Translational attenuation mediated by an mRNA intron

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Background: The unfolded protein response (UPR) is an intracellular signaling pathway that is activated by the accumulation of unfolded proteins in the endoplasmic reticulum (ER). The UPR results in an increase in transcription of ER-resident proteins that facilitate protein folding in the ER. A key regulatory step in UPR activation is the regulated splicing of *HAC1* mRNA, which encodes Hac1p, a transcription factor dedicated to this pathway. Hac1p can be detected only when the spliced form of *HAC1* mRNA (termed $HAC1^{i}$ mRNA, for *i*nduced) is produced; this was surprising because the unspliced $HAC1^{u}$ mRNA ($HAC1^{u}$ for *u*ninduced) is equally stable in cells.

Results: We show that in contrast to most other unspliced pre-mRNAs, the $HAC1^{u}$ mRNA is transported from the nucleus into the cytosol. Although $HAC1^{u}$ mRNA is associated with polyribosomes, no detectable $Hac1p^{u}$ is produced unless the intron is removed, indicating that the presence of the intron prevents mRNA translation. When it is produced, $Hac1p^{u}$ has a stability similar to that of $Hac1p^{i}$, the form of the Hac1p that is produced from the spliced mRNA and that differs from $Hac1p^{u}$ by a short carboxy-terminal tail sequence. $Hac1p^{u}$, however, is differently modified and less active in activating transcription. Interestingly, when transplanted into the 3′ untranslated region of a completely unrelated mRNA, the *HAC1* intron is sufficient to attenuate translation of the preceding open reading frame.

Conclusions: We have shown that the *HAC1* mRNA intron is both necessary and sufficient to prevent complete translation of polyribosome-associated mRNAs. To our knowledge, this identifies a new way by which translation of a mRNA can be attenuated.

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Background

The unfolded protein response (UPR) is an intracellular signal transduction pathway that connects the endoplasmic reticulum (ER) and the nucleus [1–3]. The UPR responds to changes in the protein-folding capacity of the ER lumen. Under certain stress conditions, or changes in secretory output, unfolded and/or improperly modified proteins accumulate in the ER lumen. Such conditions activate the UPR, resulting in the upregulation of transcription of genes encoding ER-resident proteins [4,5]. The transcription of many of the ER chaperones that assist in folding accumulated proteins is regulated in this way. In both mammalian and yeast cells, a promoter element required for regulation by the UPR has been identified [6–8]. In yeast, this element is a sequence of 22 base pairs (bp) that is called the unfolded protein response element (UPRE); it is necessary and sufficient for regulation by the UPR.

Three key components of this signal transduction pathway have recently been identified: Ire1p, a serine/threonine transmembrane kinase; tRNA ligase, an RNA-processing enzyme, and Hac1p, a bZIP transcription factor that binds to the UPRE [8–14]. These proteins act together in an unprecedented fashion to signal from the ER to the nucleus. Ire1p is a bifunctional enzyme located in the ER and/or inner nuclear membrane [14]. Its amino terminus lies in the ER lumen [11] (which is continuous with the nuclear envelope), where it is thought to act as a sensor, receiving information about the prevailing folding conditions (reviewed in [15]). Its carboxy-terminal portion (present in the cytosol and/or nucleoplasm) has a serine/threonine kinase domain, followed by a tail domain that functions as a site-specific endonuclease. When unfolded proteins accumulate in the ER lumen, Ire1p oligomerizes and autotransphosphorylates in a manner similar to that described for cell-surface growth factor receptors [16]. Autophosphorylation is thought to activate the endonuclease portion of Ire1p. Its substrate for cleavage is the mRNA encoding the downstream transcription factor Hac1p [10,14]. Ire1p specifically removes a 252-nucleotide intron from the 3′ end of newly synthesized $HAC1^{u}$ mRNA (*u* for *u*ninduced). The exons are then religated by tRNA ligase, which until recently was thought to be dedicated to pre-tRNA splicing [13]. This unconventional splicing reaction, carried out by only two enzymes, Ire1p and tRNA ligase, converts $HAC1^{u}$ mRNA to $HAC1^{i}$ mRNA (*i* for *i*nduced), which is translated to

produce the active transcription factor Hac1p^{i}. We consider it likely that *HAC1* mRNA in UPR-induced cells becomes spliced before or as it exits from the nucleus, which is consistent with the described localization of tRNA ligase to the nuclear pore [17].

The *HAC1* mRNA intron has no similarity to introns removed by the conventional splicing machinery [13,14]. This is not surprising in view of the fact that it is processed by different enzymes. Secondary structure predictions suggest that the cleavage sites are located in symmetrical stem–loop structures. The sequence of these predicted stems extends a small distance either side of the 252-nucleotide intron. It has been proposed that this symmetrical structure might reflect binding of Ire1p as a dimer to the *HAC1^{u}* mRNA, with each endonuclease active site recognizing a single stem–loop [14]. In each case, a single G residue is required for cleavage at that splice junction. Additional features within or adjacent to the intron that are required for recognition by the splicing machinery remain to be determined.

Unlike other pre-mRNAs that are spliced by the spliceosome, *HAC1^{u}* mRNA is stable in yeast cells [8,10]. Its sequence predicts that it encodes a protein Hac1p^{u}, differing in its carboxy-terminal tail from the active form Hac1p^{i}, the protein produced from the spliced *HAC1^{i}* mRNA. As a result of splicing of *HAC1* mRNA, the nucleotide sequence encoding the predicted Hac1p^{u} carboxy-terminal tail is removed and replaced by the sequence encoding the Hac1p^{i} tail [10]. *In vivo*, however, no Hac1p^{u} can be detected. This makes the splicing event a key regulatory step in the UPR: the required transcription factor is only present in cells that express spliced *HAC1^{i}* mRNA.

Several models could explain the lack of detectable Hac1p^{u} in yeast cells. Previous data suggested that both Hac1p^{i} and Hac1p^{u} are actively translated, as the two forms of *HAC1* mRNA co-migrate with polyribosomes when cell extracts are analyzed by sucrose density gradient centrifugation [10]. To explain this data we previously suggested that the Hac1p^{u} carboxy-terminal tail causes this form of the protein to be extremely unstable. As both forms of Hac1p are rich in PEST sequences (regions with an abundance of the amino acids Pro, Glu, Asp, Ser and Thr, which have been shown to destabilize proteins by targeting them for degradation), we predicted that this instability would be conferred by increased recognition of Hac1p^{u} by the ubiquitin–proteasome degradation system. This is supported by data showing that mutants in the ubiquitin-conjugating enzymes Ubc4p and Ubc5p activate the UPR in a *HAC1*-dependent manner [10]. This activation is independent of *IRE1*; thus it must be caused by Hac1p^{u} as no *HAC1* mRNA splicing occurs in cells lacking Ire1p.

