The cap-proximal RNA secondary structure inhibits preinitiation complex formation on HAC1 mRNA

Received for publication, September 15, 2021, and in revised form, January 20, 2022 Published, Papers in Press, January 28, 2022, https://doi.org/10.1016/j.jbc.2022.101648

Jagadeesh Kumar Uppal1, Leena Sathe1, Abhijit Chakraborty2, Sankhajit Bhattacharjee1, Anthony Thomas Pulvino1, and Madhusudan Dey1,*

From the 1Department of Biological Sciences, University of Wisconsin Milwaukee, Milwaukee, Wisconsin, USA; 2Center for Autoimmunity and Inflammation, Center for Cancer Immunotherapy, La Jolla Institute for Immunology, La Jolla, California, USA

Edited by Karin Musier-Forsyth

Translation of HAC1 mRNA in the budding yeast Saccharomyces cerevisiae is derepressed when RNase Ire1 removes its intron via nonconventional cytosolic splicing in response to accumulation of unfolded proteins inside the endoplasmic reticulum. The spliced HAC1 mRNA is translated into a transcription factor that changes the cellular gene expression patterns to increase the protein folding capacity of cells. Previously, we showed that a segment of the intron sequence interacts with the 5′-UTR of the unspliced mRNA, resulting in repression of HAC1 translation at the initiation stage. However, the exact mechanism of translational derepression is not clear. Here, we show that at least 11-base-pairing interactions between the 5′-UTR and intron (UI) are sufficient to repress HAC1 translation. We also show that overexpression of the helicase eukaryotic initiation factor 4A derepressed translation of an unspliced HAC1 mRNA containing only 11-bp interactions between the 5′-UTR and intronic sequences. In addition, our genetic screen identifies that single mutations in the UI interaction site could derepress translation of the unspliced HAC1 mRNA. Furthermore, we show that the addition of 24 RNA bases between the mRNA 5′-cap and the UI interaction site derepressed translation of the unspliced HAC1 mRNA. Together, our data provide a mechanistic explanation for why the cap-proximal UI–RNA duplex inhibits the recruitment of translating ribosomes to HAC1 mRNA, thus keeping mRNA translationally repressed.

In all living organisms, mRNA carries the information that directs protein synthesis involving ribosomes, tRNA, and other factors in a process called translation (1, 2). Translation is a tightly coordinated and regulated process that ensures that an optimal amount of protein is produced, and the production of a specific protein is increased or decreased as needed. There are two major categories of translational regulation: global and transcript specific. In global regulation, the overall rate of translation is increased or decreased by inhibiting or activating one or more general translational components. In transcript specific regulation, the translation of a given mRNA is controlled by the cis element(s) present in its UTRs and/or by the transacting regulatory protein(s) or noncoding RNA(s) (3). The cis elements include the upstream ORFs (uORFs), specific nucleotides flanking AUG start codon and the structured mRNA elements. As for example, four short uORFs control the GCN4 mRNA translation in the budding yeast Saccharomyces cerevisiae (4), and two uORFs control the activating transcription factor 4 mRNA in humans (5). Like uORFs, an RNA secondary structure located within the 5′-UTR controls the HAC1 mRNA translation in S. cerevisiae (6), whereas a tertiary-folded RNA structure, called the internal ribosomal entry site, controls the cMYC mRNA translation in humans (7).

The S. cerevisiae HAC1 mRNA exists in a translationally repressed form in the cytoplasm by a unique mechanism involving its unspliced intron that interacts with the 5′-UTR to form an RNA duplex (Fig. 1A) (6). Previously, we and others have shown that the 5′-UTR–intron (UI)–RNA duplex inhibits the initiation of HAC1 translation (6, 8). Under stress conditions, such as when unfolded proteins overaccumulate inside the endoplasmic reticulum (ER), HAC1 mRNA colocalizes with an ER-resident RNase Ire1 that cleaves out the inhibitory intron (9, 10). tRNA ligase then ligates two cleaved exons (11), thus generating a matured mRNA that yields Hac1 protein (12, 13). Hac1 is a basic leucine-zipper transcription factor that binds to unfolded protein response element present in promoters of many ER-resident enzyme and chaperone genes (14), resulting in activation of their transcriptional program. Ultimately, the protein folding capacity of the cell is enhanced by this altered transcriptional program, which is collectively known as the unfolded protein response (15).

The intron in HAC1 mRNA functions as a cis-acting modulator of its mRNA’s translation (Fig. 1A). Removal of this intron by the RNase Ire1 derepresses its translational initiation (6, 8). However, the exact mechanism of translational derepression at the initiation stage is not yet clear. Typically, several eukaryotic translation initiation factors (eIFs), ribosomes, and the initiator methionyl-tRNA (Met-tRNA\textsubscript{Met}) work in concert to ensure that Met-tRNA\textsubscript{Met} finds the correct cognate start codon to initiate translation (16). Initiation of translation, according to the classical view, begins at the m\textsuperscript{7}G-cap of mRNA with the formation of a

* For correspondence: Madhusudan Dey, deym@uwm.edu.
ribonucleoprotein complex known as the preinitiation complex (PIC). PIC then travels along the 5' UTR in search of a start codon in a process known as the ribosomal scanning. However, under certain conditions, cap-dependent translation initiation occurs without ribosomal scanning (17). Therefore, it is not yet clear how and to what extent the UI–RNA duplex in HAC1 mRNA affects the assembly of ribosomes and other factors to form the PIC or its movement along the 5'-UTR.

In the present study, we reanalyzed both transcriptome and translatome data published for the budding yeast S. cerevisiae (18). In addition, we did the in silico analysis of RNA–RNA interaction between the 5'-UTR and intronic sequences. Along with these computational analyses, we provide in vivo evidence that the UI–RNA duplex sterically blocks the ribosomal access to the 5'-UTR near the m7G-cap of HAC1 mRNA to form the PIC, thus keeping mRNA translationally repressed.

Results

Single base changes in the 5'-UTR derepress translation of the unspliced HAC1 mRNA

Ruegsegger et al. (6) reported that a long-range base-pair interaction between sequences of the 5'-UTR (nucleotide positions from -38 to -20) and the intron (nucleotide positions from 764 to 782) keeps HAC1 mRNA translationally repressed (Fig. 1A). We show that a single base-pair interaction between the RNA base C(-27) at the 5'-UTR and the G771 at the intronic region is important to keep the HAC1 mRNA translationally repressed (8). Translational repression is released when the ER-resident endonuclease Ire1 cleaves the intron at 28S rRNA.
positions G661 and G913 (19). Still, the detailed mechanisms underlying the translational repression and derepression of HAC1 mRNA are not clear. In an effort to understand the mechanisms, we screened for single mutation(s), which would activate translation from the unspliced HAC1 mRNA. For the mutational screen, we transformed an Escherichia coli XL1-Red mutator strain with a plasmid bearing a hemagglutinin-tagged HAC1 gene and purified the mutated plasmid pool. The mutated plasmid pool was then introduced in the splice-deficient hac1Δire1Δ strain and screened for yeast colonies that were resistant to an ER stress–inducing agent tunicamycin. From the tunicamycin-resistant colonies, individual plasmids were rescued, restested, and sequenced to identify mutations. Apart from the C(-27)G mutation that we reported earlier (8), two single mutations (i.e., C(-23)G and C(-32)A) were identified within the UI interaction site (Fig. 1A), which conferred resistance to tunicamycin (Fig. 1B, rows 3 and 9).

