

HacA-Dependent Transcriptional Switch Releases *hacA* mRNA from a Translational Block upon Endoplasmic Reticulum Stress[∇]

Harm J. Mulder* and Igor Nikolaev

Genencor, Danisco, Archimedesweg 30, 2333 CN Leiden, The Netherlands

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Activation of the unfolded protein response (UPR) in eukaryotes involves the splicing of an unconventional intron from the mRNA encoding the transcriptional activator of the pathway. In *Saccharomyces cerevisiae* a 252-nucleotide (nt) unconventional intron is spliced out of the transcript of *HAC1*, changing the 3' end of the *HAC1* open reading frame and relieving the transcript from a translational block in a single step. The translational block is caused by the base pairing of part of the unconventional intron with the 5'-untranslated region (5'UTR). In *Aspergillus niger* and other aspergilli, the unconventional intron in *hacA* mRNA is only 20 nt long. Since this intron is part of a stable stem-loop structure, base pairing with the 5'UTR, in contrast to the case with yeast *HAC1*, is not possible. However, analysis of the *hacA* mRNA revealed a GC-rich inverted repeat (18 base pairings). Upon the activation of the UPR, the 5'UTR of *hacA* mRNA is truncated by 230 nt, removing the left part of this inverted repeat. This implies a similar release of a translational block as in the case of *S. cerevisiae HAC1* but in two steps. The mechanism behind the 5' truncation, which does not take place in either yeast *HAC1* or mammalian *xbp1* mRNA, has been hitherto unknown. Here we show that during secretion stress in *A. niger*, *hacA* transcription starts from a new start site closer to the ATG, relieving the transcript from translational attenuation. This transcriptional switch is mediated by HacA itself and the unfolded protein response element 2 (UPRE2) in the *hacA* promoter.

In eukaryotic cells, the majority of secreted and membrane proteins are folded, assembled, and modified in the endoplasmic reticulum (ER). Chaperones and foldases assist in folding the newly synthesized proteins (4), and correctly folded proteins are transported to the Golgi compartment, where further modification takes place. The secretory proteins are transported in vesicles from the Golgi apparatus to the cell membrane, where the content of the vesicle is released into the medium. When the amount of newly synthesized proteins exceeds the folding capacity of the ER, or if folding in the ER is impaired by other means, the eukaryotic cell triggers an intracellular signaling pathway in order to counteract the resulting secretion stress. This response is known as the unfolded protein response (UPR) (11, 25, 31, 37) and leads to the increased synthesis of ER-resident foldases and chaperones as well as many other components of the secretory pathway (34). Genomic analysis of the UPR in *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Aspergillus nidulans*, *Trichoderma reesei*, and *Aspergillus niger* all linked large sets of genes (several hundred) to the UPR, affecting all stages of the secretory pathway (2, 5, 16, 33, 34). The central activation step of the UPR involves the splicing of an unconventional intron from the mRNA encoding the transcriptional activator of the UPR. It is conserved from lower eukaryotes like yeasts to higher eukaryotes like mammalian cells and is mediated by Ire1p/IreA, a transmembrane kinase/RNase residing in the ER membrane. With its luminal domain, Ire1p/IreA senses the “folding state” of proteins in the ER, and when necessary, a signal is transmitted over the ER

membrane by the activation of the RNase domain, which in turn processes the messenger encoding the transcriptional activator of the UPR.

In *S. cerevisiae*, the Ire1p-mediated splicing of a 252-nucleotide (nt) unconventional intron from *HAC1* mRNA relieves the transcript from a translational block (12, 28). *HAC1* encodes a basic leucine zipper-type transcription factor that activates its target genes by a direct interaction with upstream activating sequences. In mammalian cells, the *HAC1* homologue *XBPI* is activated in a similar way by the splicing of a 26-nt unconventional intron (15), whereas *Trichoderma reesei hac1* and *Aspergillus nidulans* and *A. niger hacA* are activated by the splicing of a 20-nt unconventional intron (20, 29). To distinguish the unspliced *hacA* mRNA form the spliced form, they are referred to as *hacA^u* (uninduced) and *hacAⁱ* (induced), respectively.

Taking into account the magnitude of the UPR and the risk that the accumulation of unfolded proteins in the ER pose onto the cell, it seems plausible that natural selection has favored a strict controllability of the pathway. Although the UPR and its central activation step are conserved in all eukaryotic organisms, there are differences in the molecular mechanisms underlying the signaling pathway that give the different organisms different ways of control over the response. In mammalian cells, the UPR consists of three distinct pathways that operate in parallel and include general attenuation of translation, apoptosis, and increased folding and degradation of unfolded proteins (6). The mammalian UPR is triggered by at least three different ER stress sensors, all ER transmembrane proteins. This concerns (i) IRE1, a nuclease that splices the unconventional intron from *XBPI* mRNA (38); (ii) ATF6, which migrates to the Golgi compartment in response to ER stress (in the Golgi compartment, its cytosolic domain, which encodes a transcrip-

* Corresponding author. Mailing address: Genencor, Danisco, Archimedesweg 30, 2333 CN Leiden, The Netherlands. Phone: 31 715686142. Fax: 31 715686130. E-mail: harm.mulder@danisco.com.

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tion factor, is proteolytically released and relocated to the nucleus, where it upregulates its target genes) (9); and (iii) a protein kinase-like ER kinase (PERK) that phosphorylates translation initiation factor 2 α in response to ER stress, resulting in a general inhibition of translation (8).

In contrast to mammalian cells, in *S. cerevisiae*, the only known sensor of the UPR is Ire1p. In addition to the on/off switch of yeast UPR, several other factors influencing the UPR circuit have been described. The transcription of HAC1 was shown to be autoregulated (23), and the translation of the unspliced HAC1^u mRNA was blocked by long-range base pairing (28). Furthermore, a second ER-to-nucleus signaling pathway was found, which leads to an increased level of production of Hac1p combined with the production or activation of a putative UPR modulating factor (14). Both signals are required for super-UPR activation. Finally, Gcn4p and its activator, Gcn2p, were found to be required for the induction of the majority of the UPR target genes (26).

Integrated with the Ire1p pathway, these elements turn the UPR from a simple switch into a more complex response and give the yeast cell more fine-tuned control over the UPR.

The UPR in *A. niger* shares some characteristics with the *S. cerevisiae* response, but there are some differences. As saprophytic organisms, filamentous fungi are capable of secreting large amounts of proteins to recruit nutrients. This characteristic might have shaped the secretory pathway and have set specific demands to the stress responses, which might explain the differences with *S. cerevisiae* UPR.

First, in addition to the unconventional intron splicing, the 5'-untranslated regions (5'UTR) of the *hacA* transcripts of *A. niger*, *A. nidulans*, and *T. reesei* are truncated upon ER stress (20, 29). This has not been reported for yeast HAC1 or mammalian *XBPI* homologues. Second, a novel type of transcriptional downregulation mechanism was described for filamentous fungi, which was termed repression under secretion stress (1, 24). Upon ER stress, it downregulates the transcription of some genes encoding secreted proteins, thereby lowering the burden on the ER. Third, three UPR elements (UPREs) are present in the *hacA* promoters of *A. niger*, *A. nidulans*, and *A. fumigatus* (19). These elements could play a role in the upregulation of *hacA* by the activator protein itself, which was experimentally shown for *A. niger* (20). The position of the UPREs in the *hacA* promoter in relation to start sites of the full-length and truncated *hacA* mRNAs could also implicate a role for them in the truncation of the *hacA* transcript.

Here we show the mechanism behind the truncation of the 5'UTR of the *A. niger hacA* mRNA and the involvement of the UPREs in the regulation of the response.

