Nuclear Import of Upf3p Is Mediated by Importin-α/-β and Export to the Cytoplasm Is Required for a Functional Nonsense-Mediated mRNA Decay Pathway in Yeast

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Manuscript received April 19, 2002
Accepted for publication June 5, 2002

ABSTRACT

Upf3p, which is required for nonsense-mediated mRNA decay (NMD) in yeast, is primarily cytoplasmic but accumulates inside the nucleus when *UPF3* is overexpressed or when *upf3* mutations prevent nuclear export. Upf3p physically interacts with Srp1p (importin-α). Upf3p fails to be imported into the nucleus in a temperature-sensitive *srp1-31* strain, indicating that nuclear import is mediated by the importin-α/β heterodimer. Nuclear export of Upf3p is mediated by a leucine-rich nuclear export sequence (NES-A), but export is not dependent on the Crm1p exportin. Mutations identified in NES-A prevent nuclear export and confer an Nmd$^-$ phenotype. The addition of a functional NES element to an export-defective *upf*$^-$ allele restores export and partially restores an Nmd$^+$ phenotype. Our findings support a model in which the movement of Upf3p between the nucleus and the cytoplasm is required for a fully functional NMD pathway. We also found that overexpression of Upf2p suppresses the Nmd$^-$ phenotype in mutant strains carrying *nes-A* alleles but has no effect on the localization of Upf3p. To explain these results, we suggest that the mutations in NES-A that impair nuclear export cause additional defects in the function of Upf3p that are not rectified by restoration of export alone.

EUKARYOTIC cells from a variety of organisms including yeast, nematodes, mice, and humans rapidly eliminate mRNAs that contain a premature termination codon (Losson and Lacroute 1979; Leeds *et al.* 1991; Pulak and Anderson 1993; Perlick *et al.* 1996). Nonsense mRNA degradation occurs through a pathway called nonsense-mediated mRNA decay (NMD), which serves two purposes. One is in RNA surveillance in which nonsense mRNAs arising through errors in gene expression are rapidly eliminated to prevent the accumulation of deleterious truncated proteins (Pulak and Anderson 1993; Cali and Anderson 1998; Li and Wilkinson 1998; Frischmeyer and Dietz 1999). A second purpose is to control the abundance of a subset of endogenous wild-type mRNAs containing built-in signals that can trigger premature termination of translation, which leads to faster decay as part of the normal repertoire of gene expression (Lelivelt and Culbertson 1999). NMD requires a mechanism to distinguish a premature nonsense codon from the normal wild-type termination signal. In yeast, this involves the presence of a degenerate downstream sequence element (DSE) located 3′ of a premature nonsense codon (Peltz *et al.* 1993; Hagan *et al.* 1995; Zhang *et al.* 1995; Czaplinski *et al.* 1999). mRNAs containing a premature termination codon but lacking a DSE fail to be degraded by the NMD pathway (Peltz *et al.* 1993; Ruiz-Echevarria *et al.* 1996, 1998).

In yeast, the three factors Upf1p, Upf2p, and Upf3p are required for NMD (Leeds *et al.* 1991, 1992; Cui *et al.* 1995; He and Jacobson 1995; Lee and Culbertson 1995). Mutations in the *UPF* genes stabilize nonsense mRNAs, resulting in rates of decay similar to those of the corresponding wild-type mRNAs. In addition, the efficiency of translation termination at premature stop codons is decreased in strains carrying *upf*$^-$ mutations while the overall efficiency of nonsense mRNA translation increases (Muhlrad and Parker 1999; Bidou *et al.* 2000). These effects, combined with the increase in mRNA stability, contribute to the ability of *upf*$^-$ mutations to suppress nonsense and frameshift mutations (Culbertson *et al.* 1980; Leeds *et al.* 1991, 1992; Maderazo *et al.* 2000).

Homologs of the yeast Upf proteins have been identified in *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, mice, and humans (Page *et al.* 1999; Lykke-Andersen *et al.* 2000; Mendell *et al.* 2000; Aronoff *et al.* 2001; Serin *et al.* 2001). Human hUpf1p and hUpf2p were identified as homologs of yeast Upf1p and Upf2p (Perlick *et al.* 1996; Applequist *et al.* 1997). A mutation in the conserved helicase domain of hUpf1p confers a dominant-negative phenotype in yeast (Leeds *et al.*

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1992) and partially inactivates the NMD pathway in monkey COS and human HeLa cells (Sun *et al.* 1998). Several homologs of yeast Upf3p were identified in humans (Lykke-Andersen *et al.* 2000; Serin *et al.* 2001). These proteins are derived from two genes, each of which expresses several isoforms due to alternative splicing. These studies suggest that the function of the Upf proteins in identifying and targeting nonsense mRNAs for rapid decay is conserved among eukaryotes.

Translation is required for the rapid decay of nonsense mRNAs. Nonsense mRNAs are stabilized by the presence of 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 cofractionates with polyribosomes (Atkin *et al.* 1997). Physical interactions between the yeast Upf proteins suggest that they act in concert to promote NMD on polyribosomes (He and Jacobson 1995; He *et al.* 1997). The Upf proteins copurify with release factor eRF3 and Upf1p also copurifies with release factor eRF1 (Czaplinski *et al.* 1998; Wang *et al.* 2001).

Upf1p associates with polyribosomes in the absence of Upf2p or Upf3p, and it appears to facilitate the dissociation of Upf2p with polyribosomes. Upf3p is required for the association of Upf2p with polyribosomes (Atkin *et al.* 1997). On the basis of these results, it was proposed that Upf3p recruits Upf2p to polyribosomes (Atkin *et al.* 1997; Culbertson 1999, 2001). Upf2p may then facilitate NMD by interacting with Upf1p (He *et al.* 1996). Upf1p could be recruited to polyribosomes through an association with release factors (He *et al.* 1997; Czaplinski *et al.* 1998).

It is still unclear how nonsense mRNAs are initially identified as substrates for NMD. The Upf proteins are not associated with all translating ribosomes (Atkin *et al.* 1995; Maderazo *et al.* 2000). Upf1p is at least 100-fold less abundant than ribosomes. Furthermore, Upf2p and Upf3p are 10- to 20-fold less abundant than Upf1p. The low abundance of the Upf proteins suggests the existence of a mechanism that serves to specifically recruit the Upf proteins to the site of premature translation termination of nonsense mRNAs.

We showed previously that Upf3p contains a functional nuclear export sequence, suggesting that Upf3p may function in an early step in nonsense mRNA recruitment (Shirley *et al.* 1998). However, it was not known at that time whether nuclear entry and exit is required for NMD. Nuclear localization was recently demonstrated for the human homologs of Upf3p. Several isoforms of hUpf3p were shown to shuttle between the nucleus and the cytoplasm in cell culture heterokaryons (Lykke-Andersen *et al.* 2000; Serin *et al.* 2001). In this article, we demonstrate that the import of yeast Upf3p into the nucleus is mediated by the importin-α/β heterodimer. We further demonstrate that the export of Upf3p from the nucleus via a leucine-rich nuclear export sequence is required for a fully functional NMD pathway. Our results support the hypothesis that Upf3p functions in one of the initial steps necessary to earmark a nonsense mRNA for recruitment as a substrate in the NMD pathway. This occurs prior to or during export of a nonsense mRNA from the nucleus to the cytoplasm.

MATERIALS AND METHODS

Strains and plasmids: Strain RSy5 (*MAT*α *ade2-1 leu2-1 tyr7-1 can1-100 upf3-Δ1 trp*$^-$ *ura*$^-$ *his3*$^-$ *GAL2*$^+$) was used for immunofluorescence microscopy and to assay suppression of the *leu2-1*, *tyr7-1*, and *can1-100* nonsense mutations. Strain LRSy323 (*MAT***a** *his4-38 SUF1-1 trp1-Δ1 upf3-Δ1 ura3-52 leu2-Δ1*) was used for the experiment shown in Figure 1C. Strain PJ69-4A (*MAT***a** *trp1-901 leu2-3,112 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*; obtained from E. Craig) was used for the two-hybrid assay (James *et al.* 1996). The localization of Upf3p-HA was examined in strain PSY730 (*MAT***a** *srp1-31 leu2-3,112 his3*$^-$ *ade2*$^-$ *trp1*$^-$ *ura3-52*; Tabb *et al.* 2000; strain obtained from P. Silver). The effects of *CRM1* on Upf3p localization were examined in strains MNY12 (*MAT***a** *CRM1::Kan*^{r} *leu2*$^-$ *his3*$^-$ *trp1*$^-$ *ura3*$^-$ *[pDC-CRM1T539C-GFP]*) and MNY8 (*MAT***a** *CRM1::Kan*^{r} *leu2*$^-$ *his3*$^-$ *trp1*$^-$ *ura3*$^-$ *[pDC-CRM1T539C-HA]*; Neville and Rosbash 1999). The *CRM1T529C* allele confers sensitivity to leptomycin B.

Plasmids are listed in Table 1. All plasmids were created using techniques and reagents as described previously (Shirley *et al.* 1998). pAF8 was constructed by ligating the *Xho*I-*Sac*I fragment from pUZ178 (Atkin *et al.* 1997) containing *UPF2* into the same sites in pRS423. Plasmids expressing green fluorescent protein (GFP) fusion constructs were made as follows. Oligomers (Operon, Alameda, CA) were designed to amplify the *UPF3* promoter and 5′-untranslated region (UTR), using pLS17 as a template. The product was subcloned into a shuttle vector using unique restriction sites engineered by PCR. Next, sequences encoding the GFP open reading frame (ORF) without the termination codon were amplified from plasmid pRSETB (courtesy of L. Robinson). Restriction sites were engineered into the PCR product to allow insertion immediately downstream of the *UPF3* promoter. Finally, the *UPF3* ORF and sequences corresponding to the *UPF3* 3′UTR were amplified via PCR using pLS17 as a template. Using unique restriction sites, the PCR product was subcloned downstream of the GFP ORF. The entire cassette was cloned into pRS316 and pRS426, creating pNE36 and pNE39, respectively. The plasmid expressing Upf3p-Triple-GFP was constructed by recombinational cloning. pNE36 was digested with *Hin*dIII and *Bse*RI. Sequences encoding the *upf3-Triple* ORF were PCR amplifed from template pRLS125 and transformed into strain RSy5 along with linearized pNE36. Recombined plasmids were rescued and sequenced. The *upf3-Triple-GFP* cassette was subcloned into pRS426, creating pAF14. To create pAF51, *upf3-Triple-REV-GFP*, the *Bse*RI-*Kpn*I fragment of pRLS141 was purified and subcloned into the same sites of pAF14. The construction of additional plasmids is described below or was described previously (Shirley *et al.* 1998).

