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Purification and characterization of the Upf1 protein: A factor involved in translation and mRNA degradation

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ABSTRACT

mRNA degradation is an important control point in the regulation of gene expression and has been shown to be linked to the process of translation. One clear example of this linkage is the observation that nonsense mutations in a gene can accelerate the decay of the corresponding mRNA. In the yeast *Saccharomyces cerevisiae*, the product of the *UPF1* gene, harboring zinc finger, NTP hydrolysis, and helicase motifs, was shown to be a *trans*-acting factor in this decay pathway. A *UPF1* gene disruption results in stabilization of nonsense-containing mRNAs and leads to a nonsense suppression phenotype. As a first step toward understanding the molecular and biochemical mechanism of nonsense-mediated mRNA decay, we have purified Upf1p from a yeast extract and characterized its nucleic acid-dependent NTPase activity, helicase activity, and nucleic acid binding properties. The results presented in this paper demonstrate that Upf1p contains both RNA- and DNA-dependent ATPase activities and RNA and DNA helicase activities. In the absence of ATP, Upf1p binds to single-stranded RNA or DNA, whereas hydrolysis of ATP facilitates its release from single-stranded nucleic acid. Based on these results, the role of Upf1p's biochemical activities in mRNA decay and translation are discussed.

Keywords: ATPase; helicase; nonsense-mediated mRNA degradation; Upf1 protein

INTRODUCTION

Many investigations have demonstrated that mRNA turnover plays a vital role in regulating gene expression (reviewed in Peltz et al., 1991, 1994; Peltz & Jacobson, 1993; Ross, 1995). Studies on the mechanisms of mRNA turnover have identified *cis*-acting sequences and *trans*-acting factors that are involved in controlling mRNA decay (Peltz & Jacobson, 1993; Decker & Parker, 1994; Peltz et al., 1994). In addition, it is now well established that the processes of mRNA turnover and translation are intimately linked, and that understanding this connection is critical for elucidating the mechanisms of mRNA decay (reviewed in Peltz et al., 1991, 1994; Peltz & Jacobson, 1993; Ross, 1995). One clear example of this relationship is the observation that premature termination of translation by a nonsense mutation can dramatically destabilize the transcript produced from that aberrant gene (reviewed in Peltz et al., 1994). Reduced mRNA levels or decreased stabilities of nonsense-containing transcripts have been observed in prokaryotes as well as eukaryotes (reviewed in Peltz et al., 1991, 1994).

Our laboratory has focused on the nonsense-mediated mRNA decay pathway in the yeast *Saccharomyces cerevisiae* in order to further understand the link between translation and decay as well as to gain insights into the mechanisms of mRNA turnover. Several *trans*-acting factors involved in nonsense-mediated mRNA decay have been identified. A genetic screen for translational frameshift suppressors led to the identification of a class of mutants called *upf* (for *up* frameshift; Culbertson et al., 1980; Leeds et al., 1992). Subsequent analysis of these alleles demonstrated that mutations in *UPF1* (*MOF4*), *UPF2* (*SUA1*), and *UPF3* (*SUA6*) genes result in an increased accumulation of nonsense-containing mRNAs while having no effect on the abundance of most wild-type transcripts (reviewed in Peltz et al., 1994; He et al., 1993; Dinman & Wickner, 1994; Peltz et al., 1993a). The increased concentration of these aberrant transcripts was a direct result of the greater stability of these RNAs (see Peltz et al., 1994). Both the *UPF1* and *UPF2* genes have been cloned and their gene products have been demonstrated to interact geneti-

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cally (Leeds et al., 1992; Cui et al., 1995; He & Jacobson, 1995). The cloning of the *UPF3* gene has not been described. In addition to stabilizing aberrant transcripts, mutations in the *UPF1*, *UPF2*, and *UPF3* genes cause suppression of certain nonsense and frameshift alleles (Leeds et al., 1992; Cui et al., 1995). The Upf1p has been shown to co-sediment with polysomes in sucrose gradients (Peltz et al., 1993b; Atkins et al., 1995).

The *UPF1* gene encodes a peptide with a predicted molecular weight of 109 kDa and a cysteine-rich region harboring multiple zinc finger domains as well as the seven conserved motifs common to the members of helicase superfamily I (Altamura et al., 1992; Koonin, 1992). Several genes from this family of helicases have been identified from both *Escherichia coli* and *S. cerevisiae* and their protein products have been purified and demonstrated to contain both NTPase and helicase activities (Abdel-Monem et al., 1976, 1977; Scott et al., 1977; Taylor & Smith, 1980; Wood & Matson, 1987; Roman & Kowalczykowski, 1989; Lahaye et al., 1991; Rong & Klein, 1993; Tsaneva et al., 1993). In addition, based on sequence comparisons, numerous other gene products encoded within genomes of several RNA viruses as well as genes from mouse cells have been identified as members of this family, although the helicase activity of these proteins has not been reported (Gorbalenya et al., 1988). The *UPF1* gene is homologous to the *SEN1* gene in the yeast *S. cerevisiae* and the *Mov-10* gene in mouse cells, both of which are members of the helicase superfamily group I (Altamura et al., 1992; Koonin, 1992; Leeds et al., 1992).

As a first step toward understanding the molecular and biochemical mechanism of nonsense-mediated mRNA decay, we have purified Upf1p from yeast extracts and characterized its nucleic acid-dependent NTPase activity, helicase activity, and nucleic acid-binding properties. The results presented in this paper demonstrate that Upf1p contains both RNA- and DNA-dependent NTPase activity, as well as RNA and DNA helicase activity and, in the absence of ATP, binds to single-stranded RNA or DNA, whereas hydrolysis of ATP facilitates its release. Based on these results, the role of Upf1p's biochemical activities in mRNA decay and translation are discussed.

RESULTS

Purification of the Upf1p

The *FLAG-UPF1* allele, which harbors an epitope tag at the amino terminus of its protein coding region (Peltz et al., 1993b) was utilized in order to detect and purify Upf1p from yeast cells. Cells harboring the *FLAG-UPF1* allele had the same mRNA turnover and nonsense suppression phenotypes as cells harboring the wild-type *UPF1* gene (Peltz et al., 1993b; Cui et al., 1995). In order to obtain higher levels of Upf1p expression, the protein coding region of the *FLAG-UPF1* allele was subcloned into a 2-μ plasmid downstream of the strong constitutive glyceraldehyde-3-phosphate-dehydrogenase promoter (G3PD). This plasmid was transformed into a yeast strain (BJ5457) that is deficient for processing of several cellular proteases (*pep4*; Jones, 1991).

The Upf1p purification protocol is shown in Figure 1A and an SDS-PAGE gel of the purification is shown in Figure 1B. Cytoplasmic extracts were prepared, applied to a DEAE Sephacel column, and protein fractions were subsequently eluted with a salt gradient of 150–700 mM KCl. The fractions containing Upf1p eluted at 250 mM KCl as detected by immunoblotting (data not shown). These fractions were diluted with an equal volume of DEAE Sephacel loading buffer lacking KCl

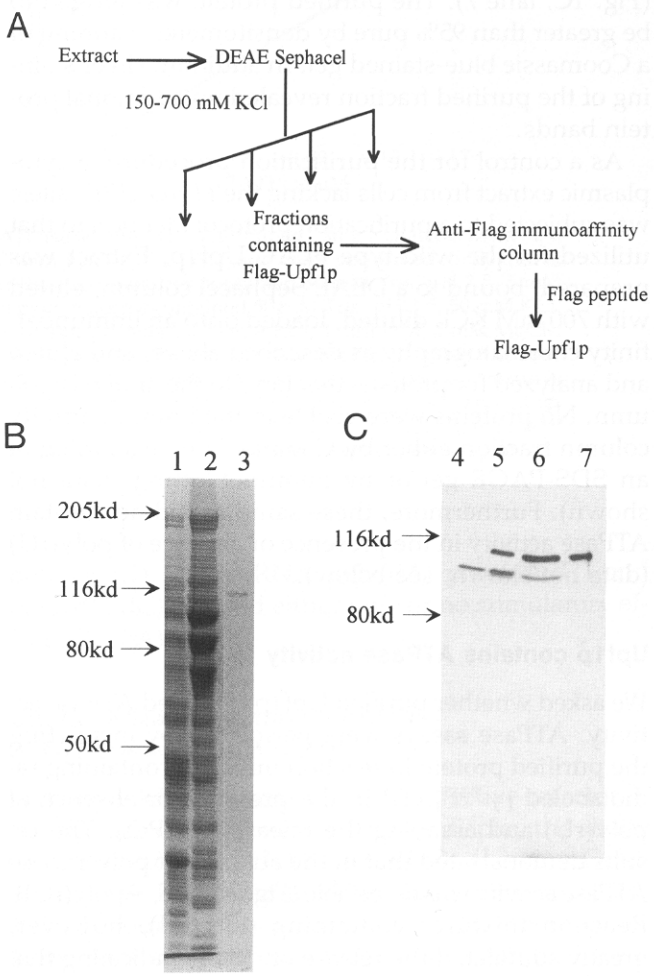

FIGURE 1. Purification of Upf1p. **A:** Purification scheme. A cytoplasmic extract was prepared from cells harboring the *FLAG-UPF1* allele and loaded onto a DEAE Sephacel column and eluted with a salt gradient. Fractions containing Upf1p were purified on a FLAG monoclonal antibody column. Fractions from the column were analyzed by SDS-PAGE and either (**B**) stained with Coomassie blue or (**C**) immunoblotted using the same antibody in the immunoaffinity column. Lanes 1 and 5, extract from BJ5457 cells transformed with pG-1FLAG-UPF1 (50 μg); lanes 2 and 6, DEAE Sephacel fraction (20 μg); lanes 3 and 7, immunoaffinity chromatography fraction (250 ng); lane 4, extract from BJ5457 transformed with pG-1 vector.

