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tRNA Ligase Is Required for Regulated mRNA Splicing in the Unfolded Protein Response

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Summary

The accumulation of unfolded proteins in the endoplasmic reticulum (ER) triggers an intracellular signaling pathway, the unfolded protein response (UPR), that leads to increased transcription of genes encoding ER-resident proteins. Transcriptional activation is mediated by a dedicated transcription factor, Hac1p, whose activity is controlled by regulated splicing of its mRNA. We have identified a mutation in tRNA ligase that disrupts the UPR in the yeast Saccharomyces cerevisiae. In this mutant, splicing of *HAC1* mRNA, but not tRNA, is blocked. In contrast, *HAC1* mRNA splicing is not impaired in cells that are blocked in spliceosome-mediated mRNA splicing. Furthermore, the splice junctions of *HAC1* mRNA do not conform to the consensus sequences of other yeast pre-mRNAs. Our results suggest that the regulated splicing of *HAC1* mRNA occurs by a novel pathway, involving tRNA ligase and bypassing the spliceosome.

Introduction

When unfolded proteins accumulate in the endoplasmic reticulum (ER), cells respond by increasing the transcription of genes encoding ER-resident proteins that assist in protein folding. This unfolded protein response (UPR) pathway monitors the concentration of unfolded proteins in the ER lumen and transduces a signal to the transcriptional apparatus in the nucleus. In Saccharomyces cerevisiae, the ER-resident proteins that are known to be induced upon activation of the UPR are Kar2p (or BiP encoded by *KAR2*), Pdi1p (endoded by *PDI1*), Eug1p (a PDI-like protein, encoded by *EUG1*), and Fkb2p (a peptidyl–prolyl *cis-trans* isomerase) (reviewed by McMillan et al., 1994; Shamu et al., 1994; Sweet, 1993).

Experimentally, the accumulation of unfolded proteins in the ER can be induced by various treatments, as follows: first, by preventing protein glycosylation with the addition of drugs such as tunicamycin (Tm); second, by preventing disulfide bond formation with reducing agents; and third, by expressing mutant secretory proteins that do not fold properly and thus accumulate in the ER. The genes encoding ER-resident proteins that are coordinately regulated by the UPR share a common upstream activating sequence, the unfolded protein response element (UPRE). This element is both necessary and sufficient to activate transcription in response to the accumulation of unfolded proteins in the ER (Kohno et al., 1993; Mori et al., 1992).

In addition to the UPRE, two other components have been identified in the yeast S. cerevisiae that are required for this signaling pathway: *IRE1* (encoding a transmembrane serine–threonine kinase) and *HAC1* (encoding a DNA binding protein with homology to the leucine zipper family of transcription factors) (Cox et al., 1993; Cox and Walter, 1996 [this issue of *Cell*]; Mori et al., 1993). Ire1p lies in the ER or inner nuclear membrane (or in both) and transmits the UPR signal across the ER membrane by a mechanism similar to those found in transmembrane kinases in the plasma membranes of higher eukaryotic cells (Shamu and Walter, 1996). Hac1p binds to the UPRE in the promoters of ER-resident chaperone genes activating their transcription. We recently found that Hac1p activity is controlled by regulated splicing of its mRNA. Upon induction of the UPR, a 252 bp nucleotide intron is removed, which leads to production of a new form of Hac1p containing a different C-terminal tail. This new tail renders Hac1p resistant to the rapid destruction that is observed in uninduced cells. The regulated processing of *HAC1* mRNA requires a functional and activated Ire1p transmembrane kinase. Thus, activation of the pathway results in a stable form of Hac1p that binds to the UPRE of target genes and increases their transcription (Cox and Walter, 1996).

Neither *IRE1* nor *HAC1* is required for viability of cells grown on rich medium; however, they are essential under conditions that induce accumulation of unfolded proteins in the ER (Cox et al., 1993; Cox and Walter, 1996; Mori et al., 1993). This can be achieved in several ways, including reducing the cellular levels of ER chaperones (Beh and Rose, 1995; Craven et al., 1996). Here, we describe the results of a genetic screen for synthetic lethality that is based on this observation. We report the identification of a new component of the UPR, namely tRNA ligase. We propose that tRNA ligase is directly involved in the regulated splicing of *HAC1* mRNA.

Results

Isolation of Mutants Defective in the UPR

ER-resident proteins are retained in the ER by virtue of their conserved C-terminal ER retention signal, HDEL. The four amino acid peptide mediates the retrieval from the early Golgi apparatus of the ER-resident proteins that have exited the ER. When the HDEL sequence is deleted, ER-resident proteins are secreted from cells (Hardwick et al., 1990; Pelham, 1989, 1990). Cells that express an HDEL-less version of Kar2p (BiP), encoded by *kar2–ΔHDEL*, as the only version of Kar2p grow at normal rates, but induce the UPR to increase the synthesis of Kar2p (Hardwick et al., 1990). This compensates for the loss of Kar2p from the ER owing to secretion. Indeed, this activation of the UPR is important, because no viable spores bearing both *Δire1* and the *kar2–ΔHDEL* alleles are produced from diploid cells heterozygous in both loci (Beh and Rose, 1995). To identify new components of the UPR pathway, we therefore designed a genetic screen to isolate mutations that, like *Δire1*, are lethal when combined with the *kar2–ΔHDEL* mutation.

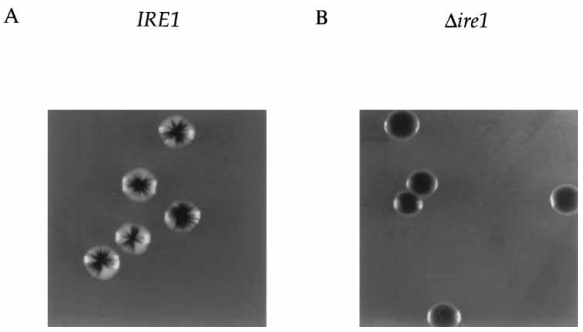

Figure 1. Mutants in the UPR Are Nonsectoring

A haploid *ade2 ade3* yeast strain carrying a *kar2–ΔHDEL* allele as the genomic copy and containing a centromeric plasmid with wild-type *KAR2* and *ADE3* genes was used in the screen (CF109). Cells were plated on low adenine–containing plates (2 μg/ml) and incubated at 30°C for 5–7 days. Strains with a functional UPR pathway can lose the plasmid as indicated by the appearance of white sectors in the colony owing to the *ade2 ade3* genotype (A). In contrast, strains that cannot induce the UPR, such as the congenic *Δire1* strain shown (CF110), cannot lose the *ADE3*-containing plasmid, and therefore nonsectoring red colonies are formed (B).