Two-hybrid assay: The two-hybrid vectors used were pGBDU-C1,C2 and pGBDU-C1,C2 (Table 1; James *et al.* 1996). Most of the translational fusions between ORFs of interest and the Gal4 activation domains (AD) or binding domains (BD) were generally constructed as follows. Primers were designed to amplify the entire ORF of interest from plasmid DNA containing the gene sequence. The primers were used to engineer unique restriction sites and the 5′ and 3′ ends

TABLE 1

Plasmids

Plasmid	Vector	Description
pRS316[a]	—	*CEN URA3 ARS4*
pRS423[b]	—	*2μ ori HIS3*
pRS426[b]	—	*2μ ori URA3*
pAF8	pRS423	*2μ ori UPF2*
pLS17[c]	pRS316	*UPF3*
pLS51[c]	pRS316	*UPF3-HA*
pLS73[d]	pRS426	*2μ ori UPF3-HA*
pRLS125[d]	pRS316	Two tandem copies of *upf3-Triple-HA* (L88A, I90A, L93A)
pRLS134[d]	pRS316	UPF3-Rev
pRLS144[d]	pRS316	Two tandem copies of *upf3-Triple-HA-M10*
pRLS145[d]	pRS316	Two tandem copies of *upf3-Triple-HA-Rev*
pRLS207	pRS423	*2μ ori GAL1-cre*
pRR2	pRS316	*UPF3-loxP ADE2 loxP-Rev*
pRRNES2	pRS316	*upf3-nes2*
pRRNES2-Rev	pRS316	*upf3-nes2-Rev*
pRRNES3	pRS316	*upf3-nes3*
pRRNES3-Rev	pRS316	*upf3-nes3-Rev*
pRRNES5	pRS316	*upf3-nes5*
pRRNES5-Rev	pRS316	*upf3-nes5-Rev*
pRRNES6	pRS316	*upf3-nes6*
pRRNES6-Rev	pRS316	*upf3-nes6-Rev*
pRRNES7	pRS316	*upf3-nes7*
pRRNES7-Rev	pRS316	*upf3-nes7-Rev*
pUZ177	pRS316	*UPF2*
pGBDU-C1,C2[e]		*2μ ori URA3 GAL4* binding domain
pGAD-C1,C2[e]	—	*2μ ori LEU2 GAL4* activation domain
pAF10	pGBDU-C2	*2μ ori UPF3-BD*
pAF11	pGBDU-C2	*2μ ori upf3-Triple-BD*
pAF12	pGAD-C1	*2μ ori UPF2-AD*
pAF20	pGBDU-C1	*2μ ori SRP1-BD*
pAF23	pGBDU-C1	*2μ ori UPF2-BD*
pAF25	pGBDU-C2	*2μ ori UPF3(1-98)-BD*
pAF26	pGBDU-C2	*2μ ori UPF3(99-387)-BD*
pAF27	pGBDU-C2	*2μ ori UPF3(1-220)-BD*
pAF28	pGAD-C2	*2μ ori UPF3-AD*
pAF29	pGAD-C2	*2μ ori upf3-Triple-AD*
pAF30	pGAD-C2	*2μ ori UPF3(1-98)-AD*
pAF31	pGAD-C2	*2μ ori UPF3(99-387)-AD*
pAF33	pGAD-C2	*2μ ori UPF3(99-220)-AD*
pAF34	pGAD-C2	*2μ ori upf3-nes2-AD*
pAF35	pGAD-C2	*2μ ori upf3-nes3-AD*
pAF36	pGAD-C2	*2μ ori upf3-nes5-AD*
pAF37	pGAD-C2	*2μ ori upf3-nes6-AD*
pAF38	pGAD-C2	*2μ ori upf3-nes7-AD*
pRLS211	pGBDU-C2	*2μ ori upf3-nes2-BD*
pRLS212	pGBDU-C2	*2μ ori upf3-nes3-BD*
pRLS214	pGBDU-C2	*2μ ori upf3-nes5-BD*
pRLS215	pGBDU-C2	*2μ ori upf3-nes6-BD*
pRLS216	pGBDU-C2	*2μ ori upf3-nes7-BD*
pNE36	pRS316	*2μ ori UPF3-GFP*
pNE39	pRS426	*2μ ori UPF3-GFP*
pAF14	pRS426	*2μ ori upf3-Triple-GFP*
pAF51	pRS426	*2μ ori upf3-Triple-Rev-GFP*

[a] Sikorski and Hieter (1989).
[b] Christianson *et al.* (1992).
[c] Atkin *et al.* (1997).
[d] Shirley *et al.* (1998).
[e] James *et al.* (1996).

of the PCR products. After amplification using high-fidelity polymerase, the PCR products were digested with the specific enzymes and ligated into similar sites in multiple cloning sites in the two-hybrid vectors.

To construct plasmids expressing Upf3p-Gal4 fusions, internal restriction sites were used to delete certain sequences in *UPF3*. pAF25 and pAF30, expressing Upf3p(1-98)-BD and Upf3p(1-98)-AD, respectively, were constructed by digesting pAF10 with *Bam*HI and *Pst*I. The resulting fragment was ligated into the same sites of pGBDU-C2 and pGAD-C2. Upf3p (1-220) was fused to the Gal4 binding domain by digesting pAF10 with *Bam*HI and *Pvu*II. The resulting fragment was ligated into plasmid pGBDU-C2, which was first digested with *Bgl*II, blunt ended using T4 DNA polymerase, and digested with *Pst*I. This created plasmid pAF27. The plasmids expressing Upf3p(99-387)-BD and Upf3p(99-387)-AD, pAF26 and pAF31, respectively, were created by digesting plasmid pAF10 with *Pst*I. The resulting fragment was ligated into similar sites in pGBDU-C2 and pGAD-C2. Upf3p(99-220) was fused to Gal4-AD by digesting pAF10 with *Pst*I and *Pvu*II. The *Pst*I-*Pvu*II DNA fragment was ligated into pGAD-C2, which was digested with *Bgl*II, blunt ended using T4 DNA polymerase, and digested with *Pst*I to generate plasmid pAF33.

To construct plasmid pAF12, which expresses a translational fusion between Upf2p and Gal4-AD, an *Xho*I-*Bgl*II fragment from pAF6 was ligated into *Sal*I and *Bgl*II sites in pGAD-C1. This fragment contains the sequence coding for *UPF2* but lacks the intron. pAF12, which carries *UPF2*, was digested with *Xho*I and *Bgl*II and ligated into *Sal*I and *Bgl*II sites in pGBDU-C1 to create pAF23.

Translational fusions between Crm1p/Xpo1p and the Gal4p-BD were constructed by digesting pKW442 (*CRM1*; Stade *et al.* 1997) with *Eco*RI and *Bam*HI. This fragment was ligated into pGAD-C1 and pGBDU-C1 to generate plasmids pAF41 and pAF21, respectively. As a positive control for expression of the Crm1p fusion proteins, translational fusions between Rip1p, Gal4p-BD, and Gal4p-AD were used.

Plasmids expressing two-hybrid fusion proteins were transformed into strain PJ69-4A, which contains *GAL1-HIS3* (James *et al.* 1996). Expression of the *HIS3* reporter indicates an interaction. The strength of the interaction was assessed in the presence of 3-aminotriazole (3-AT), which inhibits His3p enzymatic activity. The specificity of the interactions between all the fusion proteins was tested by assaying for an interaction between each fusion protein and a fusion between the Gal4p activation domain and amino acids 615–753 of Exo84p or a fusion between the Gal4p binding domain and amino acids 756–931 of Prp8p (Kuhn and Brow 2000).

Cellular localization: To study the effects of *srp1-31* on localization, we examined Upf3p-HA in strain PSY730 carrying plasmid pLS73. Cells were prepared for immunofluorescence as described previously (Shirley *et al.* 1998) except for the addition of 3.5% formalin for 5 min prior to fixing with formaldehyde. To inhibit the function of Crm1p in strains MNY8 and MNY12, cells grown to $OD_{600} = 0.5$ in YEP were pelleted and resuspended in YEP plus 100 ng/ml of leptomycin B (provided by M. Yoshida; Kudo *et al.* 1999) and incubated at room temperature for 0.5–2 hr. After formaldehyde fixation, the cells were washed with PBS containing 1% Triton X-100 and 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) followed by a final wash with PBS.

Assays for function: The function of the *UPF* genes was assayed by suppression of nonsense mutations and accumulation of *CYH2* pre-mRNA. Nonsense suppression monitors all effects of *UPF* mutations simultaneously, including function in translation termination and NMD, whereas *CYH2* pre-mRNA accumulation monitors the function of the *UPF* genes only in NMD.

Suppression was monitored in strains carrying the *leu2-1* (UAA) and *tyr7-1* (UAG) nonsense mutations, which prevent growth on medium lacking leucine and tyrosine. Loss-of-function mutations in any of the *UPF* genes confer growth of a *leu2-1 tyr7-1* strain on medium lacking leucine and tyrosine. The *can1-100* (UAA) nonsense mutation confers resistance to canavanine in a Upf^+ strain, whereas mutations in any of the *UPF* genes suppress *can1-100* and prevent growth in the presence of canavanine. Growth was assayed by plating serial dilutions of cells (Shirley *et al.* 1998).

NMD was monitored by measuring the accumulation of *CYH2* pre-mRNA using Northern blotting. *CYH2* (ribosomal protein L29) contains an intron that is inefficiently spliced from the pre-mRNA (Kaufer *et al.* 1983). The presence of an in-frame stop codon at position 19 in the intron triggers rapid decay of unspliced pre-mRNA, which is exported to the cytoplasm, associates with polyribosomes, and is degraded by the NMD pathway (He *et al.* 1993). The pre-mRNA accumulates to a four- to sixfold higher level in Nmd^- strains due to a corresponding increase in the pre-mRNA half-life but without any change in the mature mRNA half-life. The accumulation of *CYH2* pre-mRNA has routinely been used to monitor the activity of the NMD pathway (for example, see Zhang *et al.* 1997; Zuk and Jacobson 1998).

An antisense RNA probe complementary to nucleotides 572–959 of *CYH2* pre-mRNA was used to detect pre-*CYH2* and mature mRNA (Lelivelt and Culbertson 1999). To assess the relative accumulation, the *CYH2* pre-mRNA/mRNA ratio in the strain to be analyzed was normalized to the ratio in the *UPF3* strain to calculate the fold change in the accumulation of the pre-mRNA. The fold changes were averaged across all trials and standard deviations (SD) were derived. To calculate *P* values, the statistical similarities of the average fold change in mRNA accumulation between pairwise sets of strains were assessed using a two-tailed *t*-test assuming equal variances at $\alpha = 0.05$.

PCR/oligonucleotide-directed mutagenesis and selection for mutations: Multiround PCR was used to generate mutations in sequences coding for residues L88, I90, L92, and L93 of the Upf3p NES-A. Oligonucleotide RR03 [caaccgagaatgaag gatttaag (a,c,g)(a,c,t,g)(a,c) gtt (a,c,g)(a,c,t,g)(a,c) aga (a,c,g)(a,c,t,g)(a,c) (a,c,g)(a,c,t,g)(a,c) cctccaaatttgactgcagatg; mutated codons underlined] contains a degenerate sequence for the 12 bases that specify each of the 4 amino acids, allowing each of the three base codons to specify 15 different amino acids without resulting in a nonsense codon. In the first round of PCR, oligonucleotides RRO3 and LSO183 and template pLS17 generated DNA that contained mutations in NES-A and included a recognition site for *Sna*BI. The fragment generated in the first round of PCR was purified and used as a megaprimer in the second round. The megaprimer and oligonucleotide T7 (taatacgactcactataggg) and template pLS17 generated DNA that contained mutations in NES-A and included recognition sites for *Sna*BI and *Sac*II. The third round of PCR amplified the DNA isolated from the second round. This fragment was digested with *Sac*II and *Sna*BI and used to replace the *Sac*II-*Sna*BI fragment from pRR2 that contained the wild-type NES-A element. Plasmid pRR2 contains a translational fusion between *UPF3* and a *loxP* site from bacteriophage P1 (*UPF3-loxP*) that is separated from another *loxP* site fused to sequences coding for the wild-type HIV-1 Rev NES (*loxP-Rev*) by the *ADE2* gene. The above ligation mixture was used to transform XL10-Gold ultracompetent cells (Stratagene, La Jolla, CA) and plated onto LB medium containing 100 μg/ml ampicillin. DNA was isolated from a pool of ~8600 transformants.

A two-step strategy was devised to select for mutations that confer loss of Upf3p function followed by restoration of func-

tion after a functional NES was recombined *en masse* into a pool of PCR-mutagenized genes. In step 1, strain RSy5 was cotransformed with a pool of pRR2 plasmids containing mutations in NES-A (pRR2Mut) in *UPF3-loxP* and 2μ plasmid pRLS207, which carries the *cre* recombinase gene under the control of the *GAL1* promoter. Expression of the *cre* recombinase promotes recombination between the *loxP* sites, resulting in a translational fusion between *UPF3* and *loxP* and a functional HIV-1 Rev NES. Transformants were plated on SD medium without uracil, histidine, adenine, leucine, and tyrosine to select for the presence of the plasmids pRR2Mut and pRLS207 and for mutations that suppress *leu2-1* and *tyr7-1*. Plates were incubated 4–5 days at 30°. In step 2, colonies were replica plated to SD-uracil, histidine-containing galactose to induce expression of the *cre* recombinase gene. Colonies were replica plated to SD-uracil, histidine-containing canavanine to select for canavanine resistance due to loss of suppression of the *can1-100* nonsense allele. A total of 84,000 transformants were plated in step 1 and 389 survived step 2.

