Evidence That Base-pairing Interaction between Intron and mRNA Leader Sequences Inhibits Initiation of HAC1 mRNA Translation in Yeast*

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Background: Hac1 protein, encoded by a cytoplasmically spliced mRNA, activates the unfolded protein response to maintain cellular protein homeostasis and alleviate endoplasmic reticulum stress.

Results: Under non-stress conditions, translation initiation on the HAC1 mRNA is repressed.

Conclusion: Base-pairing interaction between the 5′ leader and intron represses translation initiation on the HAC1 mRNA.

Significance: A unique mechanism of intron-mediated inhibition of ribosomal scanning.

The Hac1 transcription factor in yeast up-regulates a collection of genes that control protein homeostasis. Base-pairing interactions between sequences in the intron and the 5′-untranslated region (5′ UTR) of the HAC1 mRNA represses Hac1 protein production under basal conditions, whereas cytoplasmic splicing of the intron by the Ire1 kinase-endonuclease, activated under endoplasmic reticulum stress conditions, relieves the inhibition and enables Hac1 synthesis. Using a random mutational screen as well as site-directed mutagenesis, we identify point mutations within the 5′ UTR-intron interaction site that derepress translation of the unspliced HAC1 mRNA. We also show that insertion of an in-frame AUG start codon upstream of the interaction site releases the translational block, demonstrating that an elongating ribosome can disrupt the interaction. Moreover, overexpression of translation initiation factor eIF4A, a helicase, enhances production of Hac1 from an mRNA containing an upstream AUG start codon at the beginning of the base-paired region. These results suggest that the major block of translation occurs at the initiation stage. Supporting this interpretation, the point mutations that enhanced Hac1 production resulted in an increased percentage of the HAC1 mRNA associating with polysomes versus free ribosomal subunits. Thus, our results provide evidence that the 5′ UTR-intron interaction represses translation initiation on the unspliced HAC1 mRNA.

In eukaryotic cells mRNA translation begins with the ordered assembly of the small (40S) ribosomal subunit, initiator methionyl-tRNA (Met-tRNA\textsuperscript{Met}), and initiation factors at the 5′-end of an mRNA (1, 2). The resulting preinitiation complex (PIC)\textsuperscript{3} then travels along the 5′ mRNA leader in search of a translation start codon in a process referred to as ribosomal scanning. Typically, the first AUG codon encountered by the scanning PIC is selected as the translation start site in part due to base-pairing interactions between the anticodon loop of the Met-tRNA\textsuperscript{Met} and the AUG codon. After selection of the start codon, initiation factors dissociate from the PIC, and the 60S ribosomal subunit joins yielding an 80S ribosomal complex (2). The 80S complex then enters the elongation phase of translation and decodes the mRNA to produce a polypeptide.

In addition to alterations in the activity of initiation factors, translation is also controlled by cis-acting mRNA elements (3). For example, binding of the translation factor eIF4E to the 5′-m\textsuperscript{7}G (7-methylguanosine)-cap and the poly(A)-binding protein PABP to the 3′-poly(A) tail facilitates ribosome recruitment to an mRNA (4, 5). In addition, nucleotide sequence context (the Kozak consensus motif) flanking an AUG or non-canonical start codon impacts the efficiency with which a scanning ribosome selects a translation start site (3). Moreover, secondary structure in the 5′ UTR can interfere with PIC binding or scanning and thereby block translation (3, 6–8). Translation of mRNAs with more secondary structure shows a greater requirement for the RNA helicase eIF4A (1). Additionally, out-of-frame AUG start codons and upstream open reading frames (ORFs) in the 5′ leader can restrict access of the ribosome to the main ORF. A classic example is the yeast GCN4 mRNA in which short upstream ORFs potentiate translation of the main GCN4 ORF and confer heightened dependence on translation factor activity (9). In addition to these important roles of sequences in the 5′ UTR, sequence elements in the 3′ UTR can also modulate translation. Interaction of RNA-binding proteins such as the GAIT complex (10) and sex-letal, 3The abbreviations used are: PIC, preinitiation complex; ER, endoplasmic reticulum; WCE, whole cell extract; NAT, nourseothricin-resistance marker genemarker gene; Q-PCR, quantitative PCR; SD, synthetic dextrose; Tm\textsuperscript{R}, tunicamycin-sensitive; Tm\textsuperscript{S}, tunicamycin-resistant; UPR, unfolded protein response.
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Smaug, or CPEB (11, 12), with elements in the 3′ UTR of select mRNAs represses translation.

A unique strategy is observed in regulating translation of the yeast HAC1 mRNA involving the 5′ UTR and the intron (13). The intron (spanning nucleotides G661 to G913, with the adenine of AUG start codon assigned as 1) is not spliced in the nucleus by the spliceosome but instead is retained in the mRNA that is exported to the cytoplasm (14). Previous work established that base-pairing interactions between elements in the intron and the 5′ UTR repress translation of the unspliced HAC1 mRNA (13). Under conditions of endoplasmic reticulum (ER) stress, the endoribonuclease Ire1 is activated and cleaves both exon-intron boundaries of the HAC1 mRNA in the cytoplasm (15, 16). The two exons are then ligated by tRNA ligase (17), resulting in an altered ORF with a new codon starting at nucleotide G661 and a UAG stop codon at nucleotide 963 (see Fig. 1A). Finally, the 3′ UTR has been reported to positively regulate translation of the spliced HAC1 mRNA (18). The Hac1 protein produced from the spliced mRNA is a transcription factor and primary effector of the unfolded protein response (UPR) (19, 20) that activates expression of a set of genes involved in maintaining protein homeostasis and alleviating ER stress (16, 21–24). As the Hac1 proteins produced from both the spliced and unspliced mRNA are functional translation factors (15), a key element in the UPR is repression of translation of the unspliced Hac1 mRNA.

