A factor required for nonsense-mediated mRNA decay in yeast is exported from the nucleus to the cytoplasm by a nuclear export signal sequence

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Accepted 28 August; published on WWW 14 October 1998

SUMMARY

In Saccharomyces cerevisiae, Upf3p is required for nonsense-mediated mRNA decay (NMD). Although localized primarily in the cytoplasm, Upf3p contains three sequence elements that resemble nuclear localization signals (NLSs) and two sequence elements that resemble nuclear export signals (NESs). We found that a cytoplasmic reporter protein localized to the nucleus when fused to any one of the three NLS-like sequences of Upf3p. A nuclear reporter protein localized to the cytoplasm when fused to one of the NES-like sequences (NES-A). We present evidence that NES-A functions to signal the export of Upf3p from the nucleus. Combined alanine substitutions in the NES-A element caused a re-distribution of Upf3p to a subnuclear location identified as the nucleolus and conferred an Nmd⁻ phenotype. Single mutations in NES-A failed to affect the distribution of Upf3p and were Nmd⁺. When an NES element from HIV-1 Rev was inserted near the C terminus of a mutant Upf3p containing multiple mutations in NES-A, the cytoplasmic distribution typical of wild-type Upf3p was restored but the cells remained phenotypically Nmd⁻. These results suggest that NES-A is a functional nuclear export signal. Combined mutations in NES-A may cause multiple defects in protein function leading to an Nmd⁻ phenotype even when export is restored.

Key words: mRNA decay, Saccharomyces cerevisiae, Nuclear export

INTRODUCTION

It has been observed in a variety of eukaryotes including yeast, nematodes, mice and humans that mRNAs containing a premature stop codon (nonsense mRNAs) decay at an accelerated rate relative to their wild-type counterparts (Leeds et al., 1991; Pulak and Anderson, 1993; Perlick et al., 1996; Applequist et al., 1997). The accelerated decay of nonsense mRNAs is referred to as nonsense-mediated mRNA decay (NMD).

In Saccharomyces cerevisiae, intrinsic mRNA decay proceeds through a series of highly ordered steps that initiate with the shortening of the poly(A) tail at the 3' end of the mRNA (Decker and Parker, 1993; Muhlrad et al., 1994). Following deadenylation, the 7-methyl guanosine cap at the 5' end of the mRNA is removed by the decapping enzyme Dcp1p which leads to rapid 5'→3' degradation of the mRNA by the exoribonuclease Xrn1p. Deadenylation-dependent decapping is thought to be the most common decay pathway for degrading wild-type yeast mRNAs (Caponigro and Parker, 1996). In contrast, nonsense mRNAs are immediately decapped and degraded prior to the shortening of the poly(A) tail. The accelerated decay of nonsense mRNAs is achieved at least in part through bypassing of one of the rate-determining steps in the intrinsic mRNA decay pathway (Muhlrad and Parker, 1994).

The three trans-acting factors Upf1p, Upf2p, and Upf3p, are required for NMD in S. cerevisiae (Leeds et al., 1991, 1992; Cui et al., 1995; He and Jacobson, 1995; Lee and Culbertson, 1995). Loss-of-function mutations in any of the three UPF genes inactivates the NMD pathway. In a upf⁻ genetic background, the decay rates of nonsense mRNAs are stabilized to a rate that equals or closely approaches their wild-type counterparts. Our laboratory is focused on elucidating the basis by which nonsense mRNAs are selectively recruited as substrates for NMD.

All previous evidence in yeast indicates that both the recruitment and decay of nonsense mRNAs occurs in the cytoplasm in association with translating ribosomes. Cytoplasmic decay was suggested by the observation that nonsense mRNAs were detected in the cytoplasm in situ only in a strain lacking the NMD pathway (Long et al., 1995). Several results have implicated translation in NMD. Nonsense mRNAs are stabilized by nonsense tRNA suppressors (Losson and Lacroute, 1979) and they are recruited into polyribosomes (Leeds et al., 1991; He et al., 1993; Zhang et al., 1997). In addition, a portion of the total cellular pool of Upf1p, Upf2p and Upf3p is found associated with polyribosomes (Leeds et al., 1991; He et al., 1993; Atkin et al., 1995, 1997; Zhang et al., 1997).

Physical interactions have been observed between some of the Upf proteins. Strong physical interactions were detected between Upf1p and Upf2p and between Upf2p and Upf3p.
using the two-hybrid system (He and Jacobson, 1995; He et al., 1997). An interaction between Upf1p and Upf3p was shown to be considerably weaker and dependent upon the presence of Upf2p. These results lead to the suggestion that Upf2p mediates an interaction between Upf1p and Upf3p, allowing for the formation of a heterotrimeric complex which was assumed to assemble on polyribosomes (He et al., 1997).

However, a more complicated scenario may exist. Upf proteins are not present in equimolar amounts, which limits the extent to which a heterotrimeric complex could form, and Upf1p associates with polyribosomes independently of Upf2p and Upf3p (Atkin et al., 1997). In addition, Upf3p is required for the association of Upf2p with polyribosomes while Upf1p appears to facilitate the dissociation of Upf2p. Based on these results, it was proposed that the Upf proteins may form a stepwise series of bipartite complexes rather than a heterotrimeric complex (Atkin et al., 1997). Upf3p could bind to Upf2p in order to recruit Upf2p into polyribosomes. Once associated with polyribosomes, Upf2p could facilitate NMD by interacting with Upf1p to form a Upf1p-Upf2p complex. Evidence suggests that the interaction between Upf1p and Upf2p is required for the NMD pathway (He et al., 1996). These interpretations of existing data suggest that Upf3p performs an early step in the selective recruitment of nonsense mRNAs into the NMD pathway.

The association of the Upf proteins with polyribosomes suggests that at least a portion of the total cellular pool of the Upf proteins resides in the cytoplasm. Indirect immunofluorescence microscopy and cell fractionation has shown that the majority of Upf1p resides in the cytoplasm associated with polyribosomes (Atkin et al., 1995). While a portion of Upf2p and Upf3p are associated with polyribosomes and therefore at least partly cytoplasmic, both of these proteins contain sequence motifs that resemble bipartite nuclear localization signals (NLSs) (He and Jacobson, 1995; Lee and Culbertson, 1995). The presence of these sequences suggest that Upf2p or Upf3p may also enter the nucleus.

In this paper we show that while the majority of Upf3p is localized in the cytoplasm, Upf3p enters and exits the nucleus. The export of Upf3p was examined in detail using a genetic approach. We found that export is mediated by a leucine-rich nuclear export signal (NES) that resembles the NESs found in Gle1p and Kap95p (Murphy and Wente, 1996; Iovine and Wente, 1997). Our results suggest that Upf3p may be required for a function in the NMD pathway that occurs in the nucleus. This is the first indication that the nucleus may have a role in the NMD pathway in yeast.

**MATERIALS AND METHODS**

**Strains, media, and genetic methods**

*S. cerevisiae* strain YM4126 (*MATa* ura3-52 his3-A200 ade2-101 lys2-801 trpl-903 leu2-3-112 canR GAL2*), a gift from M. Johnston, was used for immunofluorescence microscopy. Strain LR5323 (*MATa* his4-38 SUF1-1 trpl-1Δ1 upf3-A1 ura3-52 leu2-Δ1) was used for immunofluorescence microscopy and the *his4-38/SUF1-1* allosuppression assay. Strains were constructed, grown, and maintained as described previously (Sherman, 1991). All yeast transformations were done by electroporation (Grey and Brendel, 1992).

**Plasmid constructions**

The plasmids used in this study are listed in Table 1. Plasmid DNA was isolated from *Escherichia coli* strain DH5α using either a QiAprep spin miniprep kit (Qiagen, Santa Clarita, CA) or the method of Lee and Rasheed (1990). All restriction endonucleases were purchased from New England Biolabs (Beverly, MA). DNA sequence analysis was performed using the ABI sequenase kit from Perkin Elmer (Foster City, CA) and an ABI 377 automated fluorescent sequencer. Oligonucleotides were purchased from Operon (Alameda, CA). All oligonucleotides except those used for sequence analysis are listed in Table 2. Polymerase chain reactions were performed using cloned *Pfu* polymerase (Stratagene, La Jolla, CA).

