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Human Upf Proteins Target an mRNA for Nonsense-Mediated Decay When Bound Downstream of a Termination Codon

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Summary

Nonsense-mediated decay (NMD) rids eukaryotic cells of aberrant mRNAs containing premature termination codons. These are discriminated from true termination codons by downstream *cis*-elements, such as exon–exon junctions. We describe three novel human proteins involved in NMD, hUpf2, hUpf3a, and hUpf3b. While in HeLa cell extracts these proteins are complexed with hUpf1, in intact cells hUpf3a and hUpf3b are nucleocytoplasmic shuttling proteins, hUpf2 is perinuclear, and hUpf1 cytoplasmic. hUpf3a and hUpf3b associate selectively with spliced β-globin mRNA in vivo, and tethering of any hUpf protein to the 3′UTR of β-globin mRNA elicits NMD. These data suggest that assembly of a dynamic hUpf complex initiates in the nucleus at mRNA exon–exon junctions and triggers NMD in the cytoplasm when recognized downstream of a translation termination site.

Introduction

Eukaryotic cells have evolved mechanisms to ensure the fidelity of gene expression. One such mechanism, called mRNA surveillance, makes certain that only mRNAs with full coding potential are available for translation in the cytoplasm (reviewed in Maquat, 1995; Jacobson and Peltz, 1996; Li and Wilkinson, 1998; Culbertson, 1999; Frischmeyer and Dietz, 1999; Hentze and Kulozik, 1999; Hilleren and Parker, 1999a, 1999b). Aberrant mRNAs containing premature termination codons can arise from a failure in processing or by mutation. They are recognized by the mRNA surveillance machinery and subjected to nonsense-mediated decay (NMD). Thus, potentially deleterious truncated proteins are not expressed at high levels.

Many human genetic disorders resulting from frameshift or nonsense mutations are believed to be rendered recessive by the NMD pathway. A well studied example is the human β-globin gene, where premature termination codons in the first two exons are recessive, but nonsense mutations in the third exon produce decay-resistant mRNAs causing a dominant form of β-thalassemia (Baserga and Benz, 1988; Thein et al., 1990; Enssle et al., 1993; Hall and Thein, 1994). mRNA surveillance is of particular importance to lymphocytes, where immunoglobulin and T cell receptor genes undergo somatic recombination and mutation to generate versatility in the immune response. Truncated open reading frames are abundant by-products, and premature termination codons in the T cell receptor-β (*TCR*-β) gene lead to extraordinarily strong downregulation of the mRNA (Carter et al., 1995, 1996; Li and Wilkinson, 1998).

The process of NMD has been well studied in yeast. An mRNA containing a premature termination codon is subjected to deadenylation-independent decapping by Dcp1p, followed by 5′ to 3′ decay by the exonuclease Xrn1p (Muhlrad and Parker, 1994; Beelman et al., 1996). A downstream sequence element (DSE) of low conservation, believed to appear throughout most coding regions but never in the 3′UTR, allows recognition of a termination codon as premature (Peltz et al., 1993; Hagan et al., 1995; Zhang et al., 1995). Recently, a heterogeneous nuclear ribonucleoprotein (hnRNP), Hrp1p, was shown to interact with a DSE in vitro and may thus provide a downstream "mark" for a premature termination codon (González et al., 2000).

Nuclear as well as cytoplasmic events are involved in mammalian NMD. Not only are most mRNAs with premature termination codons less abundant in both compartments than the corresponding wild-type mRNAs (Cheng and Maquat, 1993; Belgrader and Maquat, 1994; Belgrader et al., 1994; Cheng et al., 1994; Kessler and Chasin, 1996; Zhang and Maquat, 1996; Zhang et al., 1998a), but pre-mRNA splicing is clearly involved. A termination codon is recognized as premature if located upstream of an exon–exon junction (Cheng et al., 1994; Carter et al., 1996; Thermann et al., 1998; Zhang et al., 1998a, 1998b), with a distance of at least 50 nucleotides generally required (Cheng et al., 1994; Carter et al., 1996; Thermann et al., 1998; Zhang et al., 1998a, 1998b). Importantly, introns are rare in the 3′UTR of mammalian genes, and when they exist are nearly always within 50 nucleotides of the end of the open reading frame (Nagy and Maquat, 1998). Both general and mRNA-specific inhibition of translation blocks NMD (Belgrader et al., 1993; Carter et al., 1995; Thermann et al., 1998).

These observations raise the question of how a mammalian cell determines that an intron, which was removed in the nucleus, was present downstream of a termination codon, which is later recognized by the ribosome and translation release factors in the cytoplasm. It has been hypothesized that splicing leaves a "mark" at or near exon–exon junctions that persists and acts post translation termination (Cheng et al., 1994; Maquat, 1995; Carter et al., 1996; Thermann et al., 1998; Zhang et al., 1998a; Hentze and Kulozik, 1999). Proteins that could constitute such a mark have recently been identified in HeLa nuclear extracts (Kataoka et al., 2000; Le Hir et al., 2000; McGarvey et al., 2000).

Three extensively studied proteins in NMD are the yeast polysome-associated Upf proteins: Upf1p, Upf2p/Nmd2p, and Upf3p (Leeds et al., 1991, 1992; Atkin et al., 1995, 1997; Cui et al., 1995; He and Jacobson, 1995; Lee and Culbertson, 1995). Upf1p is a cytoplasmic ATP-dependent RNA helicase; mutations affecting its heli-

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case activity also impair NMD (Czaplinski et al., 1995; Weng et al., 1996a, 1996b, 1998). Upf1p interacts in two-hybrid assays with Dcp2p/Nmd1p (He and Jacobson, 1995), a Dcp1p-interacting protein involved in cap removal, suggesting a direct link between Upf1p and mRNA decapping (Dunckley and Parker, 1999). In two-hybrid assays, Upf2p interacts with both Upf1p and Upf3p, and deletion of its Upf1p-interacting domain disrupts NMD (He et al., 1996, 1997). Upf3p contains nuclear import and export signals and has been localized to the nucleus (Shirley et al., 1998; Culbertson, 1999). In addition to their role in NMD, yeast Upf proteins are involved in translation termination. Their deletion enhances nonsense suppression (Leeds et al., 1992; Weng et al., 1996a, 1996b, 1998; Maderazo et al., 2000), and Upf1p interacts with translation release factors eRF1 and eRF3 (Czaplinski et al., 1998). In *C. elegans* seven *Smg* genes are involved in NMD (Pulak and Anderson, 1993; Cali et al., 1999), with SMG-2 being a clear homolog of Upf1p (Page et al., 1999).

Here we identify and characterize human homologs of yeast Upf2 and Upf3 proteins, hUpf2, hUpf3a, and hUpf3b. Previously, a homolog of yeast Upf1p, hUpf1/Rent1, had been reported to be involved in mammalian NMD (Perlick et al., 1996; Applequist et al., 1997; Sun et al., 1998), and very recently the human homolog of Upf2 was identified and found to interact with hUpf1 (Mendell et al., 2000). We demonstrate that binding of any hUpf protein downstream of a translation termination codon triggers NMD and that hUpf3a and hUpf3b interact specifically with spliced mRNA in vivo. We find distinct subcellular locations for the human Upfs, suggesting that they do not simply form a complex that scans the mRNA after translation termination. Rather, a more dynamic surveillance complex begins assembly on the mRNA in the nucleus and then directs NMD, probably at the perinuclear surface, when encountered downstream of a site of translation termination.

