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Characterization of the biochemical properties of the human Upf1 gene product that is involved in nonsense-mediated mRNA decay

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ABSTRACT

The Upf1 protein in yeast has been implicated in the modulation of efficient translation termination as well as in the accelerated turnover of mRNAs containing premature stop codons, a phenomenon called nonsense-mediated mRNA decay (NMD). A human homolog of the yeast *UPF1*, termed HUpf1/RENT1, has also been identified. The HUpf1 has also been shown to play a role in NMD in mammalian cells. Comparison of the yeast and human UPF1 proteins demonstrated that the amino terminal cysteine/histidine-rich region and the region comprising the domains that define this protein as a superfamily group I helicase have been conserved. The yeast Upf1p demonstrates RNA-dependent ATPase and 5′ → 3′ helicase activities. In this paper, we report the expression, purification, and characterization of the activities of the human Upf1 protein. We demonstrate that human Upf1 protein displays a nucleic-acid-dependent ATPase activity and a 5′ → 3′ helicase activity. Furthermore, human Upf1 is an RNA-binding protein whose RNA-binding activity is modulated by ATP. Taken together, these results indicate that the activities of the Upf1 protein are conserved across species, reflecting the conservation of function of this protein throughout evolution.

Keywords: ATPase; helicase; NMD; RNA binding; Upf1

INTRODUCTION

The nonsense-mediated mRNA decay (NMD) pathway is a cellular quality control mechanism that degrades aberrant mRNAs harboring premature termination codons (Losson & Lacroute, 1979; Gozalbo & Hohmann, 1990; Maquat, 1995; Caponigro & Parker, 1996; Jacobson & Peltz, 1996; Ruiz-Echevarria et al., 1996; Weng et al., 1997). This mRNA surveillance pathway functions in all eukaryotic systems examined and appears to have evolved to recognize premature termination events during the protein synthesis process (Pulak & Anderson, 1993; Weng et al., 1996a, 1996b, 1998; Czaplinski et al., 1998, 1999).

Several genes required for NMD have been identified in the yeast *Saccharomyces cerevisiae*. Mutations in the *UPF1*, *UPF2*, *UPF3*, *MOF2/SUI1*, *MOF5*, *MOF8*, and *HRPI* genes were shown to selectively stabilize mRNAs containing early nonsense mutations without affecting the decay rates of most wild-type mRNAs (Leeds et al., 1991, 1992; Cui et al., 1995, 1999; He & Jacobson, 1995; Lee & Culbertson, 1995; Gonzalez et al., 2000). Subsequently, Upf1p, Upf2p, and Upf3p were shown to form a complex (He & Jacobson, 1995; Weng et al., 1996b; He et al., 1997). In other studies using *Caenorhabditis elegans*, *Smg* genes were identified whose products were shown to be involved in NMD and a subset of those are homologous to the yeast *UPF* genes (Pulak & Anderson, 1993; Cali & Anderson, 1998).

The yeast *UPF1* gene and its protein product have been the most extensively investigated factor in the NMD pathway (Altamura et al., 1992; Koonin, 1992; Leeds et al., 1992; Atkin et al., 1995, 1997; Czaplinski et al., 1995, 1999; Cui et al., 1996; Weng et al., 1996a, 1996b, 1998). The Upf1p contains a cysteine- and histidine-rich region near its amino terminus and all the motifs of the superfamily group I helicases. The yeast

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Upf1p has been purified and demonstrated to have RNA-binding activity, RNA-dependent ATPase activity, and RNA helicase activity (Czaplinski et al., 1995, Weng et al., 1996a, 1996b). Disruption of the *UPF1* gene results in stabilization of nonsense-containing mRNAs and suppression of certain nonsense alleles (Leeds et al., 1991; Cui et al., 1995; Czaplinski et al., 1995; Weng et al., 1996a, 1996b). A set of mutations was isolated in the *UPF1* gene that separated its mRNA decay function from its activity in modulating translation termination at a nonsense codon (Weng et al., 1996a, 1996b). Consistent with the view that the Upf1p is involved in modulating translation termination, recent results have shown that it interacts with the translation termination release factors eRF1 and eRF3 (Czaplinski et al., 1998, 1999). These data suggest that the NMD and translation termination pathways are linked (reviewed in Czaplinski et al., 1998, 1999). Based on these observations, a "surveillance complex" consisting of at least Upf1p, Upf2p, Upf3p, and the release factors has been suggested to modulate translation termination and NMD (Czaplinski et al., 1998, 1999).

Homologs of the Upf1p have been identified in humans cells (the HUPF1/RENT gene; Perlick et al., 1996, Applequist et al., 1997) and in *C. elegans* (Page et al., 1999; Jacobson & Peltz, 1996; WORMPEP: Y48G8A 3304.a). It is evident that the Upf1p plays a conserved role in NMD (Leeds et al., 1991; Weng et al., 1996a, 1996b; Sun et al., 1998). The conserved domains of Upf1 include a cysteine- and histidine-rich region followed by a superfamily group I helicase domain. The extreme amino- and carboxyl-termini are divergent among all of the Upf1-like proteins. The conserved cysteine-rich region and helicase domain of the *UPF1* gene suggest an important function of these domains. Previous results have demonstrated that understanding the helicase activity of yeast Upf1p has provided powerful insight into the role of Upf1p in these processes (Weng et al., 1996a, 1996b; Czaplinski et al., 1998, 1999). The results presented here describe the purification and characterization of the helicase, ATPase, and RNA-binding activities of the human Upf1 protein (HUpf1 hereafter) with the long term goal of understanding the mechanism of NMD in mammalian systems.