To construct translational fusions between the *UPF3* NLS sequences and the *lacZ* gene, DNA fragments containing the putative NLS sequences were amplified by PCR using oligonucleotides Lso107 and Lso108 for NLS1, Lso109 and Lso110 for NLS2, and Lso111 and Lso112 for NLS3 (Table 2). Oligonucleotides Lso107-Lso112 contain recognition sites for *BanHI* or *SalI* at their ends. The resulting PCR products containing sequences coding for NLS1, NLS2 and NLS3 were digested with *BanHI* and *SalI* and then ligated into the same sites in *psJ101*, which contains *lacZ*, creating plasmids pLS95, pLS96, and pLS97 respectively (Table 1). Constructs were confirmed by restriction endonuclease mapping and DNA sequence analysis.

Two putative NES elements, NES-A and NES-B were identified in Upf3p by analyzing the amino acid sequence using Findpatterns (Genetics Computer Group, Inc.). Translational fusions were made between the two elements and a NLS-*lacZ* fusion contained on pDU254 (Ursic et al., 1995). The NLS in this fusion is from yeast *SEN1*, which codes for a putative nuclear RNA helicase (DeMarini et al., 1992). Complementary oligonucleotides (Lso103-Lso106, Table 2) were synthesized containing the *UPF3* NES-A and NES-B sequences. Oligonucleotides Lso103-106 also contained overhanging ends for the restriction sites *NheI* and *SphI*. The respective oligonucleotides (Lso103/Lso104 and Lso105/Lso106) were annealed and ligated at the same sites in pDU254, yielding plasmids pLS93 and pLS94, respectively (Table 1). Following a similar strategy, plasmids pLS102, pLS103, and pLS104, which contain point mutations in NES-A (Table 1) were generated using complementary oligonucleotides Lso208/Lso209, Lso210/Lso211 and Lso212/Lso213 respectively (Table 2). All constructs were confirmed by DNA sequence analysis.

Plasmid pLS51 contains a translational fusion between sequences encoding the Influenza virus hemagglutinin (HA) epitope recognized by the monoclonal antibody 12CA5 and *UPF3* (Atkin et al., 1997). The insertion of the HA epitope occurs immediately following the codon corresponding to the 4th amino acid in Upf3p (site 1, Fig. 1A). To construct a similar allele on a multi-copy 2μ plasmid, a *SpeI-KpnI* fragment from pLS51 containing the epitope-tagged *UPF3* allele was cloned into the same sites in the 2μ vector pRS426 yielding plasmid pLS73. These constructs, along with pLS17 or the vector alone, were transformed separately into strain LRSy323, which contains upf3-A1 (Lee and Culbertson, 1995). Western blotting of SDS-polyacrylamide gels was used to confirm that the epitope-tagged protein was expressed and migrated at a position corresponding to the predicted size of 49 kDa (see Results).

Base substitutions in *UPF3* and *UPF3-HA* designed to change conserved leucine and isoleucine residues in the NES-A element to alanines were generated using the Chameleon™ Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Oligonucleotides MLO1-MLO3 and MLO14 (Table 2) and pLS17 template DNA were used to construct plasmids pML4, pML6, pML5, and pML7, respectively (Table 1). Oligonucleotides MLO1-MLO3 (Table 2) and pLS1 template DNA were used to construct plasmids pML11, pML9 and pML14, respectively (Table 1). Similarly, oligonucleotides RSO39-RSO41 (Table 2) and pML14 template DNA were used to construct plasmids pRLS160, pRLS159 and pRLS158.
respectively (Table 1). All mutations were confirmed by DNA sequence analysis.

Sequences coding for the wild-type HIV-1 Rev NES and the M10 Rev NES (Meyer and Malim, 1994; Fischer et al., 1995) were fused with UPF3-HA and mutant upf3-HA alleles by synthesizing complementary oligonucleotides (RS031–RS034; Table 2) that contain the HIV-1 Rev NES and the M10 Rev NES sequences. The annealed oligonucleotides RS031/RS032 and RS033/RS034 also produced overhanging ends compatible with the restriction site NotI. The heterologous NES sequences were ligated near the N terminus of Upf3p-HA (site 1, Fig. 1A) at the NotI sites in plasmid pLS51, thereby replacing the fragment containing the HA epitope, and yielding plasmids pRLS132 and pRLS133 (Table 1). Following a similar strategy, oligonucleotides RS031/RS032 and RS033/RS034 were ligated at the NotI sites in pML14 to yield plasmids pRLS127 and pRLS128 (Table 1).

Sequences coding for the NES of Kap95p and NES-A from Upf3p were fused with UPF3-HA and upf3-Triple-HA by synthesizing

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*Sikorski and Hieter, 1989.
‡ Christianson et al., 1992.
§ Atkin et al., 1997.
|| Ursic et al., 1995.

The heterologous NES sequences for the HIV-1 Rev NES and M10 NES were also inserted into a NorI site that was created after the last sense codon in UPF3 (site 2, Fig. 1A). The NorI site was created in pLS17 by Chameleon mutagenesis using oligonucleotide RSO30 (Table 2). Oligonucleotides RSO31/RSO32 and RSO33/RSO34 were ligated into the newly created NorI site creating plasmid pLS17-Rev and pLS17-M10. A BseRI-XbaI fragment from pLS17-Rev was replaced with BseRI-XbaI fragments from pML14, pLS51, pRLS158, pRLS159 and pRLS160 to yield plasmids pRLS140, pRLS141, pRLS164, pRLS165 and pRLS168, respectively (Table 1). Following a similar strategy, the BseRI-XbaI fragment from pLS17-Rev was replaced with BseRI-XbaI fragments from pML14 and pLS51 to yield pRLS142 and pRLS143, respectively (Table 1).

Sequences coding for the NES of Kap95p and NES-A from Upf3p were fused with UPF3-HA and upf3-Triple-HA by synthesizing
complementary oligonucleotides (RSO35-RSO38; Table 2) that contain the Upf3p NES-A and Kap95p NES sequences. Annealed oligonucleotides RSO35/RSO36, which contain the NES-A sequence, and RSO37/RSO38, which contain the Kap95p NES sequence, were inserted at site 1 and site 2 of Upf3p-HA and Upf3p-Triple-HA and RSO37/RSO38, which contain the Kap95p NES sequence, were annealed to complementary oligonucleotides (RSO35-RSO38; Table 2) that contain the Upf3p NES-A and Kap95p NES sequences. Annealed oligonucleotides RSO35/RSO36, which contain the NES-A sequence, and RSO37/RSO38, which contain the Kap95p NES sequence, were inserted at site 1 and site 2 of Upf3p-HA and Upf3p-Triple-HA following the strategy described above for the HIV-1 Rev NES and M10 NES.

In some experiments, mutant Upf3p-HA proteins containing triple amino acid substitutions in NES-A were analyzed in strains carrying two tandem copies of the corresponding mutant alleles on a plasmid. To construct the appropriate plasmids, pML14, pRLS140 and pRLS142 were digested with KpnI and the 3′ end overhangs removed using T4 DNA polymerase (New England Biolabs, Beverly, MA). The linearized plasmids were then digested with BstXI to release a fragment containing the entire Upf3p-HA coding region. The fragments were ligated back into their respective plasmids that had been first digested with SacI, blunt-ended using T4 DNA polymerase, and subsequently digested with BstXI to create plasmids pRLS125, pRLS145 and pRLS144, respectively (Table 1).

**Assay for NMD by allosuppression**

The effects of mutations on the NMD pathway were assayed by the his4-38/SUFI-1 allosuppression assay (Culbertson et al., 1980; Leeds et al., 1991). Strains carrying the his4-38 frameshift mutation cannot grow on medium lacking histidine. The SUFI-1 frameshift suppressor tRNA allows readthrough of the his4-38 frameshift mutation sufficient to confer growth on medium lacking histidine at 30°C but not at 37°C. Since the his4-38 frameshift mutation brings into register a downstream nonsense codon, his4-38 mRNA levels are decreased due to the NMD pathway. Inactivation of the NMD pathway by a loss-of-function mutation in any of the three Upf genes stabilizes the his4-38 mRNA. The increased levels of his4-38 mRNA and the presence of the SUFI-1 frameshift suppressor tRNA allow a his4-38 SUFI-1 upf- strain to grow on medium lacking histidine at both 30°C and 37°C.