Results

Identification of Human Homologs of Yeast Upf2 and Upf3 Proteins

To identify proteins involved in mammalian NMD, we searched the nucleotide databases for human homologs of yeast Upf2 and Upf3 proteins. The sequences of several expressed sequence tags (ESTs) were then used to design RT-PCR reactions, resulting in isolation of cDNAs encoding proteins with limited similarity to yeast Upf2p and Upf3p. Human hUpf2, hUpf3a, and hUpf3b are schematized in Figure 1A. hUpf2 contains several putative bipartite nuclear localization signals (NLS) close to its N terminus, as well as regions similar to eIF4G also found in yeast Upf2p (Mendell et al., 2000; Ponting, 2000). hUpf3a and hUpf3b contain a conserved domain with some similarity to an RNA recognition motif (RRM), indicating that they may be RNA binding proteins. The RNP-2 motif within the RRM-like sequence is the most conserved portion of all Upf3 proteins in the database (our unpublished observations). Interestingly, the nuclear export sequence identified in yeast Upf3p (Shirley et al., 1998) overlaps with RNP-2, suggesting that nuclear export and RNA association could be linked.

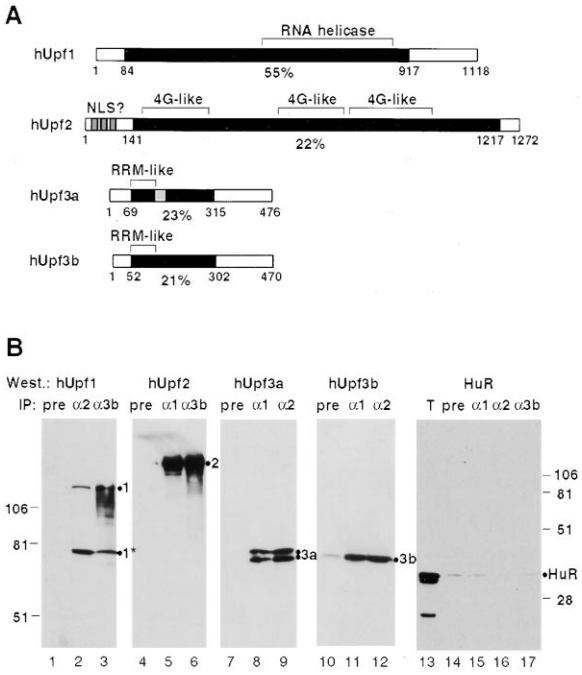

Figure 1. Human Upf Proteins Interact in HeLa Cell Extracts

(A) Schematic representation of human Upf proteins. Black boxes represent regions of similarity to yeast Upf proteins, with the position within the protein (in amino acids) and the percent identity given below. N and C termini, not similar to the corresponding yeast Upf proteins, are shown as white boxes. The RNA helicase domain of hUpf1 is indicated. Putative nuclear localization signals (NLS) close to the N terminus of hUpf2 are depicted as gray boxes and eIF4G-like sequences, also present in yeast Upf2p (Ponting, 2000), are indicated. The location of RRM-like sequences (20% identical to RRM-2 of ASF/SF2) in hUpf3a and hUpf3b are indicated and an alternative 99 nucleotide exon within hUpf3a is represented by a gray box. Sequences of hUpf2, hUpf3a, and hUpf3b are deposited in GenBank (accession numbers: AY013249, AY013250 and AY013251, respectively). Homologs of Upf2 and Upf3 can also be identified in the completed genome sequences of *Caenorhabditis elegans* and *Drosophila melanogaster*, as well as in *Arabidopsis thaliana* and *Schizosaccharomyces pombe* (our unpublished observations). (B) Coimmunoprecipitation of hUpf proteins from HeLa cell extracts using anti-hUpf antibodies. hUpf proteins were immunoprecipitated from HeLa cell extracts using rabbit preimmune (pre; lanes 1, 4, 7, 10 and 14), anti-hUpf1 (α1; lanes 5, 8, 11 and 15), anti-hUpf2 (α2; lanes 2, 9, 12 and 16), or anti-hUpf3b antibodies (α3b; lanes 3, 6 and 17). Immunoprecipitates, as well as one lane with 2.5% total extract (T, lane 13), were electrophoresed on a 10% polyacrylamide/SDS gel and, after transfer to a nitrocellulose membrane, probed with mouse polyclonal anti-hUpf1 (lanes 1–3), anti-hUpf2 (lanes 4–6), anti-hUpf3a (lanes 7–9), anti-hUpf3b (lanes 10–12), or monoclonal anti-HuR antibodies (lanes 13–17). The hUpf1 (1), hUpf2 (2), hUpf3a (3a), hUpf3b (3b), and HuR proteins are identified on the right of each panel. Coelectrophoresed molecular weight markers are indicated on the left for lanes 1–12 and on the right for lanes 13–17. An ~75 kDa degradation product of hUpf1, also present in the starting extract, is indicated by 1*. The two bands observed in lanes 8 and 9 are most likely splice variants of hUpf3a that differ by 33 amino acids, as depicted in (A). hUpf3a and hUpf3b migrate unusually slowly for their size (~52 kDa), probably due to a K/E-rich domain (Graceffa et al., 1992).

To demonstrate that hUpf proteins, like their yeast homologs, interact in cell extracts, rabbit and mouse polyclonal antibodies were raised against recombinant hUpf1, hUpf2, hUpf3a, and hUpf3b. hUpf proteins immu-

noprecipitated from freshly prepared RNase-treated HeLa cell extracts using rabbit anti-hUpf antibodies were subjected to Western blotting with mouse anti-hUpf antibodies (Figure 1B). Relative to the amounts present in the total extract (not shown) ~2% of hUpf1 was coimmunoprecipitated with antibodies against hUpf2 (lane 2), hUpf3a (not shown), or hUpf3b (lane 3), while 20% of hUpf2, hUpf3a (both forms), and hUpf3b coimmunoprecipitated with each of the other hUpf proteins (lanes 5, 6, 8, 9, 11, 12). This is consistent with Upf1p being about 10-fold more abundant than Upf2p and Upf3p in yeast (Maderazo et al., 2000). Blotting with a control monoclonal antibody against the RNA binding protein HuR (Gallouzi et al., 2000) revealed that coimmunoprecipitation is specific (lanes 13–17). Antibodies against hUpf3a and hUpf3b show low but significant crossreaction, so it was not possible to test whether these two proteins associate with each other.

Human Upf1, Upf2, and Upf3 Proteins Are Concentrated in Different Cellular Locations

The polyclonal antibodies raised against hUpf1, hUpf2, hUpf3a, and hUpf3b were affinity purified and used to localize endogenous hUpf proteins in HeLa cells. As reported by Applequist et al. (1997), hUpf1 is evenly distributed throughout the cytoplasm (Figure 2A, panel 1). In contrast, antibodies to hUpf2 show strong perinuclear staining. hUpf3a and hUpf3b appear predominantly nuclear with a minor fraction in the cytoplasm (Figure 2A, panels 2–4).