A prediction from this model is that Hac1p^{u} should be stabilized in a *Δubc4 Δubc5 Δire1* mutant yeast strain. When this prediction was tested, however, only trace amounts of Hac1p^{u} could be detected and, moreover, this low level of expression depended upon overexpression of *HAC1^{u}* mRNA [10]. We therefore decided to reinvestigate the regulation of Hac1p^{u} production and degradation. Here we show that degradation of Hac1p^{u} is not the primary way in which Hac1p^{u} levels are kept limiting in normally growing cells when the UPR is not induced. Rather we find that, because of the presence of the *HAC1* intron, the translation of *HAC1^{u}* mRNA is severely attenuated.

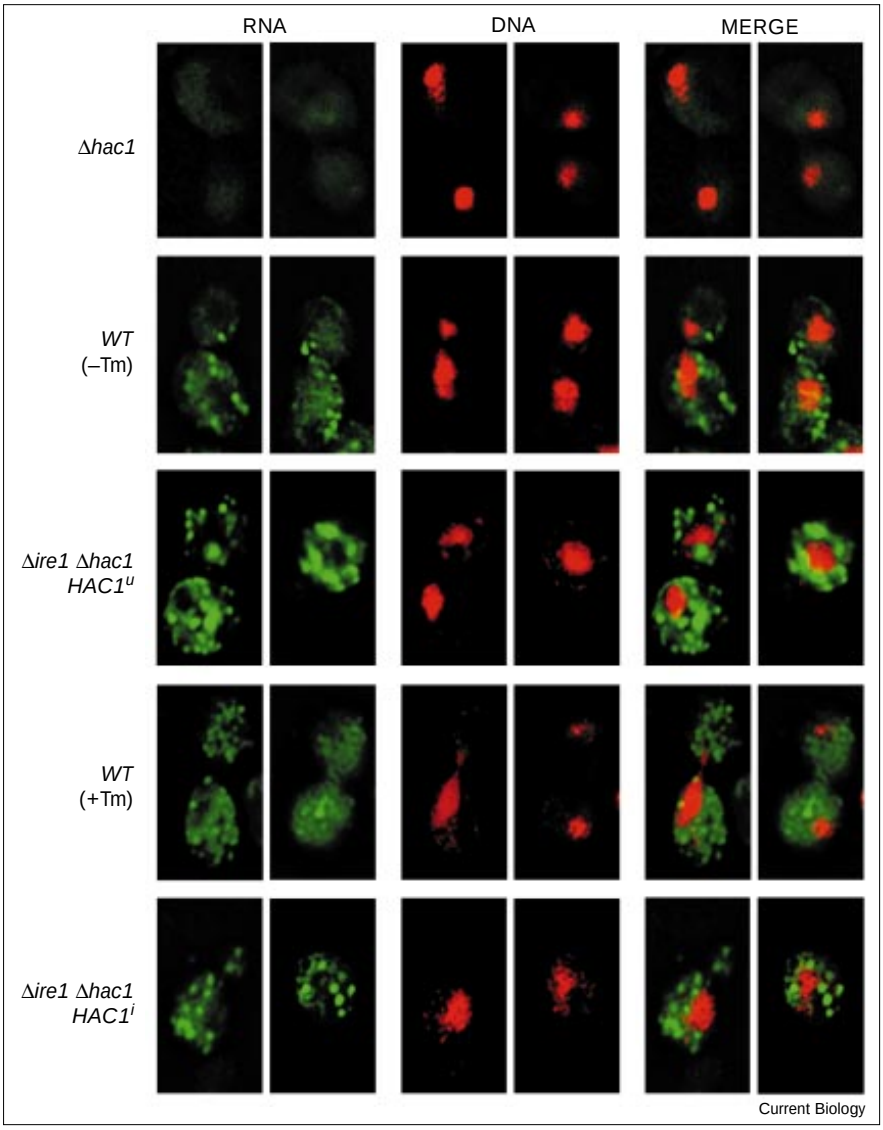
Results

Under normal growth conditions, unspliced *HAC1^{u}* mRNA is present in yeast cells in approximately the same amount as spliced *HAC1^{i}* mRNA in induced cells. However, no Hac1p^{u}, the predicted translation product of the unspliced mRNA, can be detected [8,10]. The lack of Hac1p^{u} could be the result of regulation at one or more of the following levels. First, unspliced *HAC1^{u}* mRNAs could be prevented from exiting the nucleus and hence remain sequestered from cytoplasmic ribosomes. Second, *HAC1^{u}* mRNA could exit from the nucleus but production of Hac1p^{u} could be regulated at the level of translational initiation or elongation. Third, *HAC1^{u}* mRNA could exit from the nucleus and be translated normally, but the Hac1p^{u} produced might be extremely unstable.

To determine whether *HAC1^{u}* mRNA is sequestered in the nucleus, we decided to determine its intracellular localization directly. To this end, we used *in situ* hybridization, employing a technique that was recently successfully adapted to the analysis of yeast cells [18]. We used digoxigenin-labeled antisense *HAC1* RNA as a probe. In normally growing cells we detected *HAC1^{u}* mRNA in punctate structures that are scattered throughout the cytoplasm [Figure 1, WT (–Tm)]. An indistinguishable pattern was observed when *HAC1^{u}* mRNA was expressed from a low-copy plasmid in *Δire1 Δhac1* cells (Figure 1, *Δire1 Δhac1 HAC1^{u}*). In both cases, *HAC1^{u}* mRNA was largely excluded from the nucleus as visualized by DAPI staining of the DNA (Figure 1, compare RNA and DNA in the MERGE pictures). Only a low level of background fluorescence was observed in *Δhac1* cells, indicating that the observed signals are specific for *HAC1* mRNA (Figure 1, *Δhac1*).

The mRNA localization pattern of *HAC1^{u}* mRNA was also indistinguishable from that of *HAC1^{i}* mRNA produced on induction of the UPR by tunicamycin treatment of the cells [Figure 1, WT (+Tm)] or when spliced *HAC1^{i}* mRNA was expressed in *Δire1 Δhac1* cells (Figure 1, *Δire1 Δhac1 HAC1^{i}*). The punctate cytoplasmic structures observed for the *HAC1^{u}* and *HAC1^{i}* mRNAs are similar in appearance to those seen for other translated mRNAs

Figure 1



Both forms of *HAC1* mRNA are released from the nucleus. Logarithmically growing *Δhac1* (JC408), wild-type [W303-1B grown in the absence (–Tm) or presence (+Tm) of 1 μg ml^{-1} tunicamycin], *Δire1 Δhac1 HAC1^u* (JC416 + pJC316) and *Δire1 Δhac1 HAC1^i* (JC416 + pRC43) cells were fixed and probed for *HAC1* mRNA by *in situ* hybridization. DNA was visualized by staining with DAPI. Images were produced using an inverted Olympus microscope and deconvolved using DeltaVision software.