The survivors potentially include alleles in which the function of Upf3p is restored by the addition of the HIV Rev1-1 NES or they could include alleles that confer partial suppression where the addition of the NES makes no phenotypic difference. Upon further testing of a subset of alleles, we found that none of them exhibited improved function when the NES was added and all of them conferred partial suppression of nonsense mutations. DNA fragments from five transformants each containing the mutated NES-A region were generated by PCR using template DNA from yeast spheroplasts and oligonucleotides T7 and RSO62 gactgaggctgagaggagttg (Klebanow and Weil 1999). The fragments were sequenced to characterize the mutations. Fragments were digested with *Spe*I and either *Sna*BI or *Bse*RI and ligated into the same sites in pLS17 and pRLS134, replacing the wild-type NES and creating plasmids pRRNES2, pRRNES3, pRRNES5, pRRNES6, pRRNES7 and plasmids pRRNES2-Rev, pRRNES3-Rev, pRRNES5-Rev, pRRNES6-Rev, pRRNES7-Rev, respectively (Table 1).

RESULTS

The nuclear import of Upf3p is mediated by the importin-α/importin-β heterodimer: The nuclear import of proteins containing either an SV-40-like nuclear localization signal (NLS) element (Dingwall and Laskey 1991) or a bipartite NLS element (Robbins *et al.* 1991) involves binding of the NLS-bearing protein to a heterodimer composed of importin-α (Impα) and importin-β (Impβ; Yano *et al.* 1992, 1994; Enenkel *et al.* 1995; Gorlich *et al.* 1995). Impα binds to the NLS-containing proteins through recognition of the nuclear localization signal and serves as an adapter between the import cargo and Impβ.

Three NLS motifs in Upf3p that were shown previously to direct reporters to the nucleus are referred to as NLS1, NLS2, and NLS3. They correspond to amino acids 15–31, 58–74, and 284–300, respectively (Figure 1A; Lee and Culbertson 1995; Shirley *et al.* 1998). The presence of NLS motifs in Upf3p prompted us to test whether Impα/β heterodimer mediates the import of Upf3p into the nucleus. To accomplish this, we determined whether Upf3p interacts with the *Saccharomyces cerevisiae* homolog of Impα, Srp1p, and whether the localization of Upf3p is altered in a temperature-sensitive *srp1-31* strain.

The two-hybrid system was used to assess whether Srp1p interacts with Upf3p (Figure 1B). A 2μ plasmid carrying the *SRP1-BD* allele codes for a fusion protein that contains the Gal4p DNA binding domain and full-length Srp1p. This plasmid was cotransformed into yeast strain PJ69-4A (James *et al.* 1996) with a 2μ plasmid expressing *UPF3-AD*, which codes for a fusion protein containing full-length Upf3p and the Gal4p activation domain. Coexpression of *SRP1-BD* and *UPF3-AD* resulted in robust growth on medium lacking histidine, indicating an interaction. The growth on selective medium was specific to transformants expressing the Upf3p and Srp1p fusion proteins. These results indicate that Upf3p and Srp1p interact in the two-hybrid system.

To characterize the regions of Upf3p that contribute to the interaction, we generated 2μ plasmids expressing Gal4p-AD fused in-frame to different fragments of Upf3p (Figure 1A). *UPF3(1-98)-AD* codes for a fusion protein that includes both NLS1 and NLS2. *UPF3(99-387)-AD* codes for a fusion protein that contains NLS3. *UPF3 (99-220)-AD* codes for a fusion protein that does not contain NLS1, NLS2, or NLS3.

Transformants carrying *SRP1-BD* and *UPF3(1-98)-AD* grew more robustly on medium lacking histidine than did transformants expressing the full-length Upf3 protein (Figure 1B). To determine the strength of the interaction between Srp1p-BD and Upf3p(1-98)-AD, we tested growth of the transformants on media lacking histidine and containing varying concentrations of 3-AT. The expression of full-length *UPF3-AD* and *SRP1-BD* failed to promote growth on SD medium without histidine that contained ≥3 mM 3-AT. In contrast, transformants expressing *SRP1-BD* and *UPF3(1-98)-AD* grew robustly on SD medium without histidine with up to 30 mM 3-AT, indicating that the region of Upf3p containing NLS1 and NLS2 interacts strongly with Srp1p.

Coexpression of *SRP1-BD* and *UPF3(99-387)-AD* promoted growth on SD medium lacking histidine to the same extent as the transformants expressing *SRP1-BD* and full-length *UPF3-AD* (Figure 1B). Transformants carrying *SRP1-BD* and the *UPF3(99-220)-AD* fusion failed to grow on medium lacking histidine. Taken together, these results indicate that regions of Upf3p that include the NLS1 and NLS2 or NLS3 motifs contribute to the interaction of Upf3p with Srp1p. The internal region of Upf3p from residue 99 to 220 that does not contain an identifiable NLS motif does not interact.

To determine whether the nuclear import of Upf3p is mediated by the Impα/β heterodimer, we examined the localization of epitope-tagged Upf3p (Upf3p-HA) in a strain carrying the *srp1-31* temperature-sensitive allele (Figure 1C). Other studies show that the nuclear import of substrates in the *srp1-31* strain diminishes with time following a shift to 37° with ~95% of protein import blocked after 6 hr at the nonpermissive temper-

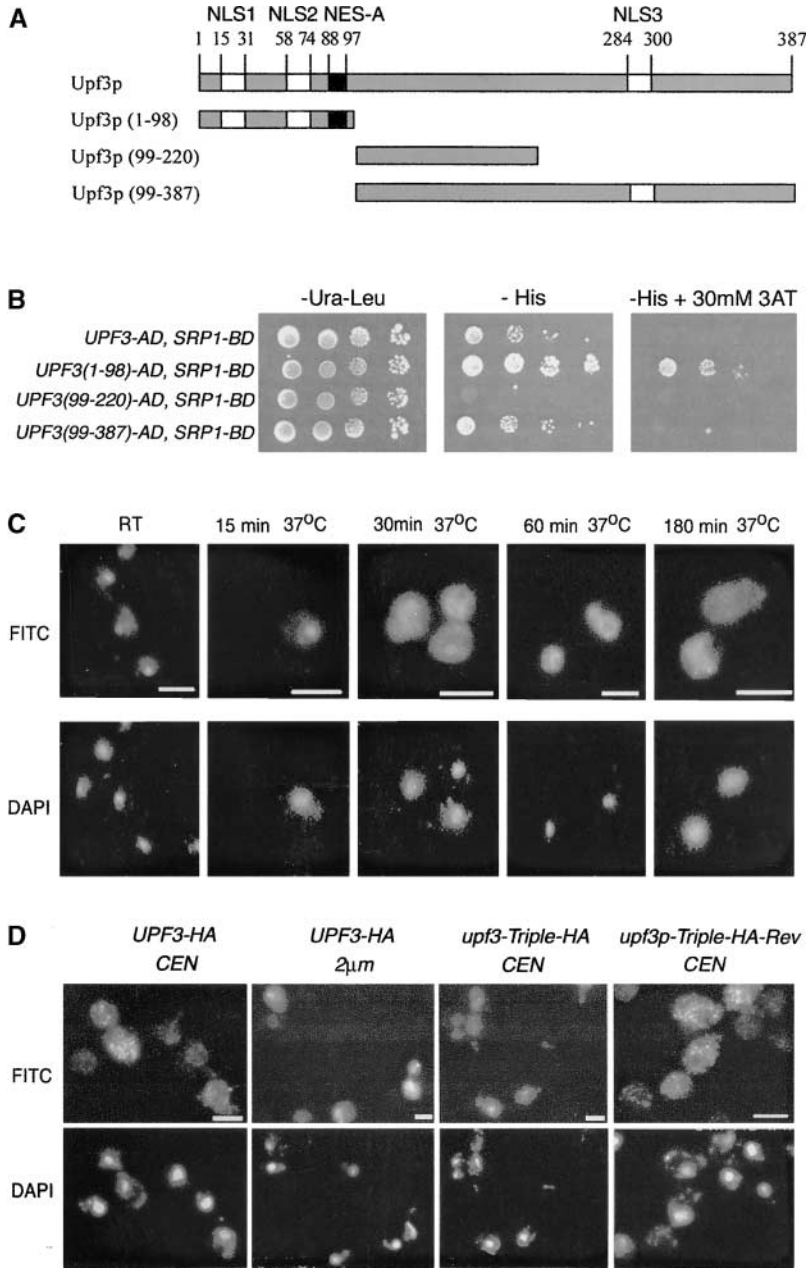

FIGURE 1.—Srp1p mediates the import of Upf3p into the nucleus. (A) Amino acid segments of Upf3p fused in-frame to the Gal4 activation or binding domain used in all two-hybrid experiments. (B) Strain PJ69-4A was transformed with plasmids, resulting in strains with the genotypes indicated to the left. Growth was monitored using serial drop tests by plating 10^0, 10^1, 10^2, and 10^3 serial dilutions (left to right) of log phase cultures (see MATERIALS AND METHODS) on SD without leucine and uracil (left), SD without histidine (middle), and SD without histidine with 30 mM 3-AT (right). (Left) The relative growth under conditions that select for the presence of the plasmids carrying the *SRP1-BD* and *UPF3-AD* alleles is shown. (Middle and right) The relative growth of the same strain under conditions in which the extent of growth is proportional to the strength of the interaction between the proteins is shown. (C) Subcellular localization of Upf3p-HA expressed from a 2μ plasmid in the temperature-sensitive *srp1-31* strain PSY730. Cells carrying *srp1-31* were grown at 25° and then shifted to 37°. Cells were removed at 0, 15, 30, 60, and 180 min after the shift to 37°. The detection of FITC staining in representative cells from each time point is shown at the top. (D) Subcellular localization of epitope-tagged Upf3p-HA expressed from centromeric and 2μ plasmids (left and middle) and Upf3p-Triple-HA expressed from a centromeric plasmid in strain LRSy323 (right) is shown. (C and D, bottom) DAPI staining marks the nucleus. Bar, 2.5 μm.

ature (TABB *et al.* 2000). A 2μ plasmid expressing *UPF3-HA* was transformed into strain PSY730 (*srp1-31*). The localization of Upf3p-HA was determined by indirect immunofluorescence microscopy at several time points following a shift to 37° and compared to DAPI staining. As controls (Figure 1D), we assessed the localization of wild-type Upf3p-HA expressed from centromeric and 2μ plasmids and a mutant version of Upf3p-HA expressed from a centromeric plasmid carrying a defective nuclear export sequence (Upf3p-Triple-HA). Overexpressed Upf3p-HA and export-defective Upf3p-HA expressed at a normal level accumulate in the nucleolus, whereas wild-type Upf3p-HA expressed from a centromeric plasmid accumulates only in the cytoplasm (SHIRLEY *et al.* 1998).

At the permissive temperature, Upf3p-HA accumulates in a distinct area of the nucleus adjacent to the area stained by DAPI as well as throughout the cytoplasm (Figure 1C, RT). The area of staining within the nucleus corresponds to the nucleolus (SHIRLEY *et al.* 1998) and serves as an indicator of import into the nucleus. After 15 min following the shift of the *srp1-31* strain to 37°, the localization of Upf3p-HA was indistinguishable from its localization at room temperature. However, after ≥30 min Upf3p-HA was not detected in the nucleolus and was visible only in the cytoplasm. We also examined the distribution of Upf3p-HA expressed from a 2μ plasmid in an *SRP1* strain. Even after 180 min following the shift to 37°, Upf3p-HA localized to the nucleolus and cytoplasm in a pattern indistinguishable from its local-

ization at room temperature. The gradual decrease in the nucleolar accumulation of Upf3p-HA at the nonpermissive temperature in the *srp1-31* strain indicates that Srp1p is necessary for the import of Upf3p into the nucleus. These results indicate that nuclear import of Upf3p is mediated by the Impα/β heterodimer.

