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ATP is a cofactor of the Upf1 protein that modulates its translation termination and RNA binding activities

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ABSTRACT

The nonsense-mediated mRNA decay pathway decreases the abundance of mRNAs that contain premature termination codons and prevents suppression of nonsense alleles. The *UPF1* gene in the yeast *Saccharomyces cerevisiae* was shown to be a *trans*-acting factor in this decay pathway. The Upf1p demonstrates RNA-dependent ATPase, RNA helicase, and RNA binding activities. The results presented here investigate the binding affinity of the Upf1p for ATP and the consequences of ATP binding on its affinity for RNA. The results demonstrate that the Upf1p binds ATP in the absence of RNA. Consistent with this result, the TR800AA mutant form of the Upf1p still bound ATP, although it does not bind RNA. ATP binding also modulates the affinity of Upf1p for RNA. The RNA binding activity of the DE572AA mutant form of the Upf1p, which lacks ATPase activity, still bound ATP as efficiently as the wild-type Upf1p and destabilized the Upf1p–RNA complex. Similarly, ATPγS, a nonhydrolyzable analogue of ATP, interacted with Upf1p and promoted disassociation of the Upf1p–RNA complex. The conserved lysine residue (K436) in the helicase motif Ia in the Upf1p was shown to be critical for ATP binding. Taken together, these findings formally prove that ATP can bind Upf1p in the absence of RNA and that this interaction has consequences on the formation of the Upf1p–RNA complex. Further, the results support the genetic evidence indicating that ATP binding is important for the Upf1p to increase the translation termination efficiency at a nonsense codon. Based on these findings, a model describing how the Upf1p functions in modulating translation and turnover and the potential insights into the mechanism of the Upf1p helicase will be discussed.

Keywords: helicase; mRNA stability; nonsense-mediated mRNA decay; RNA; termination; translation

INTRODUCTION

The pathways that specify transcript decay rates are important determinants in controlling the abundance of mRNAs in cells (reviewed in Peltz et al., 1993; Decker & Parker, 1994; Maquat, 1995; Ross, 1995; Jacobson & Peltz, 1996). In addition, it has now become clear that the processes of mRNA turnover and translation can be intimately linked (reviewed in He et al., 1993; Peltz & Jacobson, 1993; Decker & Parker, 1994; Maquat, 1995; Ross, 1995; Yun & Sherman, 1995; Jacobson & Peltz, 1996; Weng et al., 1997). The observation that premature translation termination results in accelerated degradation of mRNAs, a process that has been termed nonsense-mediated mRNA decay (NMD), is one clear example that establishes this link (reviewed in Peltz et al., 1993; Maquat, 1995; Ruiz-Echevarria et al., 1996; Weng et al., 1997). Both the *cis*-acting sequences and *trans*-acting factors involved in the NMD pathway in the yeast *Saccharomyces cerevisiae* are currently being investigated. Mutation or deletion of any one of the *UPF1*, *UPF2*, or *UPF3* genes have been shown to specifically stabilize nonsense-containing transcripts (Leeds et al., 1991, 1992; Cui et al., 1995; He & Jacobson, 1995; Lee & Culbertson, 1996). Recent results further indicate that the Upf1p, Upf2p, and Upf3p interact to form a complex (He & Jacobson, 1995; He et al., 1996, 1997; Weng et al., 1996b; reviewed in Weng et al., 1997).

The *UPF1* gene and its protein product have been characterized most extensively (Leeds et al., 1991, 1992;

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Atkin et al., 1995; Czaplinski et al., 1995; Weng et al., 1996a, 1996b). The *UPF1* gene harbors all of the motifs characteristic of members of the superfamily group I helicases (Koonin, 1992). The Upf1p has been purified and contains nucleic acid-dependent ATPase and helicase activities (Czaplinski et al., 1995; Weng et al., 1996a, 1996b).

Recent genetic studies suggest that the Upf1p is a multifunctional protein involved in nonsense-mediated mRNA decay, as well as in modulating various aspects of translation, including programmed −1 ribosomal frameshifting and translation termination at nonsense codons (Cui et al., 1996; Weng et al., 1996a, 1996b; reviewed in Weng et al., 1997). The conclusion that the Upf1p is involved in modulating multiple steps in the translation process as well as NMD was based on a set of mutations in the *UPF1* gene that altered translation termination at a nonsense codon or programmed −1 ribosomal frameshifting efficiencies (Cui et al., 1996; Weng et al., 1996a, 1996b).

A subset of mutations in the *UPF1* gene that changed the conserved amino acids located in ATPase/helicase motif I, II, and VI of the Upf1p were particularly informative in determining the role of the Upf1p in NMD and translation termination (Weng et al., 1996a). A collection of these mutations is shown in Figure 1 (Weng et al., 1996a, 1996b). The highly conserved lysine residue (K436) in the ATPase motif I (also known as the P loop) was mutagenized to glutamine (Fig. 1; this mutant is designed K436Q). Previous results have suggested that this amino acid participates in ATP binding and hydrolysis (La Court et al., 1985; Fry et al., 1986; De Vos et al., 1988; Jurnak, 1988). The K436Q *upf1* allele inactivated the nonsense-mediated mRNA decay pathway, stabilizing nonsense-containing mRNAs and allowing suppression of *tyr7-1* and *leu2-2* nonsense alleles (Fig. 1; Weng et al., 1996a, 1996b). Biochemical analysis of the K436Q of the Upf1p demonstrated that this mutation inactivated both the ATPase and helicase activities of the Upf1p (Fig. 1; Weng et al., 1996a, 1996b). The aspartic acid and glutamic acid (DE) residues in ATPase motif II were changed to alanine residues (Fig. 1; this mutant is designated DE572AA). The DE residues were shown to be highly conserved among both the helicase superfamilies group I and II and thought to participate in ATP binding and hydrolysis (Walker et al., 1982; La Court et al., 1985; Gorbalenya et al., 1989). In addition, the threonine and arginine residues in motif VI, which are highly conserved among members of helicase superfamily I, were changed to alanines (Fig. 1; this mutant is designated TR800AA). The positive charge of this motif has been demonstrated to be involved in RNA binding in both helicase superfamilies I and II (Linder et al., 1989; Pause et al., 1993; Weng et al., 1996a, 1996b). Interestingly, both the DE572AA and TR800AA *upf1* alleles separated the nonsense suppression function from its mRNA turnover function (Fig. 1; Weng et al., 1996a, 1996b). Cells harboring the DE572AA and TR800AA *upf1* alleles stabilized all of the nonsense-containing mRNAs monitored, but, unlike other *upf1* alleles, did not allow suppression of *tyr7-1* and *leu2-2* nonsense alleles (Fig. 1; Weng et al., 1996a, 1996b). Furthermore, both mutants inactivated the RNA-dependent ATPase and helicase activities of the Upf1p (Weng et al., 1996a, 1996b). The DE mutant form of the Upf1p was an interesting protein because it still was capable of forming a complex with RNA (Weng et al., 1996). Interestingly,

