tion, the ribosomes will dissociate from the mRNA before they reach the legitimate termination codon, and thus they will fail to remove the remaining Y14-hUpf3 complex. This remaining complex, because it likely contains hUpf3, could recruit hUpf2 and hUpf1, probably together with components of the termination complex (eRF1 and eRF3), and trigger degradation of this mRNA. There may be additional functions for the exon-exon junction in the cytoplasm, such as influencing the efficiency of translation [which for some mRNAs depends on the splicing pattern of the mRNA (*47*)] and the localization of mRNAs. Determining the complete composition and functions of individual components of this complex will shed further light on post-splicing gene regulation.

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33. The cDNAs encoding hUpf3a and hUpf3b were isolated from 293T cells by reverse transcription polymerase chain reaction (RT-PCR) using the primers that were designed according to the previously reported sequences AF318575 and AF318576 (*29*). Primers for hUpf3a are 5′-CGAGGATCCATGCTGTCGGCCCTAGAAGTGCAGTTCCAC-3′ (sense; underlined nucleotides represent the Bam HI site) and 5′-CGTACTCGAGTCACTCTGCCTCTTCCCTCTTCTCAGGACC-3′ (antisense; underlined nucleotides indicate the Xho I site). Primers for hUpf3b are 5′-CGAGGATCCATGAAGGAAGAGAAGGAGCACAGGCC-3′ (sense; underlined nucleotides represent the Bam HI site) and 5′-CGTACTCGAGTTATCACTCCTCTCCTCCTTCTTTTCTATGGC-3′ (antisense; underlined nucleotides indicate the Xho I site). The PCR products were digested with Bam HI and Xho I and inserted into Flag-pcDNA3, which contains a single Flag epitope between the Hind III and Bam HI sites. The resulting *hUpf3a* gene lacks 12 nucleotides that encode amino acids 255 to 258 (numbering based on AF318575). The *hUpf3b* gene that we isolated was identical to AY013251, which lacks 39 nucleotides of exon 8 from AF318576.
34. Subcellular fractionations of HeLa and 293T cells and immunoprecipitations were done in RSB-100 buffer [10 mM tris-HCl (pH 7.5), 100 mM NaCl, 2.5 mM $MgCl_2$, 35 μg/ml digitonin, and one tablet of protease inhibitor cocktail (Roche) per 50 ml of buffer]. Y14 mAbs 4C4 and 1F12 were used for immunoprecipitation and Western blotting, respectively (*14*). Aly/REF mAb 11G5 was described in (*19*). Flag mAb M2, conjugated on agarose beads, and polyclonal rabbit antibody to Flag were purchased from Sigma and used for immunoprecipitation and Western blotting, respectively. For RNase A treatment, RNase A (5.9 mg/ml, U.S. Biochemical) was added to a final concentration of 10 μg/ml and preincubated on ice for 15 min before immunoprecipitation.
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38. hUpf3a and hUpf3b proteins as well as hnRNP A1 and hnRNP K proteins were translated in vitro using reticulocyte lysate (Promega) from plasmids Flag-hUpf3a, Flag-hUpf3b, myc-A1 (*49*), and myc-K (*50*); 4 μg of GST-fusion proteins were used for each binding. Binding buffer consisted of 10 mM tris-HCl (pH 7.5), 200 mM NaCl, 10% glycerol, 0.1% Triton X-100, RNase A (2 μg/ml), and 1 tablet of protease inhibitor cocktail (Roche) per 50 ml of buffer. Washing was done in the same buffer without RNase A.
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42. For preparation of templates for in vitro transcription, pCDC (previously referred to as pSP14-15) and pAd2 were linearized with Sma I and Xba I, respectively (*51*). The in vitro splicing reaction with HeLa and 293T cell extracts was performed as described (*51*, *52*) with the following modifications: 293T cells were transfected with either Flag-hUpf3b or Flag-pCDNA3. After 24 hours, whole-cell extract was prepared from transfected 293T cells in buffer D [10 mM Hepes-KOH (pH 7.9), 100 mM KCl, 0.2 mM EDTA] by sonication followed by centrifugation. Each 10 μl of splicing reaction contained 1 μl of HeLa nuclear extract and 5 μl of 293T whole-cell extract.
43. A 40-μl portion of the splicing reaction mixture was treated with 1 unit of RNase H (Promega) and 5 to 7 μM of oligonucleotides in a total reaction volume of 50 μl for 20 min (see figure legends) at 30°C, as described (*19*).
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53. We thank members of our laboratory, especially H. Deng and L. Abel, for excellent technical assistance; W. Friesen, A. Gubitz, and J. Dostie for critical reading of the manuscript; G. Daly for secretarial assistance; and R. Lührmann for cap mAb H20. Supported by NIH grant 5 R01 GM37125.14 and by the Human Frontier Science Program. G.D. is an Investigator of the Howard Hughes Medical Institute.

