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The hnRNA-Binding Proteins hnRNP L and PTB Are Required for Efficient Translation of the Cat-1 Arginine/Lysine Transporter mRNA during Amino Acid Starvation▽†

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The response to amino acid starvation involves the global decrease of protein synthesis and an increase in the translation of some mRNAs that contain an internal ribosome entry site (IRES). It was previously shown that translation of the mRNA for the arginine/lysine amino acid transporter Cat-1 increases during amino acid starvation via a mechanism that utilizes an IRES in the 5′ untranslated region of the Cat-1 mRNA. It is shown here that polypyrimidine tract binding protein (PTB) and an hnRNA binding protein, heterogeneous nuclear ribonucleoprotein L (hnRNP L), promote the efficient translation of Cat-1 mRNA during amino acid starvation. Association of both proteins with Cat-1 mRNA increased during starvation with kinetics that paralleled that of IRES activation, although the levels and subcellular distribution of the proteins were unchanged. The sequence CUUUCU within the Cat-1 IRES was important for PTB binding and for the induction of translation during amino acid starvation. Binding of hnRNP L to the IRES or the Cat-1 mRNA in vivo was independent of PTB binding but was not sufficient to increase IRES activity or Cat-1 mRNA translation during amino acid starvation. In contrast, binding of PTB to the Cat-1 mRNA in vivo required hnRNP L. A wider role of hnRNP L in mRNA translation was suggested by the decrease of global protein synthesis in cells with reduced hnRNP L levels. It is proposed that PTB and hnRNP L are positive regulators of Cat-1 mRNA translation via the IRES under stress conditions that cause a global decrease of protein synthesis.

*C*ationic *a*mino acid *t*ransporter *1* (Cat-1) is a high-affinity Na^+-independent transporter of L-arginine and L-lysine belonging to system y+ (12, 62). Growth factors, hormones, and nutrients can modulate its expression level (20, 53). Expression of the Cat-1 gene increases during stress in a manner that involves phosphorylation of translation initiation factor 2α (eIF2α) (24). During such conditions, expression of the Cat-1 gene is regulated at the level of (i) mRNA synthesis via the transcription factor ATF4, which binds an amino acid response element in the first exon of the gene (54); (ii) mRNA stability via the binding of the nucleocytoplasmic protein HuR to an AU-rich element present within its 3′ untranslated region (UTR) (94); and (iii) translation via a cap-independent mechanism of initiation via an *i*nternal *r*ibosome *e*ntry *s*ite (IRES) (23).

IRES-dependent translation involves the recruitment of ribosomes independently of the m^7G cap at the 5′ end of the mRNA followed by initiation downstream of the ribosome binding site (46). We showed that increased translation of the Cat-1 mRNA during amino acid starvation requires the translation of a 48-amino-acid open reading frame (ORF) in the 5′ UTR of the mRNA, introducing the concept of a "dynamic IRES" (23, 93). In the absence of upstream ORF (uORF) translation, the Cat-1 IRES remains in an inactive conformation. During starvation, translation of the uORF unwinds this secondary structure, leading to formation of a conformation that has IRES activity. Previous studies have also proposed that the active conformation is stabilized by proteins (*I*RES *trans*-*a*ctivating *f*actors [ITAFs]) that are either synthesized or modified by processes that require eIF2α phosphorylation. We also showed that decreased translation elongation rates of the uORF in fed cells result in a prolonged half-life of this active remodeled structure, mimicking the role of ITAFs in amino acid-starved cells (21). Ribosome stalling within the uORF can occur physiologically during stresses that cause increased phosphorylation of elongation factor 2 and therefore decreased translation elongation rates (63). These two mechanisms of induction of Cat-1 IRES activity can therefore cooperate in achieving maximum Cat-1 protein levels during stress.

It is now evident that many eukaryotic mRNAs encoding key survival proteins can be translated via cap-independent IRES-mediated mechanisms. These mechanisms are activated under a variety of stress conditions, including starvation for growth factors/nutrients, heat shock, endoplasmic reticulum stress, hypoxia, and viral infections as well as during the G_2/M phase

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of the cell cycle and apoptosis (46, 83). The requirement for canonical factors (eIF4A, eIF4G, eIF4B, eIF2α, etc.) for IRES-mediated initiation on these mRNAs also varies. Translation driven by the c-*myc* and BiP IRESs is resistant to cleavage of eIF4G, but eIF4A activity is required (85). Furthermore, translation from several IRESs (14, 32, 33, 37, 74, 82) is maintained during programmed cell death accompanied by the caspase-dependent cleavage of initiation factors (11).

Besides canonical translation initiation factors, ITAFs that modulate IRES functions have been identified (80). These ITAFs can modulate IRES activity by affecting ribosome recruitment and/or modifying IRES structures. For example, polypyrimidine tract binding protein (PTB), unr, La autoantigen, and heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP C1/C2) stimulate the activity of several IRES elements (35, 36, 44, 56), whereas HuR inhibits p27 IRES activity (48). Many of these "noncanonical" translation initiation factors belong to a family of RNA binding proteins known as hnRNPs, such as A1, C1/C2, I, E1/E2, L, and K. hnRNPs are involved in a variety of cellular functions including transcription and pre-mRNA splicing, as well as mRNA export, translation, localization, and stabilization (4, 19, 47, 67). The noncanonical translation initiation factors which have been shown to regulate IRES activity include PTB (also known as hnRNP I), hnRNP C1/C2, La autoantigen, poly(rC) binding proteins 1 and 2, and unr. Nonetheless, different IRESs are regulated by different sets of these ITAFs (46, 83). Some of the ITAFs can also have dual functions, such as hnRNP A1. hnRNP A1 was recently shown to interact with the fibroblast growth factor 2 (FGF-2) IRES and increase FGF-2 IRES activity (2). This is in contrast to the negative regulation of XIAP IRES activity (50). ITAFs are also reported to shuttle between the nucleus and the cytoplasm (5), and levels in the cytoplasm vary under different stress conditions and in different cell types (49). Indeed, hnRNP C1/C2 was found to translocate from the nucleus to the cytoplasm during the G_2/M phase of the cell cycle, resulting in an increase in IRES-dependent expression of the c-myc protein (43). IRES activity can also be regulated in a cell/tissue-specific manner as documented for FGF-2 and c-myc during murine embryogenesis (16, 17).

Amino acid starvation is a stress that activates the GCN2/eIF2α phosphorylation program (34, 61). Similar to the reaction to other stresses (endoplasmic reticulum stress, hypoxia, UV radiation, etc), cells respond by decreasing global protein synthesis and increasing translation of mRNAs that code for proteins important for adaptation to stress, including the Cat-1 protein (1). Translation of the Cat-1 mRNA during starvation is induced by binding of ITAF(s) to the Cat-1 IRES (93). In this study we identified two ITAFs involved in the starvation-induced expression of Cat-1. The data support the role of hnRNP L and PTB in Cat-1 mRNA translation and a direct role of PTB in regulation of Cat-1 IRES activity during nutritional stress. It is shown that hnRNP L and PTB associate with the Cat-1 IRES in vitro and in vivo and that these associations are well correlated with increased Cat-1 IRES activity. We propose a model in which, when eIF2α is phosphorylated during stress, the 40S ribosome, PTB, and hnRNP L are part of a complex with IRES-containing mRNA leaders that allows efficient translation initiation of stress response mRNAs such as Cat-1.

MATERIALS AND METHODS

Cell culture and transfections. Culture of C6 rat glioma cells and depletion of amino acids were performed as described previously (23). Mouse embryonic fibroblasts (MEFs) with wild-type (S/S) or mutant (S51A) eIF2α (A/A) were from Randall Kaufman (University of Michigan). Plasmid DNAs were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For transfections with small interfering RNAs (siRNAs) (Dharmacon), cells were seeded at 2×10^5/well in 35-mm wells, transfected after 1 day with 50 nM siRNA using 5 μl Lipofectamine RNAiMax transfection reagent (Invitrogen) in 1 ml growth medium according to the manufacturer's protocol, and used for experiments after 48 h or as indicated. This protocol resulted in a 70 to 90% decrease of target protein levels. In order to achieve a 25% decrease of target protein, the same protocol was applied with cells seeded at 10^5/well.

Plasmids. Bicistronic vectors containing the chloramphenicol acetyltransferase (CAT) ORF followed by the 5′ UTR of Cat-1 mRNA and the luciferase (LUC) ORF were generated as described previously (22, 93). A bacterial expression vector encoding FLAG-tagged human hnRNPL (pf-hnRNPL) was created from pf-TBP (gift of Cheng-Ming Chiang, University of Texas Southwestern University) and pcDNA3-hnRNPL (gift of Gideon Dreyfuss, University of Pennsylvania) using standard methods. The hnRNP L-green fluorescent protein (GFP) fusion protein containing GFP connected to the C terminus of hnRNP L via the linker GGISGGGSGGGGS was expressed from the cDNA in pcDNA3. A bacterial expression vector for His-tagged PTB (pET28-PTB) was from Thomas A. Cooper (Baylor College of Medicine, TX).