and were subsequently applied to an anti-FLAG immunoaffinity chromatography column. The column was washed three times with 15 column volumes of column buffer containing 150, 700, and 150 mM KCl, respectively. The Upf1p was subsequently eluted from the antibody column by inclusion of the FLAG peptide in the column buffer. As shown in the stained SDS-PAGE gel (Fig. 1B), fractions eluted from the antibody column contained only one band with an apparent molecular weight of approximately 110 kDa (Fig. 1B, lane 3). Furthermore, the purified protein was able to react with the anti-flag antibody as detected by immunoblotting (Fig. 1C, lane 7), consistent with the purified protein being encoded by the *FLAG-UPF1* gene. An endogenous protein of about 95 kDa cross-reacted with the FLAG antibody on a western blot (Fig. 1C, lane 4) and was not detected in the purified Upf1p fraction (Fig. 1C, lane 7). The purified protein was judged to be greater than 95% pure by densitometer scanning of a Coomassie blue-stained gel. In addition, silver staining of the purified fraction revealed no additional protein bands.

As a control for the purification procedure, a cytoplasmic extract from cells lacking the *FLAG-UPF1* allele was subjected to a purification protocol identical to that utilized for the wild-type FLAG-Upf1p. Extract was prepared, bound to a DEAE Sephacel column, eluted with 700 mM KCl, diluted, loaded onto an immunoaffinity chromatography as described above, and eluted and analyzed for proteins that bind to the antibody column. No proteins were visible in the immunoaffinity column fraction either by Coomassie blue staining of an SDS-PAGE gel or by immunoblotting (data not shown). Furthermore, these samples did not contain ATPase activity in the presence or absence of poly(rU) (data not shown, see below).

Upf1p contains ATPase activity

We asked whether purified Upf1p exhibited ATPase activity. ATPase assays were performed by incubating the purified protein in reaction mixtures containing radiolabeled [γ-^{32}P]ATP in the presence or absence of poly(rU) and assaying the release of $^{32}PO_4$. The results demonstrated that in the absence of poly(rU) no ATPase activity was detectable (Fig. 2A,B,C, −poly(rU)). Reaction mixtures containing poly(rU), however, greatly stimulated the release of $^{32}PO_4$, indicating that Upf1p harbored a nucleic acid-dependent ATPase activity (Fig. 2A,B,C, +poly(rU)). Concentrations of poly(rU) at or above 330 nM maximally stimulated the ATPase activity of the Upf1p (data not shown).

A variant of Upf1p in which the highly conserved lysine in the A-box (GxxGxGKT) of the NTP binding and hydrolysis motif was changed to a glutamic acid (K436Q) (Y. Weng, K. Czaplinski, & S.W. Peltz, data not shown) was purified by the protocol outlined in Figure 1A. This mutation inactivates the nonsense-mediated mRNA decay activity of the Upf1p and allows suppression of certain nonsense alleles (Y. Weng, K. Czaplinski, & S.W. Peltz, unpubl. results). The ATPase activity of the mutant protein in the presence or absence of poly(rU) was assayed as delineated above. Unlike wild-type Upf1p, the mutant protein did not contain any detectable RNA-dependent ATPase activity, even when reaction mixtures contained fivefold more of the mutant Upf1p (data not shown, see Fig. 7). This result confirms that the conserved lysine residue described above is essential for the Upf1p ATPase activity and demonstrates further that the purified protein is Upf1p.

Characterization of the Upf1p ATPase activity

The conditions for optimal ATPase activity were determined by varying the concentration of the components in the reaction mixture. The results shown in Figure 2A were from an experiment that varied the KCl concentration in the reaction mixture. The ATPase activity of Upf1p functioned best without the addition of KCl, although considerable ATPase activity was observed at concentrations between 0 and 150 mM KCl. The nucleic acid-dependent ATPase activity of Upf1p was inhibited by KCl at concentrations above 200 mM (Fig. 2A).

The $MgCl_2$ requirement for the Upf1p-associated ATPase activity was determined. The $MgCl_2$ concentration in the reaction mixtures was varied and the ATPase activity was monitored as described above. The results demonstrated that $MgCl_2$ enhanced the Upf1p ATPase activity (Fig. 2B). Optimal ATPase activity was observed when the reaction mixture contained 2 mM $MgCl_2$ (Fig. 2B). Increasing the concentration of $MgCl_2$ in the reaction mixture did not dramatically affect its ATPase function; the ATPase activity was constant between a range of 2 and 10 mM (Fig. 2B). Addition of 5 mM EDTA into the reaction mixture completely inhibited the nucleic acid-dependent ATPase activity (data not shown).

The pH optimum for the ATPase activity of the Upf1p was examined. The pH of the reaction mixture was varied from 5.5 to 11 and the ATPase activity was monitored. As shown in Figure 2C, the ATPase activity was active in a broad pH range between 5.5 and 9, with the highest activity observed at pH 8.0. Above pH 9.0, the ATPase activity decreases rapidly.

The K_m for the Upf1p ATPase activity was determined. Based on the experiments shown in Figure 2, the reaction mixture contained 50 mM KCl, 3 mM $MgCl_2$, 20 mM Tris, pH 8.0, 1 mM DTT, and 330 nM poly(rU). The ATPase hydrolysis rate for the Upf1p was determined at various ATP concentrations and the K_m of ATP binding was determined using a double reciprocal plot of the velocity of ATP hydrolysis versus