For the screen, we used a reporter strain carrying a *kar2–ΔHDEL* allele and *ade2 ade3* mutations in the genome and containing a plasmid carrying both wild-type *KAR2* and *ADE3* genes. The *ade2* mutation results in formation of a red pigment, which is no longer produced when the function of *ADE3* is also impaired. Thus, growth of this strain on plates containing low concentrations of adenine gives rise to sectoring colonies (Figure 1A), because spontaneous loss of the plasmid, and therefore the *ADE3* marker, results in cells that no longer develop red pigment (Koshland et al., 1985). As expected, when *IRE1* was disrupted in this strain, only red nonsectoring colonies were observed (Figure 1B), because cells having lost the plasmid will not grow to produce a white sector.

In contrast with previous reports that monitored spore germination, we found, however, that *Δire1 kar2–ΔHDEL* double mutant cells were viable (data not shown). Thus, formally the combination of the *Δire1* and *kar2–ΔHDEL* mutations is not synthetically lethal. For the purposes of this genetic screen, however, their growth rate was sufficiently reduced to yield a nonsectoring phenotype in our assay (Figure 1B).

Cells were mutagenized by irradiation with UV light (15% survival). From 20,000 colonies screened, we isolated 17 mutants that gave rise to nonsectoring colonies. Mutants were then tested in a secondary screen for lack of induction of the UPRE–*lacZ* reporter upon treatment with Tm. Of three mutants that were unable to induce this reporter, one belonged to a novel complementation group, as diploids heterozygous for this mutation and either *Δire1* or *Δhac1* exhibit the ability to induce the UPR. Additional complemetation tests showed that the other two isolated mutants were allelic to *ire1*. For reasons outlined below, we henceforth refer to the novel mutant as *rlg1-100*.

Characterization of *rlg1-100* Mutant Cells

rlg1-100 cells grew at normal rates and, like *ire1* and *hac1* mutants, were impaired in the induction of endogenous *KAR2* mRNA upon Tm treatment (Figure 2A; Cox and Walter, 1996) and were unable to grow on plates lacking inositol (Figure 2B). Thus, they showed phenotypes indistinguishable from cells carrying mutations in other components of the UPR.

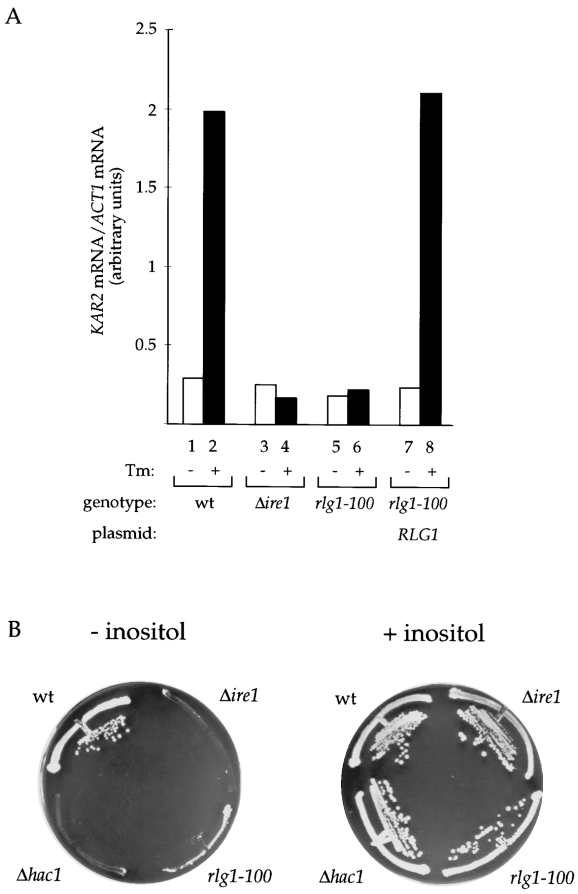

Figure 2. Characterization of the *rlg1-100* Mutant Strain

(A) Northern hybridization was performed on RNA isolated from the following strains: wt (JC102); *Δire1* cells (CS243); *rlg1-100* cells (CF181); and *rlg1-100* cells carrying a centromeric vector pCF138 containing wild-type *RLG1*. Each strain was grown to mid-log phase and incubated in the presence or absence of Tm for 2.5 hr. Total RNA was extracted and analyzed by Northern hybridization using DNA specific probes for *KAR2* and *ACT1*. The data was quantitated and *KAR2* mRNA levels were normalized to *ACT1* mRNA levels.

(B) Cells from wt (JC102), *Δire1* (CS243), *Δhac1* (JC402), and *rlg1-100* (CF181) strains were streaked for single colonies on plates containing either 100 μg/ml inositol (+inositol) or no inositol (−inositol). Plates were incubated at 30°C for 2 days and photographed.

To identify the mutant gene, we cloned genomic DNA fragments that complemented in low copy the UPR defects in *rlg1-100* cells. Subcloning revealed one gene, *RLG1*, that restored the induction of *KAR2* mRNA transcription upon Tm treatment (Figure 2A, lanes 7 and 8). The identification of this gene was surprising, because *RLG1* encodes tRNA ligase, a previously characterized essential protein that is required for pre-tRNA splicing (Phizicky et al., 1992). To ascertain whether *rlg1-100* is indeed a mutant allele of *RLG1*, we cloned the mutant gene. Sequencing revealed a single point mutation (C442T) that is predicted to change a conserved histidine at position 148 in tRNA ligase to tyrosine. To prove unambiguously that the H148Y mutation is responsible