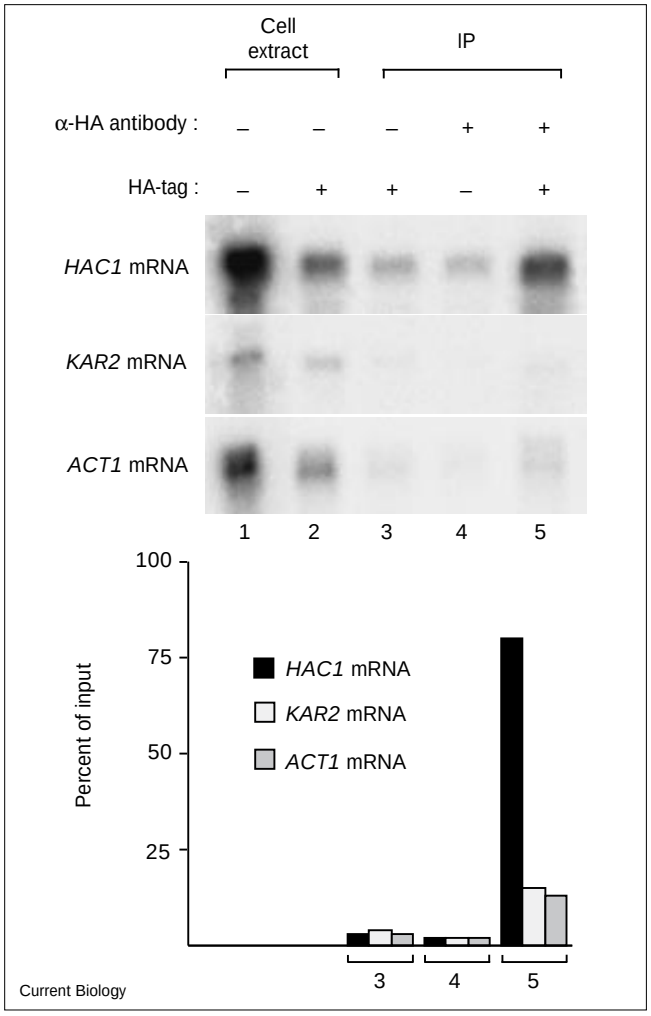
using this technique [18] and presumably represent cytoplasmic clusters of polyribosomes. These data show that *HAC1^u* mRNA, unlike other spliced mRNAs, is efficiently released from the nucleus in its unspliced form.

Previous data indicated that *HAC1^u* mRNA co-migrates with polyribosomes on sucrose density gradients [10]. This, together with the cytoplasmic localization of the mRNA shown in Figure 1, suggests that translation of *HAC1^u* mRNA is properly initiated. However, despite this apparent association of *HAC1^u* mRNA with ribosomes, no Hac1p^u is detectable in cell extracts (see Figure 3b, lane 1), even upon prolonged exposure of the blot. One possible explanation (which we previously favored [10]) is that Hac1p^u is extremely unstable and thus is degraded as soon as it is produced. Alternatively, however, the presence of the intron in *HAC1^u* mRNA could somehow attenuate translational elongation. According to computer algorithms, the intron is predicted to fold into a tight secondary structure that ribosomes may have difficulties traversing (C. Patil and P.W., unpublished observation).

If actively translating ribosomes are indeed stalled by the *HAC1^u* mRNA intron, then each ribosome should have a nascent Hac1 peptide associated with it. It is unlikely that these chains would be detected in whole-cell extracts by western blotting, as they would be low in number and heterogeneous in size. Thus, to ask whether nascent Hac1 peptides are present, we used an indirect approach. Extracts from uninduced cells expressing a version of Hac1p tagged at the amino terminus with a hemagglutinin (HA) epitope [10] and, as a control, untagged Hac1p, were

subjected to immunoprecipitation using a monoclonal anti-HA antibody (Figure 2). If nascent Hac1 peptide chains are present, the antibody should bind and precipitate them together with the ribosomes and the mRNA to which they are tethered. Indeed, northern analysis shows that *HA-HAC1*^{u} mRNA could be selectively precipitated (Figure 2, lane 5). Quantitation of the northern blot shows that 80% of the *HA-HAC1* mRNA present in the cell extract was precipitated, as compared with only 15% and 13% of *KAR2* and *ACT1* mRNAs, respectively (Figure 2, bottom). As expected, only background levels of *HAC1*^{u} mRNA were precipitated from extracts of cells bearing untagged *HAC1* (Figure 2, lane 4). This data suggests that most of the cellular *HAC1*^{u} mRNA is indeed functionally engaged with ribosomes, at least some of which have synthesized the amino terminus of Hac1p^{u}. This is consistent with the co-sedimentation of *HAC1*^{u} mRNA with poly-ribosomes [10].

