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Replacement of the yeast *TRP4* 3′ untranslated region by a hammerhead ribozyme results in a stable and efficiently exported mRNA that lacks a poly(A) tail

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ABSTRACT

The mRNA poly(A) tail serves different purposes, including the facilitation of nuclear export, mRNA stabilization, efficient translation, and, finally, specific degradation. The posttranscriptional addition of a poly(A) tail depends on sequence motifs in the 3′ untranslated region (3′ UTR) of the mRNA and a complex *trans*-acting protein machinery. In this study, we have replaced the 3′ UTR of the yeast *TRP4* gene with sequences encoding a hammerhead ribozyme that efficiently cleaves itself in vivo. Expression of the *TRP4*-ribozyme allele resulted in the accumulation of a nonpolyadenylated mRNA. Cells expressing the *TRP4*-ribozyme mRNA showed a reduced growth rate due to a reduction in Trp4p enzyme activity. The reduction in enzyme activity was not caused by inefficient mRNA export from the nucleus or mRNA destabilization. Rather, analyses of mRNA association with polyribosomes indicate that translation of the ribozyme-containing mRNA is impaired. This translational defect allows sufficient synthesis of Trp4p to support growth of *trp4* cells, but is, nevertheless, of such magnitude as to activate the general control network of amino acid biosynthesis.

Keywords: 3′ untranslated region; general control; hammerhead ribozyme; mRNA processing; poly(A) tail; *Saccharomyces cerevisiae*

INTRODUCTION

The poly(A) tail serves different purposes for mRNAs on their way to being translated into protein. Polyadenylation is thought to be necessary for efficient export of mRNA from the nucleus (Ullman et al., 1997; Hilleren et al., 2001) and plays a role in the maintenance of mRNA stability in the cytoplasm (Decker & Parker, 1993; Brown & Sachs, 1998). Moreover, efficient mRNA translation also requires a poly(A) tail (Gallie, 1991; Iizuka et al., 1994; Jacobson, 1996). Lack of a poly(A) tail can, therefore, result in unstable poorly translated mRNA and reduced amounts of gene product.

The 3′ untranslated region (3′ UTR) of an mRNA plays an important role in 3′ end processing of eukaryotic transcripts. Sequence motifs, located in the 3′ UTR, are necessary for cleavage at a specific site and the addition of a poly(A) tail (for reviews, see Wahle & Rüegsegger, 1999; Zhao et al., 1999). The sequence motifs of yeast transcripts that direct cleavage and polyadenylation are not as conserved as in higher eukaryotes. Several yeast 3′ untranslated regions were shown to carry degenerate motifs that can at least partially replace one another (Egli et al., 1995; Guo et al., 1995). Deletion of the 3′ UTR usually results in a significant reduction of the amount of gene product. A deletion of the 3′ UTR of the *TRP4* gene led to a reduced amount of gene product without affecting cellular growth rates (Düvel et al., 1999).

A large multiprotein complex performs cleavage and polyadenylation of pre-mRNAs in eukaryotes (Keller & Minvielle-Sebastia, 1997; Zhao et al., 1999). A subset of these factors affects only the polyadenylation efficiency whereas others are necessary for both cleavage and polyadenylation. A complex pattern of interactions

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connects the different components with each other and cooperative interactions between the processing factors have been observed (Kessler et al., 1997). As a consequence, cleavage and polyadenylation are coupled processes in vivo. However, an uncoupling of cleavage and polyadenylation was achieved in vivo in yeast by using an artificial hammerhead ribozyme as a mRNA 3′ end processing signal (Egli & Braus, 1994).

Ribozymes are RNAs with an enzymatic activity for RNA cleavage (Altman, 1990; Cech, 1990). Hammerhead ribozymes are a subset of self-cleaving ribozymes that were originally isolated from plant viroids (Symons, 1992). Hammerhead ribozymes consist of three stem-loop-forming regions and two highly conserved single-stranded sequences. Cleavage occurs at a triplet sequence, usually GUC. Hydrolysis of the phosphodiester bond results in a 2′-3′-cyclic phosphate and a 5′-hydroxyl product (Tanner, 1999). This is in contrast to enzymatic cleavage by processing factors that generally result in a 3′-hydroxyl and a 5′-phosphate. Because the catalytic action of ribozymes has been studied thoroughly, new ribozymes were constructed providing applications in biotechnology and medicine. *Trans*-acting ribozymes, for example, cleave target mRNAs at specific cleavage motifs, and are widely used to decrease the amount of the mRNA of genes of interest (Birikh et al., 1997; Tanner, 1999).

The hammerhead ribozyme used in this study cleaves itself efficiently in yeast (Egli & Braus, 1994). This prompted us to determine whether a hammerhead ribozyme could replace the normal mechanism of 3′ end formation in an authentic yeast transcript. We have now found that replacing the 3′ UTR of the *TRP4* gene with a hammerhead ribozyme results in a stable, nonpolyadenylated transcript that is capable of being exported from the nucleus, but unable to promote normal efficiencies of translation.