Despite much progress in understanding the regulation of HAC1 mRNA translation, the molecular mechanism is not yet clear. To gain mechanistic insights into HAC1 mRNA translation, we conducted a genetic screen to identify intragenic mutations that enable a splicing-deficient and translationally inert HAC1 mRNA (i.e. HAC1-G661C) to produce a Hac1 protein capable of stimulating the ER stress response. From this screen we identified a single base mutation (i.e. G771A) in the intron that is predicted to disrupt the base-pairing interaction with the 5′ UTR (13), suggesting that disruption of the 5′ UTR-intron interaction leads to translation of the HAC1-G661C,G771A mRNA without splicing. Consistent with the notion that the 5′ UTR-intron interaction interferes with ribosomal scanning and translation initiation on the HAC1 mRNA, introduction of an upstream in-frame AUG codon before the 5′ UTR-intron base-pairing region relieved the translational block on the HAC1 mRNA. Taken together, in contrast to previously proposed models, our data reveal a unique mechanism of intron-mediated inhibition of ribosomal scanning.

**Experimental Procedures**

Yeast Strains and Plasmids—The ire1Δ (MATa his3-Δ1 leu2-Δ0 met5-Δ0 ura3-Δ0 ire1::KanMX) and hac1Δ (MATa his3-Δ1 leu2-Δ0 met5-Δ0 ura3-Δ0 hac1::KanMX) yeast strains were obtained from the yeast genome deletion collection. Strain J751 (MATa his3-Δ1 leu2-Δ0 met5-Δ0 ura3-Δ0 ire1::Nat) was derived by replacing KanMX4 in the ire1Δ strain with the nourseothricin-resistance (Nat) marker gene. The hac1Δ ire1Δ (MATa his3-Δ1 leu2-Δ0 met5-Δ0 ura3-Δ0 ire1::Nat hac1::KanMX) double deletion strain J772 was constructed by replacing HAC1 with KanMX4 in strain J751. Strain J1167 (MATa his3-Δ1 leu2-Δ0 met5-Δ0 ura3-Δ0 ire1::Nat HAC1-G661C,G768C) was constructed by integration of plasmid pC4070 (linearized with Ascl) into strain J751. After selection of segregants on 5-fluoroorotic acid, the replacement of the WT HAC1 allele with the HAC1-G661C,G768C intron mutant was confirmed by PCR. For the UPR assay, yeast growth was tested on SD medium containing the ER stress inducer tunicamycin (0.4 μg/ml). Mutations were created by site-directed mutagenesis or by fusion PCR. The plasmids used in this study are listed in Table 1.

**Reverse Transcription (RT) and Quantitative (Q) PCR Analyses**—RNA isolation and RT-PCR analyses were performed essentially as described previously (25). Yeast cells were grown in synthetic complete medium at 30 °C till the A600 reached ~0.5–0.6. When desired, DTT (5 mM) was added to the medium to induce ER stress, and cells were grown for an additional 4 h. Total RNA was isolated using an RNaseasy kit (Qiagen), and first-strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and random primers. The synthetic cDNA was subjected to RT-PCR and Q-PCR analyses.

| Plasmid | Description | Reference |
|---------|-------------|-----------|
| pRS316  | Low copy URA3 vector | (46)       |
| pc3641  | Destroy SacI site in pRS316 | This study |
| pRS306  | URA3 integrating vector | This study |
| D63     | HAC1 in pRS316 (insert at Kpn1/BamHI sites) | (25)       |
| D470    | HAC1-D110C in pRS316 | This study |
| D803    | HAC1-G661C,G771A in pRS316 | This study |
| pC4058  | HA-HAC1 in pc3641 | This study |
| D1278   | HAC1-C–27G in D63 | This study |
| D1279   | HAC1-G771C in D63 | This study |
| pC4059  | HA-HAC1-C–23G in pC4058 | This study |
| pC4060  | HA-HAC1-C–23G,C–24G in pC4058 | This study |
| pC4062  | HA-HAC1-G661C in pC4058 | This study |
| pC4063  | HA-HAC1-G767C,G768C in pC4058 | This study |
| D1280   | HAC1-C–27G,G771C in D63 | This study |
| pC4070  | HA-HAC1-G767C,G768C in pRS306 | This study |
| D1116   | HAC1-A1G7/G in pRS316 | This study |
|         | HAC1-A1G | This study |
| D1118   | HAC1-A1G | This study |
|         | HAC1-A1G7/G in pRS316 | This study |
| D1119   | HAC1-A1G | This study |
|         | HAC1-A1G7/G in pRS316 | This study |
| D860    | IRE1 in pRS316 | This study |
| B3353   | TIE2 (eIF4A) in Yeplac181 (High copy LEU2) | (47)       |
| FJ2051  | TIE3 (eIF4G) in Yeplac181 | (36)       |
| B3355   | CDC33 (eIF4E) in Yeplac181 | (47)       |
| B3995   | TIE1-D170E (eIF4A-D170E) in Yeplac181 | (34)       |

For RT-PCR, the synthetic cDNA was amplified by PCR using primers specific for HAC1 (forward primer 5′-CGCAATGCATTCTCCATTACCC-3′, corresponding to nucleotides +35 to +60 of the HAC1 open reading frame (ORF), and reverse primer 5′-GGGTTAGCTTTTCTCCTGAAAGTG-3′, corresponding to nucleotides +604 to +621) or ACTI (forward primer 5′-CTGAAAGAAATTATTCGGTG-3′, corresponding to nucleotides +919 to +939 of the ACTI ORF (numbering includes the intron), and reverse primer 5′-CTTGTGGGTGAAC-ACTTTGCTATCCTACCC-3′, corresponding to nucleotides +1408 to +1428). For analysis of HAC1 mRNA splicing, the same forward primer was used along with a reverse primer (5′- CCCACACACCGATAATACGG-3′) that corresponded to...
nucleotides +1002 to +1025. PCR products were then resolved by agarose gel electrophoresis. For Q-PCR, the synthetic cDNA was amplified on a real time PCR machine (Bio-Rad CFX 3.1) using primers specific for HAC1 (forward primer 5′-CTGAACTTGGCTATCCC-3′ and reverse primer 5′-CGATATTCGAGATCTGACTCAGTGA-3′) or ACT1 (forward primer 5′-CTGAAAGAATTTGCGTGCTG-3′ and reverse primer 5′-GTGATGACCTTGGACATT-3′). Experiments were repeated twice, and each sample was run in triplicate. Data were analyzed using CFX 3.1 software, and the threshold (Ct) value was calculated. The Ct value represents the average value of triplicate samples, and these average values were used to calculate the Ct-HAC1/Ct-ACT1 ratio.