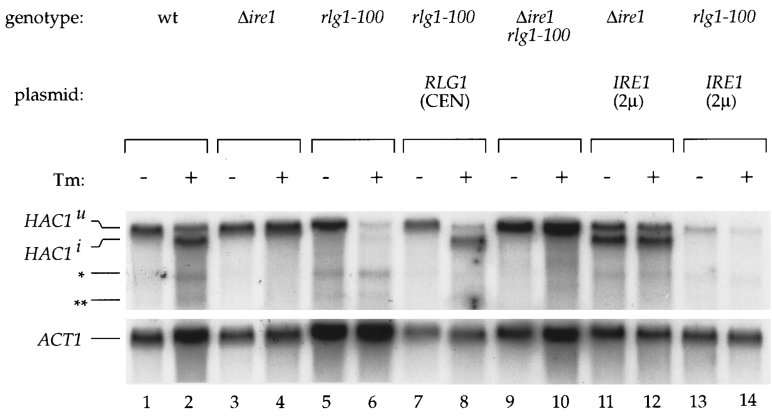

Figure 3. *HAC1* mRNA Splicing Is Blocked in *rlg1-100* Cells

Northern hybridization was performed on RNA isolated from the following strains: wt (JC102), lanes 1 and 2; Δ*ire1* (CS243), lanes 3 and 4; *rlg1-100* (CF181), lanes 5 and 6; *rlg1-100* (CF181) carrying a centromeric plasmic with wild-type *RLG1* (pCF138), lanes 7 and 8; Δ*ire1 rlg1-100* double mutant (CF203), lanes 9 and 10; Δ*ire1* carrying a high copy number (2μ) plasmid pCS122 with wild-type *IRE1,* lanes 11 and 12; and *rlg1-100* (CF181) carrying this same pCS122 vector, lanes 13 and 14. Each strain was grown to mid-log phase and incubated in the presence (even numbered lanes) or absence (odd numbered lanes) of Tm for 20 min. Total RNA was extracted and analyzed by Northern hybridization using DNA specific probes for *HAC1* and *ACT1*. Faint bands (denoted with asterisks) may represent the 5′ exon alone (two asterisks) and the 5′ exon plus the IVS (one asterisk) (Cox and Walter, 1996). It is not clear whether these bands correspond to splicing intermediates or dead-end products.

for the observed phenotype, we reconstructed the mutation using site-directed mutagenesis. Indeed, Δ*rlg1* cells bearing *rlg1*(H148Y) on a low copy plasmid grew at normal rates but were unable to induce the UPR, whereas, as expected, Δ*rlg1* cells bearing wild-type *RLG1* on the plasmid could induce the UPR (data not shown). Thus, the phenotype of *rlg1*(H148Y) cells is indistinguishable from that of *rlg1-100* cells. Based on these results, we conclude that the single identified mutation in tRNA ligase is sufficient to cause loss of the UPR and therefore named the mutant *rlg1-100,* consistent with it being a new allele of *RLG1*.

Effects of the *rlg1-100* Mutation on *HAC1* mRNA Processing

The identification of an RNA-processing enzyme as a component of the UPR was particularly intriguing in light of the discovery that a step in the pathway involves the regulated splicing of *HAC1* mRNA (Cox and Walter, 1996). Therefore, to assess directly whether tRNA ligase is involved in *HAC1* mRNA processing, we examined the fate of *HAC1* mRNA in *rlg1-100* mutant cells by Northern hybridization (Figure 3). Wild-type cells contain unspliced *HAC1*^{u} mRNA (Figure 3, lane 1), which upon induction of the UPR by addition of Tm is processed to the smaller spliced *HAC1*^{i} mRNA (Figure 3, lane 2) (Cox and Walter, 1996). This conversion is abolished in Δ*ire1* cells, which cannot transmit the signal induced by unfolded proteins across the ER membrane (Figure 3, lanes 3 and 4). Interestingly, in *rlg1-100* mutant cells, activation of the UPR leads to the disappearance of unspliced *HAC1*^{u} mRNA, but, in contrast with wild-type cells, no spliced *HAC1*^{i} mRNA is produced upon induction of the UPR (Figure 3, lanes 5 and 6). Importantly, the level of actin mRNA, which is a substrate of the conventional splicing machinery, was not affected in *rlg1-100* cells upon induction of the UPR (see ACT1 in Figure 3, lanes 5 and 6), indicating that the effect of the mutation is specific for *HAC1* mRNA.

Expression of an intron-less *HAC1*^{i} gene in the *rlg1-100* mutant strain results in constitutive activation of the pathway (Cox and Walter, 1996). Northern hybridization confirmed that the intron-less *HAC1*^{i} mRNA is stably expressed in *rlg1-100* mutant cells in both the absence and presence of Tm (data not shown). Thus, we can exclude the possibility that spliced *HAC1*^{i} mRNA is formed in the *rlg1-100* mutant but is then rapidly degraded. We therefore conclude that the *rlg1-100* mutation blocks production of spliced *HAC1*^{i} mRNA.

Because the *HAC1* mRNA splicing defect is only observed upon induction of the UPR, it must require transmission of the signal resulting from the accumulation of unfolded proteins in the ER to the splicing machinery. The proposed early role of Ire1p in the UPR, along with the lack of *HAC1* mRNA splicing in Δ*ire1* cells, predicts that Ire1p functions upstream of tRNA ligase. To establish the order of the components in the pathway directly, we determined the fate of *HAC1* mRNA in an *rlg1-100* Δ*ire1* double mutant (Figure 3, lanes 9 and 10). As expected, the double mutant strain behaved identically to the Δ*ire1* mutant, i.e., only unspliced *HAC1* mRNA was observed. Furthermore, overexpression of Ire1p, which turns on the UPR constitutively in the absence of any agents that induce unfolded proteins, results in constitutive splicing of *HAC1* mRNA in wild-type cells (Figure 3, lanes 11 and 12) and leads to constitutive degradation of *HAC1* mRNA in *rlg1-100* mutant cells (Figure 3, lanes 13 and 14). Thus, the regulatory hierarchy of the components of the UPR pathway is *IRE1→RLG1→HAC1*.

tRNA Splicing Is Not Affected in *rlg1-100* Cells

Yeast cells disrupted for *RLG1* are inviable (Phizicky et al., 1992). Thus, although the *rlg1-100* mutation blocks the formation of spliced *HAC1*^{i} mRNA completely, it must not block splicing of essential pre-tRNAs to a similar degree. It is possible that the *rlg1-100* mutation is a partial loss-of-function mutation that affects the UPR more severely than tRNA splicing. Alternatively, the *rlg1-100* allele may be a pathway-specific mutation that affects only *HAC1* mRNA splicing. To distinguish between these two possibilities, we asked whether tRNA splicing is affected in *rlg1-100* cells. To this end, we labeled cells with [^{32}P]-orthophosphate and analyzed the tRNA population by two-dimensional gel electrophoresis. Control cells bearing wild-type *RLG1* driven by a regulated promoter show a discrete and previously well characterized accumulation of tRNA halves and intervening