To test whether human Upf3 proteins shuttle in and out of the nucleus, we performed heterokaryon experiments (Pinol-Roma and Dreyfuss, 1992). HeLa cells transiently transfected with plasmids expressing C-terminally FLAG-tagged hUpf3a or hUpf3b were fused with untransfected mouse L929 cells in the presence of the translation inhibitor cycloheximide (Figure 2B). Four hours after fusion, the hUpf3a-FLAG protein migrated partially from the HeLa cell nucleus to the mouse nucleus (identified by spotted Hoechst staining and indicated by an arrow, panels 1–3) and was equally distributed between the two nuclei by 16 hr after fusion (not shown). The hUpf3b-FLAG protein was not yet detectable in the mouse nucleus 4 hr after fusion (not shown), but had fully equilibrated by 16 hr (panels 4–6). In control experiments, Myc-tagged hnRNP A1, a shuttling protein, migrated to the mouse nucleus within 4 hr (panels 7–9), whereas Myc-tagged hnRNP C1, a nonshuttling protein, was absent from the mouse nucleus even after 16 hr (panels 10–12). Shuttling of the hUpf3 proteins provides them with the potential to complex with the cytoplasmic hUpf1 and hUpf2 proteins.

Human Upf3 Proteins Trigger mRNA Decay When Tethered to the 3′UTR of β-Globin mRNA

Since hUpf3a and hUpf3b are nucleocytoplasmic shuttling proteins and contain an RRM-like domain, they could be part of a mark that targets an mRNA for NMD when located downstream of a translation termination codon. We therefore tested whether tethering the hUpf3 proteins to the 3′UTR of wild-type β-globin mRNA (not containing a premature termination codon) would result in NMD. hUpf3a (long form) and hUpf3b were fused to

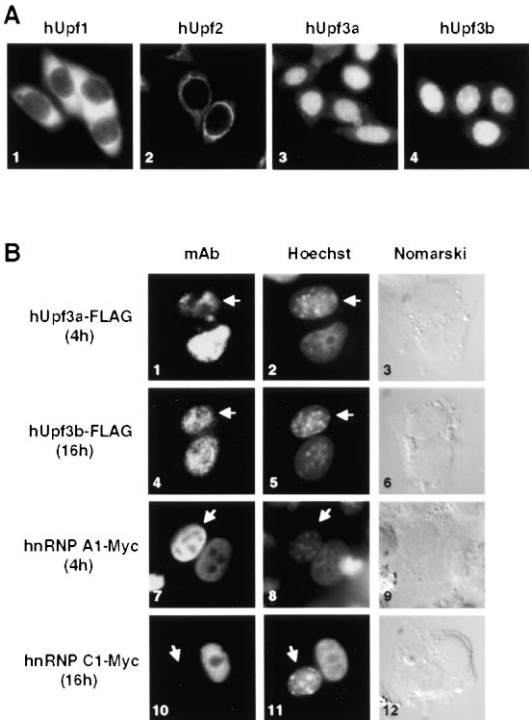

Figure 2. Localization of hUpf Proteins in HeLa Cells

(A) Immunocytochemical staining of fixed, permeabilized HeLa cells with affinity-purified rabbit anti-hUpf1 (panel 1), rabbit anti-hUpf2 (panel 2), mouse anti-hUpf3a (panel 3), or mouse anti-hUpf3b (panel 4) antibodies. Protein localizations were visualized using Texas Red conjugated anti-rabbit or Alexa 488 conjugated anti-mouse IgG antibodies and confirmed using affinity purified antibodies from both rabbit and mouse. (B) hUpf3a and hUpf3b are nucleocytoplasmic shuttling proteins. HeLa cells, transiently transfected with plasmids producing FLAG-tagged hUpf3a (panels 1–3), FLAG-tagged hUpf3b (panels 4–6), Myc-tagged hnRNP A1 (panels 7–9), or Myc-tagged hnRNP C1 (panels 10–12), were fused with mouse L929 cells using 50% PEG-3350. After 4 hr (panels 1–3 and 7–9) or 16 hr (panels 4–6 and 10–12) of incubation in the presence of cycloheximide, cells were fixed and stained with monoclonal anti-FLAG (panels 1 and 4) or anti-Myc (panels 7 and 10) antibodies. Protein localizations were visualized using Alexa 488 conjugated anti-mouse IgG antibodies. Human were distinguished from mouse nuclei (indicated by arrows) by staining with Hoechst dye 33258.

the N or C terminus of an MS2 coat polypeptide defective in oligomerization (LeCuyer et al., 1995). Vectors expressing these fusion proteins were cotransfected into HeLa cells with constructs carrying the wild-type human β-globin gene (with two introns), but containing in its 3′UTR two to six copies of an MS2 coat protein binding site (starting 95 nucleotides downstream of the termination codon; Figure 3A, β-2bs, β-4bs and β-6bs). Expression levels were controlled by inclusion of a third vector encoding β-globin mRNA with an extended 3′UTR (without MS2 coat protein binding sites; βG). After 24 hr, cytoplasmic RNA was isolated and analyzed for β-globin mRNA by Northern blotting (Figure 3B).

The levels of β-globin mRNAs with increasing numbers of MS2 coat protein binding sites in their 3′UTRs progressively decreased in cells coexpressing the hUpf3 fusion proteins, up to 2-fold in the case of hUpf3a (Figure 3B, lanes 2, 3, 7, 8, 12, and 13) and 5- to 6-fold with

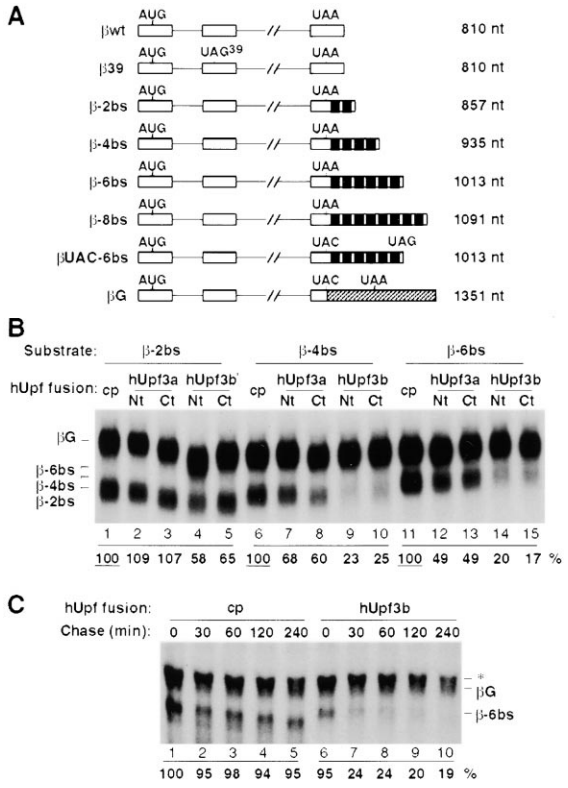

Figure 3. hUpf3a and hUpf3b Trigger NMD When Tethered to the 3′UTR of β-Globin mRNA