RESULTS

Expression and purification of recombinant human Upf1

The human *UPF1* gene encodes a polypeptide with a predicted molecular weight of 130 kDa and shows a strong homology to the cysteine-rich region as well as the helicase superfamily I domain of the yeast *UPF1* gene (Perlick et al., 1996; Applequist et al., 1997). The yeast Upf1p demonstrates a nucleic-acid-dependent ATPase activity and $5' \rightarrow 3'$ helicase activity (Czaplinski et al., 1995). These biochemical activities of the Upf1p were utilized to examine the mechanism of Upf1 function in translation termination and mRNA turnover. The conservation of the helicase domain suggests that HUpf1 protein might have similar biochemical properties to the yeast Upf1p. Thus, the goal of the experiments described below is to perform a biochemical characterization of the HUpf1 protein.

The HUpf1 protein was purified by constructing a human *UPF1* allele harboring a Flag epitope at the amino terminal end of the human *UPF1* gene. This allele was inserted into a Baculovirus transfer vector and the protein was purified from insect cell extracts (High Five). A prominent band with the expected size of human Upf1 protein was present specifically in High Five extracts expressing HUpf1 protein, indicating the feasibility of the large scale purification (Fig. 1A, compare second and third lanes). The cell extracts were incubated with anti-FLAG immunoaffinity resin at 4 °C. The beads, recovered after centrifugation, were washed extensively and the HUpf1 was subsequently eluted by inclusion of the FLAG peptide in the appropriate buffer (see Materials and methods). Fractions eluted contained predominantly one band with a molecular weight of ~130 kDa (Fig. 1B) that reacted with the anti-flag antibody as detected by immunoblotting (Fig. 1C). The purified protein was aliquoted and stored in 10% glycerol-containing buffer at −70 °C with no apparent loss of activity over several months. A nonionic detergent (Triton X-100) was used at 0.01% to stabilize the protein during storage. We utilized this purified HUpf1 protein to characterize its biochemical properties.

HUpf1 demonstrates nucleic-acid-dependent ATPase activity

We asked whether purified HUpf1 protein exhibited ATPase activity. ATPase assays were performed by incubating the purified protein in reaction mixtures containing radiolabeled [γ-^{32}P]-ATP in the presence of nucleic acid (poly(rU) as in Fig. 2A,B) and assaying the release of $^{32}PO_4$ (see Materials and methods). The salt and pH conditions for optimal ATPase activity were determined by varying these components in the reaction mixture. The ATPase activity was monitored in reactions where the pH was varied from 4.0 to 10.0. The results demonstrated that the ATPase activity was most active from pH 4.0 to 6.0, with the maximum activity being observed at pH 5.0 (Fig. 2A). Above pH 6.0, the ATPase activity decreased (Fig. 2A). No salt dependence was demonstrated at pH 5.0 (data not shown). However, small differences in ATPase activities were observed at pH 7.0 when salt concentrations were varied. The ATPase activity of HUpf1 functioned without the addition of KOAc, and a slight increase in activity

