GRS1: A yeast tRNA synthetase with a role in mRNA 3’ end formation

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Running title: GRS1 and Transcription Termination
SUMMARY

Transcription termination and 3’end formation are essential processes necessary for gene expression. However, the specific mechanisms responsible for these events remain elusive. A screen designed to identify trans–acting factors involved in these mechanisms in Saccharomyces cerevisiae identified a temperature-sensitive mutant that displayed phenotypes consistent with a role in transcription termination. The complementing gene was identified as GRS1, which encodes the S. cerevisiae glycyl tRNA synthetase. This result, although unusual, is not unprecedented given that the involvement of tRNA synthetases in a variety of cellular processes other than translation has been well established. A direct role for the synthetase in transcription termination was determined through several in vitro assays using purified wild type and mutant enzyme. First, binding to two well-characterized yeast mRNA 3’ ends was demonstrated by crosslinking studies. In addition, it was found that all three substrates compete with each other for binding to GlyRS enzyme. Next, the affinity of the synthetase for the two mRNA 3’ ends was found to be similar to that of its “natural” substrate, glycine tRNA in a nitrocellulose filter binding assay. The effect of the grs1-1 mutation was also examined and found to significantly reduce the affinity of the enzyme for the three RNA substrates. Taken together, these data indicate that not only does this synthetase interact with several different RNA substrates, but also that these substrates bind to the same site. These results establish a direct role for GRS1 in mRNA 3’ end formation.
INTRODUCTION

The formation of a eukaryotic mRNA 3’ end is a complex process involving factors that recognize, cleave and process the pre-mRNA as well as those that facilitate the dissociation of RNA polymerase II from the DNA template. Although many of these factors have been identified, a comprehensive model of how the termination event interfaces with the processing activities remains elusive. The favored model suggests that termination is dependent on the recognition of a polyadenylation signal that then triggers a change in the polymerase, resulting in dissociation of the enzyme, followed by the termination event(s) (1). The most likely alteration in the actively transcribing enzyme would be a result of the “offloading” of 3’ end processing factors that associate with the carboxyl terminal domain (CTD) of the largest subunit of RNAP II. In addition to factors involved in RNA processing interacting with the transcription machinery, recent data demonstrates that transcription factors may also be part of the polyadenylation machinery (2,3). Thus, this apparent cross talk of both complexes reinforces the notion that the transcription and processing reactions are tightly coupled \textit{in vivo}.

The requirement for coordination of termination and processing suggests the involvement of additional factors that are likely to influence one or both events. As noted, changes to the polymerase, including dephosphorylation of the CTD are likely to influence the processivity of the enzyme (4). In addition, there may be other termination factors that either (i) directly alter the actively transcribing complex to elicit dissociation from the template or (ii) are involved in alternative mechanisms to promote termination, such as chromatin remodeling (5). For example, mutations in a proly isomerase gene, \textit{ESS1}, have resulted in defects in transcription termination (6). The termination region may also have inherent properties that contribute to the termination event, as indicated by the fact that the \textit{S. cerevisiae} RNAP II elongation complex, like those of \textit{E. coli}, are destabilized an RNA hairpin structure (7). Finally, co-transcriptional cleavage at the poly (A) site does not seem to be an absolute requirement for termination (8-10) supporting the notion that while passage through the poly (A) site may affect the processivity of the enzyme, it
does not dissociate from the complex until it is well past the poly (A) site.

While transcription complexes as well as cleavage and polyadenylation reactions can be reconstituted from purified components, the complexity of factors suggests the possibility of additional interactions in vivo. Further attempts to define the mechanism of transcription termination have led to the identification of additional interactions between components of the transcriptional machinery as well as the involvement of unexpected trans-acting factors. One such factor, the *S. cerevisiae* GRS1 gene, was identified in a genetic screen for mutations that cause RNAP II to read through an otherwise functional 3’ end signal (11). *GRS1* encodes both the cytoplasmic and mitochondrial glycyl tRNA synthetase (12). Cells containing only the *grs1-1* allele display both a termination defect and a slow-growth phenotype at the non-permissive temperature. The *grs1-1* mutation was identified as a proline to phenylalanine change at amino acid 552, located in the C-terminal domain of the protein (11).

