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Hepatitis C Virus Internal Ribosome Entry Site-Dependent Translation in *Saccharomyces cerevisiae* Is Independent of Polypyrimidine Tract-Binding Protein, Poly(rC)-Binding Protein 2, and La Protein

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Translation initiation of some viral and cellular mRNAs occurs by ribosome binding to an internal ribosome entry site (IRES). Internal initiation mediated by the hepatitis C virus (HCV) IRES in *Saccharomyces cerevisiae* was shown by translation of the second open reading frame in a bicistronic mRNA. Introduction of a single base change in the HCV IRES, known to abrogate internal initiation in mammalian cells, abolished translation of the second open reading frame. Internal initiation mediated by the HCV IRES was independent of the nonsense-mediated decay pathway and the cap binding protein eIF4E, indicating that translation is not a result of mRNA degradation or 5′-end-dependent initiation. Human La protein binds the HCV IRES and is required for efficient internal initiation. Disruption of the *S. cerevisiae* genes that encode La protein orthologs and synthesis of wild-type human La protein in yeast had no effect on HCV IRES-dependent translation. Polypyrimidine tract-binding protein (Ptb) and poly-(rC)-binding protein 2 (Pcbp2), which may be required for HCV IRES-dependent initiation in mammalian cells, are not encoded within the *S. cerevisiae* genome. HCV IRES-dependent translation in *S. cerevisiae* was independent of human Pcbp2 protein and stimulated by the presence of human Ptb protein. These findings demonstrate that the genome of *S. cerevisiae* encodes all proteins necessary for internal initiation of translation mediated by the HCV IRES.

Most eukaryotic mRNAs are translated by a 5′-end-dependent mechanism, involving recruitment of the 40S ribosomal subunit by a series of interactions organized around the cap-binding protein eIF4E and the 5′-cap structure of the mRNA. Initiation of translation of some viral and cellular mRNAs is 5′-end independent and occurs by internal binding of the 40S ribosomal subunit to an internal ribosome entry site (IRES) (52). The IRES of hepatitis C virus (HCV) is a highly structured RNA that includes the 5′ untranslated region (5′UTR) of the viral mRNA and sequence downstream of the initiating AUG (58, 67, 70). The 40S ribosomal subunit binds directly to the HCV IRES independently of known translation initiation proteins (37, 54). Initiation proteins eIF2 and eIF3 are required for the formation of the 80S ribosome and subsequent translation (37, 62). Cellular proteins considered necessary for efficient HCV IRES-dependent initiation in mammalian cells include La protein (3), polypyrimidine tract-binding protein (Ptb) (2), poly(rC)-binding protein 2 (Pcbp2) (64), and Nsap1 (35). These proteins were found to interact with the HCV IRES by in vitro binding and UV cross-linking assays. Depletion and reconstitution experiments subsequently demonstrated a requirement for La, Ptb, and Nsap1 in HCV IRES-dependent initiation. It has been suggested that La protein, Ptb, Pcbp2, unr, Mpp-1, and Nsap1 act as chaperones, maintaining the RNA in an appropriate structure for binding to ribosomes and translation initiation proteins (31).

The capacity of an RNA to direct internal initiation may be functionally determined by demonstrating that the sequence directs the translation of the second open reading frame of a bicistronic mRNA, independent of translation of the first open frame (31). An in vivo functional assay can be used to directly identify proteins required for IRES-mediated initiation. The yeast *Saccharomyces cerevisiae* has been used as a model organism to understand 5′-end-dependent initiation (23). The rapid growth, ease of genetic manipulation, and completely sequenced genome of this organism make it ideal for identifying cell proteins that participate in IRES-mediated translation. Initiation dependent upon the HCV and poliovirus IRESes has been observed in cell extracts from yeast (4, 26, 27). IRES-dependent initiation occurs in yeast that have undergone carbon starvation (51) or when 5′-end-dependent initiation is compromised (38, 65). At least one gene of *S. cerevisiae*, *URE2*, harbors an IRES (38).

To determine whether internal initiation mediated by the HCV IRES occurs in wild-type yeast, a bicistronic mRNA was designed in which the IRES was inserted between the yeast *ADE3* and bacterial *lacZ* genes. *S. cerevisiae* was transformed with a plasmid encoding the bicistronic mRNA. β-Galactosidase activity was observed in strains producing bicistronic mRNAs containing the HCV IRES plus the first 120 amino acids of the polyprotein. Introduction of a single base change in the HCV IRES, known to abrogate internal initiation in

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mammalian cells, abolished production of β-galactosidase. Disruption of *UPF1*, a component of the nonsense-mediated decay pathway (40), had a similar effect on both HCV IRES-mediated and 5′-dependent initiation. These findings, coupled with the results of RNA analyses, revealed that production of β-galactosidase is not a consequence of translation of fragmented RNAs. A single amino acid alteration within eIF4E renders the protein temperature sensitive and unable to bind the 5′-cap structure of the mRNA at the restrictive temperature. At the permissive temperature, the altered protein binds the 5′-cap structure with reduced affinity (6). At the nonpermissive temperature, internal initiation mediated by the HCV IRES decreased minimally compared to the decline in 5′-dependent initiation. These results demonstrate that translation of the second open reading frame of the bicistronic RNA is independent of translation of the first open reading frame, fulfilling the definition of an IRES.

The absence of yeast orthologs of Ptb, Pcbp2, and Nsap1 suggests that these proteins are not necessary for HCV IRES-dependent internal initiation in this organism. Production of Ptb in yeast slightly stimulated production of β-galactosidase dependent on the HCV IRES. Neither disruption of three yeast genes that encode La protein orthologs nor synthesis of human La had an effect on HCV IRES-dependent translation. These findings demonstrate that the genome of *S. cerevisiae* encodes all the proteins necessary for internal initiation of translation mediated by the HCV IRES.

MATERIALS AND METHODS

Yeast. Yeast strains W1368-8BH (*MAT*α *ade2*Δ *ade3*Δ *can 1-100 his 3-11,15 leu2-3,112 trp1-1 ura3-1*) and W1588-4A (*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) were provided by Rodney Rothstein, Columbia University. Yeast strains 1230 (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) and 1231 (*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) were provided by Aaron P. Mitchell, Columbia University. Yeast strain AAY273, a W303a derivative [*MAT***a** *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1*Δ*5(LEU2)*] was provided by Audrey L. Atkin, University of Nebraska, Lincoln. Yeast strains CY1 (*MAT***a** *ura3 lys2 ade2 trp1 his3 leu2 LHP1*) and YSS238 (*MAT*α *ura3-52 lys2-801 ade2-101 his3*Δ*200 leu2*Δ*1 slf*::*HIS3 sro9*::*URA3 lhp1*::*LEU2*) were both courtesy of Sandra Wolin, Yale University. YSS328 was grown in the presence of 5-fluoroorotic acid to identify YSS238B (*MAT*α *ura3-52 lys2-801 ade2-101 his*Δ*200 leu2*Δ*1 slf*::*HIS3 sro9*Δ *lhp1*::*LEU2*). Yeast stains CW04 (*MAT***a** *ade2-1 leu2-3,112 his3-11,15 trp1-1 cdc33*::*LEU2* < *4E wt TRP1*>) and 4-2 [(eIF4E –ts) *MAT***a** *ade2-1 leu2-3,112 his3-11,15 trp1-1 cdc33*::*LEU2* < *4E −4-2 TRP1*>] were provided by Michael Altmann, University of Bern.

Plasmids. The yeast *cen* shuttle plasmid pRS416 (New England BioLabs, Inc.) containing the *ADH1* promoter and transcription termination signal was used to encode the bicistronic mRNA. The *ADE3* gene from *S. cerevisiae* and the *lacZ* gene of *Escherichia coli* were inserted into the plasmid, either singly (monocistronic plasmids) or together (bicistronic plasmid). The HCV IRES of genotype 1a (courtesy of Charles Rice, Rockefeller University) was placed between the *ADE3* and *lacZ* genes as a fusion of the first 5 or the first 120 amino acids of the viral polyprotein with the second amino acid of β-galactosidase. Mutations of the HCV IRES and removal of the AUG codon of the *ADE3* gene were carried out by PCR with specific oligonucleotide primers. DNA encoding amino acids 3 through 354 of Ure2p was amplified from total DNA isolated from strain 1231 of *S. cerevisiae* and placed between the *ADE3* and *lacZ* genes as a fusion with the second amino acid of β-galactosidase. Human *PCBP2* and *La* DNAs amplified by PCR from a HeLa cell mRNA library (Invitrogen, Inc.) were inserted into the yeast *cen* shuttle plasmids pRS413 and pRS415 (New England Biolabs, Inc.) containing the *ADH1* promoter and transcription termination signal. A six-histidine tag was encoded within the 3′ primer used in the amplification of *PCBP2*. Human *PTB* DNA amplified by reverse transcription and PCR from total RNA isolated from S3 HeLa cells was inserted into the yeast *cen* shuttle plasmid pRS413 containing the *ADH1* promoter and transcription termination signal. A six-histidine tag was encoded within the 3′ primer used in both reverse transcription and amplification of *PTB*.