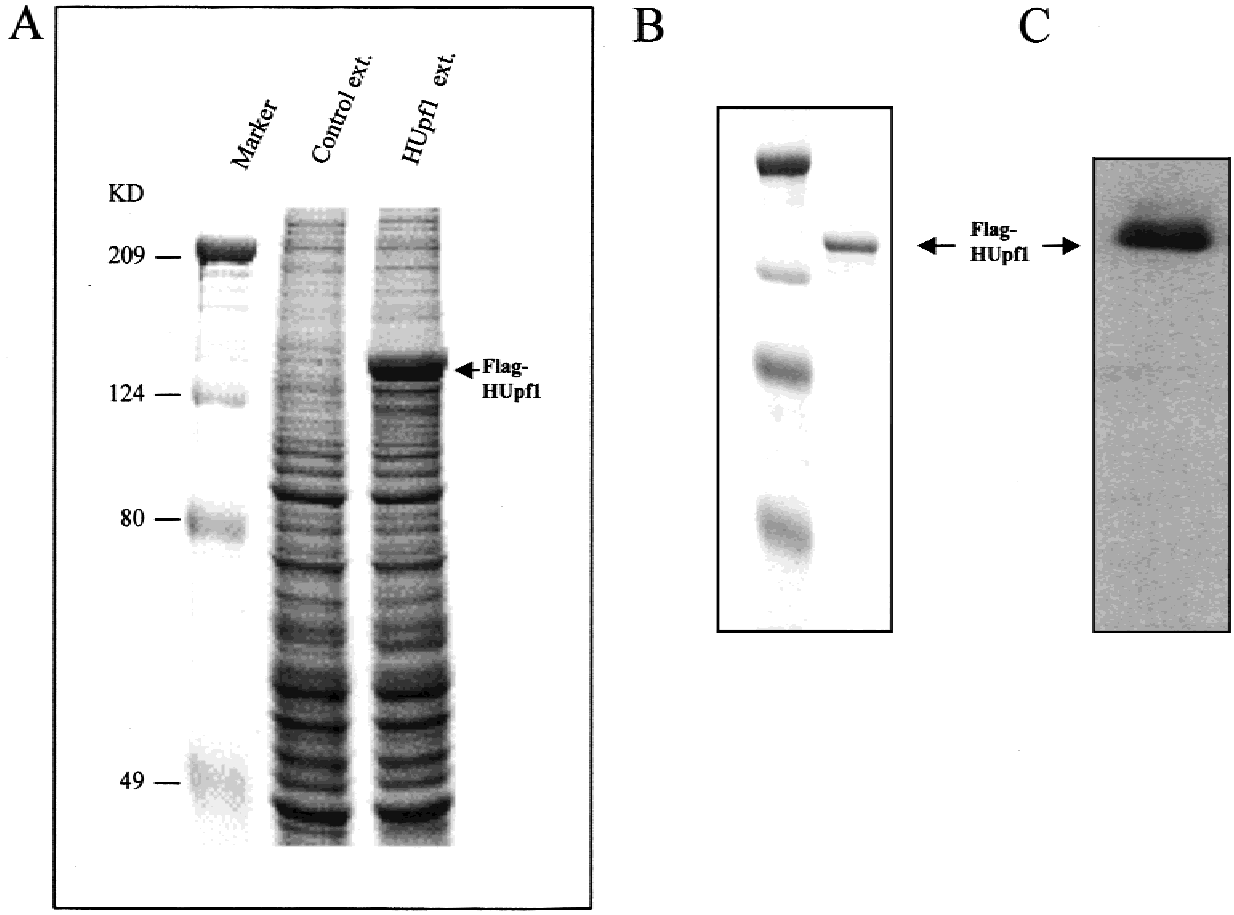

FIGURE 1. Expression and purification of HUpf1. **A**: Overexpression of recombinant HUpf1p in insect cell line as detected by Coomassie-stained SDS-PAGE. High Five cell extract from uninfected cells is shown in the second lane. The prominent band in the third lane represents the recombinant HUpf1 being expressed in High Five cells. Molecular weight markers are shown in the first lane. **B**,**C**: Purification scheme. Total extract was prepared from High Five cells harboring the *FLAG-UPF1* allele. The anti-FLAG M2 affinity resin was used to obtain the pure Hupf1 protein. The purified protein was checked on SDS-PAGE gel by Coomassie staining (**B**) and by western blot analysis (**C**). A monoclonal FLAG antisera was used as the primary antibody, and anti-mouse IgG antibody conjugated to horseradish peroxidase as the secondary antibody.

was observed between 0 and 40 mM KOAc (Fig. 2B). Minor decreases in activity were observed with KOAc concentrations ranging from 40 to 300 mM KOAc (Fig. 2B).

We next characterized the effect of other nucleic acids on the ATPase activity of HUpf1 protein. The poly(rU) in the reaction mixture was omitted or replaced with one of the following, ribohomopolymers poly(rA), poly(rC), poly(rG), poly(rA:rU), and poly(rG:rC), total RNA from yeast (which is predominantly rRNA), tRNA, or deoxyribohomopolymers poly(dA), poly(dI), poly(dT), poly(dA:dT), and poly(dC:dG). The ATPase activity of HUpf1 protein was determined at pH 5.0 and 50 mM KOAc, as described above. The results indicated that poly(dT) and poly(dA) stimulated the HUpf1 ATPase activity to the greatest extent (Fig. 2C). Poly(rA), poly(rA:rU), poly(rU), and poly(dA:dT) exhibited a more moderate stimulatory effect on the HUpf1 ATPase activity while poly(dI), poly(rG), poly(rG:rC), rRNA, tRNA and poly(dG:dC) showed either little or no effect (Fig. 2C). Reactions lacking nucleic acid polymers resulted in extremely low ATPase activity, demonstrating the nucleic acid dependence of this activity. Thus, poly (dT) was routinely used for subsequent ATPase assays.

A mutant HUpf1 in which the highly conserved aspartic and glutamic acids (DE636) in motif II of the NTP-binding and hydrolysis motifs were changed to alanines (DE636AA) was constructed by site-directed mutagenesis. The corresponding mutation in the yeast Upf1p (DE572AA) inactivated the NMD activity of the Upf1p and demonstrated no ATP hydrolysis activity (Weng et al., 1996a). The $HUpf1_{DE636AA}$ mutant protein was purified as described above and its ATPase activity was determined. The results demonstrated that, unlike wild-type HUpf1, the mutant protein did not contain