24 May 2001; accepted 20 July 2001

Communication of the Position of Exon-Exon Junctions to the mRNA Surveillance Machinery by the Protein RNPS1

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In mammalian cells, splice junctions play a dual role in mRNA quality control: They mediate selective nuclear export of mature mRNA and they serve as a mark for mRNA surveillance, which subjects aberrant mRNAs with premature termination codons to nonsense-mediated decay (NMD). Here, we demonstrate that the protein RNPS1, a component of the postsplicing complex that is deposited 5′ to exon-exon junctions, interacts with the evolutionarily conserved human Upf complex, a central component of NMD. Significantly, RNPS1 triggers NMD when tethered to the 3′ untranslated region of β-globin mRNA, demonstrating its role as a subunit of the postsplicing complex directly involved in mRNA surveillance.

The maturation of mRNA in eukaryotes involves nuclear processing before cytoplasmic translation. The quality of the mRNA is assessed at different stages. For example, export of mRNA requires successful pre-mRNA splicing, and mRNAs

that fail to be fully spliced are retained in the nucleus (*1*). A final quality-control step is mRNA surveillance. This process detects mRNAs with truncated open reading frames and subjects them to nonsense-mediated mRNA decay (NMD) [reviewed in (*2–9*)]. NMD thus prevents the synthesis of potentially deleterious truncated proteins and is responsible for rendering a large fraction of human disease mutations recessive.

The process of NMD has been intensively studied in several organisms. In *Saccharomyces cerevisiae*, three Upf proteins, Upf1p, Upf2p/Nmd2p, and Upf3p, are essential for NMD (*8–10*). In *Caenorhabditis elegans*, seven *smg* genes, three encoding homologs of the yeast Upf proteins, are required (*11*, *12*). In mammals, functional homologs of the three Upf proteins have recently been identified (*13–16*). NMD depends on active translation, and the translation release factors, eRF1 and eRF3, interact with the Upf proteins (*17*, *18*). In mammals, a premature termination codon is detected when present in the mRNA more than 50 nucleotides upstream of the last splice junction (*3*, *5*, *6*). In yeast, where few genes have introns, a downstream sequence element (DSE) plays a similar role (*19*). To explain how the former presence of an intron, which was removed in the nucleus, can be recognized during translation termination in the cytoplasm, it was hypothesized that a "mark" deposited by pre-mRNA splicing would travel with a mammalian mRNA to the cytoplasm (*3*, *5*, *6*).

We recently demonstrated that tethering of any of three interacting human Upf proteins, hUpf1, hUpf2, or hUpf3 (isoforms *a* or *b*), to the 3′ UTR (untranslated region) of β-globin mRNA leads to NMD (*13*). Thus, hUpf proteins mimic the role of introns, which trigger NMD when present downstream of a termination codon. Yet, the mechanism linking the hUpf proteins to pre-mRNA splicing has remained obscure. Recently, a multiprotein postsplicing complex that is deposited in a sequence-independent manner 20 to 24 nucleotides upstream of exon-exon junctions has been characterized (*20*). One of the proteins in this complex, REF/Aly, interacts with the mRNA export receptor TAP to facilitate nuclear export of mRNA (*21–23*). Three other deposited proteins, RNPS1, DEK, and SRm160, have been implicated in pre-mRNA splicing (*24–26*), and a cytoplasmic role for a fourth protein Y14 is suggested by its association with mRNA in both the nucleus and cytoplasm (*27*, *28*).