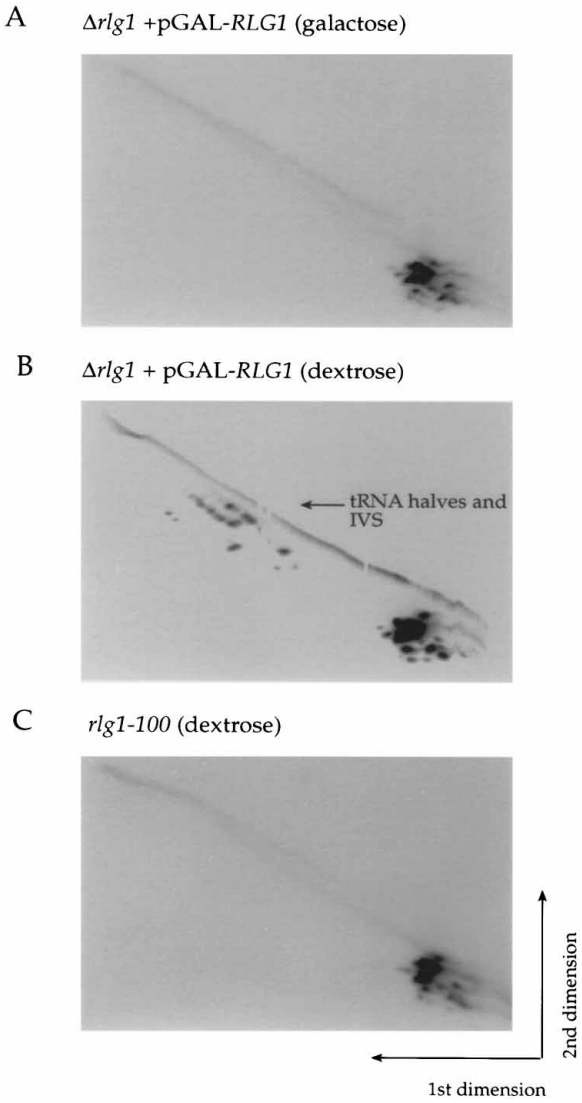

Figure 4. tRNA Splicing Is Not Affected in the *rlg1-100* Mutant Strain

The indicated strains were grown to logarithmic phase in the appropriate growth media and labeled for 30 min with [^{32}P]-orthophosphate as described in Experimental Procedures. Total RNA was extracted and displayed on two-dimensional polyacrylamide gels. First dimension is from right to left; second dimension is from bottom to top. The cluster of spots that is unique to (B) has been previously well characterized, and the identity of individual spots is known (Phizicky et al., 1992). As indicated, they correspond to tRNA halves and IVS.

sequences when expression of *RLG1* is turned off (Figure 4B). In contrast, *rlg1-100* cells do not accumulate any detectable tRNA splicing intermediates and thus behave indistinguishably from cells expressing wild-type *RLG1* (compare Figures 4C and 4A). These results suggest that the *rlg1-100* allele affects the UPR pathway specifically.

Splicing of *HAC1* mRNA Is Not Blocked in *prp2^{ts}* or *prp8^{ts}* Mutant Cells

The data presented so far suggest that tRNA ligase plays a role in *HAC1* mRNA splicing. This suggests that *HAC1* mRNA splicing may occur by a different molecular mechanism than conventional mRNA splicing. This conjecture is further supported by the lack of consensus sequences flanking the splice junctions of *HAC1* mRNA (Figure 5A). In particular, a number of bases that are highly or absolutely conserved in the splice junctions of other yeast mRNAs are not found in *HAC1* mRNA (Figure 5A, indicated by asterisks). Because splice junctions of other mRNAs are recognized by direct base-pairing with snRNAs in the spliceosome, this considerable divergence makes *HAC1* mRNA an unlikely substrate for the conventional splicing machinery. To address this conjecture experimentally, we asked whether mutations that block splicing of other mRNAs also affect splicing of *HAC1* mRNA.

prp2^{ts} cells are mutant in an essential splicing factor, an RNA-dependent ATPase that is required for the first catalytic step of mRNA splicing. In the *prp2^{ts}* mutant, splicing of pre-mRNAs is severely blocked at the nonpermissive temperature, as shown for actin mRNA in Figure 5B (compare lanes 3 and 7). In contrast, splicing of *HAC1* mRNA is not blocked, nor is the total amount of both forms of *HAC1* mRNA noticeably diminished (Figure 5B, lanes 4 and 8). Similarly, when a *prp8^{ts}* mutant strain is shifted to the nonpermissive temperature, actin mRNA splicing is blocked, whereas splicing of *HAC1* mRNA is not affected (Figure 5B, compare lanes 10 and 12). Prp8p functions during both catalytic steps of pre-mRNA splicing, where it is involved in mediating the association of two snRNPs in the spliceosome.

Unexpectedly, we observed that splicing of *HAC1* mRNA was induced even in the absence of tunicamycin at the nonpermissive temperature in both *prp2^{ts}* and in *prp8^{ts}* but not in wild-type cells (Figure 5, compare lanes 7 and 11 with lane 3). This indicates that defects in conventional mRNA splicing lead to induction of the UPR, presumably because some ER protein requires splicing for its biosynthesis. Consistent with this notion, we found that transcription of *EUG1* (encoding a PDI-like ER-resident protein) is induced at the restrictive temperature in both *prp2^{ts}* and *prp8^{ts}* but not in wild-type cells (data not shown). *EUG1* is one of the target genes of the UPR and was selected because it does not contain a heat shock element in its promoter and because its transcription is therefore insensitive to the shift to the nonpermissive temperature of the *prp* mutants.

Because spliced *HAC1^{i}* mRNA is produced at the nonpermissive temperature in both *prp2^{ts}* and *prp8^{ts}* strains even in the absence of Tm, it became important to rule out the possibility that the observed *HAC1^{i}* mRNA was produced prior to the block in Prp2p or Prp8p function. To address this concern, we determined the half-life of *HAC1^{i}* mRNA. We used a strain bearing a temperature-sensitive mutation in RNA polymerase II, in which no new mRNA transcripts are produced at the nonpermissive temperature. Thus, it is possible to measure the rate of mRNA decay by Northern hybridization. As shown in Figure 5C, *HAC1^{i}* mRNA decays with a half-life of ~20 min. The total incubation time in the previous experiment of the *prp2^{ts}* and *prp8^{ts}* strains at the nonpermissive temperature was 2.7 hr. This is longer than the time required to block mRNA splicing in both *prp2^{ts}* and *prp8^{ts}*

cells (1 hr; Jackson et al., 1988; Lee et al., 1984), and considerably longer than the half-time of *HAC1*^{i} mRNA. We therefore conclude that the population of *HAC1*^{i} mRNA observed in Figure 5B (lanes 7, 8, 11, and 12) must have originated after conventional mRNA splicing is blocked. This suggests that splicing of *HAC1* mRNA bypasses the block in spliceosome-mediated splicing that is induced by these mutants.

Discussion

We have identified an additional component that is required for the UPR pathway in yeast, tRNA ligase. We have shown that a single amino acid substitution in tRNA ligase is sufficient to cause a complete loss of the UPR without causing other defects in cell growth. By all criteria examined, the defects of the *rlg1-100* mutant are indistinguishable from strains carrying null mutations in the other two previously identified components of the pathway, *IRE1* and *HAC1*.