Export of Upf3p via a leucine-rich nuclear export sequence is required for a fully functional NMD pathway. We showed previously that the export of Upf3p from the nucleus requires a leucine-rich signal sequence called NES-A (Figure 1A; Shirley *et al.* 1998). The substitution of alanine for two leucine and one isoleucine residue in NES-A (*upf3-Triple-HA*) causes a redistribution of the protein from the cytoplasm to the nucleolus (Figure 1D). This allele impairs the function of Upf3p-Triple as indicated by allosuppression of the NMD-sensitive *his4-38* frameshift mutation and by increased accumulation of nonsense mRNAs. The insertion of the wild-type NES from HIV-1 Rev at the C terminus of Upf3p-Triple-HA (Upf3p-Triple-HA-Rev) restores the export of Upf3p-Triple-HA-Rev from the nucleus; however, we found that strains carrying the *upf3-Triple-HA-Rev* allele were phenotypically indistinguishable from a *upf3-Triple-HA* strain when assayed by the *his4-38/SUF1-1* allosuppression assay (Shirley *et al.* 1998). These initial results precluded the demonstration that retaining Upf3p in the nucleus directly affects the function of Upf3p.

Since allosuppression of the *his4-38* frameshift mutations is complex and requires the presence of a tRNA frameshift suppressor, we reasoned that direct suppression of a nonsense mutation is likely to be a more sensitive indicator of perturbations in the function of Upf3p. We used nonsense suppression assays to reexamine the function of *upf3-Triple-HA* and *upf3-Triple-HA-Rev* in strains carrying *leu2-1* (UAA), *tyr7-1* (UAG), and *can1-100* (UAA). In the presence of wild-type *UPF* genes, strains carrying these nonsense mutations fail to grow on SD medium without leucine and tyrosine but are able to grow when canavanine is added. Mutations that impair the function of the Upf proteins confer growth on SD medium without leucine and tyrosine and prevent growth on SD medium plus leucine, tyrosine, and canavanine.

Strain RSy5 (*leu2-1 tyr7-1 can1-100 upf3-Δ1*) was transformed with a centromeric plasmid containing two tandem copies of *upf3-Triple-HA* (pRLS125). The presence of two gene copies compensates for the approximately twofold underexpression of protein expressed from *upf3-Triple* (Shirley *et al.* 1998). To create the *upf3-Δ1*, *UPF3*, and *UPF3-HA* strains, RSy5 was transformed with an empty vector (pRS316) and with centromeric plasmids containing *UPF3* (pLS17) and *UPF3-HA* (pLS51).

The *upf3-Δ1* transformant grew on SD medium without leucine and tyrosine but not in the presence of canavanine (Figure 2). The transformants expressing wild-type *UPF3* or epitope-tagged *UPF3-HA* failed to grow on SD medium without leucine and tyrosine but grew on canavanine-containing medium. Transformants carrying *upf3-Triple-HA* grew on SD medium without leucine and tyrosine and on the canavanine-containing medium but not as robustly as either the *upf3-Δ1* strain or the *UPF3* strain, respectively. These results indicate that the level of suppression in the *upf3-Triple-HA* strain is reduced compared to that of the *upf3-Δ1* strain. Reduced suppression of *leu2-1*, *tyr7-1*, and *can1-100* indicates that the three alanine substitutions in NES-A cause decreased function of the Upf3p-Triple protein.

The above transformants were also assayed for the activity of the NMD pathway by determining the relative levels of accumulation of *CYH2* pre-mRNA (He *et al.* 1993; Figure 3; Table 2). The amount of *CYH2* pre-mRNA was 5.29 ± 0.57-fold more abundant in a *upf3-Δ1* strain than in a transformant carrying wild-type *UPF3*. The relative accumulation in transformants carrying *upf3-Triple-HA* is 3.44 ± 0.30. This accumulation was significantly less than that observed in a *upf3-Δ1* strain ($P < 0.005$), indicating that the alanine substitutions in NES-A seriously impair but do not completely abolish the function of Upf3p.

The Upf3p-Triple-HA protein exhibits impaired nuclear export, and as a result it redistributes from the cytoplasm to the nucleolus (Figure 1D). To further examine the export of this protein, we transformed strain RSy5 with a plasmid containing either two tandem copies of *upf3-Triple-HA-Rev* (pRLS145) or tandem copies of *upf3-Triple-HA-M10* (pRLS144). The *upf3-Triple-HA-Rev* and *upf3-Triple-HA-M10* alleles code for Upf3-Triple-HA that contains either the wild-type Rev NES or the export-defective M10 Rev NES at the C terminus (Meyer and Malim 1994; Fischer *et al.* 1995). Consistent with previous results (Shirley *et al.* 1998), we found that the wild-type Rev NES but not the export-deficient M10 NES restores the nuclear export of Upf3p-Triple-HA (Figure 1D).

To examine the effect that restoration of nuclear export has on the function of the mutant Upf3-Triple-HA-Rev protein, we compared the level of nonsense suppression in transformants expressing *upf3-Triple-HA-Rev* with those expressing *upf3-Triple-HA*. The growth of the *upf3-Triple-HA-Rev* strain on SD medium without leucine and tyrosine was less than that of the *upf3-Triple-HA* strain (Figure 2). When canavanine was present, the *upf3-Triple-HA-Rev* strain grew more robustly than the *upf3-Triple-HA* strain (Figure 2). These results demonstrate that the level of suppression of the *leu2-1*, *tyr7-1*, and *can1-100* nonsense mutations is lower in the transformants expressing *upf3-Triple-HA-Rev* than in those expressing *upf3-Triple-HA*. The growth of transformants carrying *upf3-Triple-HA-M10* was indistinguishable from the growth of a *upf3-Triple-HA* strain on both types of selective media (Figure 2). These data indicate that only the export-competent Rev NES element improves the function of the Upf3-Triple protein.

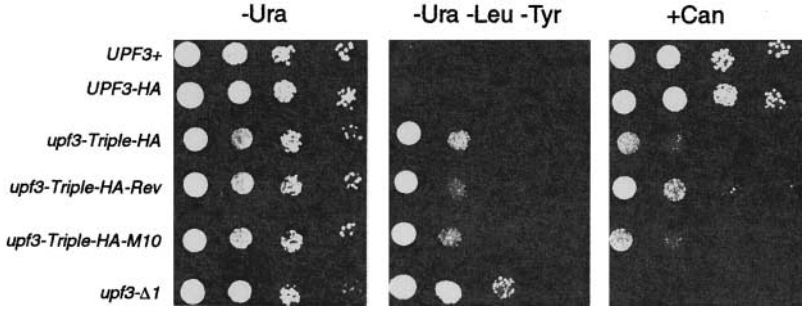

FIGURE 2.—Effect of the HIV-1 Rev nuclear export sequence on the function of Upf3p-Triple-HA. Suppression of *leu2-1*, *tyr7-1*, and *can1-100* was used as an indicator of Upf3p function in which growth was monitored on SD-leucine, tyrosine, and SD medium containing canavanine, using serial drop tests (Figure 1; MATERIALS AND METHODS). Strain RSy5 (*leu2-1 tyr7-1 can1-100 upf3-Δ1*) was transformed with centromeric plasmids, resulting in strains with the genotypes indicated at the left. Relative growth rates were compared as described in Figure 1 on SD-uracil (left), SD-leucine, tyrosine (middle), and SD medium containing canavanine (right). (Left) Relative growth under conditions that select for the presence of the *URA3* plasmids carrying the *upf3* alleles is shown. (Middle) Relative growth of the same strains under conditions in which the extent of growth is proportional to the extent of impairment of Upf3p function is shown. (Right) Relative growth in the presence of canavanine in which growth is inversely proportional to the extent of impairment of Upf3p function is shown.

A comparison of the relative levels of *CYH2* pre-mRNA accumulation in transformants carrying *upf3-Triple-HA-Rev* (2.98 ± 0.29) and *upf3-Triple-HA* (3.44 ± 0.30) indicates that the addition of the exogenous Rev NES significantly decreases the accumulation of *CYH2* pre-mRNA ($P < 0.05$; Figure 3; Table 2). This decrease was specific to the wild-type Rev NES since the relative level of *CYH2* pre-mRNA accumulation in the *upf3-Triple-HA-M10* strain (3.56 ± 0.29) was statistically indistinguishable from the *upf3-Triple-HA* strain ($P > 0.90$). These results indicate that rescuing the export of Upf3p-Triple-HA by insertion of the Rev NES improves the ability of Upf3p to function in NMD. We conclude, therefore, that impairing the export of Upf3p causes an impairment of NMD. This indicates that the export of Upf3p is required for a fully functional NMD pathway.

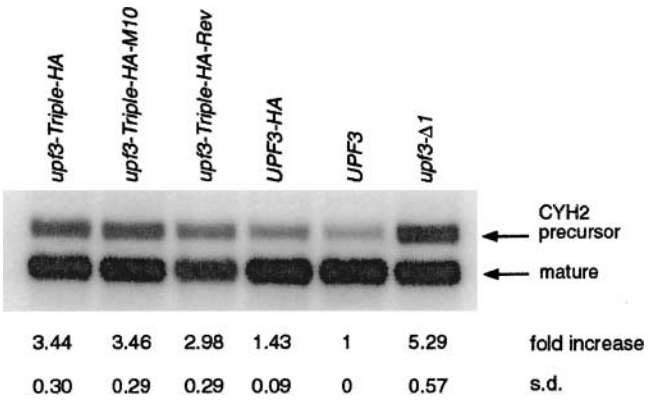

FIGURE 3.—Effect of the wild-type and M10 Rev NES on the function of Upf3p-Triple-HA in NMD. Northern blotting was used to assess the effect of inserting sequences coding for the wild-type and M10 Rev NES in *upf3-Triple-HA* on the accumulation of *CYH2* pre-mRNA. Strain RSy5 was transformed with centromeric plasmids expressing the genes indicated at the top of each lane. The plasmids expressing the *upf3-Triple* alleles indicated in the figure contained duplicate copies of each gene. The representative hybridization signal specific to the precursor and mature forms of *CYH2* RNA is shown. The relative accumulation of *CYH2* pre-mRNA expressed as the fold increase and the associated SD was calculated on the basis of five experiments. The calculation of relative accumulation and the statistical treatment of the data are described in MATERIALS AND METHODS.

Nonconservative amino acid substitutions in NES-A impair Upf3p function more severely than alanine substitutions: We isolated five new *upf3-nes* alleles containing less conservative amino acid changes in NES-A than the alanine substitutions encoded by the *upf3-Triple* allele. Transformants carrying the mutant *upf3-nes* alleles were assayed for suppression of the *leu2-1*, *tyr7-1*, and *can1-100* nonsense mutations and for the accumulation of *CYH2* pre-mRNA. The cellular distribution of two of the mutant proteins was also examined.

The growth of transformants carrying centromeric plasmids expressing the *upf3-nes* alleles on SD medium without leucine and tyrosine and the canavanine-containing medium was indistinguishable from the growth of a strain expressing *upf3-Triple-HA* (Table 3). Similarly, the accumulation of *CYH2* pre-mRNA in the five *upf3-nes* strains was similar to that found in transformants carrying *upf3-Triple-HA*. These results indicate that the amino acid substitutions in the Upf3-nes proteins cause impaired function. The staining patterns of epitope-tagged Upf3p-nes2-HA and Upf3p-nes7-HA were indistinguishable from the nucleolar distribution of Upf3p-Triple-HA, indicating that export of the mutant proteins is impaired (data not shown).