Bacterial expression of recombinant hnRNP L and PTB. Recombinant hnRNP L and PTB were expressed in *Escherichia coli* BL21(DE3) from pf-hnRNPL and pET28-PTB vectors. Cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h and collected by centrifugation. FLAG-tagged hnRNP L was purified essentially as described previously (8). For PTB purification, cell pellets were lysed by three freeze-thaw cycles in 20 mM HEPES-KOH, pH 7.9, 1 M NaCl, 10 mM imidazole, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, and centrifuged for 15 min at $15{,}500 \times g$. His-tagged PTB was purified from the supernatant using nickel-nitrilotriacetic acid spin columns (Qiagen) according to the manufacturer's protocol. Protein purity and integrity were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

In vitro transcription. In vitro transcription was performed with 2 μg DNA template and 20 U of T7 RNA polymerase (New England Biolabs) for 2 h at 37°C according to the manufacturer's protocol. Radiolabeled RNA was prepared in the same way except that reaction mixtures contained 0.1 mM CTP and 20 μCi [α-^{32}P]CTP. Biotinylated RNA was made using 0.5 mM biotin-14-CTP (Invitrogen) and 0.5 mM CTP. The RNAs were purified on polyacrylamide gels before use.

Preparation of cytoplasmic extracts and RNA affinity pulldown assays. To purify proteins that interact with Cat-1(−270) RNA, C6 cells were lysed in cytoplasmic extract buffer (CEB) (10 mM HEPES-KOH, pH 7.6, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.25% NP-40, EDTA-free Complete Mini protease inhibitor, 1 mM NaF, and 1 mM Na_3VO_4). The lysates were centrifuged at $16{,}200 \times g$ for 15 min at 4°C, and the supernatants were used for RNA affinity pulldown. In vitro-transcribed RNA and biotinylated DNA oligonucleotide complementary to the 3′ end of the RNA (150 pmol each) were annealed in CEB. Cytoplasmic extracts (3 mg protein) were incubated with biotinylated DNA-RNA complexes and 400 U/ml of RNase inhibitor RNasin (Roche) in 600 μl of CEB at 4°C for 30 min. The complexes were captured by incubating mixtures for 30 min at 4°C on 150 μl Dynabeads (Dynabeads M-280 streptavidin; Invitrogen). The beads were washed five times with 800 μl CEB, and the associated proteins were eluted in 100 μl 1× SDS-polyacrylamide sample buffer. Eluates (30 μl) were run on a 10% SDS-polyacrylamide gel. The regions of interest were excised from Coomassie blue-stained gels and digested overnight at room temperature with trypsin (20 ng/μl) in 50 mM ammonium bicarbonate. The peptides were extracted with 50% acetonitrile-5% formic acid and analyzed by liquid chromatography-mass spectrometry (Cleveland Clinic Foundation Mass Spectrometry Facility).

Polyribosome analysis. C6 cells transiently transfected with siRNAs were starved for amino acids, and polyribosomes were analyzed as described previously (52). rRNAs and proteins were analyzed on agarose gels and by Western blotting, respectively. mRNAs were analyzed by quantitative real-time reverse transcriptase PCR (qRT-PCR) as described previously (52).

Immunoprecipitations. The interactions of hnRNP L and PTB with endogenous mRNAs in vivo were detected by immunoprecipitation followed by RT-PCR analysis. Cytoplasmic extracts (500 μg protein in 10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.25% NP-40), nuclear extracts (200 μg protein

in 20 mM HEPES, pH 7.9, 0.45 M NaCl, and 1 mM EDTA), or ribosomal extracts (200 μg protein in 10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.25% NP-40) were incubated with EDTA-free Complete Mini protease inhibitor (Roche), 0.2 U/μl RNase inhibitor (Roche), 4 μg mouse immunoglobulin G (IgG), and 20 μl protein G beads (Sigma). The samples were centrifuged, and the supernatants were incubated with 4 μg primary antibody for 1.5 h at 4°C. Protein G beads (20 μl) were added, and the samples were incubated for 4 h at 4°C. The mixtures were washed five times with 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.03% NP-40, and RNA was extracted with 300 μl Trizol reagent (Invitrogen) and 60 μl chloroform-isoamyl alcohol. The RNA was precipitated with an equal volume of isopropanol, and the pellets were washed with 70% ethanol and suspended in 20 μl diethyl pyrocarbonate-treated water. Total RNA was also isolated from the extracts. mRNAs were detected by RT-PCR according to the manufacturer's protocol (SuperScript III; Invitrogen). RNA (3 μl of the immunoprecipitate or 1 μg total RNA) was used in the reverse transcriptase reactions using specific primers (see Table S1 in the supplemental material) in 20 μl. The cDNA products were amplified by PCR using specific primers.

UV cross-linking assays and EMSAs. Recombinant proteins (0.2 to 0.4 μg) were incubated with radiolabeled RNA (2×10^5 to 5×10^5 cpm) in 10 mM HEPES-KOH, pH 7.9, 2.5 mM $MgCl_2$, 100 mM KCl, 0.5 mM DTT, 0.5 mM EDTA containing 0.05 μg/μl yeast tRNA in a 25- to 30-μl final volume for 20 min at room temperature. Either the mixtures were loaded on 6% native polyacrylamide gels for electrophoretic mobility shift assay (EMSA), or the samples were irradiated under UV light (SpectroLinker UV Cross-linker XL-1000; Spectronics Corporation) for 20 min on ice. These samples were digested with RNase A and analyzed by 10% SDS-PAGE, and the radioactivity was detected with a Molecular Dynamics PhosphorImager or autoradiography.

Preparation of ribosome-enriched fractions. Ribosome-enriched fractions were collected essentially as described previously (92). C6 cells were homogenized in 20 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 300 mM KCl, 10 mM DTT, 100 U/ml RNasin, and 100 μg/ml cycloheximide. After centrifugation at 10,000 × *g* for 15 min at 4°C, the supernatant was layered over 20% (wt/vol) sucrose in the above buffer containing cycloheximide and centrifuged at 60,000 rpm for 2 h in a Beckman Optima TLX ultracentrifuge. Pellets were collected and suspended in 10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.25% NP-40, and EDTA-free Complete Mini protease inhibitor. The fractions were used for immunoprecipitation and for mRNA analysis by RT-PCR.

Other methods. Cells were metabolically labeled with [^{35}S]Met for 1 h, and the incorporated radioactivity was measured as described previously (21). Assays of LUC and CAT activity and determination of protein concentration were performed as described previously (22). Cytosolic and nuclear extracts were prepared as described previously (90). Transport system y+ activity was measured by following the uptake of L-[^{3}H]arginine (0.1 mM, 7.5 μCi/ml) for 30 s at 37°C in Earle's balanced salt solution in the presence of sodium and 2 mM leucine, which prevents uptake through system y+L (88). Proteins were detected on Western blots with antibodies to Cat-1 (51), murine PTB (Zymed), hnRNP L (Immunoquest), tubulin (Sigma), ATF3 and ATF4 (Santa Cruz Biotechnology), PCNA (Cell Signaling), γ-actin (Chemicon International), and rpS5 (US Biological) and with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgGs as secondary antibodies. Antibodies were visualized by enhanced chemiluminescence (Perkin-Elmer).

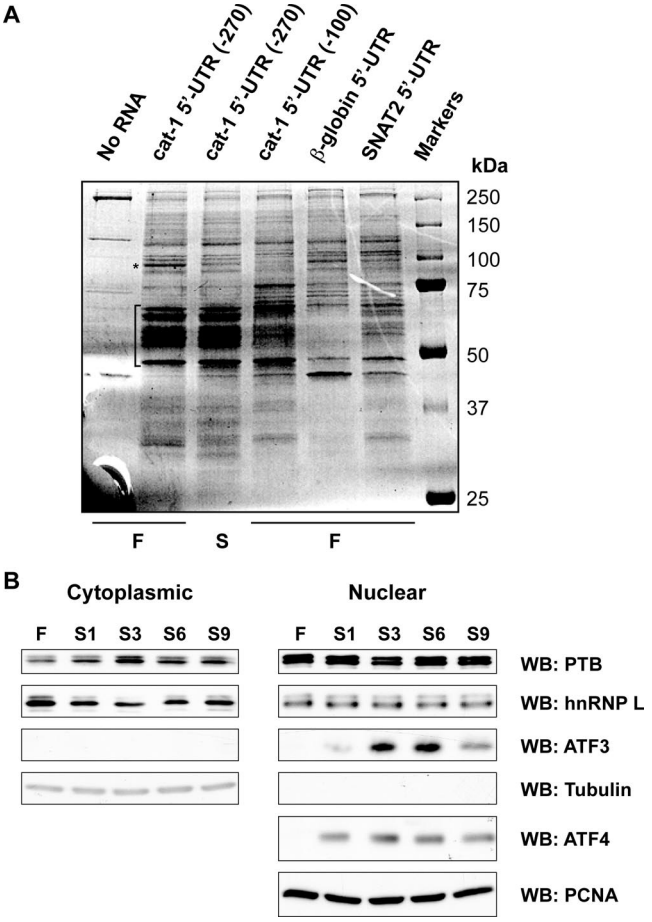


FIG. 1. (A) Identification of proteins that bind to the 5′ UTR of the Cat-1 mRNA. Cytoplasmic extracts from fed (F) and 9-h-starved (S) C6 cells were incubated with streptavidin beads coated with the indicated RNAs, and the bound proteins were eluted, resolved by SDS-PAGE, and stained with Coomassie brilliant blue as described in Materials and Methods. Bands from starved cells within the indicated bracket and the asterisk were subjected to mass spectrometry analysis. (B) C6 cells were cultured under fed (F) conditions or starved of amino acids for 1 to 9 h (S1 to S9). Cytoplasmic and nuclear extracts were prepared and analyzed by Western blotting. Tubulin and PCNA were included as cytoplasmic and nuclear markers, respectively.