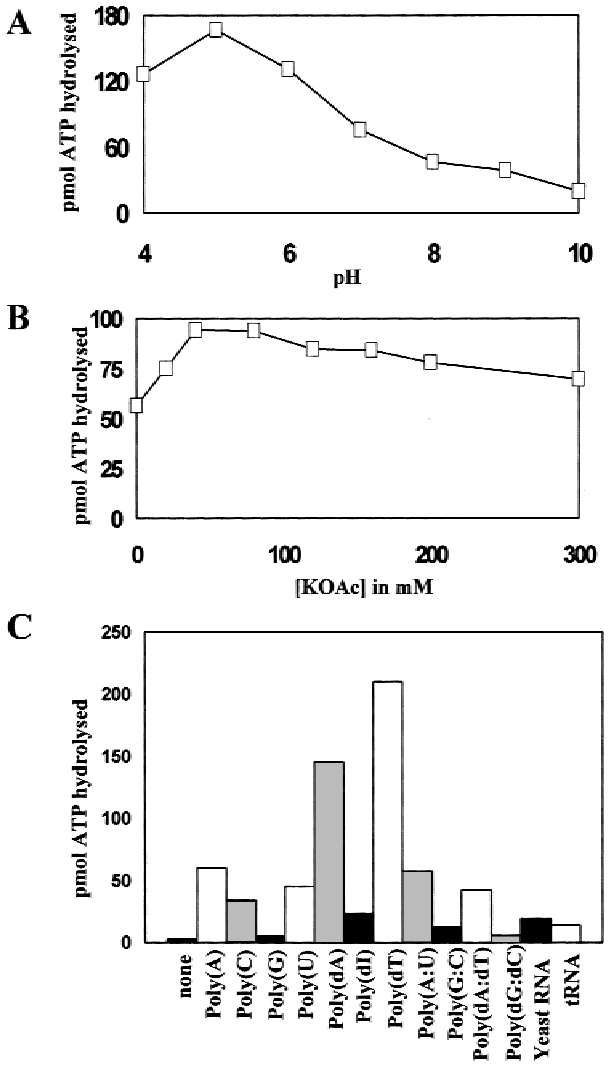

FIGURE 2. Optimization of ATPase assays. The ATPase activity of HUpf1 was determined using a charcoal assay. Experiments in **A** and **B** contained 100 ng of purified HUpf1, 100 μM ATP, and 1 μCi [γ-^{32}P]-ATP (3,000 Ci/mmol), and were performed for 30 min at 37 °C in the presence or absence of poly(rU). **A:** pH dependence. Reaction mixtures contained 50 mM KOAc, 2.5 mM $Mg(OAc)_2$, 0.1 mg/mL BSA, and 50 mM buffer as follows: MES, pH 4, MES, pH 5, MOPS, pH 6, MOPS, pH 7.0, Tris, pH 8.0, Tris, pH 9.0, Glycine, pH 10.0. The optimal pH was found to be around 5. **B:** Effect of KOAc. Reaction mixtures contained 50 mM MES, pH 5, 2.5 mM Mg(OAc)2, 0.1 mg/mL BSA, and KOAc concentrations as indicated. The KOAc concentration did not have a drastic effect on ATPase activity. The optimal concentration was found to be 50 mM. **C**: Effect of nucleic acids on ATPase activity. Reaction mixtures contained 50 mM MES, pH 5, 50 mM KOAc, 2.5 mM $Mg(OAc)_2$, 0.1 mg/mL BSA, and various nucleic acids as indicated. Poly(dT) and poly(dA) have the highest stimulation of ATPase activity.

any detectable ATPase activity, confirming that the conserved DE residues described above are essential for ATPase activity (Fig. 3). This further demonstrates that the ATPase activity detected is specific to the wild-type HUpf1 protein and not the result of a contaminating activity (Fig. 3).

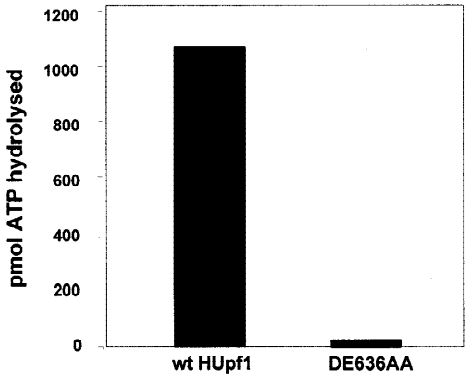

FIGURE 3. ATPase activity of wild-type HUpf1 and the DE636AA mutant. ATPase assays were carried out as described in Figure 2. Reaction mixtures contained 50 mM MES, pH 5, 50 mM KOAc, 2.5 mM $Mg(OAc)_2$, 0.1 mg/mL BSA, and poly(dT). Purified protein (100 ng) was used for both wild-type and DE636AA mutant. The DE636AA mutant shows drastically reduced ATPase activity compared to the wild type. The higher activity of the wild-type HUpf1 observed (when compared to Fig. 2) is the result of the difference in the specific activity of the protein.

HUpf1 exists as a monomer in solution

Density gradient centrifugation analysis was performed to correlate the ATPase activity of the purified fraction with that of the HUpf1 protein. The yeast Upf1p sediments as a monomer in solution (Czaplinski et al., 1995). Many helicases, however, can be found that function in multimeric states (Reha-Krantz & Hurwitz, 1978; Finger & Richardson, 1982; Dykstra et al., 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). Therefore, to determine whether HUpf1 functions as a monomer, purified HUpf1 was loaded on a 15–35% glycerol gradient. In parallel, proteins of known molecular weights 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 indicated that the HUpf1 peak was predominantly located in fractions 15, 16, and 17 of the glycerol gradient (Fig. 4B). The ATPase assays demonstrated that maximal RNA-dependent ATPase activity was observed in fractions 15, 16, and 17, whereas residual amounts of activity were present in the side fractions (Fig. 4A). These observations demonstrate that purified HUpf1 and the RNA-dependent ATPase activity peaks coincide, strongly indicating that this activity is associated with HUpf1 rather than a contaminating protein.

The size of the HUpf1 was determined by comparing its location within the gradient to the location of the molecular weight markers (Fig. 4B). The peak fractions containing HUpf1 lie in between the 220- and 78-kDa

