Evidence that phosphorylation of human Upf1 protein varies with intracellular location and is mediated by a wortmannin-sensitive and rapamycin-sensitive PI 3-kinase-related kinase signaling pathway

MAHADEB PAL,^{1,3} YASUHITO ISHIGAKI,^{1,2} ESZTER NAGY,^{1,4} and LYNNE E. MAQUAT^{1,2}

¹Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, New York 14263, USA

²Department of Biochemistry and Biophysics, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642, USA

ABSTRACT

Human Upf1 protein (p), a group 1 RNA helicase, has recently been shown to function in nonsense-mediated mRNA decay (NMD) in mammalian cells. Here, we demonstrate that the estimated 3×10^6 copies of hUpf1p per exponentially growing HeLa cell are essentially equally distributed among polysomal, subpolysomal, and ribosome-free fractions. We also demonstrate that hUpf1p binds RNA and is a phosphoprotein harboring phosphoserine and phosphothreonine. hUpf1p is phosphorylated to the highest extent when polysome-associated and to the lowest extent when ribosome free. We find that serum-induced phosphorylation of hUpf1p is inhibited by wortmannin at a concentration that selectively inhibits PI 3-kinase related kinases and, to a lesser extent, by rapamycin. These and other data suggest that phosphorylation is mediated by a wortmannin-sensitive and rapamycin-sensitive PI 3-kinase-related kinase signaling pathway. Comparisons are made of hUpf1p to Upf1p and SMG-2, which are the orthologs to hUpf1p in *Saccharomyces cerevisiae* and *Caenorhabditis elegans*, respectively.

Keywords: human Upf1 protein; nonsense-mediated mRNA decay; phosphorylation; P1 3-kinase-related kinase; RNA helicase

INTRODUCTION

Cells have evolved numerous pathways to recognize and eliminate functionally abnormal RNAs. One such pathway is exemplified by the recently discovered charging of tRNA in the nucleus: although the bulk of tRNA aminoacylation takes place in the cytoplasm, the small fraction of tRNA aminoacylation that takes place in the nucleus provides a proofreading mechanism as a requirement for efficient tRNA export to the cytoplasm (Lund & Dahlberg, 1998). Another such pathway is exemplified by nonsense-mediated mRNA decay (NMD): in mammalian cells, although the bulk of mRNA trans-

lation takes place in the cytoplasm, data indicate that the small fraction of translation that takes place in association with nuclei, possibly but not assuredly by cytoplasmic ribosomes during mRNA export to the cytoplasm, provides a proofreading mechanism for the presence of premature termination codons and ensures that the bulk of cytoplasmic mRNA is nonsense free (reviewed in Maquat, 1995, 1996, 2000; Li & Wilkinson, 1998; Hentze & Kulozik, 1999). NMD prevents the accumulation of defective and potentially deleterious proteins that could function in new or dominant-negative ways. NMD in mammalian cells is also likely to regulate the abundance of functionally important transcripts, as has been demonstrated for alternatively spliced SRp20 and SRp30 transcripts of *Caenorhabditis elegans* (Morrison et al., 1997), and transcripts that influence telomere length (Lew et al., 1998) or the levels of a kinetochore subunit (Dahlseid et al., 1998) in *Saccharomyces cerevisiae*. In fact, studies indicate that expression of at least 225 of the ~6000 genes in *S. cerevisiae* is affected either directly or indirectly by NMD (Lelivelt & Culbertson, 1999).

Reprint requests to: Lynne E. Maquat, Department of Biochemistry and Biophysics, School of Medicine and Dentistry, University of Rochester, 601 Elmwood Avenue, Box 712, Rochester, New York 14642, USA; e-mail: lynne_maquat@urmc.rochester.edu.

³Present address: The Cleveland Clinic, Department of Molecular Biology, Lerner Research Institute, 9500 Euclid Avenue, Cleveland, Ohio 44195, USA.

⁴Present address: Intercell, Inc., Dr Bohr Gasse 1R, A-1030 Vienna, Austria.

Overexpression of human (h) Upf1 protein (p) harboring an arginine-to-cysteine change at position 844 (R844C) within the putative helicase domain has been shown to have a dominant-negative effect on NMD in mammalian cells (Sun et al., 1998), making hUpf1p the only *trans*-acting factor known to date to promote NMD in mammalian cells. hUpf1p, which consists of 1,118 amino acids, was first identified based on similarities to its well-characterized ortholog in *S. cerevisiae* (Perlick et al., 1996; Applequist et al., 1997). Yeast Upf1p demonstrates binding to RNA and single-stranded DNA, RNA- and DNA-dependent ATPase activity, and 5' → 3' helicase activity (Czapinski et al., 1995; Weng et al., 1996a). Disruption of the yeast *UPF1* gene selectively stabilizes mRNAs containing early nonsense codons without affecting the decay rates of most wild-type mRNAs (Leeds et al., 1991; Weng et al., 1996a, 1996b, 1998). Because disruption of either the yeast *UPF2* or *UPF3* gene does likewise (Cui et al., 1995; He & Jacobson, 1995; Lee & Culbertson, 1995), all three *UPF* gene products are thought to be integral components of the NMD pathway. In support of this idea, Upf1p, Upf2p, and Upf3p colocalize with 80S ribosome particles and polysomes (Peltz et al., 1993; Atkin et al., 1995, 1997), where NMD takes place (Zhang et al., 1997), and Upf2p interacts with Upf1p and Upf3p (He & Jacobson, 1995; He et al., 1997; Weng et al., 1996b).

In addition to functioning in NMD, yeast Upf1p has been shown to enhance translation termination (Leeds et al., 1992; Weng et al., 1996a, 1996b, 1998; Maderazo et al., 2000). Results indicating that certain mutations within the yeast *UPF1* gene affect the process of programmed -1 frameshifting used by viruses to control viral gene expression (Cui et al., 1996), together with the finding that yeast Upf1p interacts with eukaryotic translation termination factors (eRF) 1 and 3 (Czapinski et al., 1998), have led to the proposal that the association of Upf1p with peptidyl release factors may be key to the assembly of a putative surveillance complex that sequentially enhances translation termination, identifies premature termination events, and promotes NMD (Ruiz-Echevarría et al., 1996; Czapinski et al., 1998; Weng et al., 1998). Upf3p has also been proposed to constitute the surveillance complex at least at the point of translation termination since deletion of the *UPF3* gene also affect programmed -1 frameshifting (Ruiz-Echevarría et al., 1998).

The finding that overexpression of hUpf1p harboring the R844C mutation abrogates NMD two- to threefold in mammalian cells harboring the wild-type *hUPF1* gene (Sun et al., 1998) plus the finding that overexpression of yeast Upf1p harboring the corresponding mutation abrogates NMD two- to threefold in yeast cells harboring the wild-type *UPF1* gene (Leeds et al., 1991, 1992) suggest that mammalian and yeast Upf1p share at least some functions. Consistent with this idea, hUpf1p is detected primarily, if not exclusively in the cytoplasm of

ethanol-fixed (Applequist et al., 1997) or formaldehyde-fixed (Serin et al., 2001) human cells using immunofluorescence and in postnuclear fractions of human cell extracts by western blot hybridization (Applequist et al., 1997), in agreement with localization of yeast Upf1p to the cytoplasm (Atkin et al., 1995, 1997). Furthermore, hUpf1p flanked by the extreme N- and C-termini of yeast Upf1p complements Upf1p-deficient yeast in a frameshift allosuppression assay, indicating function in translation termination (Cui et al., 1995; Perlick et al., 1996). Finally, in vitro-synthesized hUpf1p, like in vitro-synthesized yeast Upf1p, interacts with in vitro-synthesized eRF1 and eRF3 (Czapinski et al., 1998).