Figure 2

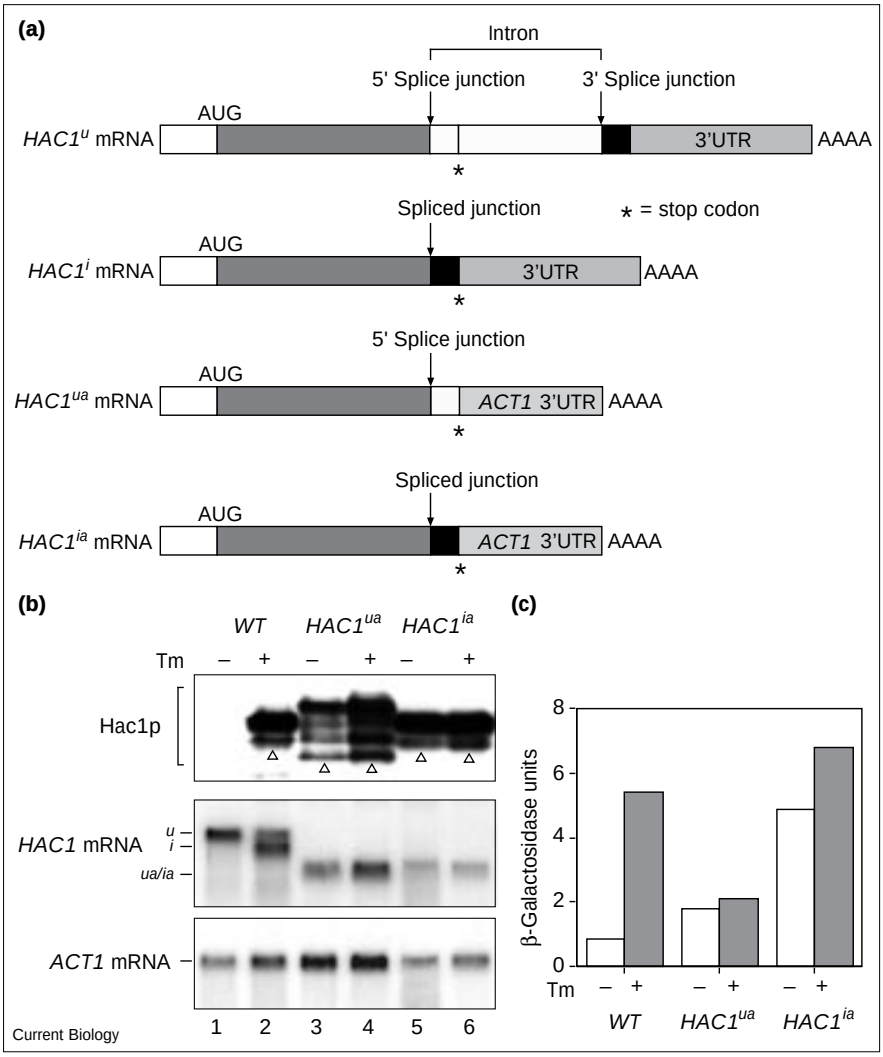


The amino-terminal peptide of Hac1p^{u} is synthesized. Extracts were prepared from wild-type cells (lanes 1,4) and from *Δhac1* cells expressing HA-tagged *HAC1*^{u} mRNA from a plasmid (pJC316) (lanes 2,3,5). Immunoprecipitations were carried out using α-HA antibody as described in Materials and methods. RNA was extracted from both the starting material (cell extract) and the immunoprecipitations (IP) and subjected to northern analysis. Note that the extracts from the HA-tagged and untagged strains were of different concentrations. For each sample, however, the material in lanes 3–5 was derived from exactly twice the amount loaded in lanes 1 and 2, respectively. The data were quantitated using a Biorad PhosphorImager (lower panel). The blot was stripped after probing for *HAC1* mRNA and *KAR2* mRNA and reprobed for *ACT1* mRNA. The small amount of remaining *HAC1* signal was subtracted before quantitating the *ACT1* mRNA signal, as they have similar mobilities.

To determine whether the *HAC1*^{u} mRNA intron blocks translation of full-length Hac1p^{u}, we expressed a chimeric mRNA encoding Hac1p^{u}, in which sequences corresponding to most of the intron and the entire 3′ untranslated region (UTR) were replaced by the unrelated *ACT1* mRNA 3′ UTR (Figure 3a, *HAC1*^{ua} mRNA). Induction of the UPR with tunicamycin has no effect on the mobility of the RNA (Figure 3b, middle panel, lanes 3,4), indicating that the 5′ splice site that remains in *HAC1*^{ua} mRNA is not recognized by the activated Ire1p endonuclease. Surprisingly, we found that cells expressing *HAC1*^{ua} mRNA abundantly produce Hac1p^{u}, which forms a heterogeneous population that can be resolved into four discrete bands on SDS–PAGE (Figure 3b, top panel, lanes 3,4). In contrast, two different species are consistently observed for Hac1p^{i}, either when expressed from a wild-type *HAC1* mRNA in UPR-induced cells (Figure 3b, top panel, lane 2) or when expressed constitutively from a corresponding chimeric mRNA in which the spliced *HAC1*^{i} coding sequence is followed by the *ACT1* 3′ UTR (Figure 3b, top panel, lanes 5, 6). A preliminary characterization of these bands using *in vivo* phosphate labeling and phosphatase treatments suggests that the different forms represent differently phosphorylated species of the primary Hac1p translation products, indicated with arrow heads in Figure 3b (R.E.C. and P.W., unpublished observations). These phosphorylation events occur independently of the status of the UPR as they are observed in equal prominence in untreated and tunicamycin-treated cells (Figure 3b, lanes 3–6). These forms are independent of UPR activation, as they are also observed in *IRE1*-deleted cells (data not shown). Although the Hac1p^{u} is produced in similar abundance as Hac1p^{i}, the UPR is only partially induced in these cells (Figure 3c, compare *HAC1*^{ua} to *HAC1*^{ia}), indicating that the transcriptional activation activity of Hac1p^{u} is constitutively lower, possibly owing to the additional phosphorylation events.

Thus, while the multiplicity of the different protein species leads to an unexpectedly complex expression pattern, it is nevertheless clear from these data that Hac1p^{u}, when produced, is present at significant concentrations in cells. This suggests that Hac1p^{i} and Hac1p^{u} may have similar stabilities, and argues against our previously proposed model that Hac1p^{u} is an exceedingly unstable