Western Blot Analysis—Yeast cells were grown in synthetic complete medium to A600 = 0.6. When desired, 5 mM DTT was then added to the medium to induce ER stress, and cells were incubated an additional 4 h before harvesting. Cells were harvested by centrifugation, mixed with 2 volumes of 20% (w/v) trichloroacetic acid, and then broken by agitation with glass beads. Proteins were extracted with SDS Loading Buffer (2% SDS, 2 mM EDTA, 50 mM Tris-HCl (pH 6.8), 10% glycerol, 0.01% bromphenol blue), and then neutralized with 1 M Tris base. Samples were boiled for 5 min and then subjected to SDS-PAGE and immunoblot analysis using antibodies raised against recombinant Hac1 protein or yeast eIF2α or using anti-HA antibodies (Cell Signaling Technology or Roche Applied Science).

Polysome and Northern Analysis—Cells were grown to mid-log phase, treated with cycloheximide (100 μg/ml), incubated for 5 min, and harvested. Cell pellets were resuspended in 5 ml of Polysome Buffer (20 mM Tris-HCl (pH 8.0), 140 mM KCl, 5 mM MgCl2, 100 μg/ml cycloheximide), pelleted, and then resuspended in 1 ml of Polysome Buffer containing 1% Triton X-100 (Sigma), 1 mM EDTA, EDTA-free Protease Inhibitor tablet (Roche Applied Science) and 4 μl of RNaseOUT (Invitrogen). Cells were broken by vigorous mixing with glass beads at 4 °C using a vortex mixer. WCEs were clarified by centrifugation and the equivalent of 500 μg of protein was loaded per lane. Gradients were fractioned, the fractions were then mixed with ethanol and washed with 70% ethanol, and resuspended in 30 μl of loading dye (1% SDS, 20% glycerol, 100 mM Tris-HCl (pH 6.8), 2% bromphenol blue). Samples were loaded onto a 15% polyacrylamide gel and run for 1.5 h at 200 V. After electrophoresis, the gel was stained with SYBR Green (Molecular Probes) and imaged. The RNA was imaged using phosphorimaging.

Results

Genetic Screen Identifies a Single Mutation in the HAC1 Intron That Bypasses the Requirement for mRNA Splicing to Derepress HAC1 Production—Under conditions of ER stress, the endonuclease Ire1 cleaves the phosphodiester bonds after G661 (see Fig. 1B, lane 1), showing that Hac1 function is essential for the ER stress response (21, 22). Mutation of the first Ire1-cleavage site (i.e. G661C) impaired yeast cell growth on tunicamycin medium (Fig. 1B, row 3), suggesting that the G661C mutation reduced the HAC1 mRNA level, mRNA splicing, and/or translation.

To determine whether the G661C mutation caused a reduction in HAC1 mRNA levels and/or mRNA splicing, total RNA was extracted from these cells and then used as a template to amplify the HAC1 mRNA as well as an endogenous ACT1 mRNA (housekeeping control) by RT-PCR as described under “Experimental Procedures.” As shown in Fig. 1D, similar amounts of ACT1 mRNA were detected in each RNA sample (lanes 1–3). As expected, no HAC1 mRNA was detected in the hac1Δ strain transformed with an empty vector (Fig. 1D, lane 1).
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To determine whether the G661C mutation impaired mRNA splicing, RT-PCR was performed using primers that readily distinguish unspliced HAC1 mRNA from spliced HAC1 mRNA. The m7G (7-methylguanosine) cap, 5' and 3' UTRs (black dotted lines), exons (dark blue boxes), intron (solid orange line), and poly(A) tail (An) are shown. The intron spans nucleotides G661 to G913 and interacts with the 5' UTR. The nucleotide sequence of the 5' UTR-intron interaction is shown at the top. The adenine of the HAC1 AUG start codon is assigned as position +1 with positive and negative values for the downstream and upstream nucleotides, respectively. Two in-frame HAC1 UAG stop codons are shown; one located in the intron (nucleotide positions +691 to +693) and the second located in exon 2 (nucleotide positions +967 to +969). The yeast growth assay. Transformants of hac1Δ or ire1Δ yeast strains carrying an empty vector or expressing the indicated HAC1 allele were grown in SD medium to saturation, and 5 μl of serial dilutions (of A600 = 1.0, 0.1, 0.01, and 0.001) were spotted on SD medium or SD medium containing 0.4 μg/ml tunicamycin and incubated 3 d at 30 °C. The m7G cap of each sample was immunoblotted with polyclonal antibody raised against recombinant Hac1 protein. Strains described in panel B were grown in SD medium and treated with 5 mM DTT to induce ER stress, and WCEs were prepared and subjected to SDS-PAGE followed by Western blot analysis using a polyclonal antibody raised against recombinant Hac1 protein. Lanes are numbered according to row numbers in panel B, D, analysis of HAC1 mRNA levels. Total RNA was extracted from the hac1Δ strains expressing the indicated HAC1 alleles and grown under non-stress condition. The RNA was used as a template for RT and Q-PCR analyses of HAC1 and ACT1 mRNAs as described under “Experimental Procedures.” The ratio of threshold values (Ct) for HAC1 and ACT1 mRNAs were shown. E, RT-PCR analysis of HAC1 mRNA splicing. A hac1Δ strain carrying an empty vector or expressing WT HAC1 or the HAC1-G661C mutant was grown in SD medium and treated with 5 mM DTT, and then total RNA was extracted and used as a template for RT-PCR analysis of 28S rRNA or of unspliced (HAC1u) and spliced (HAC1+) HAC1 transcripts as previously described (25).