MATERIALS AND METHODS

Strains and transformations. *A. niger* AB4.1 (*cspA1 pyrG*) and transformants thereof were grown on minimal medium plates containing (per liter) 6 g NaNO₃, 1.5 g KH₂PO₄, 0.5 g MgSO₄ · 7H₂O, 0.5 g KCl, trace elements (35), 1.5% agar, and 20 g fructose as a carbon source (pH 6.0). A total of 5 mM uridine was added in case of auxotrophy. Protoplasts were transformed according to standard procedures (13). *Agrobacterium tumefaciens*-mediated transformation of *A. niger* was performed according to previously described methods (3). Transformation plates were incubated at 18°C.

Construction of an *A. niger* Δ *hacA* strain. A 4.5-kb fragment containing *hacA* with 2 kb of its 5'-flanking region and 1.1-kb 3'-flanking region was PCR amplified from *A. niger* genomic DNA using primers P1 and P2 and cloned into

pCR-Blunt II-TOPO (Invitrogen). The resulting plasmid, pHM58, was cut with BglII, filled in with Klenow fragment, and cut with SalI. This removed the major part of the *hacA* gene from the plasmid, which was replaced with the *hygB* cassette from pAN7-1 (GenBank accession number Z32698). For this purpose, pAN7-1 was cut with XbaI, filled in with Klenow fragment, and cut with XhoI. A 6.6-kb KpnI-XhoI fragment containing the *hygB* cassette flanked by the upstream and downstream regions of *hacA* was isolated from the resulting plasmid. The fragment was cloned between the left and right borders of *A. tumefaciens* plasmid pSDM14 (22). This resulted in plasmid pHM60, which was used for the replacement of *hacA* in *A. niger* by *A. tumefaciens*-mediated transformation.

Construction complementation plasmid. A 3.2-kb FspI-BamHI fragment from plasmid pHM58 was ligated into the HincII-BamHI sites of pBluescriptSK(+), resulting in plasmid pHacA. For selective targeting of the plasmid to the *pyrG* locus of *A. niger*, the 3.9-kb XbaI fragment of pABpyrG*Not (kindly provided by Peter Punt, TNO Zeist, The Netherlands) was inserted into the SpeI site of pHacA, resulting in plasmid pHacA-pyrG*. The *pyrG** gene contains a filled-in BamHI site disrupting the open reading frame (ORF). Only the homologous integration of the plasmid at the *pyrG* locus will restore uridine prototrophy, enabling the selective targeting of the construct to the *pyrG* locus.

Site-directed mutagenesis. For purposes of mutagenesis of the elements present in the *hacA* promoter and 5'UTR of *hacA* mRNA, a 623-bp fragment containing these elements was PCR amplified from pHacA with primer pair P3 and P4 and cloned into pCR-BluntII-TOPO (Invitrogen), resulting in pCR-Blunt-PhacA. Site-specific mutations were introduced into the various elements using the QuikChange site-directed mutagenesis kit (Stratagene). The primers are listed in Table 1. The mutations were verified by sequencing performed with the BigDye sequencing kit (Applied Biosystems) and analyzed with the ABIPrism 3100 genetic analyzer (Applied Biosystems). A 493-bp BstEII-AspI fragment of pHacA was replaced with the corresponding but mutated fragment from plasmid pCR-Blunt-PhacA.

The unconventional intron was removed by digesting pHacA with PstI followed by religation. The *hacA* variant with the unspliceable unconventional intron was constructed by ligation of the annealed oligonucleotide pair P10-P11 into PstI-digested plasmid pHacA. For mutation of the long-range inverted repeats, a 1,756-bp PCR fragment was generated in several successive PCR and assembly steps, which involved primers P3, P4, P12, P13, P14, and P15 (Table 1). The resulting fragment was cloned into pCR-BluntII-TOPO, and after sequencing, a 1,351-bp BstEII-BglII fragment was cloned into pHacA.

After introduction of the mutations into pHacA, the XbaI *pyrG** fragment was cloned into the SpeI site of pHacA. Constructs having the *pyrG** gene in the same orientation as that of wild-type plasmid pHacA-pyrG* were selected for transformation to *A. niger* Δ *hacA*.

Electrophoretic mobility shift assay (EMSA). A total of 100 pmol oligonucleotide primers was annealed in 100 μ l 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl by heating the mixtures to 95°C for 5 min and cooling them down to room temperature by placing the tubes on the work bench. A total of 1 pmol of annealed oligonucleotide was end labeled in a 20- μ l reaction mixture with 5 μ l [γ -³²P]ATP and 20 units T4 polynucleotide kinase (New England Biolabs). The reaction mixture was incubated for 1 h at 37°C, and the radiolabeled oligonucleotide was purified on Biogel P30 spin columns (Bio-Rad). Binding reactions (20- μ l mixtures) were performed using a buffer containing 25 mM Tris (pH 8.0), 5 mM dithiothreitol (DTT), 100 mM KCl, 4 mM spermidine, 0.1 μ g/ μ l poly(dI-dC), 0.25% bovine serum albumin, 5% glycerol, and included 1 nM labeled probe, and 200 nM purified HacA protein (20). Binding was allowed to proceed on ice for 15 min. The reaction mixtures were loaded onto a 6% polyacrylamide gel and electrophoresed at 100 V in 0.25 \times TBE (89 mM Tris, 89 mM boric acid, 20 mM EDTA [pH 8.3]).

Southern blot analysis. Genomic DNA for Southern blot analysis was isolated by adding 800 μ l buffer (100 mM Tris [pH 8.0], 50 mM EDTA, 1 mM DTT, 35 mM sodium dodecyl sulfate) to 0.1 g of ground mycelium. The DNA was purified by phenol-chloroform extraction according to standard procedures (30). The DNA pellet was resuspended in H₂O and incubated with DNase-free RNase (Roche Molecular Biochemicals). For each digest, 10 μ g of genomic DNA was incubated for 6 h at 37°C with 20 U of an appropriate restriction enzyme. The reaction products were separated on 0.8% TBE agarose gels. Blotting and hybridization were done according to standard procedures (30).

Northern blot analysis. For Northern blot analysis, cultures were grown overnight at 30°C on minimal medium supplemented with 0.2% (wt/vol) yeast extract. Aliquots of 0.5 g wet mycelium were transferred into 50 ml fresh medium, and the cultures were incubated for 3 h before provoking ER stress by the addition of either 20 μ g/ml tunicamycin or 20 mM DTT. Equal amounts of either dimethyl sulfoxide or water were added to the control cultures. Samples from the stressed and unstressed cultures were taken 3 h after applying the stress agents.

TABLE 1. PCR oligonucleotide primers used in this study^a

Oligonucleotide	Sequence	Description
P1	5'-GGAGAACCAAGGTGCTATTG	-2,071-bp <i>hacA</i> start codon
P2	5'-GTTTAGGAGCCTTTACCTCC	+1,152-bp <i>hacA</i> stop codon
P3	5'-ATTTACCGTACGGTCAATTGGGGC	-503-bp <i>hacA</i> start codon
P4	5'-CCCTGCCTGTACTGACGAGTCATC	+120-bp <i>hacA</i> start codon
P5	5'-GGCTGTATCTGAACACG GACGCCTTTAAAGAGT	UPRE1
P6	5'-TCCCCGTTATGACACGGAC GCCTGTGTTCTCTGT	UPRE2
P7	5'-ATGGTTCTTAAGGAC CACTCCTTCTTGGCCCT	UPRE3
P8	5'-TCTTTTATTGTTCTCTGGTTCTTAAGGACACC	ATG uORF
P9	5'-CGACCTACATCCCGTCCCA ACGTCAGCGGTTAAGATAAGGCTCATAGT AAATCGATTG	5' splice site
P10	5'-TAGTGCCGTCCTCTGCGATCTTCA	Unspliceable intron us
P11	5'-TGAAGATCGCAGAGGACGGCACTA	Unspliceable intron ls
P12	5'-GCCTGGGTTAGCGCCCGTCAAGCCCGTTATGACACGGTGGCCTGTGTTT	Inverted repeat 5'UTR
P13	5'-GACAGGTAATTCCTGCCCCATGACTTTCTCTTCTTACAGG	Inverted repeat <i>hacA</i> gene
P14	5'-TCCCCGTTATGACACGGTGGCCTGTGTTCTCTGT	-168-bp <i>hacA</i> start codon
P15	5'-TCAAACCGCTCAAGATTCTGTTT	+1,253-bp <i>hacA</i> start codon
P16	5'-CGTCGAGAACGTCAAAGGCGAACCCGTC	For 5'RACE <i>hacA</i>
P17	5'-TTCGATGACAAGGATGTCCAGA	For RT-PCR <i>bipA</i>
P18	5'-GGGGATGAGCTTGGTCATGA	For RT-PCR <i>bipA</i>
P19	5'-ATTTAAATACCCTCTCCCATCGTCTCTC	For RT-PCR <i>pdiA</i>
P20	5'-CCTCCTCGGCGGTGCAGTCAACCTTCA	For RT-PCR <i>pdiA</i>

^a Mutations are represented in boldface type.