Despite similarities in the mechanism by which Upf1p promotes NMD in yeast and mammalian cells, (1) hUpf1p is only 51% identical to yeast Upf1p, with the largest differences being 63 additional amino acids at the N-terminal end and 83 additional amino acids at the C-terminal end, (2) full-length hUpf1p fails to function in either nonsense suppression or NMD in yeast (Perlick et al., 1996; Czapinski et al., 1998), and (3) hUpf1p flanked by the extreme N- and C-termini of yeast Upf1p functions in nonsense suppression but not NMD in yeast (Perlick et al., 1996; Czapinski et al., 1998). Because only three of the seven SMG factors required for NMD in *C. elegans* (Pulak & Anderson, 1993; Morrison et al., 1997; Cali & Anderson, 1998; Page et al., 1999; R. Aronoff, pers. comm.; S. Kuchma and P. Anderson, pers. comm.) currently have identifiable counterparts in *S. cerevisiae* (Leeds et al., 1992; Cui et al., 1995; He & Jacobson, 1995; Lee & Culbertson, 1995), differences in NMD between species may extend beyond sequence differences between orthologs.

Here we undertake biochemical characterizations of hUpf1p in HeLa and HEK293 cells to draw comparisons of hUpf1p its orthologs in *S. cerevisiae* and *C. elegans*. We find that hUpf1p is present at $\sim 3 \times 10^6$ copies per exponentially growing HeLa cell, binds RNA, and is essentially equally distributed among polysomal, subpolysomal, and ribosome-free fractions. We demonstrate that hUpf1p is a phosphoprotein. Polysome-associated hUpf1p contains the most phosphate and ribosome-free hUpf1p contains the least phosphate. Phosphate was detected at serine and threonine residues but not tyrosine residues. Furthermore, serum-induced hUpf1p phosphorylation is inhibited by wortmannin and, to a lesser extent, rapamycin. These and other data suggest involvement of a PI 3-kinase-related kinase signaling pathway.

RESULTS

Upf1p is hyperphosphorylated when associated with polysomes

Phosphorylation and dephosphorylation are often important means by which cellular proteins can be func-

tionally regulated in a reversible fashion. As one of many examples, the high degree of phosphorylation characteristic of SR proteins *in vivo* is required for their function in spliceosome assembly and splicing catalysis, and virus-induced dephosphorylation in late adenovirus-infected cells inactivates SR-protein function as either splicing enhancers or repressors (Kanopka et al., 1998 and references therein). hUpf1p harbors many potential sites of phosphorylation according to internet searches for phosphorylation motifs. To examine the possibility that hUpf1p is a phosphoprotein, cytoplasmic extract from untransfected HeLa cells was prepared either in the presence or absence of two phosphatase inhibitors, sodium ortho-vanadate and sodium fluoride. Extract prepared in the presence of inhibitors was incubated further without calf intestinal alkaline phosphatase (CIP), and extract prepared in the absence of inhibitors was incubated further with CIP. CIP effectively dephosphorylates proteins containing phosphoserine and phosphothreonine, which together account for >97% of protein-bound phosphate in eukaryotic cells (Coligan et al., 1997). The extract was then subjected to two-dimensional gel electrophoresis and analyzed by western blot hybridization using anti-hUpf1p antibody.

In extract treated with phosphatase inhibitors, hUpf1p appears as multiple spots (Fig. 1), each of which is potentially a different phosphorylated isoform. The finding that the more acidic isoforms were either not present or decreased in abundance in extracts treated with CIP instead of phosphatase inhibitors (Fig. 1) indicates that the isoforms differ, at least in part, by the extent of phosphorylation. Therefore, hUpf1p is a phosphoprotein (see below).

Because an estimated 80% of yeast Upf1p is polysome associated and the rest is polysome free (Atkin et al., 1995), we reasoned that the phosphorylation status of hUpf1p could differ with cellular location and, conceivably, function. To examine the intracellular distribution of hUpf1p, the cytoplasm of untransfected HeLa cells was layered over 30% sucrose and centrifuged at

FIGURE 1. hUpf1p is a phosphoprotein. Cytoplasmic extract from untransfected HeLa cells was prepared in the presence or absence of phosphatase inhibitors (NaV + NaF). Extract prepared in the presence of inhibitors was subsequently incubated without calf intestinal alkaline phosphatase (CIP), and extract prepared in the absence of inhibitors was subsequently incubated with CIP. Constituent proteins were separated by isoelectric focusing (1st D) followed by electrophoresis in SDS-polyacrylamide (2nd D). The positions of hUpf1p in the resulting two-dimensional gels were analyzed by Western blot hybridization and ECL (Amersham) using anti-hUpf1p antibody.

130,000 $\times g$ for 2.5 h at 4°C (Brewer & Ross, 1990). Four hours prior to harvest, half of the cells were incubated with 5 $\mu\text{g/mL}$ of cycloheximide to stabilize polysome structure. RNA-free protein and, as a control, protein-free RNA were purified from the postpolysomal cytosol (supernatant), subpolysomes (sucrose pad), and polysomes (pellet). The finding that 18S and 28S rRNA were detected by ethidium bromide staining only in subpolysomal and polysomal fractions (data not shown) corroborated that the supernatant was free of ribosomal subunits and polysomes as expected. Consistent with this conclusion, western blot hybridization of ribosomal protein (rp) L10 revealed that 80% and 19% of rpL10 were present in the pellet and pad, respectively, and the stabilization of polysomes with cycloheximide resulted in 95% of rpL10 copurifying with polysomes and only 5% copurifying with the pad (Fig. 2B). The results of western blotting using anti-hUpf1p antibody indicated that hUpf1p is essentially equally distributed among polysomal, subpolysomal, and ribosome-free HeLa-cell fractions in the absence of cycloheximide (Fig. 2A). In validation of this finding, the stabilization of polysomes with cycloheximide resulted in a slight increase in the amount of polysome-associated hUpf1p and a slight decrease in the amount of ribosome-free hUpf1p (Fig. 2A).

To determine if the degree of hUpf1p phosphorylation differs with cellular location, hUpf1p in polysomal, subpolysomal, and ribosome-free fractions was subjected to two-dimensional gel electrophoresis and western blot hybridization using anti-hUpf1p antibody. Results revealed that polysome-associated hUpf1p is phosphorylated to a larger extent than subpolysome-associated hUpf1p, and ribosome-free hUpf1p is phosphorylated to a smaller extent than subpolysome-associated hUpf1p (Fig. 3A). A simple mixing experiment in which polysomal and ribosome-free fractions were incubated prior to two-dimensional gel electrophoresis and western blot hybridization indicated that the hypophosphorylated state of ribosome-free hUpf1p relative to polysomal hUpf1p was not attributable to experimentally induced dephosphorylation but accurately reflects a difference in intracellular phosphorylation (Fig. 3B). Notably, ribosome-free hUpf1p does harbor some phosphate as evidenced by a decrease in mobility in the first dimension after CIP treatment (data not shown).