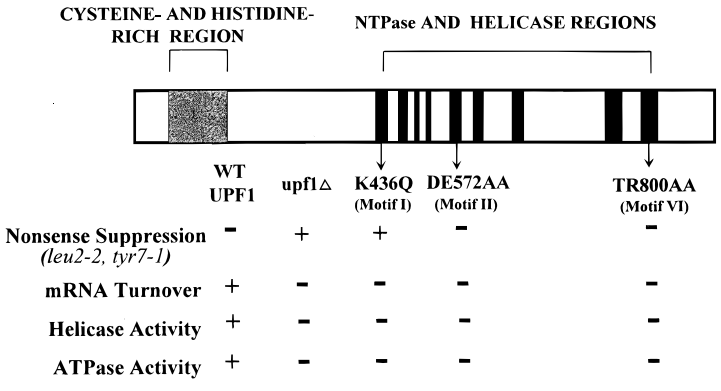

FIGURE 1. Properties of the Upf1 helicase domain mutants. A schematic representation of the Upf1 protein. The shaded area near the amino terminus represents the cysteine- and histidine-rich region. Black bars represent NTPase and helicase motifs. Mutations in the conserved lysine residue (K436Q) and aspartic and glutamic residues (DE572AA) in motif I and II of the ATPase/helicase region were designated as indicated. Below the schematic of the *UPF1* gene is a summary of the in vivo nonsense suppression phenotypes of cells harboring *upf1* alleles and the biochemical activities of mutant forms of Upf1p. Nonsense suppression phenotypes were determined in yeast strain PLY146, which harbors *leu2-2* and *tyr7-1* nonsense alleles. −, Nonsense suppression was not observed; +, nonsense suppression was observed. Activity of the nonsense-mediated mRNA decay pathway was determined by monitoring the abundance of the the leu2-2, tyr7-1, and nonsense-containing pgk1 mRNAs, as well as CYH2 precursor in cells harboring various *upf1* alleles in comparison to their abundance in *UPF1*$^+$ and *upf1*Δ strains. +, Nonsense-mediated mRNA decay pathway was active; −, nonsense-mediated mRNA decay pathway was inactive. The ATPase and helicase assays were performed as described previously (Czaplinski et al., 1995) −, ATPase or helicase activities of these mutant forms of the Upf1p were not detectable by charcoal assay; +, ATPase and helicase activities of the Upf1p were readily detectable. Data in Figure 1 is summarized from Weng et al. (1996a).

addition of ATP to the reactions promoted disassociation of the Upf1p–RNA complex. Taken together, these results suggested that the ability to bind ATP is important for Upf1p to function in translation termination, but its ATPase and helicase activities were dispensable. The ATPase and helicase activities were, however, required in the subsequent step that identifies the transcript as aberrant and degrades the nonsense-containing transcript.

Based on the potential significance of ATP as an important regulator of Upf1p activity, the interaction of ATP with Upf1p was characterized and its affect on the affinity of Upf1p for RNA was analyzed. The results presented here demonstrate that ATP binds to the wild-type or mutant DE572AA and TR800AA forms of the Upf1p, in the absence of RNA, with a K_d of approximately 9 μM. Further, addition of ATP to the Upf1p–RNA complex promotes its rapid disassociation. The reduced stability of the Upf1p–RNA complex when ATP was bound to the Upf1p was a consequence of its binding, independent of its hydrolysis, because ATPγS, a nonhydrolyzable analogue of the ATP, bound to the Upf1p, albeit with reduced affinity, and promoted disassociation of the Upf1p–RNA complex. Consistent with this view, addition of ATP to Upf1p–RNA complex that contains the DE mutant form of the Upf1p that lacks ATPase activity still promoted disassociation of the Upf1p–RNA complex. Taken together, these results indicate that ATP functions as a cofactor of the Upf1p to modulate the stability of the Upf1p–RNA complex. The significance of these results will be discussed in context of the role of the Upf1p in translation termination and mRNA decay.

RESULTS

Characterization of the effects of ATP on the RNA binding properties of the wild-type and mutant forms of the Upf1p

Mutations in the conserved helicase motifs of the *UPF1* gene were introduced in the *FLAG-UPF1* allele, which functioned as the wild-type *UPF1* gene in preventing nonsense suppression and promoting nonsense-mediated mRNA decay (Czaplinski et al., 1995; Weng et al., 1996a). The wild-type, K436Q, DE572AA, and TR800AA forms of the Upf1p were purified by immunoaffinity chromatography directed against the Flag epitope as described previously (Czaplinski et al., 1995; Weng et al., 1996a). As reported previously (Weng et al., 1996), the various mutant forms of the Upf1p eluted from the antibody column are shown in an SDS-PAGE gel in Figure 2A. A single band with an apparent molecular weight of approximately 110 kDa that reacted with the anti-Flag antibody as determined by immunoblotting was observed (Fig. 2B). Using a uniformly radiolabeled in vitro-prepared 78-nt transcript as a substrate (Fig. 2C, lane 1; see Materials and Methods), the RNA binding characteristics of the wild-type and mutant forms of the Upf1p were investigated by gel shift assay as described previously (Czaplinski et al., 1995; Weng et al., 1996a). Consistent with what was observed previously (Weng et al., 1996a, see Fig. 8; presented for comparison with other results), the TR800AA form of the Upf1p did not bind to RNA, consistent with this motif being required for RNA binding (Fig. 2C, lane 2). In the absence of ATP, the wild-type, K436Q, and DE572AA forms of the Upf1p resulted in a slower migrating band, indicating formation of a Upf1p–RNA complex (Fig. 2C, lanes 3, 5, and 7; see Weng et al., Fig. 8). Addition of ATP to the reaction mixture promoted disassociation of both the wild-type Upf1p–RNA and Upf1p$_{DE572AA}$–RNA complexes (Fig. 2C, lanes 4 and 8). Interestingly, although the DE572AA form of the Upf1p does not have ATPase activity (Weng et al., 1996a), ATP binding was still capable of modulating its ability to complex with RNA. ATP addition to the reaction mixture containing the K436Q form of the Upf1p had no affect on the stability of the Upf1p–RNA complex (Fig. 2C, lane 6).