Since tunicamycin is a more specific agent to induce ER stress, Northern blots of these treatments are shown, where possible. For the analysis of the UPREs and *hacA* processing, the DTT blot is shown, as DTT resulted in much more pronounced *hacA* splicing.

For RNA isolation, mycelium was ground with 1-mm glass beads using the Fast Prep FP120 system (Bio 101 Savant), and total RNA was isolated using the RNeasy plant total RNA kit (Qiagen). In order to prevent the degradation of the RNA during the purification procedure, 1.5-ml screw-cap tubes were filled with approximately 0.5 ml 1-mm glass beads and 700 μ l RLC buffer, which was provided with the RNeasy plant total RNA kit. The filled tubes were frozen in liquid nitrogen prior to the harvesting of the mycelia. The mycelium was harvested rapidly and dried thoroughly between tissue paper, and a small piece of mycelium (100 to 200 mg) was added to a pre-frozen tube and refrozen in liquid nitrogen. The frozen tubes were then placed into the Fast Prep FP120 apparatus and run for four cycles of 20 s at a speed of 6.5, which allowed the thawing and simultaneous grinding of the mycelia. For Northern analysis, 10 μ g of total RNA was separated on a 1.5% (wt/vol) agarose gel containing 10 mM phosphate buffer (pH 7) and blotted onto a Hybond N membrane (Amersham Pharmacia Biotech). Blotting and hybridization were done according to standard procedures (30). Probes were generated by labeling DNA fragments with deoxycytidine 5'-[α -³²P]triphosphate using the Prime-It II labeling kit (Stratagene) and purified on Biogel P30 spin columns (Bio-Rad). Exposures were made using Curix HC-S Plus X-ray films (Agfa). Quantification of the Northern blot signals was done by phosphorimager analysis (Instant Imager; Packard).

5'RACE and RT-PCR. For 5' rapid amplification of cDNA ends (5'RACE) and reverse transcriptase PCR (RT-PCR), first-strand cDNA was made from 1 μ g of total RNA using the Smart RACE cDNA amplification kit (Clontech). The 5' end of the *hacA* transcript was amplified by PCR with primer P16 using cycling conditions recommended by the kit's supplier. For the transcription analysis of *bipA* and *pdiA*, cDNA was amplified using primer pairs P17-P18 and P19-P20, respectively.

Microscopy. For microscopy, mycelia was grown between two glass slides in minimal medium supplemented with 0.2% yeast extract with and without the addition of 10 μ g/ml tunicamycin. Images were taken using an Olympus BX51 microscope equipped with an Olympus DP11 digital camera.

RESULTS

The *A. niger* Δ *hacA* strain. To assess the role of the *hacA* gene and the control mechanisms in the UPR of *A. niger*, we aimed to obtain a corresponding knockout strain. Recently, *A. tumefaciens*-mediated transformation was shown to be an efficient tool for gene replacement in *Aspergillus awamori* (18).

Using this transformation procedure, the *hacA* gene of *A. niger* was successfully replaced with the hygromycin resistance cassette. The construct consisted of the hygromycin cassette from pAN7-1 flanked by 2 kb of the *hacA* 5' upstream and 1.5 kb of the 3' downstream regions, respectively.

Two types of colonies were present on the transformation plates. The majority of the colonies had a regular wild-type phenotype, whereas approximately 30% consisted of small nonsporulating yellow colonies. Southern analyses performed on both types of colonies showed the absence of the *hacA* gene in the aberrant colonies, whereas the construct was ectopically integrated in transformants having a wild-type phenotype.

Thus, the deletion of *hacA* results in a distinct phenotype. Growth is severely impaired, and sporulation is almost absent compared to the wild-type strain (Fig. 1A). On minimal medium plates as well as on plates with rich medium, such as complete medium (CM) and potato dextrose agar, the Δ *hacA* strain formed small compact colonies with only few conidia. The growth differences observed were analyzed further microscopically. Under normal growth conditions, parts of the hyphae of the Δ *hacA* strain are swollen compared to the wild type, and often, these swollen hyphae are branched at the tips, forming finger-like extensions as shown in Fig. 4B. In the presence of tunicamycin, growth of the Δ *hacA* strain was nearly abolished. The hyphae are shorter than those in the wild-type strain and have swollen irregular shapes.

Thus, the application of ER stress in the form of 10 μ g/ml tunicamycin severely affected the growth of the Δ *hacA* strain, indicating that a functional UPR is necessary for coping with ER stress.

Northern analysis showed the inability of the Δ *hacA* strain to activate the UPR (Fig. 1B). In a wild-type strain, the upregulation of the chaperone *bipA* and the foldases *cypB* and *pdiA*, due to ER stress, coincides with the processing of *hacA* mRNA. In the Δ *hacA* strain, however, ER stress does not trigger a comparable response. The basal transcript levels of *bipA*, *cypB*, and

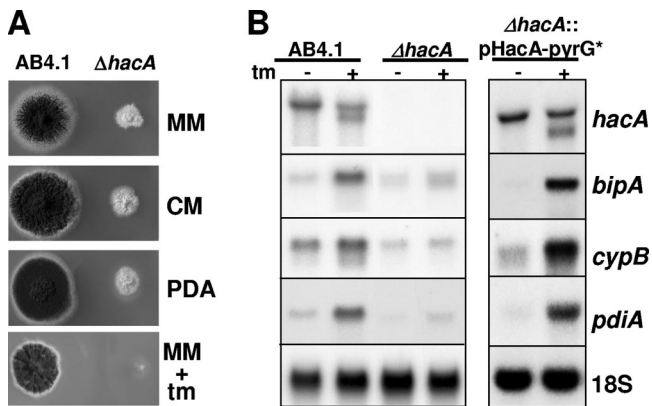


FIG. 1. Phenotypic and transcriptional analysis of the *A. niger* Δ *hacA* strain. (A) Growth of the Δ *hacA* strain compared to that of wild-type strain AB4.1 on different agar media. MM, minimal medium; CM, MM supplemented with 0.5% yeast extract; PDA, potato dextrose agar; tm, 10 μ g/ml tunicamycin. (B) Northern blot analysis showing the lack of a functional UPR in the Δ *hacA* strain and the restoration of the response by transforming the Δ *hacA* strain with plasmid pHacA-pyrG*.

pdiA are low, and in response to tunicamycin treatment, only a slight, if any, upregulation was observed. In contrast, the retransformation of the Δ *hacA* strain with the wild-type *hacA* gene fully restored the transcriptional pattern of regulation typical for a wild-type strain.

The Δ *hacA* strain was used further as a background for studying the *hacA* gene expression mechanism.