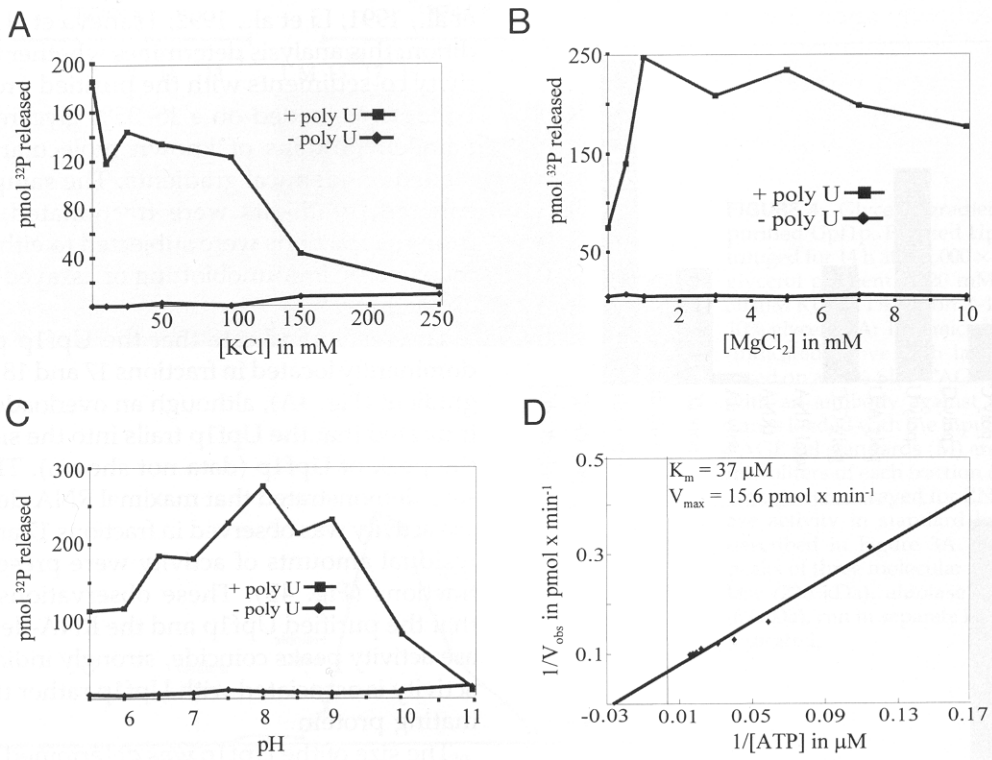

FIGURE 2. Optimization of the Upf1p ATPase activity. Upf1p ATPase activity was determined using a charcoal assay. Experiments in A, B, and C contained 3.5 ng Upf1p, 100 µM ATP with 1 µCi [γ-^{32}P] ATP and were performed for 20 min at 30 °C in the presence or absence of 330 nM poly(rU) RNA. **A:** Reaction mixtures contained 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, and KCl concentrations as indicated. **B:** Reaction mixtures contained 50 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, and $MgCl_2$ concentrations as indicated. **C:** Reaction mixtures contained 50 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, and 50 mM buffer as follows: MES, pH 5.5; MES, pH 6.0; MOPS, pH 6.5; MOPS, pH 7.0; Tris, pH 7.5; Tris, pH 8.0; Tris, pH 8.5; Tris, pH 9.0; glycine, pH 10.0; glycine, pH 11.0. **D:** Lineweaver–Burke plot of ATPase activity. Reaction mixtures contained 50 mM Tris, pH 8.0, 50 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 3.5 ng Upf1p, 330 nM poly(rU) RNA, and either 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM, 80 µM, or 90 µM ATP containing 1 µCi of [γ-^{32}P] ATP. Rate of ATP hydrolysis at each concentration was monitored and the K_m for ATP was determined by a Lineweaver–Burke plot.

the concentration of ATP. The K_m for ATP was demonstrated to be 37 µM (Fig. 2D).

Characterization of the nucleic acid specificity of the ATPase activity of Upf1p

The experiments described above demonstrated that poly(rU) greatly stimulated the Upf1p ATPase activity. We wanted to determine whether other nucleic acids could also stimulate this activity of Upf1p. The poly(rU) in the reaction mixture was replaced with either the ribohomopolymers poly(rA), poly(rC), poly(rG), poly(rA:rU), and poly(rG:rC), total RNA from yeast (which is predominantly rRNA), tRNA, or the deoxyribohomopolymers poly(dA), poly(dC), poly(dI), poly(dT), poly(dA:dT), and poly(dC:dG). The ATPase activity of Upf1p was determined as described before. The results indicate that poly(dT) and poly(rU) were the best at stimulating the Upf1p ATPase activity (Fig. 3A). Poly(rC), poly(rG:rC), poly(dC), and poly(dI) exhibited a moderate stimulatory effect on the Upf1p ATPase activity, whereas poly(rA), poly(rG), poly(rA:rU), rRNA, tRNA, poly(dA), poly(dA:dT), and poly(dG:dC) showed either little or no stimulatory effect (Fig. 3A).

Characterization of the specificity of NTPase activity of Upf1p

Although ATP is usually the most effective cofactor in the NTPase reaction many helicases display the ability to hydrolyze other rNTPs or dNTPs (Lowery-Goldhammer & Richardson, 1974; Abdel-Monem et al., 1976; Kolodner & Richardson, 1977; Kornberg et al., 1978; Arai & Kornberg, 1981; Liu & Alberts, 1981; Crute et al., 1988; Bruckner et al., 1991). Therefore, the specificity of the Upf1p NTPase activity was determined. Reaction mixtures were prepared containing the purified protein, poly(rU), and [α-^{32}P] labeled ribonucleotides or deoxyribonucleotides. The reactions were incubated at 30 °C for 60 min and the reaction products were monitored by thin layer chromatography. The results shown in Figure 3B demonstrate that Upf1p can efficiently hydrolyze ATP or dATP. The

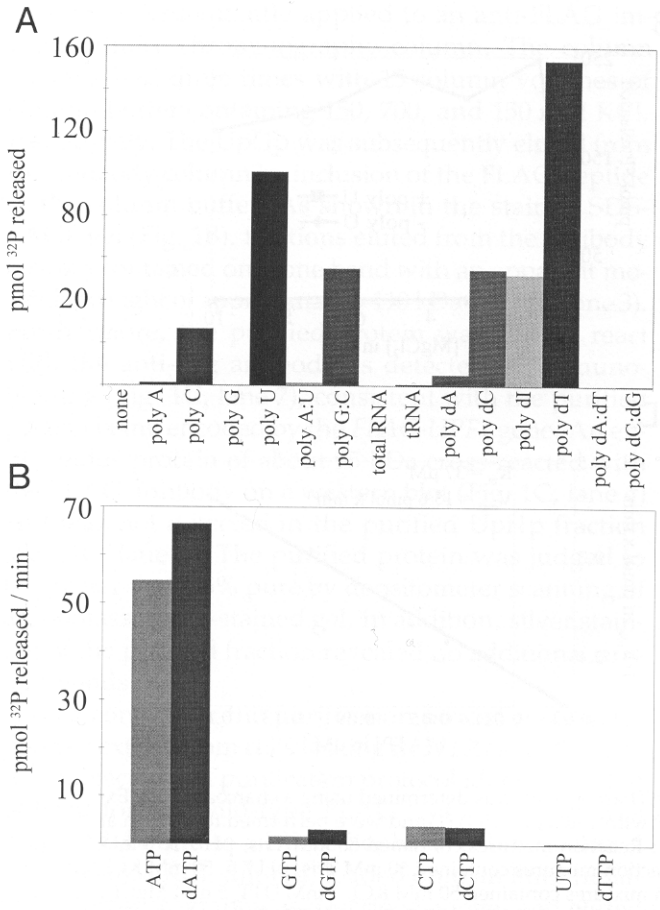

FIGURE 3. Characterization of the Upf1p NTPase activity. **A:** The ability of other polyribonucleotides and polydeoxyribonucleotides to stimulate the ATPase activity of Upf1p was monitored using a charcoal assay under standard buffer conditions (50 mM Tris, pH 8.0, 50 mM KCl, 3 mM $MgCl_2$, and 1 mM DTT). Each reaction contained 3.5 ng Upf1p and the concentration of all of the nucleotide homopolymers in the reaction was equivalent to 100 μM of individual free nucleotide. **B:** The ability of Upf1p to hydrolyze NTPs and dNTPs was tested using a TLC assay under standard buffer conditions with 3.5 ng Upf1p, 330 nM poly(rU), and 100 μM of the indicated NTP or dNTP with 1 μCi of the same [α-^{32}P] NTP or dNTP.

other ribonucleotides and deoxyribonucleotides were either hydrolyzed at a much lower rate or not at all (Fig. 3B). The Upf1p was able to hydrolyze CTP, dCTP, GTP, and dGTP, albeit 20-fold less efficiently than ATP, whereas UTP and dTTP were hydrolyzed minimally (Fig. 3B).

Characterization of Upf1p in free solution

Density gradient centrifugation analysis was undertaken in order to determine whether Upf1p forms a complex or is monomeric in solution. Many helicases function in multimeric states (Reha-Krantz & Hurwitz, 1978; Finger & Richardson, 1982; Dykstra & Kushner, 1984; Bernstein & Richardson, 1988; Mastrangelo et al., 1989; Rozen et al., 1990; Chao & Lohman, 1991; Seo et al., 1991; Li et al., 1992; Tsaneva et al., 1993). In addition, this analysis determines whether the ATPase activity co-sediments with the purified protein. Purified Upf1p was loaded on a 15–35% glycerol gradient. In parallel, proteins of known molecular weight were loaded on identical gradients. The samples were centrifuged, gradients were fractionated, and aliquots from the fractions were subjected to either SDS-PAGE followed by immunoblotting or assayed for their ATPase activity.

The results indicate that the Upf1p peak was predominantly located in fractions 17 and 18 of the glycerol gradient (Fig. 4A), although an overloaded protein blot indicated that the Upf1p trails into the side fractions of the peak of Upf1p (data not shown). The ATPase assays demonstrated that maximal RNA-dependent ATPase activity was observed in fractions 17 and 18, whereas residual amounts of activity were present in the side fractions (Fig. 4B). These observations demonstrate that the purified Upf1p and the RNA-dependent ATPase activity peaks coincide, strongly indicating that this activity is associated with Upf1p rather than a contaminating protein.

The size of the Upf1p was determined by comparing its location within the gradient to the location of the molecular weight markers (Fig. 4B). The peak fractions containing Upf1p lie in between the 67- and 158-kDa protein markers, with fractions 17 and 18 having a molecular weight of approximately 110 kDa. Thus, these results indicate that the Upf1p is monomeric in free solution (>50 mM KCl).

