (Weng et al., 1996b). The phenotype of our *nup100Δ upf1Δ* strain resembles the phenotypes of cells with the DE572AA, RR793AA, and TR800AA *upf1* alleles. Interestingly, the DE572AA, RR793AA, and TR800AA mutations in Upf1p do not affect the interaction with Nup100p (Fig. 6). Thus, the similar phenotypes are not simply due to a disruption in the Upf1p interaction with Nup100p, but suggest a more complicated mechanism.
- In the current view of NMD (reviewed in the Introduction), Upf1p is recruited to the surveillance complex by interactions with Upf2p and/or the translation release factors eRF1 and eRF3 when translation terminates upstream of the complex (Czaplinski et al., 1998; Le Hir et al., 2001). Our results suggest that Upf1p could join the complex earlier than previously thought, either during or immediately after mRNA transport, and that the interaction between Upf1p and eRF1 and eRF3 might instead be a trigger

for NMD. This is significant because it suggests a mechanism to ensure that Upf1p associates with newly synthesized mRNA as it is transported from the nucleus to the cytoplasm.

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