The aforementioned results suggested that 11 RNA bases at the 5′-UTR ranging from A(-22) to C(-32) likely form a core 11-bp RNA duplex with the intrinsic bases from U766 to G776, which tunes the HAC1 mRNA translation (Fig. 1A). Thus, we extended our studies and mutated almost all base-pairing nucleotides of the UI–RNA duplex and determined their relative contribution in translational repression. Nucleotides C(-29), C(-30), and C(-33) were individually mutated to adenine, thus generating three plasmids bearing C(-29)A, C(-30)A, and C(-33)A mutation at the 5′-UTR of HAC1 mRNA. In addition, we mutated the adenine at position -22 of the HAC1-C(-23)G derivative to thymine (T), thus generating a HAC1-A(-22)T, C(-23)G double mutant. The mutant plasmids were then individually introduced in both hac1Δ and hac1Δire1Δ strains. The resulting strains were tested for their ability to survive under a condition of ER stress stimulated by tunicamycin.

The hac1Δ strain containing a vector plasmid or the same plasmid bearing a WT HAC1 allele grew on synthetic complete (SC) medium (Fig. 1B, SC-uracil, rows 1, 2, 4, and 5). However, the hac1Δ strain grew on the SC medium containing tunicamycin only when expressed a WT HAC1 allele (Fig. 1B, tunicamycin, rows 2 and 5). These results are consistent with the previous findings that HAC1 gene provides an essential function to alleviate ER stress. Like WT HAC1, each HAC1-5′-UTR mutant [HAC1-C(-23)G, HAC1-C(-27)G, HAC1-C(-29)A, HAC1-C(-30)A, HAC1-C(-32)A, or HAC1-A(-22)T, C(-23)G] allowed the hac1Δ cells to grow on the tunicamycin medium (Fig. 1B). Western blot (WB) analysis showed that Hac1 protein was produced when cells were grown in the presence of an ER stressor DTT (Fig. 1C, lanes 2, 6, 10, 14, and 18) or tunicamycin (data not shown). These data collectively suggest that the 5′-UTR mutations have little or no effect on Hac1 protein expression induced by ER stress.

Unlike hac1Δ strain, the hac1Δire1Δ strain containing a WT HAC1 was unable to grow on the tunicamycin medium (Fig. 1B, rows 2 and 5). As expected, Hac1 protein was not detected in those cells grown in the presence of tunicamycin (data not shown) or DTT (Fig. 1C, lane 4), suggesting that the tunicamycin-sensitive phenotype was due to lack of Hac1 protein expression. Furthermore, we tested whether the loss of protein expression was due to the loss of HAC1 mRNA splicing. Both hac1Δ and hac1Δire1Δ strains containing a WT HAC1 allele on the URA3 plasmid were grown in the presence of DTT. Total RNA was isolated from the DTT-stressed cells and subjected to RT–PCR analysis to examine the spliced (HAC1s) and unspliced (HAC1u) populations of mRNA. RT–PCR analysis showed only unspliced mRNA species in the hac1Δire1Δ strain expressing a WT HAC1 allele (Fig. 1D, lanes 3 and 4). Both HAC1s and HAC1u mRNA species were observed in the hac1Δ strain grown in the presence of an ER stressor DTT (Fig. 1D, lane 2). These results are consistent with other studies that demonstrate that Ire1 function is required for HAC1 mRNA splicing. The spliced mRNA yields Hac1 protein that induces the ER stress response.

The hac1Δire1Δ strain containing the plasmid-borne HAC1 or its 5′-UTR mutant [i.e., HAC1-C(-29)A, HAC1-C(-30)A, and HAC1-C(-33)A] was unable to grow on the tunicamycin medium (Fig. 1B, rows 7, 8, and 10). In contrast, the same hac1Δire1Δ strain containing the HAC1-C(-23)G, HAC1-C(-27)G, HAC1-C(-32)A, or HAC1-A(-22)T, C(-23)G mutant was able to grow on the tunicamycin medium (Fig. 1B, rows 3, 6, 9, and 11). Notably, cells containing the HAC1-A(-22)T, C(-23)G, or HAC1-C(-27)G mutant grew more rapidly than cells containing the HAC1-C(-23)G or HAC1-C(-32)A mutant (Fig. 1B). The results suggested that Hac1 protein was likely produced from the unspliced mRNA starting at the AUG codon of exon 1 until the stop codon UGA at nucleotide 690 of the adjacent intron. This translational product of unspliced HAC1 mRNA is known as “Hac1u” protein (20). Hac1u protein, like the Hac1 protein translated from the spliced mRNA (i.e., Hac1 protein), is a transcription factor with a basic leucine-zipper domain followed by a transcription activation domain, differing in only 10 residues of its C terminus (20). However, Hac1u protein is short lived, differently modified, and less active transcription factor (19).

To determine that Hac1u protein was produced from the aforementioned 5′-UTR mutants in the ire1Δ hac1Δ strain, whole cell extracts (WCEs) were prepared from the respective strains grown in the SC medium and subjected to WB analysis. A significant amount of Hac1 protein was produced (~25% compared with WT) in the hac1Δire1Δ strain containing the HAC1-C(-27)G (Fig. 1C, lanes 5–8) and HAC1-A(-22)T, C(-23)G mutants (Fig. 1C, lanes 13–16). Consistent with our recent report (21), we also observed that Hac1u expression was induced almost twofold when cells were grown in the presence of the ER stressor DTT (Fig. 1C, lanes 5–8). However, Hac1u protein was almost undetectable in the hac1Δire1Δ strain containing the HAC1-C(-23)G or HAC1-C(-32)A mutant, even when cells were grown in the presence of DTT (Fig. 1C, lanes 11, 12, 19, and 20). It appears that a low amount of Hac1u protein was sufficient to confer a mild tunicamycin-resistant phenotype (Fig. 1B, rows 3 and 9). A possibility that we cannot rule out that the low amount of Hac1u protein produced from the unspliced HAC1-C(-23)G or HAC1-C(-32)A mRNA was likely degraded rapidly mediated by a degraders
protein, Duh1, as previously reported (22). Taken together, our data suggest that single mutations at the 5′-UTR bases C(-23), C(-27), and C(-32) derepressed translation of the unspliced HAC1 mRNA, whereas mutations at the bases C(-29), C(-30), and C(-33) did not have any noticeable effect. Together, these results suggest that RNA bases C(-23), C(-27), and C(-32) play major regulatory roles in translational repression of HAC1 mRNA.

Next, we used the IntaRNA program (23) to predict the RNA–RNA hybrid formation between sequenced of the 5′-UTR and the intron. Nineteen RNA bases at the 5′-UTR (the base position from -20 to -38) were predicted to form 15-bp interactions with the 20 intronic bases (the base position from 764 to 782) with a duplex binding energy (E) of −25.15 kcal/mol (Fig. 2, A and B). The low E value of the duplex energy indicates a thermodynamically stable hybrid, capable of forming a strong RNA secondary structure. About 13 bps were predicted when the 5′-UTR base C(-23) and C(-32) were mutated to guanine and adenine, respectively (Fig. 2). The respective E values were −21.28 kcal/mol mutant and −22.56 kcal/mol (Fig. 2). Interestingly, we found that only 11 bps were predicted when the RNA base C(-27) was mutated to guanine, and the E value was of −14.33 kcal/mol (Fig. 2). These higher E values upon mutations at RNA bases C(-23), C(-27), and C(-32) suggested that each mutation weakened the strength of base-pair interaction. Indeed, the relatively higher E values upon the C(-27)G mutation likely explains why Hac1u expression was more in cells harboring the HAC1-C(-27)G mutant than in cells harboring the HAC1-C(-23)G or HAC1-C(-32)A mutant (Fig. 1B). Concurrently, these computational data strongly corroborate with the phenotypic observations that cells containing the HAC1-C(-27)G mutant were more resistant to tunicamycin than cells containing the HAC1-C(-23)G or HAC1-C(-32)A mutant (Fig. 1B, compare rows 3, 6, and 9).