The kinetics of disassociation of the Upf1p–RNA complex after addition of the ATP was monitored. Upf1p–RNA complexes were formed by mixing Upf1p with radiolabeled RNAs (Fig. 3, lane 1). ATP was subsequently added to reaction mixtures containing the complexes and the rate of complex disassociation was monitored by the RNA gel shift assay. The results indicate that ATP addition caused rapid disassociation of the Upf1p–RNA complex (Fig. 3, lanes 2–9). Within 4 min of ATP addition, the majority of the Upf1p–RNA complex had disassociated.

Determination of the binding affinity of ATP to the wild-type and mutant forms of the Upf1p

The Upf1p$_{DE572AA}$–RNA the complex disassociated when ATP was added to the reaction mixture. Previous results demonstrated that this mutation inactivated both the ATPase and helicase activities of the Upf1p (Fig. 1). The results described above indicate that ATP binding, independent of its hydrolysis, modulated the stability of the Upf1p–RNA complex and that the Upf1p$_{DE572AA}$ was still capable of binding to ATP. To test this hypothesis, the binding constants of ATP for the wild-type and mutant forms of the Upf1p were determined by monitoring the Upf1p:ATP complex at various ATP concentration using a nitrocellulose filter binding assay. The results demonstrated that the wild-type Upf1p bound ATP efficiently, with a K_d of 9.0 μM (Table 1; Fig. 4A). The K436Q form of the Upf1p bound to ATP with a K_d of 140 μM, which is 16-fold higher than wild-type Upf1p, indicating that the ATP binding activity in this mutant has been significantly reduced (Table 1; Fig. 4B). In contrast, the DE572AA and TR800AA mutants bound to ATP with K_ds similar to the wild-type Upf1p (9.5 μM and 10.3 μM, respectively; Table 1; Fig. 4A).

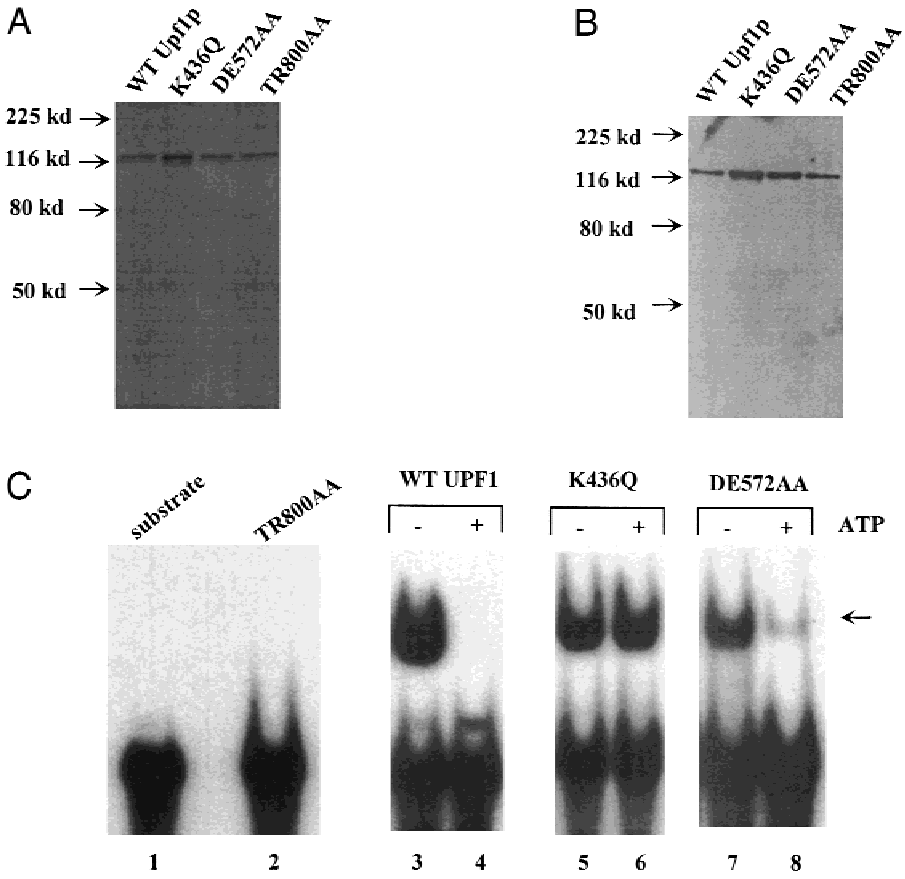

FIGURE 2. Characterization of the effect of ATP on the Upf1p–RNA complex. Wild-type and mutant forms of the Upf1p were purified as described in Materials and Methods. Purified proteins were analyzed by SDS-PAGE and either (**A**) stained with Coomassie blue or (**B**) immunoblotted using a monoclonal antibody against the Flag epitope. **C:** Effect of ATP addition on dissociation of the wild-type and mutant forms of the Upf1p–RNA complex. Binding reactions were performed as described in Materials and Methods. Where indicated, 1 mM ATP was added (ATP [+]) 5 min before the binding reactions were terminated. [−] indicates mixtures lacking ATP. Arrows indicate the position of the Upf1p–RNA complex in a native PAGE gel. Similar results are reported in Weng et al. (1996a).