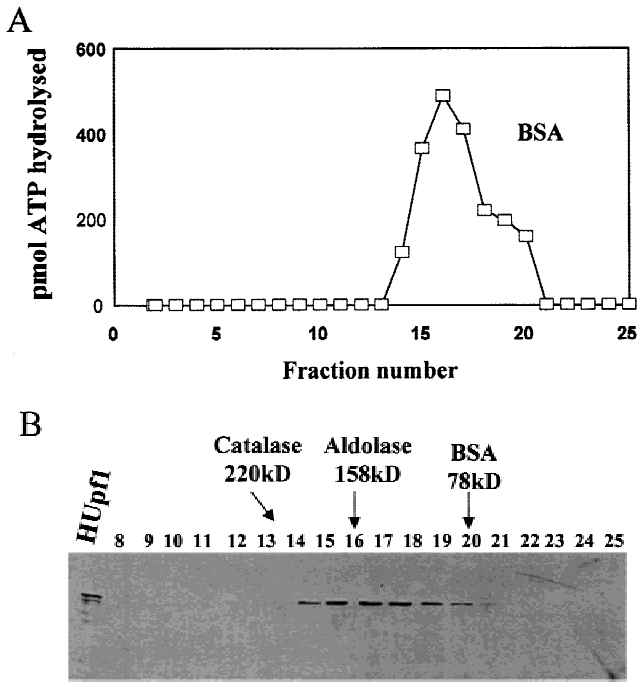

FIGURE 4. Glycerol gradient centrifugation of HUpf1. Purified HUpf1 (10 μg) was centrifuged for 14h at 157,000 $\times$ *g* through 15–35% glycerol gradient in 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.1% Triton X-100, and 10% glycerol. **A**: Ten microliters of each fraction were assayed for nucleic-acid-dependent ATPase activity in standard buffer conditions as described in Materials and methods. **B**: Five microliters of each fraction (indicated above each lane) were electrophoresed on an 8% SDS-PAGE gel, immunoblotted with HUpf1 antiserum. The first lane on the left contains HUpf1 as a positive control. The positions of three molecular weight markers, catalase (220 kDa), aldolase (158 kDa), and BSA (78 kDa), run in separate identical gradients, are indicated.

protein markers, with fractions 15–17 having a molecular weight of approximately 130 kDa. Thus, these results indicate that HUpf1 protein is monomeric in the solution.

Characterization of HUpf1 helicase activity

The ability of HUpf1p to displace partially duplexed nucleic acids was determined using a strand displacement assay as described previously (Venkatesan et al., 1982; Rozen et al., 1990; Czaplinski et al., 1998). Briefly, a radiolabeled oligodeoxyribonucleotide (28 nt) was annealed to a large single-stranded template (72 nt), forming a substrate that contains a double-stranded region bordered by a 5′ single-stranded region. The presence of the HUpf1 helicase activity is determined by monitoring the displacement of the shorter oligodeoxyribonucleotide. The substrate for assaying helicase activity is illustrated on the right side of Figure 5A. The reaction conditions used to assay helicase activity were identical to those described in Figure 2. Reaction mixtures containing increasing concentrations of HUpf1 were incubated with the substrate at 37 °C for 30 min and electrophoresed on a nondenaturing gel. As shown in Figure 5A, lane 1, the helicase substrate migrated near the top of the gel. Denaturing the substrate by boiling releases the radioactive oligodeoxyribonucleotide that migrates into the gel (Fig. 5A, lane 2). Addition of HUpf1 to the reaction mixtures displaced the 28 nt oligodeoxyribonucleotide fragment from the DNA substrate (Fig. 5A, lanes 3–5). The amount of the 28-nt oligonucleotide displaced increased concomitantly with the concentration of HUpf1 in the reaction mixture (Fig. 5A, lanes 3–5). This pattern of 3′ $\rightarrow$ 5′ strand displacement is consistent with the HUpf1 displaying a 5′ $\rightarrow$ 3′ helicase activity, as the duplex with a 5′ single-stranded region was unwound. No displacement of the 28-nt oligonucleotide was detected if ATP was omitted from the reaction mixture (Fig. 5A, lane 6), indicating that ATP is required for HUpf1 helicase activity. These results indicate that HUpf1 possesses a 5′ $\rightarrow$ 3′ helicase activity. Furthermore, the mutant Hupf1 protein that lacks the ATPase activity ($HUpf1_{DE636AA}$) was used in the experiment as described above. The results demonstrated that no unwinding was observed with the mutant protein $HUpf1_{DE636AA}$ (Fig. 5B, lanes 6–8).

Characterization of HUpf1 nucleic-acid-binding activity

Previous results indicated that ATP and RNA-binding activities of the yeast Upf1p are important in the ability of the surveillance complex to recognize premature termination codons (Weng et al., 1996a, 1996b, 1997; Czaplinski et al., 1998). In addition, the translation termination factor eRF3 and RNA compete for interaction with yeast Upf1 (Czaplinski et al., 1998, 1999). ATP binding to the Upf1p reduces its affinity for RNA but not eRF3 (Weng et al., 1996a, 1996b, 1998; Czaplinski, 1998), allowing for substrate specificity. Therefore, modulation of the RNA-binding activity by the ATP cofactor is likely to be an important property of the HUpf1 protein.

The RNA-binding characteristics of HUpf1 were investigated using a gel shift assay described previously (Czaplinski et al., 1995; Weng et al., 1996a, 1996b). A uniformly labeled 130-nt-long transcript generated from random sequence was used as a probe. The ability of HUpf1 protein to bind this RNA was assayed by monitoring the mobility of the RNA probe in a native gel. The results demonstrated that addition of HUpf1 protein retarded the mobility of the substrate RNA consistent with formation of HUpf1:RNA complexes (Fig. 6A, lane 2). We next asked whether ATP addition to the reactions reduced the HUpf1:RNA complexes. Reactions were prepared as described above and ATP was subsequently added to the mixtures. The results demonstrated that addition of ATP to the reactions markedly reduced formation of the HUpf1:RNA complexes (Fig. 6A, lane 3). Taken together, these results demon-