FLAG-hUpf1p is phosphorylated on serine and threonine residues, and phosphorylation is sensitive to inhibition by wortmannin and, to a lesser extent, rapamycin

To obtain a source of hUpf1p for biochemical analyses, hUpf1p was expressed in HeLa cells as a FLAG-tagged protein. A pFLAG-hUPF1-IRES1neo' expression vector harboring hUPF1 cDNA was constructed and stably introduced into HeLa cells. The HeLa cell line express-

FIGURE 2. HeLa-cell hUpf1p is present in polysomal, subpolysomal, and ribosome-free fractions. Cytoplasm was generated from 3×10^7 exponentially growing HeLa cells. Prior to harvest, half of the cells were incubated with 5 μ g/mL of cycloheximide (+CHX) for 4 h to stabilize polysome structure, and the remaining half were not exposed to cycloheximide (-CHX). Each half was loaded over 1.5 mL of 30% sucrose and centrifuged at 36,000 rpm and 4 °C in an SW60 rotor for 2.5 h. Protein (10 μ L, which is the equivalent of 1.5×10^6 cells) from each polysomal (pellet), subpolysomal (sucrose pad), and ribosome-free (region above the pad; S130; sup) fraction was electrophoresed in a 10% SDS-polyacrylamide gel and analyzed by western blot hybridization and ECL (Amersham) using antibody against either hUpf1p (**A**) or ribosomal protein (rp) L10 (**B**), which is a component of the large ribosome subunit. Numbers below each lane specify the percentage of total-cell hUpf1p in each fraction and are representative of three independently performed experiments. Serial dilutions of protein from a pellet were used to demonstrate that the analysis is quantitative.

ing the highest level of FLAG-hUpf1p was expanded, and FLAG-hUpf1p was purified using an anti-FLAG immunoaffinity column. Immunopurified FLAG-hUpf1p, which unexpectedly migrated more slowly than its predicted 124-kDa size, constituted >85% of Coomassie blue staining material (Fig. 4). Results of two-dimensional gel electrophoresis and Western blotting using anti-FLAG antibody indicated that the degrees of FLAG-hUpf1p and endogenous HeLa cell phosphorylation were comparable in total-cell as well as polysomal, subpolysomal, and ribosome-free fractions (data not shown).

FIGURE 3. Polysome-associated hUpf1p is hyperphosphorylated relative to ribosome-free hUpf1p. **A:** Protein (10 μ L) from the -CHX sample analyzed in Figure 2 was subjected to isoelectric focusing and analyzed for hUpf1p as described in the legend to Figure 1. **B:** As in **A** except pellet and supernatant fractions analyzed in the bottom third were mixed and incubated at room temperature for 15 min prior to isoelectric focusing. Notably, **A** and **B** were subjected to two-dimensional analysis on separate occasions, explaining the difference in protein mobility in the first dimension.

One biochemical analysis undertaken was an identification of the amino acids phosphorylated in FLAG-hUpf1p. Immunopurified FLAG-hUpf1p was pre-incubated in the absence or presence of CIP and then subjected to western blot hybridization using antiphosphothreonine, antiphosphoserine, antiphosphotyrosine or, as a control, anti-FLAG antibody. Results revealed the presence of phosphothreonine and phosphoserine in samples not exposed to CIP (Fig. 5). Failure to detect antiphosphotyrosine antibody reactivity with FLAG-

FIGURE 4. Purification of FLAG-hUpf1p. Cytoplasmic extract from the HeLa cell transfectants expressing the highest level of FLAG-hUpf1p was loaded onto an anti-FLAG immunoaffinity column (Load), the flow-through (FT) was collected, and bound material was subsequently washed three times with binding buffer containing, sequentially, 150 mM, 450 mM, and 150 mM KCl. FLAG-hUpf1p was eluted using anti-FLAG peptide (Eluate), and the specified percentages of each sample were resolved in 10% SDS-polyacrylamide and stained with Coomassie brilliant blue. Molecular weight standards (in kilodaltons) are provided at the left.

FIGURE 5. FLAG-hUpf1p is phosphorylated on serine and threonine residues. Immunopurified FLAG-hUpf1p (10 ng) was incubated with or without CIP, electrophoresed in 7.5% SDS-polyacrylamide, and analyzed by western blot hybridization using either anti-FLAG (α -FLAG), antiphosphothreonine (α -P-Thr), or antiphosphoserine (α -P-Ser) followed by ECL.

hUpf1p even though the antibody did react with total HeLa cell protein suggested that hUpf1p lacks phosphotyrosine (data not shown). However, different antiphosphotyrosine antibodies are known to react with varying efficiencies to different phosphotyrosines (A.P. Fields, pers. comm.). Therefore, it is difficult to rule out tyrosine phosphorylation, especially considering that hUpf1p harbors a match to a tyrosine kinase phosphorylation site according to the Scan Prosite program. Pre-incubation of FLAG-hUpf1p with CIP prior to western blot hybridization reduced the extent of immunoreactivity with antiphosphothreonine and antiphosphoserine antibodies (Fig. 5), consistent with the sensitivity of phosphothreonine and phosphoserine to CIP.

FLAG-hUpf1p was also transiently expressed in human embryonic kidney (HEK)293 cells as a means to examine the mechanism of hUpf1p phosphorylation. Several proteins that regulate translation have been identified as targets of phosphatidylinositol 3-kinase-related kinase (PIK-related kinase) signaling pathways (see, e.g., Thomas & Hall, 1997; Abraham, 1998; Gingras et al., 1999; Raught et al., 2000). Therefore, it is possible that hUpf1p could also be a target for signaling by one or both of these pathways. To test this possibility, the status of FLAG-hUpf1p phosphorylation after serum deprivation and, subsequently, the re-addition of serum was examined. HEK293 cells were transiently transfected with pCI-neo-FLAG-hUPF1 (Sun et al., 1998) using lipofection, deprived of serum 16 h later, and serum supplemented after an additional 36 h. Cells were lysed (Gingras et al., 1998), and FLAG-hUpf1p was immunopurified and subjected to two-dimensional gel electrophoresis. Results indicated that serum does regulate FLAG-hUpf1p phosphorylation: FLAG-hUpf1p was dephosphorylated after serum deprivation and rephosphorylated after serum addition as evidenced by its two-dimensional gel electrophoretic mobility (Fig. 6A,B). In parallel, transiently transfected HEK293 cells were deprived of serum for 36 h and then pre-treated for 20 min with either wortmannin (100 nM) or rapamycin (25 nM) before serum addition. At the concentration used, wortmannin selectively inhibits PIK-related kinases by covalently binding to their catalytic subunits (see, e.g., Sarkaria et al., 1998 and references therein). Rapamycin inhibits FKBP12-rapamycin-

FIGURE 6. Serum-induced phosphorylation of FLAG-hUpf1p is sensitive to inhibitors of PIK-related-kinases. HEK293 cells were transiently transfected with pCI-neo-FLAG-hUPF1. After 16 h, the medium was replaced with serum-free medium. After an additional 36 h, cells were pretreated for 20 min with no inhibitor (**A** and **B**), 100 nM wortmannin (**C**), or 25 nM rapamycin (**D**). Serum was either not added (**A**) or added to a final concentration of 15% (**B**, **C**, and **D**), cells were lysed after 30 min, and FLAG-hUpf1p was immunopurified and subject to two-dimensional gel electrophoresis. These results were reproduced in two independently performed experiments.

associated protein/mammalian target of rapamycin (FRAP/mTOR) kinase, a type of PIK-related kinase, by binding to the kinase together with the immunophilin FKBP12 (Abraham, 1998). Results indicated that the serum-induced phosphorylation of FLAG-hUpf1p is inhibited by wortmannin and, to a lesser extent, by rapamycin (Fig. 6C,D). Considering the features of a putative PIK-related kinase that is predicted to function in mammalian-cell NMD (see Discussion), the simplest interpretation of these results is that hUpf1p phosphorylation is mediated by a wortmannin-sensitive and rapamycin-sensitive PIK-related kinase signaling pathway. However, we cannot rule out the possibility that wortmannin sensitivity derives from contributions of a PI 3-kinase (Ui et al., 1995) that functions together with a rapamycin-sensitive PIK-related kinase.