(A) Schematic representation of β-globin mRNAs expressed from plasmids used for transient transfection in Figures 3–5 (not to scale). Exons and introns are shown as boxes and lines, respectively. Positions of translation initiation and termination codons are indicated above each sequence. In βUAC-6bs and βG, the β-globin termination codon (UAA) has been mutated to a UAC codon, as shown. Each MS2 coat protein binding site (19 nt) is depicted as a black box. A 645 nt fragment of the GAPDH ORF and 3′UTR inserted into βG is cross-hatched. The lengths in nucleotides of the spliced mRNAs (excluding their polyadenylate tails) are given on the right. (B) Tethering of hUpf3a and hUpf3b to the 3′UTR of β-globin mRNA. HeLa cells were cotransfected with plasmids expressing β-2bs (lanes 1–5), β-4bs (lanes 6–10), or β-6bs (lanes 11–15) and βG (lanes 1–15), as well as plasmids producing MS2 coat protein alone (cp, lanes 1, 6, and 11) or fused to the N terminus (Nt) or C terminus (Ct) of hUpf3a (lanes 2, 7, 12 and 3, 8, 13, respectively) or hUpf3b (lanes 4, 9, 14 and 5, 10, 15, respectively). Total HeLa cytoplasmic RNA was prepared 24 hr after transfection, fractionated in a 1.2% formaldehyde/agarose gel, and probed with an antisense β-globin cRNA after transfer to a nylon membrane. The identities of the different mRNAs are on the left. Band intensities were quantitated by phosphorimager with the relative steady state level of the mRNA normalized to the internal control (βG) and to the corresponding unfused coat protein experiment, in which mRNA levels were set at 100% (underlined). (C) A pulse–chase experiment demonstrates mRNA decay in the tethering assay. HeLa "Tet-off" cells were cotransfected with plasmids expressing β-6bs and βG from a tetracycline-responsive CMV promoter, as well as plasmids producing MS2 coat protein alone (cp, lanes 1–5) or fused to the N terminus of hUpf3b (lanes 6–10). After a 4 hr transcriptional pulse, cells were incubated for various times as indicated above, and total RNA was subjected to Northern blotting and quantitated as described above. The identities of the different mRNAs are on the right. A cross-hybridizing RNA species (*) migrating just above βG mRNA is seen in all experiments (e.g., see Figure 4A) but is especially apparent here, where the short transcriptional pulse results in much lower reporter RNA levels. These RNAs were, however, sufficiently separated to allow accurate quantitation of the control βG mRNA.

hUpf3b (Figure 3B, lanes 4, 5, 9, 10, 14, and 15). Coexpression of unfused MS2 coat protein (Figure 3B, lanes 1, 6, and 11) had no significant effect as compared to the empty vector (see Figures 4C and 5B below). Western blots using an anti-MS2 coat protein antibody (a generous gift from Professor P. Stockley) revealed that the hUpf3a fusion proteins are expressed at approximately 5- to 10-fold lower levels than the hUpf3b fusions (data not shown), which could explain why the latter are more active in the tethering assay. Immunocytochemical experiments confirmed that, like their endogenous counterparts (Figure 2), the MS2 coat protein fusions of both hUpf3a and hUpf3b are nuclear and able to shuttle (data not shown).

To test if the reduction in β-globin mRNA levels triggered by tethering hUpf3 proteins to the 3′UTR is due to mRNA decay (as opposed to some other RNA processing event), a pulse–chase experiment was performed. Plasmids containing the β-globin gene with six MS2 coat protein binding sites in the 3′UTR (β-6bs) and the control β-globin gene (βG), both under the control of a tetracycline-responsive promoter, were cotransfected with plasmids expressing either MS2 coat protein alone or in N-terminal fusion with hUpf3b into HeLa "Tet-off" cells in the presence of tetracycline (see Experimental Procedures). hUpf3b was chosen for this experiment because preliminary data showed that a strong effect was required to obtain a clear result. Sixteen hours later, a 4 hr transcriptional pulse was induced by washing away the tetracycline. After addition of tetracycline to block further transcription, total β-globin mRNA levels were monitored over time by Northern blotting.

As seen in Figure 3C, tethering hUpf3b to the 3′UTR of β-globin mRNA leads to a biphasic mRNA decay pattern: ~80% of the mRNA disappears within the first 30 min after blocking transcription (compare lanes 6 and 7), whereas the remaining ~20% of the mRNA decays with the same slow rate as the stable control mRNA (lanes 7–10). In contrast, β-globin mRNA was stable when coexpressed with the MS2 coat protein (lanes 1–5). Gradual shortening of the β-globin mRNA over time indicates that some deadenylation takes place (compare β-6bs in lanes 1–5). We conclude that tethering hUpf3b to the 3′UTR of β-globin mRNA induces rapid mRNA decay.

Tethering hUpf2 and hUpf1 to the 3′UTR Also Results in Decreased β-Globin mRNA Levels

Since the hUpf proteins interact in cell extracts (Figure 1B), we asked whether tethering hUpf2 and hUpf1 to β-globin mRNA would also result in reduced mRNA levels. Plasmids expressing these proteins fused to MS2 coat protein and β-globin mRNAs with two to eight MS2 coat protein binding sites in their 3′UTRs were cotransfected. Similar to the hUpf3 proteins, tethering hUpf2 (Figure 4A) or hUpf1 (Figure 4B) led to a 3-fold or 4-fold decreased level, respectively, of the reporter mRNA with eight binding sites. Western blots and immunocytochemical staining revealed that the hUpf1 fusion protein was cytoplasmic and expressed at high levels similar to hUpf3b, whereas the hUpf2 fusion was even lower in abundance than hUpf3a (data not shown).

Mutant hUpf2 and hUpf1 proteins were also tested.

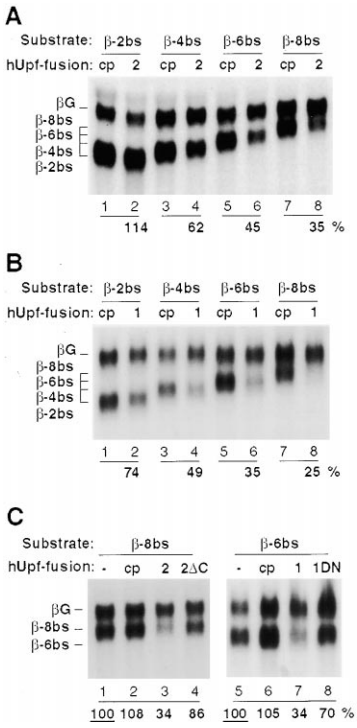

Figure 4. hUpf2 and hUpf1 Proteins Trigger NMD When Tethered to the 3′UTR of β-Globin mRNA

(A) Tethering of hUpf2 to the 3′UTR of β-globin mRNA. HeLa cells were cotransfected with plasmids expressing β-2bs (lanes 1, 2), β-4bs (lanes 3, 4), β-6bs (lanes 5, 6), or β-8bs (lanes 7, 8) and βG (lanes 1–8), as well as plasmids producing MS2 coat protein alone (cp, lanes 1, 3, 5, and 7) or N-terminally fused to hUpf2 (2; lanes 2, 4, 6, and 8). Total HeLa cytoplasmic RNA was subjected to Northern blotting and quantitated as described in the legend to Figure 3B, with mRNA identities indicated on the left. (B) Tethering of hUpf1 to the 3′UTR of β-globin mRNA. Same experiment as in (A) but with hUpf1 fused to MS2 coat protein (1). (C) Mutant hUpf2 and hUpf1 proteins are impaired in triggering NMD. HeLa cells were cotransfected with plasmids expressing β-8bs (lanes 1–4) or β-6bs (lanes 5–8) and βG (lanes 1–8), as well as an empty pcDNA3 vector (-; lanes 1, 5) or a plasmid producing MS2 coat protein alone (cp, lanes 2, 6) or coat protein N-terminally fused to hUpf2 (2; lane 3), to a hUpf2 deletion mutant lacking the C-terminal 204 amino acids (2ΔC; lane 4), to hUpf1 (1; lane 7) or to a hUpf1 R844C (dominant-negative) mutant (1DN; lane 8). Relative steady state levels of the β-globin reporter mRNAs, calculated as above, are given below, with mRNA identities indicated on the left.

