The unfolded protein response: an intracellular signalling pathway with many surprising features

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The unfolded protein response (UPR) is an intracellular signalling pathway – originating in the endoplasmic reticulum (ER) and leading to the cell nucleus – that controls transcription of genes encoding ER-resident proteins. Recent developments in this field show that this pathway utilizes unique regulatory mechanisms, including translational attenuation and a regulated mRNA splicing step catalysed by a bifunctional transmembrane kinase/endoribonuclease and tRNA ligase. This review describes the characterization of the UPR signalling pathway, focusing on the novel regulatory mechanisms that it has revealed.

The unfolded protein response (UPR) is induced following the accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER) and results in the upregulation of genes encoding ER-resident enzymes involved in protein folding[1–3]. Thus, the cell is able to increase the folding capacity of the ER according to need. The target genes of the UPR share a common upstream activating sequence in their promoters, the unfolded protein response element (UPRE), that directs their transcription upon induction of the pathway[4]. The existence of this common element suggested early on that the pathway is controlled by a common transcription factor whose activity is regulated by the conditions in the ER lumen.

Although the UPR exists in all eukaryotic cells, much of the progress in understanding this signalling pathway comes from recent studies in the yeast *Saccharomyces cerevisiae*. Starting with genetic approaches to dissect the molecular mechanism of the UPR pathway, *IRE1*[5,6] was identified as the first gene known to be required for a functional UPR. *IRE1* encodes a transmembrane protein similar in structure to mammalian growth-factor receptor kinases. Ire1p resides in the ER or inner nuclear membrane (with which the ER is continuous), with its N-terminal half facing the ER lumen, and its C-terminal half – which contains the kinase domain – facing the nucleus or cytoplasm. In this orientation, the N-terminal half is thought to serve as a sensor domain that detects the accumulation of unfolded proteins in the ER lumen, whereas its kinase domain would function in the cytoplasm or nucleus to activate downstream events in the pathway. Thus, the structure and topology of Ire1p make it ideally suited to function as the proximal sensor of the conditions in the ER lumen and to be the signal-transduction device that transmits this information across the membrane.

Upon induction of the UPR, Ire1p oligomerizes in the plane of the membrane and becomes transautophosphorylated by neighbouring Ire1p molecules[7]. How does Ire1p activation lead to induction of downstream events in the signalling pathway? By analogy to signal-transduction pathways that operate downstream of other serine/threonine kinase receptors, the expectation was that Ire1p would activate a kinase cascade leading to phosphorylation and activation of a downstream transcription factor. Recent work, however, has revealed a remarkably different signal-transduction pathway downstream of Ire1p. Here, we describe the series of unexpected results that allowed the elucidation of the unique signalling events that control the UPR.

Nonconventional splicing of *HAC1* mRNA

The discovery of the second component of the UPR, the bZIP transcription factor Hac1p, was a key step in understanding the UPR signalling cascade[8–10]. Again, genetic screens were the key to identifying *HAC1*. Hac1p was shown to bind specifically to the UPRE of target genes, inducing their transcription. A most surprising observation, however, was that Hac1p could only be detected in UPR-induced cells, suggesting that transcription is controlled by a change in Hac1p concentration, rather than by a change in its conformation as might have been expected as a possible consequence of the activation of a kinase cascade.

Perhaps the most surprising result was that the appearance of Hac1p results from regulated splicing of its mRNA (Fig. 1)[8,11]. Upon induction of the UPR, a 252-nucleotide intron is removed that is present towards the 3′-end of the open reading frame (ORF) of *HAC1*^{u} mRNA (u for uninduced). Splicing results in the production of *HAC1*^{i} mRNA (i for UPR-induced) and the subsequent production of Hac1p^{i}. Constitutive expression of *HAC1*^{i} mRNA results in the unregulated expression of Hac1p^{i} and fully induced levels of transcription of all known targets of the pathway. Thus, splicing of *HAC1* mRNA is sufficient to trigger full induction of the UPR.