To confirm that the G661C mutation eliminated Hac1 protein expression, WCEs were prepared from the cells exposed to DTT to induce ER stress (“Experimental Procedures”) and then subjected to immunoblot analysis using an antibody against the Hac1 protein. As expected, the Hac1 protein was detected in the extract obtained from cells expressing the WT HAC1 allele (Fig. 1C, lane 2). In contrast, no Hac1 protein was detected in cells expressing the HAC1-G661C mutant (lane 3). These results are consistent with the model (13, 15) that translation of the unspliced HAC1 mRNA is blocked.

To screen for intragenic suppressors of the splicing- and translation-defective HAC1-G661C allele, a plasmid bearing the HAC1-G661C variant was amplified in Escherichia coli XL-1 red cells (Stratagene) that are deficient in three DNA repair pathways. The mutagenized pool of plasmids was purified and used to transform a hac1Δ yeast strain. Transformants were screened for the ability to grow on medium containing tunicamycin. The HAC1 plasmid was isolated from the tunicamycin-resistant (TmR) colonies, re-tested in a hac1Δ strain to confirm that the TmR phenotype was associated with the plasmid, and then sequenced to detect the mutation. A single second-site suppressor mutation, G771A, was found to restore the
The G771A mutation derepressed the HAC1 mRNA translation (Fig. 1C, lane 4) or ire1Δ (Fig. 1C, lane 7) cells. These data demonstrate that a single base mutation in the intron is sufficient to eliminate translational control of the HAC1 mRNA.

Translational Control of the HAC1 mRNA Is Sensitive to Alteration of a Single Base Pair Interaction between the Intron and 5’ UTR—To gain further insights into HAC1 translational control and the importance of base-pairing contacts between the intron and the 5’ UTR of the HAC1 mRNA, mutations designed to disrupt single or double base-pairing interactions were introduced into the intron or the 5’ UTR of a human influenza hemagglutinin (HA) epitope-tagged version of HAC1. In this construct, the HA tag was inserted between residues Ser-10 and Asn-11 of the HAC1 ORF. Importantly, the HA-tagged HAC1 allele functioned like untagged WT HAC1 and complemented the TmS phenotype of a hac1Δ strain (Fig. 2A, row 2) in an IRE1-dependent manner (Fig. 2B, row 2). As shown in Fig. 2B, the single C→23G (row 3) and the double C→23G,C→24G (row 4) mutations, located in the 5’ UTR of the HA-HAC1 mRNA and designed to disrupt the base-pairing interaction with the intron (Fig. 1A), suppressed the TmS phenotype of an ire1Δ hac1Δ double-mutant strain. As no HAC1 mRNA splicing takes place in the ire1Δ hac1Δ strain, these results indicate that, like the G771A mutation in the intron (Fig. 1B, row 7), point mutations in the HAC1 5’ UTR can derepress Hac1 protein expression.

The G771A mutation derepressed HAC1 mRNA translation (Fig. 1C, lane 4) or ire1Δ (Fig. 1C, lane 7) cells. These data demonstrate that a single base mutation in the intron is sufficient to eliminate translational control of the HAC1 mRNA.
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(row 5) and G767C,G768C (row 6) mutations in HA-HAC1 conferred strong TmR phenotypes in the ire1Δ hac1Δ strain, suggesting that mutations in either the 5’ UTR or the intron allowed ribosomes to produce the Hac1 protein from the unspliced mRNA.

Immunoblot and Northern analyses were used to assess the impact of the 5’ UTR and intron mutations on Hac1 protein and mRNA levels. As the cells lack Ire1 protein and no splicing of the HAC1 mRNA will take place, extracts were prepared from cells grown in the absence of ER stress. Whereas no HA-Hac1 was detected in the extracts from cells expressing WT HA-HAC1 (Fig. 2C, upper panel, lane 2), very low or low levels of HA-Hac1 protein were observed in the extracts prepared from cells expressing the C→23G or C→23G,C→24G mutant alleles, respectively, of HA-HAC1 (Fig. 2C, upper panel, lanes 3 and 4). Thus, low level expression of HA-Hac1 protein from the HA-HAC1-C→23G allele was apparently sufficient to confer a TmR phenotype. Consistent with the more pronounced TiR phenotype, the HA-Hac1 protein was readily detected in extracts prepared from the ire1Δ hac1Δ strain expressing the G767C (Fig. 2C, lane 5) and G767C,G768C (lane 6) mutant form of HA-HAC1. Northern analyses revealed that the levels of HA-HAC1 mRNA were similar in cells expressing the WT or various mutant alleles (Fig. 2C, lower panel, lanes 3–6 versus 2), suggesting that differences in protein expression were not due to altered mRNA levels. These results provide further support for the hypothesis that mutations designed to disrupt the 5’ UTR-intron interaction derepress Hac1 production by enabling translation of the unspliced HAC1 mRNA.

The random mutational screen (Fig. 1B) revealed that the G771A mutation in the HAC1 intron derepressed Hac1 synthesis. In the unspliced HAC1 mRNA, G771 is predicted to form a 27/G771 base pair. As shown in Fig. 2D, both HAC1-C→27G (row 3) and HAC1-G771C mutants (row 4) conferred a TMr phenotype in the ire1Δ hac1Δ strain. In contrast, the HAC1-C→27G,G771C double mutant (Fig. 2D, row 5) functioned like WT HAC1 (row 2) and conferred a TMs phenotype in the ire1Δ hac1Δ strain. As expected, the Hac1 protein was detected only in the extracts obtained from cells expressing the HAC1-C→27G mutant (Fig. 2E, Western, lane 2) and the HAC1-G771C mutant (lane 4). The mutual suppression of the HAC1-C→27G and HAC1-G771C mutants in the HAC1-C→27G,G771C double mutant is consistent with the proposed base-pairing interaction between these residues and further demonstrates that repression of HAC1 expression by the 5’ UTR-intron interaction is sensitive to loss of a single base pair interaction.