Role of the small ORF and the putative 5' splice site. The 5'UTR of the full-length transcript of *hacA* contains a small ORF (uORF) of 44 codons (Fig. 2A), which is not present in the truncated *hacA* mRNA. We previously hypothesized that this region could be involved in a translational control mechanism of *hacA^u* mRNA, since translational control is often associated with short ORFs, present in the 5'UTR of mRNAs (17). The *S. cerevisiae* transcription factor Gcn4p and the mammalian transcription factor ATF4 are translationally controlled by small uORFs (7, 10). To test this hypothesis, the ATG start codon of the uORF was mutated to CTG, and the mutated *hacA* construct was introduced into the Δ *hacA* strain. However, northern analyses of the Δ uORF strain and subsequent signal quantification did not show clear effects of the mutation on the transcription levels of the foldases and/or the truncation of *hacA* mRNA upon the application of ER stress compared to the wild type (Fig. 2B and C). Note that a second ATG is present in the reading frame of the 44-codon uORF of *hacA*. This ATG, however, is located 7 nt upstream of the central spacer nucleotide of UPRE2 (Table 1) and was therefore not mutated. As a result, an 8-codon-long uORF remains intact in the 5'UTR of the *hacA^u* mRNA of this strain. Interestingly, this uORF shows remarkable sequence identity with two small uORFs present in the *hacA* transcripts of *A. nidulans* and *A. fumigatus*. The translated sequences of these uORFs of *A. niger*, *A. nidulans*, and *A. fumigatus* are MTRWPVFL, MTPWPLS, and MPTWLFL, respectively. Although this conservation could be the result solely of the fact that all three uORFs overlap with the UPRE2 sequence in each promoter, a func-

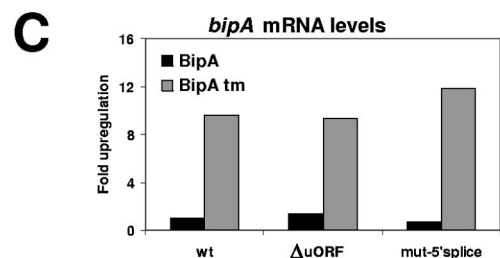
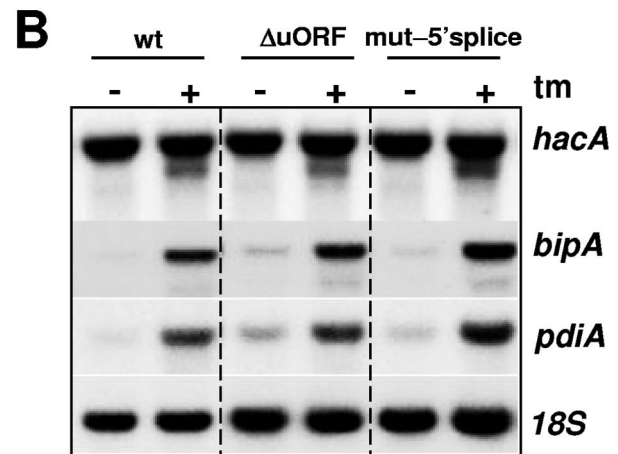
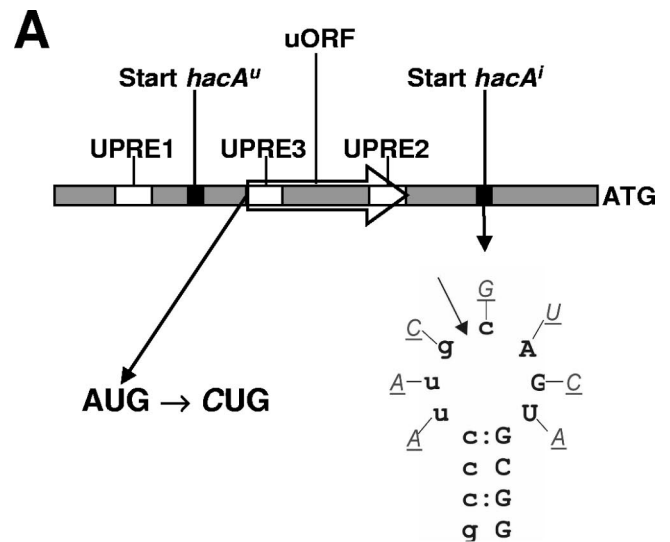


FIG. 2. Mutational analysis of the uORF and the putative 5' splice site, two elements present in the 5'UTR of *hacA^u* mRNA. (A) Schematic overview of the 5'UTR of the *hacA* gene. ATG indicates the start codon of *hacA*, UPREs are indicated by open boxes, the starts of *hacA^u* and *hacAⁱ* mRNAs are indicated by black boxes, and the 44-codon uORF is indicated by an open arrow. The mutations introduced to disrupt the uORF and the putative 5' splice site are indicated below. The putative IreA cutting site is indicated by an arrow. (B) Northern blot analysis showing the effect of ER stress imposed by tunicamycin (tm) on the transcription of *hacA*, *bipA*, and *pdiA*. 18S rRNA was used as a loading control. (C) Relative *bipA* levels in the wild type (wt), the Δ uORF strain, and the mut-5' splice-site strain in the absence (black bars) and presence (gray bars) of 20 μ g/ml tunicamycin.

tion of the uORFs in translational attenuation cannot be excluded.

Previous inspection of the sequence around the start site of *hacAⁱ* mRNA revealed a strong sequence similarity with the intron borders of the unconventional intron (20). If this would lead to a similar secondary structure, as is the case for the unconventional intron borders, this structure could be a potential target for IreA and explain the mechanism behind the truncation of *hacA*. In contrast to the splicing of the unconventional intron, which involves an IreA-mediated cut in each of the two loops followed by the religation of the two exons by tRNA ligase (32), the hypothetical IreA-mediated 5' truncation of *hacA* mRNA would involve only a cut at the recognition site. Although no RNA secondary structure incorporating the putative 5' splice site in a stable stem-loop structure could be predicted, mutational analysis was performed to test whether or not this site would be a substrate for IreA. The seven nonpaired nucleotides in the putative loop were mutated from purines to pyrimidines, or vice versa, in order to destroy a putative recognition site for IreA (Fig. 2A). If IreA indeed would be involved, this strategy should lead to the inability to form the truncated *hacA* messenger. However, Northern analysis of a strain bearing such mutations did not show any effect of the mutations in the putative 5' splice site on the processing of the *hacA* mRNA (Fig. 2B). Three hours after tunicamycin-induced stress, the truncated *hacA* mRNA was present at levels comparable to those of the wild-type strain. The upregulation of *pdiA* and *bipA* mRNA levels upon stress was also similar to the wild-type situation.

Role of UPREs in the *hacA* promoter. Previously, we identified three UPREs in the *hacA* promoter by EMSA. The presence and conservation of these three boxes in the *hacA* promoters of *A. niger*, *A. nidulans*, and *A. fumigatus* suggest a function for all of them (19). Mutational analysis was carried out to determine the contribution of each of these elements to the transcriptional regulation of *hacA*. Mutations were introduced into the core sequences of the *hacA* UPREs. The loss of binding due to the mutations was confirmed in vitro by EMSAs with the HacA protein and 34-bp double-stranded oligonucleotides comprising the different mutated UPREs. Whereas two mutations (Fig. 3B) were sufficient to completely abolish binding of HacA to UPRE2 and UPRE3, the corresponding two mutations introduced into UPRE1 did not lead to a complete loss of binding. As a result, two additional mutations were introduced into UPRE1.

None of the strains carrying mutations in the *hacA* UPREs showed phenotypic growth changes on a plate test (Fig. 4A), and these results were confirmed by light microscopy (data not shown). However, Northern analyses performed on the strains revealed an altered transcriptional pattern for *hacA* and the foldases.