A further unexpected result was the absence of consensus sequences at the splice junctions of *HAC1* mRNA that are common to all pre-mRNAs processed by the spliceosome. This provided the first hint that splicing of this mRNA utilizes a nonconventional splicing pathway[12]. Mutational analysis demonstrating that no functional spliceosome was required for processing *HAC1* mRNA confirmed this conjecture and raised the question of the identity of the machinery responsible for this uniquely regulated splicing

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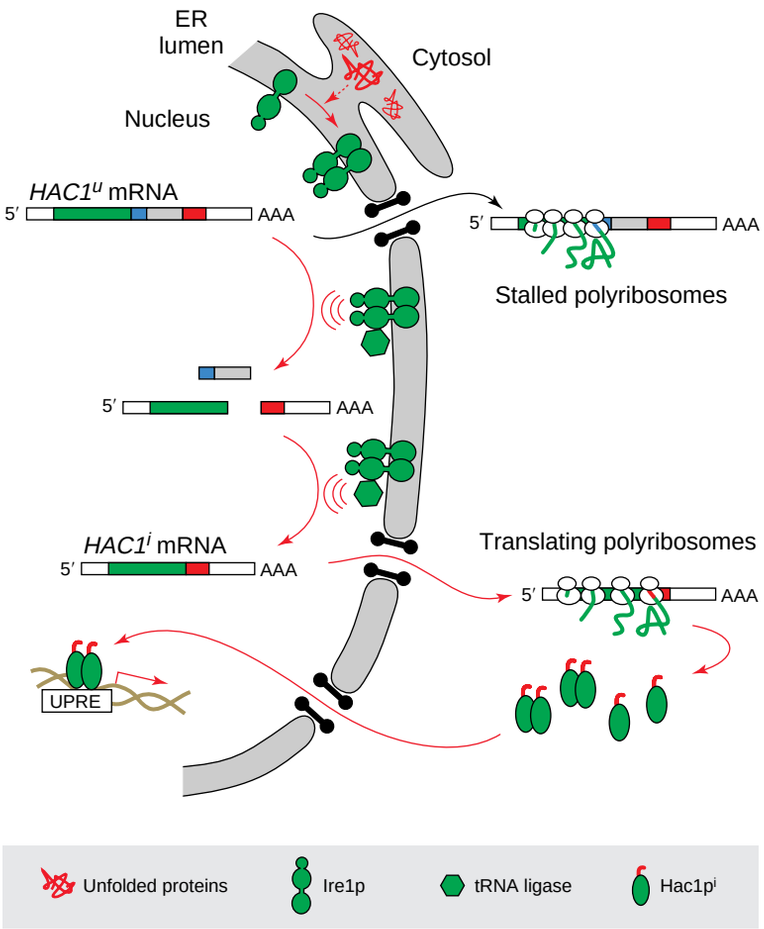

FIGURE 1

Model of the unfolded protein response (UPR) signalling pathway. Accumulation of unfolded proteins in the endoplasmic reticulum (ER) lumen triggers activation of Ire1p, which cleaves *HAC1*^{u} mRNA at both splice junctions. tRNA ligase then joins the two exons to produce the spliced form of the message, *HAC1*^{i} mRNA. Both forms of *HAC1* mRNA exit the nucleus and associate with polyribosomes. However, only the spliced form gives rise to protein, Hac1p^{i}, which then enters the nucleus and upregulates genes containing the unfolded protein response element (UPRE). Whether Ire1p is indeed a resident protein in the inner nuclear membrane as depicted in this model has not been determined experimentally and hence remains speculation. Alternatively, Ire1p might be localized solely in the ER membrane, where it would process *HAC1* mRNA immediately after its transport to the cytoplasm.

event[13]. Genetically, *IRE1* must act upstream of *HAC1* in the UPR pathway because cells deleted for *IRE1* are unable to induce splicing of *HAC1* mRNA. However, the link between Ire1p and splicing of *HAC1* mRNA remained elusive until a third component of the UPR pathway was identified.

In a screen designed to isolate mutants impaired in the UPR, a mutant allele of tRNA ligase, *rlg1-100*, was isolated[12]. tRNA ligase is a well-characterized protein that is involved in pre-tRNA splicing; specifically, it catalyses ligation of tRNA halves generated by tRNA endonuclease to generate properly spliced tRNAs. As such, it is an essential gene; the isolated mutant is therefore a pathway-specific allele that selectively inactivates the UPR without affecting pre-tRNA splicing. As tRNA ligase is an RNA-processing enzyme capable of ligating tRNA halves, it seemed possible that its function in the UPR might be to ligate the *HAC1* mRNA halves. The fate of *HAC1* mRNA observed in the *rlg1-100* mutant strongly suggested that this is indeed the case. Induction of the UPR in a *rlg1-100* mutant strain results in the disappearance of *HAC1*^{u} mRNA without a concomitant appearance of the spliced *HAC1*^{i} mRNA. This suggests that the first step of the splicing reaction, the cleavage of the intron-containing *HAC1*^{u} mRNA, takes place in the mutant strain but that ligation of the mRNA halves is blocked. The *HAC1* mRNA fragments are then degraded rapidly. This disappearance of *HAC1*^{u} mRNA is specific; it only occurs upon induction of the UPR by conditions that cause accumulation of unfolded proteins in the ER and strictly require Ire1p. These data are consistent with the model in which accumulation of unfolded proteins in the ER lumen activates Ire1p, which is required for induction of cleavage of *HAC1* mRNA. tRNA ligase is then responsible for ligation of *HAC1* mRNA halves to produce the spliced *HAC1*^{i} mRNA product.