That a tRNA synthetase can function in an activity distinct from its role in translation is not unprecedented, as tRNA synthetases have been shown to be present in the nucleus and to bind substrates other than their cognate tRNA (13-15). Further support for the notion that the synthetase may participate in interactions with the 3’ end of mRNAs was demonstrated by the presence of putative tRNA-like structures at the 3’ end of several yeast mRNAs (11). Again, this is not an uncommon finding as a tRNA-like motif is a common structure found in different types of RNA as a regulatory element (16,17). We carried out a series of experiments to elucidate the role of *GRS1* in transcription termination. The 3’ end signals from two yeast genes were examined and found to interact directly and specifically with GlyRSb in vitro, indicating that GlyRS may play a direct role in promoting efficient termination of transcription. In addition, GlyRS-P552F was found to have a reduced affinity for its cognate tRNA as well as the two other RNA substrates.
EXPERIMENTAL PROCEDURES

Reagents: Culture and media components were obtained from Difco (Detroit). Amino acids and buffer components were obtained from Sigma (St. Louis). All restriction and modifying enzymes were obtained from Promega (Madison). Radiolabeled nucleotides were obtained from Perkin Elmer Life Sciences (Boston). [U-14C] glycine was obtained from Ambion (Austin). All primers were generated by Geonsys (Houston) and primer sequences are summarized in Table 1.

Plasmids: The plasmids D16, pT7T3ADH2, pL101 (18), pT7T319u (Promega), pGCYC and pGcyc-512 (19), Yep181 (20), pBEVY (21), YCp111/GRSI and YCp111/grs1-1(11), and pL421 (22) have been previously described.

Digesting Ycp111/GRSI with XhoI and SalI and ligating the resulting 2.5 kb fragment into the SalI site of Yep181 created YEpl181/GRSI. Yep181/GRSI was converted into Yep181/grs1-1 by replacing a BglII/KpnI fragment with a BglII/KpnI fragment, containing the grs1-1 mutation, from Yep181/grs1-1.

The His6-tagged versions of Yep181/GRSI(His6) and Yep181/grs1-1(His6) were generated as described (12). Briefly, two oligos (LEH 418, LEH419) were annealed and ligated into the KpnI/EcoRI sites of Yep181/GRSI and Yep181/grs1-1.

pBEVY/GRSI(His6) and pBEVY/grs1-1(His6) were constructed by amplifying the His6-GRSI and His6-grs1-1 genes from Yep181/GRSI-1(His6) and Yep181/grs1-1(His6) respectively using primers LEH 510/LEH 511. The resulting products were digested and cloned into the BamHI/PstI sites of pBEVY-Leu.

pD16tRNAgly was generated by first annealing two oligos (LEH 506, LEH 507) to create the DNA sequence encoding S. cerevisiae glycyl-tRNA flanked by KpnI and SalI sites. The resulting product was ligated into the KpnI/SalI sites of pT7T319u to give pT7T3 tRNAgly. This
plasmid was then digested with SmaI/SphI and ligated into D16.

ADH2(G18A) was amplified from T7T3ADH2(G18A) using primers LEH 482/LEH 106 and subcloned into the KpnI/XhoI sites of D16. A similar procedure was followed using T7T3ADH2(G18A,G19A) and T7T3ADH2(G18A,G19A,C101G) as template with primers LEH 497/LEH 498 to create pD16ADH2(G18A,G19A) and pD16ADH2(G18A,G19A,C101G) respectively.

Yeast strains and Methods: All strains used are derivatives of W303, and yeast cells were cultured using standard techniques (23-25). The ß-galactosidase assay was performed as described (24).

Yeast strains used for protein expression and purification are haploid derivatives of a grs1/GRS1 strain (11). The grs1/GRS1 strain was transformed with pBEVY/GRS1(His6) and sporulated. This procedure was repeated with pBEVY/grs1-1(His6) to generate the strains grs1/pBEVY/GRS1(His6) and grs1/pBEVY/grs1-1(His6).

Protein Purification: The purification procedure for GlyRS was modified from (12). His6-GlyRS and His6-GlyRS-P552F were purified from yeast cells using nickel-nitriloacetic acid agarose (Ni-NTA, Qiagen) according to the manufacturer’s instructions and eluted with buffer containing 20 mM NaPO₄ pH 7.2, 50 mM NaCl, and 250 mM Imidazole.