Deletion of the C-terminal 206 amino acids of hUpf2, a region that in yeast Upf2p interacts with Upf1p (He et al., 1996; He et al., 1997), almost completely abolished hUpf2-triggered reduction in reporter mRNA levels (Figure 4C, lane 4). Similarly, a point mutation in the helicase domain of hUpf1 (R844C, see below) impaired its effect (Figure 4C, lane 8). Western blotting revealed that the mutant fusion proteins were expressed at levels similar to their wild-type counterparts (data not shown). In control experiments, a cytoplasmic RNA helicase, eIF4A, and two nuclear RNA binding proteins, hnRNP-A1 and hnRNP-C, had no effect when tethered to the 3′UTR of β-globin mRNA (data not shown).

These results demonstrate that, like an intron, hUpf3, hUpf2, or hUpf1 protein triggers reduced mRNA levels when positioned within the 3′UTR of β-globin mRNA. Moreover, activity requires the putative hUpf1-interacting domain of hUpf2, as well as an active helicase domain in hUpf1.

Human Upf Protein-Triggered mRNA Reduction Shows the Characteristics of NMD

We next asked if β-globin mRNA reduction mediated by tethering hUpf1, hUpf2, hUpf3a, or hUpf3b to the 3′UTR downstream of the normal stop codon is in fact NMD. Because NMD in mammalian cells requires active translation (Belgrader et al., 1993; Carter et al., 1995; Thermann et al., 1998), the translation inhibitors cycloheximide (Figure 5A) and pactamycin (not shown) were tested. Cycloheximide had a general stabilizing effect on all mRNA substrates in Figure 5A (compare – and + lanes), which was expected since most eukaryotic mRNAs are somewhat stabilized when translation is inhibited (Herrick et al., 1990; Sachs, 1993). However, both the β-globin mRNA with a premature termination codon (lanes 3, 4) and β-globin reporters destabilized by tethered hUpf proteins (lanes 7–14) were stabilized to a greater extent than control mRNAs (lanes 1, 2, 5, 6). Western blotting showed that this was not due to a decrease in the levels of fusion proteins occurring during the 2 hr incubation with translation inhibitors (data not shown). Although cycloheximide-mediated stabilization could be due to either lack of active translation or to involvement of a labile protein factor, we conclude that hUpf-mediated mRNA reduction behaves like NMD with respect to universal translation inhibition.

We then investigated the importance of the position of the termination codon relative to the tethering sites for the hUpf proteins. In β-globin mRNA, insertion of an intron into the 3′UTR had been shown to lead to NMD of an otherwise normal transcript, whereas mutation of the termination codon (resulting in translation into the 3′UTR) stabilized the transcript (Thermann et al., 1998). We therefore produced a point mutation in the β-globin mRNA termination codon (UAA to UAC) that would result in translation termination downstream of the MS2 coat protein binding sites (see Figure 3A; βUAC-6bs). When cotransfected with MS2 coat protein fusions of hUpf3 (Figure 5B), as well as hUpf2 and hUpf1 (not shown), this transcript was stable (lanes 7, 8, compare to 3, 4). Ribosomes most likely displace the tethered hUpf proteins during translation, demonstrating that mRNA reduction by hUpf tethering, similar to NMD, is a post-translational process. The transcript was not stabilized simply by an MS2 coat protein–dependent blockage of the translating ribosome, since insertion of an intron downstream of the new termination codon destabilized the transcript both in the presence and absence of an hUpf-MS2 fusion protein (data not shown).

Further evidence that hUpf1, hUpf2, and hUpf3 proteins trigger NMD was obtained using a dominant-negative hUpf1 mutant. Overexpression of this protein, which contains a point mutation (R844C) in its RNA helicase domain, was reported to yield a low (~2-fold) but reproducible increase in the levels of mRNAs containing premature termination codons (Sun et al., 1998). Constructs expressing wild-type or dominant-negative hUpf1 were cotransfected with plasmids expressing hUpf1, hUpf2,

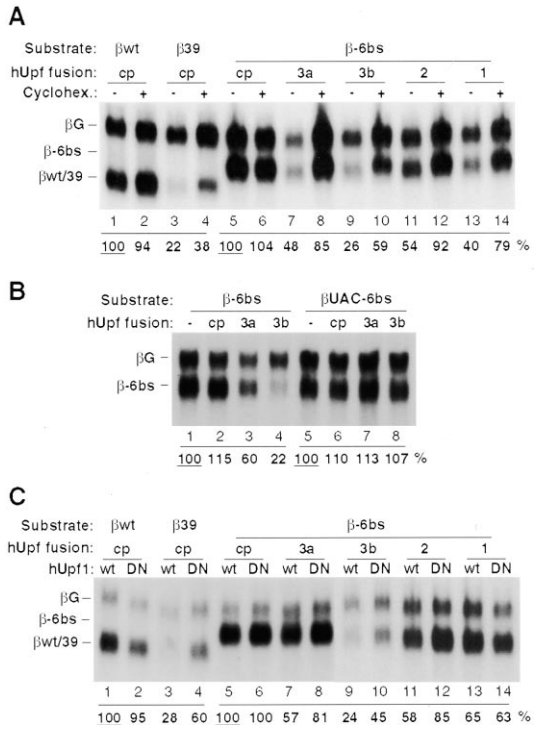

Figure 5. hUpf Protein-Triggered Decay Is Sensitive to Conditions Inhibiting NMD

(A) hUpf protein-mediated β-globin mRNA decay is inhibited by cycloheximide. HeLa cells were cotransfected with plasmids expressing βwt (lanes 1, 2), β39 (lanes 3, 4) or β-6bs (lanes 5–14) and the βG control (lanes 1–14), as well as an empty pcDNA3 vector (-; lanes 1–4) or plasmids producing MS2 coat protein alone (cp, lanes 5, 6) or coat protein N-terminally fused to hUpf3a (3a; lanes 7, 8), to hUpf3b (3b; lanes 9, 10), to hUpf2 (2; lanes 11, 12), or to hUpf1 (1; lanes 13, 14). Twenty-four hours after transfection, cells were incubated in the presence (+) or absence (-) of cycloheximide (50 μg/ml) for 2 hr. Total HeLa cytoplasmic RNA was subjected to Northern blotting and quantitated as described in the legend to Figure 3B, with mRNA identities indicated on the left. (B) Translation termination downstream of the hUpf tethering sites abrogates β-globin mRNA decay. HeLa cells were cotransfected with plasmids expressing β-6bs (lanes 1–4) or βUAC-6bs (lanes 5–8) and βG (lanes 1–8) (see Figure 3A), as well as an empty pcDNA3 vector (-; lanes 1, 5) or plasmids producing MS2 coat protein alone (cp; lanes 2, 6) or coat protein N-terminally fused to hUpf3a (3a; lanes 3, 7) or to hUpf3b (3b; lanes 4, 8). Total HeLa cytoplasmic RNA prepared 24 hr after transfection was subjected to Northern blotting and quantitation as described in the legend to Figure 3B, with mRNA identities indicated on the left. (C) hUpf2, hUpf3a and hUpf3b, but not hUpf1 protein-mediated β-globin mRNA decay is sensitive to expression of a hUpf1 dominant-negative mutant. HeLa cells were cotransfected with plasmids expressing βwt (lanes 1, 2), β39 (lanes 3, 4), or β-6bs (lanes 5–14) and βG (lanes 1–14), as well as plasmids producing MS2 coat protein alone (cp; lanes 1–6) or coat protein N-terminally fused to hUpf3a (3a; lanes 7, 8), to hUpf3b (3b; lanes 9, 10), to hUpf2 (2; lanes 11, 12), or to hUpf1 (1; lanes 13, 14). A plasmid producing hUpf1 wild-type protein (wt; odd lanes) or dominant-negative hUpf1 R844C mutant protein (DN; even lanes) was cotransfected in each case. Total HeLa cytoplasmic RNA prepared 24 hr after transfection was subjected to Northern blotting and quantitation as described in the legend to Figure 3B.