Characterization of the nucleic acid binding activity of Upf1p

The RNA- and DNA-dependent ATPase activity of the Upf1p suggests that it binds to nucleic acids. Previous results have demonstrated that some helicases can bind to DNA or RNA in the absence of ATP (Arai et al., 1981; Flores-Rozas & Hurwitz, 1992; Lee & Hurwitz, 1992), whereas others have displayed an ATP-dependent nucleic acid binding (Arai & Kornberg, 1981; Matson & Richardson, 1985). The RNA- and DNA-binding characteristics of Upf1p were investigated using a gel shift assay (Lee & Hurwitz, 1992). The substrates for the binding assays were either a uniformly radiolabeled in vitro-prepared 78-nt transcript or an end-labeled 47-nt DNA oligonucleotide. The ability of Upf1p to bind nucleic acid was determined by incubating 50 fmol of the DNA or RNA substrate with various amounts of Upf1p and assaying its binding activity by determining if the RNA or DNA substrate shifts in mobility in a nondenaturing gel.

The results demonstrate that in the absence of ATP, Upf1p was able to complex with either RNA or DNA (Fig. 5A,B). For the particular substrates assayed, Upf1p complexed more with DNA than with RNA

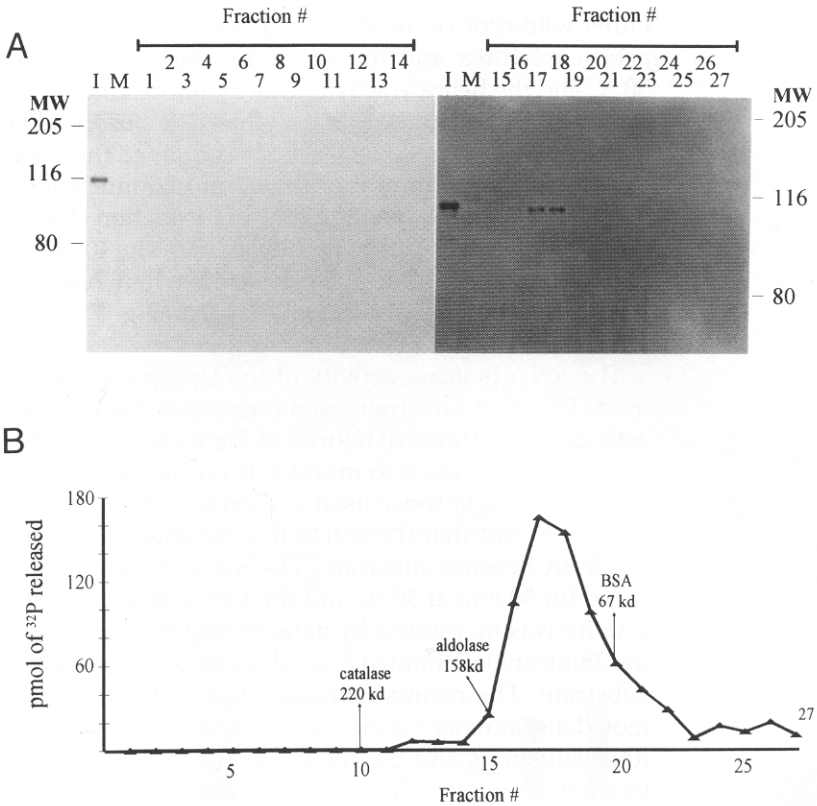

FIGURE 4. Glycerol gradient centrifugation of purified Upf1p. Purified Upf1p (3μg) was centrifuged for 14 h at 157,000 × *g* through a 15–35% glycerol gradient in 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.1% Triton X-100, 1 mM DTT, and 10% glycerol. **A:** Two microliters of each fraction (indicated above each lane) were electrophoresed on an 8% SDS-PAGE gel, immunoblotted with an antibody against the FLAG epitope. Lanes loaded with the input fraction (I) or SDS-PAGE gel standards (M) are indicated. **B:** Two microliters of each fraction (as indicated on the *x*-axis) were assayed for RNA-dependent ATPase activity in standard buffer conditions as described in Figure 3A. The positions of the peaks of three molecular weight markers, catalase (220 kDa), aldolase (158 kDa), and BSA (67 kDa), run in separate identical gradients, are indicated.

(Fig. 5A). Interestingly, at higher concentrations of Upf1p, the shifted complex migrates as two distinct bands (Fig. 5B). The two bands may reflect more than one complex molecule of Upf1p bound to the substrate. Consistent with this view, the faster migrating complex was only detected at the lower Upf1p concentrations (data not shown).

We next determined whether adding ATP to the binding mixtures altered complex formation of Upf1p with the nucleic acids. Binding mixtures were prepared as described above and then either ATP or a nonhydrolyzable analog of ATP (AppNp) was subsequently added to the mixture. Addition of ATP to the mixtures markedly reduced complex formation on both RNA and DNA, although it had a greater effect on the Upf1p:DNA complex stability (reduced to 5% of binding in the absence of ATP; Fig. 5B, compare lanes 2 and 3) than the Upf1p:RNA complex stability (reduced to 42% of binding in the absence of ATP, Fig. 5B, compare lanes 7 and 8). Although the ATP analog AppNp inhibits ATPase activity (data not shown), addition of AppNp to the binding reaction had no effect on complex formation, indicating that the hydrolysis of ATP is responsible for the decreased complex formation (Fig. 5B, lanes 4 and 9). A 25-fold excess of unlabeled substrate added to the mixture subsequent to the initial binding reaction did not affect complex formation, indicating that the Upf1p:nucleic acid complex formed was stable (Fig. 5B, lanes 5 and 10).

Characterization of the helicase activity of Upf1p

The ability of Upf1p to displace partially duplexed DNA or RNA was determined using a strand displacement assay (Venkatesan et al., 1982; Rozen et al., 1990). The reaction conditions used to assay helicase activity were identical to those described in Figure 2D. The DNA substrate for assaying helicase activity is illustrated at the bottom of Figure 6A. Two radiolabeled oligodeoxyribonucleotides (26 bp and 40 bp) were annealed to a large single-stranded template, forming a substrate containing double-stranded regions bordered by either 5′ or 3′ single-stranded regions. The directionality of Upf1p helicase activity is determined by monitoring which oligodeoxyribonucleotides are displaced. Reaction mixtures containing increasing concentrations of Upf1p were incubated with the substrate at 30 °C for 30 min and electrophoresed on a nondenaturing gel.

As shown in Figure 6A, the DNA helicase substrate migrated near the top of the gel (Fig. 6A, lane 1). Denaturing the substrate by boiling releases the two radioactive oligodeoxyribonucleotides that migrate faster and are near the bottom of the gel (Fig. 6A, lane 2). Addition of Upf1p to the reaction mixtures displaced the 40-nt oligodeoxyribonucleotide, but not the 26-nt fragment from the DNA substrate (Fig. 6A, lanes 3–8). The amount of the 40-nt oligonucleotide displaced increased concomitantly with the concentration of Upf1p in the reaction mixture (Fig. 6A, lanes 3–8). This pat-