Active Site Assay: Isolation of the E-[Gly.AMP] product was performed as modified from (26). His6-GlyRS was incubated with excess MgATP and [U-¹⁴C] glycine in buffer A (100 mM Hepes pH 7.2/10 mM MgCl₂, 30 mM KCl/0.1 mM DTT, 5 mM ß-Mercaptoethanol) for 20 minutes at 30°C in a total volume of 50 µL. The resulting protein complex was isolated on 0.45 µ HA type nitrocellulose filters (Millipore) and washed with buffer B (100 mM Hepes pH 7.2, 10 mM MgCl₂, 30 mM KCl). The filters were dried and added to 10 mL of scintillation cocktail (Sigma) for counting. To determine the total cpm added, 5 µL of each reaction was spotted on a dry filter and counted. For each sample, cpm bound was converted to µmol bound glycine (8.9E07 cpm = 1µmol glycine).

Aminoacylation Assay: A standard aminoacylation assay was performed as modified from (27).
His6-GlyRS was incubated in buffer A with the addition of 200 µM total yeast tRNA (Sigma) in a final volume of 50 µL. Reactions were incubated at 30ºC for 20 minutes, then spotted on Whatman 3mm filter paper. The filters were washed first in 300 mL cold 10% TCA for 5 minutes, followed by two washes in 300 mL cold 5% TCA for 5 minutes, and finally a 2 minute wash in 100% EtOH. The filters were dried and added to 10 mL scintillation cocktail for counting. The total cpm added was determined as described above. Cpm bound was converted to µmol tRNA charged for each sample (9.6E07 cpm = 1 µmol charged tRNA).

RNA synthesis: The template for tRNA<sup>gly</sup> was prepared by PCR, using the primers LEH 563/LEH564 on D16tRNA<sup>gly</sup> to generate T7-tRNA<sup>gly</sup>. T7-CYC1 and T7-cyc-512 were generated in a similar manner using primers LEH546/LEH 547 on pGCYC1 and pGcyc-512 respectively. Digesting T7T3ADH2 and T7T3ADH2 (G18A,G19A,C101G) with NheI generated the T7 template for ADH2 and ADH2(G18A, G19A, C101G) respectively.

Transcription reactions were performed according to the manufacturer’s instructions (Promega). Labeled transcripts were purified using P-30 Micro Bio Spin Columns (Bio-Rad). Unlabeled transcripts were first digested with 10 UDNase for 30 minutes at 37ºC before precipitation.

Crosslinking: 20 nM RNA was incubated with 1 nM His6-GlyRS for 10 minutes at 30ºC in a buffer C (100 mM Hepes pH 7.2, 10 mM MgCl<sub>2</sub>, 30mM KCl, 5 mM EDTA, 5 mM β-Merceptoethanol, and 2mg/mL salmon sperm). Reactions were UV crosslinked at 120 mJ for 90 seconds and resolved on a 10% SDS-PAGE gel. Competition assays were performed by pre-incubating the protein with varying amounts of unlabeled RNA for 10 minutes at 30ºC before adding labeled RNA. The crosslinking reaction was then continued as described above. All crosslinking gels were dried and exposed to film (Fuji).

Filter Binding: Nitrocellulose filter binding assays were based on a previously described method (28). Briefly, 1 nM His6-GlyRS was incubated with varying amounts of RNA at 30ºC for 10 minutes in buffer C, with the addition of 40 U RNasin, in a total volume of 50 µL. Reactions were filtered through a pre-soaked 0.45 µ HA type nitrocellulose filter (Millipore) and washed
with 5 mL of buffer B. Filters were dried and counted. Total cpm added was determined as described above. The amount of bound RNA in each reaction was determined by comparing the amount of cpm bound to the total cpm present in each reaction. Points used to generate binding curves were the average of three independent experiments. Binding curves were fit to a quadratic equation \( F = (A/E) * ((E+X+K)-(E+X+K)^2-(4*E*X)^0.5)/2 \) with \( E=1 \) using Sigma Plot (Jandel Scientific). The dissociation constant \( (K_a) \) is the concentration at which 50% of the RNA bound at saturation is retained on the filter.