RESULTS

Identification of proteins that form complexes with the 5′ UTR of the Cat-1 mRNA. In order to study the regulation of translation initiation from the Cat-1 IRES during amino acid starvation, we identified proteins that can form a complex(es) with the Cat-1 IRES element. This was accomplished using an RNA affinity approach. RNAs were incubated with cytoplasmic extracts from fed or starved C6 glioma cells and collected on streptavidin beads. The bound proteins were analyzed on SDS gels (Fig. 1A). An RNA with the sequence of the entire Cat-1 mRNA leader (−270 to +1; A^{+1}TG initiation codon) bound proteins with a wide range of sizes; similar binding was seen with extracts from fed and starved cells. To determine which of these proteins might be specific for the Cat-1 IRES, we analyzed binding to several control RNAs: Cat-1(−100), which does not function as an inducible IRES (93); the β-globin mRNA 5′ UTR; and the SNAT2 mRNA 5′ UTR, which contains an IRES that is not induced by amino acid starvation (25). Several proteins with molecular masses of 50 to 100 kDa bound to the Cat-1(−270) RNA but not to the controls. Proteins in this size range were excised and analyzed by mass spectrometry. Eighteen proteins were identified (see Table S2 in the supplemental material). We focused on two proteins, hnRNP L and PTB (also known as hnRNP I), because PTB is known to regulate translation initiation from other IRES elements (9, 56, 65) and hnRNP L interacts with PTB (30, 78). Alternative splicing gives rise to several isoforms of PTB (72). We have identified PTB1 as a protein interacting with the Cat-1 5′ UTR (see Table S2 in the supplemental material). Because we did not obtain any peptides for the alternatively spliced exon 9 (72), we could not determine whether PTB 2 or PTB 4 binds the Cat-1 IRES. In all subsequent studies, we

always detected both PTB 1 and PTB 2/4 proteins binding to the Cat-1 IRES (data not shown).

PTB has been shown to act as an activator (72) or repressor (15, 45) of IRES activity. In contrast, hnRNP L has not been found to regulate the activity of cellular IRESs, but it was suggested to play a role in hepatitis C virus IRES-mediated translation (31). Because translation initiation from the Cat-1 IRES increases during amino acid starvation, we tested the role of PTB and hnRNP L in this regulation. We first determined if amino acid starvation affects the subcellular distribution of these proteins, as demonstrated for other ITAFs under stress conditions (42, 50). We tested whether starvation induces changes in cytoplasmic and nuclear PTB and hnRNP L levels. Western blot analysis indicated that PTB and hnRNP L levels in the nucleus did not change during 9 h of starvation (Fig. 1B), and the cytoplasmic levels of these proteins showed very small changes. Control samples in this experiment include the transcription factors ATF3 and ATF4, important mediators of the stress response to amino acid starvation, which increased with the expected kinetics (54). In addition, tubulin and PCNA were used as loading controls for cytoplasmic and nuclear samples, respectively.

Recombinant PTB and hnRNP L bind the Cat-1 mRNA leader. In order to define where PTB and hnRNP L bind to the Cat-1 IRES, we performed UV cross-linking experiments with purified recombinant PTB 1 and hnRNP L and ^{32}P-labeled RNAs with sequences from the Cat-1 IRES (Fig. 2). We have previously shown that the Cat-1 leader (−270 to +1) and a shorter sequence (−192 to +1) are both IRESs regulated by amino acid starvation, whereas shorter RNAs are not regulated (93). hnRNP L bound to Cat-1(−270) and Cat-1(−192) but not to shorter RNAs (−192 to −110 and −116 to +1). These results demonstrate that the hnRNP L binding site is in the proximal portion of the Cat-1 mRNA leader (−192 to +1). In contrast, PTB bound to all the RNAs except Cat-1(−116). These results suggest that the PTB binding site lies in the −192 to −116 region.

As an additional test of protein binding to the Cat-1 mRNA leader, we performed cross-linking experiments in the presence of unlabeled competitor RNAs. Binding of hnRNP L to Cat-1(−192) was competed effectively by a 500-fold excess of Cat-1(−192) and Cat-1(−270) RNAs (Fig. 2C). In contrast, Cat-1(−192 to −110) and Cat-1(−116) showed only weak competition. This finding suggests that the longer RNAs assume a secondary structure required for hnRNP L binding that cannot be achieved by the shorter RNAs. As expected, PTB binding showed competition with longer RNAs but not with Cat-1(−116), further supporting the idea that the PTB binding site lies within the −192 to −116 region.

Because PTB and hnRNP L can interact in vivo (78), we determined if these proteins bind to independent sites on the Cat-1 mRNA leader. We tested the binding of PTB and hnRNP L to the Cat-1 IRES using an EMSA with ^{32}P-labeled Cat-1(−192) RNA. Shifted complexes formed in the presence of PTB (C1) or hnRNP L (C2) alone (Fig. 2D). When both proteins were present, a complex (C3) that migrated more slowly than C1 or C2 was seen, indicating that hnRNP L and PTB can bind to the same Cat-1 IRES. To confirm this conclusion, supershift experiments were performed. Antibodies to PTB or hnRNP L both bound to the C3 complex (Fig. 2E) as

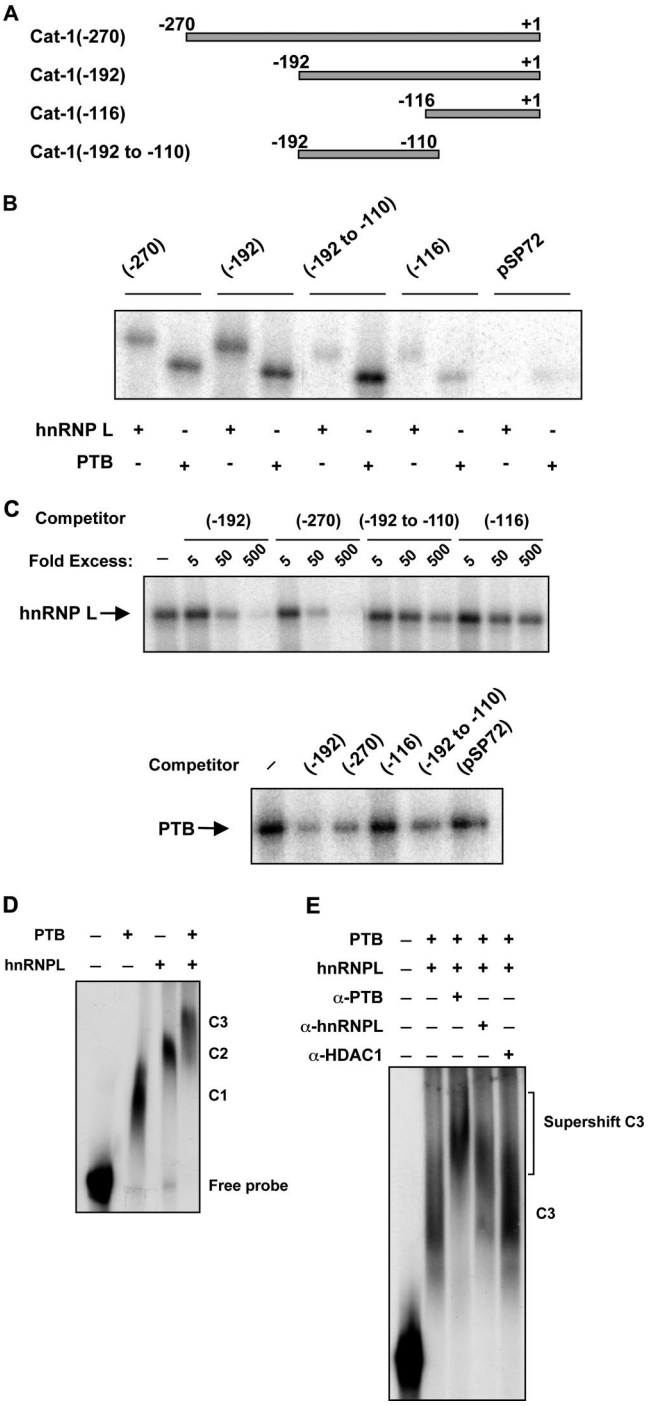


FIG. 2. Recombinant hnRNP L and PTB bind to the Cat-1 IRES. (A) Schematic of RNAs with sequences from the Cat-1 5′ UTR. (B) UV-cross-linking experiments were performed with recombinant PTB or hnRNP L and the ^{32}P-labeled RNAs. After cross-linking, samples were treated with RNase A and analyzed by SDS-PAGE. pSP72 RNA (72 nucleotides of Bluescript RNA) was used as a control. (C) Unlabeled competitor RNAs with the indicated sequences from the Cat-1 5′ UTR were added to cross-linking experiment mixtures containing PTB or hnRNP L and ^{32}P-labeled Cat-1(−192) mRNA. (D and E) EMSAs with ^{32}P-labeled Cat-1(−192) RNA, recombinant PTB and hnRNP L, and the indicated antibodies.