or hUpf3 fusion proteins and the β-globin reporter constructs (Figure 5C). The lower amounts of hUpf fusion protein plasmids used in these transfections (see Experimental Procedures) resulted in less reduction in mRNA levels in some cases (compare Figure 5C to Figures 3, 4, 5A, and 5B). Nevertheless, the dominant-negative hUpf1 protein reproducibly enhanced (about 1.5- to 2-fold) the levels of the reporter β-globin mRNA in the presence of hUpf2- (lanes 11, 12) or hUpf3-fusions (lanes 7–10), whereas no effect was observed when the MS2 coat protein alone was tethered to the 3′UTR (lanes 5, 6). When hUpf1 instead was tethered downstream, overexpression of the dominant-negative hUpf1 R844C mutant protein had no effect (lanes 13, 14). Together these results show that hUpf1, hUpf2, and hUpf3 proteins are involved in NMD and that a termination codon is recognized as premature when any of these proteins binds downstream in the mRNA.

hUpf3a and hUpf3b Interact Specifically with Spliced mRNA In Vivo

Our observation that hUpf proteins direct NMD when tethered downstream of a translation termination codon strongly suggests that these proteins normally interact with exon–exon junctions in cellular mRNAs. To test if hUpf3 proteins bind selectively to spliced mRNAs, two constructs containing a CMV minimal promoter with tetracycline-responsive elements were made that express: (1) intron-less β-globin mRNA with a shortened 3′UTR (Δ12β2) and (2) β-globin mRNA containing intron 2, but lacking intron 1 (Δ1β) (Figure 6). They were cotransfected with plasmids expressing FLAG-tagged hUpf3a or hUpf3b into HeLa "Tet-on" cells (see Experimental Procedures) that contain a doxycycline-inducible tetracycline transactivator. Twenty-four hours after transfection, doxycycline was added to induce β-globin mRNA expression, and cycloheximide was included to block translation as well as late steps in NMD that might lead to dissociation of the hUpf-mRNA complex. Cell extracts were prepared and RNAs immunoprecipitated by monoclonal anti-FLAG antibodies were analyzed by RNase protection for β-globin mRNA.

The results in Figure 6 demonstrate that hUpf3b (lane 5) and hUpf3a (lane 6) interact selectively with β-globin mRNA that has undergone splicing. Approximately 6% and 1% of spliced Δ1β mRNA coprecipitated with hUpf3b and hUpf3a, respectively (compare lanes 5 and 6 with 5% of the supernatant, lane 3). The lower activity of hUpf3a we believe is due to 5- to 10-fold lower expression levels of FLAG-tagged hUpf3a than FLAG-hUpf3b (data not shown). In contrast, after correction for background (lane 4), approximately 8- to 10-fold less of the intron-less Δ12β2 mRNA was found in each of the anti-hUpf3 immunoprecipitates (lanes 5, 6) and Δ1β pre-mRNA was undetectable (a band deriving from the probe alone, also present in lane 2, runs just below). Although the spliced and unspliced RNAs might differ in their rate of nuclear export, both are readily detectable in the supernatant fraction (lane 3). We conclude that hUpf3a and hUpf3b interact specifically with mRNAs containing exon–exon junctions in the absence of translation.

Discussion

We have characterized three novel human proteins involved in NMD, hUpf2, hUpf3a, and hUpf3b. They inter-

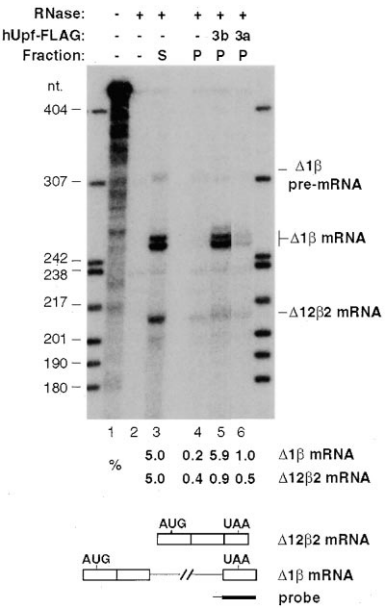

Figure 6. hUpf3a and hUpf3b Specifically Interact with Spliced β-Globin mRNA In Vivo

HeLa "Tet-on" cells were cotransfected with plasmids expressing Δ12β2 and Δ1β RNAs (lanes 3–6; RNAs outlined below) and an empty vector (-; lanes 3, 4) or a vector expressing FLAG-tagged hUpf3b (3b; lane 5) or hUpf3a (3a; lane 6). 5% of total RNA from the supernatant (S, lane 3) and 100% from the pellet (P; lanes 4–6) after anti-FLAG immunoprecipitation (see Experimental Procedures) were subjected to RNase protection assays for β-globin mRNA. RNase-treated samples were separated in a 5% polyacrylamide/8M urea gel alongside control reactions of undigested (lane 1; 0.5% loaded) and digested probe (lane 2; 100% loaded). The probe is schematized below as a black bar (intron sequence thin). The identities of the different RNAs are indicated on the right. Δ1β-mRNA routinely gives rise to a doublet in the RNase protection assay. Band intensities were quantitated by phosphorimager, and the relative levels of the RNA species, given below, were normalized to the supernatant fraction (lane 3), where 5% was loaded. Supernatants corresponding to the pellet fractions in lanes 5 and 6 were indistinguishable from that shown in lane 3 (data not shown).

act in cell extracts with hUpf1, a previously identified protein in mammalian NMD (Perlick et al., 1996; Applequist et al., 1997; Sun et al., 1998; Mendell et al., 2000). We observe that β-globin reporter mRNAs are subjected to NMD when the hUpf proteins are tethered to their 3′UTRs via MS2 coat protein fusions, and that hUpf3 proteins interact selectively with spliced β-globin mRNA in vivo. The surprising finding that hUpf proteins exhibit distinct subcellular locations suggests a model for the assembly and action of the hUpf proteins on an mRNA targeted for NMD.

Human Upf Proteins Direct NMD When Bound Downstream of a Nonsense Codon

We used tethering via the MS2 coat protein to demonstrate that human Upf proteins trigger NMD when bound downstream of the termination codon of β-globin mRNA. Three criteria established that the lower levels of β-globin mRNA observed upon hUpf tethering can be ascribed to NMD: (1) pulse–chase experiments demonstrated that tethering of hUpf3b results in mRNA decay (Figure 3C), (2) the hUpf-dependent reduction in reporter mRNA levels is a posttranslational process because it depends on active translation and because the tethering site, like NMD *cis*-elements, must be downstream of the termination codon (Figures 5A and 5B), and (3) overexpression of a dominant-negative hUpf1 mutant (Sun et al., 1998) impairs the effects of tethering hUpf2 or hUpf3 (Figure 5C).