FLAG-hUpf1p binds RNA

RNA binding was also analyzed using a gel shift assay (Lee & Hurwitz, 1992; Czapinski et al., 1995). RNA binding was determined to be the measurement of choice because it is central to hUpf1p function in NMD. In particular, mutations in yeast Upf1p that disrupt RNA binding also disrupt NMD (Weng et al., 1996a). Furthermore, FLAG-hUpf1p has considerably more RNA-binding activity than either ATPase or helicase activity (data not shown).

A uniformly 32 P-labeled, 78-nt transcript was synthesized in vitro and used as substrate for binding. Compared to comparable cell equivalents of immunopurified material from untransfected HeLa cells (Fig. 7A, lane 2), FLAG-hUpf1p shifted the transcript to a more slowly migrating position (Fig. 7A, lane 4; com-

FIGURE 7. FLAG-hUpf1p binds RNA. A gel shift assay was used to determine the ability of FLAG-hUpf1p to bind to single-stranded RNA. Increasing amounts (37.5 and 125 ng) of immunopurified FLAG-hUpf1p, protein from untransfected HeLa cells (–), or no protein (NP) (A) or 37.5 ng of immunopurified FLAG-hUpf1p (+) or protein from untransfected HeLa cells (–) (B) were incubated for 15 min with $4\text{--}6 \times 10^4$ cpm (10 fmol) of uniformly ^{32}P -labeled, 78-nt transcript. Where indicated, 100 μM ATP (ATP) or 1 μL of anti-FLAG M2 antibody (Ab) was added during the last 5 min of incubation. Free RNA specifies unreacted transcript (that appears here and elsewhere as two bands because it was not boiled prior to the binding reaction), bandshift specifies transcript bound with one or more molecules of FLAG-hUpf1p, and supershift specifies bandshift that is bound by anti-FLAG antibody. Results are the average of three independently performed experiments, which did not differ by more than 5%.

pare positions of free RNA and bandshift), indicating formation of a FLAG-hUpf1p:RNA complex. An increased concentration of FLAG-hUpf1p, unlike an increased concentration of immunopurified protein from untransfected cells, resulted in formation of complexes having decreased mobility (Fig. 7A, compare lanes 2 and 3 to lanes 4 and 5), suggesting the formation of complexes having more than one FLAG-hUpf1p molecule at higher concentrations of FLAG-hUpf1p. Multimerization upon nucleic acid binding is typical of some (Finger & Richardson, 1982; Mastrangelo et al., 1989; Chao & Lohman, 1991) but not all (Hirling et al., 1989; Lee & Hurwitz, 1992; Shuman, 1992) helicases. In fact, we found that multimerization upon RNA binding is evident for FLAG-hUpf1p only at concentrations greater than 30 ng/20 μL or 12 μM (Fig. 7; data not shown), which greatly exceeds our estimated intracellular concentration of 1 μM [considering 2×10^8 packed cells occupy 1 mL and there are 0.7 pg of hUpf1p per cell (see below)]. The findings for yeast that (1) Upf1p is monomeric in solution (Czapinski et al., 1995) and (2) multiple Upf1p molecules bind to RNA provided there is an adequate concentration of Upf1p and the RNA is larger than 20 nt led to the conclusion that a single yeast Upf1p binding site consists of ~ 17 nt (Weng et al., 1996b). Our finding that FLAG-hUpf1p did not bind ef-

ficiently to a 65-nt transcript (data not shown) may indicate a larger binding site for hUpf1p.

Addition of anti-FLAG antibody but not mouse IgG after the binding reaction resulted in a supershift of the complexes formed with FLAG-hUpf1p (Fig. 7B, compare lanes 2 and 4; data not shown for IgG), offering proof that hUpf1p is a component of the complexes. Addition of 100 μM of ATP after the binding reaction reduced FLAG-hUpf1p:RNA complex formation (Fig. 7B, compare lanes 2 and 3). The ability of ATP to dissociate FLAG-hUpf1p:RNA complexes was also demonstrated for yeast FLAG-Upf1p (Weng et al., 1996a, 1998; Czapinski et al., 1998).

There are an estimated 3×10^6 molecules of hUpf1p per HeLa cell

Considering that the majority of hUpf1p associates with ribosomes, insight into its mode of action in translation termination and NMD may derive from determinations of its cellular abundance. To this end, the concentration of FLAG-hUpf1p immunopurified from the HeLa-cell transfectant expressing the highest level of FLAG-hUpf1p was derived relative to a known concentration of commercially available FLAG-bacterial alkaline phosphatase (BAP) by using western blot hybridization and

anti-FLAG antibody (Fig. 8A). The reactivities of known amounts (12, 6, 3, and 1.5 ng) of FLAG-BAP were compared to the reactivities of serial dilutions of FLAG-hUpf1p (Fig. 8B,C). The three samples of FLAG-hUpf1p that fell within the linear range of the FLAG-BAP samples revealed a FLAG-hUpf1p concentration of 19.5 ± 1.8 ng/ μ L. Next, the reactivities of known amounts (2, 1, 0.5, and 0.25 ng) of FLAG-hUpf1p were compared to the reactivities of serial dilutions of extract from 800 exponentially growing HeLa cells by using western blot hybridization and anti-hUpf1p antibody (Fig. 8D). The three samples of cell extract that fell within the linear range of the FLAG-hUpf1p samples revealed a hUpf1p concentration of $3.1 \pm 0.3 \times 10^6$ molecules per HeLa cell (Fig. 8E,F).

DISCUSSION

Upf1p is essentially equally distributed among polysomal, subpolysomal, and ribosome-free fractions in exponentially growing HeLa cells (Fig. 2), which is compatible with the finding that most of Upf1p in yeast colocalizes with polysomes (Atkin et al., 1995). Both hUpf1p and FLAG-hUpf1p are phosphoproteins (Figs. 1 and 5), as is the *C. elegans* ortholog to hUpf1p, SMG-2 (Page et al., 1999). The importance of phosphorylation to NMD in *C. elegans* is illustrated by the finding that *smg-1*, *-3*, *-4*, *-5*, *-6*, and *-7* mutants, which are defective in NMD, are defective in either the phosphorylation or dephosphorylation of SMG-2 (Page et al., 1999). Data demonstrating that hUpf1p is a phosphoprotein

are consistent with the recent identification of human orthologs to SMG-3, SMG-4 (G. Serin et al., submitted) and SMG-1 (G. Denning and A.P. Fields, pers. comm.). SMG-1 is predicted to be a PIK-related kinase (Page et al., 1999). hSmg1p is also predicted to be a PIK-related kinase (G. Denning and A.P. Fields, pers. comm.), consistent with our findings that the serum-induced phosphorylation of hUpf1p is inhibited by wortmannin (Fig. 6). Thus, it is more likely that the wortmannin sensitivity of serum-induced hUpf1p phosphorylation derives from inhibition of hSmg1p rather than inhibition of a PI 3-kinase. However, we cannot rule out the possibility that a PI 3-kinase is also involved. At this point, it is difficult to determine if the rapamycin sensitivity derives from hSmg1p or another protein. It is tempting to speculate that hSmg1p could directly phosphorylate hUpf1p because hUpf1p phosphorylation is predominantly on serine and threonine residues (Fig. 5). Other PIK-related kinase family members such as DNA-dependent protein kinase and FRAP/mTOR preferentially phosphorylate (S/T)P and (S/T)Q motifs (Bannister et al., 1993; Fadden et al., 1997), suggesting that any of the 22 SQ, 6 TQ, 2 SP, and 3 TP motifs within hUpf1p might be targets for hSmg1p. Notably, Upf1p in *S. cerevisiae* is also likely to be a phosphoprotein given that *C. elegans* SMG-3 and SMG-4 are thought to be orthologous to, respectively, *S. cerevisiae* Upf2p and Upf3p (R. Aronoff, pers. comm.; S. Kuchma and P. Anderson, pers. comm.). Consistent with the possibility of Upf1p phosphorylation in *S. cerevisiae*, epitope-tagged Upf1p appears to be posttran-