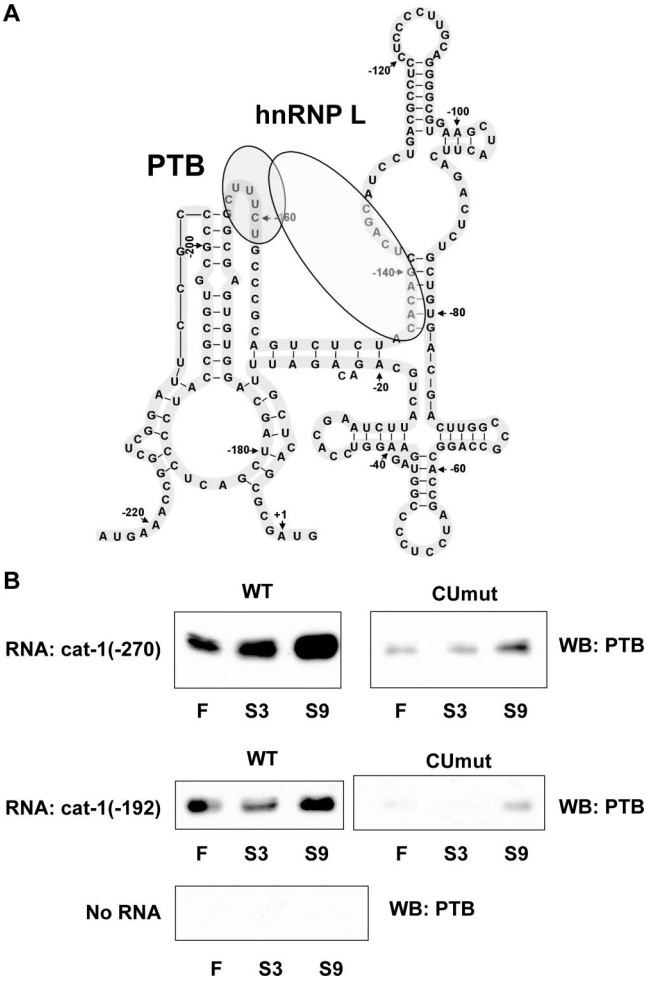


FIG. 3. PTB associates with the Cat-1 mRNA leader via a CU-rich element. (A) Structure of the Cat-1 mRNA leader (70) demonstrating the PTB binding site and the PTB-interacting protein hnRNP L. (B) Cat-1(−192) or Cat-1(−270) RNAs containing either the wild-type sequence or a mutation in the CU-rich element were incubated with cytoplasmic extracts from fed or amino acid-starved cells. The complexes were collected on streptavidin beads and analyzed by SDS-PAGE and Western blotting as described in Materials and Methods.

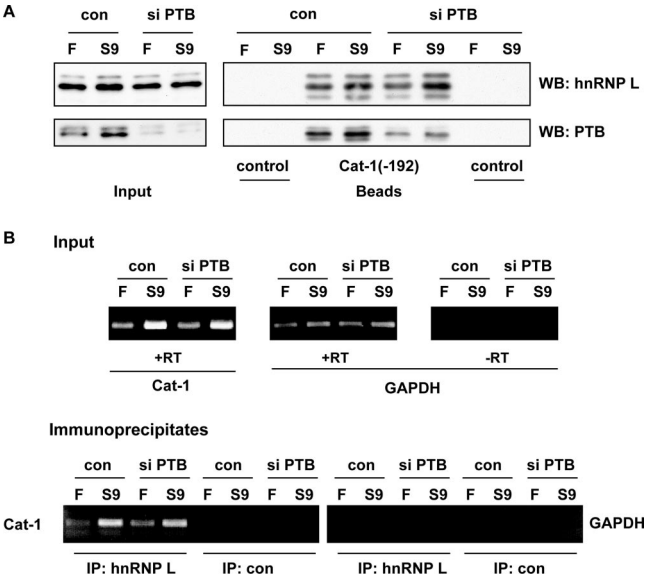


FIG. 4. (A) Treatment of C6 cells with siRNA against PTB reduces binding to the Cat-1 IRES. C6 cells were transfected with siRNA to PTB for 2 days and then incubated under amino acid-fed or -starved conditions. Cytoplasmic extracts were incubated with Cat-1(−192) RNA, and the complexes were collected on streptavidin beads. The bound proteins were analyzed by SDS-PAGE and Western blotting as described under Materials and Methods. (B) The cytoplasmic extracts used in panel A were immunoprecipitated with anti-hnRNP L antibody or control IgG. Cat-1 and GAPDH mRNAs in the immunoprecipitates were analyzed by RT-PCR.

indicated by the disappearance of C3 and the appearance of slower-migrating species. In contrast, control antibody to histone deacetylase 1 did not supershift C3. These data demonstrate that hnRNP L and PTB can both bind to the same Cat-1 mRNA leader and that the two proteins can bind independently.

PTB associates with the Cat-1 mRNA leader via a CU-rich element. In order to identify the PTB binding site in the Cat-1 mRNA leader, we examined the sequence −116 to −192 for CU-rich motifs which were described previously as consensus PTB binding sites (57). The importance of the element $^{-164}$CUUUCU^{-159} was tested first (Fig. 3A) (21). Binding experiments were performed using Cat-1(−270) and Cat-1(−192) RNAs and cytoplasmic extracts from fed and starved cells. Both wild-type RNAs and mutants with the UUCU sequence changed to CCGC (CUmut) were analyzed. The RNA was isolated on streptavidin beads, and the binding of PTB was analyzed by Western blotting (Fig. 3). PTB bound to wild-type RNAs. Moreover, increased binding was seen with extracts from starved cells. In contrast, the binding of PTB to mutant RNAs was greatly reduced, suggesting that the PTB binding site includes the CU-rich element at −164 to −159. The small amount of binding to the mutant RNAs may be due to a second CU-rich sequence ($^{-124}$CCUCCUCCCCUU^{-113}). As expected, no signal was seen in samples that did not contain RNA. These data are consistent with the idea that PTB binds to the Cat-1 mRNA leader via the CUUUCU element (Fig. 3A, IRES with PTB binding).

To test if binding of hnRNP L is independent of PTB, we performed similar binding experiments on cytoplasmic extracts from C6 cells in which the endogenous PTB protein was reduced using siRNAs specific for PTB. Western blot analysis showed that the level of PTB was reduced by >90% under both fed and starved conditions, while the level of hnRNP L was not affected (Fig. 4A). The level of PTB binding to the Cat-1 IRES in extracts from siRNA-treated cells was ~30% of control, whereas the level of hnRNP L binding was unaffected. This supports the conclusion that hnRNP L can bind to the Cat-1 IRES independently of PTB. To further support this conclusion, we determined the binding of hnRNP L to Cat-1 mRNA within cells; hnRNP L was immunoprecipitated from the extracts used in Fig. 4A, and the isolated mRNAs were analyzed by RT-PCR. Cat-1 mRNA was immunoprecipitated with hnRNP L. In addition, increased amounts were seen in extracts from starved cells (Fig. 4B). The reduction of PTB by siRNA treatment did not have a detectable effect on the

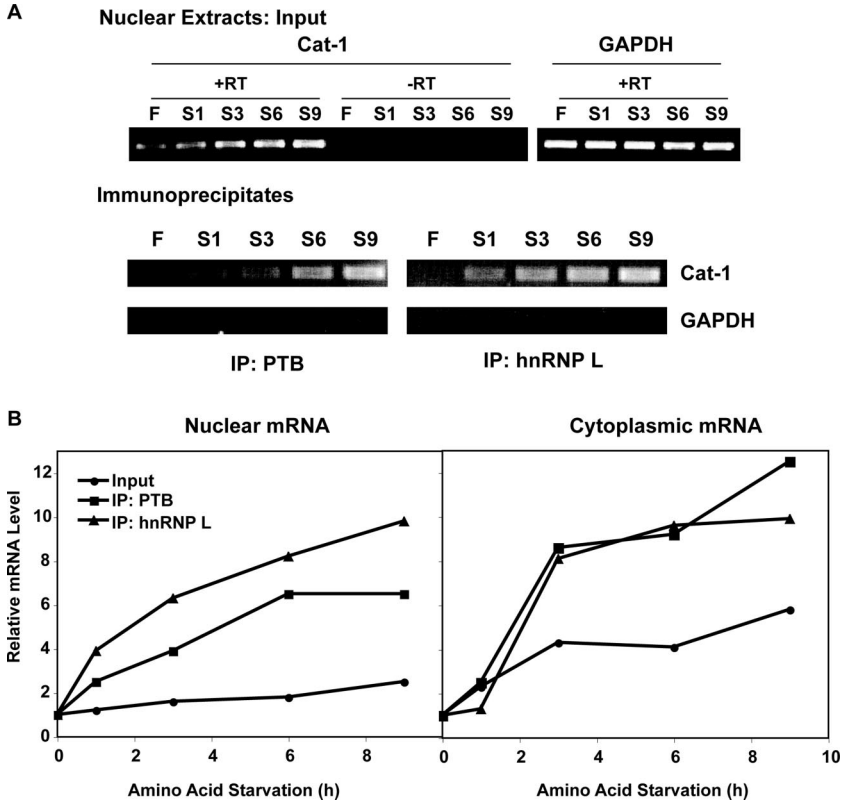


FIG. 5. Cat-1 mRNA preferentially associates with hnRNP L and PTB during amino acid starvation. C6 cells cultured under fed or amino acid-starved conditions for the indicated times. Nuclear and cytoplasmic extracts were prepared and immunoprecipitated for hnRNP L and PTB. (A) Cat-1 and GAPDH mRNA levels in input and immunoprecipitated samples analyzed by RT-PCR. (B) Relative levels of RT-PCR products in panel A and similar experiments with cytoplasmic extracts calculated from scanned gels.

amount of mRNA isolated. As control experiments, we showed that no Cat-1 mRNA was detected in immunoprecipitates with control IgG. In addition, no glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was found associated with hnRNP L. Together, these data indicate that PTB and hnRNP L associate with the Cat-1 IRES in amino acid-starved cells and that the $^{-164}$CUUUCU^{-159} element in the Cat-1 mRNA leader is a potential target for regulation of the Cat-1 IRES by PTB.