Taken together, these molecular, genetics, and computational data suggest that single mutations at the 5′-UTR [e.g., C(-23)G, C(-27)G, and C(-32)A] can partially disrupt the long-range base-pairing interaction with the intron, resulting in

**Figure 2.** Quantitative analyses of base-pair interaction between sequences of the 5′-UTR and the intron. A, predicted base-pairing interactions between sequences of the intron and the 5′-UTR or its mutants. The RNA–RNA hybrid interactions are predicted using the IntaRNA software (23). B, mutations at the 5′-UTR bases lower the duplex interaction energy. The bar diagram shows duplex energies of 5′-UTR–intron interactions.
leaky expression of Hac1\textsuperscript{u} protein. Indeed, these results lead us to speculate that ribosome was able to produce Hac1\textsuperscript{u} protein from these 5'-UTR mutants with a weak base-pair interaction, more likely utilizing an alternate mechanism for cap-dependent translation initiation.

**Eleven base-pairing interactions between the sequence of the 5'-UTR and the intron are sufficient to repress HAC1 mRNA translation**

Ruegsegger et al. (6) show that the interaction between 16 RNA bases of the 5'-UTR and the intron leads to repression of \( \text{HAC1} \) mRNA translation. Consistent with this report, the IntaRNA analysis predicts 15 possible base-pair interactions (Fig. 3A). Here, we experimentally determined the minimum number of base-pair interactions required for the translational repression in HAC1 mRNA. First, we engineered the HAC1 gene by mutating five DNA bases coding for five 5'-UTR RNA bases as follows. Four RNA bases at positions -35 to -38 (AACC) were replaced by RNA base UUGG, and cytosine at the position -20 was replaced by a guanine (Fig. 3A). This derivative, herein referred to as HAC1\textsuperscript{11bp}, was expected to preserve only a stretch of 11 continuous UI base-pair

![Figure 3. About 11-bp interactions between sequence of the 5'-UTR and the intron are sufficient to repress HAC1 mRNA translation.](image)

- **A**, the base-pair interaction between the sequences of the 5'-UTR (black) and intron (orange) in WT and mutant HAC1 mRNAs. Base-pair interactions are shown by dots. The nucleotide numbers are shown at the top and bottom. **B and C**, analysis of yeast growth. The hac1\textsuperscript{Δ} or hac1\textsuperscript{Δ}ire1\textsuperscript{Δ} strains expressing the WT and HAC1\textsuperscript{9bp} or HAC1\textsuperscript{11bp} mutant were tested for growth on the synthetic complete (SC) medium and the same SC medium containing the indicated concentration of tunicamycin (Tm). **D**, Tm-induced endoplasmic reticulum (ER) stress activates splicing of HAC1\textsuperscript{11bp} mRNA. The hac1\textsuperscript{Δ} strain expressing WT or mutant HAC1\textsuperscript{11bp} was grown in the presence (+) and absence (−) of Tm. Total RNA was isolated (28S rRNA is shown) and subjected to RT–PCR analysis to monitor the spliced (HAC1\textsuperscript{s}) and unspliced (HAC1\textsuperscript{u}) mRNA populations. Experiment was repeated twice, and average ratios are shown. **E**, expression of Hac1 protein from the HAC1\textsuperscript{9bp} mutant without ER stress. The hac1\textsuperscript{Δ} and hac1\textsuperscript{Δ}ire1\textsuperscript{Δ} strains expressing WT HAC1 and HAC1\textsuperscript{9bp} or HAC1\textsuperscript{11bp} mutant were grown in the presence (+) and absence (−) of DTT. Whole cell extracts were prepared and subjected to Western blot analysis using anti-Hac1 and anti-Pgk1 antibodies. pgk1, phosphoglycerate kinase 1.
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interactions (Fig. 3A). Second, we further engineered the HAC111bp allele by mutating two more DNA bases coding for the RNA bases at positions -22 and -23, thus generating a mutant that was expected to preserve only 9 bp interactions, herein referred to as HAC19bp derivative (Fig. 3A). We expressed individually the HAC111bp and HAC19bp mutants in both hac1Δ and hac1Δire1Δ strains and examined how these mutations affected the cell’s ability to adapt to and overcome ER stress.

The hac1Δ strain expressing the HAC111bp mutant grew on the SC medium and the same medium containing a low (0.2 μg/ml) or high (0.5 μg/ml) concentration of tunicamycin (Fig. 3B, row 3 and Fig. 3C, row 3). RT–PCR analysis showed both unspliced and spliced mRNA in cells expressing HAC111bp mutant, like in cells expressing a WT allele, when they were grown in the presence of DTT (Fig. 3D). These data suggest that the HAC111bp mutant neither affect the fitness of cells to grow under an ER stress condition nor impair HAC1 mRNA splicing. Interestingly, we observed that the hac1Δire1Δ strain harboring the HAC111bp mutant grew slowly on the SC medium containing tunicamycin at a low concentration (0.2 μg/ml, Fig. 3B). Growth was almost abolished at a high concentration of tunicamycin (0.5 μg/ml, Fig. 3B), WB showed that Hac1 protein was almost undetectable in cells harboring HAC111bp mutant even when they were grown in the presence of DTT (Fig. 3E, WB, lanes 5 and 6). These results suggested that mutations weakened the strength of interaction between the 5’-UTR and intronic sequences, resulting in leaky translation of Hac1 protein and partial survival of cells under a mild ER stress condition. These results further suggested that a low amount of Hac1 protein was sufficient to confer the tunicamycin-resistant phenotype and that Hac1 protein produced from the unspliced mRNA was likely degraded rapidly as reported earlier (22). In contrast, the hac1Δire1Δ strain harboring the HAC19bp mutant grew normally on the SC medium containing tunicamycin at concentration of 0.5 μg/ml (Fig. 3C, row 4). Growth was correlated with the expression of Hac1 protein (Fig. 3E, WB, lanes 7 and 8). These results suggested that mutations at both positions -22 and -23 of HAC111bp allele eliminated the base-pair interaction between the UI, or 9 bp interactions between the UI were not sufficient to repress translation. Nevertheless, these results suggest that minimally the regions predicted to have 11 base-pairing interactions are sufficient and near threshold for inhibition of HAC1 mRNA translation.

An RNA hairpin of 11-bp stem inhibits translation of the matured HAC1 mRNA

The RNAfold software (24) predicts that the minimum free energy (MFE) of the 11-bp RNA hairpin in HAC1 mRNA is approximately −22 kcal/mol. It has been shown previously that an RNA hairpin (MFE = −30 kcal/mol) was capable of inhibiting in vitro translation when positioned at 12 nucleotides away from the 5’-cap but not when positioned 52 nucleotides away (25). In contrast, an iron-responsive element with higher free energy (MFE = −5.86 kcal/mol) combines with iron regulatory proteins and inhibits translation initiation when positioned at <60 nucleotides away from the 5’-cap (26). Thus, we sought to determine how and to what extent, a similar 11-base-paired RNA hairpin modulated the general protein synthesis under physiological conditions. Specifically, we monitored translation from an intron-less HAC1 mRNA by adding an 11 base-paired RNA hairpin near the 5’-cap.

To this end, we made two Hac1 constructs: HAC1Δ and UI-HAC1Δ. In HAC1Δ construct, the entire intron sequence was deleted from the D63 plasmid bearing the HAC1 gene under its natural promoter and terminator (see Experimental procedures section). The intron-less HAC1Δ construct was expected to constitutively express a HAC1 mRNA with a 5’-UTR (68 bases), a protein-coding region (717 bases), and a 3’-UTR (416 bases) (Fig. 4A) (18). In the UI-HAC1Δ construct, 11 complementary nucleotides of the HAC1-5’-UTR sequence (−32 to −22) were inserted at position −11 of the 5’-UTR of the HAC1Δ construct. The UI-HAC1Δ construct was expected to produce an mRNA with an extra 11-bp 5’-UTR sequence, including an 11-bp RNA hairpin positioned ~39 bases away from the 5’-cap (Fig. 4A).