Several lines of evidence suggest that tRNA ligase participates directly in the UPR. First, we found that the *rlg1-100* mutation selectively blocks the UPR. The essential function of tRNA ligase in pre-tRNA splicing appears undiminished in *rlg1-100* cells (Figure 4), thus making it unlikely that defects in pre-tRNA processing are indirectly responsible for the observed block in the UPR. Second and most important, the *rlg1-100* mutation selectively affects *HAC1* mRNA processing (Figure 3) that we have shown in the accompanying paper to be an important regulatory step in the UPR (Cox and Walter, 1996). We have shown here that *HAC1* mRNA is specifically degraded in *rlg1-100* cells in a reaction that depends on activation of Ire1p. Third, mutations in *PRP2* and *PRP8* that block spliceosome-mediated processing of pre-mRNAs (Jackson et al., 1988; Lee et al., 1984; Teem et al., 1983) fail to impair *HAC1* mRNA splicing (Figure 5B). Fourth, the splice junctions of *HAC1* mRNA diverge considerably from the consensus sequences found in other pre-mRNAs (Figure 5A; Rymond and Rosbash, 1992). Finally, tRNA ligase is a known RNA-processing enzyme with a well characterized role in pre-tRNA splicing (Greer et al., 1983; Westaway et al., 1988). Based on these observations, we propose a model in which tRNA ligase catalyzes the obligate religation of *HAC1* mRNA halves that are produced upon activation of the UPR (Figure 6). In contrast with tRNA halves, we propose that the *HAC1* mRNA halves are rapidly degraded if they are not religated. This provides a plausible explanation as to why we do not observe the accumulation of stable intermediates. Intriguingly, our model implies that *HAC1* mRNA splicing is catalyzed, at least in part, by components for which no role in mRNA splicing has yet been described. Thus, we propose that *HAC1* mRNA splicing occurs by an unprecedented mechanism that bypasses components of the conventional mRNA splicing machinery.

The *cis* elements in *HAC1* mRNA that make it a substrate for Ire1p-regulated splicing remain to be determined. They are likely to provide invaluable clues that may help identify other mRNAs that may also use this alternative splicing pathway. Also, we note that sequences within the intervening sequence (IVS) of *HAC1* mRNA resemble, but do not match precisely, the branch point consensus sequence for conventional mRNA splicing, UACUAAC. If these sequences prove to be functionally important for *HAC1* mRNA splicing, it would suggest that both the conventional and this new pathway for mRNA splicing share some common features or components (or both).

An important question is how the H148Y mutation in tRNA ligase can exhibit a complete loss-of-function phenotype for the UPR, yet lead to no detectable defects in pre-tRNA processing. tRNA ligase is a multifunctional enzyme that contains three distinct enzymatic activities: a polynucleotide kinase, a cyclic phosphodiesterase, and adenylate synthetase. These activities are arranged in distinct functional domains on a single polypeptide chain encoded by *RLG1*, and catalyze a series of sequential reactions that together result in the joining of tRNA halves (Apostol et al., 1991; Greer et al., 1983; Phizicky et al., 1986; Xu et al., 1990). Although histidine 148 is conserved in tRNA ligase in other yeast species, it maps toward the C-terminal end of the adenylate synthetase domain, distant from residues that are important for the catalytic activity. Its location therefore sheds no immediate light onto its possible function. The absence of observable tRNA splicing defects in the *rlg1-100* mutant strain suggests that the enzymatic activities of the mutant protein required for pre-tRNA splicing are intact. Indeed, extracts of the *rlg1-100* strain have wild-type activity in pre-tRNA splicing (Apostol and Greer [UC Irvine], personal communication). Thus, *HAC1* mRNA splicing must pose structural or functional requirements on tRNA ligase that are dispensable for tRNA splicing.

The function of tRNA ligase in pre-tRNA splicing is preceded by that of tRNA endonuclease, a membrane-bound multisubunit enzyme that cleaves pre-tRNAs at both exon–intron junctions during the first splicing steps (Peebles et al., 1983). Enzymatic analyses suggest that tRNA halves produced by tRNA endonuclease are directly channeled to tRNA ligase to be rejoined. Furthermore, tRNA ligase binds pre-tRNAs with high affinity (Apostol and Greer, 1991). Thus, it has been suggested that tRNA ligase is in a physical complex with tRNA endonuclease in which it binds pre-tRNA substrates, presents them to the endonuclease, and then ligates the halves.

Considering these steps in pre-tRNA processing, several models could explain the exclusive effects of the *rlg1-100* mutation on *HAC1* mRNA splicing. First, the *rlg1-100* mutation may affect an uncharacterized enzymatic activity in tRNA ligase that is required for *HAC1* mRNA splicing but dispensable for pre-tRNA splicing. Second, the *rlg1-100* mutation may abolish a putative obligate interaction of tRNA ligase with *HAC1* mRNA. In this scenario, *HAC1* mRNA and pre-tRNAs may bind, for example, to different sites on tRNA ligase. Finally, the *rlg1-100* mutation may impair an interaction of tRNA ligase with other proteins that are required for the UPR but not for pre-tRNA splicing. Although we cannot formally distinguish between these possibilities, we favor the latter two scenarios, which could explain how *HAC1* mRNA splicing is regulated whereas pre-tRNA splicing occurs constitutively. Further support for the model that the known enzymatic activities of tRNA ligase are required for processing of *HAC1* mRNA comes from the

