Functions of hUpf3a and hUpf3b in nonsense-mediated mRNA decay and translation

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ABSTRACT

The exon–junction complex (EJC) components hUpf3a and hUpf3b serve a dual function: They promote nonsense-mediated mRNA decay (NMD), and they also regulate translation efficiency. Whether these two functions are interdependent or independent of each other is unknown. We characterized the function of the hUpf3 proteins in a λN/boxB-based tethering system. Despite the high degree of sequence similarity between hUpf3b and hUpf3a, hUpf3a is much less active than hUpf3b to induce NMD and to stimulate translation. We show that induction of NMD by hUpf3 proteins requires interaction with Y14, Magoh, BTZ, and eIF4AIII. The protein region that mediates this interaction and discriminates between hUpf3a and hUpf3b in NMD function is located in the C-terminal domain and fully contained within a small sequence that is highly conserved in Upf3b but not Upf3a proteins. Stimulation of translation is independent of this interaction and is determined by other regions of the hUpf3 protein, indicating the presence of different downstream pathways of hUpf3 proteins either in NMD or in translation.

Keywords: exon–junction complex; nonsense-mediated mRNA decay; Upf3; tethering

INTRODUCTION

Nonsense-mediated mRNA decay (NMD) is a gene expression surveillance mechanism reported in all eukaryotic organisms analyzed. In humans, the activity of NMD modulates the phenotype of acquired and hereditary disorders (Hall and Thein 1994; Wilschanski et al. 2003; Holbrook et al. 2004) by degrading transcripts with premature termination codons arising from errors in transcription or splicing, from mutated genes, or from genes that are physiologically regulated by NMD (He et al. 2003; Mendell et al. 2004). Thus, NMD is of general biological and medical importance. Mechanistically, NMD depends on splicing, translation, and a number of *trans*-acting protein factors, such as the Upf and Smg proteins (Gonzalez et al. 2001; Schell et al. 2002; Maquat 2004).

According to current models of mammalian NMD, the exon–junction complex (EJC) is deposited on the mRNA during splicing at a position 20–24 nt upstream of exon–exon boundaries (Le Hir et al. 2001). Components of the EJC, namely Y14 (RBM8A), Magoh, RNPS1, eIF4AIII (DDX48, Nuk34), and Barentsz (BTZ, MLN51, CASC3), are involved in the definition of the exon–intron junctions during NMD (Lykke-Andersen et al. 2001; Fribourg et al. 2003; Gehring et al. 2003; Chan et al. 2004; Degot et al. 2004; Ferraiuolo et al. 2004; Palacios et al. 2004; Shibuya et al. 2004). Recently, we have shown that these EJC components define two functionally distinguishable EJC subgroups: RNPS1-type EJCs require normal levels of UPF2 to trigger efficient NMD, whereas Y14-Magoh-eIF4AIII-BTZ-type EJCs even activate NMD in UPF2-depleted cells and thus tolerate very low UPF2 levels (Gehring et al. 2005). In the cytoplasm, the translating ribosome removes EJCs and associated NMD factors within the open reading frame, which validates the mRNA for the pool of stable translated mRNAs. If, however, the ribosome terminates at a stop codon upstream of an EJC, it is thought to recruit hUpf1, possibly via an interaction of hUpf1 with the peptide release factors. hUpf1 is required for the degradation of all known NMD substrates, which is thought to involve both decapping and deadenylation (Lejeune et al. 2003).

EJCs play a role not only in NMD, but in several other post-transcriptional steps of gene expression (Tange et al. 2004).

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The EJC's role in nucleocytoplasmic transport is indicated by the interaction of REF/Aly with export factors such as TAP/p15 (Stutz et al. 2000; Zhou et al. 2000). The EJC has also been shown to enhance translation when deposited within an ORF by increasing the proportion of mRNAs associated with polysomes (Lu and Cullen 2003; Nott et al. 2003, 2004).