When UPRE2 was mutated separately, in combination with UPRE3, or in combination with UPRE1 and UPRE3, the truncation of *hacA* mRNA did not occur anymore in response to ER stress (Fig. 3C). Although only the full-length transcript was formed in the UPRE2 mutants 3 h after DTT treatment, *bipA* and *pdiA* transcripts were upregulated to the same extent as in the wild-type strain, indicating that the truncation of *hacA* is not a necessity for the induction of the pathway. In contrast, the mutation of UPRE1 or UPRE3 separately did not lead to

apparent changes in the splicing of *hacA* mRNA under ER stress conditions. However, a mutation of UPRE1 did result in lower basal levels of *hacA* mRNA (Fig. 3D). All three strains carrying mutations in UPRE1 had approximately half of the *hacA* mRNA levels compared to those of the wild type and the other mutant strains. This is in line with the finding that *hacA* is subject to autoregulation (20). Another observation is, however, more puzzling. In the wild-type strain, the amount of the full-length *hacA* messenger had dropped significantly 3 h after the addition of DTT to the culture. This effect was also observed for the mutants Δ uORF, mut-5'splice, and Δ IR (mutated inverted repeat) (see below) upon DTT stress (data not shown). However, except for Δ UPRE2, this effect was not observed for the other UPRE mutants. On the contrary, full-length *hacA* levels were slightly induced by ER stress in strains with a mutation in either UPRE1, UPRE3, or both elements. The observation that UPRE1 seems to repress the transcription of full-length *hacA* mRNA when HacAⁱ is abundantly present implies a dual function for UPRE1. Under normal conditions, when the concentration of HacAⁱ is low, UPRE1 functions as an activator site, whereas it functions as a repressor site during DTT stress. The underlying mechanism of this possible dual function of UPRE1 is unclear and will be the subject of further investigation.

The transcription start sites of the three single UPRE mutants were determined by 5'RACE on RNA samples of non-induced and DTT-induced cultures to check whether mutations in the UPREs had an effect on the exact start site of *hacA^u* and *hacAⁱ* mRNA. As in the wild type, in all three mutants (UPRE1, UPRE2, and UPRE3), the full-length *hacA* started at 303 nt upstream of the ATG. In DTT-stressed cultures of the UPRE1 and UPRE3 mutants, the transcription start site of the truncated *hacA* mRNA was found 73 nt upstream of the ATG, as in the wild type.

Long-range base pairing and translational block. Long-range base pairing was shown to prevent the uninduced form of the yeast *HAC1* homologue from being efficiently translated (28). Part of the 252-nt intron of yeast *HAC1^u* mRNA forms a stable double-stranded structure by base pairing with the 5'UTR, thereby preventing the ribosomes from reading through. Unconventional intron splicing removes the 252-nt intron and with it the right half of the inverted repeat, thereby releasing *HAC1^u* mRNA from its translation block.

An identical mechanism is not possible for *A. niger hacA^u* mRNA. The unconventional intron of *hacA* is only 20 nt long, and a stable stem-loop structure is predicted for this sequence, which leaves no nucleotides available for a putative long-range base pairing with the 5'UTR. However, sequence analyses revealed a 26-nt-long inverted repeat in *hacA^u* mRNA (Fig. 5A). This repeat contains 18 bp, 12 bp of which are GC pairs. In comparison, the yeast *HAC1* inverted repeat stretches out over 19 nt and has 16 bp, 11 bp of which are GC pairs. The left half of the inverted repeat in *A. niger hacA* is located in the 44-codon uORF, and the right half is located in the *hacA* ORF close to the ATG. Truncation upon ER stress removes approximately 230 nt of the 5'UTR of *hacA* mRNA including the uORF and thereby the left half of the inverted repeat, possibly releasing *hacA* from a translational block analogous to the yeast *hac1* mechanism.

To determine the effect of the putative base pairing on the

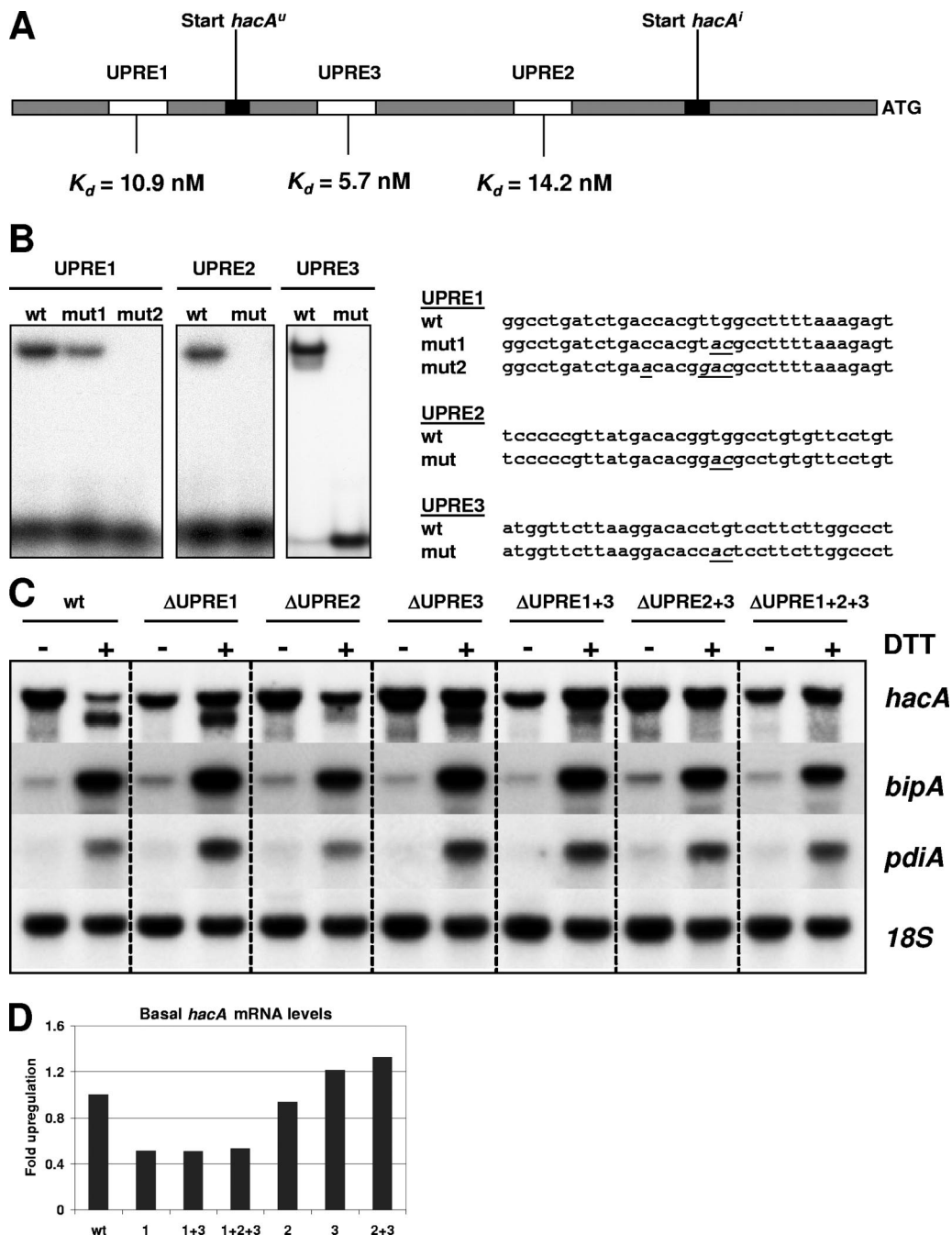


FIG. 3. Role of the UPREs in regulation of *hacA*. (A) Schematic overview of the promoter region of *hacA*. The transcription start points of *hacA'* and *hacA'* are indicated by black boxes. The UPREs are represented by open boxes. The binding constant of each UPRE is shown at the bottom. (B) EMSAs showing the inability of HacA to bind to the mutated UPREs. The sequences of the 34-bp oligonucleotides representing the UPREs are shown, and mutations introduced into each UPRE to abolish binding by HacA are indicated in italics and are underlined. wt, wild type. (C) Northern analysis showing the effect of mutated UPREs on transcription levels of *hacA*, *bipA*, and *pdiA*. ER stress was imposed by the addition of 20 mM DTT to the medium. (D) Relative *hacA* mRNA levels in the different UPRE mutants under nonstress conditions.

regulation of *hacA*, both half-sites of the inverted repeat were mutated. Since both half-sites are located in coding regions, only silent mutations were introduced to disrupt the putative base pairing (Fig. 5A). Although this strategy left 6 bp intact, it is likely that a stable base pairing of both half-sites is no longer possible. Analysis of the Δ IR strain by Northern blots showed that under normal growth conditions, approximately

three-times-more *bipA* and *pdiA* transcripts were present than in the wild type (Fig. 5B), indicating higher basal levels of HacA in this strain. Under ER stress, the transcriptional pattern of *hacA*, *bipA*, and *pdiA* was similar to that of the wild-type strain.