Growth at 37°C was assayed by plating serial dilutions of cells from culture. Primary cultures were grown to an OD600 = 0.4-0.6 in media lacking uracil to select for the presence of the URA3 plasmids. The cultures were first diluted back to an OD600=0.18-0.22 and tenfold dilutions were plated as 5 μl drops on plates containing media lacking uracil to assay growth at 37°C when cells are selected to harbor the URA3 plasmids. Identical cultures were plated on media lacking both uracil and histidine to assay the extent of growth due to mutations in UPF3. Plates were incubated at 37°C between 48 and 72 hours.

**Northern and western blotting**

Total RNA was isolated by hot phenol extraction as described by Leeds et al. (1991). Northern blotting was performed as described previously (Atkin et al., 1997), with all samples denatured by treatment with glyoxal and DMSO. The DNA probe used to detect CYH2 pre-mRNA and mRNA is complementary to nucleotides 15-780 of CYH2 which includes both the ORF and intron. The DNA probes used to detect HIS4 and actin mRNAs are complementary to nucleotides 1-1000 of HIS4 mRNA and 6-1428 of ACT1 mRNA, respectively. Total protein was isolated for western blotting by solubilizing cell pellets from 10 ml mid-log phase cultures as described by Atkin et al. (1995). The soluble lysate was quantitated with a BCA protein assay (Pierce, Rockford, IL). Protein electrophoresis, western transfer and quantitative detection of Upf3p-HA were performed as described previously (Atkin et al., 1997). The primary antibodies, 12CA5, were incubated and subsequently washed in TBS, pH 7.5, followed by brief washes in TBS, pH 9.5. Incubation and subsequent washes of radio-labeled secondary antibodies (Amersham SJ. 431) were performed in TBS, pH 9.5. Both northern and western blots were quantitated with Molecular Dynamics Phosphorimager Model 425 (Sunnyvale, CA).

**Cellular localization**

To localize fusions to β-galactosidase, indirect immunofluorescence microscopy was performed as described previously (Ursic et al., 1994). Since expression of β-galactosidase hybrid proteins is under control of the GAL10 promoter, yeast strains were grown in the presence of 2% galactose for 10-16 hours to induce expression. To detect β-galactosidase, cells were decorated first with antibodies raised in rabbits against β-galactosidase (D. Ursic, personal communication) at a dilution of 1:40 and subsequently with a 1:600 dilution of fluorescein-conjugated goat F(ab')2 fragment specific for rabbit IgG(H+L) (Boehringer Mannheim, Indianapolis, IN). Protein electrophoresis, western transfer and quantitative detection of Upf3p-HA were performed as described previously (Atkin et al., 1997). The primary antibodies, 12CA5, were incubated and subsequently washed in TBS, pH 7.5, followed by brief washes in TBS, pH 9.5. Incubation and subsequent washes of radio-labeled secondary antibodies (Amersham SJ. 431) were performed in TBS, pH 9.5. Both northern and western blots were quantitated with Molecular Dynamics Phosphorimager Model 425 (Sunnyvale, CA).

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The primary antibodies were decorated with unconjugated affinity-purified goat antibodies specific to rat IgG (H+L) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) used at a dilution of 1:200. The secondary antibodies were detected by affinity-purified fluorescein (FITC)-conjugated donkey antibodies specific for goat IgG (H+L) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) used at a dilution of 1:200. To detect Rpa190p, rabbit antibodies specific for Rpa190p (provided by M. Nomura) were used at a dilution of 1:50 in conjunction with 1:200 dilution of affinity-purified Cy®5-conjugated donkey antibodies specific to rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). All antibodies were incubated with cells for 1-2 hours at room temperature. Diamidino-2-phenylindole (DAPI) was used to stain DNA.

**RESULTS**

**Epitope-tagged Upf3p is functional in NMD**

In order to determine whether an epitope-tagged Upf3p is suitable for inferring the localization of wild-type Upf3p, we assayed the ability of UPF3-HA to provide wild-type function. UPF3-HA codes for a Upf3 protein that has three copies of the HA epitope inserted at the N terminus (see Materials and Methods; site 1, Fig. 1A). The function of UPF3-HA was assayed in strains carrying the chromosomal disruption upf3-ΔI by three methods: allosuppression, his4-38 mRNA accumulation and CYH2 pre-mRNA accumulation.

Using the allosuppression assay (see Materials and Methods and Fig. 1), the ability of Upf3p-HA to function in NMD was determined by comparing the growth of a strain carrying UPF3-HA to strains carrying either the null or wild-type UPF3 allele. In this assay, vigorous growth is indicative of impaired Upf3p function while lack of growth signifies wild-type Upf3p function. Strain LRSy323 (his4-38 SUF1-1 upf3-ΔI) was transformed separately with centromeric plasmids expressing UPF3 or UPF3-HA. The upf3-ΔI strain grew robustly at 37°C on medium lacking histidine (Fig. 1B1). In contrast, a transformant carrying the wild-type UPF3 allele failed to grow at 37°C (Fig. 1B7). The growth of a transformant carrying UPF3-HA was also significantly reduced at 37°C although not as diminished as compared to the wild-type UPF3 strain (Fig. 1B6).

his4-38 mRNA and CYH2 pre-mRNA are both substrates for the NMD pathway (Leeds et al., 1991; He et al., 1993). his4-38 is a +1 frameshift mutation that brings into register a downstream nonsense codon. CYH2, which codes for ribosomal protein L29 (Kaufer et al., 1983) contains an intron that is inefficiently spliced from the pre-mRNA (Swida et al., 1986). When CYH2 pre-mRNA is exported to the cytoplasm, it is rapidly degraded by the NMD pathway due to the presence of stop codons in the intron (He et al., 1993). Since the levels of accumulation of both of these nonsense mRNAs depend on the ability of the Upf proteins to function in the NMD pathway, we used quantitative northern blotting to assess the function of UPF3-HA.

The relative levels of accumulation of his4-38 mRNA in UPF3, UPF3-HA and upf3-ΔI strains were 1.0, 1.0 (±0.3), and 3.2 (±1.4), respectively (Fig. 2A). Upf3p-HA functioned 104% (±13%) as compared to wild-type Upf3p in reducing the accumulation of his4-38 mRNA. The relative levels of CYH2 pre-mRNA in UPF3, UPF3-HA, and upf3-ΔI were 1.0, 1.6 (±0.3), and 5.2 (±1.2), respectively (Fig. 2B). Upf3p-HA functioned 86% (±8%) as well as wild-type Upf3p in reducing the accumulation of the CYH2 pre-mRNA.