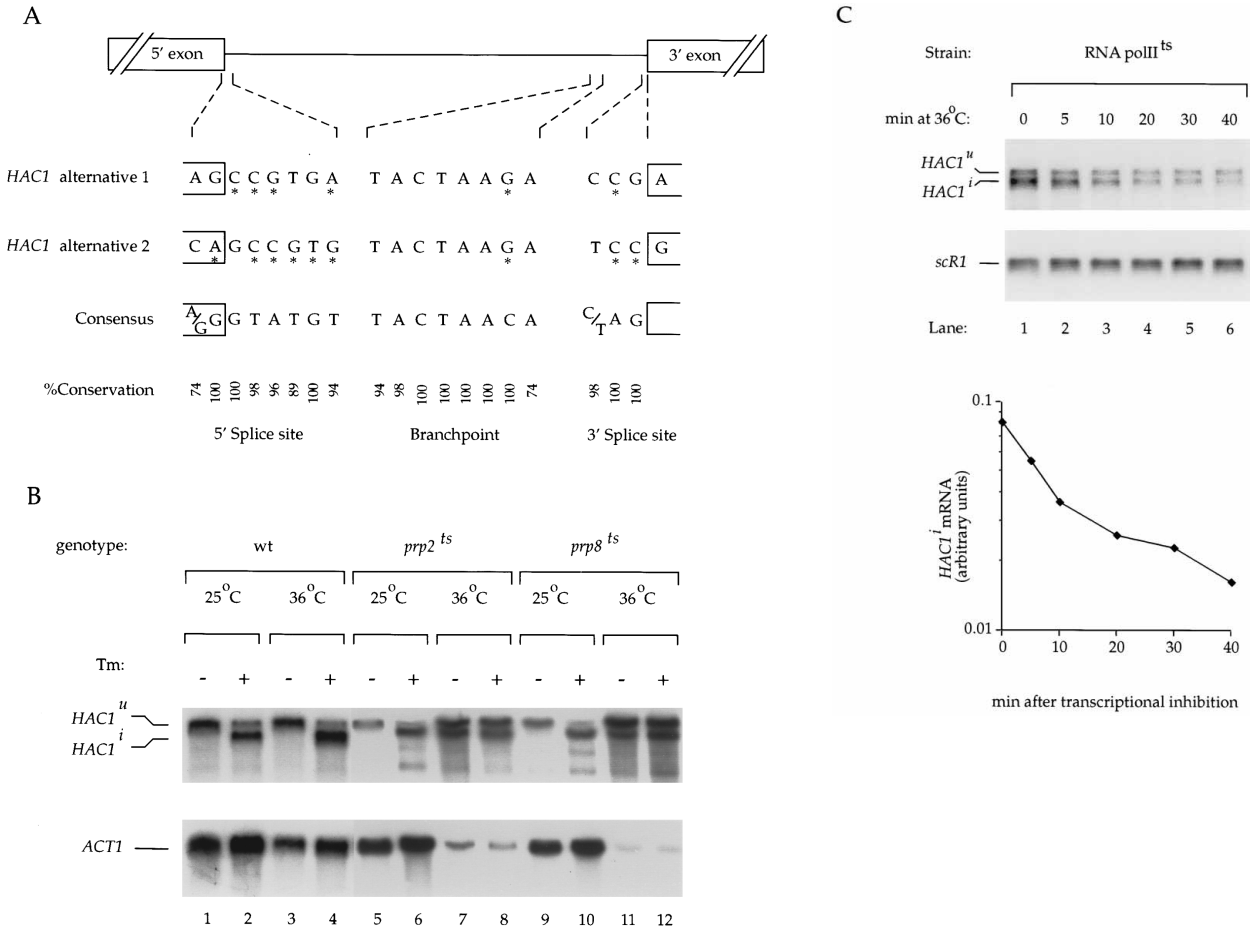

Figure 5. Splicing of *HAC1* mRNA Is Not Affected in Conditional Mutants That Block Spliceosome-Mediated Pre-mRNA Processing

(A) An alignment of the nucleotide sequences flanking the splice junctions in *HAC1* mRNA and the consensus sequences found for spliceosome-mediated mRNA splicing is shown (Rymond and Rosbash, 1992). Spliced *HAC1*^{i} mRNA contains a single G-residue at the exon–exon junction. We can presently not distinguish whether this G-residue is derived from the 5′ or 3′ exon. Thus, there are two possibilities for the exon/IVS junctions that are indicated by the solid and dashed boxes, respectively. The percentage of conservation of nucleotides at the splice junctions and branch point is indicated. The bases in *HAC1* mRNA that differ from well conserved consensus bases are marked by asterisks.

(B) Northern hybridization was performed on total RNA isolated from wild type (JC102), lanes 1–4; *prp2-1* (EJS42), lanes 5–8; and *prp8-1* (YEJS17), lanes 9–12; cells. Each strain was grown at the permissive temperature (25°C) or restrictive temperature (36°C) for 2 hr and then treated with (even numbered lanes) or without (odd numbered lanes) 10 μg/ml Tm for an additional 40 min. RNA was extracted and analyzed by Northern hybridization using DNA-specific probes for *HAC1* and *ACT1*.

(C) JC218 cells bearing a temperature-sensitive allele of *RBP1* encoding RNA polymerase II were treated with Tm for 20 min and then shifted to the nonpermissive temperature. At different time points after temperature shift, RNA was extracted and analyzed by Northern hybridization. Blots were probed for *HAC1* mRNA and, as a control, for the RNA polymerase III transcript *SCR1* RNA. *HAC1*^{i} mRNA was quantitated and normalized to *SCR1* RNA.

observation that a tRNA ligase mutant (K119S, changing an essential lysine residue required for adenylate synthetase activity), which is inactive for joining of tRNA halves, fails to complement the *HAC1* mRNA processing defects of the *rlg1-100* mutant strain (Apostol and Greer [UC Irvine], personal communication).

HAC1 mRNA splicing is initiated by an Ire1p-mediated event that leads to nucleolytic cleavage. In principle, tRNA endonuclease could perform one or both of the required *HAC1* mRNA cleavage steps. According to this view, Ire1p would somehow regulate access of the substrate to the constitutively active endonuclease. Alternatively, tRNA ligase may interact with another endonuclease, different from tRNA endonuclease, that cleaves *HAC1*^{u} mRNA. A particularly exciting possibility is suggested by sequence similarities between the essential C-terminal tails of Ire1p kinase and mammalian RNase L, a nuclease that also contains a kinase domain and is, like Ire1p, activated by oligomerization (Bork and Sander, 1993; Dong and Silverman, 1995; Zhou et al., 1993). Based on these structural and functional similarities, we speculate that Ire1p may itself be the endonuclease that cleaves *HAC1*^{u} mRNA and thus initiates the splicing reaction directly. Oligomerization of Ire1p kinases could, for example, activate the putative C-terminal RNase domain by phosphorylation, or inactivate putative RNase inhibitors. We are currently developing in vitro assays to test this hypothesis directly.