Transformation. Yeast cells were transformed with different plasmids using standard cation transformation (29) and plated on selective medium (synthetic complete lacking uracil, uracil and histidine, or uracil and tryptophan).

β-Galactosidase assays. Transformants were assayed by colony filter assay (12). Colonies were transferred to a nitrocellulose filter and frozen for 25 min at −80°C. The frozen filter was placed with the colonies face up on top of a Whatman 3MM filter presoaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 50 mM $MgSO_4$, 50 mM, β-mercaptoethanol, 2.7 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside dissolved in *N*,*N*-dimethylformamide) and incubated 8 h at 30°C until blue color develops. The Miller solution assay was used to quantitate β-galactosidase expression (49). Five randomly picked transformants from three individual transformations were grown in broth under selection (synthetic complete minus uracil, minus uracil and histidine, or minus uracil and tryptophan) for 40 h, diluted, and harvested during mid-logarithmic growth. An optical density at 600 nm of 1 was collected and assayed for β-galactosidase activity, the amount of substrate, o-nitrophenyl-β-D-galactopyranoside (ONPG), hydrolyzed per minute (Miller units). Transformants of strains CW04 and 4-2[(eIF4E –ts) were grown in broth at room temperature for 40 h, diluted, and then grown at either room temperature or 37°C for 5 hours. An optical density at 600 nm of 1 was collected and assayed for β-galactosidase activity as the amount of ONPG hydrolyzed per minute (Miller units).

Southern hybridization analysis. Cultures of wild-type yeast or yeast transformed with different plasmids were grown in rich broth (YePD) or under selection (synthetic complete minus uracil) for 40 h, diluted, and harvested during mid-logarithmic growth. The culture was collected and washed and total cellular DNA was isolated (13). DNA was cleaved with StuI, fractionated in a 0.8% agarose gel buffered with 0.5X Tris-borate-EDTA, and transferred to a GeneScreen plus nylon membrane (PerkinElmer Analytical and Life Sciences). Membranes were hybridized with a randomly radiolabeled DNA probe produced by PCR amplification of *lacZ* nucleotides 321 to 640, the HCV IRES, plus nucleotides encoding the first 120 amino acids of the polyprotein, or the entire open reading frame of *ADE3*.

Northern hybridization analysis. Cultures of wild-type yeast or yeast transformed with different plasmids were grown in rich broth (YePD) or under selection (synthetic complete minus uracil) for 40 h, diluted, and harvested during late-logarithmic growth. The entire culture was collected and washed with water, and total RNA was isolated (13). RNA was fractionated in a 1.5% agarose-formaldehyde gel, and transferred to GeneScreen plus nylon membrane (PerkinElmer Analytical and Life Sciences). Membranes were hybridized with a randomly radiolabeled DNA probe produced by PCR of *lacZ* nucleotides 321 to 640 or the HCV IRES plus nucleotides encoding the first 120 amino acids of the polyprotein.

Reverse transcription-PCR. Cultures of wild-type yeast or yeast transformed with the plasmid encoding the bicistronic RNA were grown in YePD or under selection for 40 h, diluted and harvested during late-logarithmic growth. The entire culture was collected and washed with water and total RNA was isolated (13). Primer 1, which is complementary to nucleotides 50 to 80 of *lacZ* was annealed to 250 ng of total RNA in the presence of 10% dimethyl sulfoxide and extended by Superscript reverse transcriptase (Invitrogen, Inc) at 42°C to generate a cDNA copy of the RNA. RNase H was added after 1 h to remove the RNA template. The cDNA product was diluted 1:50 and amplified by polymerase reactions primed with either primers 2 and 3 or primers 4 and 1. The initiating and terminating codons of *ADE3* are encoded within primers 2 and 3. Primer 4 anneals 300 nucleotides upstream of the terminating codon of *ADE3*.

Western blot analysis. Cultures of wild-type yeast or yeast transformed with the plasmid encoding human PBCP2, PTB, or La were grown in rich broth (YePD) or under selection (synthetic complete minus histidine or synthetic complete minus tryptophan) for 40 h, diluted, and harvested during late-logarithmic growth. The entire culture was collected, washed with water, resuspended in pretreatment buffer (20 mM Tris, pH 7.8, 0.1 M β-mercaptoethanol, 1 M sorbitol) and incubated at 30°C for 10 min. Following pretreatment, cultures were collected, resuspended in treatment buffer (1 M sorbitol, 10 mM potassium phosphate, pH 6.0) containing 0.1 mg/ml zymolase (zymolase 20T; Seikagaku, Inc., Tokyo, Japan) and incubated at 30°C for 1 h. Approximately 90% of the cells were converted to spheroplasts as judged by microscopy. All cells were collected, resuspended in 50 μl of hypotonic solution (100 mM HEPES, 10 mM NaCl, 1 mM dithiothreitol) containing protease inhibitors (Roche Applied Science), and 0.1 g glass beads (Sigma), transferred to disposable glass tubes and vortexed for 30 seconds three times.

The extracts were transferred to cold microcentrifuge tubes and the glass beads were washed with an additional 50 μl of hypotonic solution containing

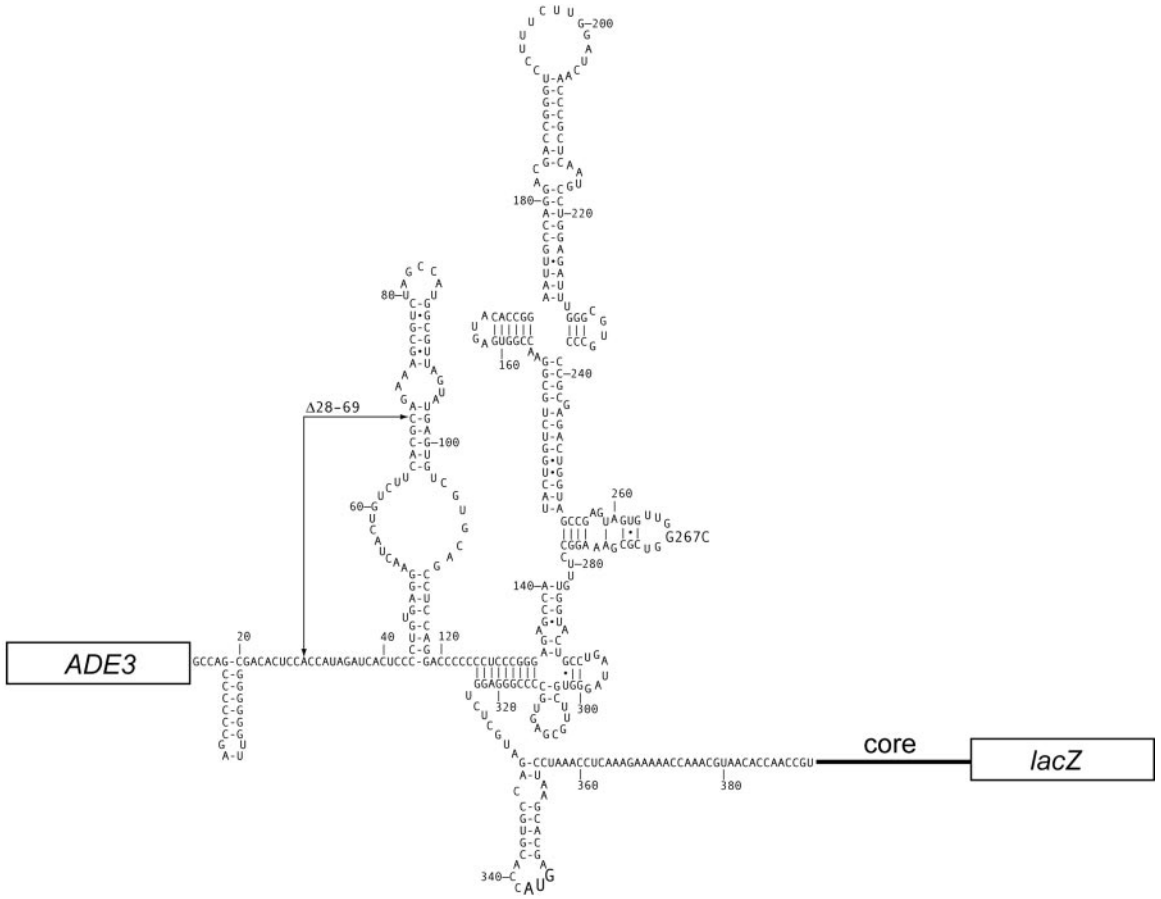

FIG. 1. Structure of bicistronic mRNA. DNA encoding the HCV genotype 1a 5′ untranslated region (nucleotides 1 to 342) and nucleotides encoding the first 5 or the first 120 amino acids of the polyprotein (core) was inserted between the yeast *ADE3* and the *E. coli lacZ* genes. The locations of genetic alterations are indicated: deletion of bases 28 to 69, and mutation of G267 to C. The secondary structure of the HCV IRES is from reference 44.