**Fig. 1. Effect of mutant upf3 alleles on allosuppression of his4-38.** (A) A schematic representation of Upf3p. Upf3p is predicted to be a 45 kDa protein of 387 amino acids. The solid black boxes correspond to amino acids 15-31, 58-74, and 284-300 and represent the three bipartite NLS motifs (Lee and Culbertson, 1995). The solid gray boxes correspond to amino acids 88-97 and 151-160 and represent the two NES motifs. NES-A and NES-B were identified by scanning the Upf3p amino acid sequence in both directions using Findpatterns (Genetics Computer Group). Open triangles above the bar represent the insertion sites for the HA epitope and heterologous and homologous NES sequences. Site 1 and site 2 correspond to insertion sites after the 4th amino acid and the 387th amino acid of Upf3p, respectively. The domain of Upf3p identified to interact with Upf2p corresponds to amino acids 78-278 (He et al., 1997). (B,C) Mutations in the NES-A element of UPF3 were tested for their ability to complement the null allele upf3-ΔI. The mutant upf3 alleles were introduced on centromeric plasmids into strain LRSy323 (his4-38 SUF1-1 upf3-ΔI). Complementation was assayed by monitoring growth due to allosuppression of the his4-38 frameshift mutation (see Materials and Methods). Relative growth rates were compared by plating 10 2, 10 3 serial dilutions (left to right) of log phase cultures on medium lacking uracil (left panels) and medium lacking uracil and histidine (right panels) at 37°C. The left panels in B and C show relative growth under conditions that select for the presence of URA3 plasmids carrying alleles of UPF3. The right panels show the relative growth of the same strains under conditions where the extent of growth is proportional to the extent of impairment of Upf3p function. (B) Epitope-tagged versions of mutant upf3 alleles. Strain LRSy323 (his4-38 SUF1-1 upf3-ΔI) was transformed with plasmid pRS316 (B1), pML9 (upf3-L93A-HA) (B2), pML11 (upf3-L88A-HA) (B3), pML14 (upf3-Tripe-HA) (B4), pRLS125 (2x-upf3-Tripe-HA) (B5), pLS51 (UPF3-HA) (B6) and pLS17 (UPF3) (B7). (C) Untagged versions of mutant upf3 alleles. Strain LRSy323 was transformed with plasmid pRS316 (C1), pLS17 (UPF3) (C2), pMLA (upf3-L88A) (C3), pML6 (upf3-L93A) (C4), and pML5 (upf3-Tripe) (C5).
Based on these results, we conclude that the presence of the HA epitope slightly diminishes the function of Upf3p-HA in NMD. Diminished function, which most likely reflects partial loss of function, was indicated by the increased growth rate in the allosupression assay and the increased level of CYH2 pre-mRNA as compared to the wild-type *UPF3* strain. If function is partially lost, we expect the remaining function of Upf3p-HA to reflect that of wild-type Upf3p and the localization of Upf3p-HA and wild-type Upf3p to be the same.

The distribution of Upf3p-HA between the nucleus and the cytoplasm depends on gene dosage

The distribution of Upf3p-HA was examined in cells of strain LRSy323 (*upf3*-Δ1) transformed with either a centromeric plasmid expressing *UPF3-HA* (pLS51) or a 2μ plasmid expressing *UPF3-HA* (pLS73). Upf3 protein distribution was visualized by indirect immunofluorescence microscopy (Fig. 3, left column). The nucleus was identified using DAPI which stains DNA (Fig. 3, right column). Upf3p-HA expressed from the centromeric plasmid was detected in a granular distribution with local foci of intense staining throughout the cytoplasm. Substantially less staining was observed within the nucleus (Fig. 3B). The staining pattern was specific to Upf3p-HA since cells lacking the *UPF3-HA* allele displayed no detectable staining under identical conditions (Fig. 3A).

A different distribution of Upf3p-HA was observed when Upf3p-HA was expressed from the 2μ plasmid. Quantitative

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**Fig. 2.** Effect of mutant *upf3* alleles on RNA accumulation. Quantitative northern blotting was used to access the effects of mutations in the NES-A element of *UPF3* on the accumulation of his4-38 mRNA and CYH2 precursor mRNA. LRSy323 (his4-38 *upf3*-Δ1) was transformed with the following plasmids resulting in the corresponding genotypes: pRS316 (*upf3*-Δ1, lane 1), pLS17 (*UPF3*, lane 2), pML4 (*upf3*-L88A, lane 3), pML6 (*upf3*-L93A, lane4), pML5 (*upf3*-Triple, lane 5), pLS51 (*UPF3-HA*, lane 6), pML11 (*upf3*-L88A-HA, lane 7), pML9 (*upf3*-L93A-HA, lane 8), and pML14 (*upf3*-Triple-HA, lane 9). Quantitative values were derived from four to six independent trials each performed in duplicate. (A) The figure shows representative hybridization signals specific to his4-38 mRNA (upper panel) and actin (lower panel). Following quantitation of each trial, his4-38/actin ratios were normalized against the his4-38/actin ratio from the strain carrying *UPF3* (lane 2) to calculate the fold increase in his4-38 mRNA accumulation associated with each genotype relative to *UPF3*. The average fold increase from all trials were determined along with their associated sample standard deviations (s.d.). The sample standard deviation associated with *UPF3* is arbitrarily calculated as zero due to the necessity of normalizing before averaging and does not adequately represent the normal variation associated with this sample. (B) The figure shows the representative hybridization signal specific to precursor and mature forms of CYH2. The fold increase from each trial refers to average fold increase of the CYH2 precursor/mature ratio relative to the fold increase from the *UPF3* strain (lane 2) for each sample. Calculations were performed as in A.

**Fig. 3.** Subcellular localization of Upf3p-HA. The localization of epitope-tagged Upf3p-HA was determined by indirect immunofluorescence microscopy. Strain LR Sy323 (*upf3*-Δ1) was transformed with centromeric plasmid pRS316 (A), centromeric plasmid pLS51 (*UPF3-HA*) (B) and 2μ plasmid pLS73 (*UPF3-HA*) (C). Cells were decorated with 3F10 rat monoclonal antibodies specific to the HA tag. The primary antibodies were detected with unconjugated secondary antibodies in conjunction with fluorescein-conjugated tertiary antibodies. The left set of panels shows the detection of fluorescein staining in representative cells from each transformant. DAPI staining which marks the nucleus is shown in the right set of panels. Bar, 2.5 μM
western blots show that the abundance of Upf3p-HA increased eightfold when expressed from the 2μ plasmid as compared to expression from a centromeric plasmid (see Fig. 8). Upf3p-HA expressed from the 2μ plasmid was detected throughout the cytoplasm with a much less granular distribution than observed when Upf3p-HA was expressed from the centromeric plasmid. In addition, an intense staining was observed in a location adjacent to the area stained by DAPI which, as shown below, corresponds to the nucleolus (Fig. 3C).

In order to determine whether a portion of Upf3p-HA accumulates in the nucleolus, cells carrying UPF3-HA on either the centromeric plasmid or the 2μ plasmid were double-labeled with antibodies to detect Upf3p-HA and Rpa190p. RPA190 codes for the nucleolar-localized large subunit of RNA polymerase I (Memet et al., 1988; Oakes et al., 1993). The differentially labeled proteins were visualized by confocal microscopy.

The image of Upf3p-HA (green) (Fig. 4A,D) and the image of Rpa190p (red) (Fig. 4B,E) were overlaid (Fig. 4C,F) to determine the amount of co-distribution between the two proteins. Any overlap in staining between the two proteins is indicated by yellow. Little overlap in staining was seen between Upf3p-HA and Rpa190p when Upf3p-HA was expressed from the centromeric plasmid (Fig. 4A-C). In contrast, the staining pattern of Upf3p-HA significantly overlapped the staining pattern of Rpa190p when Upf3p-HA was expressed from the 2μ plasmid (Fig. 4D-F). This indicates that a portion of the total cellular pool of Upf3p-HA localizes to the nucleolus when Upf3p-HA is overexpressed.

Fig. 4. Co-localization of epitope-tagged Upf3 proteins with a nucleolar protein. Cells expressing UPF3-HA and mutant upf3-HA alleles containing mutations in NES-A were double labeled with antibodies against the HA epitope and Rpa190p. The differentially labeled proteins were visualized by confocal microscopy. Strain LRSy323 (upf3-Δ1) was transformed with pLS51 (UPF3-HA, Cen) (A-C), pLS73 (UPF3-HA, 2μ) (D-F), pML9 (upf3-L93A-HA) (G-I), pML11 (upf3-L88A-HA) (J-L), and pRLS125 (2×upf3-Triple-HA) (M-O). The fluorescein-stained Upf3-HA proteins shown in green (A,D,G,J,M) and the Cy5-stained Rpa190p shown in red (B,E,H,K,N) were overlaid to determine the amount of co-distribution between the two proteins (C,F,I,L,O). Overlap in staining is shown in yellow. Bar, 2.5 μM.
Sequence elements in Upf3p direct reporter proteins to the nucleus

Upf3p contains three separate regions that resemble bipartite nuclear localization signal (NLS) sequences (Lee and Culbertson, 1995) which could function to direct Upf3p into the nucleus (Fig. 1A). The three sequences, referred to as NLS1, NLS2, and NLS3, correspond to amino acids 15-31, 58-74, and 284-300, respectively. Each NLS motif was tested for its ability to function independently as a nuclear localization signal by evaluating whether they could function to direct β-galactosidase to the nucleus. 2μ plasmids carrying GAL10-NLS1-lacZ, GAL10-NLS2-lacZ, and GAL10-NLS3-lacZ and GAL10-lacZ (Table 1) were separately transformed into a GAL2+ strain (YM4126). The transformants were grown in galactose to induce expression of the fusion proteins. Following induction, the proteins were localized by indirect immunofluorescence microscopy (Fig. 5, left column). The localization of each NLS-β-galactosidase fusion protein was compared to the distribution of β-galactosidase alone. DAPI was used to locate the nucleus (Fig. 5, right column).