Association of the Cat-1 mRNA with PTB and hnRNP L increases during amino acid starvation in the nucleus and cytoplasm. If PTB and hnRNP L function as ITAFs during the enhanced translation of the Cat-1 mRNA during amino starvation, their association with the Cat-1 mRNA could increase under this stress. Furthermore, because PTB and hnRNP L are mainly nuclear proteins, association with target RNAs may occur in the nucleus before transport to the cytoplasm. To test this hypothesis, we immunoprecipitated RNP complexes with anti-PTB and hnRNP L from nuclear and cytoplasmic extracts and analyzed the isolated mRNAs by RT-PCR. In agreement with earlier studies, the amount of Cat-1 mRNA in the extracts increased during starvation in both nuclear (Fig. 5A and B) and cytoplasmic (Fig. 5A and B) extracts. The amount of Cat-1 mRNA associated with PTB and hnRNP L also increased during starvation, and the increase in the association was two- to threefold greater than the increase in total Cat-1 mRNA in both the nucleus and the cytoplasm (Fig. 5B). As expected, the amount of GAPDH mRNA did not increase during starvation and very small amounts were found in the immunoprecipitates. The increased association of Cat-1 mRNA with PTB and hnRNP L during starvation is consistent with the idea that these proteins may play a role in translation initiation from the Cat-1 IRES.

We also examined the association of the Cat-1 mRNA with PTB in ribosomal pellets as a test of the importance of this association during translation. Starvation did not cause a change in the level of PTB or rpS5 (loading control) in the ribosomal fraction (Fig. 6A). The association of mRNAs with PTB in the pellet was examined by immunoprecipitation followed by RT-PCR. The amount of Cat-1 mRNA associated with PTB increased ninefold at 9 h of starvation (Fig. 6B and C), compared to the sixfold increase in the level of cytoplasmic Cat-1 mRNA (Fig. 5B). These data support the hypothesis that PTB plays a role in the increased activity of the Cat-1 IRES during amino acid starvation. Furthermore, the fact that increased association was observed at 9 h and not at 3 h (data not shown) supports earlier findings that there is a lag between Cat-1 mRNA accumulation and Cat-1 mRNA translational activation (21).

PTB and the PTB binding site in the Cat-1 mRNA are required for increased Cat-1 IRES activity during amino acid starvation. Our results suggest that PTB binds to the Cat-1 IRES via the CU element in the mRNA leader and that the extent of binding increases during amino acid starvation. To

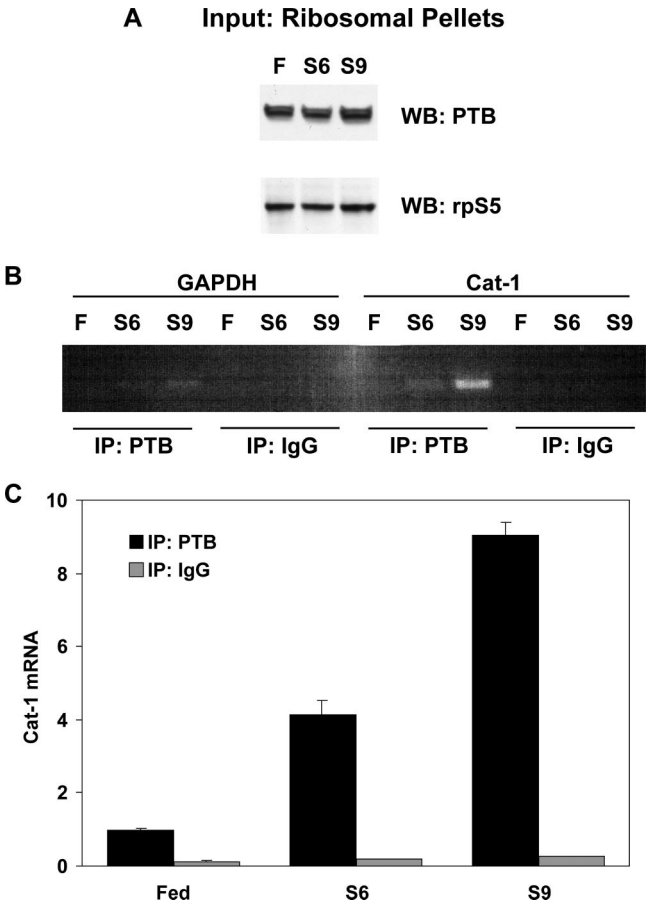


FIG. 6. Preferential association of Cat-1 mRNA with PTB in ribosomal fractions during amino acid starvation. C6 cells were incubated under fed or amino acid-starved conditions for 6 or 9 h, and ribosomal fractions were prepared. (A) PTB and ribosomal protein rpS5 were analyzed by Western blotting. (B and C) Samples were immunoprecipitated with anti-PTB antibody or control IgG. Cat-1 and GAPDH mRNAs in the immunoprecipitates were monitored by RT-PCR (B) or quantitative real-time PCR (C).

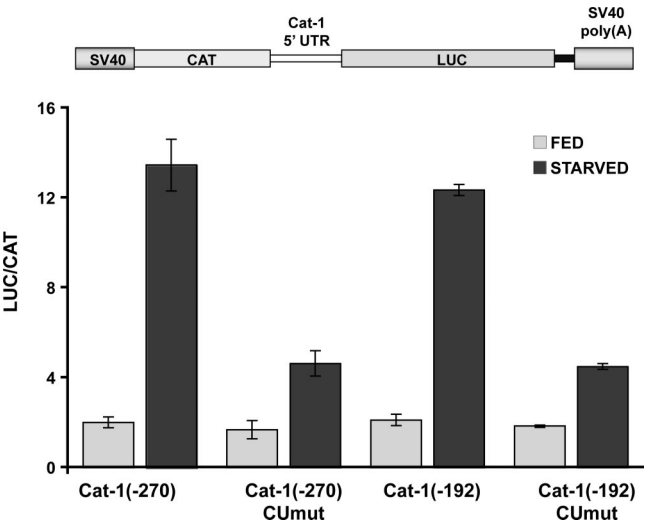


FIG. 7. The CU-rich element in the Cat-1 mRNA leader is required for IRES function. C6 cells were transiently transfected with bicistronic expression vectors containing the wild-type or CU-mutant Cat-1 5′ UTRs shown in the schematic. After 36 h, cells were cultured for 9 h under amino acid-fed or -starved conditions. The cell lysates were assayed for LUC and CAT activities. The graph shows means ± standard errors of the means for three independent experiments. SV40, simian virus 40.

test whether this binding is important for IRES-mediated translation initiation, we tested the IRES activity using bicistronic mRNAs (Fig. 7). C6 cells were transfected with vectors that express mRNAs encoding both CAT and LUC reporter proteins. The CAT reporter is translated from the 5′ end of the mRNA via cap-dependent translation initiation, whereas the LUC reporter is translated only if there is an active IRES in the region between the two ORFs; the LUC/CAT ratio is a measure of IRES activity (93). We have used these vectors to characterize the properties of the Cat-1 IRES. Vectors containing the entire Cat-1 mRNA leader (−270) or bases −192 to +1 were analyzed (Fig. 3A). These vectors had either wild-type or mutant CU sequences to test the importance of the PTB binding site in the IRES. All the constructs gave similar LUC/CAT ratios in fed cells, indicating that the PTB binding site is not required for basal expression from the IRES. Amino acid starvation caused a large increase in the LUC/CAT ratio with the two wild-type mRNAs, but a much smaller increase was seen with the CUmut mRNAs. These results demonstrate the importance of the PTB binding site in the induction of Cat-1 IRES activity during amino acid starvation.

As an additional test of the importance of PTB in IRES activation during amino acid starvation, we examined the effect of reducing PTB levels using siRNAs (Fig. 8A). For these experiments, we used C6 cells stably transfected with the bicistronic vector containing the Cat-1(−270) IRES. LUC expression from the Cat-1 IRES was stimulated after 9 h of amino acid starvation both in cells treated with a control siRNA and in untreated cells (Fig. 8B). In contrast, reducing PTB levels by siRNA treatment nearly abolished the increase in Cat-1 IRES activity caused by amino acid starvation. The specificity of this effect is supported by the observation that siRNA treatment did not affect basal IRES activity in fed cells. In addition siRNA treatment did not affect total protein synthesis, as measured by the incorporation of [^{35}S]Met into protein (Fig. 8C). In contrast, hnRNP L-depleted cells showed a decrease in total protein synthesis when hnRNP L levels were decreased by 70% (Fig. 8C). However, by showing that PTB and its binding site in the Cat-1 IRES are required for the stimulation of IRES activity during amino acid starvation, these experiments provide strong support for a role for PTB in IRES activation.