In vertebrates, two homologs of the yeast Upf3 protein exist, termed hUpf3a and hUpf3b (Lykke-Andersen et al. 2000) or hUpf3 and hUpf3X, respectively (Serin et al. 2001). Two splice variants of hUpf3a, hUpf3aL and hUpf3aS, have been described that either retain or skip exon 4. Each hUpf3 mRNA isoform and splice variant is expressed in all adult tissue types examined (Serin et al. 2001). hUpf3a and hUpf3b are predominantly nuclear and shuttle between nucleus and cytoplasm (Lykke-Andersen et al. 2000; Serin et al. 2001). Both hUpf3 proteins preferentially associate with spliced rather than unspliced mRNA, indicating that the process of splicing and EJC recruitment facilitates their interaction with mRNAs (Lykke-Andersen et al. 2000). hUpf3aL and hUpf3b can bind hUpf2 (Lykke-Andersen et al. 2000; Serin et al. 2001), an interaction that is conserved from yeast to humans. The cocrystal structure of the interacting domains of hUpf2 and hUpf3b revealed that the N-terminal RNP domain (ribonucleoprotein-type RNA-binding domain, also known as RNA recognition motif, RRM) of hUpf3b interacts hydrophilically with the C-terminal MIF4G (middle portion of eIF4G) domain of hUpf2 (Kadlec et al. 2004). Although the interaction between Upf3 and Upf2 is evolutionarily widely conserved, it is not required for the NMD activity of tethered hUpf3b, suggesting either that tethering of hUpf3b to the RNA bypasses the hUpf2-requiring step of NMD or that the interaction with hUpf2 is a redundant step within the NMD process (Gehring et al. 2003, 2005). By contrast, the C-terminal domain of hUpf3b is essential for NMD and is needed to assemble a complex containing the Y14/Magoh heterodimer. This domain is also present in hUpf3a, explaining its coprecipitation with Y14 (Kim et al. 2001; Lykke-Andersen et al. 2001). Functionally, both hUpf3a and hUpf3b destabilize a reporter mRNA if tethered downstream of a physiological termination codon (Lykke-Andersen et al. 2000). This mRNA degradation reflects characteristic features of NMD such as dependency on translation and hUpf1 (Lykke-Andersen et al. 2000; Gehring et al. 2003). In contrast to hUpf3aL, hUpf3aS lacks a part of the N-terminal RNP fold and does not coprecipitate hUpf2 (Ohnishi et al. 2003). In gel filtration experiments, hUpf3aL cofractionates with hUpf2 and elutes at a higher molecular weight than hUpf3aS, which associates with Smg7 but not hUpf2 (Ohnishi et al. 2003; Schell et al. 2003). These differences in the protein–protein interactions suggest different functions of the various hUpf3 isoforms. Therefore, we have analyzed the different activities of hUpf3a and hUpf3b in NMD and in translation stimulation. Immunoprecipitated complexes of hUpf3a and hUpf3b contain a similar set of proteins, including Y14, Magoh, eIF4AIII, and BTZ. However, hUpf3a is much less efficient than hUpf3b in triggering NMD and in stimulating translation. NMD and translation stimulation by hUpf3 proteins require different protein regions and interacting factors, representing separable and likely independent functions of hUpf3a and hUpf3b.

RESULTS AND DISCUSSION

The NMD activities of hUpf3a and hUpf3b are markedly different

hUpf3aL has previously been shown to destabilize a reporter mRNA if tethered downstream of the physiological termination codon employing a MS2-based tethering system (Lykke-Andersen et al. 2000). Similarly, tethering of hUpf3a with the λN/boxB system decreased the abundance of the globin 5boxB reporter mRNA (Fig. 1B, lanes 3,4). We compared the effects of tethering hUpf3b with hUpf3aL and hUpf3aS. Other hUpf3a variants, which we identified from HeLa cDNA (data not shown) but cannot safely be assigned to a protein product by Western blotting, were not analyzed. In keeping with previous studies (Lykke-Andersen et al. 2000; Gehring et al. 2003), tethered hUpf3b decreased the amount of the reporter mRNA to less than one-third compared to the unfused λN-peptide (Fig. 1B, lanes 7,8). In contrast, tethered hUpf3aL displayed weak NMD activity and the abundance of reporter mRNA was reduced to only 71% (Fig. 1B, lane 3). Neither deletion of the hUpf2-interacting domain between amino acids 66 and 140 (Fig. 1B, lane 5) nor of exon 4 (hUpf3aS; Fig. 1B, lane 4) changed the weak activity of hUpf3a. If, however, a C-terminal stretch of 14 amino acids (residues 434–447, construct hUpf3aΔY14) was deleted, the abundance of reporter RNA remaining with tethered hUpf3aΔY14 was clearly increased compared to tethered full-length hUpf3aL (Fig. 1B, lanes 3,6): In each of nine independent experiments the ratio of reporter RNA was at least 1.45 times higher with hUpf3aΔY14 than with hUpf3aL (mean, 1.70-fold; SD, 0.38), indicating that the small reduction in mRNA abundance with hUpf3aL is a specific albeit weak effect of hUpf3aL. This stretch of amino acids (434–447) is highly homologous to residues 421–434 of hUpf3b, which are required to form a complex containing Y14 (Gehring et al. 2003). We conclude that hUpf3a is only marginally NMD active in tethered function analysis, despite its high degree of homology with hUpf3b. Nonetheless, the same protein module is required for the partial NMD activity of hUpf3a and the strong NMD activity of hUpf3b.