β-Galactosidase lacking an NLS was detected throughout the cell in both the nucleus and the cytoplasm (Fig. 5A). The NLS1- and NLS2-β-galactosidase fusion proteins were detected primarily in the nucleus in a region overlapping DAPI-stained nuclear DNA (Fig. 5B,C). The area of staining of these two reporter proteins extended beyond the area of DAPI staining, suggesting that both of the fusion proteins localize throughout the nucleoplasm. The NLS3-β-galactosidase fusion protein was also detected within the nucleus, but the staining pattern differed from the other NLS fusions (Fig. 5D). The area of staining was restricted to a subnuclear location adjacent to the area of DAPI staining. The nuclear localization of the NLS1-, NLS2- and NLS3-β-galactosidase fusion proteins indicates that any of the three NLS motifs identified in Upf3p could potentially function to direct Upf3p to the nucleus.

To examine whether NLS3-β-galactosidase localized to the nucleolus, cells were double-labeled with antibodies to detect β-galactosidase and Nop1p. Nop1p, the yeast homolog of mammalian fibrillarin, is an abundant nucleolar protein (Aris and Blobel, 1988; Tollervey et al., 1991). The relative distribution of NLS3-β-galactosidase and Nop1p were analyzed by indirect immunofluorescence microscopy. The image of β-galactosidase (green) (Fig. 6A) and the image of Nop1p (red) (Fig. 6B) were overlaid to determine the amount of co-distribution between the two proteins (Fig. 6C). Any overlap in staining is indicated by yellow.

There was significant overlap in the staining patterns of NLS3-β-galactosidase and Nop1p (Fig. 6C). The region of overlap corresponds to the nucleolus and is separate but adjacent to the region stained by DAPI (blue) (Fig. 6D). The co-distribution between the two proteins was confirmed by visualizing the differentially labeled proteins using confocal microscopy (data not shown). The similar distribution of the NLS3-β-galactosidase fusion protein and Nop1p indicates that sequences from Upf3p which include the 3rd NLS can function to direct a reporter protein to the nucleolus.

A sequence element in Upf3p directs a nuclear reporter protein out of the nucleus

Upf3p contains two separate regions that resemble leucine-rich nuclear export signal (NES) sequences known to promote the export of HIV-1 Rev, PKIα in humans, and Gle1p and Kap95p in yeast (Meyer and Malim, 1994; Fischer et al., 1995; Wen et al., 1995; Murphy and Wente, 1996; Iovine and Wente, 1997) (Fig. 7A). The two putative NES elements identified in Upf3p

![Fig. 5. Subcellular localization of NLS-β-galactosidase fusion proteins.](image-url)
are referred to as NES-A (amino acids 88-97) and NES-B (amino acids 151-160).

In order to test whether NES-A and NES-B can function independently as signals for nuclear export, the NES elements were fused to a reporter protein consisting of a NLS derived from the yeast Sen1p protein (DeMarini et al., 1992) and β-galactosidase (see Materials and Methods). The NLS in Sen1p causes the wild-type protein and reporter fusions containing the NLS to localize exclusively to the nucleus with no staining in the cytoplasm (Ursic et al., 1995). The ability of NES-A and NES-B to function as signals for nuclear export was assayed by examining the localization of the NLS-NES-A- and NLS-NES-B-β-galactosidase fusion proteins.

2μ plasmids carrying GAL10-NLS-lacZ, GAL10-NLS-NES-A-lacZ and GAL10-NLS-NES-B-lacZ were separately transformed into strain YM4126. Following galactose induction, the cellular distribution of each NLS-NES-β-galactosidase fusion protein was compared to the distribution of the NLS-β-galactosidase fusion protein lacking a NES. The fusion proteins were detected by indirect immunofluorescence microscopy (Fig. 7B, left column). The cells were also stained with DAPI (Fig. 7B, right column).

The staining pattern for the NLS-β-galactosidase fusion overlapped with DAPI staining (Fig. 7B1), indicating that this fusion is targeted primarily to the nucleus. The staining pattern for NLS-NES-B-β-galactosidase was indistinguishable from NLS-β-galactosidase (data not shown). However, the staining pattern for NLS-NES-A-β-galactosidase fusion protein was distributed throughout the nucleus and the cytoplasm (Fig. 7B2). These results suggest that NES-A signaled the export of the fusion protein from the nucleus into the cytoplasm. Either NES-B does not function as a nuclear export signal, or it fails to export enough of the fusion protein to the cytoplasm to be immunologically detected.

Cytoplasmic accumulation of NLS-NES-A-β-galactosidase could also occur if NES-A interferes with nuclear import rather than promoting nuclear export. To distinguish between these possibilities, we examined the effects of mutations in NES-A on the distribution of NLS-β-galactosidase. Alanine substitutions were constructed at three sites chosen on the basis of previous functional studies of NES elements in other proteins (Fischer et al., 1995; Wen et al., 1995; Murphy and Wente, 1996). Alanine was used to replace two conserved leucine residues corresponding to positions 88 (L88A) and 93 (L93A) in full-length Upf3p and a semi-conserved isoleucine residue corresponding to position 90 (I90A). The mutations in NES-A were made singularly as well as in triple combination.

The NES-A elements containing the single amino acid substitutions L88A and L93A and the triple amino acid substitution L88A·I90A·L93A (Triple) were inserted into GAL10-NLS-lacZ. The three plasmids carrying the NLS-NES-A-lacZ fusions were transformed into strain YM4126. Following growth in galactose, cells were stained to detect β-galactosidase. NLS-β-galactosidase containing NES-A-L88A (Fig. 7B3), NES-A-L93A (Fig. 7B4), and NES-A-Triple (not shown) were retained in the nucleus. The nuclear staining pattern observed for each of these fusions was indistinguishable from the staining pattern of NLS-β-galactosidase (Fig. 7B1). These results suggest that NES-A does not interfere with nuclear import but instead is capable of directing the export of β-galactosidase from the nucleus to the cytoplasm.

**Mutations in NES-A cause nucleolar accumulation of Upf3p-HA**

The presence of the NLS-like and NES-like sequences in Upf3p led us to explore whether Upf3p enters the nucleus and is subsequently exported from the nucleus into the cytoplasm. To examine this, we determined the localization of Upf3p-HA produced by alleles containing the single mutations L88A or L93A, and an allele containing the three mutations L88A, I90A, and L93A (Triple) in the NES-A element.

Centromeric plasmids expressing upf3-L88A-HA, upf3-L93A-HA and upf3-Triple-HA were transformed separately into the upf3-D1 strain LRSy323. To monitor expression, total yeast protein was extracted from cells expressing Upf3p, Upf3p-HA, Upf3p-L88A-HA, Upf3p-L93A-HA and Upf3p-Triple-HA and analyzed on a western blot probed with mouse monoclonal antibodies specific to the HA epitope (Fig. 8). The level of protein accumulation was quantified by PhosphorImager Analysis using 35S-conjugated secondary antibodies and is displayed as the level of expression relative to Upf3p-HA. The level of Upf3p-HA expressed from the centromeric plasmid has been defined as 100%. All upf3-HA alleles containing mutations in NES-A produced proteins that possessed identical electrophoretic mobility as Upf3p-HA (Fig. 8). The accumulation of Upf3p-L88A-HA (111%±25%) and Upf3p-L93A-HA (133%±60%) was similar to the accumulation of Upf3p-HA (100%±16%) expressed from the centromeric plasmid. The level of Upf3p-Triple-HA was reduced to 60% (±16%) of the level of Upf3p-HA expressed from the centromeric plasmid.