In order to define the role of hnRNP L in Cat-1 IRES activity, we tested whether overexpression of this protein can activate the Cat-1 IRES. We observed a fourfold induction of Cat-1 IRES activity in C6 cells (data not shown) and a 2.5-fold increase in MEFs (Fig. 9A) transfected with a vector expressing a fusion protein of hnRNP L with GFP. We have shown earlier that activation of translation from the Cat-1 IRES requires a signaling pathway that involves phosphorylation of the initiation factor eIF2α. We had hypothesized that this phosphorylation induces the expression or modification of ITAFs

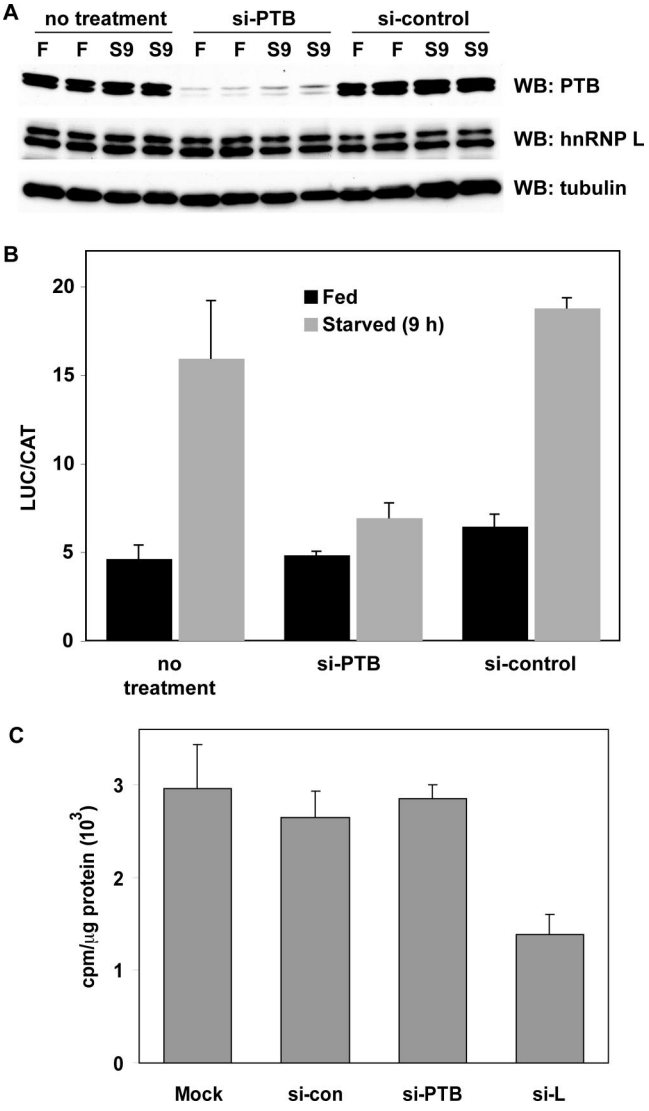


FIG. 8. A C6 cell line that was stably transfected with the bicistronic Cat-1(−270) vector was transiently transfected with the indicated siRNAs. After 48 h, cells were cultured for 6 or 9 h under amino acid-fed or -starved conditions. (A and B) Cell extracts were analyzed by Western blotting (A) and for LUC and CAT activities (B). (C) The C6 cell line stably transfected with the bicistronic expression vector was transfected with the indicated siRNAs. After 48 h, the incorporation of [^{35}S]Met was measured as described in Materials and Methods. Means ± standard errors of the means from triplicate determinations are shown.

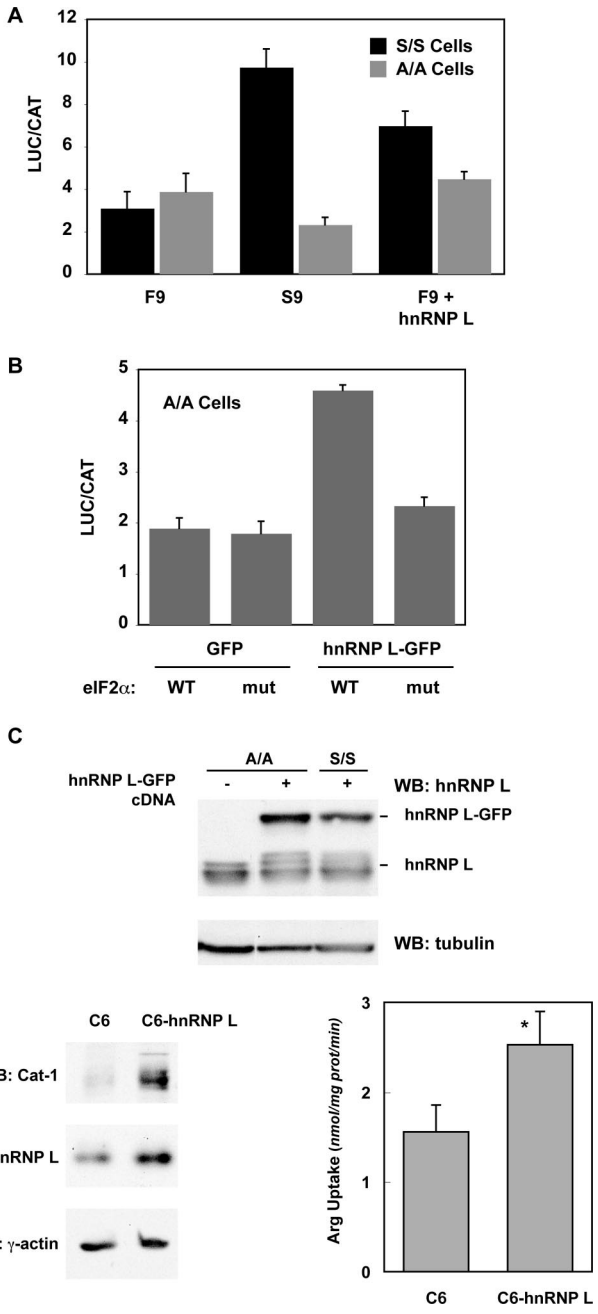


FIG. 9. hnRNP L induction of Cat-1 IRES activity is dependent on eIF2α phosphorylation. (A) MEFs (wild type [S/S] and S51A mutants [A/A]) were transfected with a bicistronic Cat-1(−270) vector and a vector expressing an hnRNP L-GFP fusion protein. Cell extracts were analyzed for LUC and CAT activities from fed and starved cells as indicated. (B) A/A MEFs were cotransfected with a vector expressing the hnRNP L-GFP fusion protein or GFP alone and vectors expressing the indicated eIF2α proteins. Analysis was performed as described for panel A. (C) Transfected and control cells were immunoblotted for hnRNP L to demonstrate the expression level of transfected hnRNP L-GFP. Tubulin is included as a loading control. (D) Control C6 cells and cells expressing hnRNP L from a stably transfected construct (pCDNA3-hnRNP L) were analyzed for Cat-1 expression by Western blotting (left) and L-arginine transport as described in Materials and Methods. Means ± standard errors of the means for triplicate determinations are shown (*, $P < 0.02$).

that stimulate IRES expression. We therefore tested if the induction of Cat-1 IRES activity by hnRNP L requires eIF2α phosphorylation. This was accomplished by comparing IRES activity in wild-type MEFs and in A/A cells, which have a homozygous mutation in the phosphorylation site of eIF2α (S51A). As expected, amino acid starvation induced activity of the Cat-1 IRES in S/S but not in A/A cells (Fig. 9A). Similarly, transfection of hnRNP L stimulated IRES activity to a greater extent in wild-type cells than in mutant cells, suggesting that eIF2α phosphorylation and hnRNP L act synergistically to stimulate IRES activity. To confirm this finding, we transfected

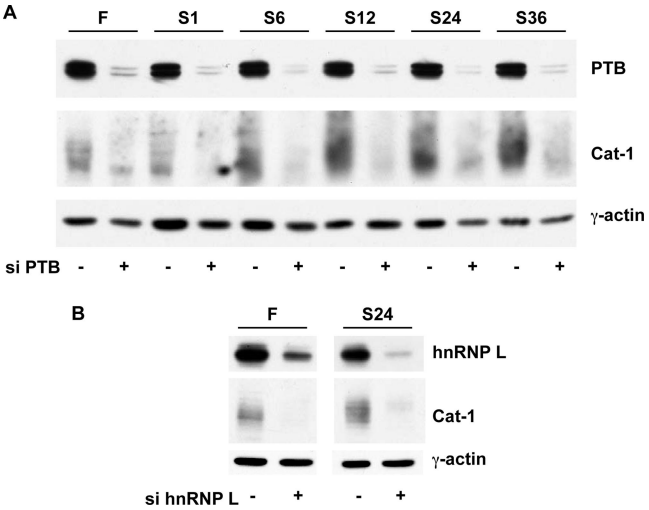


FIG. 10. Depletion of PTB (A) or hnRNP L (B) diminishes Cat-1 protein accumulation in amino acid-starved cells. C6 cells were transfected with the indicated siRNAs. After 48 h, cells were cultured for the indicated times (hours) under amino acid-fed or -starved conditions and the indicated proteins were analyzed by Western blotting. γ-Actin was used as a loading control.