ATPγS caused disassociation of the Upf1p–RNA complex

These results indicated that ATP binding, independent of its hydrolysis, promoted disassociation of the Upf1p–RNA complex. To test this hypothesis, increasing concentrations of the nonhydrolyzable analogue of ATP, ATPγS, were added to reaction mixtures containing the radiolabeled RNA and the wild-type form of the Upf1p. Complex formation was monitored by RNA gel shifts as described above. The results of this experiment demonstrated that ATPγS promoted dissociation of Upf1p–RNA complex when 10 mM ATPγS was added to the reactions (Fig. 5, lane 7). Lower concentrations of ATPγS were not able to promote disassociation of the Upf1p–RNA complex (Fig. 5, lanes 4–6). Another analogue of ATP, AMP-PNP, which was previously shown to inhibit the ATPase activity of the Upf1p (Czaplinski et al., 1995), did not promote disassociation of the Upf1p–RNA complex (Fig. 5, lanes 10 and 11).

Preparations of ATPγS can be contaminated with as much as 5% ADP. To ensure that ATPγS, rather than ADP, promoted disassociation of the RNA–Upf1p complex, ADP was added to the RNA binding reaction and its affect on Upf1p–RNA complex formation was monitored. ADP addition to the reaction mixture had no affect on the stability of the Upf1p–RNA complex, with the complex being equally stable in the presence or absence of ADP (Fig. 5, lanes 8 and 9).

The ATPγS added to the reaction mixtures was prepared as an Li^+ salt. To determine whether the stability of the Upf1p–RNA complex was affected by the addition of Li^+, reaction mixtures containing or lacking 40 mM of LiCl were prepared and Upf1p–RNA complex formation was monitored as described above. This Li^+ concentration added was equivalent to the Li^+ concentration when 10 mM of ATPγS was added to the reaction mixture. The results demonstrated that addition of Li^+ to the reaction did not promote disassociation of the Upf1p–RNA complex (Fig. 5, lane 18). We also tested whether trace amounts of ATP in the ATPγS sample could account for the observed disassocia-

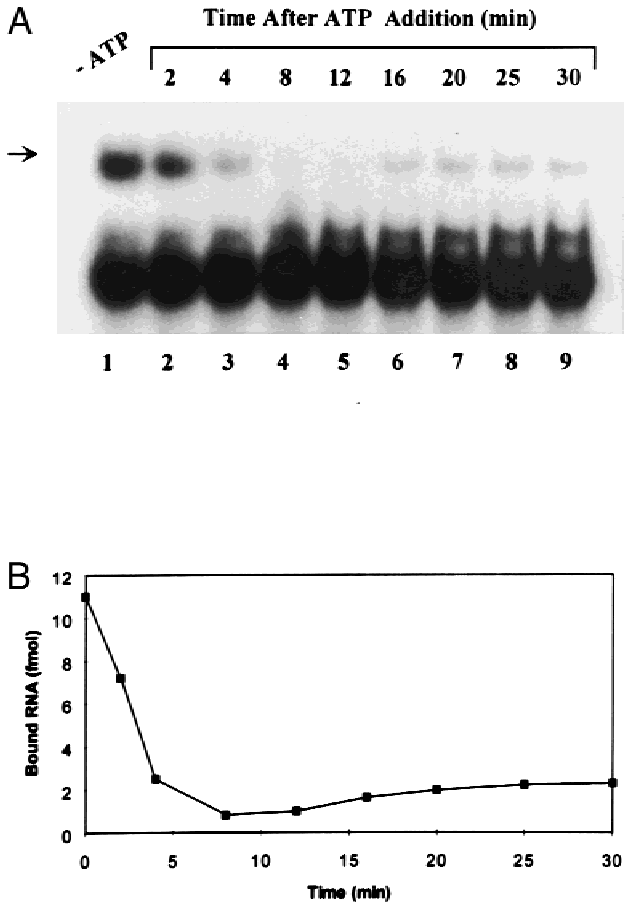

FIGURE 3. Kinetics of Upf1p disassociation of Upf1p–RNA complex. RNA binding assays were performed as described in Materials and Methods using the wild-type form of the Upf1p in the absence of ATP (lane 1) or in the presence of 0.05 mM ATP. **A:** An autoradiograph is shown. Arrow indicates the position of the Upf1p–RNA complex in native PAGE gels. **B:** Quantitation of the results from A.

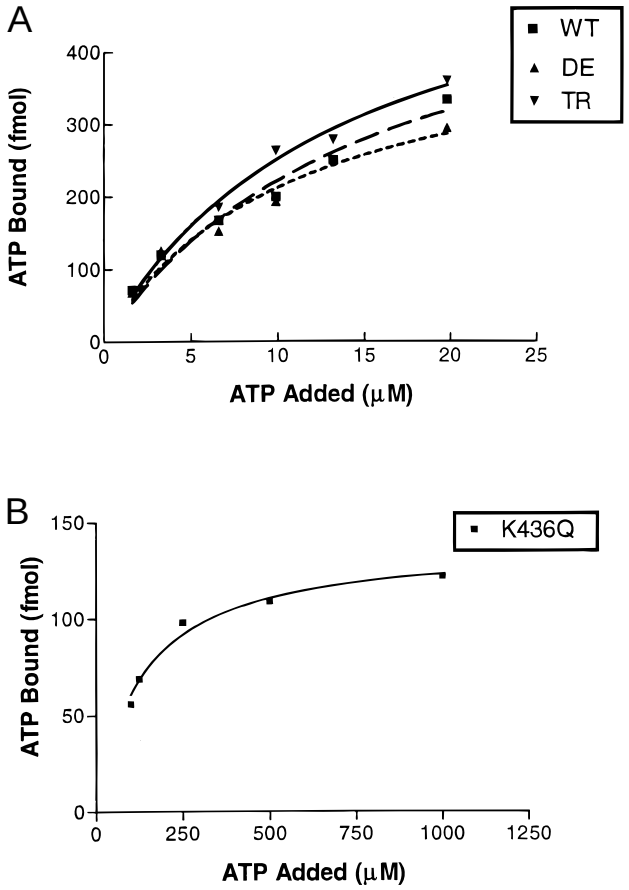

FIGURE 4. Wild-type and mutant Upf1p ATP binding. The amount of ATP bound for the wild-type, DE572AA, TR800AA, and K436Q mutant Upf1 proteins was determined using increasing amounts of ATP using a nitrocellulose filter binding assay as described in Materials and Methods. **A:** Plot of the data for the wild-type, DE572AA, and TR800AA mutant proteins. **B:** Plot of the data for the K436Q mutant protein. Data were plotted and disassociation constants (Table 1) were determined using Prism 2.0.