The different NMD activities of hUpf3a and hUpf3b are determined by their C termini

To find the basis for the observed differences in NMD activity between hUpf3a and hUpf3b, we compared

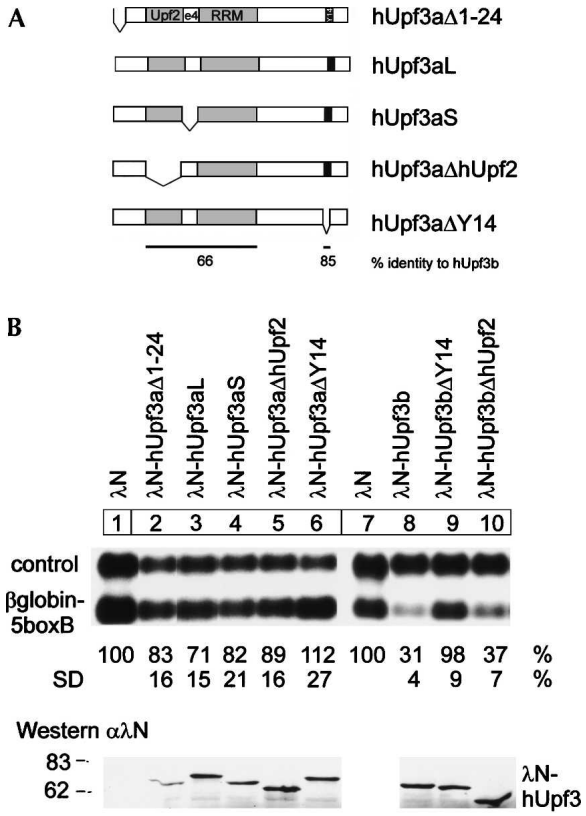

FIGURE 1. hUpf3a elicits NMD less efficiently than hUpf3b. (*A*) Schematic representation of hUpf3a mutants used in tethered function analysis: The Y14 interacting site homologous to hUpf3b is shown in black, and the other conserved region containing the hUpf2-interacting site and the RRM-like domain is depicted in gray. The alternatively spliced exon 4 is in light gray (e4). The Δ1–24 construct corresponds to an N-terminally truncated variant of hUpf3a reported by Serin at al. (2001). Full-length hUpf3a and the splice variant lacking exon 4 are designated hUpf3aL and hUpf3aS, respectively. hUpf3aΔhUpf2 lacks part of the proposed hUpf2 interaction site (amino acids 66–140); hUpf3aΔY14 lacks the proposed Y14-interaction site (amino acids 434–447). (*B*) HeLa cells were transfected with the globin 5boxB reporter, a control for transfection efficiency and the indicated λN-tagged hUpf3-construct. As a positive control, hUpf3b constructs were used (*right* panel). The amount of reporter mRNA was quantified relative to the unfused λN-peptide. Data are means from five independent experiments. To control for protein expression levels, lysates were analyzed by immunoblotting with an anti-λN-antibody.

C-terminal sequences of Upf3 proteins from several vertebrates (Fig. 2). In all Upf3b proteins deposited in databases, a C-terminal sequence required for complex formation with Y14 is highly conserved (Gehring et al. 2003). The corresponding sequence is more variable and in some species, such as rat and mouse, even absent in Upf3a proteins. Single amino acid changes within this region of hUpf3b impair its NMD activity (Gehring et al. 2003). Notably, the homologous position of arginine R419, which has been shown to be important for hUpf3b activity, is replaced by an alanine residue in hUpf3a (A432). We therefore reasoned that substituting A432 in hUpf3a for an arginine, i.e., changing hUpf3a to the Upf3b consensus, might improve the NMD activity of hUpf3a. Indeed, the hUpf3aL 432R point mutant is more active than the hUpf3a wild type and displays a NMD activity similar to that of tethered hUpf3b (Fig. 3B, lanes 2,3,5). When we exchanged the C-terminal 150 amino acids of hUpf3aL for the corresponding sequence of hUpf3b, we obtained a hybrid protein that was as active as hUpf3b (Fig. 3B, lanes 4,5). These data demonstrate that the NMD activity of hUpf3 proteins is determined by the C-terminal sequence in general and is largely affected by the single amino acid position R419 in hUpf3b and A432 in hUpf3a, respectively.

hUpf3a-dependent NMD requires interaction with Y14/Magoh but not with hUpf2

In order to test the functional relevance of known protein–protein interactions of hUpf3 proteins, we analyzed the ability of mutant Upf3a proteins to coprecipitate hUpf2 and Y14/Magoh (Fig. 4A), proteins that have previously been shown to interact with hUpf3 proteins (Lykke-Andersen et al. 2000, 2001; Kim et al. 2001; Serin et al. 2001; Gehring et al. 2003). Full-length hUpf3aL and hUpf3b precipitated both hUpf2 and Y14/Magoh. By contrast, hUpf3aS and the mutant hUpf3aΔhUpf2 (amino acids 66–140 deleted) did not precipitate hUpf2, probably because they lack the N-terminal or the C-terminal part of the Upf2-binding RNP-fold, respectively (Kadlec et al. 2004). hUpf3aL, hUpf3aS, and the hUpf3aΔhUpf2 mutant all display a similar partial NMD activity when compared to hUpf3aΔY14 (Fig. 1, lanes 3–5,6), which demonstrates that hUpf3a's ability to bind hUpf2 does not significantly modulate its NMD function. In contrast, the ability to form a complex with Y14/Magoh correlates with NMD activity: All hUpf3 proteins containing the C-terminal Y14/Magoh interaction sequence were at least partially active in triggering NMD. When Y14/Magoh binding is abrogated, NMD activity is completely lost. However, this does not explain the difference in activity between hUpf3aL, hUpf3aL 432R, hUpf3a+3bCterm, and hUpf3b. Although in our immunoprecipitation experiments hUpf3a proteins had a tendency to coprecipitate less Y14, Magoh, and eIF4AIII compared to hUpf3b (Fig. 4A, lanes 2,3,7; Fig. 5, lanes 2–4), this effect was only gradual and could not reflect the clear differences in NMD activity. We conclude that the interaction of Upf3 proteins with Y14/Magoh is necessary but not sufficient for NMD function. This has previously been shown for tethered hUpf3b using siRNA-mediated depletion of Y14 (Gehring et al. 2003). However, the marginal reduction of reporter mRNA did not permit us to apply the same strategy for tethered hUpf3a.