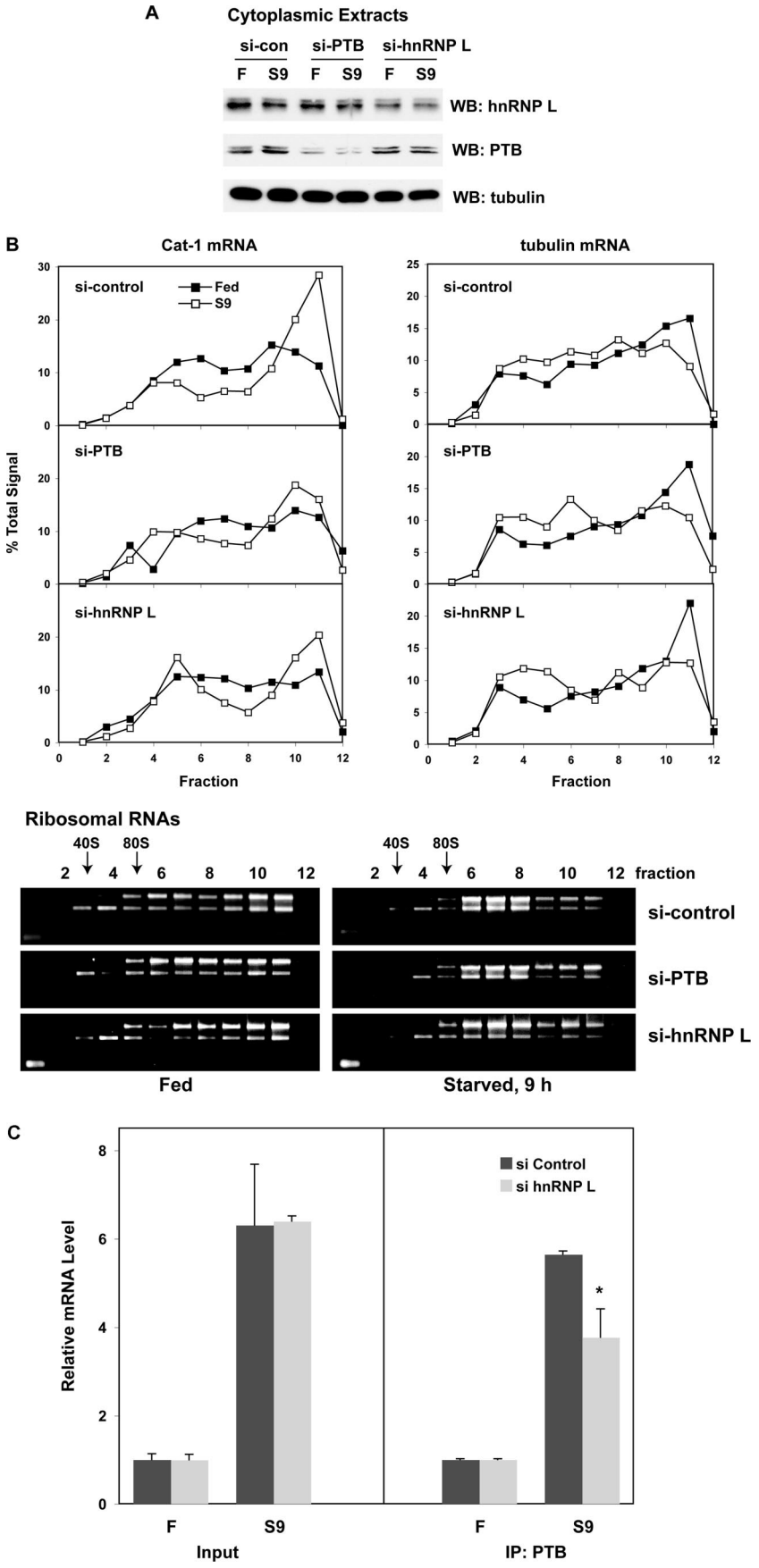
A/A cells with wild-type or mutant eIF2α and the hnRNP L-GFP fusion (Fig. 9B). Western blotting showed that the chimeric hnRNP L-GFP was expressed at similar levels in S/S and A/A cells (Fig. 9C). Overexpression of hnRNP L-GFP stimulated Cat-1 IRES activity in A/A cells only if wild-type eIF2α was also expressed. These results demonstrate that hnRNP L stimulates IRES activity in a process that requires eIF2α phosphorylation. Because our previous results suggest that this phosphorylation has indirect effects, this synergy may be via other proteins whose expression requires eIF2α phosphorylation. As an additional test of hnRNP L function at the Cat-1 IRES, we examined the effect of overexpressing this protein on expression of the endogenous Cat-1 mRNA. Overexpression of hnRNP L in stably transfected C6 cells led to an increase in both Cat-1 protein and arginine transport (Fig. 9D). These data provide strong evidence for the role of hnRNP L in Cat-1 protein synthesis.

PTB and hnRNP L are required for the efficient translation of the Cat-1 mRNA in C6 cells in vivo. It was important to determine the roles of PTB and hnRNP L in the regulation of Cat-1 mRNA translation during starvation. To test this, we examined the effect of depletion of PTB and hnRNP L on the levels of the Cat-1 protein in amino acid-starved cells. Depletion of PTB from C6 cells abolished induction of Cat-1 protein levels during amino acid starvation (Fig. 10A). Interestingly, depletion of PTB decreased Cat-1 protein levels in starved cells below the levels in control untreated cells. We also tested the levels of Cat-1 protein in hnRNP L-depleted cells. As we mentioned earlier, depletion of hnRNP L by 70% decreased protein synthesis rates by 50% (Fig. 8C). It is shown here that Cat-1 protein levels were dramatically decreased under these conditions (Fig. 10B). Levels of γ-actin did not change during siRNA treatments for both proteins (Fig. 10A and B). The decrease of protein synthesis by hnRNP L depletion was not due to a stress response. We did not observe an increase in stress-induced genes such as CHOP (not shown). Furthermore, treatment of cells with either siRNAs against PTB or hnRNP L did not change the control or starvation Cat-1 mRNA levels (not shown). We conclude that PTB and hnRNP L are required for induction of Cat-1 protein levels during amino acid starvation. However, in contrast to PTB, which regulates translation of specific mRNAs, hnRNP L has a more global role in mRNA translation. The latter finding merits further investigation.

To further confirm that PTB and hnRNP L influence the translation efficiency of the Cat-1 mRNA during amino acid starvation, we analyzed ribosomal profiles in control and siRNA-treated cells and characterized the association of the Cat-1 mRNA with polyribosomes. We developed siRNA treatment conditions that reduced hnRNP L levels by 25% (Fig. 11A) without affecting the polyribosome profile (Fig. 11B) or total protein synthesis rates (data not shown). Treatment with siRNAs against PTB and hnRNP L lowered the levels of the corresponding proteins by 75 and 25%, respectively, without one protein affecting the level of the other (Fig. 11A). Cytosolic extracts from fed and amino acid-starved cells were separated by sucrose gradient centrifugation, and the mRNAs in equal volumes of each fraction were analyzed by qRT-PCR (Fig. 11B). Analysis of rRNAs (Fig. 11B) and of the S5 protein of the 40S ribosomal subunit (not shown) demonstrated that the amount of heavy polyribosomes (fractions 10 to 12) is reduced during amino acid starvation, consistent with the global inhibition of protein synthesis.

The amount of Cat-1 mRNA in heavy polyribosomes showed a 50% increase during amino acid starvation, consistent with increased translation from the IRES under these conditions. In contrast, starvation caused smaller increases (~25%) in the amounts of Cat-1 mRNA in samples from cells depleted of PTB or hnRNP L. We have shown that depletion of PTB does not alter the association of Cat-1 mRNA with hnRNP L (Fig. 4). We did a similar experiment examining the effect of hnRNP L depletion on the amount of mRNA that immunoprecipitated with PTB (Fig. 11C). Interestingly, decreasing the hnRNP L levels reduced the association of the cytoplasmic Cat-1 mRNA with PTB in starved cells (Fig. 11C). Because PTB can bind directly to the Cat-1 mRNA leader, it is likely that hnRNP L modulates the PTB-mRNA interaction in vivo. These results demonstrate that these proteins are required for efficient translation of the Cat-1 mRNA during amino acid starvation.

As a control, the distribution of tubulin mRNA, which is translated by cap-dependent initiation, was also analyzed in this experiment (Fig. 11B). As expected, this mRNA showed very different behavior. In control cells, amino acid starvation caused a 25% decrease in the amount of tubulin mRNA in heavy polyribosomes, consistent with the inhibition of cap-dependent translation initiation. In addition, starvation caused similar inhibition in siRNA-treated cells, suggesting that PTB and hnRNP L have specific functions in translation initiation at the Cat-1 IRES but not a cap-dependent mRNA. Consistent with the stress of amino acid starvation, a more dramatic shift to lighter polyribosomes was observed for the 5′-terminal oligopyrimidine mRNAs, the rpL27 and rpL32 mRNAs (data not shown). The latter is in agreement with inhibition of transla-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tion of these mRNAs under conditions of amino acid starvation that cause inhibition of mTOR activity (71, 84).

DISCUSSION

IRES-mediated translation prevails under conditions when cap-dependent translation is compromised (27, 81). More interestingly, IRES-mediated translation is less sensitive to reduced active ternary complexes, a condition associated with eIF2α phosphorylation (10, 69, 79). We have previously shown that Cat-1 gene expression increases under stress conditions that cause eIF2α phosphorylation (24), mostly because of increased translation initiation at the IRES (22). We suggested earlier that ITAFs are involved in the regulation of IRES activity (21, 93). In the present study we identified ITAFs that regulate the Cat-1 IRES during amino acid starvation by forming a complex with the IRES. Several lines of evidence support the conclusion that PTB and hnRNP L, the two proteins studied here, are important for increased Cat-1 mRNA translation during amino acid starvation: (i) these proteins bound to the Cat-1 IRES in vitro; (ii) depletion of either hnRNP L or PTB decreased the efficiency of Cat-1 mRNA translation, as shown by the redistribution of the mRNA from heavy polyribosomes to lighter polyribosomes and decreased levels of Cat-1 protein during stress; (iii) a CU-rich element within the Cat-1 IRES bound PTB and was required for the induction of IRES activity during stress; and (iv) interaction of both proteins with the Cat-1 mRNA increased in the nucleus and the cytoplasm during stress.