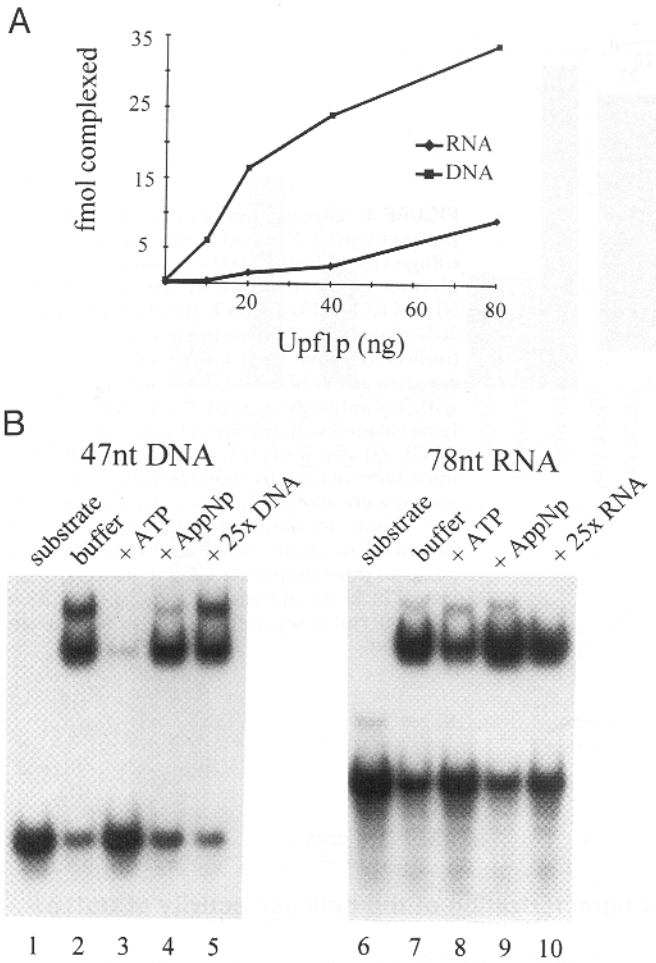

FIGURE 5. Nucleic acid binding of the Upf1p. A gel shift assay was used to determine the ability of the Upf1p to bind to either single-stranded DNA or RNA. **A:** Fifty femtomoles of either RNA or DNA binding substrate were incubated under standard buffer conditions (see Fig. 3A) with increasing amounts of Upf1p, and incubated for 15 min at room temperature. Reactions were then electrophoresed in a 4.5% PAGE gel (30:0.5 acrylamide:bisacrylamide, in 0.5× TBE containing 5% glycerol). Following electrophoresis, gels were dried and quantitated using a Bio-Rad model G-250 molecular imager. **B:** All reactions except lanes 1 and 6 contained 150 ng Upf1p and were carried out as in A, except that, after 15 min, 1 mM ATP (lanes 3 and 8), 1 mM AppNp (lanes 4 and 9), 25× unlabeled DNA (lane 5) or RNA (lane 10) or buffer (lanes 2 and 7) was added to the reactions. The mixtures were incubated for another 5 min and subsequently analyzed by gel electrophoresis. The gels were dried and autoradiographed.

tern of strand displacement is consistent with the Upf1p displaying a 5′ → 3′ helicase activity, because a duplex with a 5′ single-stranded region was unwound. No displacement of the 40-nt oligonucleotide was detected if ATP was omitted from the reaction mixture (Fig. 6A, lane 9), indicating that ATP is required for Upf1p helicase activity.

The DNA helicase activity of Upf1p purified from cells harboring the K436Q *upf1* allele was also monitored as described above and compared with the helicase activity of wild-type Upf1p. An equal amount of either wild-type or mutant Upf1p was added to the reaction mixtures and incubated for various times at 30 °C and the helicase activity was monitored on a nondenaturing gel. Analogous to what was observed in Figure 6A, when helicase reactions contained the wild-type Upf1p, the amount of the 40-nt oligonucleotide displaced increased over the time of incubation (Fig. 7, lanes 3–6). No displacement, however, of the 40-nt oligodeoxyribonucleotide was detected when reaction mixtures contained the K436Q Upf1p (Fig. 7, lanes 8–11).

The RNA helicase activity of the Upf1p was examined. The RNA substrate used for monitoring helicase activity is illustrated at bottom of Figure 8A. The reaction conditions used to monitor RNA helicase assays were identical to those used to monitor DNA helicase activity except that the reactions contained 50 fmol of the RNA helicase substrate. The reactions were incubated for 30 min at 30 °C and the Upf1p RNA helicase activity was monitored by determining the amount of the 28-nt single-stranded RNA displaced from the RNA substrate. The results demonstrated that Upf1p promoted displacement of the radiolabeled double-stranded RNA substrate, and the amount of the 28-nt oligoribonucleotide displaced increased concomitantly with greater Upf1p concentrations (Fig. 8). The RNA helicase activity was dependent on ATP; reaction mixtures containing a high concentration of Upf1p but no ATP lacked RNA helicase activity (Fig. 8, −ATP). Furthermore, no RNA helicase activity was observed when an RNA substrate containing 3′ single-stranded ends was used in the reaction mixture (data not shown). Taken together, these results indicate that Upf1p possesses a 5′ → 3′ RNA helicase activity.

DISCUSSION

Because the Upf1p plays an important role in both nonsense-mediated mRNA decay and in translation, we decided to investigate the biochemical activities of this protein in greater detail. The studies presented here have focused on the purification and initial characterization of the biochemical properties of Upf1p. These results will be discussed below in relation to how the Upf1p functions in nonsense-mediated mRNA decay and in translation.

Purification and characterization of the nucleotide hydrolysis activity of the Upf1p

An epitope tagged Upf1p was purified from yeast cells using a two-step procedure that utilized ion exchange and immunoaffinity chromatography (Fig. 1). The Upf1p was demonstrated to contain a nucleic acid-dependent ATPase activity with a K_m for ATP of 37 μM, indicating that it binds ATP quite well compared to other helicases in this superfamily (Abdel-Monem et al., 1976, 1977;

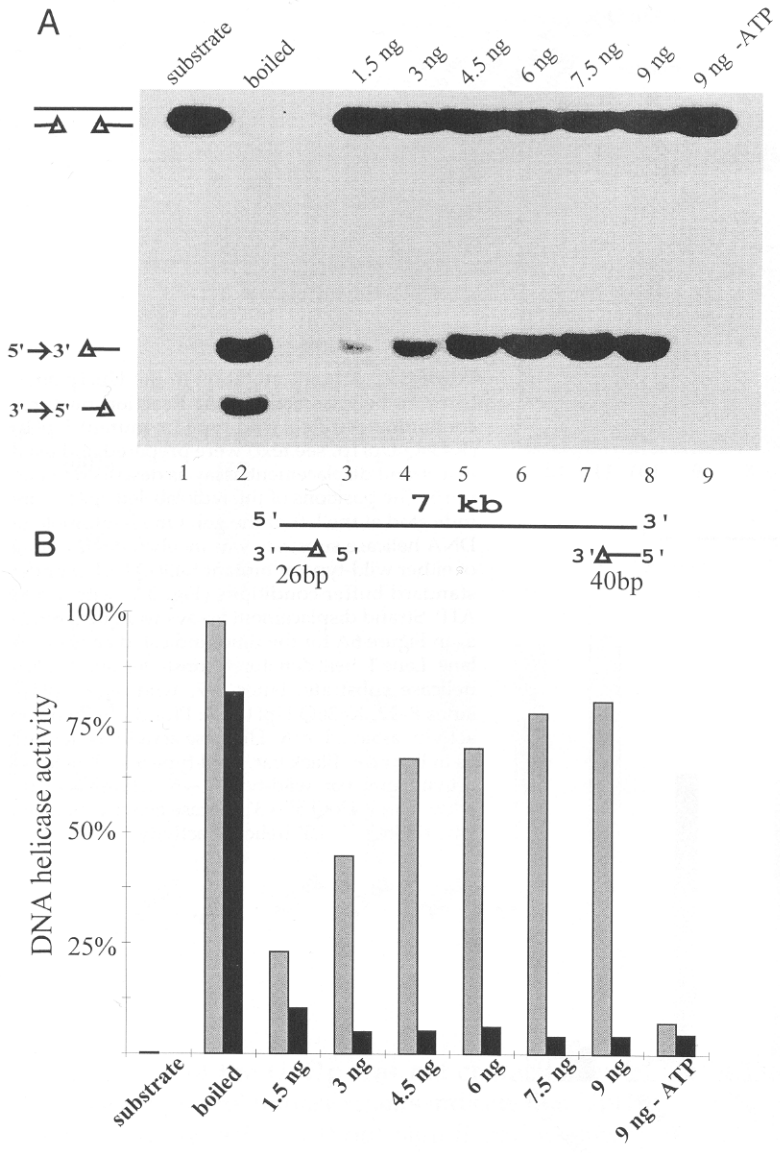

FIGURE 6. Upf1p is a 5′ → 3′ DNA helicase. **A:** A strand displacement assay was used to determine if Upf1p has DNA helicase activity. The substrate used is illustrated at the bottom of A. Displacement of the labeled 40-nt oligonucleotide indicates 5′ → 3′ DNA helicase activity, whereas displacement of the labeled 26-nt oligonucleotide indicates 3′ → 5′ DNA helicase activity. The positions of the radiolabeled species are indicated at the left of the gel. One femtomole of helicase substrate was incubated under standard buffer conditions (see Fig. 3A) with 1 mM ATP and increasing amounts of Upf1p as indicated above each lane. After 30 min at 30 °C, reactions were stopped by addition of helicase stop buffer and the samples were electrophoresed for 3 h in a 17% PAGE gel (30:1 in 1× TBE). The dried gel was autoradiographed and quantitated in a Bio-Rad model GS-670 imaging densitometer. Lane 1, helicase substrate; lane 2, heat-denatured helicase substrate; lane 3–8, reactions with the indicated amount of Upf1p; lane 9, reaction with indicated amount of Upf1p lacking ATP. **B:** Plot of the helicase activity assayed in A. DNA helicase activity is defined as the fraction of radioactivity in the displaced oligo relative to the total amount of radioactivity, with activity in the heat denatured lanes taken to be 100%. Gray bar, 5′ → 3′ helicase activity; black bar, 3′ → 5′ helicase activity.