We next tested several hUpf3 mutants for their ability to coprecipitate hUpf1, hSmg5, and RNPS1 (Fig. 4B). Two of the proteins used are NMD-active (hUpf3b, hUpf3bΔhUpf2 with amino acids 49–143 deleted), two

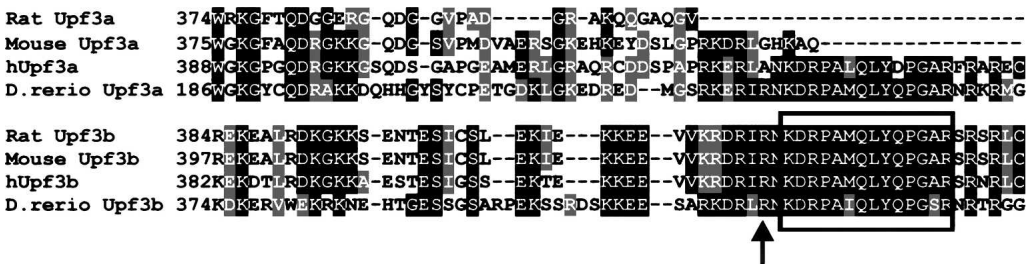

FIGURE 2. The C terminus of Upf3b but not Upf3a is conserved. C-terminal sequences for Upf3 proteins from rat (GenBank accession nos. Upf3a NM_001012159, Upf3b XP_233312), mouse (Upf3a XP_356061, Upf3b XM_110787), man (Upf3a NM_080687, Upf3b AY013251), and zebrafish (Upf3a XP_694916, Upf3b NP_957248) were aligned (ClustalW [http://www.ebi.ac.uk], Boxshade [http://www.ch.embnet.org]). Sequences were chosen to contain the Y14 interaction site (boxed) or its corresponding sequences in the other species. The arrow indicates position 432 in hUpf3a and position 419 in hUpf3b, respectively. The zebrafish Upf3a sequence is part of an unusually short Upf3 protein of unknown biological function.

are partially active (hUpf3aL and -S), and one is inactive (hUpf3bΔY14 with amino acids 421–414 deleted); some mutants cannot interact with hUpf2 (hUpf3aS, hUpf3bΔhUpf2) or with Y14/Magoh (hUpf3bΔY14). All of the proteins tested coprecipitate hUpf1, hSmg5, and RNPS1, indicating that the interactions of hUpf3 with any of these proteins does not suffice to elicit NMD. Notably, we observed that hUpf3aS precipitated the most hUpf1 when compared to the other hUpf3 protein variants. These data demonstrate that efficient immunoprecipitation of hUpf1 by hUpf3 does not depend on hUpf2 and that binding of the NMD factors hUpf1, hSmg5, and RNPS1 does not discriminate between NMD-active and NMD-inactive hUpf3 variants.