To ask whether any component of the postsplicing complex constitutes a downstream "mark" for NMD, we tested the ability of each of the identified proteins to trigger mRNA decay when tethered to the 3′ UTR of β-globin mRNA via fusion to the MS2 coat protein (Fig. 1A). To assure that any effect could be attributed to NMD, we cotransfected a wild-type or a dominant-negative mutant hUpf1 protein (hUpf1 R844C), which stabilizes NMD substrates 150 to 200% (*13*, *16*). Of the five postsplicing complex proteins tested (Fig. 1A, lanes 5 to 14), RNPS1 produced a striking down-regulation of β-globin mRNA to 25% of normal (lane 7; 22 to 34% in five independent experiments). This was comparable to that of the positive control, hUpf3b (lane 3), in contrast to the negative controls TAP and hnRNPA1 (lanes 15 to 18). Importantly, the down-regulation caused by tethering RNPS1 or hUpf3b was partially blocked (150 to 170% up-regulation in five experiments) by coexpression of the dominant-negative hUpf1 protein (compare lane 8 to 7 and 4 to 3). Tethering Y14 also resulted in a small, but reproducible, down-regulation of reporter mRNA levels (56 to 72%, six experiments) that was likewise relieved by the dominant-negative hUpf1 (lanes 5 and 6; 130 to 150%, six experiments). In contrast, no effect was observed upon tethering DEK (lanes 9 and 10), SRm160 (lanes 13 and 14), or REF2-I (lanes 11 and 12), nor with two splice variants of another REF isoform [REF1-I/Aly and REF1-II (*29*)] (*30*). As an important additional control for NMD, we determined that the amount of a β-globin mRNA substrate with its translational terminator moved downstream of the tethering sites was unaffected by the presence of the same MS2 coat protein fusions (Fig. 1B) (*31*). Thus, the down-regulation caused by tethering hUpf3b, RNPS1, and Y14 must be posttranslational.

Taken together these results demonstrate that RNPS1, and to a lesser extent Y14, both components of the postsplicing complex, trigger NMD when bound downstream of a translation termination codon. The recent demonstration that Y14, but not REF/Aly, migrates with the mRNA to the *Xenopus* oocyte cytoplasm (*28*) is consistent with a role for Y14 in cytoplasmic NMD. Negative results with the other postsplicing complex proteins could reflect inactivity of their fusion proteins or some non-NMD function.

The identification of RNPS1, a nuclear splicing activator (*25*), as a downstream signal for NMD predicts that it should exist transiently in the cytoplasm. We therefore asked whether RNPS1, like hUpf3 proteins (*13*, *15*) and Y14 (*27*, *28*), is a nucleocytoplasmic shuttling protein. The heterokaryon experiments in Fig. 2 show that a green fluorescent protein (GFP)–RNPS1 fusion migrates from the human nucleus to the mouse