tion of the Upf1p–RNA complex. To test this possibility, an aliquot of ATPγS was incubated with glucose and hexokinase for 15 min to dephosphorylate any contaminating ATP. The ATPγS-treated sample was added to the RNA binding reaction and complex formation was monitored as described above. The results demonstrated that the hexokinase-treated ATPγS was able to promote disassociation of the Upf1p–RNA complex (Fig. 5, lanes 15–17). These results indicate that contaminating ATP in the ATPγS did not account for its ability to modulate the stability of the Upf1p–RNA complex.

The concentration of ATPγS required to disassociate the Upf1p–RNA complex was 200-fold higher than the concentration of ATP required to promote disassociation (i.e., 0.05 mM versus 10 mM; Figs. 3, 5). To determine whether the high concentration of ATPγS required to disassociate the Upf1p–RNA complex reflected a lower affinity for Upf1p, the binding constants of ATP and ATPγS for Upf1p were determined by monitoring the Upf1p–ATPγS complex at various ATPγS concentrations using a filter binding assay as described above. Both the wild-type and DE572AA mutant forms of the Upf1p bound ATPγS much less effectively than ATP, with K_ds of 650 μM and 700 μM, respectively (Table 1). The decreased affinity of Upf1p for ATPγS, compared to ATP, accounts for the increased concentration of ATPγS required to promote disassociation of the Upf1p–RNA complex (Fig. 5).

DISCUSSION

A mutational analysis of the *UPF1* gene suggested that the ATPase and helicase activities of the Upf1p were required to promote decay of a nonsense-containing mRNA, but were not required for its ability to enhance translation termination at a nonsense codon (Weng et al., 1996a). Interestingly, however, genetic results indicated that the ATP-bound form of the Upf1p was needed to enhance translation termination at a nonsense codon (Weng et al., 1996a, 1996b).

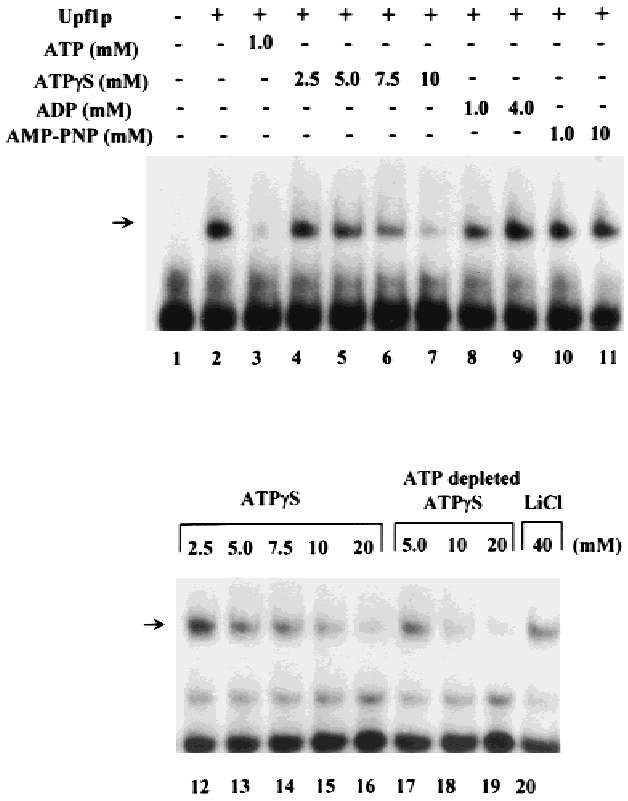

FIGURE 5. ATPγS promotes disassociation of the Upf1p–RNA complex. RNA binding reactions were performed using a gel shift assay as described in Materials and Methods. The presence (+) or absence (−) of the Upf1p, ATP, ATPγS, ADP, or AMPPNP is shown above the autoradiograph. Numbers represent the final concentration (mM) of the nucleotide or salt added to the reaction. Arrows indicate the position of the Upf1p–RNA complex in native PAGE gels.

Based on these observations, it became clear that understanding how ATP interacted with Upf1p, and the biochemical consequences of this interaction, would be important in elucidating how this protein functioned in modulating translation and mRNA turnover. Thus, the goal of the experiments presented here was to characterize the ATP–Upf1p interaction in more detail and to determine the affect of this interaction on the ability of the Upf1p to complex with RNA. The results demonstrated the following. (1) The Upf1p binds ATP in the absence of RNA with a K_d on the order of 10 μM. (2) Consistent with the observation that Upf1p–RNA complex was not required for ATP to interact with Upf1p, the TR800AA mutant form of the Upf1p, which no longer binds to RNA (Weng et al., 1996a), still bound ATP with a K_d on the order of 10 μM (Table 1). (3) The conserved lysine residue (K436) of the helicase motif Ia in the *UPF1* gene (Fig. 1) was shown to be important for ATP binding, because the K436Q form of the Upf1p mutation demonstrated greater than a 15-fold reduction in its affinity for ATP, with a K_d of 140 μM (Table 1). (4) ATP addition promoted rapid disassociation of the Upf1p–RNA complex (Fig. 3). (5) ATPγS, a nonhydrolyzable analogue of ATP, bound to Upf1p, albeit with reduced affinity (Table 1), and promoted disassociation of the Upf1p–RNA complex. This result is important because it demonstrates that ATP binding, independent of its hydrolysis, modulates the affinity of the Upf1p for RNA. (6) Consistent with the view that ATP binding, without hydrolysis, promotes rapid disassociation of the Upf1p complex, the DE572AA mutant form of the Upf1p, which does not hydrolyze ATP, still bound ATP with a K_d of approximately 10 μM. In addition, ATP bound to this mutant protein still promoted disassociation of the Upf1p–RNA complex. These results have several implications concerning how the Upf1p functions in both translation termination and mRNA turnover and will be discussed in more detail below.