To determine if the wild-type Rev NES can restore function to these proteins, the sequence coding for the Rev NES was inserted in the *upf3-nes2*, *-nes3*, *-nes5*, *-nes6*, and *-nes7* alleles. Transformants carrying centromeric plasmids expressing these alleles were assayed for suppression of the *leu2-1*, *tyr7-1*, and *can1-100* nonsense mutations and for the accumulation of *CYH2* pre-mRNA. The growth of transformants carrying the *upf3-nes-Rev* alleles on SD medium without leucine and tyrosine and on the canavanine-containing medium resembled the growth of strains containing the identical mutations but without the insertion of HIV-1 Rev. Similarly, the insertion of the HIV-1 Rev NES did not affect the accumulation of *CYH2* pre-mRNA (Table 4). These results indicate that, unlike Upf3p-Triple-HA, more drastic amino

TABLE 2

Effect of HIV Rev NES on *CYH2* pre-mRNA accumulation

Comparison between strains		Average fold change in mRNA accumulation ±SD[a]		H_O: u(A) = u(B)[b] H_A: u(A) ≠ u(B)	
A	B	A	B	*P* value	α = 0.05
upf3-Triple-HA	*upf3-Triple-HA-M10*	3.44 ± 0.30	3.46 ± 0.29	0.93	Accept
upf3-Triple-HA	*upf3-Triple-HA-Rev*	3.44 ± 0.30	2.98 ± 0.29	0.038	Reject
upf3-Triple-HA-M10	*upf3-Triple-HA-Rev*	3.46 ± 0.29	2.98 ± 0.29	0.031	Reject
upf3-Triple-HA	*upf3-Δ1*	3.44 ± 0.30	5.29 ± 0.57	0.0002	Reject

Accept, RNA accumulation was statistically the same in strain A *vs.* strain B; Reject, RNA accumulation was statistically different in strain A *vs.* strain B.

[a] mRNA accumulation was determined by Northern blotting.

[b] The relative accumulation expressed as the average fold change and the statistical treatment of data are described in MATERIALS AND METHODS.

acid changes in NES-A impair Upf3p function in a manner that is not corrected by HIV-1 Rev-directed restoration of nuclear export.

Overexpression of *UPF2* partially restores impaired function of mutant Upf3p: To further examine mutations in NES-A, we tested whether overexpression of *UPF1* or *UPF2* modifies the phenotypes of the NES-A mutations. Overexpression of *UPF1* in a strain carrying *upf3-Triple-HA* had no phenotypic effect as measured by the level of nonsense suppression (data not shown). However, coexpression of the *upf3-Triple-HA* allele with a 2μ plasmid carrying *UPF2* led to observable changes in the suppression of *leu2-1*, *tyr7-1*, and *can1-100* and in the accumulation of *CYH2* pre-mRNA (Figure 4; Table 4).

Transformants carrying both *upf3-Triple-HA* and 2μ *UPF2* grow slightly less than the *upf3-Triple-HA* strain on SD medium without tyrosine and leucine (Figure 4A). The same transformants grew better than the *upf3-Triple-HA* strain on the canavanine-containing medium. These results suggest that increasing the expression of *UPF2* in a *upf3-Triple-HA* strain decreases the extent of nonsense suppression, which indicates improved function. The effect caused by the overexpression of *UPF2* was specific to the *upf3-Triple-HA* allele because overexpression of *UPF2* had no effect on nonsense suppression in a strain carrying a *upf3-Δ1* disruption (data not shown). The decrease in nonsense suppression in a *upf3-Triple-HA* strain caused by the overexpression of *UPF2* was approximately the same as the decrease observed upon addition of the Rev NES element to Upf3p-Triple-HA.

The strains were assayed for the effect of overexpress-

TABLE 3

Phenotypes of *upf3* alleles containing mutations in NES-A

UPF3 allele	NES-A amino acid substitutions[a]	Nonsense suppression[b]	Average fold change in CYH2 pre-mRNA accumulation ±SD[c]	Subcellular localization[d]
UPF3	None	—	1 ± 0	Cytoplasm
upf3-Δ1	NA	++++	5.29 ± 0.57	ND
upf3-Triple-HA	L88A I90A L93A	++	3.44 ± 0.30	Nucleolus/cytoplasm
upf3-nes2	L88D I90A L92G L93S	++	3.28 ± 0.86	Nucleolus/cytoplasm
upf3-nes3	L88N I90D L93R	++	ND	ND
upf3-nes5	L88R I90L L92G L93S	++	4.17 ± 0.81	ND
upf3-nes6	L88G I90V L92P L93K	++	3.79 ± 0.36	ND
upf3-nes7	L88Q I90R L92V L93G	++	4.19 ± 0.82	Nucleolus/cytoplasm

ND, not done; NA, not applicable.

[a] The wild-type amino acid sequence for NES-A: ^{88}LVIRLLPPNL^{97}. The column lists the amino acid substitutions in NES-A in each of the *upf3* alleles.

[b] The degree of suppression of *leu2-1*, *tyr7-1*, and *can1-100* is indicated as follows: —, failure to suppress the nonsense; ++++, full suppression of the nonsense alleles indicated by robust growth on SD-leucine, tyrosine medium, and no growth on media SD + canavanine; ++, partial suppression of the nonsense alleles.

[c] The relative level of accumulation of CYH2 pre-mRNA as determined by Northern blotting. The relative accumulation expressed as the average fold change and the statistical treatment of data are described in MATERIALS AND METHODS.

[d] The localization of the Upf3p-HA proteins containing amino acid substitutions in the NES-A element.

TABLE 4

***CYH2* pre-mRNA accumulation in strains carrying *upf3-nes* alleles**

Strain comparisons		Nonsense suppression[a]		Average fold change in mRNA accumulation ± SD[b]		H_O: u(A) = u(B)[c] H_A: u(A) ≠ u(B)	
A	B	A	B	A	B	*P* value	α = 0.05
upf3-Triple	*upf3-Triple-Rev*	++	+	3.44 ± 0.30	2.98 ± 0.29	0.038	Reject
upf3-nes2	*upf3-nes2-Rev*	++	++	3.28 ± 0.86	3.30 ± 0.81	0.98	Accept
upf3-nes3	*upf3-nes3-Rev*	++	++	ND	ND	NA	NA
upf3-nes5	*upf3-nes5-Rev*	++	++	4.17 ± 0.81	3.64 ± 0.64	0.43	Accept
upf3-nes6	*upf3-nes6-Rev*	++	++	3.79 ± 0.36	3.71 ± 0.72	0.88	Accept
upf3-nes7	*upf3-nes7-Rev*	++	++	4.19 ± 0.82	3.95 ± 0.73	0.72	Accept
upf3-Triple	*upf3-Triple 2μ UPF2*	++	+	3.87 ± 0.52	3.03 ± 0.43	0.012	Reject
upf3-nes2	*upf3-nes2 2μ UPF2*	++	+	3.15 ± 0.28	2.97 ± 0.14	0.38	Accept
upf3-nes3	*upf3-nes3 2μ UPF2*	++	+	ND	ND	NA	NA
upf3-nes5	*upf3-nes5 2μ UPF2*	++	+	3.72 ± 0.27	3.15 ± .022	0.046	Reject
upf3-nes6	*upf3-nes6 2μ UPF2*	++	+	3.58 ± 0.22	2.83 ± 0.08	0.005	Reject
upf3-nes7	*upf3-nes7 2μ UPF2*	++	+	2.96 ± 0.10	2.55 ± 0.16	0.018	Reject

ND, not done; NA, not applicable. Accept, RNA accumulation was statistically the same in strain A *vs.* strain B. Reject, RNA accumulation was statistically different in strain A *vs.* strain B.

[a] ++ and + represent varying degrees of partial suppression of *leu2-1, tyr7-1,* and *can1-100.*

[b] mRNA accumulation was determined by Northern blotting.

[c] The relative accumulation expressed as the average fold change and the statistical treatment of data are described in MATERIALS AND METHODS.

ing *UPF2* on NMD (Figure 4B). The relative accumulation of *CYH2* pre-mRNA was 3.87 ± 0.52 in a transformant carrying *upf3-Triple-HA.* Overexpression of *UPF2* in a *upf3-Triple-HA* strain caused a significant decrease in the relative accumulation of *CYH2* pre-mRNA to 3.03 ± 0.43 ($P < 0.05$; Figure 4; Table 5). By comparison, the relative accumulation of *CYH2* pre-mRNA in the *upf3-Triple-HA* strain carrying overexpressed *UPF2* was not significantly different from the 3.12 ± 0.39-fold accumulation observed in a *upf3-Triple-HA-Rev* strain ($P > 0.5$). The results indicate that the overexpression of *UPF2* suppresses the Nmd^- phenotype in a *upf3-Triple-HA* strain by the same magnitude as the addition of Rev NES to Upf3p-Triple-HA.

When *UPF2* is overexpressed, nonsense suppression was decreased in strains carrying *upf3-nes2, upf3-nes3, upf3-nes5, upf3-nes6,* and *upf3-nes7* (Table 4). Overexpression of *UPF2* caused a significant decrease in the relative accumulation of *CYH2* pre-mRNA in transformants carrying *upf3-nes5, upf3-nes6,* and *upf3-nes7.* The overexpression did not cause a statistically significant change in the accumulation of *CYH2* pre-mRNA in the transformants carrying *upf3-nes2.* Although the insertion of the Rev NES has no effect on the function of the Upf3-nes5, Upf3-nes6, and Upf3-nes7 proteins in NMD, the overexpression of *UPF2* partially suppressed the Nmd^- phenotype in strains carrying these *upf3-nes* alleles. Taken together, these data demonstrate that the effects of the NES-A substitutions on the function of the mutant Upf3 proteins can be partially suppressed by overexpression of *UPF2.*

We asked whether simultaneously adding the Rev NES and overexpressing *UPF2* fully restores function to Upf3p-Triple. To accomplish this, centromeric plasmids expressing *upf3-Triple-HA-M10* and *upf3-Triple-HA-Rev* were separately cotransformed with the 2μ *UPF2* plasmid into strain RSy5. The transformants were assayed for nonsense suppression and for the accumulation of *CYH2* pre-mRNA (Figure 4; Table 5).

Overexpression of *UPF2* caused reduced suppression of nonsense mutations to the same extent in strains carrying either *upf3-Triple-HA-M10* or *upf3-Triple-HA* (Figure 4A). In a strain carrying *upf3-Triple-HA-Rev,* overexpression of *UPF2* caused a slight reduction in suppression of nonsense mutations compared to a strain carrying *upf3-Triple-HA-Rev* without 2μ *UPF2* and a strain carrying *upf3-Triple-HA* with 2μ *UPF2.* These results show that the insertion of the Rev NES in combination with the overexpression of *UPF2* causes only slightly more nonsense suppression than that which occurs due to the Rev NES or *UPF2* overexpression alone.