In this study we identified 18 proteins that bind to the Cat-1 IRES (see Table S2 in the supplemental material). The majority of the identified proteins are affiliated with mRNA translation. We chose to study PTB and hnRNP L because of the broad function of PTB as an ITAF (72) and because hnRNP L interacts with PTB (30, 77). We found that Cat-1 mRNA associates with PTB and hnRNP L in the nucleus and continues to associate in the cytoplasm, consistent with the fact that PTB and hnRNP L are nuclear proteins that shuttle between the nucleus and the cytoplasm (42). The mechanism that causes increased PTB and hnRNP L binding in the nucleus is not known. However, it is likely that amino acid starvation increases the interactions of PTB and hnRNP L with other nuclear proteins, thus increasing binding to the Cat-1 mRNA. Our data suggest that a multiprotein complex that assembles on the Cat-1 IRES in the nucleus is active in ribosome recruitment in the cytoplasm, supporting the hypothesis that some IRESs require a nuclear experience (73) in order to be active in the cytoplasm.

How do ITAFs regulate IRES activity? The mechanism of ITAF-mediated recruitment of the 40S ribosome to IRESs in cellular mRNAs is not known (28), although it is likely that ITAFs assemble into complexes on IRESs that interact with ribosomes. Studies of IRESs of myc family mRNAs suggest that translation is regulated by multiprotein complexes that may determine the tissue- and cell state-specific function of the IRES (13). It has also been suggested that ITAF binding promotes ribosome recruitment by remodeling the structure of mRNA leaders. For example, the binding of PCBP1 and PTB to the BAG-1 (Bcl-2-associated athanogene 1) IRES unwinds a structured RNA region close to the translation initiation codon, allowing recruitment of the ribosome (66). A similar mechanism has been proposed for the action of the ITAFs unr and PTB on the Apaf-1 (apoptotic protease activating factor 1) IRES (58).

We show here that PTB and hnRNP L are important ITAFs for Cat-1 mRNA translation under stress. We have previously proposed that the basal conformation of the Cat-1 IRES inhibits ribosome scanning or recruitment to the Cat-1 initiation codon; translation of a small ORF within the mRNA leader shifts the IRES into an active conformation (93). Our data suggest that hnRNP L is important for translation of the Cat-1 mRNA in unstressed cells. A possible explanation is that binding of hnRNP L to the Cat-1 IRES destabilizes the inactive mRNA conformation, thus allowing uORF translation and formation of the active IRES. Alternatively, hnRNP L binding may increase cap-dependent translation of the uORF, thus increasing translation of the Cat-1 ORF by reinitiation.

We also showed that the recruitment of hnRNP L and PTB to the Cat-1 mRNA is associated with efficient translation under stress conditions. We propose that hnRNP L is a core factor in a multiprotein complex on the Cat-1 IRES that recruits the ribosome; depletion of hnRNP L during amino acid starvation limited binding of PTB to the IRES, but depletion of PTB did not affect binding of hnRNP L. Such cooperative action of hnRNP L and PTB was described for the lipopolysaccharide/gamma interferon-induced stability of inducible nitric oxide synthase mRNA stability through a mechanism involving PTB and hnRNP L binding to the 3′ UTR (77). hnRNP L has been shown to have diverse functions (39). In a recent collaborative work (70), it was shown that the regulated binding of hnRNP L at the 3′ UTR of the human vascular endothelial growth factor A mRNA mediates a conformational change within the RNA, causing a switch from a translationally permissive to a translationally silent state.

How do PTB and hnRNP L mediate ribosome recruitment to the Cat-1 mRNA? The interaction could be with one of these proteins or another member of the complex. We found that hnRNP K, YB-1, and DDX1 form complexes with the Cat-1 IRES (see Table S2 in the supplemental material). These proteins are known to interact with one another (7, 60, 75) and with hnRNP L and PTB (13, 29, 40). The recruitment of the

FIG. 11. The role of PTB and hnRNP L in translational control of Cat-1 mRNA during amino acid starvation. C6 cells were transfected with the indicated siRNAs. After 48 h, cells were cultured for 9 h under amino acid-fed or -starved conditions. (A) Effect of siRNA treatment on protein expression was analyzed by Western blotting. (B) Cytoplasmic extracts were fractionated on 10 to 50% sucrose gradients as described in Materials and Methods. The tops of the gradients are on the left. Fractions (equal volumes) were analyzed for Cat-1 and tubulin mRNA expression by qRT-PCR (top) and rRNA expression on agarose gels (bottom). The positions of 40S and 80S are indicated. (C) Cytoplasmic extracts were prepared from control and si-hnRNP L-treated fed and starved cells and immunoprecipitated for PTB. Cat-1 mRNA levels in input and immunoprecipitated samples were analyzed by RT-PCR.

ribosome may involve interactions of these proteins with ribosome-associated proteins such as RACK1 (59). The facts that hnRNP L cosediments with polyribosomes during amino acid starvation (data not shown) and that depletion of hnRNP L inhibits global translation suggest a role for this protein in these interactions.

Changes in availability of ITAFs have been proposed as a mechanism to regulate translation. For example, cytoplasmic PTB levels are increased by apoptosis (3) or with toxic drugs (18), thus affecting activity of specific IRESs. Cytoplasmic accumulation of the ITAF hnRNP A1 (a translational repressor) in response to UV radiation has also been described (50). We show here that neither PTB nor hnRNP L levels changed in the cytoplasm of amino acid-starved cells. Therefore, Cat-1 IRES activation by hnRNP L and PTB should involve changes in the levels of other ITAFs or in the modifications of existing proteins. This proposal is consistent with the possibility that eIF2α phosphorylation promotes translation of components of the ITAF complex that are essential for IRES activation in the cytoplasm. This hypothesis is currently being tested.

We found that C^{-164}UUUCU within the Cat-1 IRES is the PTB binding site and is important for induction of IRES activity. PTB was initially described as a splicing factor (26) and splicing repressor (89) that binds pyrimidine-rich sequences (57). The optimal PTB-1 binding site was defined as 5′-CAGCCUGGUG CCUCUCUUUCGG-3′ using SELEX (76). Other studies defined the PTB recognition sequence as UCUU or UCUUC within a pyrimidine-rich sequence (64) or $(CCU)_n$ as part of a pyrimidine-rich tract in a stem-loop (57). PTB binding sites within IRES elements have these sequence motifs. However, the CUUUCU element of the Cat-1 IRES is not flanked by a pyrimidine-rich sequence, consistent with our idea that interaction with other proteins such as hnRNP L (57) may be important in PTB recruitment.

hnRNP L was first characterized as a nuclear protein containing three RNA recognition motifs (68). It binds to CA repeats and regulates pre-mRNA splicing (38). However, there are no reports on the involvement of hnRNP L in cellular IRES-mediated translation. The Cat-1 IRES does not contain any extensive CA repeats, and the binding of hnRNP L requires the entire inducible IRES (Fig. 2). Therefore, hnRNP L probably binds to an RNA structure in the Cat-1 IRES. We previously showed that induction of IRES activity requires a stem structure formed by C^{-182}UAGCA and G^{-10}CUCAGC (93). It is possible that hnRNP L requires this stem for binding, explaining the finding that hnRNP L binding requires the 5′ and 3′ ends of the Cat-1 mRNA leader. The PTB binding site that was described in this report is within a loop 11 nucleotides downstream of the 5′ part of the stem. This proximity to the stem may be important in stabilizing the active IRES conformation for recruitment of the ribosome. In addition, the second consensus PTB binding motif in the Cat-1 5′ UTR, C^{-116} CUCCUCCCCUU, may be inactive in IRES induction because it is distant from the stem.

There are few studies of how signaling pathways affect IRES activity and ITAFs (41). Phosphorylation of eIF2α, which is triggered by many stress conditions (91) and physiological processes such as cell division (27, 86), has a permissive role in cellular IRES-mediated translation. We have shown previously that increased Cat-1 IRES activity during amino acid starvation requires eIF2α phosphorylation (24). We show here that hnRNP L binds the Cat-1 IRES and is essential for Cat-1 protein synthesis in amino acid-sufficient and -starved cells. More important is the finding that hnRNP L increases Cat-1 IRES activity only in cells with eIF2α that can be phosphorylated. Although we do not know the mechanism of this phenomenon, it is likely that eIF2α phosphorylation is required for the expression of an hnRNP L-interacting protein or a modifier. The transcription factors ATF4, ATF5, and GADD45 are the only well-known proteins whose translation is increased during eIF2α phosphorylation via a cap-dependent mechanism (6, 55, 87, 95). Similar mechanisms may be used to regulate the synthesis of ITAFs. Future studies will focus on the search for ITAFs that are regulated in this way.

In conclusion, we have advanced our understanding of the mechanism of Cat-1 IRES induction by identifying ITAFs, a feature of all cellular IRESs (46, 83). PTB and hnRNP L are the first ITAFs described for Cat-1. Although PTB is known to function in the expression of other IRESs, hnRNP L is a novel ITAF for cellular mRNA translation under stress. The emerging view is that multiprotein ITAF complexes are important in the control of IRES-mediated translation. A crucial question for future studies is how stress signals control these complexes to regulate mRNA translation in a stress- and cell type-specific manner. As shown in this study, the cellular compartment and timing of assembly of these complexes may be important factors in regulation of IRES activity.

ACKNOWLEDGMENTS

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