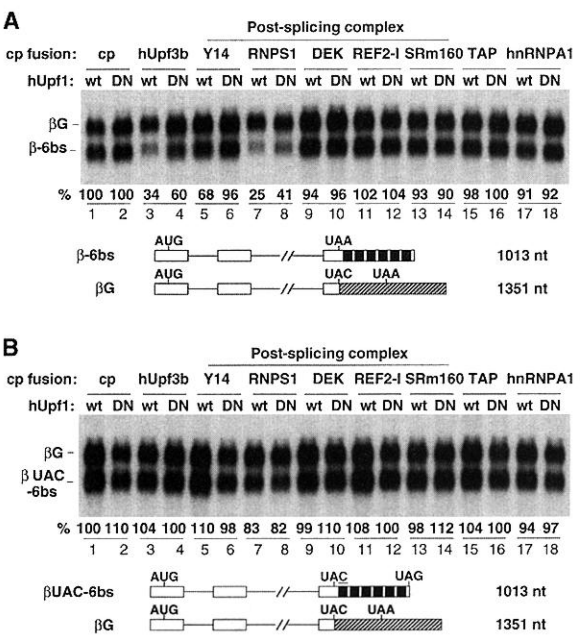

Fig. 1. Tethering of RNPS1 to the β-globin mRNA 3′ UTR triggers nonsense-mediated decay. (**A**) Northern blot analysis of β-globin mRNA levels in HeLa cells transiently expressing a β-globin reporter mRNA and various MS2 coat protein fusions. HeLa cells were transiently cotransfected with plasmids (*13*) expressing β-globin mRNA with six binding sites for MS2 coat protein in the 3′ UTR (β-6bs, 0.25 μg, schematized below with binding sites for MS2 coat protein indicated by black boxes), a control β-globin mRNA with an extended 3′ UTR (βG, 0.25 μg), wild-type hUpf1 (wt, 1.5 μg) or dominant-negative hUpf1 R844C (DN, 1.5 μg), and MS2 coat protein alone (cp) or NH_2-terminally fused to hUpf3b, Y14, RNPS1, DEK, REF2-I, SRm160, TAP, or hnRNPA1 (0.6 μg) (*35*). The amount of reporter mRNA in each lane (β-6bs), normalized to that of the corresponding internal control mRNA (βG), is given below with the level in lane 1 set at 100%. (**B**) Same as (A), except that the β-globin reporter mRNA (βUAC-6bs, schematized below) contains a point mutation in the termination codon (UAA to UAC), moving translation termination downstream of the binding sites for MS2 coat protein.

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nuclei (panel 1; two are present in the heterokaryon shown), whereas the nonshuttling control, hnRNPC, does not (panel 2). No nucleocytoplasmic shuttling was detected for GFP-tagged SRm160 (panel 5) and DEK (panel 9), consistent with their inability to activate NMD. We conclude that RNPS1 is a nucleocytoplasmic shuttling protein.

A role in NMD also predicted that RNPS1 should interact with the hUpf protein complex. We therefore tested the coimmunoprecipitation of FLAG-tagged postsplicing complex proteins with endogenous hUpf proteins from RNase-treated HEK293 cell extracts (Fig. 3A). Strikingly, high levels of hUpf3a, hUpf3b (two splice variants each), hUpf2, and hUpf1 specifically coimmunoprecipitated with FLAG-RNPS1 (lane 5). FLAG-hUpf1 (lane 1, two upper panels) and FLAG-hUpf3b (lane 1, three lower panels) served as positive controls. Two RNA-binding proteins with no known role in NMD, hnRNPD (panel 5, four isoforms) and HuR (*29*), were not detected in immunoprecipitates with any of the FLAG-tagged proteins. With the exception of FLAG-DEK, the postsplicing complex proteins, as well as FLAG-TAP, reproducibly coimmunoprecipitated very small amounts (about one-tenth the amount of FLAG-RNPS1) of both hUpf3a and hUpf3b (lanes 3, 6, 7, and 8; hUpf3b produced a smeared band in some lanes), perhaps because they interact with RNPS1. It is interesting that the short splice variant of hUpf3a (upper panel, lower band), which lacks an evolutionarily conserved 33–amino acid region (*13*, *15*), is selected by both FLAG-hUpf1 (lane 1) and FLAG-RNPS1 (lane 5) but not by the other postsplicing complex proteins (lanes 3, 6, 7, and 8). These results demonstrate that FLAG-RNPS1 associates with the hUpf complex in human cell extracts. We tried but were unable to demonstrate by far Western blotting a specific direct interaction between bacterially expressed RNPS1 and hUpf3 proteins.

The FLAG antibody immunoprecipitates were also analyzed for the presence of the mRNA export receptor TAP (Fig. 3B) and Y14 (*29*). Every FLAG-tagged postsplicing complex protein (except DEK) coimmunoprecipitated both TAP and Y14, suggesting their incorporation into endogenous postsplicing complexes that function in mRNA export as well as NMD. DEK either may be inactivated by tagging or perhaps is not a true subunit of the postsplicing complex in vivo.