protease inhibitors. Extracts from strains synthesizing human PTB were incubated with Ni-agarose beads (QIAGEN, Inc.) at 4°C overnight. The Ni-agarose beads were washed once with 10 mM imidazole in phosphate-buffered saline and eluted in 1 M imidazole in phosphate-buffered saline. Protein concentration was determined by Bradford assay, and equal amounts of protein were loaded on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel, and electrophoresed at 80 V for 2 h. Proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore) by semidry transfer at 20 V for 1 h, which was then incubated at room temperature in 5% nonfat milk dissolved in blot buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h. To detect synthesis of human PCBP2 or PTB, a mouse monoclonal antihistidine antibody (Invitrogen, Inc.) was diluted 1 to 3,000 in blot buffer containing 5% nonfat milk and incubated with the membrane at room temperature overnight. A mouse monoclonal anti-La antibody (provided by Nahum Sonenberg, McGill University) diluted 1 to 3,000 in blot buffer containing 5% nonfat milk and incubated with the membrane at 4°C overnight was used to detect synthesis of La protein in yeast. After incubation with antibody, membranes were washed three times in blot buffer. Goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Promega, Inc.) was diluted 1 to 3,000 in blot buffer containing 2.5% nonfat milk and incubated with the membrane at room temperature for 1 h. Membranes were washed three times in blot buffer and proteins were visualized by LumiGlo chemiluminescent substrate system (Kirkegaard and Perry Laboratories Inc.).

RESULTS

Translation of a bicistronic RNA in yeast. The capacity of an RNA to direct internal initiation may be functionally determined by demonstrating that the sequence directs the translation of the second open reading frame of a bicistronic mRNA, independent of translation of the first open reading frame (31). Internal initiation in yeast was assessed by transforming yeast with a plasmid encoding a bicistronic mRNA in which the HCV IRES is inserted between the yeast *ADE3* and the bacterial *lacZ* genes (Fig. 1). Transformants were assayed for β-galactosidase by colony filter assay (12). Blue colonies were observed when the HCV IRES plus sequence encoding the first 120 amino acids of the viral polyprotein was included in the intercistronic region, but not when only the first 5 amino acids of the HCV polyprotein were present (not shown).

These results were confirmed by assaying β-galactosidase activity in liquid cultures (Miller assay) (Fig. 2). A blue yeast colony containing the plasmid ADE3-HCV C120-*lacZ* was isolated, and the plasmid was recovered and introduced into fresh yeast cells. These transformants produced β-galactosidase as determined by colony and Miller assays (data not shown). No changes were present in the DNA sequence of the IRES and 200 5′- and 3′-flanking nucleotides in the plasmid recovered from a blue yeast colony. The same blue colony was also grown in the presence of 5-fluoroorotic acid to remove the plasmid (11), which resulted in loss of β-galactosidase activity. When

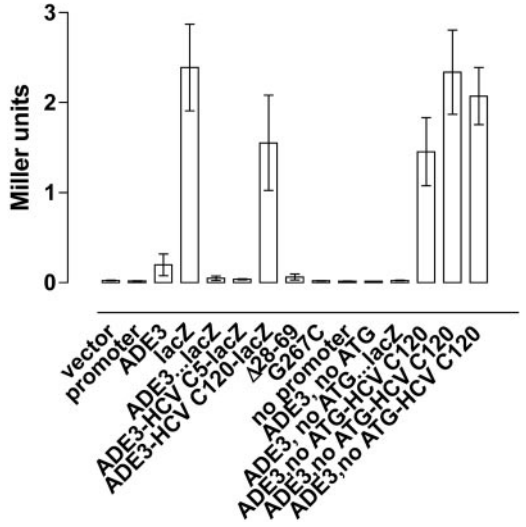

FIG. 2. Liquid β-galactosidase assays of yeast transformed with plasmids encoding bicistronic mRNAs. Yeast strain W1536-8BH (wild type) was transformed with different plasmids, and transformants were assayed for β-galactosidase synthesis. Labels on the *x* axis indicate the structure of the plasmid that was introduced into yeast. Vector: no promoter or DNA insert; promoter: *ADH1* promoter only; *ADE3*: *ADE3* gene only; *lacZ: lacZ* gene only; *ADE3*. . . *lacZ: ADE3* and *lacZ* genes with no IRES; ADE3-HCV C5-*lacZ: ADE3* gene, HCV IRES, amino acids 1 to 5 of the polyprotein, and *lacZ*; ADE3-HCV C120-*lacZ: ADE3* gene, HCV IRES, amino acids 1 to 120 of the polyprotein, and *lacZ*; Δ28-69: ADE3-HCV C120-*lacZ* lacking nucleotides 28 to 69 of the HCV 5′ untranslated region; G267C: ADE3-HCV C120-*lacZ* with a point mutation at nucleotide 267 of the HCV 5′ untranslated region; no promoter: ADE3-HCV C120-*lacZ* without the *ADH1* promoter; *ADE3*, no ATG: *ADE3* gene only without an initiator ATG; *ADE3*, no ATG. . *.lacZ: ADE3* and *lacZ* genes with no IRES without an initiator ATG of *ADE3*; *ADE3*, no ATG-HCV C120: ADE3-HCV C120-*lacZ* without an initiator ATG of *ADE3*. The last plasmid was studied in three independent colonies. *y* axis, β-galactosidase activity in Miller units as determined by solution assay.

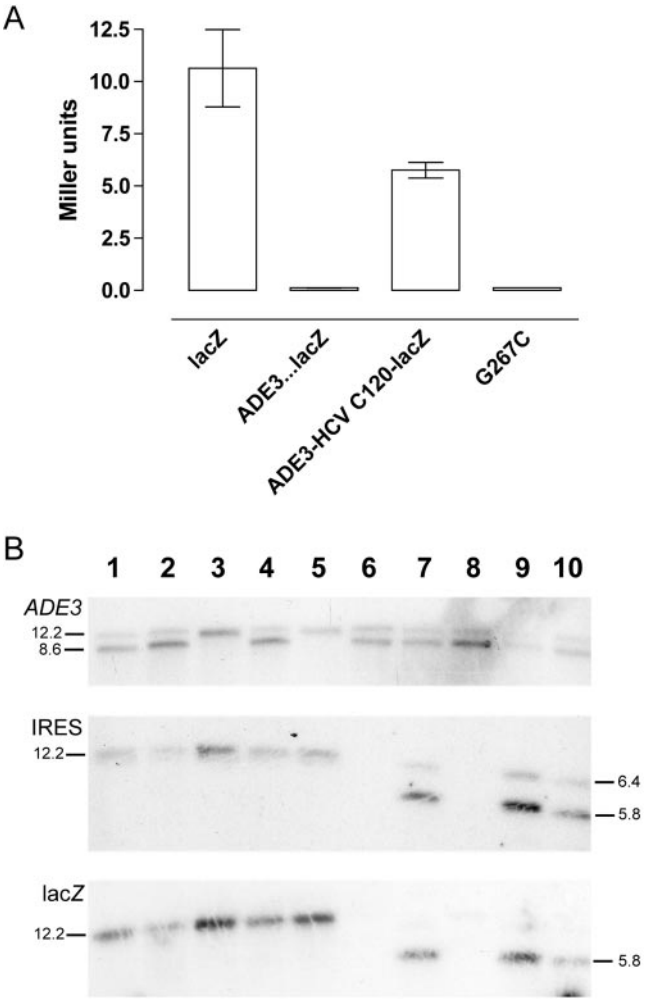

FIG. 3. Liquid β-galactosidase assays and Southern blot hybridization analysis of yeast transformed with plasmids encoding bicistronic mRNAs. Yeast strain 1231 (wild type) was transformed with different plasmids, and transformants were assayed for β-galactosidase synthesis and analyzed by Southern blot hybridization. A. Liquid β-galactosidase assays. Labels on the *x* axis indicate the structure of the plasmid that was introduced into yeast. *lacZ*: *lacZ* gene only; *ADE3*. . *.lacZ*: *ADE3* and *lacZ* genes with no IRES; ADE3-HCV C120-*lacZ*: *ADE3* gene, HCV IRES, amino acids 1 to 120 of the polyprotein, and *lacZ*; G267C: ADE3-HCV C120-*lacZ* with a point mutation at nucleotide 267 of the HCV 5′ untranslated region. B. Southern blot hybridization analysis of plasmids in yeast strains from part A. Lanes 1 to 5, DNA from strains containing plasmid ADE3-HCV C120-*lacZ*; lanes 6 to 10, DNA from strains containing plasmid G267C. DNA was cleaved with StuI. Membranes were hybridized with DNA specific for the *ADE3* gene, the HCV IRES, or *lacZ*. Expected sizes in kb are indicated at left and right.

this strain was transformed with plasmid ADE3-HCV C120-*lacZ*, β-galactosidase activity was detected. These findings confirm that β-galactosidase activity is dependent on the presence of the plasmid ADE3-HCV C120-*lacZ*.