The hac1Δire1Δ strain harboring the HAC1Δ construct was able to grow on both SC and tunicamycin media (Fig. 4B) because of constitutive expression of Hac1 protein (Fig. 4C). The slow growth of the cells on the SC medium (Fig. 4B) suggested that the constitutive expression of Hac1 protein reduced the cell growth. Unlike the HAC1Δ construct, the chimeric UI-HAC1Δ construct did not allow the hac1Δire1Δ strain to grow on the tunicamycin medium (Fig. 4B). Similar levels of HAC1 mRNA were observed in cells containing HAC1Δ and chimeric UI-HAC1Δ constructs (Fig. 4C); however, Hac1 protein expression was almost undetectable in cells containing the chimeric UI-HAC1Δ construct (Fig. 4C). These data suggest that the UI-HAC1Δ construct likely produces an mRNA containing a 5’-UTR sequence that might self-fold to form an 11-bp RNA hairpin near the 5’-cap, which is inhibitory to translation initiation. These results suggest that translation inhibition by the UI–RNA duplex is mediated by a unique mechanism; however, this is in line with the published report that the secondary structures (MFE ≤−22 kcal/mol) decrease translational efficiency (27). Collectively, these results further support our observation that a stretch of 11-bp interactions in the UI–RNA duplex is sufficient to repress HAC1 mRNA translation (Fig. 3).

Translational derepression of unspliced HAC111b mRNA by helicase elf4A

Along with ribosomes, a set of initiation factors coordinate assembly on the 5’-cap to initiate translation (16). Therefore, it is possible that the UI interaction may prevent the assembly of one/two of these components. To test this possibility, we examined if HAC1 mRNA translation could be tuned or boosted by high dose of any of these translational components. Using the high-copy-number plasmids, we overexpressed a few translational components, in particular, two key components of the scanning ribosome (i.e., elf1 and elf1A), a subunit of ternary complex (i.e., elf2a), a subunit of the elf3 complex (i.e., elf3a), subunits of the cap-binding complex elf4F (i.e.,
eIF4E and eIF4A), the GTPase-activating protein eIF5 and GTPase eIF5B (16). None of these translational factors was able to derepress translation of unspliced HAC1 mRNA and promote growth of the ire1Δ strain on the tunicamycin medium (data not shown), suggesting that overexpression of each of these translational components was insufficient to derepress HAC1 mRNA translation when the intron was not spliced.

Then, we overexpressed the aforementioned translational components in the hac1Δire1Δ strain expressing the crippled HAC111bp mutant. Interestingly, we observed that overexpression of the helicase eIF4A enhanced cell growth on the tunicamycin medium (Fig. 5A, rows 6 and 9). Consistent with the increased tunicamycin-resistant phenotype, the Hac1 protein expression was detected in cells grown in the absence or the presence of DTT (Fig. 5B, lanes 2 and 5). However, Hac1 expression was almost undetected when eIF5A was overexpressed (Fig. 5B, lanes 3 and 6). It was surprising that a major prominent band below the Hac1 protein consistently appeared on the WB of cell lysates obtained from the ire1Δhac1Δ strain (Fig. 5B, lanes 1, 3, 4, or 6). Specifically, we observed a prominent WB band of whole cell lysate prepared from the ire1Δhac1Δ strain harboring a URA3 plasmid carrying the HAC1 gene and a high-copy LEU2 plasmid (Fig. 5B, lane 1 and Fig. 5C, lane 1). However, such prominent band was not observed on the WB of cell lysate prepared from the ire1Δhac1Δ strain harboring only the URA3-based plasmid carrying the HAC111bp allele (Fig. 3E, lanes 5 and 6). Currently, we do not have any specific explanation but can speculate that unspliced HAC1 mRNA is constitutively be translated from an alternate start site in the presence of the high-copy LEU2 plasmid. Nonetheless, these results suggest that overexpression of eIF4A was able to unwind the crippled RNA secondary structure in the HAC111bp mutant, and Hac1 protein was produced from the unspliced mRNA (Fig. 5C).

The DEAD-box helicase eIF4A and a related helicase Ded1 are known to melt the 5′-UTR secondary structure and significantly contribute to PIC formation, ribosomal scanning, and start codon selection (28, 29). Therefore, to further determine if these helicases eIF4A and Ded1 could overcome the inhibitory effect of RNA secondary structure (Fig. 5A) and promote translation from the UI-HAC1s mRNA, we overexpressed them from a high-copy-number plasmid in hac1Δire1Δ cells containing the UI-HAC1s construct. Interestingly, we observed that the helicase eIF4A, but not Ded1, enhanced the tunicamycin-resistant phenotype (Fig. 5A, row 12). Consistent with the increased growth on the tunicamycin medium, we observed expression of Hac1 protein in those cells grown in the presence or the absence of DTT (Fig. 5C, lanes 2 and 5), suggesting that a high dose of eIF4A was able to overcome the inhibitory effect of the RNA secondary structure. These data suggest that eIF4A plays an important role for translational derepression of HAC1 mRNA containing a secondary structure at 5′-UTR.

Insufficient room for ribosome and helicase recruitment on the 5′-UTR of HAC1 mRNA

We reanalyzed the budding yeast S. cerevisiae transcriptome data (18) and observed that 23% of mRNAs contain a 5′-UTR...
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of ≤30 nucleotides and most mRNAs (48%) have a 5′-UTR of ≥55 nucleotides, and the median length of 5′-UTRs is of 55 nucleotides (Fig. 6A). Specifically, we found that the length of the HAC1 5′-UTR is 68 nucleotides long, and the UI–RNA duplex is positioned at about 30 RNA bases away from the 5′-cap (Fig. 6B). The RNAfold WebServer (24) software did not indicate that these 30 RNA bases adopt any specific secondary structure. Combining these analyses with the Ribo-Seq information that an individual ribosome typically covers ~30 bases in an mRNA molecule (30, 31), we posit three possible scenarios for the RNA duplex-mediated translational repression of HAC1 mRNA. First, a sequence of 30 RNA bases might not provide a sufficient landing space for the assembly of a PIC. Second, the UI–RNA duplex sterically blocks the assembly of PIC on the 5′-UTR. Third, the PIC may assemble on the 5′-UTR but be unable to unwind the nearby UI RNA duplex.

To test the aforementioned hypotheses, we increased the 5′-UTR length, particularly the length between the 5′-cap and UI–RNA duplex, under the assumption that the longer the space the more available room will exist for translation factors binding, resulting in less inhibition of translation by the nearby RNA duplex. Therefore, we added 12 bases (ACGACAAACAACC, taken from position -7 to -18) at the position -43 of the 5′-UTR, thus increasing the 5′-UTR length by 12 bases and creating a new HAC1 mutant referred to as HAC1 12b mutant (Fig. 6B). Similarly, we further increased the length by 24 and 36 RNA bases adding the extra 12 (ACGACAAACAACC) and 24 bases (ACGACAAACAACC ACGACAAACAACC), thus generating HAC1 24b and HAC1 36b mutants, respectively (Fig. 6B). Then, we monitored the expression of Hac1 protein from these HAC1 derivatives in the splice-deficient ire1Δ hac1Δ strain.