Transformants carrying *upf3-Triple-HA-M10* and 2μ *UPF2* displayed a relative accumulation of *CYH2* pre-mRNA of 3.13 ± 0.52 (Figure 4B). By comparison, the accumulation level was 3.93 ± 0.77 in the absence of overexpressed *UPF2.* We expected these values to be different because the M10 NES is nonfunctional, but in this experiment the difference was of borderline statistical significance ($P > 0.05$; Table 5). Transformants carrying *upf3-Triple-HA-Rev* and 2μ *UPF2* displayed a relative accumulation of 2.82 ± 0.41. The accumulation level was 3.12 ± 0.39 in the absence of 2μ *UPF2.* These

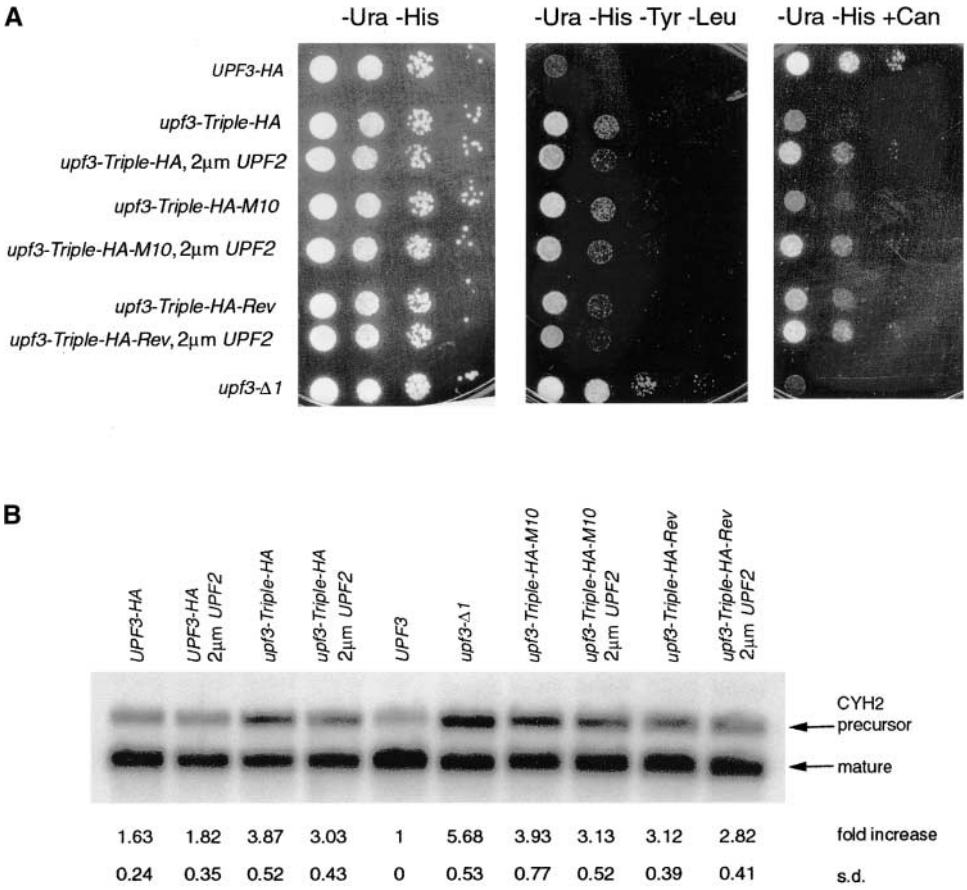

FIGURE 4.—Effects of Upf2p overexpression in transformants expressing *upf3-Triple-HA* and *upf3-Triple-HA-Rev*. (A) Suppression of *leu2-1*, *tyr7-1*, and *can1-100* was assayed by serial drop tests as described in Figure 1 and MATERIALS AND METHODS. Strain RSy5 was transformed with sets of plasmids, resulting in strains with the genotypes indicated to the left. (B) Northern blotting was used to assess the accumulation of *CYH2* pre-RNA. Strain RSy5 was transformed with sets of plasmids, resulting in strains with the genotypes indicated at the top. The representative hybridization signal specific to the precursor and mature forms of *CYH2* RNA is shown. The relative accumulation of *CYH2* pre-mRNA expressed as the fold increase and the associated SD was calculated on the basis of six experiments. The calculation of relative accumulation and the statistical treatment of the data are described in MATERIALS AND METHODS.

values are not significantly different. Similarly, the accumulation of *CYH2* pre-mRNA in transformants carrying *upf3-Triple-HA* and 2μ *UPF2* (3.03 ± 0.43) was not significantly different from the *upf3-Triple-HA-Rev* strain carrying 2μ *UPF2*. Taken together, these data demonstrate that while insertion of the Rev NES and overexpression of *UPF2* separately improve the function of Upf3p-Triple, insertion of the Rev NES in combination with overexpression of *UPF2* does not further restore function. The effects are nonadditive.

We explored the possibility that improving the interaction between Upf2p and Upf3p-Triple might increase the efficiency of export of Upf3p-Triple and therefore improve the function of the mutant Upf3 protein. If Rev NES and Upf2p have synonymous or related functions, this hypothesis would account for the lack of addi-

TABLE 5

Combined effect of HIV Rev NES and *UPF2* overexpression on CYH2 pre-mRNA accumulation

Comparison between strains		Average fold change in mRNA accumulation ± SD[a]		H_0: u(A) = u(B)[b] H_A: u(A) ≠ u(B)	
A	B	A	B	*P* value	α = 0.05
upf3-Triple-HA	*upf3-Triple-HA* 2μ *UPF2*	3.87 ± 0.52	3.03 ± 0.43	0.012	Reject
upf3-Triple-HA-M10	*upf3-Triple-HA-M10* 2μ *UPF2*	3.93 ± 0.77	3.13 ± 0.52	0.060	Accept
upf3-Triple-HA-Rev	*upf3-Triple-HA-Rev* 2μ *UPF2*	3.12 ± 0.39	2.82 ± 0.41	0.23	Accept
upf3-Triple-HA-M10	*upf3-Triple-HA-Rev*	3.93 ± 0.77	3.12 ± 0.39	0.043	Reject
upf3-Triple-HA	*upf3-Triple-HA-Rev*	3.87 ± 0.52	3.12 ± 0.39	0.018	Reject
upf3-Triple-HA 2μ *UPF2*	*upf3-Triple-HA-Rev* 2μ *UPF2*	3.03 ± 0.43	2.82 ± 0.41	0.41	Accept

Accept, RNA accumulation was statistically the same in strain A *vs.* strain B; Reject, RNA accumulation was statistically different in strain A *vs.* strain B.

[a] mRNA accumulation was determined by Northern blotting.

[b] The relative accumulation expressed as the average fold change and the statistical treatment of data are described in MATERIALS AND METHODS.

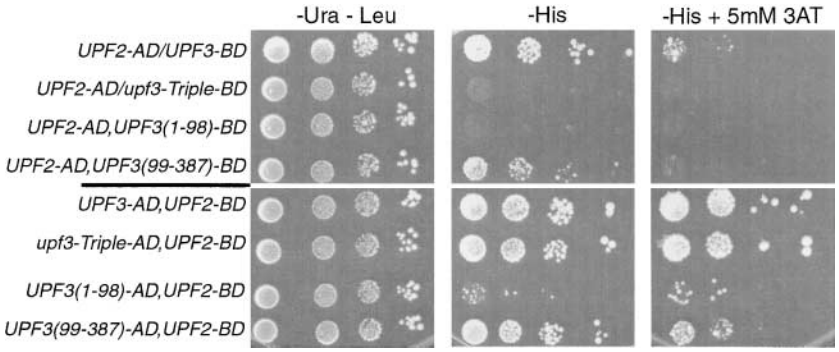

FIGURE 5.—Contribution of the NES-A element of Upf3p to the Upf3p/Upf2p interaction. Strain PJ69-4A was transformed with the plasmids expressing the genes indicated on the left. Growth was monitored by serial drop tests (Figure 1; MATERIALS AND METHODS). The interaction between the Gal4 activation domain and Gal4 binding domain fusion proteins was monitored by growth on SD-histidine (middle) and SD-histidine with 5 mM 3-AT (right).

tivity when Rev NES and Upf2p are combined. We examined the distribution of Upf3p-Triple-HA using indirect immunofluorescence in a strain overexpressing *UPF2*, but saw no change in the distribution of the mutant protein. Upf3p-Triple-HA was still trapped in the nucleolus (data not shown). Furthermore, the distribution of Upf3p-HA did not change in *upf2-Δ1* cells (data not shown). These results suggest that overexpression of Upf2p improves the function of the Upf3p-Triple by a mechanism unrelated to the ability of the mutant protein to export from the nucleus to the cytoplasm.

Effects of *nesA*$^-$ alleles on the physical interaction between Upf3p and Upf2p: Amino acids 78–278 of Upf3p, which include the NES-A element, are necessary for the interaction between Upf3p and Upf2p (HE *et al.* 1997). Since one way to compensate for an altered interaction between two proteins is by overexpressing one of the interacting components (PHIZICKY and FIELDS 1995), we speculated that overexpression of *UPF2* might improve an impaired interaction between Upf2p and Upf3p-Triple.

The Gal4p DNA binding domain was fused in-frame to full-length, wild-type Upf3p and the Gal4p DNA activation domain was fused in-frame with Upf2p. The plasmids were separately transformed into strain PJ69-4A. Coexpression of *UPF3-BD* and *UPF2-AD* resulted in robust growth on SD medium without histidine containing up to 5 mM 3-AT (Figure 5). Next we made plasmids carrying genes coding for Upf3p-Triple or Upf3p-nes fused to the Gal4p binding domain. Coexpression of *upf3-Triple-BD* and *UPF2-AD* failed to confer growth on selective medium. In addition, coexpression of the five *upf3-nes-BD* alleles with *UPF2-AD* also failed to promote growth on selective medium (data not shown). RSy5 expressing *upf3-Triple-BD* suppresses the *leu2-1*, *tyr7-1*, and *can1-100* alleles to the same extent as the *upf3-Triple-HA* allele (data not shown). The lack of interaction between Upf3-Triple-BD and Upf2p is therefore not due to gross misfolding and/or severe instability of the fusion protein. These results suggest that amino acid substitutions in the NES-A region of Upf3p diminish the interaction between Upf3p-Triple-BD and Upf2p-AD.

We also examined two-hybrid interactions using fusions in which the Gal4p activation and DNA binding domains were reversed. Similar to the results described above, coexpression of *UPF3-AD* and *UPF2-BD* conferred growth on selective medium (Figure 5). However, a stronger interaction was detected since growth was observed on SD-histidine medium in the presence of up to 25 mM 3-AT (data not shown). In contrast to the opposite orientation of the fusions, coexpression of *upf3-Triple-AD* and *UPF2-BD* conferred growth in the presence of 5 mM 3-AT (Figure 5) and 25 mM 3-AT (data not shown). Increasing the concentration of 3-AT failed to result in detectable differences between transformants expressing *UPF3-AD* or *upf3-Triple-AD* with *UPF2-BD*. Similar results were observed when the five Upf3-nes-AD proteins were tested. Upf3p-Triple-AD suppresses the *tyr7-1* and *can1-100* nonsense alleles to the same extent as Upf3p-Triple, indicating that the fusion does not have a gain-of-function mutation allowing for an interaction between Upf2p-BD and Upf3p-Triple-AD (data not shown). More likely, the difference between the two orientations is due to the weaker interaction observed between the Upf3p-BD and Upf2p-AD fusion proteins. The weaker interaction in this orientation may provide for a more sensitive assay that allows detection of smaller changes in the binding affinities between Upf3p-Triple and Upf2p.