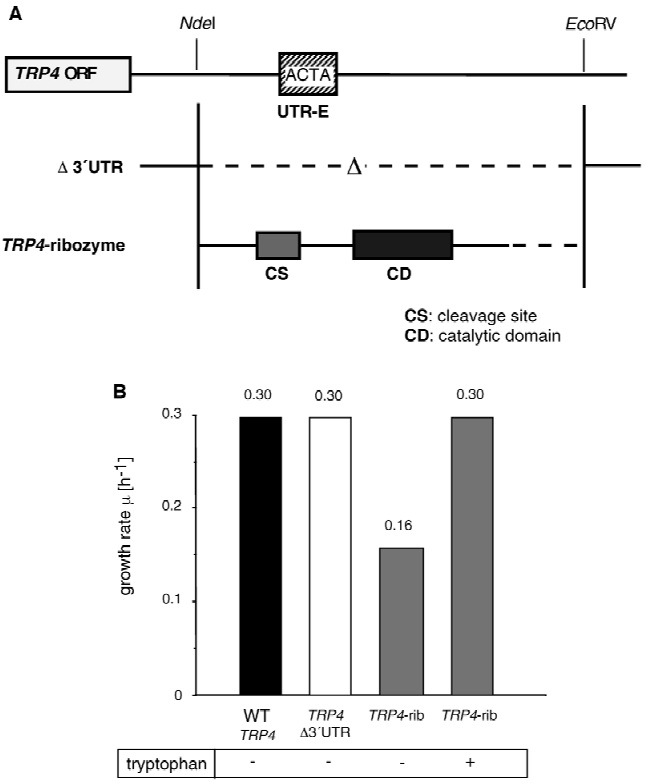

FIGURE 1. A: Schematic presentation of the different *TRP4* alleles used in this study: the *TRP4* gene carrying its own 3′ UTR, a *TRP4* gene with a deletion of the 3′ UTR (Δ3′ UTR), and a *TRP4* gene fused to an artificial hammerhead ribozyme (*TRP4*-ribozyme). The region between the *Nde*I and the *Eco*RV restriction sites spans 147 bp and includes the processing efficiency motif, UTR-E, and all poly(A) sites. The hammerhead ribozyme consists of 80 bp and carries a catalytic domain (CD) that is necessary for processing at the cleavage site (CS). The entire *TRP4* gene as well as the modified alleles were cloned into a single-copy plasmid. **B:** Growth rate (μ) of strains carrying the WT *TRP4* gene, the *TRP4* Δ3′ UTR, and the *TRP4*-ribozyme construct, respectively. The strains were grown on minimal medium with or without supplementation of tryptophan, indicated below. The values represent the average of four independent measurements. The standard deviations did not exceed 20%.

RESULTS

The *TRP4* gene of yeast encodes anthranilate-phosphoribosyl-transferase (PR-transferase, E.C. 2.4.2.18), the enzyme that catalyzes the second step in the biosynthesis of tryptophan (Furter et al., 1986). The 3′ UTR of the *TRP4* mRNA include a single-sequence motif that is strictly necessary for 3′-end processing (Egli et al., 1997). Deletion of 147 bp of the *TRP4* 3′ UTR, including this sequence motif and all wild-type poly(A) sites, leads to a two- to threefold reduction of mRNA level and Trp4p enzyme activity (Düvel et al., 1999). In this study, the *TRP4* 3′ UTR was replaced with sequences encoding an artificial 80-bp self-cleaving hammerhead ribozyme (Fig. 1A). The aim of this work was to test if a hammerhead ribozyme can act as an alternative 3′-end processing element and be capable of increasing *TRP4* gene expression compared to a construct containing deletion of the *TRP4* 3′ UTR (*TRP4* Δ3′ UTR).

The *TRP4*-ribozyme allele partially complements a *trp4* deletion in yeast

The *TRP4*-ribozyme construct was cloned into the single copy vector pRS315 (Sikorski & Hieter, 1989) and transformed into the yeast strain RH2063 carrying a deletion of the entire *TRP4* gene, including the 3′ UTR. In addition to the deletion of the chromosomal *TRP4* locus, RH2063 also contains the *gcn4-101* allele that lacks functional Gcn4p. *GCN4* encodes the transcriptional activator for the general control of amino acid biosynthesis and induces transcription of the *TRP4* gene during starvation for amino acids. As a consequence, these cells cannot react to tryptophan starvation by activating the general control system.

To compare the growth of the strain bearing the *TRP4*-ribozyme allele with strains carrying wild-type (WT)

TRP4 or the *TRP4* Δ3′ UTR allele, cultures were grown in minimal medium with or without tryptophan supplementation. All strains tested were able to grow. However, when the strain bearing the *TRP4*-ribozyme allele was grown in medium lacking tryptophan, its growth rate was approximately half that of the wild-type strain, with growth rates of 0.3 h^{-1} and 0.16 h^{-1} respectively (Fig. 1B). As reported previously, the growth rate of the *TRP4* Δ3′ UTR strain was similar to that of the wild-type cells (Düvel et al., 1999; Fig. 1B). Addition of tryptophan to the medium restored the growth rate of cells with the *TRP4*-ribozyme allele to that seen for wild-type cells (Fig. 1B). Therefore, the slow growth phenotype observed for cells expressing the *TRP4*-ribozyme allele is due to tryptophan starvation.