FIGURE 8. hUpf1p is present at $\sim 3 \times 10^6$ molecules per exponentially growing HeLa cell. **A:** Serial dilutions of 12 ng of FLAG-bacterial alkaline phosphatase (FLAG-BAP; Sigma) and an unknown amount of immunopurified FLAG-hUpf1p (indicated by wedge) were analyzed by western blot hybridization using monoclonal anti-FLAG-M2 antibody and ECL, and the resulting band intensities were quantitated by autoradiographic densitometry. **B,C:** The three dilutions of FLAG-hUpf1p that fell within the linear range of the FLAG-BAP dilutions revealed a FLAG-hUpf1p concentration of 19.5 ± 1.8 ng/ μ L. **D:** Subsequently, serial dilutions of 2 ng of FLAG-hUpf1p and extract from 800 exponentially growing HeLa cells were similarly analyzed except that anti-hUpf1p antibody was used in the western blot hybridization. **E,F:** The three dilutions of cell extract that fell within the linear range of the FLAG-hUpf1p dilutions revealed a hUpf1p concentration of $3.1 \pm 0.3 \times 10^6$ molecules per HeLa cell.

scriptionally modified because it has been observed to migrate as a doublet in acrylamide (Atkin et al., 1997).

Our finding that hUpf1p and FLAG-hUpf1p are maximally phosphorylated in association with polysomes and minimally phosphorylated when ribosome free (Figs. 2 and 3) indicates that there could be differences in hUpf1p specific activities at different cellular sites. Considering that RNA binding is required for RNA helicase activity and NMD, hUpf1p may be best poised for function in NMD when associated with polysomes. This makes sense in view of the proposal for yeast that polysome-associated, ATP-bound Upf1p switches upon ATP hydrolysis from function in translation termination, which involves binding to release factors (Czapinski et al., 1998), to function in NMD, which involves binding to RNA (Weng et al., 1998). Consistent with this view, evidence exists that the substrate for NMD in yeast is polysome associated (Zhang et al., 1997). Evidence already exists that phosphorylation plays a regulatory role at or around the time of mammalian-cell translation termination, considering that the catalytic subunit of protein phosphatase 2A (PP2A) associates with eRF1, presumably providing a means to recruit PP2A into polysomes (Andjelković et al., 1996). Additionally, at least in yeast, mutation of a PP1-like phosphatase can influence translation termination by enhancing the efficiency of nonsense suppression (Vincent et al., 1994).

Insight into the function of a protein that is active in translation can derive from determinations of copy number and assays for preferential associations with a particular ribosomal subunit. Our calculation of $\sim 3 \times 10^6$ copies of hUpf1p per HeLa cell (Fig. 8) indicates that there is one molecule of hUpf1p per ~ 3 ribosomes, based on our calculation of 50 pg of RNA per HeLa cell, 80% of which is rRNA. This is consistent with the possibility that hUpf1p normally functions to increase the efficiency of translation termination, as Upf1p does in yeast (Weng et al., 1998), but distinct from estimates of a yeast Upf1p:ribosome ratio of either $\sim 1:100$ (based on quantitation of Upf1p and ribosomal protein mRNAs and assumptions that mRNA and protein half-lives as well as mRNA translation rates are comparable; Atkin et al., 1995) or $\sim 1:200$ (based on comparisons of western blot hybridization intensities of epitope-tagged Upf1p present in crude extracts from a fixed number of cells to intensities of purified epitope-tagged Upf1p; Madrazo et al., 2000).

Results of experiments examining the association of hUpf1p with ribosomal subunits revealed that there is no clear-cut association of hUpf1p with one or the other ribosomal subunit: $\sim 15\%$ of hUpf1p was associated with 40S ribosomal subunits, $\sim 50\%$ of hUpf1p was associated with 60S ribosomal subunits, and the rest was associated with lower molecular weight components of the gradient (data not shown). An association of hUpf1p with 60S ribosomal subunits is consistent with findings in yeast that Upf1p interacts in a two-

hybrid screen with Nmd3p (He & Jacobson, 1995), an essential cytoplasmic protein that is required for a late cytoplasmic assembly step of the 60S subunit but is not a ribosomal protein (Ho & Johnson, 1999). However, because yeast Nmd3p is not present in polysomes (Ho & Johnson, 1999) and mammalian cells have an ortholog to Nmd3p (E.N. & L.E.M., unpubl. data), the polysome association of yeast Upf1p or hUpf1p is probably not via an association with, respectively, Nmd3p or its human ortholog.

MATERIALS AND METHODS

Plasmid constructions

pFLAG-hUPF1-IRES1neo' harboring the hUPF1 gene was generated by ligating the 3.4-kbp *NheI*-*NotI* fragment from pFLAG-hUPF1-neo (Sun et al., 1998) that includes the entire FLAG-hUpf1p open reading frame to the 5.3-kbp *NsiI*-*NsiI* fragment from pIRESNeo (CLONTECH) so that the FLAG open reading frame was adjacent to the hCMV promoter. pCI-neo-FLAG-hUPF1 was generated as described in Sun et al. (1998), where it was called pFLAG-hUPF-neo.

Cell transfections

Human HeLa cells were propagated in Dulbecco's modified Eagle medium (DMEM) containing 15% fetal calf serum, and 1×10^7 cells were stably transfected by using calcium phosphate (Wigler et al., 1979) and 40 μ g of pFLAG-hUPF1-IRES1neo' DNA. Fresh medium was added 24 h after transfection and, after an additional 24 h, cells were diluted 20-fold, replated, and cultured in the presence of 500 μ g/mL of G418 (Life Technologies, Inc.). Single colonies were isolated and expanded after 14 days in selective medium.

HEK293 cells were propagated in DMEM containing 10% fetal calf serum, and 2×10^6 cells were transiently transfected by using Lipofectamine Plus (Gibco) with 10 μ g of pCI-neo-FLAG-hUPF1. After 16 h, the medium was replaced with serum-free DMEM. After an additional 36 h, cells were pretreated for 20 min with no inhibitor, wortmannin (100 nM), or rapamycin (25 nM). Serum was then added to a final concentration of 15% to all but one of the inhibitor-lacking samples, cells were lysed after 30 min, and FLAG-hUpf1p was immunopurified and subject to two-dimensional gel electrophoresis (see below).