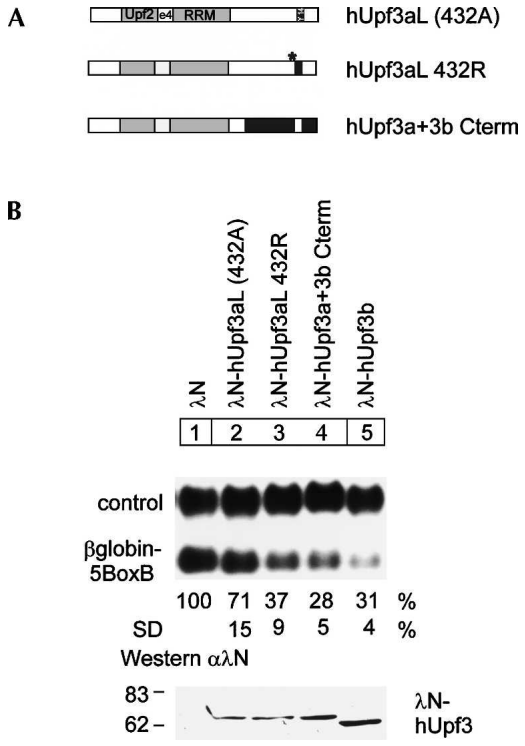

FIGURE 3. The differences in NMD activity of hUpf3a and hUpf3b reside in the C terminus. (*A*) Schematic representation of hUpf3a constructs used for tethered function analysis. Wild-type hUpf3aL (432A) was mutated to hUpf3aL 432R to match the Upf3b consensus. In construct hUpf3a+3b Cterm the entire C terminus (from position 332 in hUpf3a) was replaced by that of hUpf3b (333–483). (*B*) HeLa cells were transfected with the β-globin 5boxB reporter, a control for transfection efficiency, and the indicated λN-tagged hUpf3-construct. As a positive control, hUpf3b was transfected (lane 5). The amount of reporter mRNA was quantified relative to the unfused λN. Data are means from five independent experiments. To control for expression levels, protein lysates were analyzed by immunoblotting with an anti-λN-antibody for transfected proteins.

NMD-active hUpf3 proteins form a complex containing Y14/Magoh, eIF4AIII, and BTZ

PYM/p29, eIF4AIII, and BTZ are proteins that have been reported to interact with Y14/Magoh (Bono et al. 2004; Ferraiuolo et al. 2004; Palacios et al. 2004; Shibuya et al. 2004). We analyzed whether they are part of the NMD activating complex containing hUpf3, Y14, and Magoh. All hUpf3 proteins that show at least partial NMD activity and precipitate Y14/Magoh also precipitate eIF4AIII and BTZ (Fig. 5, lanes 2–4,6,7). In contrast, the NMD-inactive C-terminal deletion mutant hUpf3bΔY14 fails to precipitate Y14/Magoh, eIF4AIII, or BTZ (Fig. 5, lane 5). Thus, only NMD-active hUpf3 proteins engage in a large complex containing Y14/Magoh, eIF4AIII, and BTZ. All deletions of hUpf3 that disrupt complex formation with Y14/Magoh/eIF4AIII/BTZ also cause a loss of NMD function of hUpf3a and hUpf3b (Gehring et al. 2003; Fig. 1, lanes 6,9). The difference in NMD activity between hUpf3a and hUpf3b might be due to a gradual difference in affinity to the Y14/Magoh/eIF4AIII/BTZ complex that cannot be detected in immunoprecipitation experiments. Alternatively, hUpf3a might fulfill functions unrelated to NMD or represent a regulatory component of the hUpf3/Magoh/eIF4AIII/BTZ complex.

Interestingly, PYM/p29, which has previously been shown to interact with the Y14/Magoh heterodimer, is not precipitated by any hUpf3 protein or by eIF4AIII, but coprecipitates with Y14 (Fig. 5, cf. lanes 2–7,9 and lane 8)

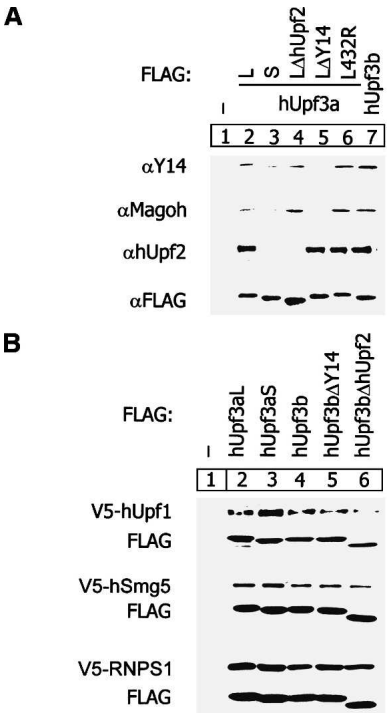

FIGURE 4. The NMD activity of hUpf3 proteins is determined by their interaction with Y14 and Magoh, but not with hUpf2, hUpf1, hSmg5, or RNPS1. (*A*) FLAG-hUpf3 proteins were expressed in HeLa cells and precipitated using anti-FLAG (M2) agarose beads; coprecipitated endogenous proteins were detected by Western blotting with antibodies directed against Y14, Magoh, and hUpf2. Unfused FLAG served as negative control (lane *1*). To control for equal loading, the membrane was reprobed with a FLAG-specific antibody (M2). (*B*) Ten micrograms (10 μg) of FLAG-hUpf3 fusion constructs were cotransfected with V5-hUpf1 (2 μg), V5-Smg5 (10 μg), or V5-RNPS1 (10 μg) and FLAG-proteins precipitated using anti-FLAG (M2) agarose beads. Coprecipitated V5 fusions were detected by Western blotting using an anti-V5 antibody. To control for equal loading, the membranes were reprobed with a FLAG-specific antibody (M2).

as previously demonstrated (Bono et al. 2004). Hence, it is unlikely that PYM/p29 plays a role in NMD activated by proteins of the Upf3 family.