Expression of the *TRP4*-ribozyme allele results in high amounts of nonpolyadenylated RNA but reduced enzyme activity

Expression of the *TRP4*-ribozyme allele was assessed by northern hybridization experiments using total RNA. The steady-state level of the *TRP4*-ribozyme mRNA was compared to that of WT *TRP4* and *TRP4* Δ3′ UTR mRNA derived from strains with a *gcn4-101* background (Fig. 2A). The *TRP4*-ribozyme mRNA was 85% that of WT *TRP4,* which was almost threefold more than the amount detected for the *TRP4* Δ3′ UTR mRNA (Fig. 2A). The use of cells bearing the *gcn4-101* allele ensured that the high amount of *TRP4*-ribozyme mRNA

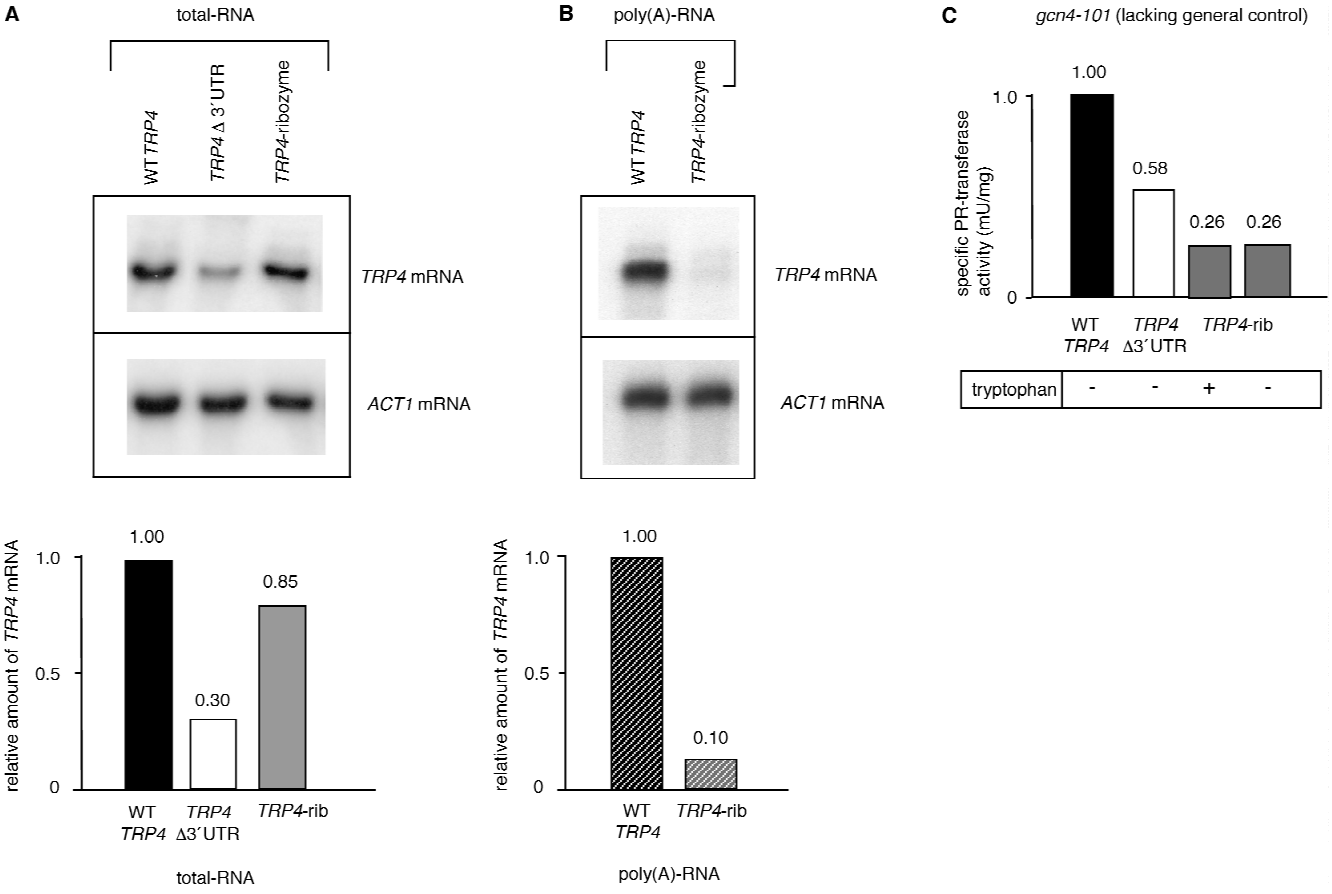

FIGURE 2. A: Northern hybridization analysis of total RNA from strains bearing the WT *TRP4*, *TRP4* Δ3′ UTR, or *TRP4*-ribozyme constructs in a *gcn4-101* background. Cells were cultivated in minimal medium without tryptophan supplementation. To detect the *TRP4* mRNA, a 1.5-kb *Mlu*I genomic DNA fragment spanning the entire *TRP4* gene was used as probe. The *ACT1* mRNA visualized by a 0.4-kb *Cla*I fragment of the *ACT1* ORF served as constitutive control. Quantification of hybridizations of at least five different RNA isolations was performed by phosphorimager analysis and is shown below. **B:** Northern hybridization analysis with poly(A)-enriched RNA of WT *TRP4* and *TRP4*-ribozyme strains. The probes to detect *TRP4* and *ACT1* mRNA were the same as those in **A**. The quantification represents four independent hybridization experiments. **C**: Specific PR-transferase activity (mU/mg protein) of yeast strains bearing WT *TRP4*, *TRP4* Δ3′ UTR, or *TRP4*-ribozyme, respectively, in a *gcn4-101* background. Supplementation of tryptophan is indicated below. The data represent the average of four independent measurements whose standard deviations did not exceed 15%.

is not the result of Gcn4p-activated transcription caused by tryptophan starvation. In support of this conclusion, there were no differences in the levels of *TRP4* mRNA in cells that were grown in the presence or absence of tryptophan (data not shown). These results indicate that there is no additional mechanism to react to tryptophan starvation that would result in an increase in *TRP4* mRNA levels.