When expressed in a hac1Δ strain (IRE1 intact in the chromosome), the HAC1-C→27G, HAC1-G771C, and HAC1-C→27G,G771C mutants, like WT HAC1, conferred a TMr phenotype (Fig. 2F, rows 2–5). Based on the results of these mutational analyses, we conclude that 5’ UTR-intron interaction and, in particular, the C→27/G771 base pair plays an important role in repressing HAC1 mRNA translation.

Introduction of an In-frame AUG Start Codon Upstream of the Secondary Structure in the 5’ UTR Derepresses HAC1 Synthesis—The secondary structure in the 5’ UTR could impair Hac1 synthesis by blocking ribosomal scanning to the start codon or, as has been proposed (13), by impairing translation elongation. To differentiate between these models, AUG start codons were inserted at three sites in the 5’ UTR of the HAC1 gene (Fig. 3A). All three of the introduced AUG codons were in-frame with the HAC1 ORF and thus would be predicted to encode Hac1 proteins with N-terminal extensions. To exclusively monitor translation initiating at the inserted AUG codon, the adenine of the authentic HAC1 start codon was mutated to guanine, generating the HAC1-A1G allele, and the adenine of the AUG codon encoding Met3 was likewise altered to guanine to generate the HAC1-A7G mutation (Fig. 3A). As shown in Fig. 3B, the HAC1-A1G,A7G mutant allele failed to complement the TmR phenotype of a hac1Δ strain (row 3). As the A1G,A7G mutation did not reduce HAC1 mRNA levels (Fig. 3D, lane 3), this result is consistent with the notion that, even following splicing, scanning ribosomes fail to initiate at the HAC1 start site (a GUG codon in the A1G mutant), and no Hac1 protein was produced (Fig. 3C, lane 2).

We first characterized the effect of replacing nucleotides −42/UA−40, immediately upstream of the 5’ UTR-intron interaction (see Figs. 1A and 3A), with an AUG codon to generate the HAC1-AUG−42,A1G,A7G allele. As shown in Fig. 3B, the HAC1-AUG−42,A1G,A7G allele conferred a TMr phenotype when expressed in a hac1Δ strain (row 4), suggesting that a functional Hac1 protein with an extra 14 amino acids at the N terminus was expressed from the AUG−42 codon. To directly examine Hac1 production, Western analyses were performed on WCEs prepared from ER-stressed cells using an antibody prepared against recombinant Hac1 protein. Consistent with the introduction of 14 extra N-terminal residues, the Hac1 protein in the HAC1-AUG−42,A1G,A7G cells was of higher molecular weight than the WT Hac1 protein (Fig. 3C, lanes 1 and 3). It is important to note that these experiments were performed using a hac1Δ strain in which the chromosomal IRE1 gene was intact. Thus, it is likely that the HAC1-AUG−42,A1G,A7G mRNA was spliced in these cells, and we conclude that a 14-residue N-terminal extension does not impair Hac1 production or the ability of Hac1 to promote yeast cell growth on medium containing tunicamycin. However, the intent of these experiments was to determine whether the introduced AUG−42 start codon could promote translation of the unspliced HAC1 mRNA. Therefore, we expressed the HAC1-AUG−42,A1G,A7G allele in an ire1Δ strain where the mRNA will not be spliced. As shown in Fig. 3B, the HAC1-AUG−42,A1G,A7G allele failed to complement the TMr phenotype of the ire1Δ strain (Fig. 3B, row 5), and Western analyses showed that the Hac1 protein was not produced (Fig. 3D, lower panel, lane 2). These results indicate that the unspliced HAC1-AUG−42,A1G,A7G mRNA is translationally repressed, and we propose that the adjacent secondary structure from the 5’ UTR-intron interaction may preclude scanning ribosomes...
from accessing the AUG\(^{-42}\) start codon or may impede formation of a productive 80S ribosome on the AUG\(^{-42}\) codon.

To further test the ability of inserted AUG start codons to overcome translational repression on the unspliced HAC1 mRNA, two additional mutants were constructed. First, the nucleotides \(^{33}\text{CCU}^{31}\) in the HAC1-A1G,7G allele were substituted by an AUG triplet to generate the HAC1-AUG\(^{-33}\),A1G,7G allele (Figs. 1A and 3A). As this mutation is predicted to disrupt two base pair interactions between the 5′ UTR and the intron (Fig. 1A) and as we previously saw that disruption of a single base pair interaction was sufficient to derepress HAC1 mRNA translation due to the close proximity of AUG\(^{-33}\),A1G,7G to the 5′ UTR (Fig. 1A), we expected to see derepressed HAC1 expression. Accordingly, as shown in Fig. 4A, row 3, and in contrast to WT HAC1 (row 1), the HAC1-AUG\(^{-33}\),A1G,7G allele conferred a Tm\(^{R}\) phenotype in the ire1Δ hac1Δ strain, and Hac1 protein was detected in extracts from cells expressing the mutant but not the WT protein (Fig. 4B, lane 3 versus 1). These experiments with the HAC1-AUG\(^{-33}\),A1G,7G mutant provide further support for the hypothesis that disruption of the secondary structure formed by the base-pairing interaction between the 5′ UTR and intron is sufficient to derepress translation of the unspliced HAC1 mRNA.