One possible explanation for β-galactosidase activity in yeast containing the plasmid ADE3-HCV C120-*lacZ* is interplasmid recombination leading to loss of sequences upstream of the *lacZ* gene. Five independent transformants were therefore examined for β-galactosidase activity by Miller assay (Fig. 3A), and the structure of the plasmid was examined by Southern blot hybridization analysis (Fig. 3B). All transformants producing monocistronic or bicistronic mRNAs displayed β-galactosidase activity (Fig. 3A). Southern blot hybridization analysis DNA cleaved with StuI was performed using DNA hybridization probes derived from the *ADE3* gene, the HCV IRES, and *lacZ*. No rearrangement or deletion of the bicistronic plasmid (Fig. 3B) or the monocistronic *lacZ* plasmid (data not shown) was observed. Southern blot hybridization analysis revealed the absence of sequences related to the HCV IRES or *lacZ* within the yeast genome (data not shown). HCV IRES-dependent initiation was unaffected in yeast strains defective in homologous recombination (data not shown). Therefore β-galactosidase activity is due to the presence of the intact plasmid ADE3-HCV C120-*lacZ* and appears to be dependent upon internal initiation mediated by the HCV IRES.

Genetic alteration of bicistronic RNA. Replacement of the HCV IRES with a 23-nucleotide spacer sequence was used to confirm that the presence of the HCV IRES is required for translation of the second open reading frame. β-Galactosidase was not detected in yeast producing a bicistronic mRNA lacking the HCV IRES (Fig. 2 and 3A), confirming that internal initiation is dependent upon the presence of the HCV IRES.

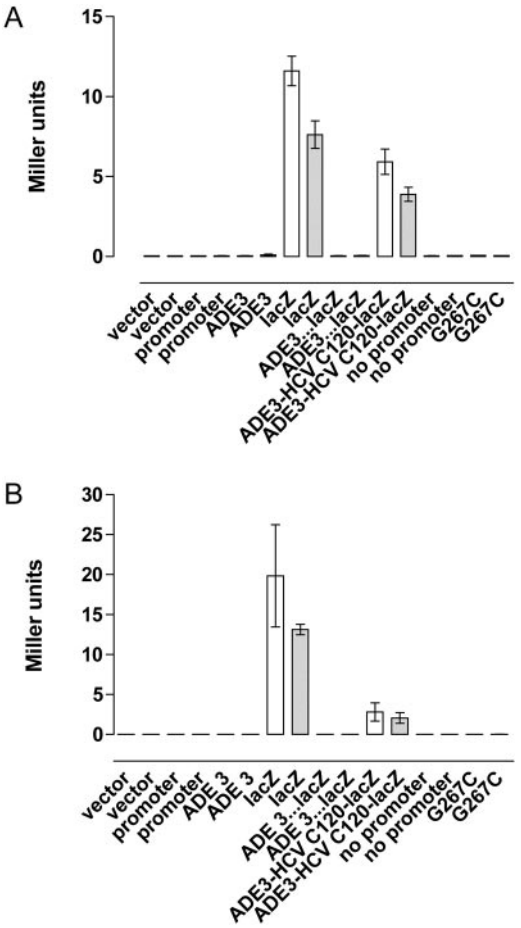

FIG. 4. Liquid β-galactosidase assays of *UPF1* null yeast transformed with plasmids encoding bicistronic mRNAs. Yeast strains AAY273 (panel A) and strain BY4741 (panel B) were transformed with different plasmids, and transformants were assayed for β-galactosidase synthesis. Labels on the *x* axis indicate the structure of the plasmid that was introduced into yeast strain. Clear bars, isogenic wild-type yeast strain; gray bars, mutant yeast strain. Vector: no promoter or DNA insert; promoter: *ADH1* promoter only; *ADE3*: *ADE3* gene only; *lacZ*: *lacZ* gene only; *ADE3*. . .*lacZ*: *ADE3* and *lacZ* genes with no IRES; ADE3-HCV C120-*lacZ*: *ADE3* gene, HCV IRES, amino acids 1 to 120 of the polyprotein, and *lacZ*; no promoter: ADE3-HCV C120-*lacZ* without the *ADH1* promoter; G267C: ADE3-HCV C120-*lacZ* with a point mutation at nucleotide 267 of the HCV 5′ untranslated region. *y* axis, β-galactosidase activity in Miller units as determined by solution assay.

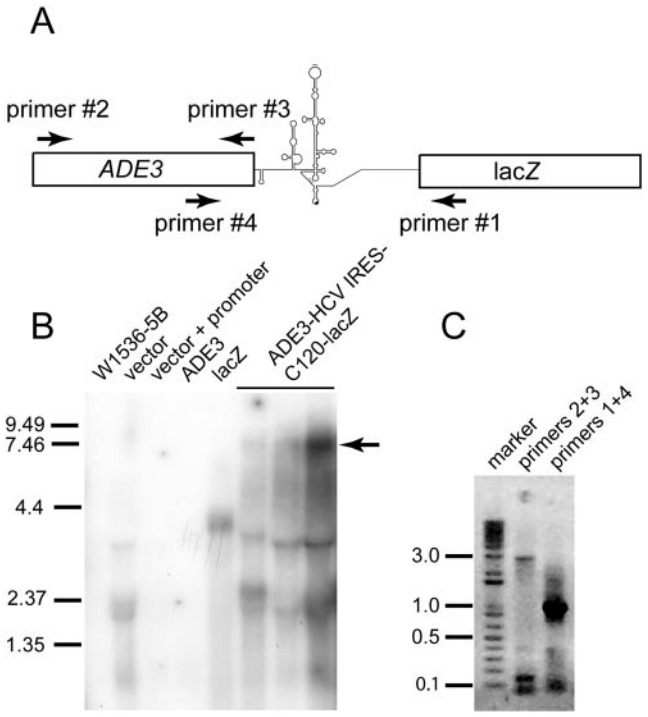

FIG. 5. Analysis of bicistronic RNA in yeast. A. Schematic diagram of the predicted bicistronic mRNA from plasmid ADE3-HCV C120-*lacZ*. The HCV IRES is shown between the open reading frames for Ade3p and β-galactosidase. The locations of oligonucleotide primers used in reverse transcription and PCRs (C) are shown. B. Northern blot hybridization analysis of total RNA from wild-type yeast W1536-5BH or from yeast containing vector, vector with *ADH1* promoter, vectors that produce monocistronic *ADE3* or *lacZ* RNA, or the bicistronic ADE3-HCV C120-*lacZ* RNA. For cells containing the HCV IRES, RNA from three colonies was prepared. The hybridization probe was a 300-nucleotide *lacZ* DNA. The arrow indicates 7.4-kb bicistronic mRNA. The sizes of RNA molecular size markers in kb are shown at left. C. Reverse transcription-PCR products fractionated in a 1% agarose gel. Primer 1 was used to prime reverse transcription of total yeast RNA from cells containing the ADE3-HCV C120-*lacZ* plasmid. Lane 1, DNA markers; lane 2, PCR product obtained with primers 2 and 3; lane 3, PCR product obtained with primers 1 and 4.

Two mutations that abrogate HCV IRES-dependent internal initiation in mammalian cells were introduced into the HCV IRES to provide further evidence that β-galactosidase activity is a consequence of internal ribosome entry. Deletion of nucleotides 28 through 69 (24, 59) or introduction of a point mutation (G267C) in stem loop IIId (32, 34) (Fig. 1) resulted in loss of β-galactosidase activity (Fig. 2 and 3A). Southern analysis of total cellular DNA from yeast containing plasmid Δ28-69 (data not shown) or G267C demonstrated no rearrangement or deletion of the plasmid (Fig. 3B). The different patterns observed with the wild-type and mutant IRESes (lanes 1 to 5 and 6 to 10) are due to the presence of a second StuI site within the HCV IRES that is more efficiently recognized when the G267C mutation is present. Therefore, mutations that abolish HCV IRES-dependent internal initiation in mammalian cells also eliminate internal initiation in yeast.