A northern hybridization analysis with poly(A)-enriched RNA was performed to determine whether the self-cleaved transcript of the *TRP4*-ribozyme allele carries a poly(A) tail (Fig. 2B). The *TRP4*-ribozyme RNA revealed only a very faint signal, suggesting that the transcripts were not efficiently polyadenylated. Quantification of the poly(A)-enriched mRNA compared to the signal intensities of total RNA hybridization demonstrated that no more than 10% of the *TRP4*-ribozyme transcripts were polyadenylated. In comparison, the polyadenylation efficiency of the WT *TRP4* mRNA was very high (Fig. 2B). Rapid amplification of cDNA ends (RACE) experiments, performed with a poly(T)- and a *TRP4*-specific primer did not result in any detectable *TRP4*-ribozyme cDNAs, regardless of whether total RNA or poly(A)-enriched RNA was used as a template. We conclude that the presence of the ribozyme greatly reduces the polyadenylation efficiency of this transcript, resulting in an mRNA population largely lacking poly(A) tails.

To compare gene expression at the protein level, specific PR-transferase activities derived from the different *TRP4* alleles were measured in crude cell extracts. In the *gcn4-101* strain, *TRP4*-ribozyme expression resulted in PR-transferase activity that was 26% of that produced by WT *TRP4* (Fig. 2C). Compared to enzyme levels expressed by the *TRP4* Δ3′ UTR allele, this activity was reduced more than twofold. In this strain, there was no difference whether cells were grown with or without tryptophan (data not shown). Thus, the reduced specific PR-transferase activity produced from the *TRP4*-ribozyme allele did not reflect the nearly wild-type amounts of mRNA observed.

Reduced enzyme activity is caused by low rates of translation initiation

Reduced expression of PR-transferase from *TRP4*-ribozyme mRNA could result from either failure of the mRNA to be exported from the nucleus or from a decrease in translation. To distinguish between these possibilities, cytoplasmic extracts were prepared from cells containing either WT *TRP4* or the *TRP4*-ribozyme allele and fractionated on sucrose gradients (Fig. 3). RNA was isolated from each fraction and analyzed by northern blotting. Across the entire gradient, the relative amount of *TRP4*-ribozyme mRNA was only slightly reduced (90%) compared to WT *TRP4* mRNA, suggesting that the *TRP4*-ribozyme mRNA was efficiently exported from the nucleus. However, whereas the *TRP4* mRNA was predominately localized to the polyribosomes (Fig. 3A), the distribution of the *TRP4*-ribozyme RNA was shifted to lighter fractions and spread throughout the gradient (Fig. 3B). Quantitation of the mRNAs that are specifically engaged with ribosomes (fractions 1 to 5 only) indicated that the level of *TRP4*-ribozyme RNA was reduced to less than half (45%) that of WT *TRP4*. We conclude from these results that the *TRP4*-ribozyme mRNA is a poor substrate for translation initiation.

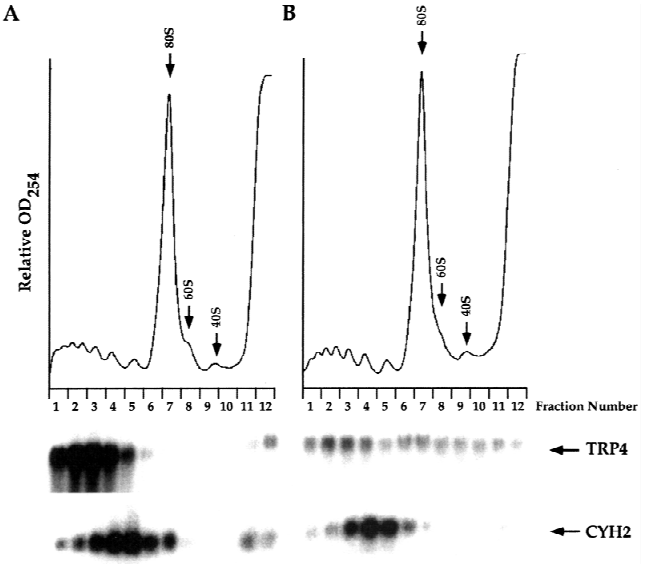

FIGURE 3. Assessment of polyribosome association of *TRP4* mRNA. Cell extracts were prepared and fractionated on 7 to 47% sucrose gradients. **A:** WT *TRP4*. **B:** *TRP4*-ribozyme. Top: Optical density profiles were monitored at 254 nm (OD_{254}), with sedimentation from the right to the left. The positions of the 80S, 60S, and 40S peaks are indicated by arrows. Bottom: Northern analysis of RNA isolated from sucrose gradient fractions. Blots were probed for the mRNAs indicated.