Different activities of hUpf3a and hUpf3b in translation stimulation

The EJC is a key regulator of post-transcriptional mRNA metabolism (Tange et al. 2004). As such, it plays a central function in NMD, but also affects the translational efficiency of spliced transcripts. The EJC, together with the Upf proteins, stimulates translation and enhances the polysome association of the mRNA (Nott et al. 2003, 2004; Wiegand et al. 2003). Since hUpf3 proteins need to interact with Y14/Magoh/eIF4AIII/BTZ, but not with hUpf2, in order to trigger NMD, we tested whether the translation stimulation shows equivalent requirements. We coexpressed λN-hUpf3 proteins with an intronless reporter mRNA, coding for *Renilla* luciferase containing 4 boxB sites within the open reading frame (Fig. 6A). As a negative control for possible nonspecific effects of coexpressed NMD factors, we used a *Renilla* luciferase construct without boxB sites; transfection efficiency was controlled by cotransfecting a firefly luciferase reporter without boxB sequences. Firefly and *Renilla* luciferase activities were normalized against their respective mRNA levels. This analysis showed that both hUpf3aL and hUpf3aS enhance translation of the *Renilla* reporter mRNA by a factor of ~1.5-fold, whereas hUpf3b stimulates translation approximately threefold without significantly affecting mRNA levels (Fig. 6B). Surprisingly, hUpf3bΔY14, which does not recruit the Y14/Magoh/eIF4AIII/BTZ complex, and even hUpf3bΔ371–470, which lacks all of the C terminus, also stimulate translation with an efficiency similar to that of full-length hUpf3b. Thus, in contrast to its NMD activity, the translation stimulation activity of hUpf3b is independent of complex formation with Y14/Magoh/eIF4AIII/BTZ.

We tested a set of hUpf3b mutants (Fig. 5B) for their activity in translation stimulation, in order to define the region of the hUpf3b protein that mediates translation

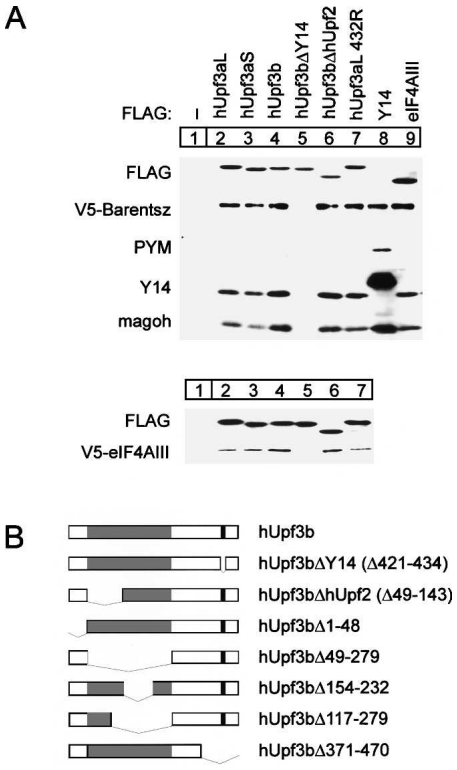

FIGURE 5. The NMD-active hUpf3 complex contains Y14, Magoh, eIF4AIII, and BTZ, but not p29/PYM. (*A*) FLAG fusions were coexpressed with V5-BTZ (*top* part) or V5-eIF4AIII (*bottom* part) and precipitated using anti-FLAG (M2) agarose beads. Unfused FLAG served as a negative control (lane *1*). Coprecipitated proteins were detected by Western blotting using antibodies directed against V5, PYM/p29, Y14, and Magoh. To control for equal loading, the membranes were reprobed with a FLAG-specific antibody (M2). (*B*) Schematic representation of hUpf3b constructs used in Figures 5 and 6.