The unconventional intron. To study unconventional intron splicing, we aimed to construct an Δ *ireA* strain. However, mul-

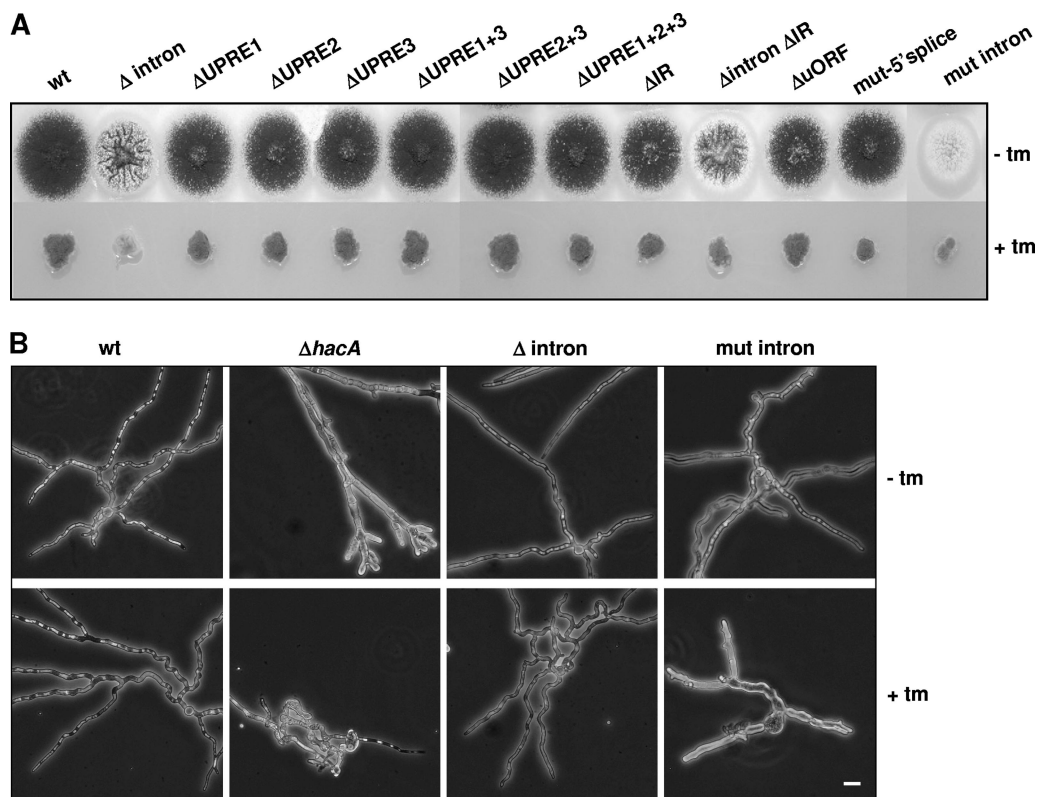


FIG. 4. Phenotypes of the strains used in this study. (A) Growth of wild-type (wt) and mutant strains on CM plates in the absence (top row) or in the presence (bottom row) of 10 μ g/ml tunicamycin (tm). (B) Microscope images of the wild-type strain, the *hacA* deletion strain (Δ *hacA*), the strain bearing the intronless *hacA* gene (Δ intron), and the strain bearing the unspliceable *hacA* variant (mut-intron).

tiple attempts and techniques to construct such a strain all failed, presumably because of the lethality of the deletion. Therefore, the importance of the unconventional intron for the UPR was determined in the following two ways: by analysis of a strain carrying the *hacA* gene lacking the unconventional intron and by analysis of a strain having a *hacA* variant with an unspliceable unconventional intron. The removal of the unconventional intron from the *hacA* gene resulted in a constitutive UPR (Fig. 5B). Both the full-length and the truncated *hacA* transcripts were abundantly present under normal growth conditions, and the levels of *bipA* and *pdiA* were 19-fold and 13-fold higher, respectively, than levels measured in the wild-type strain under nonstress conditions.

The induction of ER stress by the addition of tunicamycin had no additional effect on the response in this strain, indicating that splicing of the unconventional intron is sufficient to fully induce the pathway. Northern blot analysis of a strain containing an unspliceable *hacA* variant (mut intron) revealed that the splicing of the unconventional intron and, as a result, HacAⁱ is necessary to activate the downstream pathway (Fig. 5B). The truncation of the 5'-UTR of *hacA* mRNA in response to tunicamycin-induced stress and the accompanying upregulation of *bipA* and *pdiA* did not take place in this strain. *bipA* and *pdiA* transcripts could not even be detected on the Northern blot under both conditions. It was previously reported that the deletion of *bipA* is lethal for *Aspergillus* (27). Also, attempts to delete *pdiA* in *A. niger* were unsuccessful (21). Therefore, the apparent absence of *bipA* and *pdiA* transcripts on the

Northern blot of the strain bearing the unspliceable *hacA* variant (mut-intron strain) is most likely a result of the limited exposure time of the X-ray film. RT-PCR performed on RNA from the mut-intron strain revealed that both *bipA* and *pdiA* transcripts were indeed present in this strain albeit at lower levels than those of the wild-type strain (Fig. 5C).

Remarkably, both the Δ intron and mut-intron strains displayed clear phenotypes. When grown on agar plates, Δ intron produced fewer conidia than did the wild-type strain, whereas sporulation was almost absent in strain mut-intron (Fig. 4A). Microscopic inspection of mut-intron grown in the presence of tunicamycin revealed a phenotype slightly comparable to that of the Δ *hacA* strain: poor growth and swollen hyphae (Fig. 4B).

Apparently, both HacAⁱ and HacA^u have physiological functions in *A. niger*, and the lack of either one results in the observed phenotype. The phenotype of the strain having the intronless variant of the *hacA* gene, and therefore capable of producing only the HacAⁱ protein, is not merely a result of the permanent activation of the UPR. The overexpression of *hacA*ⁱ in a wild-type background, leading to a strain which constitutively produces the HacAⁱ protein but is also capable of producing the HacA^u protein from its endogenous *hacA* gene, did not lead to similar phenotypical changes (results not shown). This is in agreement with the previously reported observation that the amino acid sequence of the C-terminal parts of HacA^u from different aspergilli are highly conserved (20). The precise role of HacA^u in the fungus, however, is the subject of further investigation.