TABLE 1. ATP dissociation constant of wild-type and mutant forms of Upf1p.[a]

Upf1p	K_d (ATP) (μM)	K_d (ATPγS) (μM)
Wild-type	9.0	650
K436Q	140	ND
DE572AA	9.5	700
TR800AA	10.3	ND

[a]ATP binding assays of the wild-type and mutant forms of the Upf1p were performed as described in Materials and Methods. ND, not determined; ATPγS binding affinity were not assayed for these mutant forms of the Upf1p.

Upf1p bound ATP in the absence of RNA

The wild-type Upf1p bound ATP with a K_d of approximately 10 μM in the absence of RNA (Table 1). Similarly, the TR800AA mutant form of the Upf1p, which did not demonstrate any RNA binding activity, or the DE572AA mutant form of the Upf1p, which no longer demonstrated any ATPase or helicase activity, still bound to ATP with the same disassociation constant as the wild-type protein (Table 1). All three of these proteins prevented suppression at a nonsense codon, indicating that the activity of the Upf1p to modulate translation termination was still functional. Although strains harboring either the DE572AA and the TR800AA *upf1* alleles prevented nonsense suppression, they were unable to degrade nonsense-containing transcripts (Weng et al., 1996a). Conversely, strains harboring K436Q *upf1* allele demonstrated suppression of nonsense alleles and stabilization of nonsense-containing transcripts, indicating that both the NMD and translation termination functions of the Upf1p were inactivated (Weng et al., 1996a). The K436Q form of the Upf1p demonstrated a greater than 15-fold decreased affinity for ATP (Table 1). This is a particularly significant result because, unlike the loss of the ATPase, helicase, or RNA binding activities of the Upf1p, reducing the ability of the Upf1p to bind

ATP directly correlated with its inability to modulate translation termination (Fig. 1; Table 1). This result-suggests that ATP binding is important in order for the Upf1p to enhance translation termination and prevent the production of aberrant protein products (see Fig. 1; Table 1).

Upf1p-ATP bound form has a reduced affinity for single-stranded RNA

The results described above demonstrate a direct correlation between its affinity for ATP and its translation termination activity. Previous results indicated that ATP binding may affect the stability of the Upf1p–RNA complex (Weng et al., 1996a). Consistent with this observation, addition of ATP to a Upf1p–RNA complex resulted in its disassociation within 4 min (Fig. 3). Therefore, we determined whether interaction of ATP with Upf1p, independent of its hydrolysis, resulted in modulating the RNA binding activity of the Upf1p. This hypothesis was tested directly using ATPγS, a nonhydrolyzable analogue of ATP. The results demonstrated that ATPγS was able to bind to the Upf1p and promote disassociation of the Upf1p–RNA complex (Table 1; Fig. 5). Because ATPγS did not bind to the Upf1p with the same affinity as ATP (Table 1), higher concentrations of ATPγS were required to promote disassociation of the complex (Fig. 5). Nonetheless, this nonhydrolyzable analogue of ATP was still able to promote disassociation of the Upf1p–RNA complex. This is in contrast to another nonhydrolyzable analogue, AMP-PNP, which was unable to destabilize the Upf1p–RNA complex (Fig. 5). Structural differences between the two analogues probably reflect why ATPγS functions to promote disassociation of the Upf1p–RNA complex, whereas AMP-PNP did not modulate the RNA binding activity of the Upf1p. Although AMP-PNP did not affect the stability of the Upf1p–RNA complex, previous results demonstrated that, at the concentrations used here, AMP-PNP functioned as a competitive inhibitor of the ATPase activity of the Upf1p (Czaplinski et al., 1995).

The ability of ATPγS to bind to Upf1p and promote disassociation of the Upf1p–RNA complex strongly indicated that ATP binding, but not its hydrolysis, reduced the affinity of the Upf1p for RNA. Consistent with this observation, the stability of the Upf1p–RNA complex containing the mutant DE572AA form of the Upf1p was reduced in the presence of ATP (Fig. 2). Previous results demonstrated that strains harboring the *upf1*$_{DE572AA}$ allele did not allow nonsense suppression, but did not promote rapid degradation of nonsense-containing mRNAs (Weng et al., 1996a). On the other hand, the K436Q mutant form of the Upf1p, which demonstrated a greatly reduced ability to bind ATP (Table 1), bound RNA efficiently in the presence or absence of ATP (Fig. 2). Strains harboring this *upf1* allele demonstrated a nonsense suppression phenotype as well as stabilization of nonsense-containing transcripts.

Conserved DE amino acids in motif II of the helicase region of the *UPF1* gene are important for ATP hydrolysis but not for ATP binding

These results indicated that the conserved aspartic and glutamic acid residues in motif II of the superfamily group I helicases (Fig. 1) are necessary for ATP hydrolysis, but not for ATP binding. Mutating these codons to encode for alanine residues abolished the ATPase activity of the Upf1p without affecting its ability to bind ATP (Table 1; Fig. 4). Similar results have been observed for other helicases. For example, mutating the DE residues to asparagine or/and glutamine residues in the *Escherichia coli* DNA helicase II did not alter ATP binding, but reduced the ATPase activity to less than 0.5% of that observed for wild-type protein (Brosh & Matson, 1995). Similarly, mutating the DE residues in initiation factor eIF-4A resulted in the inactivation of the ATPase activity without affecting ATP binding (Pause & Sonenberg, 1992). These observations suggest that, in contrast to results observed for certain other ATPases (Walker et al., 1982; La Court et al., 1985; Gorbalenya et al., 1989), for at least Upf1p, eIF-4A, and *E. coli* DNA helicase II, motif II is necessary for ATP hydrolysis but not ATP binding.