To clarify the situation, we determined the extent to which the NES-A region contributes to the interaction with Upf2p. We made plasmids expressing fusions between the Gal4p binding domain and amino acids 1–98 or amino acids 99–387 of Upf3p (see Figure 1A). Upf3p(1-98) contains only the first 21 N-terminal amino acids, including those of NES-A, which were previously identified as part of the Upf2p interacting domain. Upf3p(99-387) contains the majority of the fragment that was previously identified as interacting with Upf2p except for the NES domain (HE *et al.* 1997). Plasmids expressing *UPF3(1-98)-BD* and *UPF3(99-387)-BD* were separately cotransformed with a 2μ plasmid expressing *UPF2-AD* into strain PJ69-4A. Coexpression of *UPF3(1-98)BD* and *UPF2-AD* did not confer growth on selective media, indicating that the NES-A region alone does not support an interaction between Upf3p and Upf2p (Figure 5). Coexpression of *UPF3(99-387)-BD* and *UPF2-*

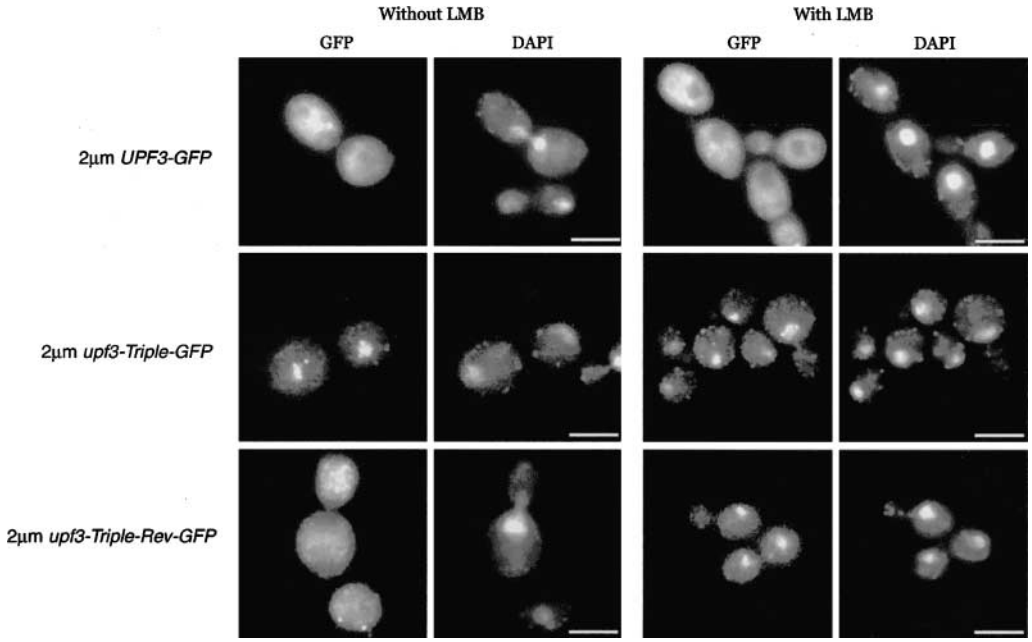

FIGURE 6.—Loss of Crm1p/Xpo1p function does not affect the export of Upf3p. The leptomycin-sensitive strain MNY8 was transformed with 2μ plasmids pNE39, pAF14, and pAF51 expressing *UPF3-GFP*, *upf3-Triple-GFP*, and *upf3-Triple-Rev-GFP*, respectively. Cells were incubated without and with leptomycin B (left and right, respectively; see MATERIALS AND METHODS). GFP fluorescence was compared with DAPI staining as indicated. Bar, 2.5 μm.

AD promoted growth but to a lesser extent than transformants expressing full-length *UPF3-BD* and *UPF2-AD*.

We reversed the fusion constructs and found that transformants expressing *UPF3(1-98)-AD* and *UPF2-BD* again did not support growth on selective media. The strain expressing *UPF3(99-387)-AD* and *UPF2-BD* grew less robustly in the presence of 5 mM 3-AT than did transformants expressing *UPF3-AD* and *UPF2-BD* (Figure 5). Therefore, consistent with our results using fusions to the Gal4p binding domain, these data indicate that NES-A alone is not sufficient for an interaction with Upf2p, but removing the NES-A region from Upf3p weakens the interaction. The NES-A region is necessary but not sufficient for an interaction.

The export of Upf3p does not require the function of Crm1p/Xpo1p exportin: Crm1p/XpoIp is a nuclear export receptor that binds to leucine-rich NES sequences (FORNEROD *et al.* 1997; STADE *et al.* 1997; IZAURRALDE and ADAM 1998; HO *et al.* 2000). We asked whether Upf3p physically interacts with Crm1p and whether Upf3p export depends on the function of this exportin. Using the two-hybrid system, we confirmed the known interaction between Crm1p-AD and Rip1p-BD (NEVILLE *et al.* 1997; HO *et al.* 2000), but were unable to detect an interaction between Crm1p and Upf3p (data not shown). To determine whether Crm1p mediates the nuclear export of Upf3p, we localized Upf3p-HA in a strain carrying a temperature-sensitive *xpo1-1* mutation. In this strain, the export of proteins by Crm1p ceases 5–15 min following a shift to the nonpermissive temperature (STADE *et al.* 1997). We were unable to detect nucleolar accumulation of Upf3p-HA expressed from a centromeric plasmid at the nonpermissive temperature (data not shown).

Proteins up to 60 kD have the potential to passively diffuse through the yeast nuclear pore complex (NPC; BORER *et al.* 1989; PANTE and AEBI 1995). Since Upf3p is a 44.9-kD protein (LEE and CULBERTSON 1995), it could potentially cross the NPC by passive diffusion. To test the passive diffusion model, we increased the size of Upf3p by fusing GFP to the Upf3p N terminus generating an ~72-kD fusion protein. The distribution of Upf3p-GFP expressed from a 2μ plasmid in the strain carrying the *xpo1-1* mutation was examined. Consistent with previous results, the distribution of Upf3p-GFP did not change upon shift to the nonpermissive temperature (data not shown). We also examined the distribution of Upf3p-GFP expressed from a 2μ plasmid in three different strains containing viable mutant alleles of *crm1*: *crm1-1*, *crm1-2*, and *crm1-3* (NEVILLE *et al.* 1997). However, we did not observe an increase in the nucleolar accumulation or a decrease in cytoplasmic staining of Upf3p-GFP in the *crm1* strains (E. NEENO-ECKWALL and M. CULBERTSON, unpublished data).

Leptomycin B (LMB), a potent inhibitor of Crm1-mediated transport, binds to mammalian and *S. pombe* Crm1p (FORNEROD *et al.* 1997; ASKJAER *et al.* 1998). Although *S. cerevisiae* Crm1p is insensitive to LMB, a T539C substitution renders Crm1p sensitive to LMB and inhibits Crm1-mediated export within 15 min after exposure to the drug (NEVILLE and ROSBASH 1999). We examined the localization of Upf3p-GFP before and after addition of the drug in strain MNY8, which carries the LMB-sensitive allele *CRM1T539C-HA*. The distribution of Upf3p-GFP expressed from a 2μ plasmid was compared to DAPI staining (Figure 6). Upf3p-GFP remained primarily cytoplasmic for 15 min to 2 hr following the addition of LMB to the medium, suggesting that the export of Upf3p is not dependent on Crm1p. The localization of Upf3p-HA was also examined using indi-

rect immunolocalization in strain MNY12, which carries the LMB-sensitive allele *CRM1T539C-GFP*. Upf3p-HA remained primarily cytoplasmic even after 2 hr of incubation in the presence of LMB.

Since the Rev NES is known to direct nuclear export through interaction with Crm1p (Fornerod *et al.* 1997; Stade *et al.* 1997; Izaurralde and Adam 1998), we compared the distribution of Upf3p-Triple-GFP, which exports independently of Crm1p, with Upf3p-Triple-Rev-GFP. Leptomycin-sensitive cells of strain MNY8 expressing *upf3-Triple-GFP* from a 2μ plasmid were incubated in the presence of LMB for 30 min to 2 hr prior to fixation with formaldehyde and visualization. As expected, Upf3p-Triple-GFP localized to a nuclear region that most likely corresponds to the nucleolus, and its localization did not change even after 2 hr of exposure to LMB (Figure 6). However, Upf3p-Triple-Rev-GFP, which is primarily cytoplasmic, redistributed to the nucleolus as early as 30 min after incubation with LMB. After 60 min, a majority of the cells showed relocalization of the protein to the nucleolus. These results indicate that wild-type Upf3p is not a substrate for export via Crm1p.

DISCUSSION

Nuclear import of Upf3p: We showed previously that Upf3p is found primarily in the cytoplasm in wild-type cells (Shirley *et al.* 1998). However, elevated expression of the *UPF3* gene results in accumulation of Upf3p in the nucleolus without causing suppression of nonsense mutations or impairment of NMD. The export-defective protein product of the *upf3-Triple* allele used in this study accumulates in the nucleolus with concomitant suppression of nonsense mutations and impairment of NMD. The product of *upf3-Triple* is ~50% less abundant than the wild-type protein (Shirley *et al.* 1998). We used plasmids that carry two copies of *upf3-Triple* because this was shown to result in a protein level that approximates the level of the wild-type protein. We therefore believe that our results showing a role for the export of Upf3p in NMD are physiologically meaningful and are not simply the consequence of overexpression. It is not currently known why Upf3p concentrates in the nucleolus *vs.* the nucleoplasm. Since ribosomal subunits assemble in the nucleolus, there may be ligands in the nucleolus to which Upf3p can bind when excess protein is present in the nucleus. Although we do not attach undue significance to accumulation in the nucleolus *vs.* the nucleoplasm, we view the nucleolar phenotype as a valid cytological indicator of perturbations in the nuclear/cytoplasmic shuttling of Upf3p.

Upf3p contains three sequence elements that resemble a classical bipartite NLS (Lee and Culbertson 1995; Shirley *et al.* 1998). NLS1 and NLS2 direct a reporter to the nucleoplasm, whereas NLS3 directs the same reporter to the nucleolus. Our results show that the importin-α/β heterodimer, which mediates the import of many NLS-containing proteins (Gorlich *et al.* 1994, 1995; Weis *et al.* 1995), is required for the nuclear import of Upf3p. The nucleolar distribution of overexpressed Upf3p diminishes with time after shift of a strain carrying *srp1-31* (Impα) to a restrictive temperature. Srp1p and Upf3p physically interact in the two-hybrid system. The strength of the interaction is comparable to the strength of the Upf3p/Upf2p interaction. Presumably Srp1p binds to the NLSs since only fragments of Upf3p that include at least one of the NLS motifs interact with Srp1p. Assuming that Upf3p imports into the nucleus by a single mechanism regardless of expression level, our results suggest that Upf3p is actively imported by a mechanism in which Srp1p(Impα) binds to Upf3p and interacts with the transport receptor Kap-95(Impβ) to mediate the import of the complex into the nucleus.

Nuclear export of Upf3p is required for NMD: A leucine-rich nuclear export sequence (NES-A) mediates the export of Upf3p from the nucleus to the cytoplasm. NES-A is a functional nuclear export signal based on the observation that an allele containing three alanine-for-leucine/isoleucine substitutions in NES-A (*upf3-Triple*) alters the distribution of Upf3p from a primarily cytoplasmic localization to a nucleolar localization (Shirley *et al.* 1998). In addition to impairing export, mutations in NES-A impair the function of Upf3p leading to suppression of nonsense mutations and changes in the accumulation of nonsense mRNAs.

In our previous studies (Shirley *et al.* 1998), we were unable to make a direct connection between the export of Upf3p and its role in NMD. It was therefore not clear at that time whether the movement of Upf3p into and out of the nucleus is required for NMD or whether it serves some purpose unrelated to NMD. In this article, we show that the insertion of a heterologous NES from HIV-1 Rev to Upf3p-Triple restores the ability of the mutant protein to export from the nucleus and this results in partial restoration of the function of Upf3p in the NMD pathway. By comparison with wild-type and null *upf3* strains, the partially reduced levels of suppression of the *leu2-1*, *tyr7-1*, and *can1-100* nonsense mutations correlate with intermediate levels of *CYH2* pre-mRNA. These results suggest that the export of Upf3p from the nucleus is necessary for Upf3p to promote NMD and associated processes such as termination of translation at premature stop codons. Several possible explanations for the lack of full restoration of NMD by exportable mutant versions of Upf3p are discussed further below.

An alternative export pathway for Upf3p: The exportin Crm1p/Xpo1p does not mediate the export of Upf3p despite the resemblance between NES-A and the leucine-rich sequences known to serve as binding sites for Crm1p (Fornerod *et al.* 1997; Stade *et al.* 1997). Upf3p does not interact with Crm1p in two-hybrid assays

and fails to be retained in the nucleus in cells where Crm1p has been inactivated by the presence of mutations in the *CRM1* gene. Leptomycin B, which blocks the function of a leptomycin-sensitive Crm1 protein, fails to disrupt the export of wild-type Upf3p but does prevent the export of a version of Upf3p carrying an HIV-1 Rev NES sequence known to bind to Crm1p. Additional results indicate that Upf3p does not passively diffuse through the NPC.