Because the AUG\(^{-42}\) insertion may have failed to derepress HAC1 mRNA translation due to the close proximity of AUG\(^{-42}\) to the 5′ UTR-intron secondary structure, the third mutant was constructed by inserting the AUG codon further upstream from the secondary structure. To avoid inserting the AUG codon too close to the 5′ end of the mRNA, where it may be susceptible to being skipped by scanning ribosomes (30, 31), the HAC1-AUG\(^{-40}\),A1G,7G allele was generated by inserting an AUG codon in the HAC1-AUG\(^{-42}\),A1G,7G mRNA failed to derepress translation.
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**A.**

| HAC1 allele | SD | Tunicamycin |
|-------------|----|-------------|
| WT          | 1  | 2           |
| AUG\(^{\text{A1G,A7G}}\) | 3  | 4           |
| AUG\(^{\text{A1G,A7G}}\) | 5  | 6           |

**B.**

- Western:
  - Lane 1: WT
  - Lane 2: AUG\(^{\text{A1G,A7G}}\)
  - Lane 3: AUG\(^{\text{A1G,A7G}}\)
  - Lane 4: AUG\(^{\text{A1G,A7G}}\)
  - Lane 5: Hac1

**FIGURE 4.** Insertion of an AUG codon at position \(-60\) derepresses translation of unspliced HAC1 mRNA. A, yeast growth assays. Transformants of an \(\text{ire1}A\) hac1\(\Delta\) strain carrying an empty vector or expressing the indicated HAC1 allele were grown in SD medium to saturation, and 5 \(\mu\)l of serial dilutions (of A\(\text{D}_{600} = 1.0, 0.1, 0.01, \text{and} 0.001\)) were spotted on SD medium or SD medium containing 0.4 \(\mu\)g/ml tunicamycin and incubated for 3 days at 30 °C. B, immunoblot analysis of Hac1 protein. Strains from panel A were grown under non-stressed conditions in SD medium, and WCEs were prepared and subjected to SDS-PAGE followed by Western analysis using polyclonal antibodies against recombinant Hac1 or Act1 protein. Lanes are numbered according to row numbers in panels 1 and 2, and an asterisk is positioned on the left side of the Hac1 protein in lanes 3 and 5.

\(\text{AUG}^{\text{A1G,A7G}}\) allele, the inserted \(\text{AUG}^{\text{A1G,A7G}}\) start codon is located \(-26\) nucleotides from the 5’ cap (32) and \(-22\) nucleotides upstream of the start of the base-paired elements.

Interestingly, when introduced into the \(\text{ire1}A\) hac1\(\Delta\) strain, the HAC1\(\text{-AUG}^{\text{A1G,A7G}}\) strain migrated more slowly in SDS-PAGE than the Hac1 protein produced in the HAC1\(\text{-AUG}^{\text{A1G,A7G}}\) strain (Fig. 4B, lane 3). Consistent with this growth phenotype, Western analysis of WCEs from the strain grown under non-ER stress conditions revealed a prominent Hac1 protein signal (Fig. 4B, lane 5). Importantly, the Hac1 protein in the HAC1\(\text{-AUG}^{\text{A1G,A7G}}\) strain migrated more slowly in SDS-PAGE than the Hac1 protein produced in the HAC1\(\text{-AUG}^{\text{A1G,A7G}}\) strain (Fig. 4B, lane 3), consistent with the longer N-terminal extension for the protein initiating at \(\text{AUG}^{\text{A1G,A7G}}\). As the HAC1\(\text{-AUG}^{\text{A1G,A7G}}\) mRNA is unspliced in the \(\text{ire1}A\) hac1\(\Delta\) strain, and thus the 5’ UTR-intron interaction is intact, the ability of \(\text{AUG}^{\text{A1G,A7G}}\) codon insertion to derepress Hac1 production leads to two important conclusions. First, in contrast to the previously proposed model of translation elongation block on the unspliced HAC1 mRNA (13), elongating ribosomes are able to translate through the 5’ UTR-intron interaction. Second, the data are consistent with a model in which the 5’ UTR-intron interaction impedes translation initiation by blocking ribosomes scanning to the native AUG start codon.

**Disruption of the 5’ UTR-Intron Interaction Results in Greater Association of HAC1 mRNA with Polysomes**—The genetic suppressor and AUG insertion mutant studies support the model that ribosomal scanning on the WT unspliced HAC1 mRNA is paused at the 5’ UTR-intron interaction site. Accordingly, the point mutations that weaken the 5’ UTR-intron interaction allow scanning ribosomes to traverse the 5’ UTR and initiate translation at the AUG start codon of HAC1. If this model is correct, few ribosomes should associate with the unspliced WT HAC1 mRNA, whereas the HAC1 mRNA containing the point mutations that disrupt the 5’ UTR-intron interaction should be readily translated and thus show a greater association with polysomes. To test this possibility, the WT chromosomal HAC1 allele (translationally repressed) in the \(\text{ire1}A\) strain J751 (HAC1 \(\text{ire1}A\), panel A) and J1167 (HAC1-G767C,G768C \(\text{ire1}A\), panel B) were resolved by velocity sedimentation in 7–47% sucrose gradients. Gradients were fractionated while scanning at A\(\text{D}_{254}\) (upper tracings), and the positions of the 40S and 60S subunits, 80S ribosomes, and polysomes are indicated. Total RNA was extracted from each fraction and subjected to Northern analysis using probes for HAC1 and ADH1 as indicated. The amounts of HAC1 mRNA in the gradient frations were quantified, and the percentage of HAC1 mRNA associating with polysomes (fractions 4–8) was calculated.

**FIGURE 5.** Disruption of the 5’ UTR-intron interaction increased the relative association of HAC1 mRNA with polysomes. WCEs from strains J751 (HAC1 \(\text{ire1}A\), panel A) and J1167 (HAC1-G767C,G768C \(\text{ire1}A\), panel B) were resolved by velocity sedimentation in 7–47% sucrose gradients. Gradients were fractionated while scanning at A\(\text{D}_{254}\) (upper tracings), and the positions of the 40S and 60S subunits, 80S ribosomes, and polysomes are indicated. Total RNA was extracted from each fraction and subjected to Northern analysis using probes for HAC1 and ADH1 as indicated. The amounts of HAC1 mRNA in the gradient frations were quantified, and the percentage of HAC1 mRNA associating with polysomes (fractions 4–8) was calculated.
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ruption of the secondary structure releases the initiation block and enables more ribosomes to translate the HAC1 mRNA, resulting in more of the mRNA associating with polyribosomes.