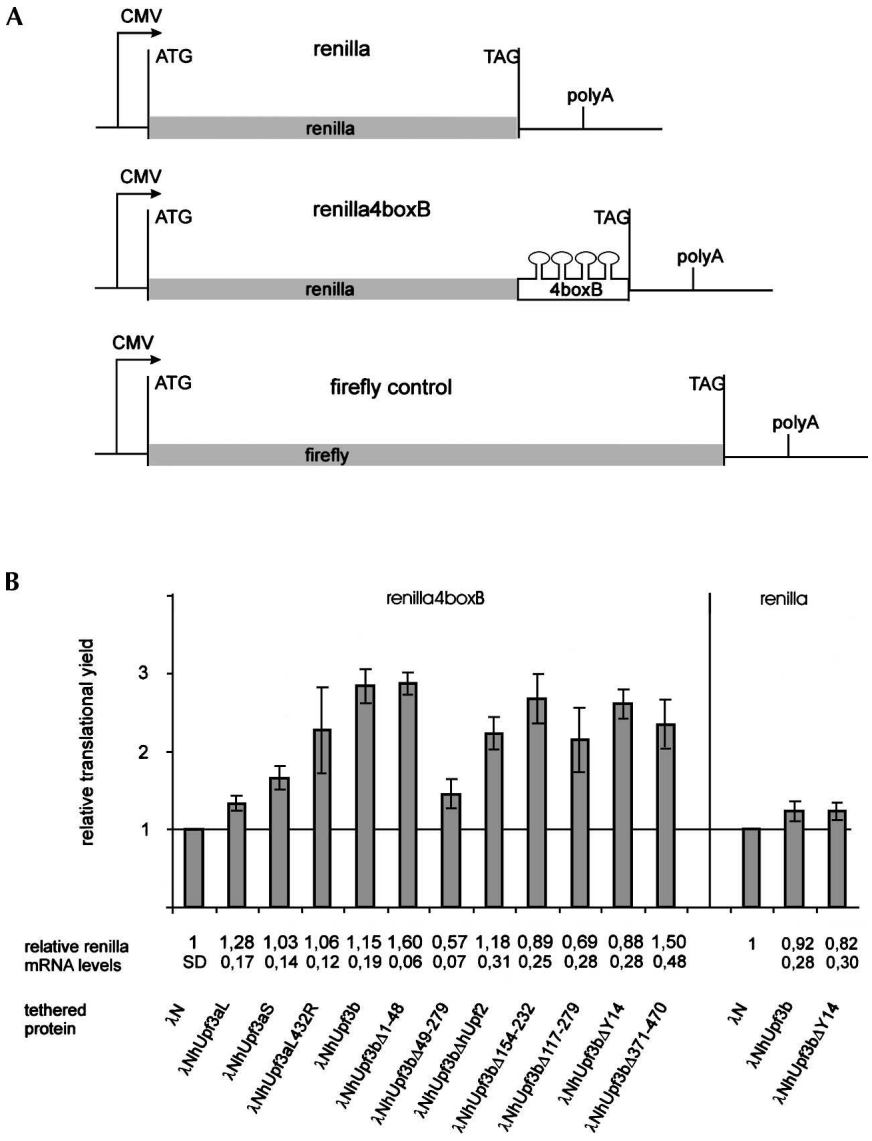

FIGURE 6. Activation of translation by hUpf3 proteins does not depend on Y14/Magoh interaction. (*A*) Schematic representation of reporter and control constructs (for details, see Materials and Methods). (*B*) Relative translational yields of *Renilla* luciferase. HeLa cells were transfected with the *Renilla* 4boxB reporter or the *Renilla* reporter without boxB sites, the firefly luciferase control, and the indicated λN-tagged hUpf3 construct (1 μg). Seventy-two hours after transfection, luciferase activities were measured, and RNA levels of reporter and control were determined by real-time PCR. *Renilla* mRNA levels and *Renilla* luciferase activities were normalized to firefly mRNA levels and firefly luciferase activity, respectively, and are represented relative to λN alone. Corresponding *Renilla* reporter mRNA levels are given *below* each bar. Data are means from at least four independent experiments; error bars represent standard errors.

(Fig. 6B). Significantly decreased translation activity was only observed for hUpf3bΔ49–279, which lacks the RNP domain including the hUpf2 interaction site. This mutant displayed the same weak activity as hUpf3a. Since the deleted region of hUpf3bΔ49–279 spans more than 200 amino acid residues, this manipulation could cause the mutant protein to adopt a misfolded, nonfunctional structure. However, hUpf3bΔ49–279 coprecipitates Y14 from HeLa extracts (data not shown), indicating that it is still able to interact with the EJC. hUpf2 binding does not seem to be required for hUpf3b function in translation because hUpf3bΔ154–232, which displays very weak hUpf2 binding (data not shown), does not significantly differ from hUpf3b wild type in translation activity. hUpf3bΔhUpf2, hUpf3bΔ117–279, and hUpf3aL432R have an intermediate activity between hUpf3a and hUpf3b, indicating that changes in these protein regions have a minor effect on the translational activity of the resulting mutant protein.

Another function of EJCs within the ORF, especially if located close to the 5′ end, is enhancing mRNA synthesis by increasing 3′ end processing efficiency (Nott et al. 2003). We designed a reporter construct with the tethering sites close to the 3′ end of the ORF to be able to examine translational effects without interfering with RNA synthesis effects. Consequently, the effect of tethered hUpf3 proteins on the abundance of the reporter RNA is small compared to the effect on reporter protein levels.

The data presented here document that hUpf3a in its splice variants is not a major NMD activating component of the EJC. The differences between hUpf3a and hUpf3b in NMD function are determined by their respective C-terminal sequences that induce assembly of a protein complex containing Y14/Magoh/eIF4AIII/BTZ, which is necessary but not sufficient for NMD. Interestingly, the role of Upf3 proteins in stimulating translation does require neither the assembly of a complex containing Y14/Magoh/eIF4AIII/BTZ nor binding of hUpf2. However, hUpf3a and hUpf3b differ in their translation stimulation activity as they do in NMD activity, indicating that the function of hUpf3a is fundamentally different from the activity of hUpf3b and not redundant as was previously thought.

We conclude that enhancing translation and activating NMD by Upf3 proteins do not involve identical protein complexes but require the recruitment of different downstream factors. Thus, different aspects of post-transcriptional mRNA metabolism represent separable functions of the EJC in general and of Upf3 proteins in particular.