β-Galactosidase activity in yeast harboring the plasmid ADE3-HCV C120-*lacZ* might be a consequence of translation of monocistronic mRNAs produced at a cryptic promoter. Deletion of the yeast *ADH1* promoter, at which transcription of the bicistronic mRNAs initiates, caused loss of β-galactosidase (Fig. 2) and Ade3p (unpublished data) and Southern blot hybridization analysis revealed no rearrangement or deletion of the plasmid (data not shown). These results demonstrate that β-galactosidase activity cannot be explained by the presence of a cryptic promoter in the bicistronic plasmid.

Internal initiation in a yeast strain defective in nonsense-mediated decay. The production of truncated proteins in eukaryotic cells is regulated by nonsense-mediated decay. This process leads to degradation of aberrant RNAs on which the ribosome has scanned past the initiating AUG codon, are improperly spliced, contain premature termination or non-

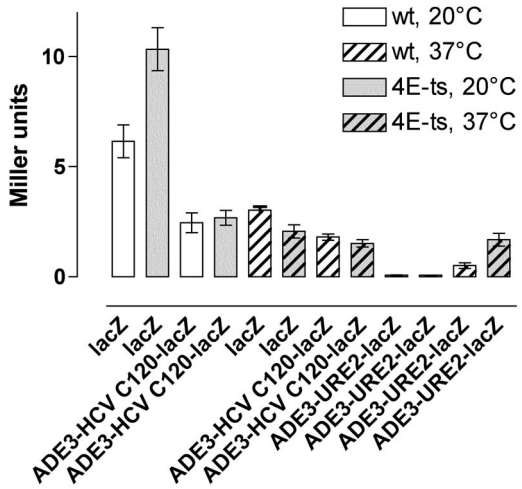

FIG. 6. Liquid β-galactosidase assays of yeast producing a temperature-sensitive eIF4E protein transformed with plasmids encoding bicistronic mRNAs. Yeast strains CW04 (wild type) and 4-2(eIF4E-ts) were transformed with different plasmids, and transformants were assayed for β-galactosidase synthesis. Labels on the *x* axis indicate the structure of the plasmid that was introduced into yeast strains. *lacZ*: *lacZ* gene only; ADE3-HCV C120-*lacZ*: *ADE3* gene, HCV IRES, amino acids 1 to 120 of the polyprotein, and *lacZ*; ADE3-URE2-*lacZ*: ADE3, nucleotides 3 to 1033 of the yeast *URE2* gene, and *lacZ*. *y* axis, β-galactosidase activity in Miller units as determined by solution assay.

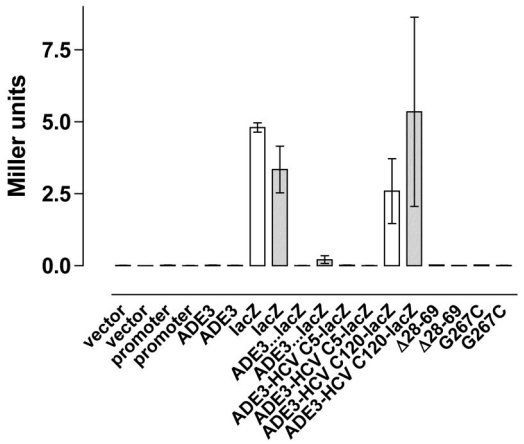

FIG. 7. Liquid β-galactosidase assays of yeast deleted for genes encoding orthologs of La protein. Yeast strains CY1 (gray bars) and YSS238B (white bars) were transformed with different plasmids, and transformants were assayed for β-galactosidase synthesis. Labels on the *x* axis indicate the structure of the plasmid that was introduced into yeast. Vector: no promoter or DNA insert; promoter: *ADH1* promoter only; *ADE3*: *ADE3* gene only; *lacZ*: *lacZ* gene only; ADE3. . .*lacZ*: *ADE3* and *lacZ* genes with no IRES; ADE3-HCV C5-*lacZ*: *ADE3* gene, HCV IRES, amino acids 1 to 5 of the polyprotein, and *lacZ*; ADE3-HCV C120-*lacZ*: *ADE3* gene, HCV IRES, amino acids 1 to 120 of the polyprotein, and *lacZ*; Δ28-69: ADE3-HCV C120-*lacZ* lacking nucleotides 28 to 69 of the HCV 5′ untranslated region; G267C: ADE3-HCV C120-*lacZ* with a point mutation at nucleotide 267 of the HCV 5′ untranslated region. *y* axis, β-galactosidase activity in Miller units as determined by solution assay.

sense codons, or have upstream open reading frames (17, 22, 53, 68, 69, 71). Bicistronic mRNA produced from the plasmid ADE3-HCV C120-*lacZ* contains two open reading frames and therefore might trigger the nonsense-mediated decay pathway. Activation of this pathway might lead to the accumulation of fragmented mRNAs, some of which might only encode *lacZ*. β-Galactosidase activity might be a consequence of 5′-end-dependent initiation from this broken mRNA.

Aberrant transcripts are stabilized in yeast that lack Upf1p, an integral component of nonsense-mediated decay (40, 72, 73). If β-galactosidase is produced by translation of aberrant RNAs generated by nonsense-mediated decay, then β-galactosidase activity should be abolished in strains lacking the *UPF1* gene. Both 5′-dependent and internal initiation was reduced 1.5-fold in a *UPF1* null strain compared to an isogenic wild-type yeast strain (Fig. 4). Southern blot hybridization analysis revealed no rearrangement or deletion of plasmids in this mutant strain (data not shown). These results indicate that β-galactosidase is not translated from fragmented mRNAs produced by nonsense-mediated decay.

Analysis of bicistronic RNA in yeast. Bicistronic mRNA produced from plasmid ADE3-HCV C120-*lacZ* is expected to be 7.4 kb in length (Fig. 5A). The results of Northern blot hybridization analysis of total RNA isolated from yeast transformed with this plasmid reveal the presence of intact bicistronic mRNA (Fig. 5B). Smaller RNAs observed in yeast containing this plasmid were also observed in yeast harboring the vector (Fig. 5B, lane 2). The bicistronic mRNA was amplified by reverse transcription primed with a *lacZ*-specific primer (Fig. 5A, primer 1). The reverse transcription product was used in PCRs to amplify the upstream open reading frame of *ADE3* and sequences of *ADE3*, the HCV IRES, and *lacZ* (Fig. 5C).

When primers complementary to *lacZ* and *ADE3* were used (primers 1 and 4, Fig. 5A), a DNA product was observed with a size consistent with the presence of the HCV IRES between the 5′ and 3′ open reading frames (Fig. 5C). The full-length open reading frame of *ADE3* was amplified using primers that overlap the initiation and termination codons (Fig. 5C). DNA product was not observed when primers complementary to the 5′ end of *ADE3* and the 5′ end of *lacZ* were used (not shown). Although the results of Northern analysis demonstrate the presence of the 7.4 kb bicistronic RNA in yeast (Fig. 5B), higher order structures may prevent amplification of a complete DNA copy. The results of RNA analysis therefore indicate that the bicistronic RNA is intact in yeast. Production of β-galactosidase is therefore not a consequence of translation of either an aberrantly spliced or fragmented bicistronic transcript.

HCV IRES-meditated internal initiation can be uncoupled from 5′-end-dependent initiation. The capacity of an RNA to direct internal initiation of the second open reading of a bicistronic mRNA should be independent of translation of the first open frame (31). Removal of the initiation codon of the *ADE3* gene, blocking translation of the first open reading frame, was used to provide further evidence for internal initiation of ADE3-HCV C120-*lacZ* bicistronic RNA. Deletion of the initiation codon of the *ADE3* gene abolished Ade3p production (not shown) but did not reduce β-galactosidase activity (Fig. 2). Therefore β-galactosidase is not synthesized by ribosomes that terminate after the *ADE3* open reading frame, traverse the IRES, and reinitiate at the HCV AUG.