Overexpression of eIF4A Restores the UPR and Derepresses Hac1 Production in Yeast Expressing the HAC1-AUG-42, A1G,A7G Variant—Whereas insertion of an AUG codon at position −60 far upstream of the secondary structure in the HAC1 mRNA led to constitutive derepression of Hac1 synthesis (Fig. 4), the insertion of an AUG codon at position −42 immediately before the secondary structure failed to derepress Hac1 synthesis in the absence of splicing (Fig. 3). As our data indicate that the secondary structure formed by the base-pairing interactions between the intron and 5′ UTR in the HAC1 mRNA interferes with translation initiation, we reasoned that scanning ribosomes lack sufficient secondary structure unwinding ability to access the AUG−42 start codon. However, because the AUG−42 codon is located just upstream of the secondary structure, the scanning ribosome may only need to partially melt the secondary structure to access the AUG−42 codon. Accordingly, we reasoned that overexpression of the translation factors that function in mRNA binding and scanning might derepress Hac1 production on the HAC1-AUG−42,A1G,A7G mRNA. Consistent with this hypothesis, overexpression of eIF4A, an RNA helicase that promotes melting of RNA secondary structures (1) conferred a tunicamycin-resistant phenotype in an ire1Δ hac1Δ strain expressing the HAC1-AUG−42,A1G,A7G allele (Fig. 6B, right panels, row 2). This derepression of HAC1 synthesis was dependent on eIF4A helicase activity as overexpression of the elf4A-D170E mutant that lacks helicase activity (33, 34) failed to support growth on tunicamycin medium (Fig. 6B, right panels, row 5). Moreover, overexpression of elf4E, the mRNA cap-binding protein (1), or elf4B, a factor that promotes elf4A activity in mRNA remodeling and scanning (1, 35, 36) (35, 36), failed to promote growth of the strain expressing the HAC1-AUG−42,A1G,A7G allele on tunicamycin medium (Fig. 6B, right panels, rows 2, 3, and 4). Consistent with these growth phenotypes, Western blot analyses demonstrated that overexpression of elf4A, but not elf4E, elf4B, or non-functional elf4A-D170E, promoted synthesis of Hac1 in from the HAC1-AUG−42,A1G,A7G mRNA (Fig. 6C). These data support the model that increasing the cellular amount of elf4A enables the scanning ribosome to melt secondary structure on the HAC1-AUG−42,A1G,A7G mRNA and gain access to the AUG−42 start codon. In contrast to the ability of elf4A overexpression to derepress translation of the HAC1-AUG−42,A1G,A7G mRNA, overexpression of elf4A failed to confer a tunicamycin-resistant phenotype in cells expressing the wild-type HAC1 mRNA (Fig. 6B, left panel, row 2). Thus, we propose that overexpression of elf4A can promote the modest level of secondary structure melting required for the scanning ribosome to access AUG−42 but is not sufficient for the ribosome to traverse through the entire base-paired region and access the wild-type HAC1 start codon.

Discussion

The Hac1 transcription factor is the primary regulator of the UPR (19, 20). Under conditions of ER stress, HAC1 expression is increased. The Hac1 protein then promotes the expression of a variety of proteins including ER chaperones that help alleviate the stress conditions. As production of Hac1 and stimulation of the UPR in the absence of ER stress would be wasteful, it is not surprising that cells have adopted a mechanism to limit Hac1 synthesis in unstressed cells. Intriguingly, in yeast the mechanism to limit HAC1 expression under non-stressed conditions is linked to the unique cytoplasmic splicing of the HAC1 mRNA (13, 15, 21). The primary HAC1 transcript containing an intron that disrupts the main ORF is exported to the cytoplasm with the intron intact (14). Translation of the unspliced HAC1 mRNA, overexpression of eIF4A failed to derepress translation of the HAC1-AUG−42,A1G,A7G mRNA, overexpression of elf4A failed to confer a tunicamycin-resistant phenotype in cells expressing the wild-type HAC1 mRNA (Fig. 6B, left panel, row 2). Thus, we propose that overexpression of elf4A can promote the modest level of secondary structure melting required for the scanning ribosome to access AUG−42 but is not sufficient for the ribosome to traverse through the entire base-paired region and access the wild-type HAC1 start codon.

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FIGURE 6. Overexpression of translation initiation factor elf4A derepressed HAC1-AUG-42,A1G,A7G mRNA translation. A, schematic diagram showing the 5′ UTR of the HAC1-AUG-42,A1G,A7G allele. Color schemes are the same as in Fig. 1A. B, yeast growth assays. An ire1Δ hac1Δ strain was transformed with a low copy number URAS plasmid expressing HAC1 (left panels) or the HAC1-AUG-42,A1G,A7G allele (right panels). The resulting strains were then transformed with high copy number (H.C.) LEU2 plasmids expressing elf4A, elf4B, elf4E, or elf4A-D170E or with a low copy-number (L.C.) LEU2 plasmid encoding IRE1. C, immunoblot analysis of Hac1 protein. Strains from the right panels in B were grown under stressed conditions in SD medium, and WCEs were prepared and subjected to SDS-PAGE followed by Western analysis using a polyclonal antibody raised against recombinant Hac1 protein. Lanes are numbered according to row numbers in panel B.
mRNA will terminate at a stop codon in the intron, whereas translation of the spliced HAC1 mRNA will generate a protein with an altered C terminus that terminates in the second exon. Previous studies (15, 27, 37) and the results in this report demonstrate that both forms of the Hac1 protein are functional and enable cells to grow under ER stress conditions. However, production of the Hac1 protein from the unspliced mRNA is inhibited by base-pairing interactions between sequences in the intron and in the 5′ UTR of the HAC1 mRNA. Within a span of 19 nucleotides, 16 form base-pairing interactions between the 5′ UTR and intron including a stretch of 11 consecutive base pairs. Splicing of the HAC1 mRNA removes the intron and thus eliminates the base-pairing interactions and enables HAC1 mRNA translation. As previously demonstrated by Rüegsegger et al. (13), replacement of the 5′ UTR base-pairing element with the sequence from the intron or replacement of the intron element with the sequence from the 5′ UTR enabled constitutive translation of the HAC1 mRNA. Moreover, when the 5′ UTR and intron elements were swapped simultaneously to allow the base-pairing interaction to occur, but with opposite orientation compared with the original context, HAC1 mRNA translational repression was restored. Although these studies clearly demonstrated that the intron played an important role in limiting HAC1 mRNA translation, the molecular mechanism of the repression was not clarified.