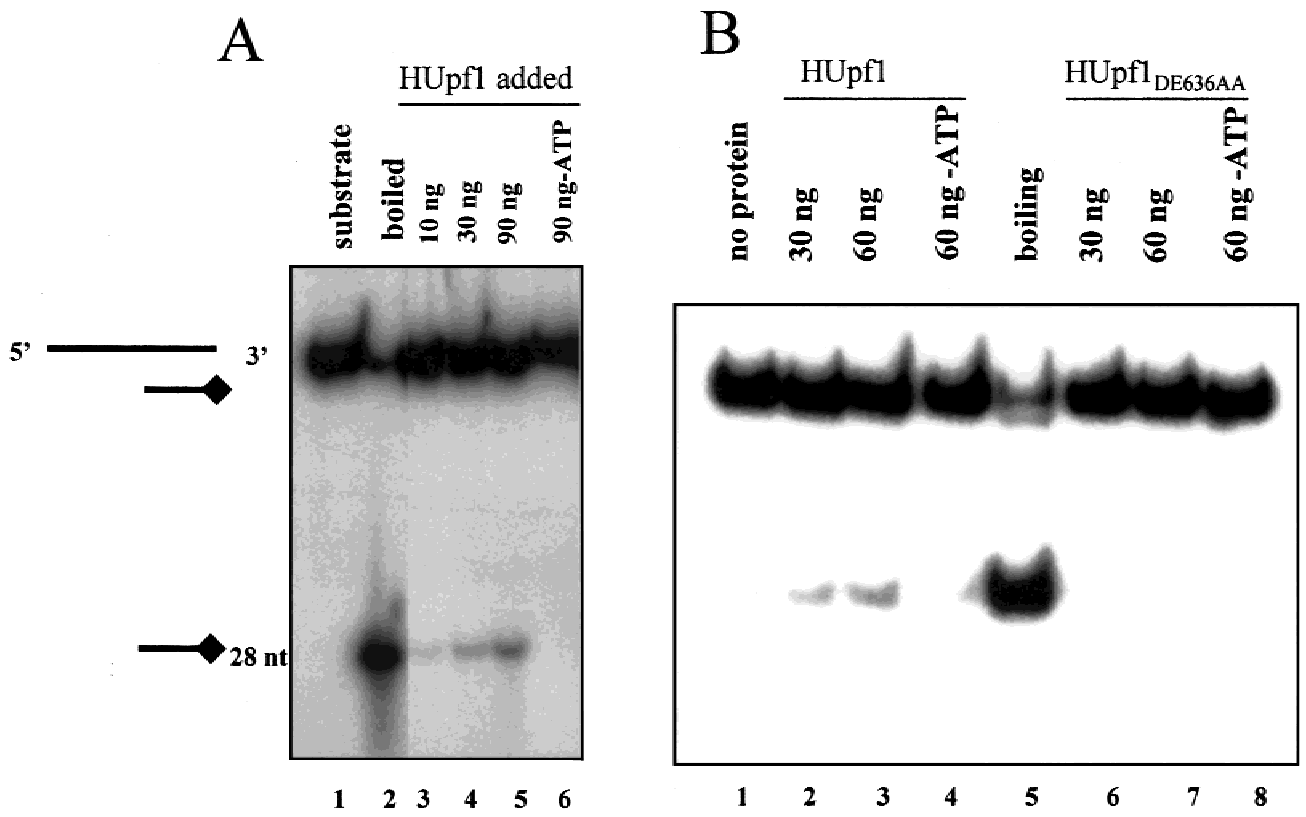

FIGURE 5. HUpf1 demonstrates 5′ → 3′ helicase activity. A strand displacement assay was used to determine whether HUpf1 has DNA helicase activity. A schematic diagram of the substrate used is illustrated on the left, marking the positions of the radiolabeled species. Displacement of the labeled 28 nt oligodeoxyribonucleotide indicates the presence of 5′-3′ DNA helicase activity. Ten femtomoles of helicase substrate were incubated under standard buffer conditions with 1 mM ATP and increasing amounts of HUpf1 as indicated above each lane. After 30 min at 37 °C, reactions were stopped by the addition of helicase stop buffer and the samples were electrophoresed for 3 h in a 17% PAGE gel. The gels were either dried and autoradiographed or directly scanned in a phosphorimager. **A**: Lane 1: helicase substrate; lane 2: heat denatured helicase substrate; lanes 3–5: reactions with indicated amounts of HUpf1; lane 6: reaction with indicated amount of HUpf1 lacking ATP. **B**: Lane 1: substrate; lanes 2–3: reactions with indicated amount of HUpf1; lane 4: reaction with no ATP added; lane 5: substrate boiled; lanes 6–8: reaction with mutant Hupf1 as indicated.

strate that ATP is a cofactor that modulates the RNA-binding activity of the HUpf1.

DISCUSSION

A large number of results have demonstrated that the yeast Upf1p plays an important role in both nonsense-mediated mRNA decay and in translation termination (reviewed in Czaplinski et al., 1999). The human *UPF1* gene has also been identified and demonstrates a high degree of conservation with the yeast *UPF1* gene (Perlick et al., 1996; Applequist et al., 1997). Preliminary data also suggested that HUpf1 is involved in modulating NMD and translation termination (Perlick et al., 1996; Applequist et al., 1997; Czaplinski et al., 1998). More recent results have shown that a dominant negative form of the HUpf1 interferes with NMD in a mammalian cell line (Sun et al., 1998). These results indicate that human Upf1 is an important factor in regulating mRNA decay and translation, and understanding its biochemical activities is important for elucidating its role in NMD and translation termination. The studies reported here describe the approach used to purify the human Upf1 protein and characterize its biochemical properties.