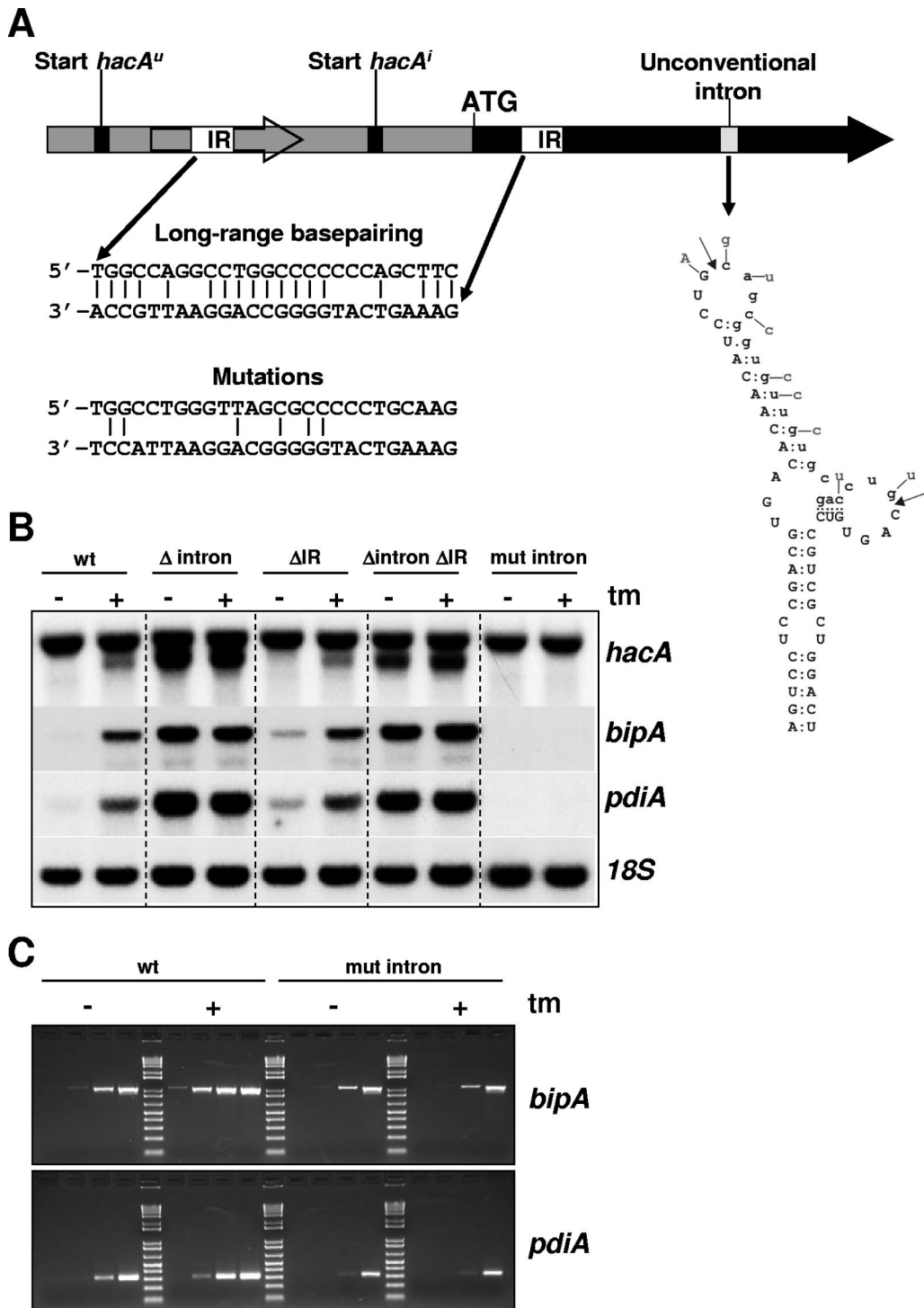


FIG. 5. Function of the unconventional intron and long-range base pairing in the regulation of the UPR. (A) Schematic overview indicating the unconventional intron and the location of the inverted repeats in relation to other elements present in *hacA*. The *hacA* ORF is indicated by a solid black arrow, and the 5'UTR is indicated by a gray box. The inverted repeats (IR) are depicted by open boxes, and the base pairing of the inverted repeats is shown at the bottom. The theoretically remaining base pairings after the introduction of silent mutations are also indicated. The structure of the unconventional intron is depicted by the stem-loop structure, and mutations introduced to prevent splicing by disrupting the structure are indicated. The intron sequence is shown in lowercase type, and cleavage sites are indicated by arrows. (B) Northern blot analysis showing the effect of the different mutations or deletions on the expression and truncation of *hacA* and on the transcript levels of *bipA* and *pdiA*. ER stress was imposed by treatment with tunicamycin (tm), and the gene coding for 18S rRNA was used as a control probe. (C) RT-PCR showing the transcript levels of *bipA* and *pdiA* in the wild-type (wt) and mut-intron strains. The four lanes under each condition correspond to 10, 15, 20, and 25 PCR cycles.

DISCUSSION

In eukaryotes, secretion stress leads to various responses, which aim at the homeostasis of ER functions. One of the secretion stress responses is the UPR, which is common to all eukaryotic cells. There are, however, differences in the molecular mechanisms underlying the UPR among different organisms. In this study, we describe some unique features of the *A. niger* UPR not reported for the *S. cerevisiae* and mammalian UPRs.

In both lower and higher eukaryotes, the activation of the UPR starts with the splicing of an unconventional intron from the *hacA* transcript, mediated by IreA. Splicing of the 20-nt intron from *A. niger hacA* mRNA is both necessary and sufficient to activate the UPR. A strain containing an unspliceable variant of *hacA* displayed poor growth and was unable to activate the pathway and upregulate the foldases in response to stress, whereas a strain containing an intronless variant of the *hacA* gene displayed a constitutive UPR (Fig. 5).

In addition to the rather drastic on/off switch of unconventional intron splicing, other regulatory elements are present in the promoter of the *hacA* gene and in the *hacA^u* transcript, which provides the fungus additional regulatory control over the response.

Long-range base pairing in the *hacA* messenger prevents the efficient translation of the full-length transcript (Fig. 5). As opposed to the mechanism in *S. cerevisiae*, where pairing takes place between the 5'UTR and the unconventional intron, the 5'UTR of *Aspergillus hacA* mRNA forms a double-stranded structure with the coding region of the transcript, resulting in translational attenuation (Fig. 5). In the case of *A. niger*, the release of the translational block upon ER stress is exerted via a switch to an alternative transcription start located approximately 230 bp downstream of the usual transcription start site. This switch is dependent on UPRE2 and on HacA itself, thus confirming the autoregulation of *hacA* transcription, as demonstrated previously (20). UPRE2 is the HacA target most proximal to the ATG and located 81 bp upstream of the start site of *hacAⁱ* mRNA. A mutation of UPRE2, either individually or in combination with UPRE1 and UPRE3, abolished the ability to synthesize the shorter transcript during ER stress (Fig. 3), indicating its importance for the transcriptional switch. The fact that the transcriptional switch depends on HacA itself was shown by two experiments. First, a strain containing the *hacA* gene without the unconventional intron, and therefore capable of producing only the active form of the HacA protein, produced both full-length and truncated *hacA* mRNAs under normal growth conditions (Fig. 5). Second, a strain carrying an unspliceable version of the *hacA* gene (cf. the mut-intron strain) (Fig. 5) and therefore unable to produce the active form of the HacA protein was unable to synthesize the truncated messenger in response to stress.