An hac1Δire1Δ strain containing an empty vector or the same vector containing a WT HAC1 or its derivative HAC1 24b was unable to grow on the tunicamycin medium (Fig. 6C, rows 1, 2, and 3). Consistent with the tunicamycin-sensitive phenotype, Hac1 protein expression was almost undetectable (Fig. 6C, WB, lanes 1, 2, and 3). In contrast, the ire1Δ Δ hac1Δ cells containing the HAC1 24b or HAC1 36b mutant were able to grow on the tunicamycin (Fig. 6A, rows 4 and 5), like the strain expressing the HAC1-C(-27)G mutant plasmid (Fig. 6A, row 6). Consistent with the tunicamycin-resistant phenotype, a significant amount of Hac1 protein was detected in these cells (Fig. 6D, WBs, lanes 4, 5, and 6). Given that the HAC1-C(-27)G mutant (Fig. 1C) allowed cells to produce Hac1 protein from the unspliced mRNA, we interpret that the longer 5′-UTR lengths in HAC1 24b and HAC1 36b mutants allow cells to translate Hac1 protein from the unspliced mRNA.

We further interpret that the PIC is likely able to form on the 5′-UTR near the cap, which starts scanning along the 5′-UTR in search for an initiation codon. The intrinsic RNA unwinding activity of the scanning ribosome (32) and eIF4A enables the unwinding of the UI–RNA duplex for translation. Of note, the 5′-UTR length of HAC1 24b mRNA before the UI RNA duplex is 54 nucleotides, similar to the predicted average length of 5′-UTR in the budding yeast S. cerevisiae. Thus, this study provides an example of a broader phenomenon that 5′-UTRs of ≥55 nucleotides in length in S. cerevisiae may be important for efficient translation of 48% mRNAs as mentioned earlier.
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Translation from the unspliced \textit{HAC1} mRNA with an increased length of 5'-UTR suggests that 30 RNA bases between the 5'-cap and UI–RNA duplex did not provide sufficient space for the assembly of the PIC. We further confirm these results using a synthetic \textit{HAC1}-AUG-42,A1G derivative, in which adenine of the normal AUG start codon was mutated to guanine and an in-frame AUG start codon was inserted by replacing the UAA RNA bases located at the 5'-UTR positions -42 to -40 (Fig. 7A). This \textit{HAC1}-AUG-42,A1G mutant, like a WT \textit{HAC1} allele, allowed the \textit{hac1Δ} strain to grow on the tunicamycin medium (Fig. 7B). Consistent with the tunicamycin-resistant phenotype, Hac1 protein was detected in cells grown in the presence of DTT (Fig. 7C), suggesting that the synthetic \textit{HAC1} mRNA behaved like a WT allele. It is to be noted that the migration of the Hac1 protein expressed from cells containing the
HAC1-AUG-42,A1G mutant was slightly slower than the Hac1 protein expressed from cells containing the WT allele (Fig. 7C, WB, compare lanes 2 and 3). The slower migration was due to expression of Hac1 protein from the upstream AUG codon, which is larger (252 amino acids) than the WT protein (238 amino acids). Unlike the hac1Δ strain, the hac1Δire1Δ strain containing the HAC1-AUG-42,A1G mutant neither grew on the tunicamycin medium (Fig. 7B, row 3) nor produced any Hac1 protein (Fig. 7D, WB, lane 3). These results suggest that either the PIC was unable to form on the 5′-UTR or the inserted AUG start codon in the 5′-UTR of unspliced mRNA remained inaccessible to the ribosomal complex because of the nearby RNA secondary structure.

To facilitate the accessibility of the AUG codon to the ribosomal complex, we added extra 24 RNA bases before the UI–RNA duplex of HAC1-AUG-42,A1G mutant, thus generating a HAC1-AUG-42,A1G24b mutant (Fig. 7A). The HAC1-AUG-42,A1G24b mutant allowed the hac1Δire1Δ strains to grow on the tunicamycin medium (Fig. 7B, row 4) and produced a detectable amount of Hac1 protein under normal condition (Fig. 7D, WB, lane 4). These data suggest that the extra 24 RNA bases before the AUG start codon allow ribosomes to form the PIC that likely starts traveling down the mRNA and finally recognizes the AUG start codon resulting in translation of the unspliced mRNA.

The helicase eIF4A is associated with the translationally repressed HAC1 mRNA

We investigated whether the UI RNA duplex would sterically block the recruitment of helicase eIF4A to the 5′-UTR. Thus, we monitored the association of helicase eIF4A with the translationally repressed and derepressed HAC1 mRNAs. WCEs were prepared from an ire1Δhac1Δ strain coexpressing untagged or FLAG-tagged eIF4A from a high-copy LEU2 plasmid and translationally repressed HAC1 or translationally derepressed HAC136b mRNA mutant from a low-copy URA3 plasmid (see the Experimental procedures section). From the WCEs, the FLAG-eIF4A protein was then pulled down by anti-FLAG agarose resin. Both pellet and supernatant fractions were collected and analyzed by WB. As expected, the FLAG-eIF4A was predominantly pulled down with the pellet fraction (Fig. 8B, WB, lanes 5 and 8). Total RNA was also isolated from both pellet and supernatant fractions. The isolated RNA from the pellet fraction was then subjected to RT–PCR analysis using HAC1-specific primers. Amplification of HAC1 complementary DNA (cDNA) was observed in the reaction mixture containing RT (Fig. 8C, lane 4) but not in the reaction without RT (Fig. 8C, lane 3), suggesting that amplification of HAC1 was based on cDNA synthesis. Interestingly, we observed that amplification of HAC1 in cells expressing the translationally repressed WT HAC1 was ~10-fold more than cells expressing the translationally derepressed HAC136b mRNA mutant (Fig. 8, D and E). These data suggest that the
translationaly repressed HAC1 mRNA is associated with the helicase eIF4A, and majority of eIF4A dissociates from the translationally active mRNA. Typically, eIF4A is a component of the cap-binding complex eIF4F, containing other two major subunits: an eIF4E and a large scaffolding protein eIF4G (3). Thus, it appears that translationally derepressed HAC1 mRNA is bound to cap-binding protein complex and exported from the nucleus.

**Discussion**

In the present study, we show that three RNA bases (C-23, C-27, and C-32) at the UI interaction site play a major role in the translational regulation of HAC1 mRNA. We also show that minimally 11 bp interactions between the UI are required to repress HAC1 mRNA translation. Furthermore, we show that overexpression of the helicase eIF4A can derepress translation of an unspliced HAC1 mRNA containing an 11-bp interaction between the 5'UTR and intronic sequences. Finally, we demonstrate that, consistent with the possibility, 30 RNA bases before the UI interaction site precludes the recruitment of the translationally competent PIC on the HAC1-5'UTR.

The rate-limiting step of translation is the initiation, which occurs typically by a cap-dependent or cap-independent manner (16). In cap-independent translation, an mRNA structure referred to as the "internal ribosomal entry site" directly binds a subset of initiation factors, the 40S ribosomal subunit and Met-tRNA$_{Met}$, resulting in the recognition of the
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start codon by Met-tRNA_{Met}^{3′}. In contrast, the start codon recognition by Met-tRNA_{Met}^{3′} in the cap-dependent translation is thought to occur in three steps. First, a 43S-PIC (a complex of the 40S ribosomal subunit, initiation factors, and the initiator Met-tRNA_{Met}^{3′}) assembles on the mRNA 3′-cap bound to an elf4F protein complex, consisting of a cap-binding protein elf4E, a scaffold elf4G, and the helicase elf4A (16, 33). Second, the 43S-PIC travels along the 5′-UTR in search of a start codon (16, 34). The ribosomal scanning is facilitated by the helicase elf4A and/or Ded1, which unwind any secondary structure present in the 5′-UTR (28). Third, the Met-tRNA_{Met}^{3′} of the 43S-PIC recognizes the start codon, and all initiation factors dissociate from the 40S ribosome. Then, the 60S ribosomal subunit joins the 40S subunit to yield an 80S ribosomal complex (35), where the Met-tRNA_{Met}^{3′} is placed right over the start codon. The 80S ribosome then proceeds to decode mRNA into a protein molecule. Consistent with this linear model of ribosomal scanning, we observed that the addition of extra space between the 5′-cap and the UI–RNA duplex allowed cells to derepress translation of the unspliced HAC1 mRNA (Fig. 6).