To gain insights into the mechanism of the translational repression of the unspliced HAC1 mRNA, we employed two mutational strategies. First, the cleavage site between exon 1 and the intron was mutated to eliminate splicing of the HAC1 mRNA, and then a second-site suppressor mutation was identified that restored Hac1 synthesis. This suppressor mutation disrupted a single base pair in the 5′ UTR-intron interaction. Likewise, using a traditional site-directed mutagenesis approach, we showed that altering a single base pair interaction between the 5′ UTR and intron was sufficient to derepress HAC1 mRNA translation in cells lacking the Ire1 endonuclease and was thus unable to splice the HAC1 mRNA. Although Rüegsegger et al. (13) showed that eliminating all 16 base-pairing interactions between the 5′ UTR and intron eliminated the translational repression, our studies reveal that loss of a single base pair interaction within or at the end of the 11 consecutive base pairs is sufficient to allow translation of the unspliced HAC1 mRNA. Thus, it appears that the HAC1 mRNA structure is poised on the threshold of strength required for translational repression, perhaps indicating that further strengthening of this interaction with additional base pair interactions might be deleterious. Likewise, it seems possible that conditions or factors that promote melting of mRNA secondary structures might be able to derepress translation of the unspliced HAC1 mRNA.

Secondary structural elements in the 5′ UTR of mRNAs have previously been shown to inhibit translation (6, 7, 38). As these structured mRNAs failed to associate with ribosomes, it was concluded that the 5′ UTR structures blocked 40S ribosome loading and/or ribosomal scanning to the start codon on the mRNA. Accordingly, the 5′ UTR-intron interaction would be predicted to impair translation initiation on the HAC1 mRNA. At odds with this hypothesis, Rüegsegger et al. (13) reported that unspliced HAC1 mRNA co-sedimented with polysomes on sucrose gradients, leading to a proposal that the 5′ UTR-intron interaction impedes translation elongation. We sought to directly test whether the 5′ UTR-intron interaction could block an elongating ribosome. Whereas inserting an AUG start codon immediately upstream of the interaction site failed to derepress translation of the unspliced HAC1 mRNA (AUG→C in Figs. 3 and 4), insertion of an AUG codon further upstream (AUG→G) enabled Hac1 synthesis in the absence of splicing (Fig. 4). In this latter construct, the inserted AUG codon was positioned ~26 residues from the cap and ~22 residues upstream of the secondary structure. These gaps between the cap, AUG codon, and secondary structure provide sufficient room for a 43S PIC to bind at the cap, scan to AUG→G start codon, and assemble a translationally competent 80S ribosome. Notably, toe-printing assays have shown that the leading edge of the ribosome is typically around 16 nucleotides downstream of the P site (39, 40), so the 22-nucleotide gap between AUG→G and the 5′ UTR secondary structure provides the space needed for an 80S ribosome. The ability of ribosomes initiating at AUG→G to elongate through the 5′ UTR-intron secondary structure is consistent with previous reports demonstrating that elongating ribosomes can melt secondary structures (7, 41). Moreover, these results demonstrate that the 5′ UTR-intron interaction does not block translation elongation, and we therefore conclude that translation of the unspliced HAC1 mRNA is blocked at translation initiation.

Polysome profiles provide an additional means to characterize translation defects. When translation initiation is impaired, fewer ribosomes associate with an mRNA, and the mRNA sediments as a free messenger ribonucleoprotein particle (mRNP) or perhaps associated with 40S subunits and smaller polysomes. On the other hand, if translation elongation is impaired, the mRNA will readily bind to ribosomes and sediment with large polysomes in sucrose gradient analyses. Rüegsegger et al. (13) found that the WT unspliced HAC1 mRNA co-sedimented with free 40S subunits as well as with polysomes, and mutation of the 5′ UTR-intron interaction resulted in loss of the association with 40S subunits and a shift of the majority of the mRNA to the polysome fractions. Likewise, Mori et al. (42) reported that the unspliced HAC1 mRNA in unstressed cells co-sedimented with ribosomal subunits, whereas the spliced HAC1 mRNA in tunicamycin-treated cells sedimented with large polysomes. In our studies we found that the association of the HAC1 mRNA with polysomes was influenced by the amount of HAC1 mRNA in the cell. Overexpression of HAC1 mRNA resulted in a relative shift in the sedimentation of the mRNA such that a greater fraction co-sedimented with polysomes. As this result did not correlate with increased synthesis of Hac1 protein, we reasoned that the overexpressed mRNA might not be translated but, rather, associated with different complexes that co-sedimented with polysomes. To avoid this possible complication, we generated isogenic strains expressing WT HAC1 or a translationally derepressed HAC1 intron mutant. Whereas the unspliced HAC1 mRNA was roughly equally dis-

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4 C. Bolinger, unpublished data.
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Introns can significantly influence the efficiency of translation initiation by regulating the interaction between the ribosome and the mRNA sequence. The presence of an intron can hinder the scanning ribosome from accessing the start codon, thus blocking translation. However, overexpression of a translation initiation factor, such as eIF4A, can help melt the beginning of the base-paired region, enabling the scanning ribosome to access a start codon at position 42. This suggests that the position of the intron in the HAC1 mRNA can be crucial for the initiation of translation, and that the presence of introns in mRNAs can provide a mechanism to control translation initiation levels. This implies that the position of introns in mRNAs can be a key regulatory element in the control of gene expression.
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