The identification of tRNA ligase as a component required for splicing of *HAC1* mRNA was another surprising step in the unravelling of the molecular mechanism of the UPR. Its involvement, and the lack of a role for the spliceosome in processing of *HAC1* mRNA, provided the first solid support for the notion that processing of *HAC1* mRNA utilizes an unusual machinery. Further evidence for an unconventional mechanism of splicing came from studies demonstrating that cleavage at either splice junction can occur independently. This is in contrast to spliceosome-mediated splicing, which occurs as a series of two transesterification reactions requiring that cleavage of the junctions be ordered. One important component remained to be identified – the endonuclease responsible for cleavage of *HAC1* mRNA.

Ire1p kinase is also an endoribonuclease

Based on the domain structure and sequence similarity between mammalian RNase L and Ire1p, it had been proposed that Ire1p had both kinase and nuclease activities[14]. RNase L, or 2′→5′-A-dependent RNase, is a soluble nonspecific ribonuclease that is activated by 5′-phosphorylated, 2′→5′-linked oligoadenylates (2–5A) that are produced upon treatment of mammalian cells with interferon[15]. Binding of 2–5A to the N-terminal domain of the RNase L molecule allows homodimerization and activation of its nuclease domain[16,17]. It is thought that binding of 2–5A causes a conformational change in RNase L that releases the inhibitory effect of the N-terminus on the catalytic domain[18]. RNase L has a number of features that are intriguingly similar to characteristics of Ire1p (Fig. 2). Like Ire1p, RNase L contains a kinase domain in its C-terminal half, followed by a C-terminal extension that shows sequence similarity to the C-terminal 133-amino-acid tail in Ire1p. The C-terminal domain of RNase L might be the nuclease domain because deletion of this domain abolishes its nuclease activity *in vitro*[18]. Deletion of the C-terminal tail of Ire1p also impairs transmission of the unfolded protein signal without affecting kinase activity[7]. Moreover, as is the case for Ire1p, the N-terminal half of RNase L is involved in sensing the signal for activation. Ligand binding to either Ire1p or RNase L is

likely to lead to its oligomerization and activation. These striking similarities in domain structure, amino acid sequence and mechanism of activation prompted a direct test of whether Ire1p might indeed be the nuclease responsible for the initial cleavage of *HAC1* mRNA.

It had previously been shown that a fusion protein consisting of glutathione *S*-transferase and the C-terminal half of Ire1p, which contains both the kinase (K) and C-terminal tail (T) domains, is an active kinase capable of phosphorylating itself *in vitro*[19]. When this fusion protein was incubated with *HAC1*^{u} RNA transcribed *in vitro*, discrete cleavage products were produced that corresponded precisely to the 5′ exon, the intron and the 3′ exon[20]. Thus, in this *in vitro* system, Ire1p is sufficient for the specific cleavage of *HAC1*^{u} mRNA at both splice junctions. Two separate reports placed the position of cleavage at both splice junctions one nucleotide apart[13,20]. While this discrepancy remains to be resolved, it does not detract from the main conclusion that Ire1p is the endoribonuclease that initiates the spliceosome-independent *HAC1*^{u} mRNA splicing event. Moreover, the entire splicing reaction could be reconstituted by adding purified tRNA ligase to the *in vitro* cleavage reaction. Thus, in contrast to spliceosome-mediated pre-mRNA splicing, which utilizes upwards of 100 different proteins and small nuclear RNA molecules, processing of *HAC1* mRNA is surprisingly simple. It requires the function of only two enzymes: the bifunctional kinase/endonuclease Ire1p and tRNA ligase (Fig. 1).