Figure 3

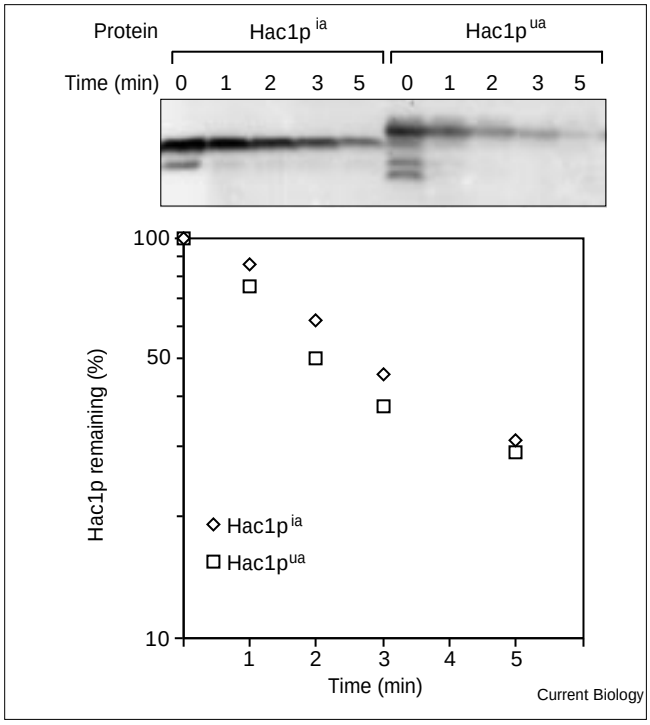


The *HAC1*^{u} mRNA intron is necessary to attenuate translation of Hac1p^{u}. (a) Schematic representation of the different forms of *HAC1* mRNA expressed. *HAC1*^{u} mRNA is the unspliced form of *HAC1* mRNA. *HAC1*^{i} mRNA is produced in *IRE1* cells by splicing of *HAC1*^{u} mRNA at the junctions indicated. *HAC1*^{ua} mRNA has most of the intron and 3′ UTR of *HAC1*^{u} mRNA replaced by the *ACT1* 3′ UTR. It retains the *HAC1*^{u} mRNA 5′ splice junction. *HAC1*^{ia} mRNA has the *HAC1*^{i} mRNA 3′ UTR replaced by the *ACT1* 3′ UTR. * Denotes the first in-frame stop codon in each mRNA. (b) Protein and RNA analysis of strains expressing the mRNAs described in (a). Log-phase *Δhac1* cells (JC408) expressing: HA-tagged *HAC1*^{u} (pJC316), *WT* lanes 1 and 2; HA-tagged *HAC1*^{ua} (pJC834), lanes 3 and 4; or HA-tagged *HAC1*^{ia} (pRC43), lanes 5 and 6; were grown for 1 h in the presence or absence of 1 μg ml^{-1} tunicamycin (Tm) and harvested. Hac1p was detected by western blot analysis using α-HA antibodies. The primary translation product in each lane is indicated with a Δ. The difference in amount of Hac1p^{u} in lane 3 compared to lane 4 is not significant and is due to unequal loading of the gel. RNA was analyzed by northern hybridization using a labeled *HAC1* probe. The different forms of the *HAC1* mRNA have different mobilities as indicated. The blot was then stripped and reprobed with a labeled *ACT1* probe as a loading control. (c) The strains used in (b) were grown for 4 h in the presence or absence of 1 μg ml^{-1} tunicamycin (Tm) before β-galactosidase levels were determined. The values represent the average of two independent experiments that were within 7% of one another.

protein [10]. To address this issue conclusively, we measured the rates of degradation of Hac1p^{i} and Hac1p^{u} directly. To this end, we treated cells expressing either protein constitutively with cycloheximide to prevent further translation and then measured the rate of decrease in protein level over time. Figure 4 shows that major forms of Hac1p^{u} and Hac1p^{i} have indistinguishable degradation rates, with a half-time of about 2 min. These data therefore rule out protein degradation as the primary cause of the marked absence of Hac1p^{u} in normally growing yeast cells, and support the alternative model suggesting that the presence of the intron in *HAC1*^{u} mRNA prevents its translation, although the *HAC1*^{u} mRNA is engaged in polyribosomes.

One obvious possibility is that ribosomes stall on *HAC1*^{u} mRNA because of potential structural barriers at the intron–exon junction. *HAC1*^{ua} mRNA, however, contains the unaltered 5′ splice junction, indicating that additional sequences in the intron are required to yield its inhibitory effects. Such sequences would not be part of the open reading frame *per se*, suggesting that it might be possible to change their position in the mRNA while maintaining the inhibitory effects of the intron. To test this intriguing possibility, we fused the *HAC1* intron (including some short flanking sequences, see Materials and methods) behind the stop codon of the spliced *HAC1*^{i} open reading frame to produce the chimeric *HAC1*$^{ia+int}$ gene (Figure 5a). In cells transformed with a plasmid bearing this synthetic gene, *HAC1*$^{ia+int}$ mRNA is produced but is not recognized as a substrate by activated Ire1p endonuclease and thus is not spliced in the presence of tunicamycin (Figure 5b, middle panel, lanes 3,4). The presence of the intron in the 3′ UTR of this mRNA, however, completely prevents the appearance of Hac1p^{i} (Figure 5b, top panel, compare lanes 1 and 2 with lanes 3 and 4). It was important to determine

Figure 4



Both forms of Hac1p have a similar rate of degradation. *Δhac1* (JC408) cells expressing either HA-tagged Hac1p^{ua} (pJC834) or HA-tagged Hac1p^{ia} (pRC43) were grown to log phase. Cycloheximide was added to a concentration of 1 mg ml^{-1} at time 0, and samples were frozen in liquid nitrogen at the times indicated. Hac1p was detected by western blot analysis using α-HA antibodies. The blots from two separate experiments (values within 5% of one another) were quantitated using NIH imaging software.

whether the chimeric *HAC1^{ia+int}* mRNA is associated with polyribosomes. To this end, we prepared extracts from cells expressing *HAC1^{ia+int}* or the control *HAC1^{ia}* gene and fractionated them by sucrose density gradient centrifugation to display polyribosome profiles (Figure 5c). The ultraviolet absorbance profile demonstrates that intact polyribosomes were present in these extracts. Moreover, both the *HAC1^{ia+int}* and *HAC1^{ia}* mRNAs were recovered near the bottom of the gradient in the same fractions as polyribosomes. This data indicates that translation of both the *HAC1^{ia+int}* and *HAC1^{ia}* mRNAs is initiated. Thus we conclude that the *HAC1* intron prevents translation of the adjacent open reading frame encoding Hac1p^{i}.

This surprising result raised the question of whether specific sequences within the *HAC1* mRNA are required for the translational attenuation afforded by the *HAC1* intron. To test this notion, we transplanted the *HAC1* intron into the 3′ UTR of a completely unrelated gene encoding the green fluorescence protein (GFP). We monitored expression of this synthetic gene and of the parent intron-less control construct by direct fluorescence microscopy. The data in Figure 6a show that GFP fluorescence is detected only in cells that express the intron-less control construct (GFP–INT). GFP fluorescence is absent in cells expressing the *HAC1* intron-containing GFP mRNA (GFP+INT). Both GFP–INT and GFP+INT mRNAs co-migrate with polyribosomes during sucrose density gradient centri-fugation (Figure 6b). Thus, these results show that the presence of the *HAC1* intron is sufficient to attenuate translation of an unrelated mRNA into which it has been inserted.