Analysis of hUpf1p distribution among polysomal, subpolysomal, and ribosome-free HeLa-cell fractions

HeLa cell fractions were prepared following Brewer and Ross (1990). Briefly, cells (3×10^7) were cultured as usual except cycloheximide (Sigma) was added to 5 μ g/mL 4 h prior to harvest where indicated. Cell pellets were resuspended in 10 mL of polysome buffer (10 mM Tris-HCl, pH 7.5, 1 mM potassium acetate, 1.5 mM magnesium acetate, and 2 mM dithiothreitol, 25 mM NaF, 1 mM Na-o-vanadate). Cells were lysed using five strokes of a loose-fitting Dounce homog-

enizer. Nuclei were pelleted by centrifugation at $10,000 \times g$ for 5 min at 4°C. Postnuclear supernatants were layered over a 30% sucrose pad in a SW60 rotor (Beckman) and centrifuged at 36,000 rpm for 2.5 h at 4°C. Pelleted material (polysomal fraction) was solubilized in 200 μ L of polysome buffer, material remaining either in (subpolysomal fraction) or above (ribosome-free fraction) the sucrose pad (S130 fraction) was concentrated to 200 μ L using a Microcon concentrator (Amicon), and 10 μ L of each fraction were analyzed by western blotting using either anti-hUpf1p antibody (see below) or anti-rpL10 antibody (Santa Cruz Biotechnology, Inc.) and autoradiographic densitometry (Alpha Innotech).

Purification of total-cell FLAG-hUpf1p

All reactions were performed at 0–4°C. Cytoplasmic lysates were prepared from $4\text{--}6 \times 10^9$ HeLa cells that were untransfected or expressed the highest level of FLAG-hUpf1p. Notably, the transfectant demonstrated the same degree of NMD as untransfected cells (data not shown). Briefly, cells were washed in ice-cold PBS and lysed in extraction buffer [20 mM HEPES, pH 7.4, 150 mM KCl, 5% glycerol, 0.5% Triton-X 100, 1 mM DTT, 1 mM PMSF, and 1 μ g/mL each of leupeptin (Sigma), pepstatin A (Sigma), and aprotinin (Sigma)]. Lysates were cleared at $10,000 \times g$ for 20 min to remove nuclei and insoluble material (Cervera et al., 1981). Cleared lysates (100 mL) were mixed with anti-FLAG immunoaffinity beads (0.75 mL; Sigma), incubated for 4 h at 4°C with gentle shaking, and poured into a column. Bound material was subsequently washed three times with 20 column volumes of binding buffer containing, sequentially, 150 mM, 450 mM, and 150 mM KCl. FLAG-hUpf1p was then eluted using 5×0.75 mL of binding buffer containing 300 μ g/mL of FLAG peptide, and analyzed by Coomassie blue and silver staining after SDS-PAGE and by western blotting using either anti-FLAG M2 or anti-hUpf1p antibody (see below). Comparisons of the relative amounts of FLAG-hUpf1p in the load, flow-through, bound [i.e., released from FLAG-antibody beads by boiling in Laemmli sample buffer (Laemmli, 1970) prior to elution], immunoeluate (released using FLAG peptide), and SDS-eluate (released using boiling in 2% SDS) fractions demonstrated that most (90%) of FLAG-hUpf1p loaded onto an immunoaffinity column binds to the column but only ~30% was eluted with FLAG peptide. Yields of FLAG-hUpf1p were determined by densitometric comparisons of serial dilutions of eluates and known quantities of FLAG-bacterial alkaline phosphatase (FLAG-BAP; Sigma) that had been electrophoresed in SDS-polyacrylamide and subjected to western blot hybridization using anti-FLAG M2 antibody.

Whole-cell lysates were prepared from 2×10^6 HEK293 cells that had been transiently transfected with pCI-neo-FLAG-hUPF1. Cells were lysed as described in Gingras et al. (1998). Cleared lysates (500 μ L of a total of 1 mL) were mixed with anti-FLAG M5 antibody (Sigma) using end-over-end rotation at 4°C for 2 h. Protein A-agarose (30 μ L; Boehringer Mannheim) and protein G-agarose (30 μ L; Boehringer Mannheim) were added and the rotation was continued for an additional 2 h. Bound material was subsequently washed two times with 1 mL of lysis buffer, then two times with RIPA buffer, and then two times with LiCl solution (Gingras et al., 1998). Bound material was resuspended in 84 μ L of Laemmli buffer and stored at –70°C until ready for use.

Western blot analysis of hUpf1p and immunopurified FLAG-hUpf1p

Protein concentration was determined by using a Micro BCA kit (Pierce) and BSA as a standard. Protein (10 μ g) was electrophoresed in 10% SDS-polyacrylamide, transferred to Hybond-C nitrocellulose (Amersham), and probed with anti-FLAG M2 antibody, polyclonal antibody against hUpf1p, polyclonal antiphosphothreonine antibody (Zymed Labs, Inc.), polyclonal antiphosphoserine antibody (Zymed Labs, Inc.) or monoclonal antiphosphotyrosine antibody (Zymed Labs, Inc.). Rabbit polyclonal antiserum was raised (Advanced ChemTech) against GST-hUpf1p that includes the C-terminal 271 amino acids of hUpf1p. Anti-hUpf1p antibody was then purified by using an ImmunoPure(G) IgG Kit (Pierce). Reactivity of the monoclonal antibodies was detected using peroxidase-conjugated rat anti-mouse antibody (Amersham), and reactivity of polyclonal antibodies against hUpf1p, phosphothreonine or phosphoserine was detected using donkey anti-rabbit antibody (Amersham). Reactivity of the secondary antibody was visualized by using enhanced chemiluminescence (ECL; Amersham).

Two-dimensional PAGE

The two-dimensional analysis of proteins was performed according to O'Farrell (1975) with modifications suggested by the manual for the 2D-Mini Protean II gel apparatus (Bio-Rad). Cytoplasmic lysates from untransfected HeLa cells or HeLa cells expressing FLAG-hUpf1p were isolated in extraction buffer in the presence or absence of 1 mM Na-o-vanadate and 50 mM NaF, and an equal volume of isoelectric focusing (IEF) sample buffer [9 M urea with Bio-lytes (Biorad)] was added. Samples were subsequently incubated with or without CIP (see below), as specified. In the first dimension, IEF was performed in the presence of a mixture of Bio-lytes, pH 3–10 and pH 5–7. In the second dimension, separation according to molecular mass was performed by electrophoresis in 10% SDS-polyacrylamide. Gels were electroblotted onto Hybond-C nitrocellulose and probed with either anti-hUpf1p antibody or anti-FLAG M2 antibody. Alternatively, 10 μ L of FLAG-hUpf1p immunopurified from HEK293 cells was similarly analyzed, except that the second dimension consisted of 7.5% SDS-polyacrylamide and anti-FLAG M5 antibody was used.

Phosphatase treatment

CIP (New England Biolabs; 10 U) was added to various hUpf1p preparations in 20 μ L of 50 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 mM MgCl₂, and 1 mM DTT, and incubated for 20 min at 30°C.

RNA binding assay

The ability of FLAG-hUpf1p to bind RNA was determined by using an electrophoretic mobility shift assay (Lee & Hurwitz, 1992; Czaplinski et al., 1995). A uniformly ³²P-labeled, 78-nt RNA was synthesized by T7 RNA polymerase (Promega) from 1 μ g of BamHI-linearized pGEM-7Zf(+) DNA (Promega) in the presence of 50 μ Ci of [α -³²P]-GTP (3,000 Ci/

mmol), 0.5 mM each of ATP, CTP, and UTP, and 50 μ M of GTP. The resulting RNA was electrophoresed in a denaturing 7.5% polyacrylamide gel and purified by elution at 4°C in 300 mM sodium acetate, pH 4.5, 1 mM EDTA, 10% phenol, and, subsequently, ethanol precipitation. RNA binding reactions were as described elsewhere (Czapinski et al., 1995). Briefly, binding was done in 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 3 mM MgCl₂, and 1 mM DTT in 20 μ L with 12.5–50 ng of FLAG-hUpf1p and 2–4 $\times$ 10⁴ cpm (10 fmol) of ³²P-labeled RNA for 15 min at 37°C. When specified, 100 μ M of ATP or 1 μ L of anti-FLAG antibody was added during the last 5 min of incubation. Reactions were mixed with 5 μ L of 10 mM EDTA, 0.1% of bromophenol blue, 0.1% of xylene cyanol, and 5% glycerol and electrophoresed in a 5% polyacrylamide (29:1 acrylamide:bis) gel. Gels were dried, and the amount of complex formation was quantitated by PhosphorImaging or autoradiographic densitometry.