Finally, we assessed the ability of the exogenously expressed FLAG-tagged proteins to interact with mRNA. The RNase protection assay in Fig. 3C shows that FLAG-tagged hUpf3b, Y14, and REF2-I (lanes 4, 5, and 8) each interacts selectively with spliced β-globin mRNA (5 to 10 times intronless) when coexpressed with β-globin reporters containing one intron (intron 2) and no introns. FLAG-tagged SRm160 showed affinity for both pre-mRNA and spliced mRNA, but not for intronless mRNA (lane 9). In contrast to the reported in vitro preference of endogenous RNPS1

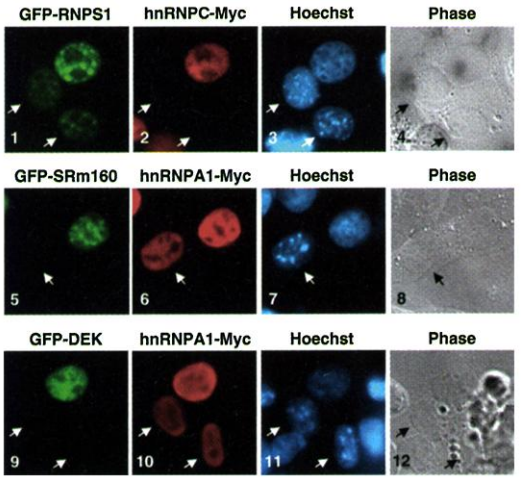

Fig. 2. RNPS1 is a nucleocytoplasmic shuttling protein. Human HEK293 cells were cotransfected with plasmids (0.8 μg) expressing GFP-RNPS1 and hnRNPC-Myc (panels 1 to 4), GFP-SRm160 and hnRNPA1-Myc (panels 5 to 8) or GFP-DEK and hnRNPA1-Myc (panels 9 to 12), as indicated and fused with untransfected mouse L929 cells (*36*).