A growing body of evidence suggests that the length of the 5′-UTR and its contextual fold determine the translational efficiency of mRNAs (36). On the 5′-UTR next to the 5′-cap, the PIC assembles and then scans for the start codons along the 5′-UTR, except for some mRNAs with extremely short 5′-UTR (<12 bases), which undergo scanning-free initiation (37). In essence, both PIC assembly and scanning are major control points of translation initiation. 5′-UTRs vary in lengths, ranging from a few to thousands of nucleotides with a median length of ~55 bases in the budding yeast S. cerevisiae and ~218 bases in humans, Homo sapiens (36). 5′-UTRs may also self-fold into a simple hairpin or more complex secondary and tertiary structures that can block the ribosomes to form the PIC (36). These secondary structures inhibit the translational efficiency. In this study, we show that minimally 11-bp interactions between the 5′-UTR and intronic sequences are required to keep HAC1 mRNA translationally repressed. Consistently, we show that addition of an 11-bp RNA hairpin inhibits Hac1 expression from an intron-less HAC1 mRNA (Fig. 4). Then, we demonstrate that the inhibitory effect of this structured mRNA is overcome by increased dosage of elf4A (Fig. 5). However, we observed that translationally repressed HAC1 mRNA is associated with the helicase elf4A (Fig. 8). Therefore, it appears that the UI RNA duplex likely interferes with the elf4A activity.

In the budding yeast S. cerevisiae, high-throughput sequencing technologies including SHAPE-Seq (selective 2′-hydroxyl acylation analyzed by primer extension) (38) and Frag-Seq (fragmentation sequencing) reveal that >90% of 5′-UTRs could form secondary structures (39). In addition, chemical probing and structural analysis of S. cerevisiae mRNAs showed that the protein-coding regions are more structured than their leader or trailer sequences (39). This is likely because most RNAs in cells are covered by ribosomes, helicases, and/or RNA-binding proteins. Indeed, these approaches significantly advance our knowledge about the structural dynamics of cellular mRNAs; however, our knowledge is still limited on how self-folded RNA elements control localization, transport, and translational efficiency of mRNA. The regulatory effects of the predicted structured mRNA on translational efficiency require an intensive compensatory mutational analysis.

Most HAC1 orthologs contain an unconventional intron that is excised to produce an active transcription factor (12, 40, 41). These introns are shorter in metazoan species (20–26 bases) compared with fungal species (>100 bases) except in some species in the fungal Clade (19–22 bases) (42, 43). Despite these differences in their lengths, sequences of both exon and intron junctions are shown to form a consensus stem–loop structure consisting of 7 bp stem and 7 bases loop containing an Ire1-cleavage motifs C xG*CxGx (* = cleavage site and x = any nucleotides) (42, 44). Introns are cleaved by atypical splicing process during ER stress, resulting in production of an active transcription factor.

Introns of HAC1 mRNA in Saccharomyces clade are long (252 bases in S. cerevisiae) (42) and uniquely associated with its 5′-UTR (Fig. 1A). This unique intron-5′-UTR association has a unique regulatory role in Hac1 protein expression at the translation level (6, 8). This class of HAC1 mRNAs with long intron is regulated not only by atypical splicing during ER stress but also coordinated recruitment of translational components along with splicing. Consistent with the aforementioned notion, we found that expression of Hac1 protein from the unspliced HAC1-C(-27)G mRNA was enhanced when cells were grown in the presence of DTT (Fig. 1C). Together, these data imply that translational derepression in HAC1 mRNA in Saccharomyces clade likely occur independent of cytosolic splicing. The question remains what would be of benefit to having two independent programs in HAC1 mRNA translation in Saccharomyces clade. Identification of translational repressor(s), if any, might help understand the regulatory events in splicing and translational derepression.

Given that HAC1 mRNA is associated with the helicase elf4A (Fig. 8C), we propose a model (Fig. 8F) that the heterotrimetric elf4F complex will form on the m’G cap and remain intact with the translationally silent HAC1 pre-mRNA. We also propose that 30 RNA bases in between the 5′-cap and the UI RNA duplex in HAC1 mRNA may not provide a sufficient space to recruit translational initiation factors, ribosomes, and Met-tRNA_{Met}^{3′}. There are 23% of mRNAs in the budding yeast containing a 5′-UTR of ≤30 nucleotides (18), and many of those mRNA make proteins normally (www.yeastgenome.org). Therefore, it is likely that the UI RNA duplex prevents the dynamic recruitment of translation factors and inhibits the 43S PIC formation, rendering mRNA translationally inert. Thus, the HAC1 mRNA translational system provides an excellent in vivo tool to study how a secondary structure near the 5′-cap modulates mRNA translation. Studies are underway to determine how the coordinated recruitment of translational factors facilitates translational repression in HAC1 mRNA and how an alternate cap-dependent translation initiation stimulates protein synthesis from HAC1 5′-UTR mutants.
Experimental procedures

Yeast strains and growth conditions

Standard yeast extract peptone dextrose (YPED) and SC with 2% dextrose media were used to grow and analyze the yeast strains of *S. cerevisiae*. Strains were grown in liquid or solid medium overnight at 30 °C. For ER stress induction, yeast cells were grown in YEPD, SC medium, or SC without appropriate amino acids at 30 °C to an absorbance value of ~0.5 to 0.6 at 600 nm. The ER-stressor DTT (5 mM) or tunicamycin (0.5 μg/ml) was added to the medium, and cells were grown further for an additional 1 h (unless otherwise indicated). In general, we prefer to use tunicamycin for growth assays on the solid medium because it is more stable than DTT. All yeast strains used in this study are listed in Table 1.

Plasmids were created using the standard gene manipulation techniques. Mutations were generated by appropriate DNA oligonucleotides, using the standard regular PCR or fusion PCR protocols. The desired mutation in each plasmid was confirmed by Sanger sequencing. All plasmids used in this study are shown in Table 1.

WCE preparation and WB analysis

Cells were cultured under an ER stress condition as stated previously. WCEs were prepared by trichloroacetic acid method as described previously (45). Proteins in WCEs were fractionated by SDS–PAGE and subjected to WB analysis using antibodies against Hac1 (generated in our laboratory), phosphoglycerate kinase 1 (catalog no.: 459250; Invitrogen) or eIF2α (catalog no.: CM-217, gift from Thomas E. Dever, National Institutes of Health, USA). All experiments were repeated at least twice.

Mutagenesis screen

Following the manufacturer’s protocol, 50 μl chemically competent XL1-Red cells (catalog no.: 200129; Invitrogen) were transformed with the plasmid D1243 containing the hemagglutinin-tagged HAC1 gene and plated on the LB medium containing ampicillin for 24 h. Cells were scraped from the LB plate, and the mutator plasmids were isolated. A hac1Δire1Δ strain was transformed with the mutated D1243 plasmids, plated on YEPD medium overnight, and then replica printed on SC-uracil medium containing tunicamycin. The mutated plasmids from 30 tunicamycin-resistant colonies were rescued from yeast cells and sequenced to identify mutations. Subsequently, single mutations were generated as required.