In yeast, tRNA ligase localizes to the nucleus, where it resides in proximity to nuclear pores and interacts with tRNA endonuclease to process pre-tRNAs[21]. Because splicing requires coupling of the cleavage and ligation reaction, we consider it likely that at least a portion of Ire1p is localized to the inner nuclear membrane to collaborate with tRNA ligase in the splicing of *HAC1* mRNA. Furthermore, it remains an open question whether Ire1p is a resident protein of the inner nuclear membrane as might be expected for an enzyme involved in mRNA processing. If so, further questions arise as to the mechanism by which it is localized; in principle, Ire1p could either be a constitutive inner nuclear membrane protein or become localized there upon activation of the UPR. Thus, the identification of the splicing machinery suggests that the route of transmission taken by the unfolded protein signal might lead directly from the ER lumen across the inner nuclear membrane into the nucleus (Fig. 1). Until the enzymatically active portion of the Ire1p molecules has been experimentally localized, however, this conjecture strictly remains speculation.

A major question that remains unanswered concerns the mechanism by which Ire1p is activated. How the presence of misfolded proteins is sensed by the N-terminus of Ire1p is not understood. One attractive model is that chaperones bind to the ER-lumenal portion of Ire1p, thereby preventing its oligomerization. Accumulation of unfolded proteins might then titrate away these inhibitory chaperones, unmasking sites that allow Ire1p molecules to bind to each other and self-activate. Furthermore, how the oligomerization and kinase activity of Ire1p regulate its nuclease activity is not known. It is possible that the kinase domain directly phosphorylates the nuclease domain thus activating it or that it simply helps stabilize a dimeric conformation that is required for nuclease activity.

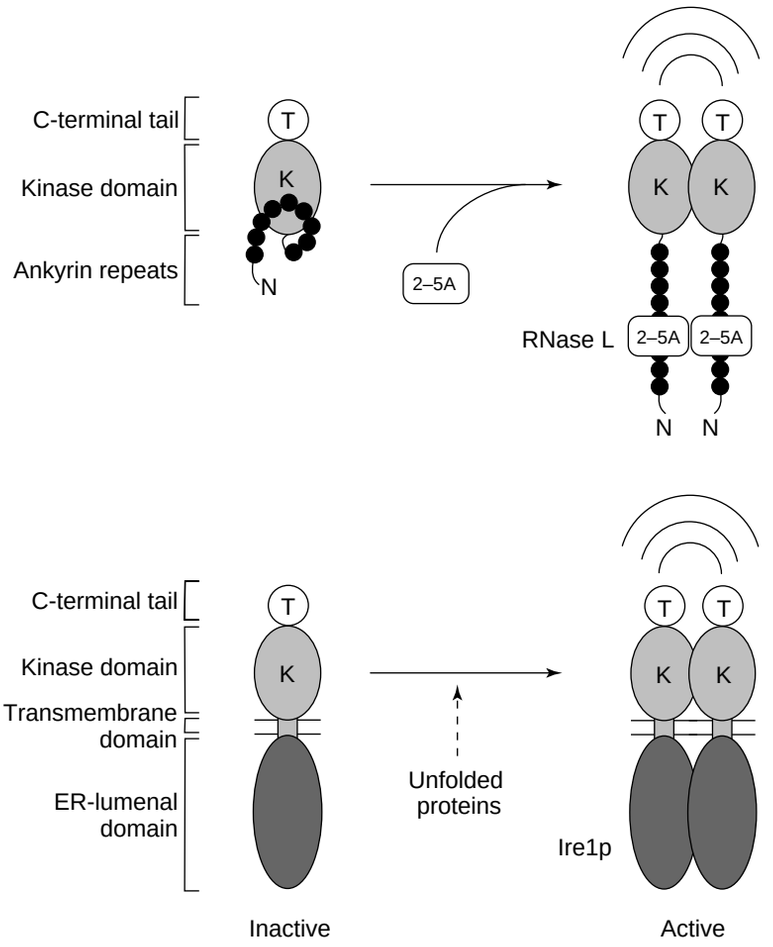

FIGURE 2

Similarities between Ire1p and RNase L. Both RNase L, a soluble enzyme, and Ire1p, a transmembrane protein, contain a kinase domain followed by a C-terminal tail domain that is required for ribonuclease activity. The kinase (K) and the C-terminal tail (T) domains show amino acid sequence similarity. The N-terminal regions of both molecules function to sense the upstream signal in their respective pathways but show no homology. Binding of 5′-phosphorylated, 2′→5′-linked oligoadenylates (2–5A) to two of the N-terminal ankyrin repeats of RNase L allows oligomerization and activation of the nuclease domain. Similarly, accumulation of unfolded proteins leads to oligomerization and activation of the kinase and nuclease activities of Ire1p. How the ER-lumenal portion of Ire1p senses the presence of unfolded proteins is not known.