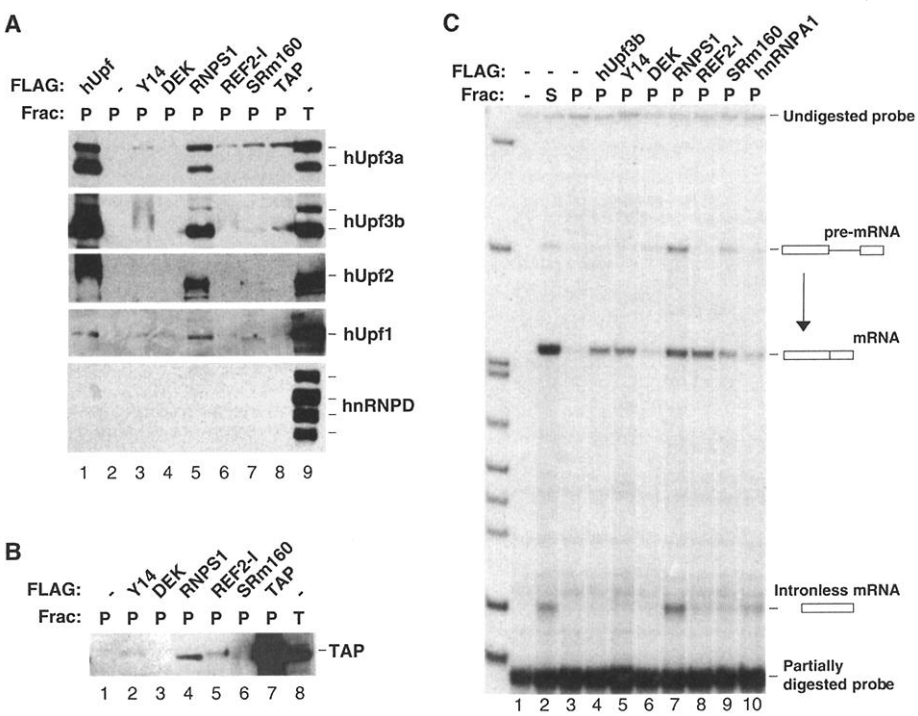

Fig. 3. Interaction between the postsplicing and hUpf complexes. (**A**) and (**B**) Western blots, probing for the presence of hUpf, hnRNPD and TAP proteins (indicated on the right) in anti-FLAG immunoprecipitates from RNase-treated cell extracts of HEK293 cells transiently expressing the indicated FLAG-tagged proteins (*37*). Pellets (P) are compared with 5% of the total extract (T). "hUpf" corresponds to FLAG-hUpf1 in the two upper panels and FLAG-hUpf3b in the three lower panels in (A). The slightly slower mobility of TAP in lanes 2 and 5 in (B) is due to a gel artifact. (**C**) RNase protection assay demonstrating association between postsplicing complex proteins and β-globin mRNA in vivo. Cell extracts were prepared of HEK293 Tet-on cells (Clontech) transiently expressing FLAG-tagged proteins (indicated above) and β-globin mRNA containing one intron ("pre-mRNA," which is converted into "mRNA" upon splicing; both indicated on the right) and intronless β-globin mRNA ("intronless mRNA"); they were immunoprecipitated with FLAG antibody, and subjected to RNase protection assays for β-globin mRNA as described (*13*). Pellets (P; lanes 3 to 10) were compared with 5% of the supernatant fraction (S; lane 2) from cells containing an empty FLAG-tag vector (– over lanes; corresponding pellet in lane 3) and to a reaction containing no cellular RNA (lane 1). In each lane, the probe was added in an almost 200-fold molar excess over total β-globin mRNA.

for spliced mRNA (*25*), FLAG-tagged RNPS1 bound all mRNA species (lane 7). This could be an artifact of tagging and overexpression or could reflect a real basal affinity of RNPS1 for RNA. Perhaps sequences that have previously been shown to act as NMD *cis*-elements but are not exon-exon junctions (*32*) are in fact high-affinity RNPS1 binding sites. FLAG-tagged DEK did not interact with any of the RNA species (lane 6), whereas FLAG-hnRNPA1, as expected, bound pre-mRNA, mRNA, and intronless mRNA (lane 10). These results argue that the FLAG-tagged proteins (except DEK) assemble at the exon-exon junctions and that the interactions documented in Fig. 3, A and B, may well occur within postsplicing complexes in vivo.

Our data explain how a dynamic postsplicing complex can function in mRNA quality control by mediating both mRNA export and mRNA surveillance (Fig. 4). As a result of pre-mRNA splicing, a multiprotein complex is deposited upstream of every exon-exon junction on nascent mRNAs (*20*). hUpf3 joins the postsplicing complex, via direct or indirect interaction with RNPS1 (and perhaps Y14). Yeast Hrp1, which interacts with both DSEs and the Upf complex (*33*), could be a functional (although not evolutionary conserved) homolog of RNPS1. The abundance of RNPS1 in mammalian cells is not known, but both hUpf3 and hUpf2 proteins are about one-tenth as abundant as hUpf1 (*13*) [measured at $\sim 3 \times 10^6$ molecules per cell (*34*)] and, therefore, about the same amount as spliceosomes, enough to transiently "mark" all nuclear mRNA exon-exon junctions. After interaction with hUpf3, the postsplicing complex binds the mRNA export receptor TAP [via REF/Aly (*21–23*)] to initiate mRNA nuclear export. Some postsplicing complex proteins, including Y14 (*28*), RNPS1, and hUpf3b, remain on the mRNA, whereas others, including REF/Aly (*28*) and SRm160, dissociate. During the first round of translation, the remaining subunits are stripped from the mRNA by the progressing ribosome and return to the nucleus. If termination occurs upstream of the last exon-exon junction, interactions between the translation release factors, eRF1 and eRF3, and the downstream postsplicing/hUpf3 complex (via hUpf2 and hUpf1) trigger mRNA decapping followed by rapid decay.