Calculating hUpf1p abundance in HeLa cells

Serial dilutions of 12 ng of FLAG-BAP and an unknown amount of immunopurified FLAG-hUpf1p were analyzed by western blot hybridization using anti-FLAG M2 antibody and ECL. Band intensities were quantitated by densitometric scanning. The three dilutions of FLAG-hUpf1p that fell within the linear range of the FLAG-BAP dilutions were used to calculate the concentration of FLAG-hUpf1p. Next, HeLa cells (2 $\times$ 10⁷) grown to 70% confluency were resuspended in 0.5 mL of PBS and boiled for 10 min at 100°C after the addition of an equal volume of 2 $\times$ Laemmli sample buffer. Serial dilutions of 2 ng of FLAG-hUpf1p and boiled extract from 800 cells were analyzed as described for the previous dilutions except that anti-hUpf1p antibody was used in the western blot hybridization. Based on experiments demonstrating 200 pg of protein per cell (data not shown), the three dilutions of cell extract that fell within the linear range of FLAG-hUpf1p were used to calculate the hUpf1p concentration per HeLa cell.

ACKNOWLEDGMENTS

The authors thank Issac Bernstein-Hanley for help purifying GST-hUpf1p, Saikat Pal for help with the kinase inhibitor studies, Josee Dostie and Nahum Sonenberg for HEK293 cells, Gabriela Denning, Alan Fields, Rachel Aronoff, Sandy Kuchma, and Phil Anderson for communicating unpublished results, and John Yates, Kevin Czapinski, Stuart Peltz, Gabriela Denning, Alan Fields, Brian Raught, and members of the Maquat lab for useful conversations. This work was supported by Public Health Service Research Grant DK33933 to L.E.M.

Received January 21, 2000; returned for revision
March 7, 2000; revised manuscript received
September 28, 2000

REFERENCES

- Abraham RT. 1998. Mammalian target of rapamycin B immunosuppressive drugs uncover a novel pathway of cytokine receptor signaling. *Curr Opin Immunol* 10:330–336.
- Andjelkovic N, Zolnierowicz S, Van Hoof C, Goris J, Hemmings BA. 1996. The catalytic subunit of protein phosphatase 2A associates with the translation termination factor eRF1. *EMBO J* 15:7156–7167.
- Applequist SE, Selg M, Raman C, Jäck H-M. 1997. Cloning and characterization of HUPF1, a human homolog of the *Saccharomyces cerevisiae* nonsense mRNA-reducing UPF1 protein. *Nucleic Acids Res* 25:814–821.
- Atkin AL, Altamura N, Leeds P, Culbertson MR. 1995. The majority of yeast UPF1 co-localizes with polyribosomes in the cytoplasm. *Mol Biol Cell* 6:611–625.
- Atkin AL, Schenkman LR, Eastham M, Dahlseid JN, Lelivelt MJ, Culbertson MR. 1997. Relationship between yeast polyribosomes and Upf proteins required for nonsense mRNA decay. *J Biol Chem* 272:22163–22172.
- Bannister AF, Gottlieb TM, Kouzarides T, Jackson SP. 1993. c-Jun is phosphorylated by the DNA-dependent protein kinase in vitro: Definition of the minimal kinase recognition motif. *Nucleic Acids Res* 21:1289–1295.
- Brewer G, Ross J. 1990. Messenger RNA turnover in cell-free extracts. *Methods Enzymol* 181:202–209.
- Cali BM, Anderson P. 1998. mRNA surveillance mitigates genetic dominance in *Caenorhabditis elegans*. *Mol Gen Genet* 260:176–184.
- Cervera M, Dreyfuss G, Penman S. 1981. Messenger RNA is translated when associated with the cytoskeletal framework in normal and VSV-infected HeLa cells. *Cell* 23:113–120.
- Chao K, Lohman TM. 1991. DNA-induced dimerization of the *Escherichia coli* Rep helicase. *J Mol Biol* 221:1165–1181.
- Coligan JE, Dunn BM, Ploegh HL, Speicher DW, Wingfield PT. eds. 1997. *Current protocols in protein science*, vol. 2. John Wiley & Sons, Inc. p 13.5.3.
- Cui Y, Dinman JD, Peltz SW. 1996. Mof4-1 is an allele of the UPF1/IFS2 gene which affects both mRNA turnover and –1 ribosomal frameshifting efficiency. *EMBO J* 15:5726–5736.
- Cui Y, Hagan KW, Zhang S, Peltz SW. 1995. Identification and characterization of genes that are required for the accelerated degradation of mRNAs containing a premature translational termination codon. *Genes & Dev* 9:423–436.
- Czapinski K, Ruiz-Echevarria MJ, Paushkin SV, Han X, Weng Y, Perlick HA, Dietz HC, Ter-Avanesyan MD, Peltz SW. 1998. The surveillance complex interacts with the translation release factors to enhance termination and degrade aberrant mRNAs. *Genes & Dev* 12:1665–1677.
- Czapinski K, Weng Y, Hagan KW, Peltz SW. 1995. Purification and characterization of the Upf1p: A factor involved in translation and mRNA degradation. *RNA* 1:610–623.
- Dahlseid JN, Puziss J, Shirley RL, Atkin AL, Hieter P, Culbertson MR. 1998. Accumulation of mRNA coding for the ctf13p kinetochore subunit of *Saccharomyces cerevisiae* depends on the same factors that promote rapid decay of nonsense mRNAs. *Genetics* 150:1019–1035.
- Fadden P, Haystead TAJ, Lawrence JC Jr. 1997. Identification of phosphorylation sites in the translational regulator, PHAS-1, that are controlled by insulin and rapamycin in rat adipocytes. *J Biol Chem* 272:10240–10247.
- Finger LR, Richardson JP. 1982. Stabilization of the hexameric form of *Escherichia coli* protein rho under ATP hydrolysis conditions. *J Mol Biol* 156:203–219.
- Gingras A-C, Kennedy SG, O'Leary MA, Sonenberg N, Hay N. 1998. 4E-BP1, a repressor of mRNA translation is inactivated by the Akt (PKB) signaling pathway. *Genes & Dev* 12:502–513.
- Gingras A-C, Raught B, Sonenberg N. 1999. eIF4 initiation factors: Effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem* 68:913–963.
- He F, Brown AH, Jacobson A. 1997. Upf1p, Nmd2p and Upf3p are interacting components of the yeast nonsense-mediated mRNA decay pathway. *Mol Cell Biol* 17:1580–1594.
- He F, Jacobson A. 1995. Identification of a novel component of the nonsense-mediated mRNA-decay pathway by use of an interacting-protein screen. *Genes & Dev* 9:437–454.
- Hentze M, Kulozik AE. 1999. A perfect mRNA: RNA surveillance and nonsense-mediated decay. *Cell* 96:307–310.
- Hirling H, Scheffner M, Restle T, Stahl H. 1989. RNA helicase activity associated with the human p68 protein. *Nature* 339:562–564.
- Ho J H-N, Johnson AW. 1999. NMD3 encodes an essential cytoplasmic protein required for stable 60S ribosomal subunits in *Saccharomyces cerevisiae*. *Mol Cell Biol* 19:2389–2399.