Translational attenuation of unspliced *HAC1* mRNA

As discussed so far, the splicing of *HAC1* mRNA is a key regulatory step in the UPR, leading directly to the production of the transcription factor Hac1p. This then prompts the question of why the unspliced form of *HAC1* RNA does not produce detectable levels of Hac1p transcription factor.

This question was first addressed in the initial paper that described the role of *HAC1* mRNA splicing in the UPR[8]. It was noted that, unlike pre-mRNAs spliced by the spliceosome, the unspliced form of *HAC1* mRNA is stable in cells. Furthermore, both the spliced and unspliced forms of *HAC1* mRNA comigrate

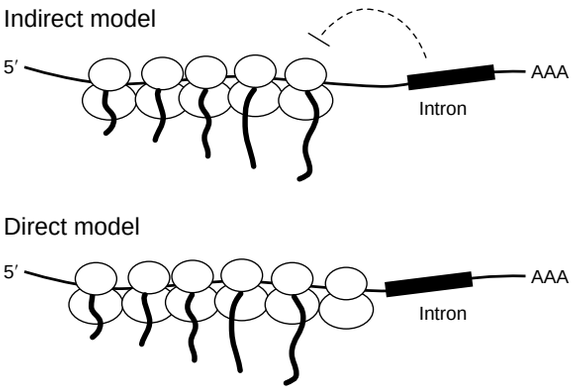

FIGURE 3

Model for translation attenuation by the *HAC1*^{u} mRNA intron. The 'indirect' model (a) predicts that ribosomes and their associated nascent chains stall upstream of the intron. Consequently, no full-length protein is produced. According to the 'direct' model (b), the first ribosome stalls upon directly encountering the intron, presumably at a region of tight secondary structure. Further ribosomes and associated nascent chains stack behind it. If the intron is localized in the 3′-untranslated region (3′-UTR) of a mRNA, ribosomes must continue moving down the 3′-UTR after protein synthesis has terminated.

with heavy polyribosomes in sucrose gradients. These data suggested that both mRNAs are actively translated. Only the spliced form of *HAC1* mRNA, however, produces detectable protein. One model to explain this observation depended on differences in the predicted proteins produced from the two forms of *HAC1* mRNA. The unspliced *HAC1*^{u} mRNA predicts a protein of 230 amino acids (Hac1p^{u}), with the 10 C-terminal amino acids encoded by the first part of the intron (Fig. 1). Splicing removes the intron and replaces this 10-amino-acid tail with a different 18-amino-acid tail encoded by the second exon, producing a 238-amino-acid protein, Hac1p^{i}. Both forms of the protein contain a PEST region, rich in the amino acids Pro, Glu, Asp, Ser and Thr, which has been shown to target proteins for ubiquitin-dependent degradation. Thus, in principle, the different C-termini could result in different stabilities of Hac1p^{u} and Hac1p^{i}. This model predicted that Hac1p^{u} is an extremely unstable protein – so unstable that it could not be detected.

More recently, however, it has been shown that the absence of Hac1p^{u} in uninduced cells is not a result of its extreme instability but rather that it is never produced[11,22]. A key experiment was to remove intron sequences 3′ to the predicted Hac1p^{u} stop codon, replacing them by unrelated 3′-untranslated sequences. These modified mRNAs encode exactly the same protein as the unspliced *HAC1* mRNA. Removal of the intron results in appearance of Hac1p^{u} *in vivo* and a partial induction of the UPR. Hac1p^{u} thus produced is present in similar amounts to Hac1p^{i}, and both proteins have indistinguishable, although very short (−2 min), half-lives. Thus, although Hac1p^{u} is an unstable protein, the activity of the UPR is not controlled by differences in the stability of Hac1p^{u} and Hac1p^{i}.

As removal of intron sequences results in appearance of Hac1p^{u}, it was clear that the intron is necessary for inhibition of protein production from *HAC1*^{u} mRNA. To determine whether the intron by itself is also sufficient for this inhibition, the *HAC1* mRNA intron was transplanted to the 3′-untranslated region (3′-UTR) of heterologous mRNAs. In all cases, presence of the intron led to disappearance of the protein encoded by the upstream ORF, demonstrating that the *HAC1* mRNA intron is both necessary and sufficient to inhibit production of Hac1p^{u}.