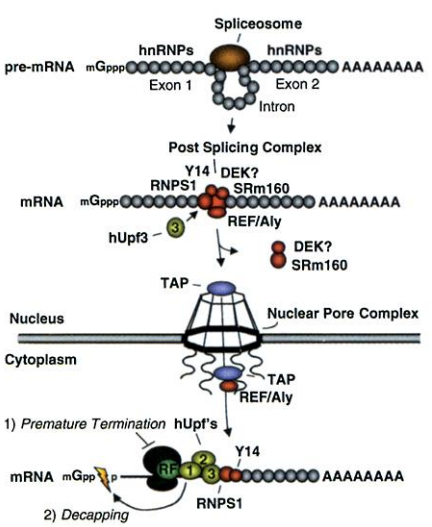

Fig. 4. How a postsplicing complex deposited upstream of each exon-exon junction tags mRNAs for nuclear export and mRNA surveillance. The pre-mRNA is coated with hnRNP proteins (gray). The spliceosome (brown) deposits a postsplicing complex (red; DEK may not be part of this complex, see text) that promotes nuclear export by interaction with TAP (blue) and mRNA surveillance by interaction with hUpf proteins (yellow). The ribosome is depicted in dark green and release factors (RF) are light green.

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30. Immunofluorescence experiments and Western blots using an antibody against MS2 coat protein (a generous gift from P. Stockley) revealed that all fusion proteins localized to the nucleus and, with the exception of the SRm160 fusion which was 3- to 5-fold lower in abundance, were expressed at similar high levels. RNPS1, Y14, REF2-I, and TAP were also tested with the MS2 coat protein fused to their COOH-termini and had the same effect on reporter mRNA levels as the NH_2-terminal fusions.
31. The slight hUpf1-independent reduction seen in the presence of the RNPS1 fusion (lanes 7 and 8) may result from RNPS1's activity as a pre-mRNA splicing factor.
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35. Twenty-four hours after transfection, total RNA was prepared using Trizol (Gibco-BRL), fractionated in 1.2% agarose/formaldehyde gels and subjected to Northern blotting using an internally ^{32}P-labeled antisense β-globin riboprobe.
36. Twenty-four hours after transfection, HEK293 cells were fused with untransfected mouse L929 cells and incubated for 4 hours in the presence of cycloheximide (*13*). Cells were fixed, permeabilized and stained using monoclonal antibody against Myc (9E10, Sigma) followed by Texas Red–conjugated antibody against mouse IgG (Molecular Probes). Nuclei were visualized by Hoechst 33258 staining (Sigma).
37. Cultured HEK293 cells (10-cm plates) were transfected (using lipofectamine, Gibco-BRL) with 40 μg of an empty vector (−) or a plasmid expressing a FLAG-tagged hUpf1, hUpf3b, Y14, DEK, RNPS1, REF2-I, SRm160, TAP, or hnRNPA1. Forty-eight hours after transfection, cells were resuspended in 400 μl hypotonic gentle lysis buffer (10 mM tris-HCl, pH 7.5, 10 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 1 μM aprotinin, 1 μM leupeptin) for 10 min on ice. NaCl was added to 150 mM and cell debris was removed by centrifugation at 10,000*g* for 10 min. RNase A was added at 200 μg/ml and after 10 min on ice, the cleared cell extract was recentrifuged. The supernatant was subsequently incubated with 100 μl anti-FLAG antibody agarose (M2; Sigma) at 4°C for 4 hours. Beads were washed 10 times with cold NET-2 (50 mM tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Nonidet-P40) and resuspended in 60 μl SDS sample buffer (50 mM tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 20% glycerol, 0.2 mg/ml bromophenol blue). A portion (20 μl) was fractionated in 10% polyacrylamide-SDS gels and subjected to Western blotting for hUpf proteins, hnRNPD (rabbit antibodies against hnRNPD kindly provided by H. Green) and TAP (rabbit antibodies against TAP kindly provided by E. Izaurralde). The FLAG-tagged proteins were all precipitated nearly quantitatively and, like the MS2 coat protein fusions, were expressed in large amounts, except for SRm160, which was 20 to 33% as much.
38. We thank E. Izaurralde, P. Stockley, and H. Green for antibodies, and K. Tycowski, I. Gallouzi, and Y. Huang for careful reading of the manuscript. This research was supported by National Cancer Institute grant CA 16038. J.L.-A was supported by fellowships from the Alfred Benzon Foundation and the Danish Natural Science Research Council. J.A.S. is an investigator of the Howard Hughes Medical Institute.

23 May 2001; accepted 13 July 2001