Whereas a clear function can be assigned to UPRE2, the roles of UPRE1 and UPRE3 in the regulation of *hacA* are somewhat more puzzling. On the whole, a mutation of UPRE1 and UPRE3 individually or together left the response and the accompanying truncation of *hacA* intact. However, these sites could provide an additional fine-tuning mechanism for *hacA* transcription. When UPRE1 was inactivated alone or in com-

ination with other sites (Δ UPRE1, Δ UPRE1 plus Δ UPRE3, and Δ UPRE1 plus Δ UPRE2 plus Δ UPRE3), the basal level of *hacA* transcription dropped to approximately 50% compared to wild-type levels (Fig. 3D). In contrast, ER stress led to a slight upregulation of the full-length *hacA* transcript in those same mutants, with the most pronounced effect in the double mutant Δ UPRE1 plus Δ UPRE3 (Fig. 3C). Since UPRE1 is most distal to the ATG of the *hacA* gene and located 50 bp upstream of the transcription start point of *hacA^u* (19), it seems plausible that UPRE1 affects the basal level of the full-length *hacA* messenger. The basal level of *hacA* transcription is, however, not dependent solely on HacA. A strain that was unable to synthesize HacAⁱ (cf. mut intron) (Fig. 5) displayed *hacA* mRNA levels comparable to those of the wild-type strain under normal growth conditions. Most likely, another, as-yet-unknown factor plays an important role in the regulation of *hacA*. In mammalian cells, the *hacA* homologue *XBPI* is upregulated by ATF6 in response to ER stress (15), and in *S. cerevisiae*, certain conditions can boost the accumulation of *HAC1* mRNA via an Ire1p-independent pathway (14). A possible candidate involved in an additional regulation of *A. niger hacA* could be CpcA. CpcA is a homologue of *S. cerevisiae* Gcn4p, which acts as the central transcription factor in response to amino acid starvation (36). In addition, *S. cerevisiae* Gcn4p plays an essential role in the UPR and is responsible for the induction of a major part of the UPR targets during ER stress (26). Its mouse homologue, ATF4, has also been shown to be involved in secretion stress (7). The *T. reesei* and *A. nidulans* homologues *cpc1* and *cpcA*, respectively, have also been linked to the UPR, as they have been shown to be upregulated in response to ER stress (2). The involvement of such a factor could explain the slight upregulation of the full-length *hacA* transcript observed upon stress in the UPRE1 mutant strains. The role of CpcA in the UPR and its effect, if any, on *hacA* transcriptional regulation, perhaps via an interaction with the UPRE1 target, and the controversial role of UPRE1 as an activator and a repressor site of *hacA* transcription remain to be established.

Whereas UPRE2 mediates the transcriptional activation of *hacA* upon stress, an opposite function could be attributed to UPRE3. Mutation analysis of UPRE3 suggests that this site could act as an HacA scavenger in order to prevent the transcriptional switch from being activated at low stress levels, at which full activation of the pathway is not necessary. The disruption of UPRE3 resulted in higher levels of *hacA* under both normal and ER stress conditions. Of the three UPREs present in the *hacA* promoter, UPRE3 possesses the highest affinity for HacA and stands apart from the other UPREs because of the missing spacer nucleotide between the two palindromic half-sites (19). Using polysomal fractionation, it has been shown that under nonstress conditions, the splicing of *hacA* still occurs but at a low level (5). The association of the spliced transcript with the polysomal fraction indicates that low levels of the HacAⁱ protein will be synthesized under normal growth conditions, which is in agreement with our previous results from UPRE-promoter reporter constructs (19). UPRE3 could therefore have the function of a "threshold" switch; only when sufficient HacAⁱ accumulates in the nucleus will the transcription of *hacA* shift to the new transcription start site, possibly initiated via the recognition of UPRE2. Although further

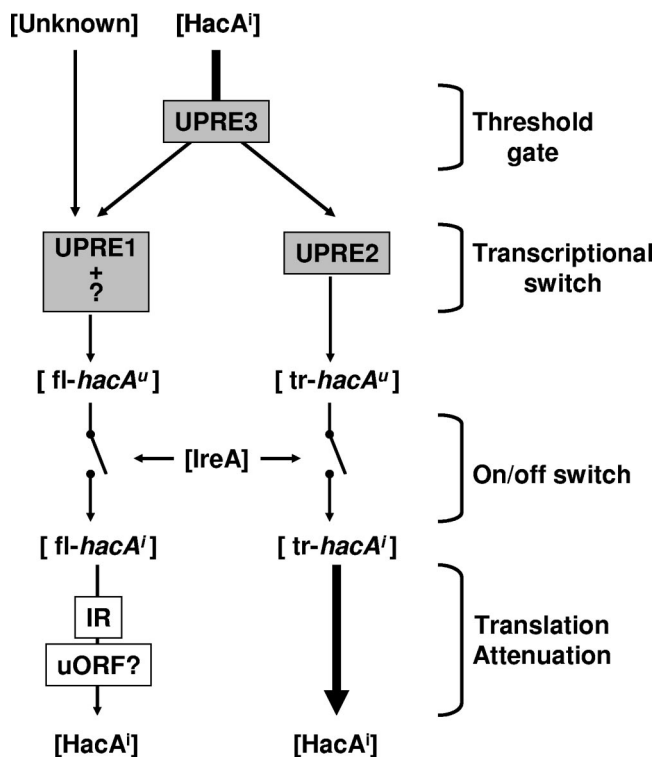


FIG. 6. Schematic overview of *hacA* regulation. The model depicts proteins and mRNAs between brackets indicating that their (active) concentration can vary. Elements present in the *hacA* promoter are in gray boxes, and elements present in the *hacA* mRNA are in white boxes. The splicing of full-length *hacA* (fl-*hacA*^u) and truncated *hacA* (tr-*hacA*^u) by IreA is depicted by the on/off switches. Translational attenuation is indicated by the thin arrow, compared to the arrow indicating nonattenuated translation. IR, inverted repeat.

analysis is needed, we suggest a mechanism in which UPRE3 intercepts HacAⁱ when the protein comes into the vicinity of the promoter, thereby preventing the activation of transcription via UPRE1 or UPRE2. Only when the HacAⁱ protein concentration reaches a certain threshold is the pathway further activated via the transcriptional switch to a new start of transcription followed by the consequent release from a translational attenuation checkpoint.

We unraveled several mechanisms behind the *A. niger* UPR, which offers the fungus controllability over and fine-tuning of the pathway. At the transcriptional level, activation occurs in two steps: intron splicing followed by a transcriptional switch to a new start site. At the translational level, long-range base pairing inhibits the efficient translation of the nontruncated *hacA* transcript. In addition to these mechanisms, our results indicate additional regulatory control of *hacA* transcription mediated via UPRE1 and HacA and most likely another transcription factor, possibly CpcA. UPRE3, finally, could function as a build-in safety mechanism to prevent unnecessary activation. The confirmation of the latter mechanisms and the integration into the regulation circuitry remains subject to further investigation. Figure 6 shows a schematic overview of our current working model. It implies that under normal growth conditions, low concentrations of active HacAⁱ will be present in the cell as a result of the limited intron splicing that constitu-

tively occurs in the fungal cell. UPRE3 acts as a threshold gate, keeping HacAⁱ away from UPRE2 until the HacAⁱ concentration reaches a certain level. The transcription of *hacA* at this stage is initiated mainly via UPRE1 and/or an additional as-yet-unknown promoter element and is mediated by HacAⁱ and an unknown protein (possibly CpcA). The result is the transcription of full-length *hacA*^u mRNA. IreA functions as the controller of an on/off switch to activate the UPR by splicing the *hacA*^u mRNA. The resulting full-length *hacA*ⁱ transcript is, however, subject to translational attenuation caused by long-range base pairing and possibly the uORF. As a result, the production of the “active” HacAⁱ protein is slow at this stage. Only during prolonged stress will enough HacAⁱ accumulate to overcome the threshold value set by UPRE3 and will the transcriptional switch be activated. The transcriptional switch is then operated by HacAⁱ, which will bind to the UPRE2 and initiate its own transcription from a new start site. This will result in the truncated *hacA*^u transcript. This messenger lacks the translational block and can be efficiently translated. However, it still contains the unconventional intron, and only after activation by IreA will it contribute to the active pool of the HacAⁱ protein and only then will the *hacA*-dependent part of the UPR be at its full strength.

As a saprophytic organism, *A. niger* secretes large amounts of enzymes into its environment to degrade complex polymeric substrates used as natural nutrients for the fungus. This characteristic might also have set special demands on the secretion machinery of the organism and on the complexity of the regulation of the UPR in comparison with, for example, *S. cerevisiae*. The activation of the UPR affects a large set of genes (2, 5, 33), and unnecessary or unnecessarily high-level activation of the UPR will be costly in terms of energy. Therefore, the presence of several subsequent checkpoints allow the fungal cell to optimally tune its secretion machinery to the environment.

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