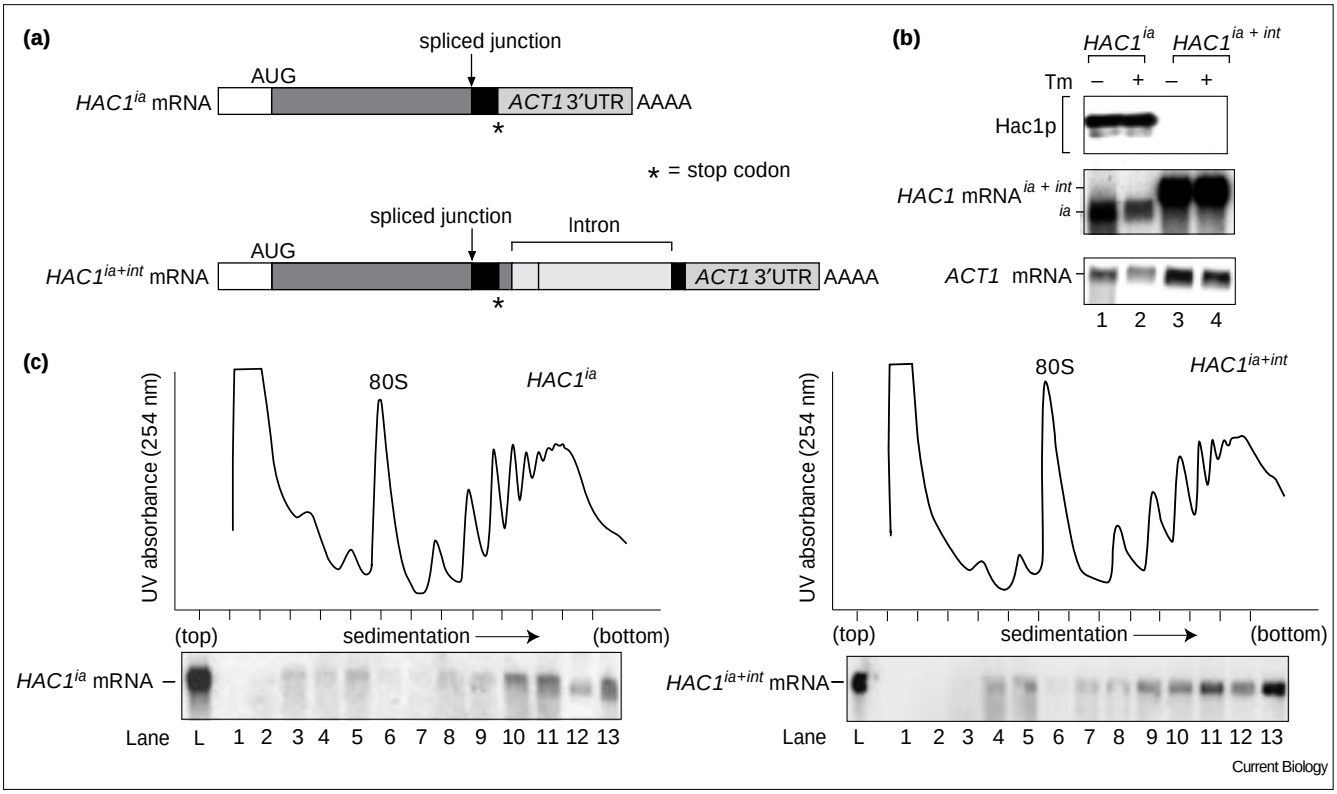
Discussion

Here we describe a new mechanism by which translation of a cytoplasmically localized mRNA can be severely attenuated. We have identified the *HAC1* mRNA intron as an element that is both necessary and sufficient to prevent synthesis of full-length protein from polyribosome-associated mRNAs. Physiologically, this mechanism has an important regulatory role by preventing synthesis of the transcription factor Hac1p in cells in which the UPR is not induced.

Data presented here show that — unlike other spliced mRNAs — the unspliced form of *HAC1* mRNA, *HAC1^{u}* mRNA, is not retained in the nucleus. This is consistent with the previous observation that most of the cellular *HAC1^{u}* mRNA is engaged on polyribosomes [10], as concluded from co-migration of *HAC1^{u}* mRNA with the heavy polyribosome fraction on sucrose density gradients and the association of the mRNA with nascent Hac1 peptides, as suggested from the data shown in Figure 2. It still remains formally possible that *HAC1^{u}* mRNA may be associated with another cytoplasmic complex of the same size as polyribosomes, although such a complex would be huge — equivalent in size to 10–20 ribosomes or 50–100 megadaltons. The results of the immunoprecipitation experiments in Figure 2 would then be explained by synthesis of a small amount of Hac1p^{u} which then associates with this large complex. If this were the case, translational attenuation would be at the level of initiation. We consider this possibility unlikely, however, and favor the notion that *HAC1^{u}* mRNA is initiated into functional translation complexes. This interpretation is also consistent with the fact that the 5′ portions of both unspliced *HAC1^{u}* mRNA and spliced *HAC1^{i}* mRNA are identical, suggesting that these mRNAs should be recognized as equivalent by translation initiation factors.

A most surprising result of our studies is that the intron still exerts its inhibitory effect when transplanted into mRNA regions beyond a stop codon, that is into the 3′ UTR of the spliced *HAC1^{i}* mRNA or into the 3′ UTR of the completely unrelated GFP mRNA. Thus the intron inhibits translation of an adjacent open reading frame, implying that translational attenuation is not as simple as

Figure 5



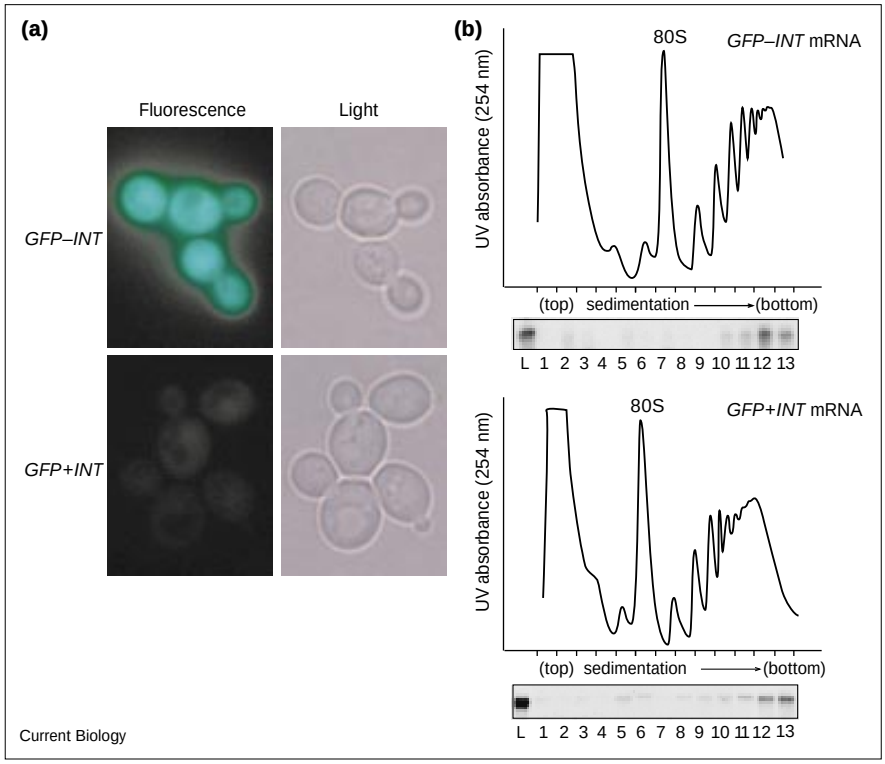
The *HAC1*^{u} mRNA intron is sufficient to prevent translation of Hac1p^{i}. (a) Schematic representation of the different forms of *HAC1* mRNA expressed. *HAC1*^{ia} mRNA is as described in Figure 3a. *HAC1*$^{ia+int}$ mRNA has the *HAC1*^{u} mRNA intron plus predicted stem structures (see Materials and methods) inserted between the *HAC*^{ia} mRNA stop codon and *ACT1* 3′ UTR. * Denotes the first in-frame stop codon in each mRNA. (b) Protein and RNA analysis of strains expressing the mRNAs described in (a). Log-phase *Δhac1* cells (JC408) expressing: HA-tagged *HAC1*^{ia} (pRC43), lanes 1,2; or HA-tagged *HAC1*$^{ia+int}$ (pRC46), lanes 3,4; were grown for 1 h in the presence or absence of 1 μg ml^{-1} tunicamycin (Tm) and harvested. RNA and protein were analyzed exactly as described in Figure 3b. (c) Northern analysis of the distribution of *HAC1*^{ia} and *HAC1*$^{ia+int}$ mRNAs in polyribosomes from extracts of the cells described in (b). Each ultraviolet (UV) profile corresponds to the northern blot directly beneath it. Cell extracts were centrifuged on 7–47% sucrose gradients and fractionated using an ISCO gradient fractionator. A UV absorbance profile was recorded by scanning at 254 nm to display the polyribosome profile. RNA was isolated from individual gradient fractions and subjected to northern analysis. The position of the 80S monosome peak and the direction of sedimentation are indicated. L, load fraction applied to the gradient.