An epitope-tagged HUpf1 was expressed in a baculovirus expression system and was purified from extracts of insect cells infected with recombinant baculovirus particles. Purification was achieved in a single step using immunoaffinity chromatography with a monoclonal antibody against the Flag-epitope tag inserted at the amino terminus of the protein. The purified HUpf1 demonstrated a nucleic-acid-dependent ATPase activity that could be stimulated by several different RNA or DNA polymers (Fig. 2). The ATPase assays were optimized and the results demonstrated that the ATPase activity of HUpf1 functioned best at pH 4–6, with increases in pH leading to reduced activity (Fig. 2A). The ATPase activity was present over a broad range of salt concentrations (0–300 mM

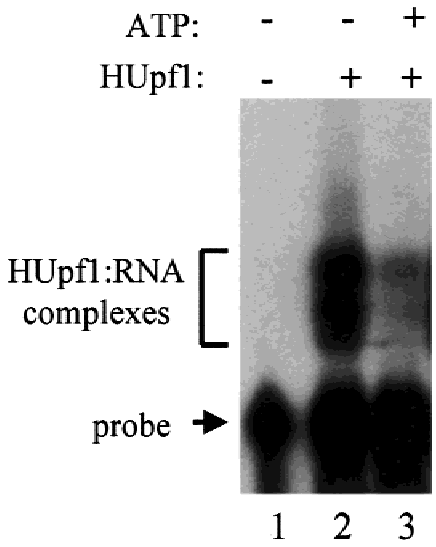

FIGURE 6. The HUpf1 is an RNA-binding protein whose binding activity is modulated by ATP. A gel shift assay was used to determine the ability of HUpf1 to bind RNA. Ten femtomoles of radiolabeled RNA were incubated under standard buffer conditions (helicase buffer) in the presence of 100 fmol of HUpf1 (lanes 2 and 3) or without HUpf1 (lane 1) and incubated for 15 min at room temperature. In lane 3, 1 mM ATP was added to the reaction after 15 min and incubated for another 10 min. Reactions were then electrophoresed in a 4.5% PAGE gel (30:0.5 acrylamide:bisacrylamide in 0.5× TBE containing 5% glycerol). Following electrophoresis, the gel was dried and autoradiographed. The positions of the RNA probe and the HUpf1:RNA complexes are indicated on the left.

KOAc; Fig. 2B). Two lines of evidence attribute the observed ATPase activity to HUpf1 rather than a contaminant. First, ATPase activity was not observed in reactions containing the $HUpf1_{DE636AA}$ harboring a mutation in motif II of NTP-binding and hydrolysis (Fig. 3). The corresponding mutation in the yeast Upf1p (DE572AA) inactivates its NMD activity and demonstrates no ATP hydrolysis (Weng et al., 1996a). Second, glycerol gradient analysis demonstrated that fractions containing HUpf1 correlated well with the ATPase activity in the gradient (Fig. 4). Taken together, these results strongly suggest that the purified HUpf1p harbors an ATPase activity.

Analogous to the yeast Upf1p, the HUpf1 was also shown to be a helicase. The HUpf1 was able to unwind a partially duplexed nucleic acid substrate. The yeast Upf1p was shown to displace duplexes containing a 5′ single-stranded extension (Czaplinski et al., 1995). The HUpf1 protein also displaced duplexes containing a 5′ single-stranded extension (Fig. 5). This observation suggests that the 5′ → 3′ ATP-dependent helicase activity is a conserved component of the NMD pathway. The RNA-binding properties of the yeast and human Upf1 proteins also appear to be conserved (Fig. 6). Interestingly, two distinct bands are observed in the RNA-binding experiments using the HUpf1 protein. This result suggests that different types of RNA:HUpf1 protein complexes are being formed. The different forms of the HUpf1p:RNA complexes formed have not been characterized further.

Understanding the biochemical activities of the yeast Upf1p has provided insight into how the Upf1p may influence translation termination and NMD (Weng et al., 1996a, 1996b, 1998; Czaplinski et al., 1998, 1999). These results showed that ATP is an important cofactor that can modulate Upf1p RNA-binding activity (Weng et al., 1998). Furthermore, the ATPase/helicase activity of the Upf1p is important for NMD, but is not required for its role in translation termination. Considering the strong conservation of sequence and function between the yeast and human Upf1, we suggest that these activities will also be important for HUpf1 to function. Future experiments will be performed to characterize the interactions of the human translation termination factors with the Upf1 and the consequence of these interactions on its biochemical activity. Furthermore, additional investigations are required to understand the role of the divergent amino- and carboxyl-terminal ends of the HUpf1. It is possible that these unique domains may be interaction domains for factors that are unique to mammalian organisms that may or may not be influencing the activities of the central domain of this translation and mRNA turnover factor.

The investigations of NMD in yeast have yielded a tremendous insight into the mechanism of this process that will most likely reflect how this process functions in all eukaryotic systems. The in vivo and in vitro studies on the yeast Upf1p have provided a solid foundation of fundamental hypotheses that can now begin to be translated into experimental systems in higher organisms. The results described here begin the biochemical studies on the mechanism of NMD in the mammalian systems.

MATERIALS AND METHODS

Expression and purification of HUpf1 and $HUpf1_{DE636AA}$ mutant

An *Xba*I-*Xho*I fragment containing the HUpf1 coding region was excised from pT7RENT1 (Czaplinski et al., 1998) and ligated into pBacPAK8, creating pBacPAK8-HUpf1. A FLAG epitope was inserted at the amino terminal of HUpf1 by PCR creating pBacPAK8-FLAG-HUpf1. The $HUpf1_{DE636AA}$ mutant was generated using Quick-Change kit (Stratagene) and appropriate oligonucleotides. The pBacPAK8-N-FLAG-HUpf1 wild-type or the DE636AA mutant were cotransfected separately into High Five cells (Invitrogen Inc.) with Baculogold DNA (Pharmingen Inc.) as per the manufacturer's instruction. Four days posttransfection, the medium containing recombinant viral particles was used to infect a flask of confluent High Five cells. After four days, the medium of the infected cells was used in a second round of High Five cell infection to establish a high titer stock of recombinant viral particles. This viral stock was subsequently used to infect High Five cells to express and purify HUpf1. On the fourth day sub-

sequent to this infection, cells were harvested by centrifugation and the cell pellet resuspended in buffer HF1 (20 mM Tris, pH 8.0, 500 mM NaCl, 0.1% Triton X-100, 5% glycerol, 1 mM PMSF, and 2μg/mL Pepstatin A, Leupeptin, and Aprotinin). Protein extracts were prepared by lysing cells using sonication at medium power for four bursts of 30 s, and clarified by centrifugation at 18K rpm for 30 min. The supernatant was removed and subsequently used for purification of recombinant HUpf1.