RT–PCR

Cells were cultured under an ER stress condition as stated previously. Cells were harvested, and total RNA was isolated using the RNeasy Mini Kit (Qiagen). Purified RNA was quantified using a Nanodrop spectrophotometer (ND-1000; Thermo Fisher Scientific). Purified RNA was used to synthesize the first-strand cDNA by a Superscript-III reverse transcriptase (catalog no.: 18080-093; Invitrogen) and a reverse primer (5’-CCCACCAACAGCGAT AAATAACGAG-3’) that corresponded to nucleotides +1002 to 1025. To assay HAC1 mRNA splicing, the synthetic cDNA was then PCR amplified using a forward primer (5’-CGCAATCG AACTTGGCTATCC CTACC-3’) that corresponded to nucleotides +35 to 60 and a reverse primer (5’-CCCACCAACAGCGATATA ACGAG-3’) that corresponded to nucleotides +1002 to 1025. The PCR-amplified products were then run on a 1.5% agarose gel to separate spliced (HAC1Δ) and unspliced (HAC1Δ) forms of HAC1 mRNA. Quantities of HAC1Δ and HAC1Δ were measured using Imagel software (NIH). Experiments were repeated at least two times.

Pulldown of eIF4A–RNA complex

The ire1Δhac1Δ strain coexpressing the untagged or FLAG-tagged eIF4A from a LEU2 plasmid and the translationally repressed HAC1Δ or translationally derepressed HAC1Δ mRNA mutant from a URA3 plasmid was grown in SC medium without uracil and leucine till the absorbance value reached ~1.0 at 600 nm. Cells (absorbance at 600 nm = ~50) were harvested and suspended in 1.2 ml of buffer A (20 mM Tris [pH 7.5], 50 mM KCl, and 10 mM MgCl2) supplemented with one EDTA-free protease inhibitor tablet per 10 ml buffer (Roche), 5 mM NaF, 1 mM DTT, 1 mM PMSF, and 1 μg/ml of the following protease inhibitors—pepsstatin A, aprotinin, and leupeptin. Cells were broken with glass beads in a vortex mixer for 10 min at 4 °C and centrifuged at 10,000 rpm for 10 min in an Eppendorf 5810R refrigerated centrifuge. The supernatant was collected, further clarified by centrifugation at 13,000 rpm 10 min at 4 °C, and clear WCE was collected.

The amount of protein in WCE was measured by the standard Bradford protein assay. About 300 μg of protein was mixed with 30 μl of anti-FLAG–agarose resin (SIGMA) in buffer A containing 0.1% of Triton X-100. After 1 h, the tube containing the aforementioned mixture was centrifuged at 2500 rpm in an Eppendorf 5415R centrifuge for 1 min at 4 °C, and both supernatant (Sup) and pellet (P) fractions were collected. The pellet fraction was washed five times with the same buffer A, and the recovered protein was dissolved in the 2× SDS dye. Proteins in the supernatant fraction were precipitated with 20% trichloroacetic acid by standard protocol. Both pellet and supernatant fractions were subjected to WB analysis using an anti-FLAG antibody. About 10% of input of WCE was also used for WB analysis.

Table 1

| Yeast strain used in this study | Genotype | Reference |
|-------------------------------|----------|-----------|
| WT (BY4741)                   | MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 | Deletion collection |
| ire1Δ                         | MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 ire1::kanMX | Deletion collection |
| hac1Δ                         | MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 hac1::kanMX | Deletion collection |
| ire1Δ hac1Δ                   | MATa his3Δ1 leu2Δ0 met5Δ0 ura3Δ0 hac1::kanMX | Lee et al. (44) |

Translational derepression of HAC1 mRNA

The yeasts were grown further for an additional 1 h (unless otherwise indicated). In general, we prefer to use tunicamycin for growth assays on the solid medium because it is more stable than DTT. All yeast strains used in this study are listed in Table 1.
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The amount of RNA in WCE was measured at an absorbance value at 600 nm using a spectrophotometer. About 300 μg of RNA was mixed with 100 μl of anti-FLAG-agarose resin (SIGMA) in buffer A containing 0.1% of Triton X-100. After 1 h, the tube containing the aforementioned mixture was centrifuged at 2500 rpm in an Eppendorf 5415R centrifuge for 1 min at 4°C. The supernatant and pellet fractions were collected. RNA was isolated from input, and supernatant and pellet fractions using the standard Trizol reagent (Invitrogen). Isolated RNA from the pellet fraction was subjected to DNase I treatment followed by RT–PCR analysis with HAC1 mRNA-specific primers as described before.

Bioinformatics analysis and RNA–RNA hybrid structure prediction

The transcriptome data were retrieved from the supporting information of an article published by Nagalakshmi et al. (18). We used the IntaRNA software (23), which predicts the RNA–RNA hybrids with duplex-binding structures by incorporating seed constraints and interaction site accessibility.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

Acknowledgments—This work was supported partly by grant from the Graduate School (Discovery and Innovation Grant), UW-Milwaukee, Milwaukee, WI, USA.

Author contributions—J. K. U., L. S., A. C., S. B., A. T. P., and M. D. methodology; J. K. U., L. S., A. C., S. B., A. T. P., and M. D. investigation; M. D., J. K. U., and A. T. P. writing—original draft.

Funding and additional information—This work was supported partly by grant from the National Institute of General Medical Sciences, National Institutes of Health, Bethesda, MD, USA (grant no.: 1R01GM124183; to M. D.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: cDNA, complementary DNA; eIF, eukaryotic translation initiation factor; ER, endoplasmic reticulum; Met-tRNA<sup>Met</sup>, initiator methionyl-tRNA; MFE, minimum free energy; PIC, preinitiation complex; SC, synthetic complete; UI, 5′-UTR—intron; uORF, upstream ORF; WB, Western blot; WCE, whole cell extract; YEPD, yeast extract peptone dextrose.

Table 2

Plasmids used in this study

| Plasmid name | Plasmid | Reference |
|--------------|---------|----------|
| D3           | pRS315, low-copy-number. LEU2 vector | Lab collection |
| D4           | pRS316, low-copy-number. 11RA3 vector | Lab collection |
| D63          | HAC1 in D4 | Sathe et al. (8) |
| D1243        | HA-tagged HAC1 in D4 | This study |
| D1310        | HAC1-C(-23)G in D4 | This study |
| D1278        | HAC1-C(-27)G in D4 | This study |
| D2367        | HAC1-C(-29)A in D4 | This study |
| D368         | HAC1-C(-30)A in D4 | This study |
| D369         | HAC1-C(-32)A in D4 | This study |
| D370         | HAC1-C(-33)A in D4 | This study |
| D387         | HAC1-A(-22)T, C(-23)G in D4 | This study |
| D812         | HAC1<sup>ab</sup> in D4 | This study |
| D2388        | HAC1<sup>ab</sup> in D4 | This study |
| D1775        | HAC1<sup>j</sup> in D4 | This study |
| D2323        | U1-HAC1<sup>j</sup> in D4 | This study |
| D1343        | HAC1<sup>ab</sup> in D4 | This study |
| D1436        | HAC1<sup>ab</sup> in D4 | This study |
| D1435        | HAC1<sup>ab</sup> in D4 | This study |
| D1118        | HAC1-AUG<sup>42</sup>A1G in D4 | Sathe et al. (8) |
| D1378        | HAC1-AUG<sup>42</sup>A1G<sup>ab</sup> in D4 | This study |
| D1041        | eIF1 in a high-copy LEU2 vector | Hinnebusch (16) |
| D1043        | eIF1A in a high-copy LEU2 vector | Hinnebusch (16) |
| D1195        | eIF2α in a high-copy LEU2 vector | Hinnebusch (16) |
| D1193        | eIF3α in a high-copy LEU2 vector | Hinnebusch (16) |
| D1045        | eIF4A in a high-copy LEU2 vector | Hinnebusch (16) |
| D1206        | eIF4B in a high-copy LEU vector | Hinnebusch (16) |
| D1197        | eIF4E in a high-copy LEU2 vector | Hinnebusch (16) |
| D1201        | eIF5 in a high-copy LEU2 vector | Hinnebusch (16) |
| D2122        | eIF5B in a high-copy LEU2 vector | Hinnebusch (16) |
| D1205        | Ded1 in a high-copy LEU2 vector | Hinnebusch (16) |
| D1420        | FLAG-eIF4A in D3 | This study |
| D1419        | eIF4A in D3 | This study |