Poor expression of the *TRP4*-ribozyme causes tryptophan starvation and activation of the general control network

The general control network is normally activated when an imbalance in the amount of available amino acids occurs. Activation of the network increases transcription of more than 40 genes involved in amino acid and purine biosynthesis (Hinnebusch, 1997). The reduced Trp4p enzyme levels in yeast cells expressing the *TRP4*-ribozyme allele might result in starvation for tryptophan. To determine if its poor expression triggers an activation of the general control network, the *TRP4*-ribozyme plasmid was transformed into the yeast strain RH2064 that carries a wild-type *GCN4* allele. When these cells were grown in minimal medium lacking tryptophan, the cell growth rate was reduced to 0.18 h^{-1} from 0.3 h^{-1} as observed for cells expressing WT *TRP4*

(Fig. 4). Addition of tryptophan to the cultures restored the growth rate of the cells expressing the *TRP4*-ribozyme construct to wild-type levels (Fig. 4). The reduced growth rate observed for the *TRP4*-ribozyme strains could be due to tryptophan deficiency resulting from insufficient Trp4p enzyme activity or, alternatively, activation of the general control network may lead to altered translation initiation efficiencies (Hinnebusch, 1997). To discriminate between these possibilities, *GCN4* cells, with either the WT *TRP4* or *TRP4*-ribozyme constructs, were grown in medium containing 10 mM 3-amino-1,2,4-triazol (3-AT) to independently activate the general control network. 3-AT causes histidine starvation by acting as a false feedback inhibitor of histidine biosynthesis. When cultured in the presence of 3-AT, growth rates for both the cells expressing the WT *TRP4* or *TRP4*-ribozyme constructs were reduced to a level similar to that observed for cells expressing the *TRP4*-ribozyme allele grown in medium lacking tryptophan (Fig. 4). This suggests that poor expression of the *TRP4*-ribozyme allele most likely results in tryptophan starvation and at least partial activation of the general control network.

To determine whether activation of the general control system is reflected in the amount of *TRP4* mRNA, a northern analysis was performed with RNA isolated from *GCN4* cells. Cells were grown either in the presence or absence of tryptophan and the amount of *TRP4*-ribozyme mRNA was compared to WT *TRP4* mRNA (Fig. 5A). Expression of the *TRP4*-ribozyme in medium with tryptophan supplementation revealed a similar amount of *TRP4* mRNA as the *TRP4*-ribozyme construct in a *gcn4-101* strain background (data not shown). However, the amount of *TRP4* mRNA increases 1.6-fold when the cells were grown without tryptophan (Fig. 5A), suggesting that the cells were starved for tryptophan and the general control network had been activated. An increase in *TRP4*-gene expression, resulting from activation of the general control network, was also observed at the enzyme level. When *GCN4* cells bearing the *TRP4*-ribozyme allele were grown in medium with tryptophan, a PR-transferase activity of 0.26 mU/mg was observed, whereas growth in the absence of tryptophan slightly increased the specific activity to 0.29 mU/mg (Fig. 5B).

To confirm that the general control system was activated in cells bearing the *TRP4*-ribozyme allele, expression of another gene (*HIS3*) regulated by general control was monitored. *HIS3* mRNA levels in yeast strains with the WT *TRP4* increased by a factor of 3.5 after 8 h of incubation with 10 mM 3-AT (Fig. 6). In *TRP4*-ribozyme strains the *HIS3* mRNA level at 0 h was already increased by a factor of three in comparison to wild type (Fig. 6). Addition of tryptophan restored the wild-type level, verifying that the general control was induced by starvation for tryptophan due to poor expression from the *TRP4*-ribozyme construct. The *HIS3* mRNA levels in *TRP4*-ribozyme strains were further increased by addition of 3-AT. After 4 h of incubation, the *HIS3* mRNA increased 4.3-fold in comparison to WT *TRP4* cells without 3-AT (Fig. 6). These data suggest that the *TRP4*-ribozyme allele is sufficiently expressed to allow growth without tryptophan supplementation. However, the reduced amount of intracellular tryptophan is perceived as a starvation signal that partially activates the entire general control network of amino acid biosynthesis.

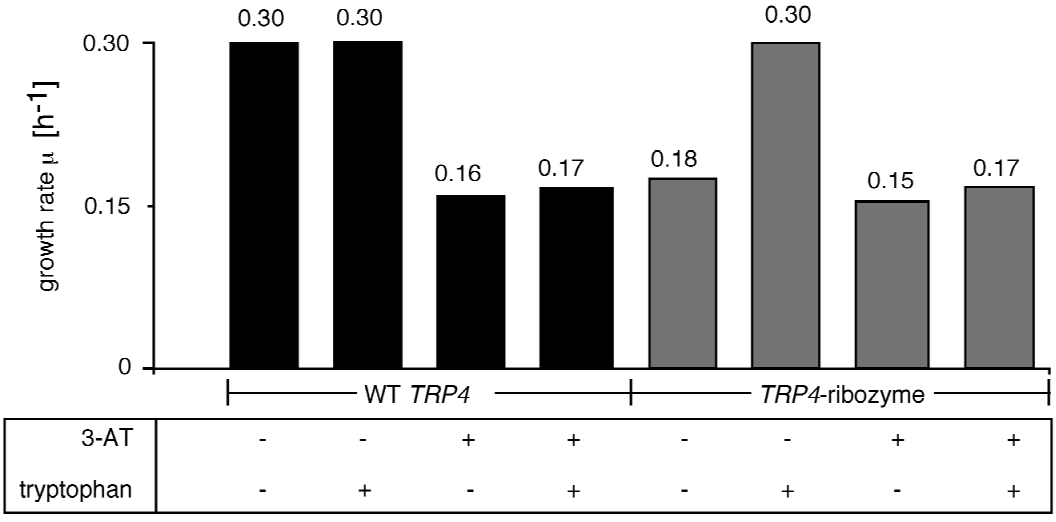

FIGURE 4. Growth rates (μ) of WT *TRP4*- and *TRP4*-ribozyme-bearing strains in a wild-type *GCN4* strain. The strains were grown in minimal medium with different combinations of tryptophan supplementation and/or 3-AT addition as indicated below by +/−. The column represents an average of four independent measurements with the standard deviations not exceeding 20%.