ribosomes stalling at the exon–intron junction or within the portions of the intron that are part of the open reading frame on *HAC1*^{u} mRNA.

We note that the transplanted intron is not sufficient to confer *IRE1*-dependent splicing on the chimeric mRNA. This suggests that additional sequences adjacent to the predicted stems are required for recognition by the splicing machinery. Once the minimal sequence required for splicing has been identified, we expect that this element will provide a useful system for experimentally regulating expression of genes in eukaryotic cells.

The molecular interactions that stall the translating ribosome remain to be determined. In principle, we can envisage two different scenarios (Figure 7). First, according to a 'distance effect' model, elements of the intron may fold into a structure that loops back towards the open reading frame of the mRNA, contacts translating ribosomes and, by an unknown mechanism, impedes their progression. Second, according to a 'direct effect' model, ribosomes may be directly halted when traversing the intron. Not all ribosomes may dissociate at the stop codon after completion of translation of the open reading frame and some may therefore continue to traverse the 3′ UTR. Structural features of the intron might inhibit progression of ribosomes that arrive there, causing following ribosomes to run into these stalled ribosomes. Eventually, the region of the mRNA preceding the block becomes filled with stationary ribosomes. Once the stop codon is covered, no more protein would be produced. Using techniques previously developed in this laboratory [19], we can now map the position of stalled ribosomes on the mRNA and thereby address these different possibilities directly.

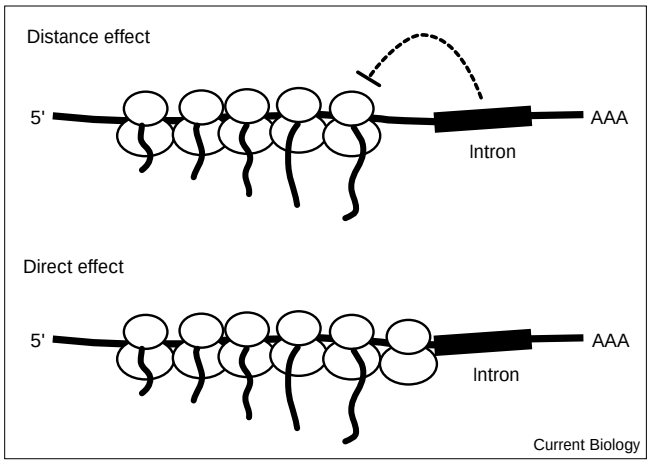
Figure 6



The *HAC1*^{u} mRNA intron is sufficient to prevent translation of the unrelated GFP message. (a) Wild-type yeast strains (W303-1A) expressing green fluorescent protein either minus (GFP–INT, pRC47) or plus the *HAC1* mRNA intron inserted into the 3′ UTR (GFP+INT, pRC48) were grown to log phase, then visualized directly using fluorescence and bright field microscopy. (b) Northern analysis of the distribution of *GFP–INT* and *GFP+INT* mRNAs in polyribosomes from extracts of cells described in (a). Polyribosomes were analyzed as described in Figure 5c.

Whatever the mechanism of translational attenuation, it must allow for a small degree of readthrough. The low amount of protein produced would be below our levels of detection, yet have sufficient activity to allow us to detect upregulation of a highly sensitive UPRE–reporter gene construct. This would explain two previously published observations [10]. First, *HAC1* was identified as a high-copy activator of the UPR in an *Δire1* strain. In this background the *HAC1* mRNA remains unspliced, so the low level of UPR activity detected must have come from leaky translation of the overexpressed mRNA. Second, a sensitive UPRE–reporter is activated in a *Δire1 Δubc4 Δubc5* strain [10]. In this strain background, we detected a low amount of Hac1p^{u} upon overexpression. Here we show that both Hac1p^{i} and Hac1p^{u} are similarly unstable proteins with a cellular half-time of only 2 min, suggesting that the very low levels of Hac1p^{u} and the Hac1p activity in the *Δire1 Δubc4 Δubc5* strain are a result of stabilization of the small amount of protein arising from translational readthrough.

Figure 7



Models for translation attenuation by the *HAC1*^{u} mRNA intron.

Most other unspliced pre-mRNAs are retained in the nucleus, where they are rapidly degraded, raising the question of why cells attenuate production of Hac1p^{u} at the level of translation of its mRNA. It is possible that translational attenuation supplies cells constitutively with a low level of Hac1p, possibly to sustain a beneficial low-level activation of UPRE-dependent transcription. When required, the activation of the *HAC1* splicing machinery enables cells to produce a large amount of active transcription factor without needing to alter the level of *HAC1* transcription.