References

1. Jackson, R. J., Hellen, C. U., and Pestova, T. V. (2010) The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat. Rev. Mol. Cell Biol.* 11, 113–127
2. Andreev, D. E., O'Connor, P. B., Loughran, G., Dmitriev, S. E., Baranov, P. V., and Shatsky, I. N. (2017) Insights into the mechanisms of eukaryotic translation gained with ribosome profiling. *Nucleic Acids Res.* 45, 513–526
3. Hershey, J. W., Sonenberg, N., and Mathews, M. B. (2012) Principles of translational control: An overview. *Cold Spring Harb. Perspect. Biol.* 4, a011528
4. Mueller, P. P., and Hinnebusch, A. G. (1986) Multiple upstream AUG codons mediate translational control of GCN4. *Cell* 45, 201–207
18. Nagalakshmi, U., Wang, Z., Waern, K., Shou, C., Raha, D., Gerstein, M., and Prats, A. C. (1997) Alternative translation of the proto-oncogene c-myc by an internal ribosome entry site. *J. Biol. Chem.* 272, 32061–32066

19. Chapman, R. E., and Walter, P. (1997) Translational attenuation mediated by a nuclear export signal. *Cell* 90, 1031–1039

20. Lee, K. P., Dey, M., Neculai, D., Cao, C., Dever, T. E., and Sicheri, F. (2008) Profile of the translation initiation factor eIF4F in mammalian cells. *Cell* 132, 89–100

21. Uppala, J. K., Bhattacharjee, S., and Dey, M. (2021) Vps34 and TOR kinase-dependent translation initiation. *Eur. J. Biochem.* 298, 101648

22. Di Santo, R., Aboulhouda, S., and Weinberg, D. E. (2016) The fail-safe mechanism of post-transcriptional silencing of unspliced HAC1 mRNA. *Elife* 5, e10069

23. Mann, M., Wright, P. R., and Backofen, R. (2017) IntaRNA 2.0: Enhanced and customizable prediction of RNA-RNA interactions. *Nucleic Acids Res.* 45, W435–W439

24. Gruber, A. R., Lorenz, R., Bernhart, S. H., Neubock, R., and Hofacker, I. L. (2008) The Vienna RNA website. *Nucleic Acids Res.* 36, W70–W74

25. Kozak, M. (1989) Circumstances and mechanisms of inhibition of translation by secondary structure in eukaryotic mRNAs. *Mol. Cell. Biol.* 9, 5134–5142

26. Goossens, B., Caughman, S. W., Harford, J. B., Klauser, R. D., and Hentze, M. W. (1990) Translational repression by a complex between the iron-responsive element of ferritin mRNA and its specific cytoplasmic binding protein is position-dependent in vivo. *EMBO J.* 9, 4127–4133

27. Weenink, T., van der Hilst, J., McKiernan, R. M., and Ellis, T. (2018) Design of RNA hairpin modules that predictably tune translation in yeast. *Synth. Biol. (Oxf.)* 3, 9sy019

28. Sharma, D., and Jankowsky, E. (2014) The Ded1/D1DX3 subfamily of DEAD-box RNA helicases. *Crit. Rev. Biochem. Mol. Biol.* 49, 343–360

29. Gao, Z., Putnam, A. A., Bowers, H. A., Guenther, U. P., Ye, X., Kingsfader, A., Hilliker, A. K., and Jankowsky, E. (2016) Coupling between the DEAD-box RNA helicases Ded1p and eIF4A. *Elife* 5, e16408

30. Laere, L. F., Hite, D. H., Hogan, G. J., and Brown, P. O. (2014) Distinct stages of the translation elongation cycle revealed by sequencing ribosome-protected mRNA fragments. *Elife* 3, e01257

31. Ingolia, N. T., Ghaemmaghami, S., Newman, J. R., and Weissman, J. S. (2009) Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. *Science* 324, 218–223

32. Takaya, S., Hickerson, R. P., and Noller, H. F. (2005) mRNA helicase activity of the ribosome. *Cell* 120, 49–58

33. Hinnenbusch, A. G., and Lorsch, J. R. (2012) The mechanism of eukaryotic translation initiation: New insights and challenges. *Cold Spring Harb. Perspect. Biol.* 4, a011544

34. Dever, T. E., Kinzy, T. G., and Pavitt, G. D. (2016) Mechanism and regulation of protein synthesis in Saccharomyces cerevisiae. *Genetics* 203, 65–107

35. Pestova, T. V., Lomakin, I. B., Lee, J. H., Choi, S. K., Dever, T. E., and Hellen, C. U. (2000) The joining of ribosomal subunits in eukaryotes requires eIF5B. *Nature* 403, 332–335

36. Leppek, K., Das, R., and Barna, M. (2018) Functional 5’ UTR mRNA structures in eukaryotic translation regulation and how to find them. *Nat. Rev. Mol. Cell Biol.* 19, 158–174

37. Elfakhès, R., and Dikstein, R. (2008) A translation initiation element specific to mRNAs with very short 5’UTR that also regulates transcription. *PloS One* 3, e3094

38. Bevilacqua, P. C., Ritchey, L. E., Su, Z., and Assmann, S. M. (2016) Genome-wide analysis of RNA secondary structure. *Annu. Rev. Genet.* 50, 235–266

39. Kertesz, M., Wan, Y., Mazor, E., Rinn, J. L., Nutter, R. C., Chang, H. Y., and Segal, E. (2010) Genome-wide measurement of RNA secondary structure in yeast. *Nature* 467, 103–107

40. Callon, M., Zeng, H., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G., and Ron, D. (2002) Ire1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* 415, 92–96

41. Wimalasena, T. T., Enjalbert, B., Guillemette, T., Plumridge, A., Budge, S., Yin, Z., Brown, A. J., and Archer, D. B. (2008) Impact of the unfolded protein response upon genome-wide expression patterns, and the role of Hac1 in the polarized growth of Candida albicans. *Fungal Genet. Biol.* 45, 1235–1247

42. Hooks, K. B., and Griffiths-Jones, S. (2011) Conserved RNA structures in the non-canonical Hac1/Xbp1 intron. *RNA Biol.* 8, 552–556

43. Irarane, E., Donovan, P. D., Ola, M., Butler, G., and Holland, L. M. (2018) Identification of an exceptionally long intron in the HAC1 gene of Candida parapsilosis. *mSphere* 3, e00532–18

44. Gonzalez, T. N., Sidrauskis, C., Dorfler, S., and Walter, P. (1999) Mechanism of non-spliceosomal mRNA splicing in the unfolded protein response pathway. *EMBO J.* 18, 3119–3132

45. Mannan, M. A., Shadrick, W. R., Bienar, G., Shin, B. S., Anshu, A., Raicu, V., Frick, D. N., and Dey, M. (2013) An ire1-phk1 chimera reveals a dispensable role of autokinase activity in endoplasmic reticulum stress response. *J. Mol. Biol.* 425, 2083–2099