We believe that tethering the hUpf proteins downstream of a termination codon recapitulates a real step in NMD, and that by fusing the hUpf proteins to the MS2 coat protein, coat protein binding sites become NMD *cis*-elements. Since exon–exon junctions act as NMD *cis*-elements, our data predict that hUpf proteins assemble onto exon–exon junctions during, or after, pre-mRNA splicing. Strongly supporting this interpretation is our finding that hUpf3a and hUpf3b specifically interact with a spliced β-globin mRNA in vivo (Figure 6). In contrast to other factors, like DEK and Y14, found deposited at or near exon–exon junctions after mRNA splicing (Kataoka et al., 2000; Le Hir et al., 2000; McGarvey et al., 2000), we were not able to demonstrate a stable interaction of hUpf3 proteins with adenovirus mRNA generated in a HeLa splicing extract (data not shown). Perhaps hUpf3 proteins join the multiprotein complex at exon–exon junctions only at a very late step in mRNA maturation prior to export, which does not occur in vitro.

A Perinuclear Surveillance Complex?

Coimmunoprecipitation experiments indicate that a stable complex containing hUpf1, hUpf2, and hUpf3 exists in RNase-treated HeLa cell extracts (Figure 1B). Surprisingly, in intact cells each of the three hUpf proteins is concentrated in a different locale. hUpf3a and hUpf3b are nucleocytoplasmic shuttling proteins, whereas hUpf2 is perinuclear and hUpf1 is cytoplasmic, arguing that the hUpf complex most likely forms at the cytoplasmic surface of the nucleus. Perhaps the reason that hUpf3 proteins require more than four hours to equilibrate fully between the two nuclei in a heterokaryon [much longer than other shuttling proteins such as hnRNP A1 and HuR (Pinol-Roma and Dreyfuss, 1992; Fan and Steitz, 1998)] is that they normally never completely escape from the nuclear surface before they return.

The idea that the hUpf complex might form in the perinuclear region helps resolve the controversy of whether NMD is a nuclear or cytoplasmic process (Maquat, 1995; Li and Wilkinson, 1998; Frischmeyer and Dietz, 1999; Hentze and Kulozik, 1999). Most mRNAs containing premature termination codons show decreased abundance in the nuclear as well as the cytoplasmic compartment (Cheng and Maquat, 1993; Belgrader et al., 1994; Belgrader and Maquat, 1994; Cheng et al., 1994; Kessler and Chasin, 1996; Zhang and Maquat, 1996; Zhang et al., 1998a). These observations would be explained if aberrant mRNAs were degraded while still attached to the nuclear surface, probably during the first round of translation. Support for such localized degradation comes from the biphasic nature of the decay seen in Figure 3C: the ~20% of the reporter mRNA that escapes initial rapid decay is as stable as wild-type mRNA. This suggests that the mRNA may be checked only once, during the first round of translation at the

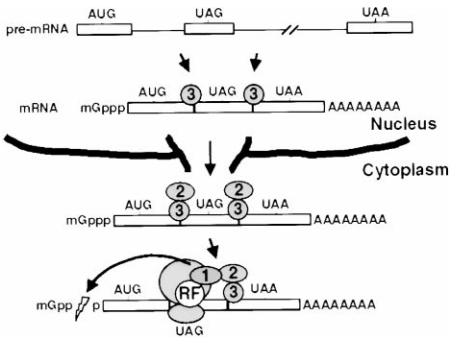

Figure 7. Model of the NMD Pathway

The order of events is described in the Discussion. An mRNA with a premature termination codon in the second exon is schematized with three boxes for exons and two lines for introns. hUpf1, hUpf2, and hUp3 proteins are shown as circles with corresponding numbers, and translation release factors are depicted by an encircled "RF." A ribosome is shown as two elipses on top of each other. Decapping of the mRNA is depicted by a lightning bolt in the cap structure; mRNA degradation ensues.

nuclear surface, and be either rapidly degraded or released to the cytoplasm for further translation.

Mechanism of NMD

In yeast, the three Upf proteins have been proposed to form a surveillance complex that, after translation termination, scans the mRNA for the presence of a downstream DSE. Our data argue that the hUpf proteins participate in a more dynamic process, whereby the surveillance complex partially assembles on the mRNA before translation. Specifically, we predict that hUpf3 first interacts with exon–exon junctions in the nucleus; second, hUpf2 interacts with hUpf3; and third, hUpf1 joins the complex, probably via translation release factors. The following observations support this scenario: (1) A dominant–negative hUpf1 mutant impairs decay when hUpf3 or hUpf2, but not hUpf1, is tethered downstream of the stop codon (Figure 5C). This argues both that only one copy of hUpf1 contributes to an active hUpf complex and that hUpf1 acts downstream of hUpf3 and hUpf2 in the NMD pathway. (2) hUpf3, hUpf2, and hUpf1 are nuclear, perinuclear, and cytoplasmic in location, respectively (Figure 2A). (3) In yeast, Upf3p interacts with Upf2p, which in turn interacts with Upf1p (He et al., 1997). (4) Polysome association of yeast Upf2p requires the presence of Upf3p but not of Upf1p, whereas Upf1p and Upf3p are each found on polysomes independent of any other Upf protein (Atkin et al., 1997).

We propose the model shown in Figure 7 for mammalian NMD, similar to one proposed earlier for yeast (Culbertson, 1999). During or after splicing, but while still nuclear, hUpf3 binds the nascent mRNA at or near exon–exon junctions. This interaction could be either direct or mediated by additional protein factors. Upon mRNA export, a perinuclear hUpf2 protein joins hUpf3. During the first round of translation, probably still at the nuclear surface, the progressing ribosome displaces any hUpf2 and hUpf3 proteins bound upstream of the termination codon. Upon translation termination, capture of hUpf1 by release factors leading to concurrent recognition of the hUpf2/3p complex bound downstream results in mRNA degradation (Figure 7). This explains why normal mRNAs, which lack exon–exon junctions in their 3′UTRs (and thus have all their hUpf3 displaced by translation of their open reading frame), are not degraded by NMD. Currently it is not clear whether hUpf1 joins the hUpf2/3p complex before, during, or after translation termination; but active translation is required for hUpf1-mediated decay in the tethering assay, so it is possible that hUpf1 interacts with the hUpf2/3p complex before termination. How the hUpf complex then relays the signal for selective decapping and decay of the aberrant mRNA remains a problem for future investigation.

Experimental Procedures

Plasmids

Reporter plasmids used in this study all contain the human β-globin gene, modified as schematized in Figure 3A (sequences available upon request), and inserted between HindIII and XbaI sites of pcDNA3 (Invitrogen).

Plasmids expressing N- and C-terminal MS2 coat protein fusions, pcNMS2 and pcCMS2, contain an oligomerization-defective MS2 coat protein mutant from the construct pKCO-MS2CP-V75E;A81G (LeCuyer et al., 1995), inserted into pcDNA3 with a modified polylinker. pcNMS2-hUpf1 and pcNMS2-hUpf1-R844C contain full-length hUpf1 cDNA, the latter with a CGG to TGT mutation in codon 844. pcNMS2-hUpf2 and pcNMS2-hUpf2ΔC contain hUpf2 cDNA extending from the translation initiation codon to the translation termination codon (codon 1273) or to codon 1066, respectively. pcNMS2-hUpf3a, pcCMS2-hUpf3a, pcNMS2-hUpf3b, and pcCMS2-hUpf3b contain full-length hUpf3a or hUpf3b cDNAs. Vectors for expression of unfused hUpf1 proteins, pcDNA-hUpf1 and pcDNA-hUpf1-R844C, were prepared by moving BamHI/NotI inserts from pcNMS2-hUpf1 and pcNMS2-hUpf1-R844C, respectively, into pcDNA3. Vectors pcDNA-hUpf3a-FLAG and pcDNA-hUpf3b-FLAG contain full-length hUpf3a and hUpf3b cDNAs with C-terminal FLAG tags inserted into pcDNA3.