It is also possible that cells have some mechanism for increasing the amount of Hac1p^{u} translated, independently of activation of Ire1p. As previously discussed [20], this might provide a quick alternative pathway for induction of

the UPR, in addition to the *HAC1* mRNA splicing reaction, which produces a potentially slower, but sustained, response. Alternatively, Hac1p might have an as yet unanticipated role(s) in the cell. In particular, we have shown here that Hac1p^u, when produced, is modified and is active in inducing the UPR in a different way from Hac1p^i. As shown for other members of the bZIP family of transcription factors (for example see [21]), Hac1p has the potential for interacting with more than one partner and thus could differentially regulate the promoters of different genes. In support of this, we have shown, for example, that the induction of UAS_{INO}-controlled genes occurs by a different mechanism and has different kinetic consequences from the induction of UPRE-controlled genes [20]. Furthermore, *HAC1* was originally identified as a gene that is upregulated at meiosis, producing an elevated level of unspliced *HAC1^u* mRNA [22]. This may suggest a still unknown role for Hac1p^u in meiosis and/or sporulation. There are thus many possible reasons why cells may attenuate the translation of *HAC1^u* mRNA and these remain to be addressed. Our studies to date, however, have revealed an unprecedented mechanism of translational regulation and thereby expanded the cellular repertoire of mechanisms for post-transcriptional gene regulation.

Materials and methods

Media and general methods

All were as described previously [10].

RNA in situ *hybridization*

This method is modified from the recently published development of this technique [18]. Yeast cultures were grown to OD_{600} 0.5–1.0, then fixed in 4% formaldehyde for 1 h. After washing twice in water, cells were spheroplasted in 0.1 M KPO_4, 0.12 M sorbitol, 30 mM 2-mercaptoethanol, 40 μg ml^{-1} zymolyase 100T, 40 U ml^{-1} RNasin for 15 min at 37°C. The spheroplasts were washed twice in 0.1 M KPO_4/0.12 M sorbitol, then placed on polylysine-coated glass slides. The slides were then washed twice in 50% formamide, 5x SSC. Prehybridization was carried out for 1 h at 60°C, using 50% formamide, 5x SSC, 1 mg ml^{-1} yeast tRNA, 100 μg ml^{-1} heparin, 1x Denhardt's solution, 0.1% Tween 20, 0.1% Triton X-100 and 5 mM EDTA. Digoxigenin-labeled *HAC1* probe was added to 0.5 μg ml^{-1}, and the slides incubated at 60°C overnight in a humidified chamber. Digoxigenin-labeled antisense-*HAC1* probe was generated using an Ambion Megascript Kit, according to the manufacturers' instructions. After incubation with probe, the slides were washed five times in 0.2x SSC, and blocked in PBS, 0.1% Triton X-100, 5% fetal bovine serum. Alkaline-phosphatase conjugated anti-digoxigenin antibody (Boehringer Mannheim) was added to the slides in blocking buffer at 1:5000, and incubated for 1 h at 37°C. Slides were washed five times in 0.1 M Tris pH 7.5, 0.15 M NaCl, 0.05% Tween-20, and twice in 0.1 M Tris pH 8.0, 0.1 M NaCl, 0.01 M $MgCl_2$. A fluorescent precipitate specific for alkaline phosphatase activity was generated using the HNPP fluorescent detection kit (Boehringer Mannheim). Reactions were carried out for 15 min, then the slides were washed twice in water. Cells were stained with 1 mg ml^{-1} DAPI in PBS for 10 min, washed, then mounted in 0.01 M Tris pH 8.4, 90% glycerol, 1 mg ml^{-1} *p*-phenylenediamine.

Northern analysis

Total cellular RNA was isolated and analyzed using previously described techniques [10]. All probes were made with [α-^{32}P]dCTP using the Ready-To-Go DNA-labeling kit (Pharmacia). The *HAC1* and *ACT1* fragments used for labeling were generated by PCR of either the complete *HAC1* open reading frame, or a 1 kb fragment of *ACT1*.

Western blot analysis

Protein was extracted from log-phase yeast as previously described [10]. Western blots were probed using a commercially available anti-HA ascities fluid (Babco) at 1:5000 in PBS 2% milk 0.5% Tween 20. Peroxidase-conjugated secondary antibody was purchased from Amersham and used at 1:5000. Signal was detected using enhanced chemiluminescence (ECL, Amersham) according to the instructions of the manufacturer.

Microscopy

For the RNA *in situ* analysis 50 optical sections (DAPI: 5 sec exposure; RNA: 1 sec exposure) 0.1 μm thick were collected using an Inverted Olympus microscope and then subjected to wide-field deconvolution 3D microscopy with a 100x 1.4 numerical aperture lens [23]. Data was analyzed using DeltaVision software (Applied Precision, Inc.). GFP was visualized using a Leica DMBL microscope with a 100x PL Fluotar objective. Images were acquired with an Optronics DEI-750 CCD camera using Scion Image software [24].

Plasmid construction

pJC834 was constructed by ligating a 400 bp *Sal*I (blunted), *Bam*HI fragment of the *ACT1* terminator from pTS210 [25] into the *Xba*I (blunted) *Bam*HI sites of pRS313. The *HAC1* promotor and open reading frame (up to the first predicted stop codon) were generated by PCR from pJC316 [10], a *HA–HAC1*-bearing plasmid, and ligated into the *Mlu*I, *Sma*I sites of the *ACT1* terminator-containing pRS313. The PCR product was sequenced. pRC43 was generated by replacing the pJC834 *HAC1^u* open reading frame (*Spe*I, *Sma*I) with the *HAC1^i* open reading frame (*Spe*I, *Sma*I) described previously [10]. pRC46 was generated by inserting a PCR fragment of the *HAC1* intron plus stem (nucleotides 625–924) behind the *HAC1^i* stop codon. The GFP open reading frame was generated by PCR and used to replace the *Spe*I, *Sma*I fragment in either pRC46 or pRC43, thus generating plasmids pRC47 and pRC48 encoding GFP in the presence and absence of the *HAC1* intron in the 3′ UTR, respectively.

Polyribosome analysis

Cell extracts and gradients were prepared as described previously [26]. Fractions were extracted three times with phenol/chloroform and RNA precipitated with isopropanol before northern analysis.

Native polyribosome immunoprecipitation

Cell extracts were prepared as described previously [26]. Cell extract (50 μl from 50 ml starting yeast culture, OD_{600} 0.8) was layered on top of a sucrose cushion [0.5 M sucrose, 25 mM Tris pH 7.0, 12 mM $Mg(OAc)_2$, 150 mM KOAc] and spun in a TLA100 Beckman ultracentrifuge at 60,000 r.p.m. for 60 min to pellet ribosomes. The ribosomal pellet was resuspended in 100 μl buffer A (10% glycerol, 0.2 M sorbitol, 25 mM Tris pH 7.0, 12 mM $Mg(OAc)_2$, 150 mM KOAc, 1000 U ml^{-1} RNasin) and precleared with 25 μl protein A–Sepharose. Anti-HA ascites fluid (2 μl) (Babco) was added, and incubated for 1 h on ice, followed by precipitation with 25 μl protein A–Sepharose for 15 min. The beads were washed twice in buffer A, then RNA extracted as described [10].

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