MATERIALS AND METHODS

Plasmids

Beta-globin 5boxB, beta-globin WT+300+e3 (control), pCIλN, pCIFLAG, and hUpf3b constructs were described previously in Gehring et al. (2003). pCIV5 was created by inserting the V5 sequence (GKPIPNPLLGLDST) into the NheI/XhoI sites of pCIneo. hUpf3a, Magoh, eIF4AIII, BTZ, RNPS1, hSmg5, hUpf1, and hUpf2 were amplified from HeLa cell cDNA by PCR (primer sequences are available upon request). The PCR products were inserted between the XhoI/NotI sites of pCIλN, pCIFLAG, or pCIV5. All deletion and point mutants were introduced by site-directed mutagenesis (sequences are available upon request).

Renilla and firefly luciferase coding sequences were amplified by PCR from pCREL (Creancier et al. 2000) and inserted between the NheI/XhoI sites of pCIneo. The 4boxB sequence was amplified by PCR with XhoI/SalI sites on the primers and inserted into the SalI site of pCIneo*renilla*. Finally, firefly, *Renilla*, and *renilla*4boxB were subcloned from pCIneo into pcDNA3.1(−) (Invitrogen) using NheI and NotI, resulting in pcDNA*renilla*, pcDNA*renilla*4-boxB, and pcDNAfirefly.

Cell culture and transfection

HeLa cells were grown in Dulbecco's modified eagle medium under standard conditions. Calciumphosphate transfection of HeLa cells was performed as previously described in Thermann et al. (1998).

For experiments shown in Figures 1 and 3, HeLa cells were cotransfected in 6-well-plates (2 mL medium) with 2 μg of beta-globin 5boxB, 0.6 μg of the control plasmid (WT+300+e3), 0.4 μg of a GFP-expression vector, and 1 μg of a λN-expression plasmid (e.g., pCI λN hUpf3a). Forty hours after transfection, cells were harvested for analysis of RNA and protein expression. Northern blotting was performed as described previously in Gehring et al. (2003).

For experiments shown in Figure 6, HeLa cells were cotransfected in 6-well-plates with 1 μg of pcDNA*renilla*4boxB or pcDNA*renilla*, 0.1 μg of pcDNAfirefly, 0.2 μg of a GFP-expression vector, and 1 μg of a λN-expression plasmid.

Luciferase measurements

Seventy-two hours after transfection, cells were harvested for analysis of luciferase activity and RNA abundance: Cells were washed two times in PBS and suspended in 350 μL of cold Passive Lysis Buffer (Promega, Dual-Luciferase Reporter Assay System). Five microliters of the lysate were used for measuring luciferase activities according to the manufacturer's recommendations in a Centro LB 960 luminometer (Berthold) with the Dual-Luciferase Reporter Assay System (Promega). The remainder of the lysate was used for RNA extraction.

cDNA synthesis and real-time PCR

cDNA was synthesized from 2 μg of cytoplasmic RNA using a mix of primers (20 μM each) (Danckwardt et al. 2004):

5′-TGCTGGTCGATGCACGTGACGC$(T)_{16}$A-3′,
5′-TGCTGGTCGATGCACGTGACGC$(T)_{16}$C-3′,
5′-TGCTGGTCGATGCACGTGACGC$(T)_{16}$G-3′.

In order to avoid amplification of plasmid DNA contaminating the cDNA preparations, an anchor-specific antisense primer (5′-TGCTGGTCGATGCACGTGACGC-3′) and a specific sense primer were used for real time PCR:

for *Renilla* luciferase 4boxB: 5′-TAGAGTCGACGCTCGCTTC-3′,
for *renilla* luciferase: 5′-TCGGACCCAGGATTCTTTTC-3′,
for firefly luciferase: 5′-GTGGATTACGTCGCCAGTCA-3′.

For quantitative PCR, the SYBR Green I Kit and the Light-cycler 2000 (Roche) were used according to the manufacturer's instructions.

Immunoprecipitation and Western blot

For immunoprecipitation experiments, HeLa cells were transfected in 10-cm dishes with 10 μg of a pCIFLAG plasmid, 2 μg of a GFP expression vector, and 10 μg of a pCIV5 plasmid, if required. Cells were harvested 40 h after transfection for immunoprecipitation: Cells were washed once with PBS, lysed in 1 mL lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.5% Triton-X 100, 0.25% deoxycholate, 1× complete protease inhibitors, 1 mM PMSF, 20 μg/mL RNase A at pH 7.2) by passing several times through a syringe, and centrifuged (10 min, 4°C, 16,000*g*). The supernatant was assayed for protein concentration by the Bradford method. Equal protein amounts were incubated for 1 h on a rotator with 15–20 μL of anti-FLAG-agarose (Sigma). The beads were washed with 4 × 1 mL lysis buffer without protease inhibitors. Bound proteins were eluted with 40 μL 200 mM glycin (pH 2.5) and analyzed by SDS-PAGE and Western blotting. Antibodies against FLAG and V5 were from Sigma. Secondary antibodies (HRP-conjugated anti-mouse and anti-rabbit) were from Sigma and used at 1:10,000 dilution for Western blotting.

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