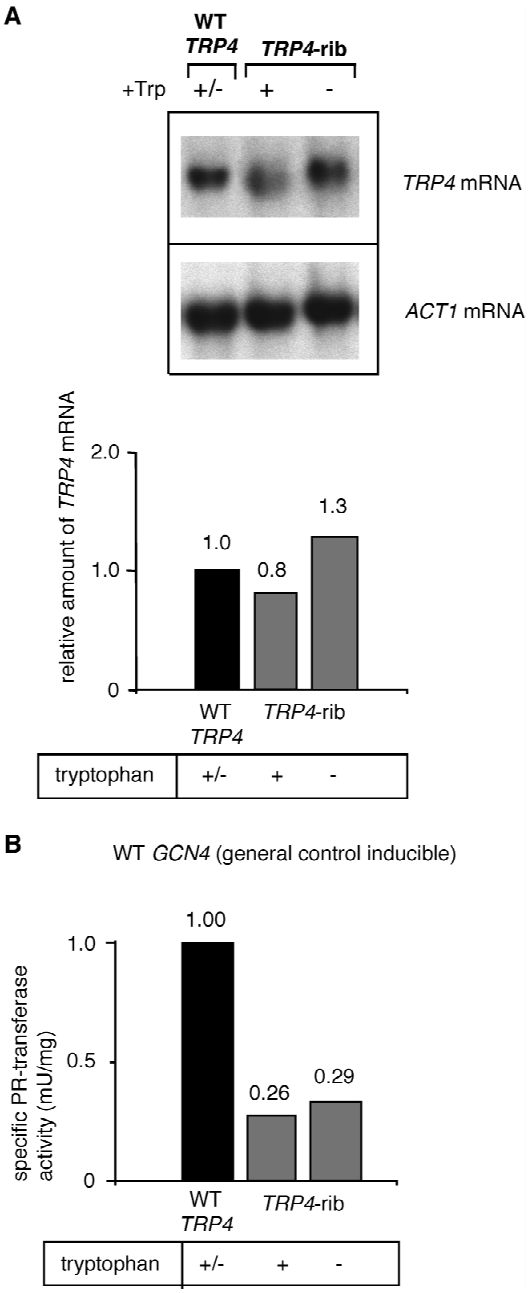

FIGURE 5. Characterization of *TRP4*-ribozyme gene expression in yeast cells with a wild-type *GCN4* background. **A:** Analysis of total RNA by northern hybridization experiments. The cultures were grown with or without tryptophan supplementation. The amount of *TRP4* mRNA in a WT *TRP4* strain with the same *GCN4* genetic background was taken as the control and set as 1.0. The amount of WT *TRP4* mRNA was the same whether the strains were grown with or without tryptophan and are indicated by +/−. The probes used are the same described in Figure 2A. Four independent experiments were quantified and the average value for each sample is shown below. **B:** Specific PR-transferase activity of *TRP4*-ribozyme levels produced in a *GCN4* genetic background with or without tryptophan. The columns represent at least four independent measurements with the standard deviations not exceeding 15%.