Previous data showed that unspliced *HAC1*^{u} mRNA comigrates with polyribosomes during sucrose density-gradient centrifugation[8]. However, it remained formally possible that this association was the result of fortuitous comigration of some other high-molecular-weight complex formed in either the nucleus or the cytoplasm and containing *HAC1*^{u} mRNA, rather than actually representing a functional engagement of the mRNA with translating ribosomes. *In situ* hybridization[22] showed directly that the vast majority of *HAC1*^{u} mRNA in the cell is present in the cytoplasm. In view of the probable nuclear localization of the splicing reaction, this result suggests that the unspliced message is not 'waiting' in the nucleus as a pool ready for splicing. Moreover, immunoprecipitation experiments showed unambiguously that *HAC1*^{u} mRNA is engaged in functional polyribosomes: antibodies recognizing an epitope tag engineered into the N-terminal end of Hac1p selectively precipitated *HAC1*^{u} mRNA. Taken together, these experiments strongly suggest that *HAC1*^{u} mRNA is recruited into functional polyribosomes in the cytosol, but that ribosomes stall during elongation and hence do not proceed to produce completed Hac1p^{u}.

How might the *HAC1* mRNA intron prevent ribosomes from synthesizing full-length Hac1p^{u}? Secondary-structure predictions show that the intron is likely to form stable stem–loop structures, which could be inhibitory for the progression of ribosomes. Exactly how this might be accomplished mechanistically is not clear, but we envision two models that differ in the position along the *HAC1* mRNA where the ribosomes are stalled (Fig. 3). In the first, 'indirect effect', model, some feature of the intron would act at a distance to stop ribosomes in their tracks before they actually reach the intron itself. The second, 'direct effect', model suggests that the intron structure directly prevents ribosomes from moving through it. As the intron can exert its inhibitory effects even when transplanted into the 3′-UTR region (i.e. behind the stop codon), this model would predict that at least some ribosomes continue moving down the mRNA even after the protein product has been released during termination. In both models, stalling of the first ribosome would cause ribosomes behind it to back up, leading to an mRNA loaded up with functionally engaged yet stationary ribosomes.

Concluding remarks

Future work will determine which, if either, of the above models for translational attenuation of *HAC1*^{u} mRNA is correct. What is clear, however, is that regulation of Hac1p production is unconventional, relying on the two novel mechanisms of regulated splicing and translational attenuation. Both the splicing machinery and the ribosome recognize elements within

the intron of *HAC1* mRNA, and it remains to be determined to what extent its sequence, structure or both are important. Secondary-structure modelling predicts similar stem–loop structures at both splice junctions in *HAC1* mRNA (Ref. 20), and Ire1p cleaves in the same position within the loop at both junctions. These stem–loop structures might represent the minimal elements required for recognition of *HAC1*^{u} mRNA by the Ire1p nuclease.

Why cells choose to regulate production of Hac1p so tightly and by mechanisms that are so different from other signal-transduction events is not clear. It is possible that translational attenuation allows cells to produce small amounts of Hac1p^{u} constitutively as a result of some intrinsic leakiness of the translational block. It is also possible that other, yet to be discovered, regulatory mechanism(s) exist that allow cells to produce more Hac1p^{u} by relief of translational attenuation. As Hac1p is already made as partially synthesized molecules on the stalled ribosomes, this would allow rapid production of Hac1p. By contrast, although splicing of *HAC1* mRNA might be slower, it builds an element of memory into the signalling pathway: once splicing is initiated, production of high levels of Hac1p^{i} continues until the spliced *HAC1*^{i} mRNA is degraded. In this way, production of Hac1p^{i} and Hac1p^{u} might correspond to two kinetically distinct routes of the UPR.

But things could be even more complicated. A putative mechanism that relieves the translational block might actually respond to a signal(s) other than that of unfolded proteins in the ER. The transcriptional activity of Hac1p^{u} might also differ, considering that Hac1p^{u} is structurally distinct from Hac1p^{i} – both with respect to the sequence of its C-terminal tail and its posttranslational modifications. In this way, the complex regulation of expression from *HAC1* mRNA could provide a mechanism for coordinating more than one signalling pathway and cellular response. Given the history of unexpected revelations in this field, it seems best to keep an open mind.

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