Conserved TR amino acids in motif VI in the helicase region of the *UPF1* gene are important for RNA binding

The TR800AA form of the Upf1p does not exhibit RNA-dependent ATPase or helicase activities (Fig. 1; Weng et al., 1996a, 1996b). Interestingly, this mutant Upf1p does not complex with RNA, but can still bind to ATP with an affinity equivalent to the wild-type Upf1p (Table 1; Figs. 2, 4). As described above, these results are consistent with the observation that the site on the Upf1p that binds to ATP is independent of the RNA binding site. Previous results demonstrated that RNA binding is necessary for the Upf1p to hydrolyze ATP (Czaplinski et al., 1995). The RNA binding motif (motif VI) of eIF-4A was also found to be necessary for ATP hydrolysis (Pause et al., 1993).

Comparison of the Upf1p with other helicases

Other superfamily group I helicase nucleic acid interactions are also modulated by ATP (reviewed in Lohman & Bjornson, 1996). The Rep helicase is probably the most well characterized superfamily group I helicase. The *E. coli* Rep 3′ $\rightarrow$5′ helicase interaction with DNA is also modulated by ATP binding (Wong &

Lohman, 1992). This protein is required for replication of a number of phages and enhances replication of the *E. coli* chromosome. The Rep protein undergoes a DNA-induced dimerization as a consequence of DNA binding, and the dimer is thought to be the active helicase (Chao & Lohman, 1991). Interestingly, ATP binding to Rep reduces the affinity for single-stranded DNA of one of the Rep monomers in the homodimer, whereas its affinity for double-stranded DNA increases (Wong & Lohman, 1992). Hydrolysis of ATP disrupts the DNA duplex and leaves the two Rep monomers bound to single-stranded DNA (Wong & Lohman, 1992). Thus, the two Rep monomers supply two DNA binding sites in which the DNA binding status is communicated to the other in response to ATP binding and hydrolysis. This "active rolling mechanism" for helicase activity is used by the Rep dimer for translocation and melting of DNA duplexes and utilizes coordinated cycles of ATP binding and hydrolysis by the two Rep monomers (reviewed in Lohman & Bjornson, 1996).

At present, a detailed mechanism of the Upf1p helicase activity has not been elucidated. The results presented here, however, begin to define potential steps in the helicase cycle of the Upf1p. Analogous to the Rep helicase, both ATP binding and hydrolysis appear to be major regulators of the helicase cycle. Further experiments are required, however, to determine whether the Upf1p functions as a monomer, a homodimer, or higher-order oligomer, or as part of a larger protein complex. The Upfp complex probably consists of at least Upf1p, Upf2p, and Upf3p (Weng et al., 1996b; He et al., 1997), and may consist of other proteins as well (He & Jacobson, 1995). The observation, however, that purified Upf1p functions independently as an RNA and DNA helicase indicates that a basal helicase activity resides in the Upf1p (Czaplinski et al., 1995). Using the Rep protein as a model for other superfamily group I helicases, it is predicted that the Upf1p may be able to interact to form a dimer (or larger complex) that is used to translocate and melt RNA duplexes.

The potential for Upf1p to complex with itself is supported by previous results (Weng et al., 1996b). Mutations in the cysteine and histidine-rich region of the *UPF1* gene were functional in promoting decay of nonsense-containing transcripts, but allowed suppression of the *leu2-2* and *tyr7-1* nonsense alleles. The characterization of the biochemical properties of the mutant proteins demonstrated wild-type RNA helicase, ATPase activities, and single-stranded RNA binding. However, the Upf1p–RNA complex formed using these mutant proteins migrated slower than the wild-type Upf1–RNA complex. Although the reason for the slower migration of the RNA–protein complex has not been elucidated, this result is consistent with these mutant forms of the Upf1 interacting with themselves. Taken together, these observations would be consistent with the active form of the Upf1p being able to assemble an oligomeric state.

Mutant forms of the Upf1p help define a mechanism for how the surveillance complex affects translation termination and nonsense-mediated mRNA decay

The results presented here place constraints on a model for how the NMD complex, or surveillance complex, enhances translation termination and promotes NMD. In particular, the following three points must be considered in developing a model on how these factors function in these processes. (1) Upf1p binds ATP in the absence of RNA. (2) An ATP–Upf1p complex has a reduced affinity for single-stranded RNA. (3) Although the ATPase and helicase activities are dispensable for modulating translation termination, the ability to bind ATP is important for the Upf1p to enhance translation termination at a nonsense codon. Based on the results presented here to demarcate certain constraints of how the NMD pathway can function, a model for how the Upf1p functions in these processes is depicted in Figure 6. We suggest that one of the initial key steps is that an ATP-bound Upf1p enhances termination at the premature stop codon. ATP is utilized as a cofactor in this step in order to enhance translation termination and prevents Upf1p from interacting with single-stranded RNA. This may be important so that the surveillance complex does not disassociate from the ribosome until translation termination has been completed. Once the termination event is completed, the ATPase/helicase activities of the Upf1p is activated and, in combination with other factors in the surveillance complex, monitors whether correct translation termination has occurred by searching 3′ of the termination codon for a sequence called a downstream element (DSE; Fig. 6; reviewed in Ruiz-Echevarria et al., 1996). If aberrant premature translation termination occurred, which is established by the surveillance complex interacting with the DSE, the RNA is rapidly degraded by the nonsense-mediated mRNA decay pathway.