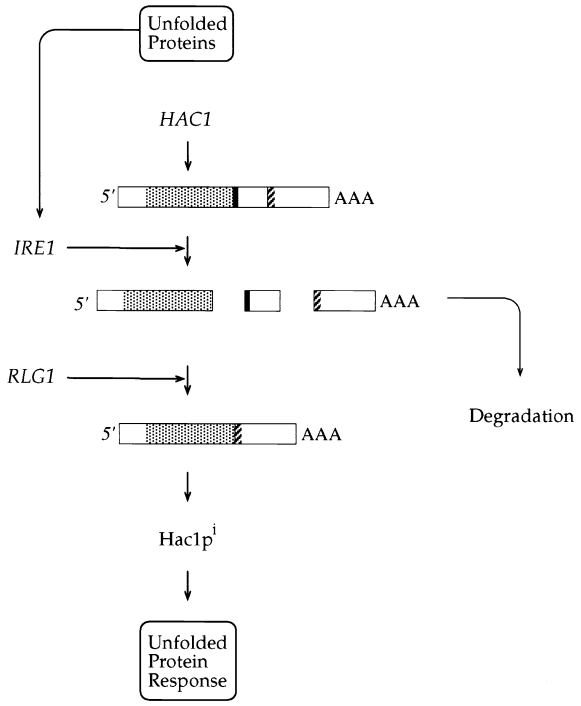

Figure 6. Model for tRNA Ligase Function in the UPR Pathway

The accumulation of unfolded proteins in the ER activates an Ire1p-dependent nucleolytic cleavage of *HAC1* mRNA. Ire1p could be directly involved in the cleavage event or, alternatively, may be required to activate a yet unknown nuclease. *HAC1* mRNA halves are then religated by tRNA ligase to generate the spliced form of this mRNA. If religation is blocked, the splicing intermediates are rapidly degraded in vivo. Spliced *HAC1* mRNA results in production of a more stable form of Hac1p that binds to the UPRE and thus activates transcription of the target genes of the UPR. Stippled, filled, and hatched boxes represent Hac1p coding information. Splicing of *HAC1* mRNA results in the removal of 10 codons from $HAC1^u$ (black) with the concomitant addition of 18 codons (diagonally striped) to produce $HAC1^i$.

In wild-type cells expressing endogenous *HAC1*, we find that about 50%–60% of the *HAC1* RNA molecules are spliced when the UPR is induced by Tm treatment for 20 min. No increase in the absolute level of spliced $HAC1^i$ mRNA was observed in cells overexpressing *HAC1* mRNA from a 2μ plasmid when Tm-treated for the same time period (data not shown). This indicates that the splicing machinery is normally saturated when the UPR is induced. In contrast, we observed that virtually all *HAC1* RNA is degraded in *rlg1-100* cells when Ire1p is activated upon Tm treatment (Figure 3, lane 6). This apparent paradox can be most easily explained if one assumes that *HAC1* mRNA ligation is the rate-limiting step of the overall splicing reaction. In *rlg1-100* mutants, the rate of nucleolytic cleavage may therefore no longer be limited by ligation, resulting in a more efficient processing, and hence degradation, of $HAC1^u$ mRNA. Thus, our data suggest that during splicing of $HAC1^u$ mRNA, cleavage and religation are mechanistically coupled.

HAC1 mRNA splicing, like the splicing of all other cytoplasmic RNAs, most likely occurs in the nucleus. Sequestering the splicing machinery in a different compartment confers the advantage, unique to eukaryotic cells, that access of ribosomes to the RNA is restricted until it has been properly processed. In the accompanying paper, we propose that both unspliced $HAC1^u$ RNA and spliced $HAC1^i$ RNA are translated. Thus, in contrast with other pre-mRNAs, $HAC1^u$ RNA is a functional mRNA. We therefore propose that the activated *HAC1* mRNA splicing machinery catches $HAC1^u$ mRNA during export from the nucleus to the cytosol. Indeed, enzymes involved in pre-tRNA splicing and modification are found in close proximity to nuclear pores, suggesting a link between export and processing (Clark and Abelson, 1987; Simos et al., 1996). Therefore, if our speculation is correct and Ire1p is directly involved in $HAC1^u$ mRNA processing, we predict that Ire1p, or at least the fraction of the Ire1p molecules that could participate in splicing, lies in the inner nuclear membrane. Ire1p would then transduce the unfolded protein signal directly from the ER lumen into the nucleus.

Experimental Procedures

Media and General Methods

Media, reagents, and general procedures are as described by Cox and Walter (1996).

Plasmid Construction

To make the *kar2–ΔHDEL* integrating vector, a synthetic oligonucleotide was used to amplify the C-terminal domain of *KAR2*, converting the C-terminus of the encoded protein from FEHDEL to FGR (Hardwick et al., 1990). The PCR fragment containing the altered C-terminal domain of *KAR2* and an actin terminator were cloned into YIplac204 (Gietz and Sugino, 1988) to generate pCF104. The wild-type *KAR2*, *ADE3*, and *URA3* sectoring plasmid pCF105 is a centromeric vector made by subcloning the *ADE3* gene into the BamHI site of pMR397 (Rose et al., 1989).

pCF138 was constructed by inserting a genomic XhoI–SacI fragment containing *RLG1* into the CEN/ARS vector pRS316 (*URA3*) (Sikorski and Hieter, 1989). Similarly, pCF157 was constructed by inserting this fragment into the CEN/ARS vector pRS313 (*HIS3*) (Sikorski and Hieter, 1989).

Yeast Strains

Yeast strains used in this study are listed on Table 1. Reporter strain CF109 was constructed by homologous recombination of pCF104 (linearized with BsmI) into the chromosomal copy of *KAR2* to generate *kar2–ΔHDEL*. Correct integration was confirmed by PCR. To generate strain JC218, strain YAS880 (a gift from A. Sachs) was backcrossed twice to JC103 (Cox and Walter, 1996).

Primary Screen

Strain CF109 was mutagenized with UV light to 15% survival. Cells were plated onto synthetic minimal plates lacking tryptophan and containing 2 μg/ml adenine. Cells were plated at a density of ~300 colonies per plate. Colonies were allowed to grow 5–7 days at 30°C. Individual nonsectoring colonies were restreaked onto fresh plates, and only those mutants that gave rise to uniformly red colonies were studied.

Secondary Screen

Mutant strains were transformed with the UPRE–*lacZ* reporter construct pCF118. This vector is a centromeric derivative of pJC005 (Cox et al., 1993). Yeast patches were replica-plated onto X-Gal/Tm indicator plates and incubated overnight at 30°C. Out of 17 mutants, 3 failed to turn blue on the indicator plates.