Blocking 5′-end-dependent initiation by altering the inter-

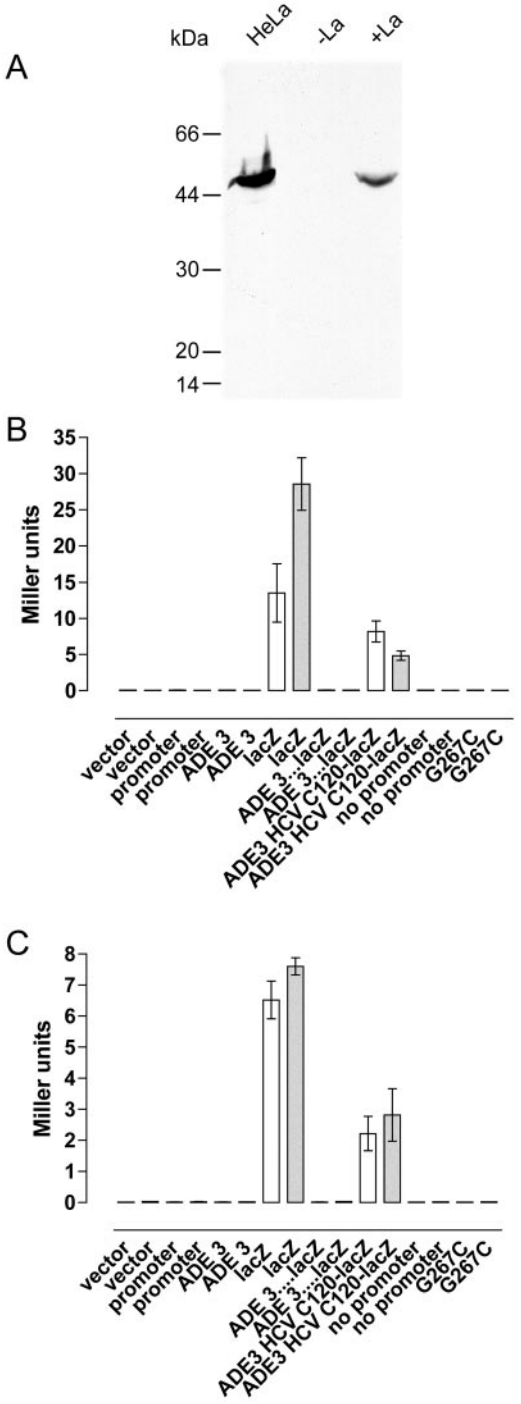

FIG. 8. Effect of human La synthesis on HCV IRES-mediated translation in yeast. A. Western blot analysis of human La synthesis in yeast. Extracts from cultures of strain 1231 (wild type) and the same strain transformed with a plasmid encoding human La protein, and HeLa cells (used as a marker for La protein) were fractionated on a 10% SDS-PAGE gel. Proteins were transferred to a polyvinylidene fluoride membrane, and human La protein (52 kDa) was detected by using a rabbit antibody specific for human La protein and the LumiGlo chemiluminescent substrate system. HeLa, extract from HeLa cells; –La, extract from untransformed wild-type yeast; +La, extract from yeast transformed with a plasmid encoding human La protein. The autoradiograph was scanned and labeled using Adobe Photoshop. B and C. Liquid β-galactosidase assays of yeast producing human La protein. Yeast strains 1231 (wild type, B) and CY1 (wild type, C) were transformed with different plasmids, and transformants were assayed for β-galactosidase synthesis. White bars, strains in the absence of human La protein; gray bars, strains synthesizing human La protein. Labels on the *x* axis indicate the structure of the plasmid that was introduced into yeast. Vector: no promoter or DNA insert; promoter: *ADH1* promoter only; *ADE3*: *ADE3* gene only; *lacZ*: *lacZ* gene only; ADE3. . .*lacZ*: *ADE3* and *lacZ* genes with no IRES; ADE3-HCV C120-*lacZ*: *ADE3* gene, HCV IRES, amino acids 1 to 120 of the polyprotein, and *lacZ*; no promoter: ADE3-HCV C120-*lacZ* without the *ADH1* promoter; G267C: ADE3-HCV C120-*lacZ* with a point mutation at nucleotide 267 of the HCV 5′ untranslated region. *y* axis, β-galactosidase activity in Miller units as determined by solution assay.

action between the 5′-cap structure and the cap binding protein, eIF4E, is another way to prevent translation of the first open reading frame of the bicistronic RNA. A single amino acid change in yeast eIF4E disrupts the interaction between eIF4E and the 5′-cap structure, renders the cells temperature sensitive, and reduces 5′-end-dependent initiation at both the permissive (20°C) and nonpermissive (37°) temperatures (5). The plasmid encoding the bicistronic mRNA ADE3-HCV C120-*lacZ* was introduced into yeast producing the altered eIF4E. Production of β-galactosidase from a monocistronic mRNA by a 5′-end-dependent mechanism was reduced fivefold at the nonpermissive temperature in the eIF4E-ts mutant (Fig. 6). In contrast, internal initiation mediated by the HCV IRES was reduced 1.9-fold in this mutant (Fig. 6). Synthesis of β-galactosidase dependent on internal initiation mediated by the HCV IRES is therefore independent of the interaction between eIF4E and the 5′-cap structure. The URE2 IRES has been shown to direct internal initiation only in the presence of altered eIF4E (38). In agreement with these findings, internal initiation dependent upon the URE2 IRES was observed only in the eIF4E-ts mutant at the nonpermissive temperature (Fig. 6), confirming that eIF4E function was compromised in these cells. These results demonstrate that β-galactosidase production is independent of translation of the upstream open reading frame, providing additional evidence for internal initiation by the yeast ribosome.

Requirement for cell proteins in internal initiation. La protein is believed to be necessary for internal initiation mediated by the HCV IRES in mammalian cells (1). Three yeast genes encoding orthologs of La protein, *LPH1*, *SRO9*, and *SLF1*, have been identified (63, 74). Plasmids encoding monocistronic *lacZ* mRNA and bicistronic mRNAs were introduced into yeast strains lacking *LPH1* or all three genes, and the effect on translation was determined. The absence of Lph1p (not shown) or all three La orthologs had no effect on HCV IRES dependent or 5′-end-dependent initiation (Fig. 7). In contrast, depletion of La protein from mammalian cells reduces internal initiation mediated by the HCV IRES (1, 16). The effect of human La protein on HCV IRES-mediated internal initiation in yeast was examined by producing this protein in two wild-type yeast strains (1231 and CY1) and the strain in which the *LPH1* gene had been disrupted (CY4). These strains contain a plasmid encoding either monocistronic *lacZ* mRNA, bicistronic mRNA encoding the wild-type HCV IRES-C120, or a 23-nucleotide spacer sequence. Synthesis of human La protein in all yeast strains was confirmed by Western blot analysis (Fig. 8A; not shown for strains CY1 and CY4). Production of human

La protein had no effect on HCV IRES-dependent or 5′-end-dependent initiation in any of the yeast strains tested (Fig. 8B and 8C; not shown for CY4).

Two additional mammalian proteins that bind the HCV IRES are Ptb and Pcbp2 (19, 35). The genome of *S. cerevisiae* does not encode orthologs of these proteins. Pcbp2 protein was produced in yeast to determine whether it would influence internal initiation. Western blot analysis confirmed that the protein was produced in yeast (Fig. 9A), yet no effect on HCV IRES-mediated internal or 5′-end-dependent initiation was observed (Fig. 9B).

Polypyrimidine tract-binding protein binds three sites within the HCV 5′UTR (2), one site each in the polyprotein open reading frame and one in the 3′UTR (30). Binding of Ptb to RNA of the HCV open reading frame is thought to repress synthesis of the viral polyprotein. Interaction of Ptb with both the 5′ and 3′ UTRs derepresses this inhibition (30). The effect of Ptb on internal initiation was determined by synthesizing the protein in yeast (Fig. 10A). The presence of Ptb stimulated HCV IRES-dependent internal initiation and repressed 5′-end-dependent initiation (Fig. 10B).

DISCUSSION

Initiation of translation is an evolutionarily conserved process in yeast and humans. Efficient initiation of translation of cellular mRNAs is dependent upon the presence of a 7-methylguanosine cap structure at the 5′ end of the RNA, a relatively unstructured 5′ untranslated region, and a poly(A) tail at the 3′ end. The 7-methylguanosine cap structure is recognized by eIF4E, the cap binding protein. This interaction facilitates the recruitment of the small ribosomal subunit and all interacting complexes to the 5′ end of the mRNA. The ribosome then scans to the initiating AUG codon. Hepatitis C virus mRNA lacks a 5′-cap structure, and the 5′ untranslated region is highly structured and contains multiple AUG codons. These characteristics suggest that translation of the viral mRNA does not occur by a 5′-end-dependent mechanism. Instead, translation of the viral mRNA occurs by internal entry of the ribosome within the 5′UTR (67). The 5′UTR of HCV mediates translation of the downstream open reading of a bicistronic transcript in poliovirus-infected cells, further demonstrating that translation does not occur by a 5′-end-dependent mechanism (70).

The identification of cell proteins required for internal ribosome binding provides insight into the mechanism of IRES-dependent initiation. In vitro binding and UV cross-linking assays have been used to identify cell proteins that may be needed for efficient internal initiation mediated by the HCV IRES, including La protein (3), polypyrimidine tract-binding protein (2), poly(rC)-binding protein 2 (64), and Nsap1 (35). The yeast *S. cerevisiae* has been used as a model organism to understand 5′-end-dependent initiation (23). The study of IRES-mediated translation in this organism should also provide insight into the mechanism of internal ribosome binding. Internal initiation mediated by the HCV IRES in *S. cerevisiae* was demonstrated by the production of β-galactosidase from a bicistronic mRNA in which the IRES was inserted between the yeast *ADE3* and bacterial *lacZ* genes. These findings establish a functional assay for identifying proteins required for IRES-mediated initiation.