Upf3p-L88A-HA, Upf3p-L93A-HA, and Upf3p-Triple-HA were localized by indirect immunofluorescence microscopy (Fig. 9, left column). DAPI was used to identify the nucleus (Fig. 9, right column). The staining patterns of Upf3p-L93A-HA (Fig. 9A) and Upf3p-L88A-HA (Fig. 9B) were nearly identical to the localization of wild-type Upf3p-HA (Fig. 3B). Both Upf3p-L88A-HA and Upf3p-L93A-HA were detected throughout the cytoplasm with little or no detectable staining in the nucleus. To examine the localization of Upf3p-Triple-HA, we constructed a centromeric plasmid that contains two tandem copies of upf3-Triple-HA (2×upf3-Triple-HA) to compensate for the approximately two-fold under expression of Upf3p-Triple-HA (see Materials and Methods). Total yeast protein was extracted from cells expressing 2×upf3-Triple-HA and quantitatively analyzed on a western blot as described previously. In cells carrying 2×upf3-Triple-HA, the accumulation of Upf3p-Triple-HA increased to 87% (±30%) of the level of Upf3p-HA expressed from the centromeric plasmid (Fig. 8). In these cells, the majority of Upf3p-Triple-HA accumulated in a subnuclear area adjacent to the DAPI-stained area (Fig. 9C). A lesser amount of Upf3p-Triple-HA was also detected in the cytoplasm. A similar less intense staining pattern was observed for Upf3p-Triple-HA in cells carrying a single copy of upf3-Triple-HA (data not shown).

To determine whether any of the mutant Upf3 proteins described above are present in the nucleolus, cells expressing upf3-L88A-HA, upf3-L93A-HA and 2×upf3-Triple-HA were double-labeled with antibodies to detect the HA epitope and Rpa190p. Confocal images of mutant Upf3-HA proteins (green) (Fig. 4GJ,M) and Rpa190p (red) (Fig. 4HKN) were overlaid to determine the amount of co-distribution between the two proteins (Fig. 4IL,LO). Upf3p-L88A-HA and Upf3p-L93A-HA showed a minimal overlap in staining with Rpa190p.
indicating that the majority of these proteins were not detected in the nucleolus. In contrast, Upf3p-Triple-HA displayed a substantial overlap in staining with Rpa190p, indicating that a significant portion of Upf3p-Triple-HA localizes to the nucleolus.

Mutations in NES-A impair the ability of Upf3p to function in NMD

We wanted to determine whether the alanine substitutions that affect the localization of Upf3p-HA also had an affect on the function of Upf3p-HA in the NMD pathway. The allosuppression assay was first used to determine whether the substitutions in NES-A effect the ability of Upf3p-HA to function in NMD (Fig. 1B,C). When transformants of strain LRSy323 carrying the mutant upf3 alleles on centromeric plasmids were examined, we found that upf3-HA alleles containing the single amino acid substitutions L88A (Fig. 1B3) and L93A (Fig. 1B2) grew slowly on medium lacking histidine and resembled the growth of a transformant carrying UPF3-HA (Fig. 1B6). However, the transformant carrying upf3-

Fig. 6. Co-localization of NLS3-β-galactosidase with a nucleolar protein. Cells expressing NLS3-β-galactosidase from plasmid pLS97 were double labeled with antibodies against β-galactosidase and the nucleolar protein Nop1p (fibrillarin). The differentially labeled proteins were detected by indirect immunofluorescence microscopy. NLS3-β-galactosidase detected by fluorescein-conjugated secondary antibodies (A, green) and Nop1p detected by rhodamine-conjugated secondary antibodies (B, red) were overlaid to determine the amount of co-distribution between the two proteins (C). Any overlap in staining is shown in yellow. DAPI staining of DNA was used to mark the nucleus (D, blue). Bar, 2.5 μM.

Fig. 7. Functional analysis of the NES elements from Upf3p (A) The sequence of NES elements. NES-A and NES-B amino acid sequences are compared with NES elements found in yeast Gle1p (Murphy and Wente, 1996), the viral protein HIV-1 Rev (Fischer et al., 1995), the human protein PK1α (Wen et al., 1995) and the yeast protein Kap95p (Iovine and Wente, 1997). Boxes denote the most conserved residues. Numbers to the left (N-terminal) and right (C-terminal) of each sequence denote the positions of the elements within each protein (B) Localization of NES-A-β-galactosidase. The fusion proteins were detected by indirect immunofluorescence microscopy as described in Fig. 5. Strain YM4126 was transformed with plasmid pDU254 expressing NLS-β-galactosidase (B1), pLS93 expressing NLS-NES-A-β-galactosidase (B2), pLS102 expressing NLS-NES-A-β-galactosidase containing the alanine substitution L88A (B3) and pLS103 expressing NLS-NES-A-β-galactosidase containing the alanine substitution L93A (B4). The fluorescein staining of β-galactosidase is shown in the left set of panels. DAPI staining of DNA marking the location of the nucleus is shown in the right set of panels. Bar, 2.5 μM.
Triple-HA (Fig. 1B4) grew robustly at 37°C and resembled the growth of a upf3-Δ1 strain (Fig. 1B1). The Nmd- phenotype conferred by upf3-Triple-HA was not due to decreased protein abundance because the transformant carrying two tandem copies of upf3-Triple-HA, which produces a nearly wild-type level of protein, also displayed an Nmd- phenotype (Fig. 1B5).

These results were confirmed by making the identical single and triple alanine substitutions directly in wild-type UPF3, which lacks the HA tag, carried on a centromeric plasmid (pLS17, Table 2). Transformants carrying upf3-L88A (Fig. 1C3), upf3-L93A (Fig. 1C4), and upf3-L90A (not shown) failed to grow at 37°C on medium lacking histidine and resembled the growth of an UPF3 strain (Fig. 1C2). In contrast, the transformant carrying upf3-Triple (Fig. 1C5) grew robustly at 37°C on medium lacking histidine and resembled the growth of an upf3-Δ1 strain (Fig. 1C1). Further genetic analysis revealed that the Nmd- phenotype conferred by upf3-Triple was recessive. A his4-38 SUF1-1 UPF3+ strain transformed with the centromeric plasmid carrying upf3-Triple failed to grow at 37°C on medium lacking histidine (data not shown).

Quantitative northern blotting was used to measure the relative levels of accumulation of his4-38 mRNA in the transformants described in the above allosuppression assay (Fig. 2A). The accumulation of his4-38 mRNA in the upf3-Δ1 strain was 3.2 (±1.4)-fold greater than in the transformant carrying the wild-type UPF3 allele. upf3-Triple showed a similar fold increase of 3.0 (±0.5). In contrast, the relative accumulation of his4-38 mRNA in transformants carrying upf3-L88A and upf3-L93A was 0.8 (±0.2) and 1.0 (±0.5), respectively. The epitope-tagged alleles UPF3-HA, upf3-L88A-HA, upf3-L93A-HA and upf3-Triple-HA had relative his4-38 mRNA accumulation levels of 1.0 (±0.3), 1.3 (±0.3), 1.1 (±0.4) and 2.6 (±0.6), respectively (Fig. 2A).

Using quantitative northern blotting with a probe that is complementary to CYH2 pre-mRNA and mature mRNA, we found that the CYH2 pre-mRNA was 5.2 (±1.2)-fold more abundant in a upf3-Δ1 strain than in a transformant carrying the wild-type UPF3 allele (Fig. 2B). The relative levels of CYH2 pre-mRNA in transformants carrying upf3-L88A and upf3-L93A were 1.3 (±0.2) and 1.1 (±0.2), respectively (Fig. 2B). CYH2 pre-mRNA in a UPF3-HA strain accumulated 1.6 (±0.3)-fold greater than in a UPF3 strain. upf3-L88A-HA and

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**Fig. 8.** Western blotting of epitope-tagged Upf3 proteins.
Quantitative western blotting was used to determine the abundance of Upf3 proteins produced by upf3 alleles containing NES-A mutations. Strain LRSy323 (upf3-Δ1) was transformed with the following plasmids resulting in the corresponding genotypes: pRS316 (upf3-Δ1, lane 1), pLS51 (UPF3-HA, lane 2), pML11 (upf3-L88A-HA, lane 3), pML9 (upf3-L93A-HA, lane 4), pML14 (upf3-Triple-HA, lane 5), pRLS125 (2x-upf3-Triple-HA, lane 6), pLS17 (UPF3, lane 7) and pLS73 (UPF3-HA on 2μ, lane 8). The epitope-tagged proteins were detected on a western blot using 12CA5 monoclonal antibodies in conjunction with 35S-labeled secondary antibodies. Quantitation is expressed as the average percentage of each lane (x bar) from five independent trials. Percentages are normalized to Upf3p-HA levels (defined as 100%) with the associated standard deviations (s.d.) displayed below.