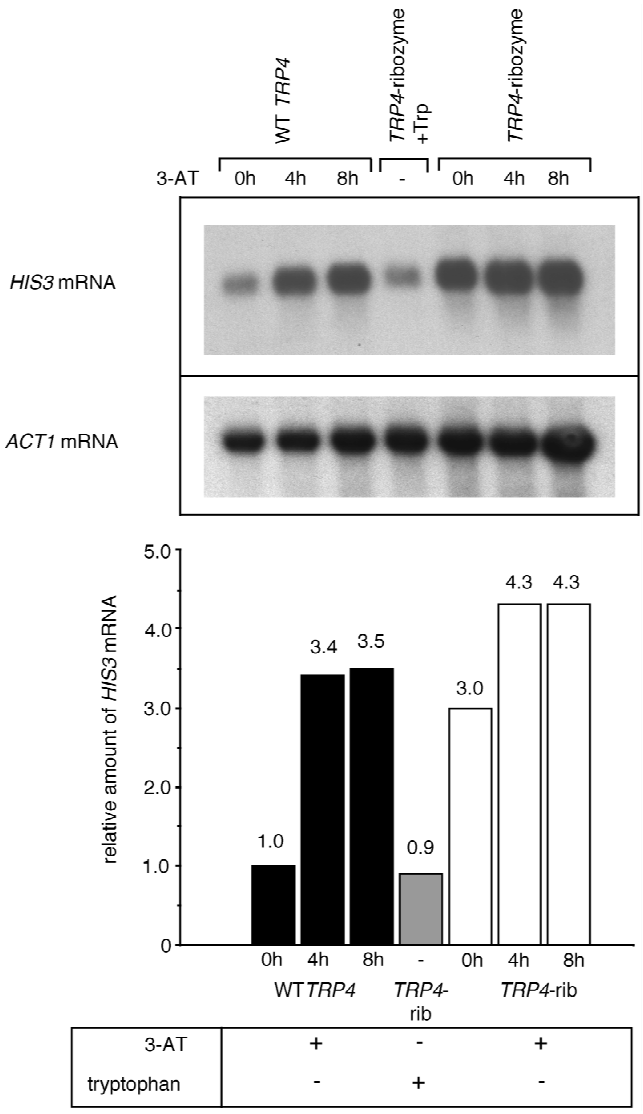

FIGURE 6. Expression of *HIS3* mRNA in yeast strains with either WT *TRP4* or the *TRP4*-ribozyme allele. The *HIS3* mRNA was visualized in northern hybridization experiments using the *HIS3* ORF as probe. The *ACT1* mRNA served as constitutive control. All yeast strains carried the wild-type *GCN4* gene. RNA was isolated immediately after addition of 3-AT (0h), and at 4 and 8 h after it (4h, 8h). In case of the *TRP4*-ribozyme-bearing strain, additional RNA isolations from cells grown in the presence of tryptophan were performed. Addition of tryptophan and 3-AT is indicated below. Each column represents the average of four independent hybridization experiments. The amount of *TRP4* mRNA in strains carrying WT *TRP4* immediately after addition of 3-AT was set as 1.0.

DISCUSSION

In this study, we have replaced the 3′ UTR of the yeast *TRP4* gene with a hammerhead ribozyme to determine whether this element can act as an alternative 3′-end processing signal. The *TRP4*-ribozyme construct produces a transcript that is efficiently cleaved but that lacks a poly(A) tail. These findings are not unexpected in light of previous studies in which cleavage products produced by hammerhead ribozymes have been used to prevent polyadenylation (Eckner et al., 1991). Such polyadenylation deficiency is most likely attributable to the coupled nature of cleavage and polyadenylation reactions and to the inability of 2′-3′-cyclic phosphate

termini generated by hammerhead cleavage to serve as substrates for poly(A) polymerase (Tanner, 1999; Zhao et al., 1999). In yeast, it has been possible to obtain polyadenylation of a ribozyme-containing transcript (Egli & Braus, 1994), an event that most likely depends on RNA sequence context, that is, triggering cryptic cleavage/polyadenylation signals. Because we were unable to detect any polyadenylated transcripts in RACE experiments we conclude that the *TRP4*-ribozyme mRNA does not contain any cryptic signals.

Although mRNA 3′-end processing and nuclear export have been shown to be linked (Eckner et al., 1991; Hilleren et al., 2001), the *TRP4*-ribozyme mRNA appears to be efficiently exported from the nucleus. It is unclear whether this occurs by pathways previously shown to be effective for nonpolyadenylated RNAs like rRNA and tRNAs (Moy & Silver, 1999; Sarkar et al., 1999; Simos & Hurt, 1999) or by some other mechanism. Unlike metazoans and their unadenylated histone mRNAs, there are no known examples of yeast RNA polymerase II transcripts that lack poly(A) tails (Goffeau et al., 1996). Future studies to assess the mechanism by which such nonpolyadenylated mRNAs are exported will be of interest.

The presence of a poly(A) tail is generally thought to be a critical determinant of mRNA stability. For some mRNAs, poly(A) shortening or removal is the rate-determining event in their decay, whereas, for others, it may be an obligate event in their degradation, but it is not the rate-determining step (Chen et al., 1995; Caponigro & Parker, 1996; Jacobson, 1996). The presence of a poly(A) tail provides an mRNA with a binding site for poly(A)-binding protein, which, in turn, serves to promote translational initiation and antagonize mRNA decapping in the cytoplasm (Gallie et al., 1989; Caponigro & Parker, 1995; Jacobson, 1996; Tarun & Sachs, 1996). In this study, the *TRP4*-ribozyme allele produces a transcript lacking a poly(A) tail and, as a consequence, it would be predicted that this mRNA would be susceptible to nucleolytic attack and considerably less abundant than its wild-type counterpart. In contrast, we found that the nonpolyadenylated ribozyme transcript accumulated to levels indicative of little, if any, change in stability. Maintenance of the stability of the poly(A)-deficient *TRP4*-ribozyme mRNA is likely to be a result of the action of the ribozyme in generation of the mRNA 3′ end, and not to some internal *TRP4* sequence features, as deletion of the *TRP4* 3′ UTR resulted in a significant decrease in the amount of *TRP4* mRNA. In support of this idea, a previous study determined that the presence of a ribozyme in an mRNA is not sufficient to render it more labile (Donahue & Fedor, 1997).

Several plausible explanations could account for the unchanged stability of *TRP4*-ribozyme mRNA. The secondary structure formed by the hammerhead ribozyme, or the terminal 2′-3′cyclic phosphate (Tanner, 1999), instead of a 3′-OH, might impede the accessibility of 3′ exonucleases to the mRNA. Alternatively, the absence of a poly(A) tail might uncouple an otherwise linked mechanism in which decapping is triggered by deadenylation (Muhlrad et al., 1994). A more likely explanation is that degradation of the *TRP4* mRNA is normally associated with its translation (Jacobson & Peltz, 1996). In a manner previously exemplified by the *MAT*α*1* mRNA (Parker & Jacobson, 1990), impaired translation of the *TRP4*-ribozyme mRNA (see below) may reduce the extent to which ribosome-associated factors act to promote decay.

Comparisons of mRNA levels, enzyme assays, and analyses of mRNA association with polyribosomes demonstrated that translation of the *TRP4*-ribozyme mRNA was reduced approximately 50–75%. Impaired utilization of this mRNA may well reflect the important role of the poly(A) tail in translation initiation (Munroe & Jacobson, 1990; Tarun & Sachs, 1995; Jacobson, 1996; Preiss & Hentze, 1998; Preiss et al., 1998). Under most circumstances, it would be expected that a poly(A)-deficient mRNA would initiate poorly in yeast (Gallie, 1991; Proweller & Butler, 1994), an event consistent with the shift of approximately half of the ribozyme-containing mRNA to the nonpolysomal fractions of the cytoplasm (Fig. 3). It is likely that the *TRP4*-ribozyme construct used here will prove valuable in further analyses of the mechanism of translation of poly(A)$^-$ mRNAs in yeast.