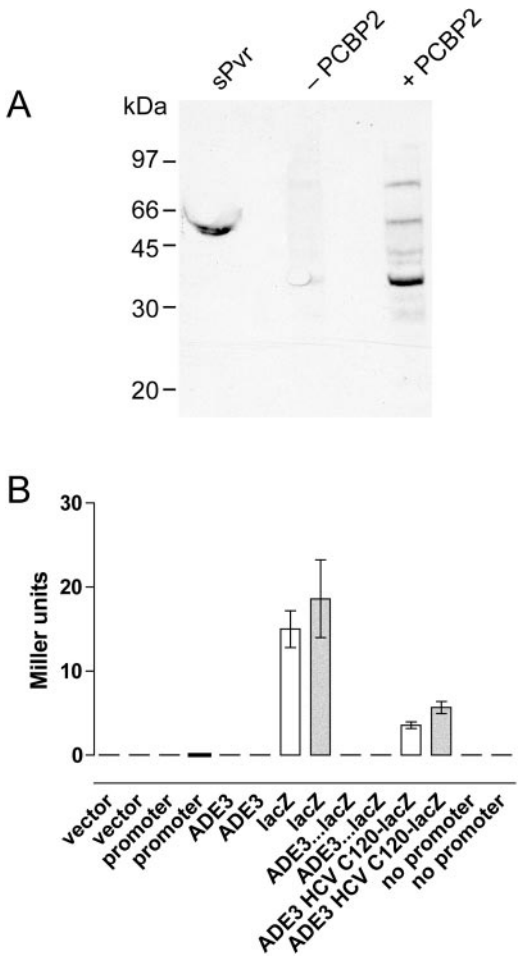

FIG. 9. Effect of human Pcbp2 synthesis on HCV IRES-mediated translation in yeast. A. Western blot analysis of human Pcbp2 synthesis in yeast. Extracts from cultures of strain 1231 (wild type) and the same strain transformed with a plasmid encoding human Pcbp2 protein, and HeLa cells producing soluble poliovirus receptor (46) (used as a marker) were fractionated on a 10% SDS-PAGE gel. Proteins were transferred to a polyvinylidene fluoride membrane, and human Pcbp2 protein (48 kDa) was detected by using a mouse monoclonal antibody against the His6 epitope and the LumiGlo chemiluminescent substrate system. sPvr, extract from HeLa cells producing soluble Pvr; –Pcbp2, extract from untransformed wild-type yeast; +Pcbp2, extract from yeast transformed with a plasmid encoding human Pcbp2 protein. The autoradiograph was scanned and labeled using Adobe Photoshop. B. Liquid β-galactosidase assays of yeast producing human Pcbp2 protein. Yeast strain 1231 (wild type) was transformed with different plasmids, and transformants were assayed for β-galactosidase synthesis. White bars, absence of human Pcbp2 protein; gray bars, strains synthesizing human Pcbp2 protein. Labels on the *x* axis indicate the structure of the plasmid that was introduced into yeast. Vector: no promoter or DNA insert; promoter: *ADH1* promoter only; *ADE3*: *ADE3* gene only; *lacZ*: *lacZ* gene only; ADE3. . .*lacZ*: *ADE3* and *lacZ* genes with no IRES; ADE3-HCV C120-*lacZ*: *ADE3* gene, HCV IRES, amino acids 1 to 120 of the polyprotein, and *lacZ*; no promoter: ADE3-HCV C120-*lacZ* without the *ADH1* promoter. *y* axis, β-galactosidase activity in Miller units as determined by solution assay.

Mutations known to abrogate HCV IRES-mediated internal initiation in mammalian cells also prevented β-galactosidase

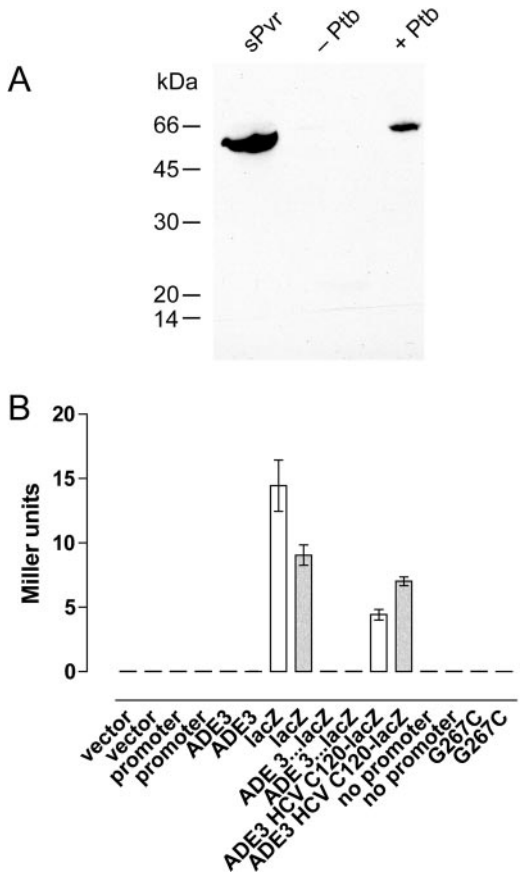

FIG. 10. Effect of human Ptb synthesis on HCV IRES-mediated translation in yeast. A. Western blot analysis of human Ptb synthesis in yeast. Extracts from cultures of strain 1231 (wild type) and the same strain transformed with a plasmid encoding human Ptb protein, and HeLa cells producing soluble Pvr (46) (used as a marker) were fractionated on a 10% SDS-PAGE gel. Proteins were transferred to a polyvinylidene fluoride membrane, and human Ptb protein (57 kDa) was detected by using a mouse monoclonal antibody against the His6 epitope and the LumiGlo chemiluminescent substrate system. sPvr, extract from HeLa cells producing soluble Pvr; –Ptb, extract from untransformed wild-type yeast; +Ptb, extract from yeast transformed with a plasmid encoding human Ptb protein. The autoradiograph was scanned and labeled using Adobe Photoshop. B. Liquid β-galactosidase assays of yeast producing human Ptb protein. Yeast strain 1231 (wild type) was transformed with different plasmids, and transformants were assayed for β-galactosidase synthesis. White bars, strain in the absence of human Ptb protein; gray bars, strains synthesizing human Ptb protein. Labels on the *x* axis indicate the structure of the plasmid that was introduced into yeast. Vector: no promoter or DNA insert; promoter: *ADH1* promoter only; *ADE3*: *ADE3* gene only; *lacZ*: *lacZ* gene only; ADE3. . .*lacZ*: *ADE3* and *lacZ* genes with no IRES; ADE3-HCV C120-*lacZ*: *ADE3* gene, HCV IRES, amino acids 1 to 120 of the polyprotein, and *lacZ*; no promoter: ADE3-HCV C120-*lacZ* without the *ADH1* promoter; G267C: ADE3-HCV C120-*lacZ* with a point mutation at nucleotide 267 of the HCV 5′ untranslated region. *y* axis, β-galactosidase activity in Miller units as determined by solution assay.

synthesis in yeast, providing genetic evidence for internal initiation. Translation of fragmented, uncapped RNAs derived from the bicistronic RNA could account for the production of β-galactosidase. Results of Northern analysis demonstrate the presence of full-length bicistronic mRNA in yeast. Even though smaller RNAs were detected in strains harboring the bicistronic plasmid, they were also present in cells containing the plasmid vector (Fig. 5). If low levels of fragmented RNAs are present, these RNAs would lack a 5′-cap structure, and perhaps a poly(A) tail, and would be poorly translated in wild-type yeast (8, 42, 60). Uncapped cellular mRNAs are found only in mitochondria in yeast and are translated in a prokaryotic-like manner with formyl-$^{met}tRNA_i$ as the initiating codon (18). The uncapped, nonpolyadenylated RNAs of the yeast L-A virus and satellite M virus are translated efficiently in the cytoplasm only when host cell transcripts are uncapped by the major coat protein of the virus, a trick to defeat the mRNA surveillance systems of the cell (45). These considerations, together with the observation that initiation via the HCV IRES in yeast is efficient (Fig. 2), make it highly unlikely that β-galactosidase activity is a consequence of translation of fragmented RNAs.

Activation of the nonsense-mediated decay pathway could lead to the production of fragmented RNAs in the cytoplasm of yeast cells. Bicistronic mRNAs contain two open reading frames and might trigger the nonsense-mediated decay pathway. If β-galactosidase is produced by translation of aberrant RNAs generated by nonsense-mediated decay, then β-galactosidase activity should be abolished in strains lacking the *UPF1* gene. Both 5′-dependent and internal initiation was reduced 1.5-fold in a *UPF1* null strain compared to an isogenic wild-type yeast strain (Fig. 4). The reduction in 5′-dependent initiation is consistent with a suggested role for Upf1p during translation initiation (21). The 1.5-fold decrease in HCV IRES-mediated initiation suggests that Upf1p is also required during internal ribosome binding.