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**Fig. 9.** Subcellular localization of Upf3p-Triple-HA, Upf3p-L88A-HA and Upf3p-L93A-HA. LRSy323 (upf3-Δ1) was transformed with plasmid pML9 expressing upf3-L93A-HA (A), pML11 expressing upf3-L88A-HA (B), and pRLS125 expressing 2x-upf3-Triple-HA (C). The localization of mutant Upf3 proteins containing either single or triple alanine substitutions in NES-A were determined by indirect immunofluorescence as described in Fig. 3. The left set of panels shows the fluorescein-stained Upf3-HA proteins in representative cells from each transformant. The right set of panels shows the DAPI staining of DNA which marks the location of the nucleus. Bar, 2.5 μM.
The HIV-1 Rev NES restores the ability of Upf3p-Triple-HA to export from the nucleus but fails to restore its function in NMD

The retention of Upf3p-Triple-HA in the nucleus suggests that NES-A containing three alanine substitutions fails to be recognized as a functional export signal. To test this hypothesis, we determined whether a heterologous leucine-rich NES sequences could restore the export of Upf3p-Triple-HA from the nucleus. Centromeric plasmids were constructed carrying one and two copies of the upf3-Triple-HA-Rev and the upf3-Triple-HA-M10 alleles (see Materials and Methods). These alleles code for a Upf3p-Triple-HA protein that contains either the wild-type HIV-1 Rev NES or the export-defective M10 Rev NES (Meyer and Malim, 1994; Fischer et al., 1995) inserted at the C terminus (site 2, Fig. 1A).

Upf3p-Triple-HA-Rev and Upf3p-Triple-HA-M10 were localized by indirect immunofluorescence in transformants carrying two copies of upf3-Triple-HA-Rev and two copies upf3-Triple-HA-M10 (Fig. 10A,B; left column). DAPI was used to locate the nucleus (Fig. 10A,B; right column). The staining pattern of Upf3p-Triple-HA-M10 (Fig. 10A) was indistinguishable from the staining pattern of Upf3p-Triple-HA (Fig. 9C). The majority of Upf3p-Triple-HA-M10 was detected in the subnuclear area adjacent to the DAPI stained area which was previously identified as the nucleolus. In contrast, the staining pattern of Upf3p-Triple-HA-Rev (Fig. 10B) resembled the staining pattern of wild-type Upf3p-HA expressed from a centromeric plasmid (Fig. 3B). The majority of the Upf3p-Triple-HA-Rev was detected in the cytoplasm with much less detectable staining within the nucleus. The cytoplasmic localization of Upf3p-Triple-HA-Rev indicates that the Rev NES restored the export of Upf3p-Triple-HA.

To determine whether the inability of Upf3p-Triple-HA to function in NMD is due to failure to export from the nucleus, transformants described above were assayed for function in NMD by allosuppression (Fig. 10C). Transformants carrying one or two copies of upf3-Triple-HA-Rev and upf3-Triple-HA-M10 (Fig. 10C5). The subcellular localization of Upf3p-Triple-HA-Rev and Upf3p-Triple-HA-M10 were determined by indirect immunofluorescence microscopy as described in Fig. 3. The fluorescein stained Upf3-HA proteins are shown in the left set of panels. DAPI staining of DNA which marks the location of the nucleus is shown in the right set of panels. Bar, 2.5 μM (C)

Allosuppression of the his4-38 frameshift mutation was used to determine the effect of the wild-type and M10 Rev NES on the function of Upf3p-HA and Upf3p-Triple-HA in NMD. Allosuppression of his4-38 was assayed as described in Fig. 1. LRSy323 (his4-38 SUF1-1 upf3-ΔI) was transformed with plasmid pRLS144 expressing 2x-upf3-Triple-HA-M10 (A) and pRLS145 expressing 2x-upf3-Triple-HA-Rev (B). The subcellular localization of Upf3p-Triple-HA-M10 and Upf3p-Triple-HA-Rev were determined by indirect immunofluorescence microscopy as described in Fig. 3. The fluorescein stained Upf3-HA proteins are shown in the left set of panels. DAPI staining of DNA which marks the location of the nucleus is shown in the right set of panels. Bar, 2.5 μM (C)

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Similar results (data not shown) were obtained when sequences coding for the wild-type and M10 Rev NES were inserted in upf3-Triple-HA at a position corresponding to site 1 (Fig. 1A). Wild-type NES sequences from yeast Kap95p and NES-A were also inserted in upf3-Triple-HA at either at site 1 or site 2 (Fig. 1A). Using the allosuppression assay, we found that the addition of either NES-A or the NES from Kap95p to Upf3p-Triple-HA also failed to restore the function of Upf3p-Triple-HA in NMD (data not shown). Since the addition of homologous and heterologous NES sequences to Upf3p-Triple-HA failed to restore its function in NMD, we were unable to confirm a direct dependency of Upf3p function in NMD on the export of Upf3p from the nucleus.

**Double alanine substitutions in NES-A are indistinguishable from the triple alanine substitution in the allosuppression assay**

The divergent phenotypes of the single and triple amino acid substitutions in NES-A suggested that pairwise combinations of single amino acid substitutions might allow us to assess the relationship between nuclear export of Upf3p and the function of Upf3p in NMD. For this reason, the function of upf3-HA alleles containing double mutations was assayed by allosuppression (Fig. 11). Centromeric plasmids carrying the double mutant alleles upf3-L88A-L93A-HA, upf3-I90A-L93A-HA and upf3-L88A-I90A-HA were transformed into strain LRSy323 (his4-38 SUF1-1 upf3-Δ1). Transformants carrying each of the three double mutant alleles (Fig. 11B,D,F) grew at 37°C on medium lacking histidine and resembled the growth of a upf3-Δ1 strain (Fig. 11A) and a upf3-Triple-HA strain (data not shown). Only wild-type UPF3-HA displayed a decreased growth rate at 37°C on medium lacking histidine (Fig. 11H). These results indicate that all three of the double mutant alleles conferred a Nmd" phenotype similar to the upf3-Triple-HA allele.

We determined whether the HIV-1 Rev NES could restore the function of the mutant upf3-HA alleles containing double mutations in NES-A. The sequences coding for the wild-type Rev NES were inserted in upf3-L88A-L93A-HA, upf3-I90A-L93A-HA and upf3-L88A-I90A-HA at a position corresponding to site 2 (Fig. 1A). Transformants carrying these alleles on centromeric plasmids were assayed by allosuppression (Fig. 11). The growth of upf3-L88A-L93A-HA-Rev (Fig. 11C), upf3-I90A-L93A-HA-Rev (Fig. 11E) and upf3-L88A-I90A-HA-Rev (Fig. 11G) on medium lacking histidine at 37°C was robust and indistinguishable from the growth of the upf3-Δ1 strain (Fig. 11A). From these results we infer that the insertion of a functional NES is not sufficient to restore the function of a mutant Upf3p harboring either double or triple mutations in NES-A.

**DISCUSSION**

Upf3p contains two sequence motifs that resemble the leucine-rich NES sequences found in HIV-1 Rev, human PKI and yeast Glep1p and Kap95p (Meyer and Malim, 1994; Fischer et al., 1995; Wen et al., 1995; Iovine and Wente, 1997). In this paper we present evidence demonstrating that one of the two NES motifs, NES-A, functions as a nuclear export signal that mediates the export of Upf3p from the nucleus to the cytoplasm. NES-A was initially identified as a functional nuclear export signal by its ability to export a nuclear localized reporter protein. In order to demonstrate that NES-A mediates the export of Upf3p from the nucleus, we analyzed the cellular distribution of epitope-tagged Upf3p containing mutations in NES-A. When conserved leucine and isoleucine residues in NES-A were changed to alanine at three positions simultaneously, the distribution of Upf3p-HA changed from a primarily cytoplasmic to a nucleolar localization. The nucleolar accumulation caused by the mutations in NES-A was prevented when a functional NES from HIV-1 Rev was inserted into Upf3p-Triple-HA. From these results, we conclude that NES-A containing three alanine substitutions is defective for nuclear export and one function of NES-A is to promote the export of Upf3p from the nucleus to the cytoplasm.