Richet & Kohiyama, 1976; Kornberg et al., 1978; Lahaye et al., 1993; Rong & Klein, 1993). The ATPase activity of Upf1p was stimulated by either RNA or DNA (Fig. 3A). The nucleotides ATP or dATP were hydrolyzed by Upf1p at a much greater rate than any of the other NTPs or dNTPs, albeit Upf1p was able to hydrolyze CTP, dCTP, GTP, or dGTP at lower levels (Fig. 3B). The concentrations of the components in the reaction mixture for optimal ATPase activity were characterized. The results demonstrated that the ATPase activity of Upf1p was inhibited by high concentrations of KCl (>200 mM, Fig. 2A) and was stimulated by magnesium (Fig. 2B). The ATPase activity of Upf1p functioned in a broad pH range (Fig. 2C).

Two lines of evidence attribute the observed ATPase activity to the Upf1p rather than to minor contaminants. Neither helicase nor ATPase activity was observed in reaction mixtures containing a mutant Upf1p purified from cells harboring a *upf1* allele in which the highly conserved lysine in the A-box (GxxGxGKT) of the NTP binding and hydrolysis motif was changed to a glutamic acid (K436Q; Fig. 7, and data not shown). Furthermore, glycerol gradient analysis demonstrated that fractions containing Upf1p correlated with the ATPase activity in the gradient (Fig. 4). Taken together, these results strongly suggest that the purified Upf1p harbors an RNA- or DNA-dependent ATPase activity.

Upf1p contains RNA and DNA helicase activity

Based on the homology of the *UPF1* gene with other members of the superfamily group I helicases, we determined whether the Upf1p was able to unwind partially duplexed RNA and DNA substrates. The only duplexes displaced by Upf1p contained a 5′ single-stranded extension. When the duplex contained a blunt end or 3′ single-stranded extension, no displacement was observed. These results argue that the heli-

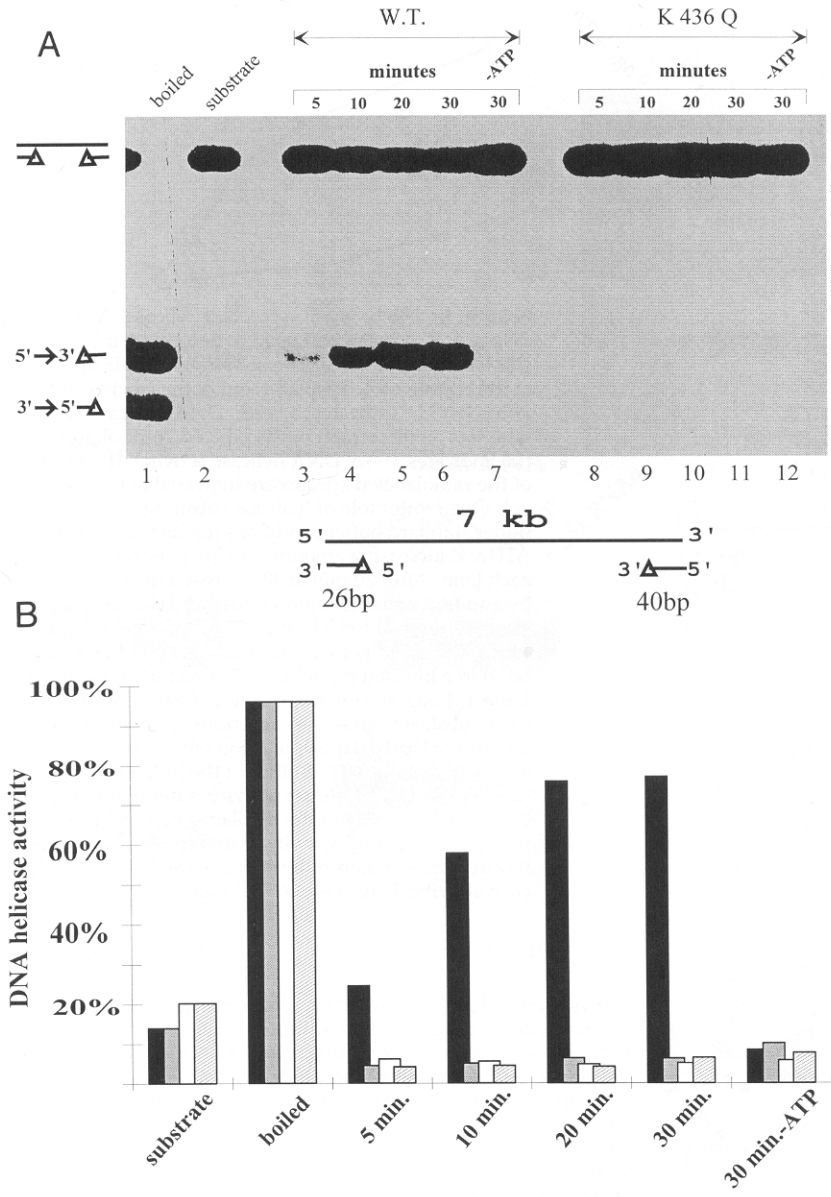

FIGURE 7. A point mutation in the Upf1p abolishes its helicase activity. **A:** Reaction mixtures containing purified wild-type or mutant Upf1p (K436Q Upf1p, see text) were prepared and used in a strand displacement assay as described in Figure 6. The positions of the radiolabeled species are indicated at the left of the gel. One femtomole of DNA helicase substrate was incubated with 14 ng of either wild-type or mutant K436Q Upf1p under standard buffer conditions (Fig. 3A) with 1 mM ATP. Strand displacement assays were performed as in Figure 6A for the times indicated above each lane. Lane 1, heat denatured substrate; lane 2, DNA helicase substrate; lanes 3–7, wild-type Upf1p; lanes 8–12, K436Q Upf1p. **B:** Plot of the helicase activity assayed in A. Helicase activity is defined as in Figure 6. Black bar, wild-type 5′→3′ helicase activity; gray bar, wild-type 3′→5′ helicase activity; white bar, K436Q 5′→3′ helicase activity; hatched bar, K436Q 3′→5′ helicase activity.

case activity begins on a 5′ single-stranded extension and proceeds in a 5′→3′ direction. The displacement of RNA and DNA duplexes is ATP dependent. The directionality of the helicase activities was the same for both RNA and DNA substrates, consistent with these activities being associated with the same protein.

Most helicases characterized previously have particular substrate specificities, being active on either DNA or RNA substrates. DNA helicases such as the Rep protein, Helicase III, and Helicase IV (HelD) only unwind duplexed DNA substrates and do not have RNA helicase activity (Matson & Kaiser-Rogers, 1990). Two other helicases have been reported to also have DNA and RNA unwinding activity. The Large T Antigen of SV40 utilizes ATP preferentially for its DNA helicase activity, whereas UTP is the most effective cofactor for its RNA helicase activity (Scheffner et al., 1989), and nuclear DNA helicase II purified from calf thymus nuclei can disrupt RNA duplexes with little nucleotide cofactor specificity (Zhang & Grosse, 1994). Many helicases can unwind an RNA:DNA duplex provided that the substrate has the appropriate 5′ or 3′ single-stranded tail (Brennan et al., 1987; Scheffner et al., 1989; Matson & Kaiser-Rogers, 1990; Lee & Hurwitz, 1992; Flores-Rozas & Hurwitz, 1993). Although the helicase activity of the Upf1p was not tested using a duplexed RNA:DNA substrate, given that Upf1p can unwind either RNA or DNA duplexes, it seems likely that it would unwind an RNA:DNA substrate.