Table 1. Yeast Strains

Strain	Genotype	Source/Reference
CF109[a]	*leu2-3,-112; his3-11,-15; trp1-1; ura3-1; ade2-1; can1-100; ade3Δ; kar2-ΔHDEL::TRP; MATα*	This study
CF110	same as CF109 except *ire1::LEU2*	This study
JC102	*trp1-1; his3-11,-15; ura3-1; ade2-1; can1-100; leu2-3,-112::LEU2 UPRE–lacZ; MATα*	Shamu and Walter, 1996
JC402	*trp1-1; ade2-1; can1-100; leu2-3,-112; his3-11,-15::HIS3 UPRE–lacZ; Δhac1::URA3; MATα*	This study
CS243	same as JC102, except *ire1::URA3*	Shamu and Walter, 1996
CF181[a]	*leu2-3,-112; trp1-1; ura3-1; ade2-1; can1-100; ade3Δ; rlg1-100; MATα* and *pUPRE–lacZ LEU2*	This study
CF203	*leu2-3,-112; his3-11,-15; trp1-1; ade2-1; can1-100; rlg1-100; ire1::URA3; MATα*	This study
EJS42	*ade1; ade2; ura1; his7; tyr1; lys2; gal1; prp2-1; MATα*	C. Guthrie
YEJS17	*ade1; ade2; ura1; his7; trp1; lys2; prp8-1; MATα*	C. Guthrie
EMPY439	*ade2-101; his3-Δ200; ura3-52; rlg1-ΔKpn1; GAL$^+$; SUC2; MATα* and pGAL–*RLG1 URA3*	Phizicky et al, 1992
JC218	*ade2; trp1; ura3; leu2-3,-112::LEU2-UPRE–lacZ; his3-11,-15::HIS3-UPRE–lacZ; rbp1*	This study

[a] Spontaneous mutation his$^-$ derivative cannot be transformed by a *HIS3* containing plasmid.

Rescue of the *rlg1-100* Mutant Phenotype

Strain CF181(*rlg1-100* first backcross) was transformed with a high copy yeast genomic library (Carlson and Botstein, 1982), and colonies were replica-plated to X-Gal/Tm plates. Colonies that turned blue were tested for their ability to grow on plates lacking inositol. Of 7,000 colonies screened, 6 turned blue on indicator plates and were also inositol prototrophs. Plasmids from these strains were rescued and sequenced. Three of them were shown to contain *HAC1*. Overexpression of *HAC1* suppresses the defects of the *rlg1-100* mutant. The remaining three plasmids had overlapping inserts, each containing the *RLG1* open reading frame. A fragment containing *RLG1* was subcloned into a low copy vector (pCF138) and shown to complement the defects of the *rlg1-100* mutant. Deletion analysis of this plasmid confirmed that the fragment carrying *RLG1* contained the complementing activity.

The *rlg1 (H148Y)* mutation also introduces a stop codon in a small open reading frame (*ORF5)* oriented in the opposite direction of *RLG1*. To confirm that the phenotype observed in the *rlg1-100* mutant strain was due to the amino acid change in the Rlg1p, we made a mutation (A612T, counted from the initiating AUG of the *RLG1* open reading frame) that introduces a stop codon in *ORF5* and that does not change the amino acid sequence of the Rlg1p. This construct restored the ability of the *rlg1-100* strain to induce the UPR, indicating that disruption of the *ORF5* is not responsible for the phenotype observed in the *rlg1-100* mutant strain. Also, a wild-type *RLG1* gene under the control of the *GAL10* inducible promoter (pBM150–RLGX, Phizicky et al., 1992) restored the ability of the *rlg1-100* strain to induce the UPR only when cells were grown in the presence of galactose. Because transcription of the small open reading frame is presumably unaffected by expression of *RLG1* by the *GAL10* promoter, we conclude that disruption of *ORF5* has no effect on the UPR.

Cloning of the Mutant *RLG1* Allele

Genomic DNA was isolated from strain CF181. The genomic copy of the *RLG1* gene was amplified with Vent Polymerase (NEB, Beverly, MA) using three overlapping pairs of oligonucleotides. The PCR fragments were subcloned using the TA cloning method (Invitrogen, San Diego, CA) and sequenced. To distinguish the original mutation from mutations introduced during the PCR procedure, two independent reactions were performed, subcloned, and sequenced for each pair of oligos. Only one mutation was found in two independent PCR reaction subclones (C442T).

Site-Directed Mutagenesis

To reconstruct the C982T mutation, an oligonucleotide ending in an Alw26I site with the desired point mutation in combination with a second oligonucleotide covering a unique HpaI site 5′ to the site of mutation was used in PCR to amplify a 400 bp fragment of *RLG1*. The amplified fragment was then digested with HpaI and Alw26I. The latter restriction enzyme allows generation of a sticky end and removal of the enzyme recognition site from the digested PCR product. Similarly, a second 600 bp PCR fragment was generated using an oligonucleotide ending in an Alw26I site in combination with a second oligonucleotide covering a downstream BglII site in *RLG1*. Digestion of both PCR products with HpaI and Alw26I and with BglII and Alw26I, respectively, left two sticky ends that when ligated restored the wild-type *RLG1* sequence at the junction. Thus, we were able to introduce the fragment with the mutation in the absence of any suitable unique restriction site in its proximity. The HpaI–BglII fragment was then subcloned into a centromeric *HIS3*-containing vector (pRS313) bearing the rest of the *RLG1* coding sequence generating pCF158 (*rlg1*[H148Y]).

Northern Analysis

Total RNA was made according to a scaled-down version of the hot phenol method (Kohrer and Domdey, 1991). RNA was analyzed by electrophoresis in a 6.7% formaldehyde, 1.5% agarose gel and transferred to a Duralon-UV membrane (Stratagene, La Jolla, CA). Hybridization was performed at 65°C overnight in 0.5 M Na_2HPO_4, 1 mM EDTA, 7% SDS [pH 7.5]. Quantitation of Northern blots was performed on a Molecular Imager System GS-363 (BioRad, Hercules, CA).

Detection of tRNA Halves and Intervening Sequences

Labeling of Cells

Strain EMPY439 was grown on minimal media lacking uracil and containing 2% galactose/2% raffinose. The culture was then diluted back into media containing either 2% dextrose or 2% galactose/2% raffinose for an additional six generations before labeling. Strain CF181 was grown on minimal media lacking leucine and containing 2% dextrose. Cells were harvested, washed, and resuspended at a density of 2×10^7 cells/ml in low phosphate medium (Warner, 1991) with the appropriate carbon source, adapted to low phosphate medium for one generation, and labeled with 0.1 mCi/ml [^{32}P]-orthophosphate for 30 min.

Analysis of RNA

Total RNA was isolated from cells and tRNA processing intermediates were displayed on two-dimensional polyacrylamide gels as previously described (Phizicky et al., 1992). Electrophoresis in the first dimension was through 10% polyacrylamide (39:1), 4 M urea in TBE, until the bromophenol blue dye front had migrated a distance of 23 cm. Electrophoresis in the second dimension was through 20% polyacrylamide, 7 M urea, until the xylene cyanol marker was just beginning to elute.

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