Although, the distribution of Upf3p-HA is primarily cytoplasmic when expressed from a centromeric plasmid, several lines of evidence indicate that Upf3p can import into the nucleus. When expressed from a centromeric plasmid, Upf3p is detected in the nucleus when it contains triple alanine substitutions in NES-A. The triple NES-A mutations most likely confer complete loss of export function since the Nmd" phenotype is recessive and the export defect caused by the NES mutations can be restored by the functional Rev NES. Nuclear accumulation of Upf3p is also observed when the abundance of wild-type Upf3p is increased due to expression from a multi-copy plasmid. Taken together, these results indicate that Upf3p is imported into the nucleus. The low level of nuclear staining observed when Upf3p is expressed from a centromeric plasmid suggests that nuclear export of Upf3p-HA is more efficient than nuclear import.

The import of Upf3p into the nucleus could be mediated by one, all or none of the putative bipartite NLS sequences identified in Upf3p (Lee and Culbertson, 1995). All three of the NLS sequences promote the import of β-galatosidase into the nucleus, but these elements have not yet been studied genetically.
to establish potential functional roles in the import of native Upf3p. In addition, sequences from Upf3p, which include the third NLS motif, direct β-galactosidase to the nucleolus after mediating its import into the nucleus. Further analysis is required to determine whether sequences from Upf3p function to direct Upf3p to the nucleolus and to assess the significance of the nucleolus in the function of Upf3p and in NMD.

We developed a nuclear export assay to test the ability of the putative NES sequences identified in Upf3p to confer cytoplasmic accumulation when fused to a nuclear localized β-galactosidase reporter. NES-A causes cytoplasmic accumulation of the β-galactosidase reporter whereas NES-B had no effect. These results suggest that NES-A functions as a nuclear export signal. The failure of NES-B to cause detectable cytoplasmic accumulation of β-galactosidase indicates either that NES-B does not function as a nuclear export signal or that it functions as a less efficient export signal. The distribution of a protein that carries both NES and NLS sequences depends on the relative rates of export and import conferred by each sequence. Therefore, NES-B might not promote sufficient accumulation of β-galactosidase in the cytoplasm for detection by indirect immunofluorescence microscopy. However, the export defect conferred by mutations in NES-A in full-length Upf3p-HA indicates that NES-B cannot substitute for loss of NES-A function in wild-type Upf3p.

The triple NES-A mutation conferred an export defect when placed in full-length Upf3p and when NES-A was fused to NLS-β-galactosidase. However, the phenotypes of single NES-A mutations differed in the two export assays. Single alanine substitutions in NES-A prevent export when fused to NLS-β-galactosidase but not when placed in full length Upf3p. The difference in the effect of single mutations in the reporter proteins when compared to Upf3p might be due to differences in the size or abundance of Upf3p relative to β-galactosidase fusions. Alternatively, the context of the export element could influence the function of NES-A. Of these, context seems the most plausible explanation. Similar variations in the effect of mutations in the NES of Gle1p were observed when the mutations in the NES were analyzed in reporter constructs and in full-length Gle1p (Murphy and Wente, 1996).

In order to evaluate how the distribution of Upf3p is related to its function in NMD, we assayed the effects of mutations in NES-A on nonsense mRNA accumulation, nonsense pre-mRNA accumulation, and allosuppression. The single alanine substitutions in NES-A, which did not significantly affect the distribution of Upf3p-HA, did not affect the function of Upf3p in NMD. Double or triple alanine substitutions in NES-A conferred an Nmd− phenotype in all three assays for NMD. The Nmd− phenotype conferred by the double or triple mutations and the export defect conferred by the triple NES mutation provides correlative evidence suggesting that export of Upf3p is required for NMD. However, the nature of the mutational defects precluded a direct demonstration of cause and effect between the export defect and the defect in NMD. Even though the heterologous NES from HIV-1 Rev restored the export of Upf3p-Triple-HA, Upf3p containing double and triple mutations in NES-A still retained an Nmd− phenotype when fused to a functional NES.

Several explanations are possible for why the Rev NES failed to restore an Nmd+ phenotype in strains carrying double or triple mutations in NES-A. The HIV-1 Rev NES may promote export through an export pathway that is not normally utilized for Upf3p. The function of Upf3p in NMD may require export through a specialized pathway. The failure of Upf3p-HA to export through its native pathway may prevent Upf3p from becoming associated with other nuclear-associated factors that are necessary for its function in NMD. Alternatively, mutations in NES-A that block export may directly affect the function of Upf3p in NMD in a manner not specifically related to export. This view is supported by the finding that wild-type NES-A from Upf3p failed to restore a Nmd+ phenotype when inserted into Upf3p-Triple-HA at the same position as the Rev NES. We propose that mutations in NES-A disrupt another aspect of Upf3p function in addition to nuclear export. This might result from changes in protein conformation that render the export-defective Upf3p unable to function in NMD. One way this could manifest itself is in the interaction between Upf3p and Upf2p (Fig. 1A). Mutations in NES-A might disrupt the interaction between Upf3p and Upf2p, which could result in a Nmd+ phenotype.

All previous evidence from yeast has indicated that the NMD pathway occurs in the cytoplasm (reviewed by Jacobson and Peltz, 1996). Our evidence demonstrates that Upf3p enters and exits the nucleus. Since the loss of Upf3p function is not known to affect processes unrelated to the NMD pathway, the ability of Upf3p to enter and exit the nucleus is the first indication in yeast that the nucleus may have a role in the NMD pathway. If so, it seems likely that Upf3p functions early in the NMD pathway, possibly at the level of nonsense mRNA recruitment. A nuclear role in NMD has already been implicated in vertebrate cells. The abundance of some nonsense containing transcripts is reduced while the mRNA remains associated with nuclear cell fractions (reviewed by Maquat, 1995, 1996; Li and Wilkinson, 1998). Since the nuclear associated decay of nonsense mRNAs also requires translation in the cytoplasm, it has been suggested that decay of nonsense mRNAs initiates during the export of the mRNA from the nucleus. In addition, the identification of nonsense mRNAs as substrates for NMD is influenced by the distance of the pre-mature termination codon to the final exon/exon boundary, which is identified following splicing events in the nucleus (Cheng et al., 1994; Maquat, 1996).

Taken together, the results from vertebrate cells suggest that the nucleus plays a role in recruiting nonsense mRNAs for degradation by the NMD pathway. Steps necessary for the recruitment of nonsense mRNAs into the NMD pathway may initiate in the nucleus prior to or concurrent with export of the mRNA to the cytoplasm. Translation and then decay of the nonsense mRNA could initiate either during or immediately following export of the mRNA to the cytoplasm. The localization of Upf3p in the nucleus raises the possibility that Upf3p functions in the initial steps necessary for recruitment of nonsense mRNAs in yeast. Upf3p could initiate the steps that are required for the accurate identification of nonsense mRNAs through association with ribosomal subunits assembled in the nucleus, with mRNP particles that export from the nucleus, or both.

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, WI, NSF grant MCB-9870313 (M.R.C.), and Public Health Service Training Grant in Genetics NIH GM07133 (R.L.S. and L.R.S.), M.J.L. and J.N.D. were supported by the NRSA Postdoctoral Fellowship NIH GM07970 and GM 17916, respectively. We thank M. Johnston for the GAL2+ yeast.
strains (YM4126). J. Aris for No1p antibodies, M. Nomura for Rpa190p antibodies, D. Ursic for β-galactosidase antibodies, S. Paddock and S. Carroll for antibodies and technical assistance with confocal microscopy and L. Olds for preparation of the figures. This is Laboratory of Genetics paper 3514.

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