Upf1p binds single-stranded RNA and DNA in the absence of ATP

The results demonstrated that, in the absence of ATP, Upf1p formed a stable complex with either single-stranded RNA or DNA (Fig. 5). The nucleic acid bind-

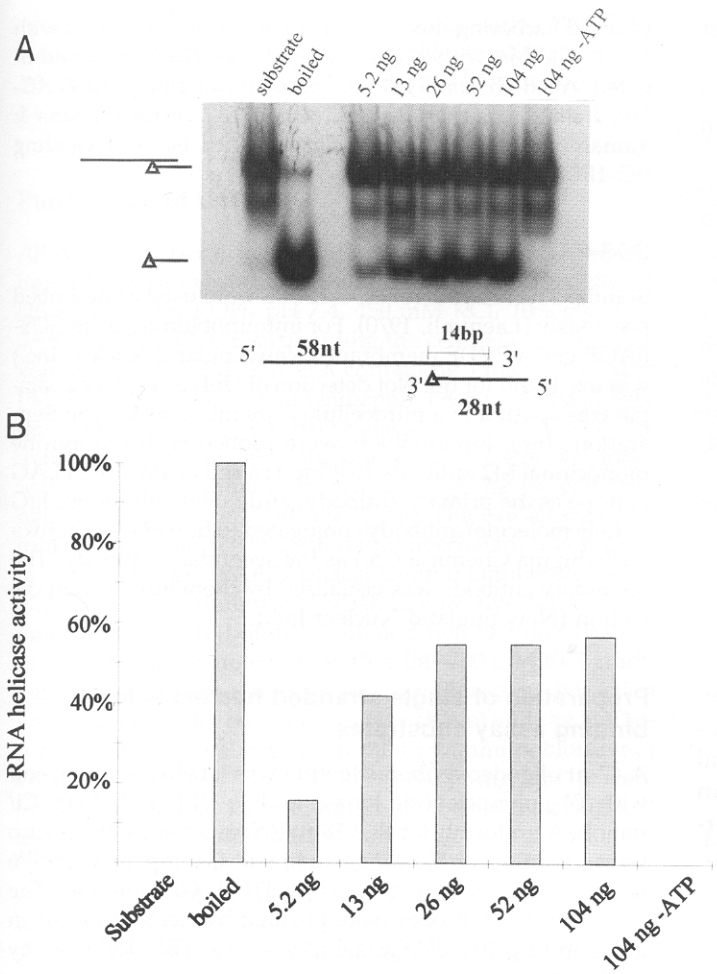

FIGURE 8. RNA helicase activity of Upf1p. **A:** Strand displacement assay was used to determine if Upf1p has RNA helicase activity. Fifty femtomoles of an RNA helicase substrate with 44-nt and 14-nt single-stranded regions 5′ of a 14-bp duplex were incubated with increasing amounts of Upf1p under standard buffer conditions with 1 mM ATP. After 30 min at 30 °C, reactions were stopped by the addition of helicase stop buffer and electrophoresed for 1.5 h in a 10% PAGE gel (30:1 in 1× TBE). The dried gel was autoradiographed and quantitated on a Bio-Rad model GS-670 imaging densitometer. **B:** Plot of the helicase activity assayed in A. RNA helicase activity was defined as the fraction of radioactivity in the displaced fragment relative to the total amount of radioactivity, with the activity in the denatured lane taken as 100%. Lane 1, RNA helicase substrate; lane 2, heat-denatured substrate; lanes 3–7, reaction containing the indicated amounts of Upf1p; lane 8, reaction with the indicated amount of Upf1p without ATP.

ing specificity of the Upf1p was not characterized in detail, although preliminary results indicate that Upf1p binds to nucleic acids without significant specificity (our unpubl. results). Addition of ATP to the binding mixture, however, destabilized the Upf1p:nucleic acid complex (Fig. 5B), having a greater effect on the Upf1p:DNA complex than on the Upf1p:RNA complex. In contrast, AppNp, a nonhydrolyzable analog of ATP that effectively inhibited the ATPase activity of Upf1p (data not shown), did not destabilize the Upf1p:RNA or Upf1p:DNA complex (Fig. 5B). This result suggests that binding of ATP to the Upf1p is not sufficient to destabilize the nucleic acid:Upf1p complex and that ATP hydrolysis is required to release the bound RNA or DNA.

The nature of the nucleic acid:Upf1p complex is not known at present. Three previously characterized RNA helicases, RNA Helicase A, p68, and vaccinia virus NPH-II protein (all DEAD/DExH family) also sediment as monomers (Hirling et al., 1989; Lee & Hurwitz, 1992; Shuman, 1992). However, many other helicases function as multimers. For example, the Rep protein of *E. coli* dimerizes upon DNA binding (Chao & Lohman, 1991). Both the SV40 Large T Antigen and the *E. coli* Rho factor function as a hexamers (Finger & Richardson, 1982; Mastrangelo et al., 1989). Other helicases such as RecBCD, RuvAB, and eIF-4A function in heteromultimeric complexes (Dykstra & Kushner, 1984; Rozen et al., 1990; Tsaneva, 1993). Although Upf1p is monomeric in solution, it is not known whether it oligomerizes in order to bind to RNA or DNA, or function as an ATPase or helicase.

Upf1p is a multifunctional protein that has functions modulating both mRNA turnover and translation

The Upf1p is a multifunctional protein that has separable activities involved in both modulating mRNA turnover and several aspects of translation (Leeds et al., 1991; Hagan et al., 1995; Y. Weng & S.W. Peltz, unpubl. results; Y. Cui, J. Dinman, & S.W. Peltz, unpubl. results). For example, it has been demonstrated that mutations in the *UPF1* gene will stabilize aberrant nonsense, frameshift, and intron-containing mRNAs and will promote suppression of certain nonsense-containing transcripts (Leeds et al., 1992; Cui et al., 1995). In a genetic analysis of the *UPF1* gene, two classes of *upf1* al-

leles were identified that separate its mRNA turnover function from its translation termination activity. Certain mutations in the helicase region of the *UPF1* gene inactivate its decay activity and, remarkably, maintain a functional translation termination activity.

The function of the Upf1p helicase activity in nonsense-mediated mRNA decay is not understood. In the current model for nonsense-mediated mRNA decay, the translating ribosome terminates at the premature stop codon and a 40S ribosomal subunit or factor scans 3′ to a "downstream element" with which the factors interact and promote decapping of the mRNA followed by rapid 5′ → 3′ degradation of the nonsense-containing mRNA (Muhlrad & Parker, 1994; Hagan et al., 1995). In this model, the helicase activity of Upf1p may be required to aid the ribosomal subunit or factors in scanning 5′ of the nonsense codon.

The biochemical analysis of Upf1p demonstrated that it has nucleic acid-dependent ATPase and helicase activities and it is an RNA- and DNA-binding protein. Nucleic acid-dependent ATPase and helicase activities participate in many aspects of DNA and RNA metabolism, including DNA replication, recombination, repair, transcription, splicing, and translation. Our goal is to understand how the activities of Upf1p function in modulating mRNA turnover and translation. In particular, it will be interesting to characterize biochemically the different classes of *upf1* alleles that separate the translation and mRNA turnover activities of the Upf1 protein and to relate these phenotypes to the biochemical properties of the Upf1p. The powerful combination of genetics and biochemistry should greatly facilitate our understanding of how the Upf1p functions in translation and mRNA turnover.

MATERIALS AND METHODS

Yeast strains, growth conditions, and transformation procedures

Cytoplasmic extracts from strain BJ5457 (*MATα, ura3-52, trp1, lys2-801, leu2-1, his3Δ200, pep4::HIS3, prb1Δ1.6R, can1, GAL*) harboring the *FLAG-UPF1* allele were prepared for purification of the Upf1p. Plasmid pG-1FLAG-UPF1 (described below) was transformed into BJ5457 cells by the lithium acetate method (Ito et al., 1983), as modified by Schiestl and Gietz (1989). Yeast media was prepared as described previously (Rose et al., 1990). Maintenance of pG-1FLAG-UPF1 was ensured by growth in minimal media lacking tryptophan. For the purification of Upf1p, 2 L of culture was grown at 30 °C until the OD_{600} reached 1.0–1.2. Cells were centrifuged at 2,500 × *g* for 10 min and stored at −70 °C.

Construction of Upf1p overexpression vector

Construction of the *FLAG-UPF1* allele has been described elsewhere (Peltz et al., 1993b). The translation start codon of the *FLAG-UPF1* allele is contained within an *Nco* I site. The plasmid harboring the *FLAG-UPF1* allele was linearized with *Nco* I, filled in with Klenow Polymerase (BRL Inc.) and ligated. A 3.6-kb *Bam*H I DNA fragment containing the *FLAG-UPF1* allele was then ligated to the pG-1 vector (Schena & Yamamoto, 1988) that was cleaved with *Bam*H I yielding pG-1FLAG-UPF1.

SDS-PAGE and protein blotting

Standard techniques for SDS-PAGE were used as described previously (Laemmli, 1970). For immunoblotting of an SDS-PAGE gel, a PVDF membrane (New England Nuclear Inc.) was used, and for dot blot detection of Upf1p, 10 μL of a sample was spotted to a nitrocellulose membrane (Micron Separations Inc). Immunoblots were probed with the murine monoclonal M2 antibody (IBI Inc.) raised against the FLAG epitope as the primary antibody, and rabbit anti-mouse IgG (whole molecule) antibody conjugated to horseradish peroxidase (Sigma Chemical Co.) as the secondary antibody. The secondary antibody was visualized by chemiluminescent detection (New England Nuclear Inc.).