Plasmids for bacterial production of recombinant hUpf fusion proteins with N-terminal glutathione S-transferase (GST) or hexahistidine (His_6) tags contain hUpf cDNAs inserted between the BamHI and NotI sites of, respectively, pGEX-4T1 (Pharmacia) and pET-His-HMK (Jensen et al., 1997) with a modified polylinker. pGEX-4T1-hUpf1-SalFS and pET-His-hUpf1-SalFS contain full-length hUpf1 cDNAs, where a frame-shift mutation was created at codon 416 by removal of a SalI site. pGEX-4T1-hUpf2Ct and pET-His-hUpf2Ct contain the C-terminal 206 codons of hUpf2 cDNA (codons 1067 to 1272). pGEX-4T1-hUpf3a, pET-His-hUpf3a, pGEX-4T1-hUpf3b, and pET-His-hUpf3b contain full-length hUpf3a and hUpf3b cDNAs.

Production and Affinity Purification of Antibodies

Rabbit and mouse polyclonal antibodies were raised against hUpf1 (amino acids 1–416), hUpf2 (C-terminal 206 amino acids), hUpf3a (full-length) and hUpf3b (full-length) proteins fused to N-terminal GST tags. Antibodies were affinity purified by adsorption to N-terminally His_6-tagged proteins, conjugated to CNBr-activated Sepharose (Amersham/Pharmacia).

Coimmunoprecipitations

HeLa spinner cells, washed twice in PBS (20 mM potassium phosphate pH 7.4, 130 mM NaCl), were incubated at 1.5×10^8 cells/ml in isotonic lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet-P40, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 μM aprotinin and 1 μM leupeptin) containing 200 μg/ml RNase A, for 16 hr at 4°C. Cell debris was precipitated by centrifugation and 100 μl of rabbit anti-hUpf antibody, or preimmune antibody, was added to 1 ml of the cleared lysate together with 1 mM of dithiothreitol and 1 mM PMSF. Cell extracts were incubated with antibodies for 1 hr at 4°C, before addition of 100 μg of protein A sepharose (Pharmacia), preswollen in NET-2 (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Nonidet-P40), followed by incubation at

4°C for four hours. Beads were then washed seven times with NET-2 and resuspended in 100 μl of SDS sample buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% sodium dodecylsulfate [SDS], 20% glycerol, 0.2 mg/ml bromophenol blue). 10 μl of each sample was subjected to SDS/polyacrylamide gel electrophoresis followed by Western blotting using mouse anti-hUpf antibodies or an anti-HuR monoclonal antibody (Gallouzi et al., 2000). hUpf proteins were visualized by ECL (Pharmacia).

Immunofluorescense Microscopy

Immunocytochemical staining of HeLa cells and preparation of heterokaryon HeLa/L929 cells was performed as described (Fan and Steitz, 1998). Anti-FLAG M2 and anti-Myc monoclonal antibodies (Sigma) were used at 10 μg/ml and at a 1:200 dilution, respectively. Affinity purified mouse and rabbit anti-hUpf antibodies were used in the range of 50–1000 ng/ml. Secondary antibodies were Alexa 488-conjugated anti-mouse IgG and Texas Red-conjugated anti-rabbit IgG antibodies (Molecular Probes), used at a concentration of 2–6 μg/ml. Hoechst dye 33258 (Sigma) was included with the secondary antibodies at 1 μg/ml to visualize the nuclei.

Transient Transfection Assays

HeLa cells were grown in DMEM medium (Gibco-BRL) containing 10% fetal bovine serum (FBS, Gibco-BRL) in 35 mm dishes and transiently transfected using Lipofectamin reagent (Gibco-BRL) according to the manufacturer's directions. For most experiments, 0.3 μg of each of the β-globin reporter plasmids were cotransfected with 1.0 μg of pcNMS2-hUpf1, -hUpf2, or -hUpf3 plasmid. In each experiment in Figure 5C, 0.3 μg of the β-globin reporter plasmids were cotransfected with 0.6 μg of one pcNMS2-hUpf plasmid and 1.5 μg pcDNA-hUpf1 plasmid. 24 hr after transfection, growth medium was removed and the cells were washed once with PBS. Cytoplasmic RNA was prepared by incubating the cells in 200 μl gentle lysis buffer (10 mM HEPES-KOH pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40) for 1 min and transferring the resulting cytoplasmic extract to 1 ml of Trizol (Gibco-BRL) for RNA isolation according to the manufacturer. One quarter (~10 μg) of total cytoplasmic RNA was run per lane on a formaldehyde-agarose gel for Northern blotting and probed with an internally ^{32}P-labeled antisense β-globin RNA covering the entire open reading frame.

For pulse–chase experiments, HeLa "Tet-off" cells (Clontech) were transfected as above, except that the β-globin reporter plasmids contained a tetracycline responsive CMV promoter. After transfection, cells were incubated in the presence of 50 ng/ml tetracycline for 16 hr. A transcriptional pulse was produced by incubating cells for 4 hr in the absence of tetracycline; 1 μg/ml tetracycline was then added and total RNA was prepared using Trizol (Gibco-BRL) at different time points.

hUpf3-RNA Coimmunoprecipitation

HeLa "Tet-on" cells (Clontech) grown in 150 mm dishes were transfected with 7.5 μg pcΔ1βwt-TET, 10 μg pcΔ12β2wt-TET and 5 μg pcDNA-hUpf3a-FLAG, pcDNA-hUpf3b-FLAG or pcDNA3 using Lipofectamin reagent (Gibco-BRL) according to the manufacturer. Twenty-four hours after transfection, doxycycline (Sigma) and cycloheximide (Sigma) were added to 1 μg/ml and 50 μg/ml, respectively, and cells were incubated for 2 hr. Cells were washed and scraped in PBS containing 50 μg/ml cycloheximide, and after pelleting they were lysed for 10 min on ice in 400 μl hypotonic lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 10 mM EDTA, 0.5% Triton-X100, 1 mM PMSF, 1 μM aprotinin, 1 μM leupeptin and 0.1 units/μl RNasin [Boehringer]). NaCl was added to 150 mM, and the cleared lysate was incubated with 100 μl of anti-FLAG-M2 agarose (Sigma) for 4 hr at 4°C. Beads were washed eight times with 1 ml cold NET-2 and FLAG-tagged proteins were eluted with 200 μl NET-2 containing 1 mg/ml FLAG peptide (Sigma) at 4°C for 2 hr. Total RNA was prepared from the supernatant and eluted fractions using Trizol (Gibco-BRL), and contaminating plasmid was removed by digestion with RQ1 DNase (Promega). RNase protections were performed by standard procedures (Fan and Steitz, 1998), except that 2% polyvinyl alcohol was included during hybridization. The probe was an internally [^{32}P]-labeled antisense β-globin RNA extending from 60 nucleotides upstream of the 3′ splice site of intron 2 to one nucleotide upstream of the β-globin polyadenylation signal.

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GenBank Accession Numbers

Sequences of hUpf2, hUpf3a, and hUpf3b have been deposited in GenBank under accession numbers AY013249, AY013250, and AY013251, respectively.