RNA splicing of the bicistronic transcript could lead to the production of RNAs that are translated by 5′-end-dependent initiation. This possibility seems highly unlikely. Less than 1% of the transcripts synthesized in *S. cerevisiae* are spliced (43). The requirements for RNA splicing in *S. cerevisiae* are much more stringent than in mammalian cells. All introns within pre-mRNA transcripts of yeast have UACUAAC sequences 20 to 55 nucleotides from their 3′ ends (39, 55), and an almost invariant GUAUGUU at their 5′ ends (50). A minimum of 40 nucleotides separate the 5′ splice site and branch point sequence (36, 66), while a stretch of U_n followed by a conserved (U/C)AG is found at the 3′ splice site (43). These sequences are not found within the ADE3-HCV C120-*lacZ* bicistronic mRNA. Furthermore, full-length bicistronic transcript was visualized by Northern analysis, and the results of reverse transcription-PCR revealed the presence of the IRES between *ADE3* and *lacZ* (Fig. 5). These findings demonstrate that internal initiation mediated by the HCV IRES is not a result of splicing of the bicistronic transcript.

The presence of a cryptic promoter in HCV-specific DNA sequences might lead to the production of short, capped *lacZ* monocistronic mRNAs that are translated by 5′-end-dependent initiation. If such cryptic promoters were present, then deletion of the *ADH1* promoter, which directs transcription of the bicistronic mRNA, should not affect the level of β-galactosidase. Deletion of the *ADH1* promoter for production of bicistronic mRNAs abolished synthesis of β-galactosidase.

It is not known why the HCV IRES mediates internal initiation in yeast when RNA encoding 120 amino acids of the viral polyprotein but not 5 amino acids was included in the bicis-

tronic mRNA. Nonviral open reading frames may influence the efficiency of internal initiation, possibly by affecting the secondary structure surrounding the initiating codon (58). Such an influence has been reported for another virus. The intergenic IRES of *Plautia stali* virus is more efficient at mediating internal initiation when viral RNA downstream of the 3′ end of the IRES is included with the downstream cistron of the bicistronic mRNA (61).

A number of cellular proteins appear to be required for efficient internal initiation mediated by the HCV IRES. La protein binds the 5′ untranslated region of hepatitis C virus around the initiating AUG codon (3). Addition of recombinant La protein to rabbit reticulocyte lysates stimulates HCV IRES-dependent internal initiation (57), and depletion of La protein from Huh7 cells by SELEX RNA decreased the ability of the HCV IRES to mediate internal initiation (1). La protein also binds the poliovirus IRES (47), and the addition of recombinant La protein to rabbit reticulocyte lysates increases the efficiency of internal initiation mediated by this IRES (48). Reduction of La protein in HeLa cells by treatment with siRNA decreased both translation and replication of poliovirus RNA, and replication of recombinant poliovirus dependent upon the HCV IRES (16).

La protein is a chaperone for small RNAs transcribed by RNA polymerase III. It binds the UUU_{OH} sequence of the 3′ ends of these RNAs and protects them from degradation (75). La protein also effects the translation of mRNAs containing 5′-terminal oligopyrimidine tracts (14). The three genes in *S. cerevisiae* that encode homologs of La protein are *LPH1*, *SRO9*, and *SLF1*. Similar to the mammalian ortholog, Lph1p is a chaperone for small RNAs transcribed by RNA polymerase III, binds the UUU_{OH} sequence of the 3′ ends of these RNAs, protects them from degradation, and facilitates the formation of RNA-protein complexes of these and other small RNAs in the cell. Lph1p is required for endonucleolytic cleavage of the 3′ trailer sequence of the $tRNA^{Ser}$ CGA. In cells lacking Lhp1p, the 3′ trailer sequence is removed by exonucleases (75). Lhp1p is required for growth when the unfolded protein response is induced by elevated temperatures (28). Sro9p and Slf1p, two cytoplasmic proteins that contain La motifs, bind RNA associated with translating ribosomes. Strains deficient in either protein are less sensitive than wild-type strains to some protein synthesis inhibitors (63).

The role of yeast La protein orthologs in HCV IRES-mediated internal initiation was determined in strains deleted for the genes encoding one or all three of these proteins. The results indicate that the efficiency of internal initiation is not affected in any of these strains. In contrast, depletion of La protein from mammalian cells reduces HCV IRES-mediated initiation (1, 16). The reason for the difference between yeast and mammalian cells is not clear. Yeast proteins other than La protein may act as RNA chaperones for the HCV IRES, or the IRES may fold properly in yeast without the need for La protein. Furthermore, the synthesis of human La protein in yeast did not stimulate internal initiation dependent upon the HCV IRES. This result was unexpected, because supplementing reticulocyte lysates with La protein stimulates HCV IRES-mediated initiation (57). La protein may act as an RNA chaperone for the HCV IRES only in a complex with other proteins that are not encoded in the yeast genome.

The human cellular protein Pcbp2, which binds poly(rC), poly(rG), and poly(rU) tracts in both pre-mRNAs and mRNAs (41), interacts with the 5′UTR of HCV (19, 35) and poliovirus (9). Depletion of Pcbp2 from HeLa cell lysates reduces both translation and replication of poliovirus RNA, and addition of recombinant Pcbp2 to the depleted extract restores translation and replication to wild-type levels (10). It has been suggested that Pcbp2 plays a role in HCV IRES-meditated initiation (64), but depletion of the protein from HeLa cell lysates had no effect on HCV IRES-mediated initiation (15). In agreement with the results of depletion experiments, HCV IRES-dependent initiation was observed in *S. cerevisiae* in the absence of Pcbp2. Furthermore, the synthesis of human Pcbp2 in yeast did not affect the efficiency of HCV IRES-mediated β-galactosidase production. It seems unlikely that Pcbp2 is required for HCV IRES-dependent internal initiation.

Polypyrimidine tract-binding protein binds three sites within the poliovirus IRES, and sites within the IRES, polyprotein coding region, and the variable X region of the 3′UTR of HCV mRNA (7, 59). Ptb binds pyrimidine-rich sequences and is involved in differential splicing of RNA polymerase II transcripts (20). Depletion of Ptb from both reticulocyte lysates and HuH7 cells by SELEX RNA reduced internal initiation mediated by the HCV IRES, and addition of recombinant Ptb restored the ability of the HCV IRES to direct internal initiation (7). In agreement with these findings, HCV IRES-mediated translation occurred in yeast in the absence of Ptb, and production of human Ptb in yeast modestly stimulated the efficiency of HCV IRES-dependent β-galactosidase production. It is not known why production of human Ptb reduced 5′-end-dependent translation in yeast.

Other human proteins believed to be required for internal initiation of translation include upstream of N-ras (unr), murine proliferation-associated protein 1 (Mpp1), and Nsap1 (25, 33, 35, 56). HCV IRES-mediated initiation in yeast is independent of these proteins, as no sequences encoding unr, Mpp1, or Nsap1 are found in the yeast genome.

Internal initiation of translation has been demonstrated both in cell-free yeast extracts (4, 26) and in living yeast cells (38, 65, 76). The intergenic IRES of cricket paralysis virus and sequences from four genes of *S. cerevisiae* have been to shown to mediate internal initiation in yeast in vivo (38, 65, 76). In yeast the cricket paralysis intergenic IRES is efficient at mediating internal initiation only in a mutant strain in which the ternary complex of eIF2-GTP-Met-$tRNA_i^{Met}$ is made artificially low (65). The IRES within the coding region of Ure2p mRNA efficiently mediates internal initiation only in a mutant strain of yeast in which the interaction between the 5′-cap structure and eIF4E is severely compromised (38). In contrast to the results obtained with the HCV IRES, internal initiation was not observed on bicistronic mRNAs containing the 5′UTRs of poliovirus, rhinovirus type 14, and the yeast gene *TFIID* (data not shown). Internal initiation of translation dependent upon the IRES of poliovirus and TFIID has been demonstrated in extracts of yeast cells (26), emphasizing the importance of using an in vivo assay to determine the requirements for internal initiation.

The unique ability of the HCV IRES to mediate internal initiation in wild-type yeast permits a genetic approach to identify cell proteins required for this process. The differences in

the requirements for La and PTB in HCV IRES-mediated initiation in yeast and mammalian systems does not exclude the possibility that other cell proteins may be needed for internal initiation. Novel proteins required for HCV IRES-dependent translation might be revealed by studies in yeast. Furthermore, the core mechanism of translation can be elucidated in yeast. For example, the requirement for specific subunits of eIF2 and eIF3 during internal initiation can be evaluated. These studies may elucidate the mechanism of internal initiation and may lead to the discovery of new antiviral agents against HCV.

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