Preparation of single-stranded nucleic acid binding assay substrates

A 47-nt oligodeoxyribonucleotide was labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P] ATP (3,000 Ci/mmol). 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). The DNA and RNA probes were purified by electrophoresis in a denaturing 10% PAGE gel (30:1 in 1× TBE) followed by electroelution.

Preparation of DNA helicase substrate

A 64-nt oligodeoxyribonucleotide complementary to the multiple cloning site of M13mp19 DNA was labeled at the 5′ end using T4 polynucleotide kinase (New England Biolabs Inc.) and [γ-^{32}P] ATP (3,000 Ci/mmol, Amersham). The labeled oligodeoxyribonucleotide was then annealed to single-stranded M13mp19 DNA in a ratio of 5:1 (oligodeoxyribonucleotide:template) by heating to 100 °C for 2 min, incubating at 60 °C for 5 min, followed by an incubation at room temperature for 2 h. The annealed substrate was labeled using Klenow polymerase, [α-^{32}P] dGTP (3,000 mCi/mmol, Amersham) and unlabeled dCTP, extracted with phenol:chloroform, and ethanol precipitated. DNA was resuspended in buffer, digested with *Sma* I, and purified by a Sepharose CL-6B spin column.

Preparation of RNA helicase substrate

A 58-nt unlabeled transcript was prepared by in vitro transcription with SP6 RNA polymerase from an *Eco*R I linearized pGEM3 plasmid. A 28-nt uniformly labeled transcript was prepared using T7 RNA polymerase in an in vitro transcription reaction containing pGEM3 plasmid linearized with *Sma* I and containing [α-^{32}P] GTP. The purified transcripts were combined and heated to 100 °C for 3 min and subsequently cooled to room temperature for 3 h. The annealed

products were electrophoresed in a 10% native PAGE gel (30:1 acrylamide:bisacrylamide in 1× TBE: 90 mM Tris, 90 mM boric acid, 10 mM EDTA) and the duplex RNA was purified by electroelution.

Purification of Upf1p

All steps were performed at 0–4 °C. A crude extract was prepared by washing a thawed aliquot of cells with buffer X (20 mM HEPES-KOH, pH 7.4, 150 mM KCl, 10% glycerol, 0.1% Triton X-100), resuspending the cell pellet in an equal volume of buffer XA (buffer X containing 1 mM DTT, 1 mM PMSF, and 1 μg/mL each of Leupeptin, Pestatin A, and Aprotinin) and lysing the cells using glass beads. The lysate was transferred to a corex tube, the beads were washed two times with an equal volume of buffer XA, and the wash supernatant was combined with the lysate. The extract was centrifuged for 1 h at 20,000 × *g* and the supernatant was applied to a 2.5 × 10 cm DEAE Sephacel column equilibrated with buffer XA. The column was washed with five column volumes of buffer XB (buffer X with addition of 1 mM PMSF). Bound proteins were eluted with a 150–700 mM KCl gradient in three column volumes. Ten microliters of each fraction was assayed by dot blot analysis (see above) and the Upf1p-containing fractions were confirmed by immunoblotting of an SDS-PAGE gel. The fractions containing Upf1p were pooled and diluted 1:2 with buffer XC (buffer X lacking KCl and containing 1 mM PMSF) and loaded onto a 1-mL anti-FLAG-M2 monoclonal antibody immuno-affinity chromatography column equilibrated with buffer XB. The flowthrough was collected and re-applied to the column. The column was washed three times with 5 mL of buffer XB, three times with 5 mL of buffer XD (buffer XB containing 700 mM KCl), and three times with 5 mL of buffer XB. Elution was performed in 10 × 1 mL aliquots of buffer XE (buffer XB containing 200 μg/mL of FLAG peptide). The eluted fractions were analyzed by immunoblotting and Coomassie blue staining of an SDS-PAGE gel. The purified Upf1p fractions were stored by dialyzing them against storage buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.1% Triton X-100, 1 mM DTT, and 50% glycerol) for 12 h with at least two changes of buffer, and fractions were stored at −20 °C. The concentration of purified Upf1p was determined from Coomassie blue-stained gels using a BioRad model GS-670 imaging densitometer with BSA as a protein standard.

Assay for NTPase activity

ATP hydrolysis was monitored using a charcoal assay (Clark et al., 1981). Reaction mixtures contained polynucleotides as indicated in the figure legends (Fig. 3), 3.5 ng of purified Upf1p, 100 μM ATP (except as indicated in Fig. 2D), and 1 μCi of [γ-^{32}P] ATP (3,000 Ci/mmol) in 20 μL total volume. Buffer conditions were as indicated in the figure legends. After incubation for 20 min at 30 °C, reactions were stopped and unreacted ATP was adsorbed by addition of 200 μL of 5% charcoal in 20 mM phosphoric acid. The charcoal was pelleted by centrifugation for 10 min at 13,200 × *g* and the $^{32}PO_4$ released was determined by counting a 100-μL aliquot of the supernatant in a scintillation counter. For each experiment, at least five controls without Upf1p were performed in order to determine the background. Values shown are the average of three separate experiments. The results of the experiments did not vary by more than 15%.

To measure NTP and dNTP hydrolysis, reactions were performed using standard reaction conditions (see Fig. 3B) and 100 μM of NTP or dNTP with 1 μCi of the appropriate [α-^{32}P] NTP or dNTP as indicated (Fig. 3B). After incubation at 30 °C for various time intervals, an aliquot (0.5 μL) was removed from the reactions and spotted on a PEI cellulose TLC plate (J.T. Baker), plates were developed with 0.75 M K_2PO_4, pH 3.5 (ATP, dATP, GTP, and dGTP), or 1 M LiCl (CTP, dCTP, UTP, and dTTP). Hydrolysis was quantitated using a Bio-Rad model G-250 molecular imager.

Glycerol gradient analysis of the Upf1p

All steps were performed at 4 °C. Three micrograms of purified Upf1p in gradient buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.1% Triton X-100, and 1 mM β-mercaptoethanol [β-ME]) containing 10% glycerol was loaded onto a 4.5-mL 15–35% glycerol gradient. Five-hundred micrograms of catalase, aldolase, or BSA in gradient buffer with 10% glycerol were loaded separately onto identical gradients as molecular weight markers. After centrifugation at 157,000 × *g* for 14 h, 200-μL fractions were collected and assayed for Upf1p by immunoblotting of an SDS-PAGE gel, and RNA-dependent ATPase activity.

Nucleic acid binding assay

The ability of Upf1p to bind nucleic acid was determined using a gel shift assay (Lee & Hurwitz, 1992). Binding reactions were performed in 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 3 mM $MgCl_2$, and 1 mM DTT in a volume of 20 μL with the amount of Upf1p as indicated in the legend of Figure 7. Fifty femtomoles of either labeled RNA or DNA (prepared as described above) was added and reactions were incubated for 15 min at room temperature and electrophoresed as described below. Alternately (Fig. 7B), after incubation for 15 min at room temperature, 1 mM ATP, 1 mM AppNp, 1.25 pmol of unlabeled RNA or DNA, or reaction buffer was added to the reactions and incubated for another 5 min at room temperature. Five microliters of gel loading buffer (50% glycerol, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) was added and the reactions were electrophoresed in a 4.5% PAGE gel (30:1 in 0.5× TBE containing 5% glycerol) at 15 mA constant current for 2–3 h. Gels were dried and the amount of RNA or DNA complexed was quantitated using a BioRad model GS-250 molecular imager.

Helicase assays

Helicase activity was examined using a strand displacement assay (Venkatesan et al., 1982; Rozen et al., 1990). Helicase assays were performed in 20 μL reactions with 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM ATP (except where otherwise indicated) with the amount of Upf1p and helicase substrate used in each experiment as indicated (see Figs. 6, 7, and 8). Helicase substrate was added to the reactions and the mixtures incubated at 30 °C for 30 min. Reactions were stopped by the addition of 5 μL stop buffer/gel loading buffer (50% glycerol, 0.5% SDS, 10 mM

EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). Samples from DNA helicase assays were electrophoresed in a 17% PAGE gel (30:1 in 1× TBE) at 15 mA constant current for 2–3 h. Samples from RNA helicase assays were electrophoresed in a 10% PAGE gel (30:1 in 1 × TBE) at 10 mA constant current for 2 h. The PAGE gels were dried and autoradiographed. Assays were analyzed using a Bio-Rad model GS-670 imaging densitometer.

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