We suggest that Upf3p is actively exported to the cytoplasm by an alternative Crm1p-independent mechanism. We define NES-A as a nuclear export sequence because mutations in the sequence impair export. However, the superficial resemblance of this NES to the canonical NES exemplified by that found in HIV-1 Rev may be simply fortuitous. Further studies will be required to unravel the mechanism of NES-A-mediated export of Upf3p.

Effects of mutations in NES-A on protein function: The *upf3-Triple* allele, which codes for a protein containing three alanine substitutions for leucine and isoleucine residues in NES-A, was chosen for detailed analysis because it is the least likely to perturb the overall conformation of the protein in domains outside of the NES. The wild-type Rev NES sequence, which mediates Crm1p-dependent export, fully restores export but only partially restores the function of the triple mutant protein. The lack of full restoration of function in NMD could be the result of Crm1p-mediated export, which appears not to be the normal route of exit for Upf3p, or it could result from the effects of the mutations on functional domains in Upf3p other than the NES.

To test these possibilities, we made additional *nes-A* alleles in which there is an exchange of hydrophobic amino acids (leucine and isoleucine) for either a charged amino acid or a polar amino acid. These nonconservative amino acid substitutions might be expected to cause more profound changes in protein conformation and the resulting proteins might be impaired more severely than the Upf3-Triple protein. When HIV-1 Rev was included in proteins encoded by these *nesA*$^-$ alleles, export was restored, but, unlike the *upf3-Triple* allele, no observable phenotypic rescue was measured by nonsense suppression or the accumulation of *CYH2* pre-mRNA. These results suggest that the nonconservative amino acid substitutions perturb the function of Upf3p in ways unrelated to export.

Interestingly, we found that overexpression of Upf2p partially suppresses mutations in NES-A and significantly decreases the abundance of *CYH2* pre-mRNA. The alleles affected by *UPF2* include *upf3-Triple* as well as the *upf3-nes* alleles containing the nonconservative amino acid substitutions in NES-A. The phenotypes caused by overexpression of *UPF2* were specific to *UPF2* since overexpression of *UPF1* had no effect in strains carrying the *upf3-nes* mutations. Allele specificity was also indicated by the finding that overexpression of *UPF2* had no phenotypic effects in a *upf3-Δ1* strain. Thus, overexpression of *UPF2* does not bypass the requirement for Upf3p in NMD and therefore most probably results from a specific interaction between Upf2p and the mutant Upf3 proteins.

One plausible model to explain the suppression of *upf3-Triple* and other *upf3nes* alleles by overexpressed Upf2p is that the increased abundance of Upf2p compensates for a diminished or altered interaction between Upf2p and mutant Upf3 proteins. The broad domain of Upf3p previously identified to interact with Upf2p includes the amino acids that define NES-A (He *et al.* 1997). In our studies, translational fusions containing fragments of Upf3p that lack NES-A display a weaker two-hybrid interaction with Upf2p as compared to the full-length Upf3p protein. These data suggest that NES-A contributes to the strength of the interaction between Upf3p and Upf2p.

The mutations in NES-A diminished the Upf3p/Upf2p interaction when Upf3p was fused to the Gal4 DNA binding domain. The diminution of the interaction, however, was not observed when Upf3p was fused to the Gal4 activation domain. It is possible that the interaction between Upf3p-BD and Upf2p-AD provides a more sensitive assay that allows detection of small changes in the binding affinities between Upf2p and mutant Upf3 proteins. This is supported by our findings that the wild-type proteins interact more strongly for the Upf3p-AD/Upf3p-BD combination than for the Upf3p-BD/Upf3p-AD combination. However, the lack of consistency between the two sets of two-hybrid results make it difficult to say conclusively that the suppression resulting from overexpression of Upf2p is due to an improved interaction with mutant Upf3p proteins. Further studies will be required using different methods of assaying protein-protein interactions to further test this model.

A Upf3p/Upf2p interaction has also been demonstrated in mammalian cells (Serin *et al.* 2001). hUpf2p co-immunoprecipitates with at least three of the human isoforms that are homologous to yeast Upf3p. Consistent with our results in yeast, mutations in the conserved NES-A-like motif of one of the human Upf3 protein isoforms eliminates the interaction with hUpf2p, indicating that the NES may contribute to the interaction between hUpf3p and hUpf2p in human cells.

To test the relationship between export of Upf3p and the interaction between Upf3p and Upf2p, we examined the consequences of simultaneously suppressing mutations in NES-A both by adding the HIV-1 Rev NES to *upf3*$^-$ alleles carrying mutations in NES-A and by overexpressing *UPF2*. Whereas partial suppression results from either one alone, the effects were nonadditive when they were combined. Despite this, we have shown that Upf2p has no effect on the export of Upf3p and its overexpression does not stabilize the Upf3p-Triple protein. Perhaps there are functions for Upf3p in addi-

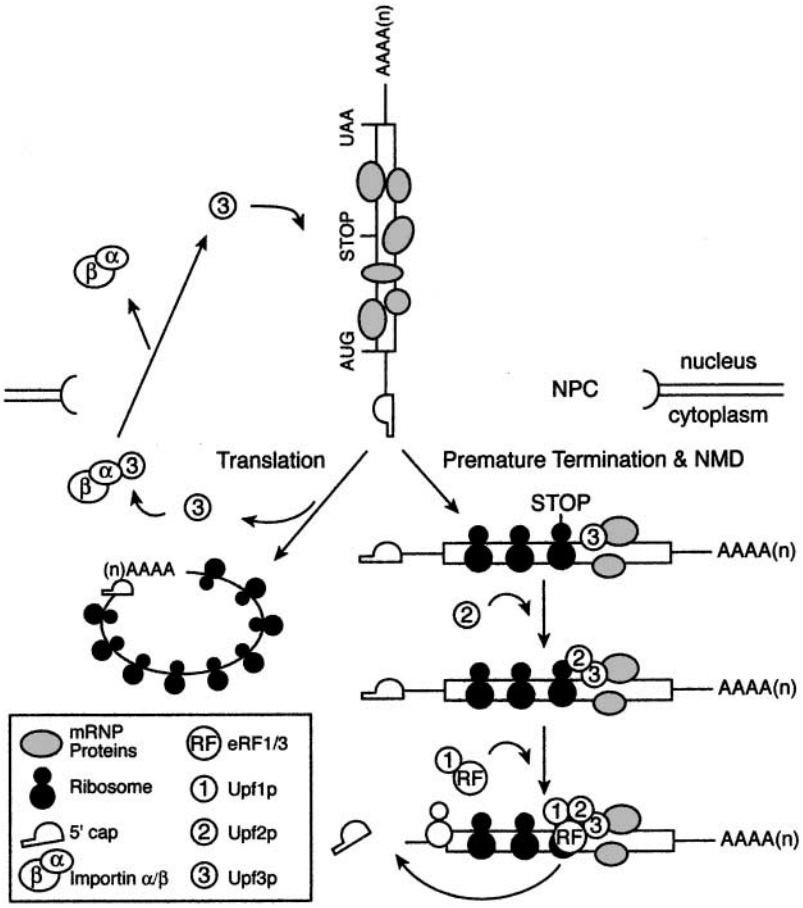

FIGURE 7.—Proposed role of Upf3p during early steps of the NMD pathway in yeast. The figure illustrates an mRNP particle exiting the nuclear pore and either undergoing complete translation or shunting into the NMD pathway after a premature translation termination event. The stepwise formation of the Upf-RF complex triggers the eventual decapping of the nonsense mRNA followed by decay.

tion to nuclear export and the Upf3p/Upf2p interaction that are also defective in the *upf3*⁻ mutants.

The role of Upf3p and Upf2p in early steps of NMD: On the basis of the following observations, we suggest that assembly of a five-member surveillance complex in yeast consisting of the three Upf proteins and the two translation termination factors is sequential and that the function of Upf3p is to initiate the formation of the complex prior to its export to the cytoplasm: (i) Upf3p imports into the nucleus; (ii) a fully functional NMD pathway requires the active export of Upf3p from the nucleus; (iii) the association of Upf2p with polyribosomes requires the presence of Upf3p, whereas Upf1p associates with polyribosomes in the absence of the other Upf proteins (ATKIN *et al.* 1997); (iv) the Upf proteins are vastly less abundant than ribosomes, suggesting that they are actively and specifically recruited to nonsense mRNAs (ATKIN *et al.* 1997; MADERAZO *et al.* 2000); (v) Upf2p and Upf3p are ~180- and 370-fold less abundant than release factors, respectively, making them unlikely to be associated with all termination complexes (MADERAZO *et al.* 2000); and (vi) Upf3p is 10- and 20-fold less abundant than Upf2p and Upf1p, respectively, making Upf3p the limiting protein in the formation of a surveillance complex (MADERAZO *et al.* 2000). Figure 7 shows a refined version of our previously published model for yeast NMD that is consistent with the observations described above (CULBERTSON 1999, 2001).

In this model, Upf3p shuttles between the nucleus and the cytoplasm and acts very early in the NMD pathway. We suggest that it may associate in the nucleus with mRNP particles through binding either to an mRNP protein or to the mRNA itself. According to the model, after full-length translation, Upf3p along with other mRNP proteins is displaced by the first translating ribosome. This promotes remodeling of the mRNP into a stable mRNA that can engage in repeated rounds of translation. If translation is halted prematurely, however, then Upf3p remains bound and seeds the formation of a Upf3p/Upf2p complex. Following translation termination, Upf1p is recruited to the paused ribosome by the release factors. Upon interaction with the Upf3p/Upf2p complex via the Upf2p/Upf1p interaction, Upf1p is activated and triggers late steps of NMD leading to mRNA decay. The Upf3p/Upf2p complex may modulate the RNA helicase activity of Upf1p (MADERAZO *et al.* 2000).

Considerable evidence suggests that NMD is accomplished by a similar mechanism in mammals. Nuclear/cytoplasm shuttling of hUpf3p has been demonstrated (LYKKE-ANDERSEN *et al.* 2000). In mammalian cells, the 3′-proximal exon-exon junction defines the boundary

that determines whether a premature stop codon promotes accelerated decay (Cheng *et al.* 1994; Nagy and Maquat 1998; Thermann *et al.* 1998; Zhang *et al.* 1998a,b; Sun and Maquat 2000; Lykke-Andersen *et al.* 2001). hUpf3 is part of a complex of proteins that binds to the last exon-exon junction in spliced mRNPs prior to nuclear export (Kim *et al.* 2001). Sequence elements that serve a function in NMD similar to that of exon-exon junctions have been identified in yeast (Peltz *et al.* 1993; Hagan *et al.* 1995; Zhang *et al.* 1995; Ruiz-Echevarria *et al.* 1996, 1998). Thus, the evidence from both yeast and mammals suggests that Upf3p can be viewed as a molecular tag on mRNP particles prior to export from the nucleus that seeds the ultimate destruction of the mRNA in the cytoplasm unless it is removed by the process of translation itself.

The authors thank the following for generously providing reagents: Judy Berman, David Brow, Elizabeth Craig, Phil James, Eric Neeno-Eckwall, Michael Rosbash, Pamela Silver, Susan Wente, and Minoru Yoshida. We thank Eric Neeno-Eckwall for critically reading the manuscript and conducting experiments with viable *crm1* alleles and Leanne Olds for preparation of figures. This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, WI; the University of Wisconsin Medical School; National Science Foundation grant 9870313 (M.R.C); and National Institutes of Health grant GM-65172 (M.R.C.). A.S.F. was supported by U.S. Department of Agriculture McIntyre-Stennis Hatch grant WIS04308 4308. R.L.S. was supported by Public Health Service Training Grant in Genetics GM-071333. This is Laboratory of Genetics paper 3576.

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Communicating editor: S. Sandmeyer