HUpf1 was purified using anti-FLAG M2 affinity resin. The protein extract was incubated with the resin in HF1 buffer for 30 min at 4 °C while rocking. After binding, the resin was washed four times in buffer X (20 mM HEPES, pH 7.4, 10% glycerol, 0.01% Triton X-100, 0.5 mM EDTA, 0.5 mM PMSF, 1 μg/mL Pepstatin A, Leupeptin, and Aprotinin). The first and second washes also contain 50 and 1,500 mM NaCl, respectively, as well as an additional 1% Triton X-100. The third and fourth washes contain 700 and 50 mM NaCl, respectively, but no additional Triton X-100. HUpf1 was eluted in several batches using buffer X supplemented with 50 mM NaCl and 250 μg/mL Flag peptide. The elutions were pooled, concentrated, and aliquoted to be stored at -70 °C.

SDS-PAGE and western 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. Immunoblots were probed with either the murine monoclonal M2 antibody (IBI Inc.) raised against the FLAG epitope (Fig. 1B), or polyclonal anti-HUpf1 anti-serum (Fig. 4B) as the primary antibody (a gift from Dr. Dietz), 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.).

ATPase assays

ATP hydrolysis was monitored using a charcoal assay (Clark et al., 1981). To demonstrate the nucleic acid dependence in those assays poly(rU) was used (Fig. 2A,B). Upon characterization poly(dT) was used routinely. A typical reaction mixture contained polynucleotides, 100 ng of purified HUpf1, 100 μM ATP, and 1 μCi of [γ-^{32}P]-ATP (3,000 Ci/mmol) in a 20-μL total reaction volume. Buffer conditions were as indicated in the figure legends. After incubation for 30 min at 37 °C, reactions were stopped and unreacted ATP was adsorbed by addition of 280 μL of 5% charcoal in 20 mM phosphoric acid. The charcoal was pelleted by centrifugation for 10 min at 13,200 $\times$ *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, values shown are the average of three separate experiments and control reactions without HUpf1 were performed to determine the background. The results of the triplicate experiments did not vary by more than 15%. The amount of ATP hydrolyzed seems to vary with the specific activity of the purified protein as observed in Figure 3 ($\sim$1,200 U) when compared to Figure 2 (100–200 range).

Glycerol gradient analysis of HUpf1

Ten micrograms of purified HUpf1 protein in gradient buffer (20 mM Tris-Cl, pH 8, 50 mM KCl, 0.1% Triton X-100, 1 mM β-mercaptoethanol) containing 10% glycerol were loaded on 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 $\times$ *g* for 14 h, 200-μL fractions were collected and assayed for HUpf1 by western analysis and for ATPase activity as described above.

Preparation of DNA helicase substrate

A 28-mer shorter oligodeoxyribonucleotide was labeled at the 5′ end using T4 polynucleotide kinase and γ-32 P ATP (3,000 Ci/mmol; Amersham). This 5′-end-labeled oligodeoxyribonucleotide was then annealed to a suitably designed 72-mer oligodeoxyribonucleotide by incubating at 100 °C for 2 min, 60 °C for 5 min, and room temperature for 2 h. The annealed substrate was then gel purified before use in the helicase assay.

Helicase assay

The helicase activity was examined using a strand-displacement assay (Venkatesan et al., 1982; Rozen et al., 1990). Helicase assays were done in 20-μL reactions containing 20 mM MES, pH 5.0, 50 mM KOAc, 2.5 mM $Mg(OAc)_2$, and 0.1 mg/mL BSA. The helicase substrate was added to the reactions and incubated at 37 °C for 30 min. Reactions were stopped by the addition of 5 μL gel loading buffer (50% glycerol, 0.5% SDS, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) and electrophoresed in a 17% PAGE gel (30:1 acrylamide:bisacrylamide in 1$\times$ TBE) at 15 mA for 2–3 h.

RNA substrate for gel shift assays

A uniformly labeled 130-nt RNA was made by in vitro transcription of *Hin*cII-digested pGEM3 containing random sequences, with T7 RNA polymerase using [α-^{32}P]UTP (3,000 Ci/mmol). The probe was purified from an 8% denaturing PAGE gel.

Gel shift assay

The ability of HUpf1 to bind nucleic acid was determined using a gel shift assay (Lee & Hurwitz, 1992). Binding reactions were performed in binding buffer (same as helicase buffer) in a 20-μL volume with or without 100 fmol of HUpf1 as indicated in Fig. 6. Ten femtomoles of labeled RNA (prepared as described above) were added, and reactions were incubated for 15 min at room temperature. Alternately, after incubation, 1 mM ATP was added to the reactions and incubated for another 10 min. Five microliters of gel loading buffer (50% glycerol, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) were added to all reactions and they were electrophoresed in a 4.5% native PAGE gel (30:0.5 acryl-

amide:bisacrylamide in 0.5 × TBE containing 5% glycerol). Gels were dried and autoradiographed.

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