The various mutant forms of the Upf1p lend support to this model and identify intermediates that affect translation termination. For example, the K436Q mutation in the *UPF1* gene reduces the ability of Upf1p to bind ATP (Table 1), and the ATPase and helicase activities are not functional (Fig. 1; Weng et al., 1996a). Consequently, this form of the Upf1p is not active in either translation termination or mRNA turnover. Conversely, both the DE572AA and TR800AA forms of the Upf1p can bind ATP as efficiently as the wild-type Upf1p protein (Table 1), and both of these proteins are active in enhancing translation termination at a nonsense codon (Fig. 1). Taken together, these results are consistent with the notion that ATP binding, independent of its

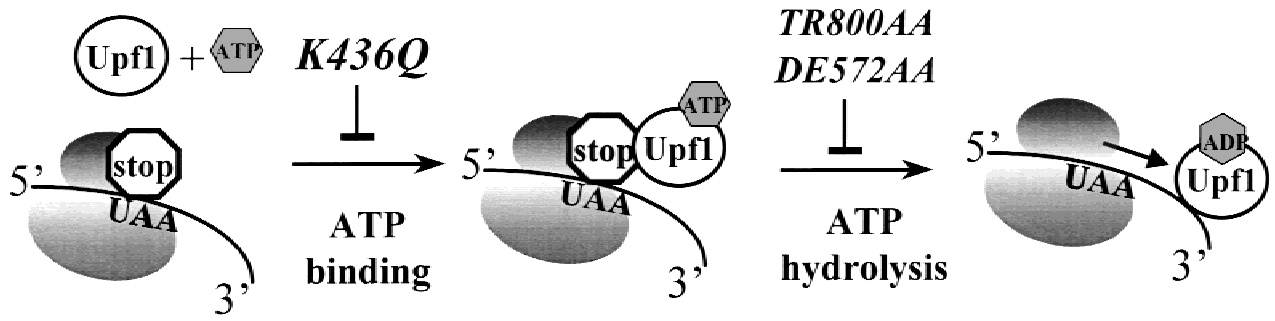

FIGURE 6. Model for formation of the Upf complex. UPF1 bound to ATP functions to enhance translation termination. Mutant protein K436Q binds ATP weakly and cannot form a functional termination complex. Subsequent to peptide hydrolysis, Upf1 hydrolyzes ATP and the Upf1p helicase activity is utilized to scan downstream to search for a downstream element. The DE572AA and TR800AA mutant proteins are unable to hydrolyze ATP, and thus cannot search for a downstream element.

hydrolysis, is required for the Upf1p to enhance the translation termination activity of the Upf1p. None of the mutant forms of the Upf1p described above, however, exhibit RNA helicase activity and none are functional in promoting nonsense-mediated mRNA decay. These results are consistent with the notion that the ATPase and helicase activities of the Upf1p are necessary for degrading aberrant mRNAs.

In summary, the results presented here indicate that ATP functions as a cofactor of the Upf1p that modulates its RNA binding activity. Clearly, this interaction has implications on how Upf1p functions in both translation and mRNA turnover. The goal of future experiments will be to elucidate the steps in the termination and decay pathway with the goal of understanding how Upf1p enhances translation termination and is then subsequently utilized to monitor whether translation termination has occurred appropriately.

MATERIALS AND METHODS

Materials

FLAG peptide, anti-FLAG M2 antibody, and anti-FLAG M2 affinity resin were obtained from IBI. Radioactive nucleotides were obtained from either NEN ([α-^{32}P]ATP, [α-^{32}P]GTP) or Amersham ([γ-35S]ATPγS). Unlabeled nucleotides were purchased from Boehringer Mannheim and SP6 RNA polymerase from BRL. ATPγS was obtained from Fluka.

Purification and nucleic acid binding assay of the Upf1p

Cytoplasmic extracts from yeast strain BJ5457 (*MATα, ura3-52*, *trp1*, *lys2-801*, *leu2-1*, *his3Δ200*, *pep4::HIS3*, *prbΔ1.6R*, *can1*, *GAL*) harboring YEp-FLAG-UPF1 alleles were prepared and the wild-type and mutant forms of the Upf1p were purified as described (Czaplinski et al., 1995; Weng et al., 1996a, 1996b). RNA binding assay of the wild-type and mutant forms of the Upf1p were determined using a gel shift assay as described previously (Lee & Hurwitz, 1992; Czaplinski et al., 1995; Weng et al., 1996a, 1996b). A uniformly labeled 78-nt RNA was made by in vitro transcription of an *Rsa* I-digested pGEM3 template with SP6 RNA polymerase using [α-^{32}P] GTP (3,000 Ci/mmol) as described previously (Czaplinski et el, 1995). Binding reactions (20 μL) containing 20 mM Hepes–KOH, pH 7.6, 50 mM KCl, 3 mM $MgCl_2$ and 2 mM DTT, 2 units of RNase inhibitor, 0.1 mg/mL bovine serum albumin, 50 fmol RNA, and 20 ng Upf1p were incubated for 20 min at room temperature. Where indicated, ATPγS, ADP, AMP-PNP, or LiCl were added to the indicated concentration. ATP was added to reaction after 15 min of incubation. The reactions were terminated by the addition of a 5$\times$ stop buffer containing 0.1 M Tris–HCl, pH 7.4, 50% glycerol, 20 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. The RNA–protein complexes formed were separated by electrophoresis in a 4.5% PAGE gel (60:1 in 0.5$\times$ TBE containing 5% glycerol) at 15 mA constant current for 2–3 h. Gels were dried and autoradiographed. For depletion of ATP from ATPγS preparation, 50 mM ATPγS, 12.5 mM glucose, 1.5 units hexokinase, 6 mM $MgCl_2$, and water were mixed together to a total volume of 40 μL and incubated on ice for 15 min. To determine kinetics of the dissociation of Upf1p–RNA complex, 50 fmol RNA substrate and 20 ng Upf1p were incubated under standard conditions for 30 min. Where indicated, ATP was added to a final concentration of 0.05 mM at 2, 4, 6, 8, 12, 16, 20, 25, or 30 min before the reactions were terminated. Upf1p–RNA complexes were visualized as described above.

ATP binding assay

The ATP binding assay was performed in a reaction mixture containing 20 ng of wild-type or mutant Upf1p in 30 mM Tris-HCl, pH 7.5, 5 mM magnesium acetate, 10% glycerol, and 1.5 mM DTT (buffer A) and varying amounts of [α-^{32}P]ATP (Pause & Sonenberg, 1992). After 5 min incubation at 4 °C, the reaction mixtures were loaded onto a nitrocellulose membrane filter in a vacuum manifold. The membrane was washed five times with 1 mL of buffer A. Bound ATP was quantitated in a scintillation counter. The background was obtained from reactions lacking protein. Binding constants were determined using Prism version 2.0 (Graph Pad, San Diego, California).

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