- Kanopka A, Mühlemann Petersen-Mahrt S, Estmer C, Öhrmalm C, Akusjärvi G. 1998. Regulation of adenovirus alternative RNA splicing by dephosphorylation of SR proteins. *Nature* 393:185–187.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Lee BS, Culbertson MR. 1995. Identification of an additional gene required for eukaryotic nonsense mRNA turnover. *Proc Natl Acad Sci USA* 92:10354–10358.
- Lee C, Hurwitz J. 1992. A new RNA helicase isolated from HeLa cells that catalytically translocates in the 3' to 5' direction. *J Biol Chem* 267:4398–4407.
- Leeds P, Peltz SW, Jacobson A, Culbertson MR. 1991. The product of the yeast UPF1 gene is required for rapid turnover of mRNAs containing a premature translational termination codon. *Genes & Dev* 5:2303–2314.
- Leeds P, Wood JM, Lee BS, Culbertson MR. 1992. Gene products that promote mRNA turnover in *Saccharomyces cerevisiae*. *Mol Cell Biol* 12:2165–2177.
- Lelivelt MJ, Culbertson MR. 1999. Yeast Upf proteins required for RNA surveillance affect global expression of the yeast transcriptome. *Mol Cell Biol* 19:6710–6719.
- Lew JE, Enomoto S, Berman J. 1998. Telomere length regulation and telomeric chromatin require the nonsense-mediated mRNA decay pathway. *Mol Cell Biol* 18:6121–6130.
- Li S, Wilkinson MF. 1998. Nonsense surveillance in lymphocytes? *Immunity* 8:135–141.
- Lund E, Dahlberg JE. 1998. Proofreading and aminoacylation of tRNAs before export from the nucleus. *Science* 282:2082–2085.
- Maderazo A, He F, Mangus DA, Jacobson A. 2000. Upf1p control of nonsense mRNA translation is regulated by Nmd2p and Upf3p. *Mol Cell Biol* 20:4591–4603.
- Maquat LE. 1995. When cells stop making sense: Effects of nonsense codons on RNA metabolism in vertebrate cells. *RNA* 1:453–465.
- Maquat LE. 1996. Defects in RNA splicing and the consequence of shortened translational reading frames. *Am J Hum Genet* 59:279–286.
- Maquat LE. 2000. Nonsense-mediated RNA decay in mammalian cells: A splicing-dependent means to down-regulate the levels of mRNAs that prematurely terminate translation. In Sonenberg N, Hershey JWB, Mathews MB, eds. *Translational Control of Gene Expression*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 849–868.
- Mastrangelo IA, Hough PVC, Wall JS, Dodson M, Dean FB, Hurwitz J. 1989. ATP-dependent assembly of double hexamers of SV40 T antigen at the viral origin of DNA replication. *Nature* 338:658–662.
- Morrison M, Harris KS, Roth B. 1997. Smg mutants affect the expression of alternatively spliced SR protein mRNAs in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* 94:9782–9785.
- O'Farrell PH. 1975. High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250:4007–4021.
- Page MF, Carr B, Anders KR, Grimson A, Anderson P. 1999. SMG-2 is a phosphorylated protein required for mRNA surveillance in *Caenorhabditis elegans* and related to Upf1p of yeast. *Mol Cell Biol* 19:5943–5951.
- Peltz SW, Brown AH, Jacobson AJ. 1993. mRNA destabilization triggered by premature translational termination depends on at least three *cis*-acting sequence elements and one *trans*-acting factor. *Genes & Dev* 7:1737–1754.
- Perlick HA, Medghalchi SM, Spencer FA, Kendzior RJ Jr, Dietz HC. 1996. Mammalian orthologues of a yeast regulator of nonsense transcript stability. *Proc Natl Acad Sci USA* 93:10928–10932.
- Pulak R, Anderson P. 1993. mRNA surveillance by the *Caenorhabditis elegans* smg genes. *Genes & Dev* 7:1885–1897.
- Raught B, Gingras A-C, Gygi SP, Imitaka H, Morino S, Gradi A, Aebersold R, Sonenberg N. 2000. Serum-stimulated, rapamycin-sensitive phosphorylation sites in the eukaryotic translation initiation factor 4G1. *EMBO J* 19:434–444.
- Ruiz-Echevarria MJ, Czapinski K, Peltz SW. 1996. Making sense of nonsense in yeast. *Trends Biochem Sci* 21:433–438.
- Ruiz-Echevarria MJ, Yasenchak JM, Han X, Dinman JD, Peltz SW. 1998. The Upf3 protein is a component of the surveillance complex that monitors both translation and mRNA turnover and affects viral propagation. *Proc Natl Acad Sci USA* 95:8721–8726.
- Sarkaria JN, Tibbetts RS, Busby EC, Kennedy AP, Hill DE, Abraham RT. 1998. Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. *Cancer Res* 58:4375–4382.
- Serin G, Gersappe A, Black JD, Aronoff R, Maquat LE. Identification and characterization of human orthologues to *Saccharomyces cerevisiae* Upf2 protein and Upf3 protein/*Caenorhabditis elegans* SMG-4. Submitted.
- Shuman S. 1992. Vaccinia virus RNA helicase: An essential enzyme related to the DE-H family of RNA-dependent NTPases. *Proc Natl Acad Sci USA* 89:10935–10939.
- Sun X, Perlick H, Dietz HC, Maquat LE. 1998. A mutated homologue of yeast Upf1 protein has a dominant-negative effect on the decay of nonsense-containing mRNAs in mammalian cells. *Proc Natl Acad Sci USA* 95:10009–10014.
- Thomas G, Hall MN. 1997. TOR signaling and control of cell growth. *Curr Opin Cell Biol* 9:782–787.
- Ui M, Okada T, Hazeki K, Haseki O. 1995. Wortmannin as a unique probe for an intracellular signaling protein, phosphoinositide 3-kinase. *Trends Biochem Sci* 20:303–307.
- Vincent A, Newnam G, Lieberman SW. 1994. The yeast translational allosuppressor, SAL6: A new member of the PP1-like phosphatase family with a long serine-rich N-terminal extension. *Genetics* 138:597–607.
- Weng Y, Czapinski K, Peltz SW. 1996a. Genetic and biochemical characterization of the mutations in the ATPase and helicase regions of Upf1 protein. *Mol Cell Biol* 16:5477–5490.
- Weng Y, Czapinski K, Peltz SW. 1996b. Identification and characterization of mutations in the UPF1 gene that affect nonsense suppression and the formation of the Upf protein complex, but not mRNA turnover. *Mol Cell Biol* 16:5491–5506.
- Weng Y, Czapinski K, Peltz SW. 1998. ATP is a cofactor of the Upf1 protein that modulates its translation termination and RNA binding activities. *RNA* 4:205–214.
- Wigler M, Sweet R, Kim GK, Wold B, Pellicer A, Lacy E, Maniatis T. 1979. Transformation of mammalian cells with genes from prokaryotes and eucaryotes. *Cell* 16:777–785.
- Zhang J, Maquat LE. 1997. Evidence that translation reinitiation abrogates nonsense-mediated mRNA decay in mammalian cells. *EMBO J* 16:826–833.
- Zhang S, Welch EM, Hogan K, Brown AH, Peltz SW, Jacobson A. 1997. Polysome-associated mRNAs are substrates for the nonsense-mediated mRNA decay pathway in *Saccharomyces cerevisiae*. *RNA* 3:234–244.