An additional translational regulatory phenomenon uncovered by these studies is suggested by the specific translational activity of the *TRP4* Δ3′ UTR mRNA (Fig. 2). Whereas the level of this mRNA is reduced more than threefold, relative to wild-type, PR-transferase activity is reduced less than half. These results imply that the deletion has removed a *TRP4* 3′ UTR sequence element normally capable of repressing translation of this mRNA. The existence of such an element is not without precedent (Gray & Wickens, 1998).

As summarized above, cells expressing the *TRP4*-ribozyme construct have decreased levels of Trp4p and are starved for tryptophan. In light of the ability of yeast cells to alter their patterns of gene expression to ensure survival in different environments, it was not surprising that the *TRP4*-ribozyme construct elicited the activation of the general control system of amino acid biosynthesis. The partial activation observed undoubtedly reflects the ability of cells harboring these constructs to synthesize modest amounts of tryptophan sufficient to support growth of *trp4* cells.

MATERIALS AND METHODS

Yeast strains

The yeast strains RH2063 (*MAT*a, *trp4*::*URA3*, *gcn4-101*, *leu2*) and RH2064 (*MAT*a, *trp4*::*URA3*, *leu2*) used in this study are

derivatives of the *Saccharomyces cerevisiae* laboratory standard strain X2180-1A (*MAT*a, *gal2*, *SUC2*, *mal*, *CUP1*). The *TRP4* open reading frame and its 3′ UTR, up to the *Eco*RV site, were replaced by the *URA3* gene. Yeast strains were cultivated on YEPD medium or on SD minimal medium with appropriate supplements at 30 °C (Rose et al., 1990).

Plasmid construction and cloning

A 3.0-kb *Sma*I/*Pst*I fragment containing the *TRP4* gene was cloned into the *Sma*I/*Pst*I sites of pRS315 (Sikorski & Hieter, 1989). The resulting plasmid pME1571 is designated as WT *TRP4* in this study. The plasmid pME1574 (*TRP4* Δ3′ UTR), carrying a 147-bp deletion of the *TRP4* 3′ UTR, was constructed by *Nde*I/*Eco*RV digestion, making blunt ends, followed by religation (Düvel et al., 1999). To construct the *TRP4*-ribozyme allele, the 147-bp *Nde*I/*Eco*RV fragment of the *TRP4* 3′ UTR was replaced by an 84-bp *Cla*I/*Bam*HI, blunt-ended fragment containing a hammerhead ribozyme (Eckner et al., 1991).

RNA analysis

Total RNA from *S. cerevisiae* was isolated according to Cross and Tinkelenberg (1991). Poly(A) enrichments were performed using the oligotex® Kit (Qiagen). For northern hybridization analysis, 20 μg of total RNAs were separated on a formaldehyde agarose gel and transferred to a positively charged nylon membrane (Biodyne B, PALL) by electroblotting. Hybridization with ^{32}P-labeled DNA fragments was performed as previously described (Egli et al., 1995). The DNA fragments were randomly radiolabeled using the HexaLabel DNA Labelling Kit from MBI Fermentas. Band intensities were visualized by autoradiography and quantified using a BAS-1500 Phosphorimaging scanner (Fuji).

Polyribosome analysis

Cells containing either WT *TRP4* or the *TRP4*-ribozyme allele were grown at 30 °C in 200 mL of SC-leu medium to an optical density at 600 nm (OD_{600}) of 0.8. Cell extracts were prepared by glass bead lysis in the presence of cycloheximide, and 10 OD_{260} units were fractionated on 7 to 47% sucrose gradients as described previously (Mangus & Jacobson, 1999). RNA from each fraction was extracted, electrophoresed, and analyzed by northern blotting using radiolabeled probes (Mangus & Jacobson, 1999).

Anthranilate-phosphoribosyl-transferase assay (E.C. 2.4.2.18)

All enzyme activities were determined *in situ* after permeabilization with Triton X-100 (Miozzari et al., 1978). Anthranilate-phosphoribosyl-transferase (PR-transferase, E.C. 2.4.2.18) activity was determined by measuring fluorometrically the decrease of anthranilate in the presence of N-(5′-phosphoribosyl-1)-anthranilate isomerase (Furter et al., 1988). The intrinsic PR-transferase activity of the helper strain RH218 (*TRP1*/*Eco*RI circle) was always less than 10% of the activity of the cell extract that was assayed, and was deduced from respective values in the assays. To determine the specific enzyme activities, protein contents were analyzed of whole Triton X-100-permeabilized cells as previously described (Herbert et al., 1971).

Rapid amplification of cDNA ends (RACE)

RACE experiments were performed based on the description in Frohman et al. (1988). A total of 100 ng of RNA was reverse transcribed using the M-MuLV Reverse Transcriptase (MBI Fermentas) and a poly-18 dT primer. In a following PCR reaction, the 3′ ends of the *TRP4*-ribozyme mRNA were amplified with the *TRP4* specific Primer TRP-RTF (5′-ATTATC TAGACCGGCAAGTACCACCTTGGC-3′).

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