**Site-Directed Mutagenesis: T7T3ADH2(G18A), T7T3ADH2(G18A,G19A), and T7T3ADH2(G18A,G19A,C101G)** were generated using the Stratagene Quickchange kit. Primers LEH426/LEH427 were used in a mutagenic PCR reaction with T7T3ADH2 to generate T7T3ADH2(G18A). Primers LEH477/LEH478 were used with template T7T3ADH2(G18A) to generate T7T3ADH2(G18A,G19A), which was then used as template with LEH252/LEH509 to generate T7T3ADH2(G18A,G19A,C101G). Mutations in \( ADH2 \) were confirmed by DNA sequencing.
RESULTS

Purification and characterization of GlyRS and GlyRS-P552F. In order to examine the interaction of GlyRS with the 3’ end of mRNA, it was first necessary to obtain active protein to use in the in vitro assays. The addition of a His₆ tag to the C-terminal domain of GlyRS and GlyRS-P552F proteins (see methods) facilitated purification from yeast cells. The addition of the C-terminal His₆ tag does not interfere with the function of the enzyme (12) and His₆-GlyRS is able to complement the growth defect of the grsI-I strain (data not shown). The purified enzymes were run on a 10%SDS-PAGE gel and visualized by coomasie stain (Fig. 1A). The yield for each purification was determined by comparing the intensity of the GlyRS band with a known quantity of BSA run on the same gel. The calculated yield was then verified by measuring the OD₂₈₀ of the GlyRS fraction. Activity of the purified His₆-GlyRS and His₆-GlyRS-P552F proteins was then determined by testing the ability of the enzyme to carry out both steps of the two-part aminoacylation reaction (Fig. 1B and C). First, the ability of His₆-GlyRS to form the glycine-AMP intermediate was determined through an active site assay (26). Equal amounts of GlyRS and GlyRS-P552F bind similar amounts of glycine in an ATP-dependent manner, indicating that both enzymes are functional (Fig. 1B). The amount of glycine bound can then be used to calculate the amount of active protein in each reaction assuming each monomer of GlyRS binds one molecule of glycine. The amount of active protein was compared to the total yield and both GlyRS and GlyRS-P552F preparations were found to contain 90% active protein. The active site assay was also performed with a protein extract made from cells transformed with empty vector (Fig. 1A, lane 1) and, as expected, does not have a significant amount of glycine binding activity (Fig. 1B).

Next the full aminoacylation reaction was performed (Fig. 1C). Due to differences in the concentration of the enzymes, the amount of tRNA charged in each assay was normalized to the amount of active protein added to the reaction (as determined from the active site assay). After a 20 minute incubation, the GlyRS reaction produced an increased amount of charged tRNA as
compared to the mutant. These data establish that both the His-GlyRS and His-GlyRS-P552F proteins are active \textit{in vitro}, although GlyRS-P552F is less efficient at charging tRNA.

**GlyRS protein crosslinks directly to the 3’ end of ADH2 and CYC1.** In order to explore the mechanism by which GlyRS is involved in transcription termination, His-GlyRS was examined for its ability to interact directly with the 3’ end of yeast mRNAs through UV crosslinking. The \textit{ADH2} and \textit{CYC1} sequences are well characterized with regard to 3’ end formation (29,30). The region of \textit{ADH2} and \textit{CYC1} proceeding the poly(A) site (160 bp and 150bp respectively) was transcribed in the presence of \([\alpha^{32}P]UTP\) and crosslinked to GlyRS. In addition, a mutant of the \textit{CYC1} 3 end, \textit{cyc-512}, was also tested. The \textit{cyc-512} 3 end contains a 38 bp deletion and is unable to direct proper transcription termination (29). The known substrate of GlyRS, tRNA\textsubscript{gly}, was also tested in the crosslinking assay in order to determine the effectiveness of the assay. For each sample, the reaction was crosslinked in the absence (-) or presence (+) of 1 nM GlyRS. A crosslinked product can be seen only in the presence of the GlyRS protein, which crosslinks to its own tRNA as well as to the 3’ end of \textit{ADH2} and \textit{CYC1}(Fig. 2). The migration of these products is consistent with the expected size of in a SDS-PAGE gel of a GlyRS-RNA crosslink. For example, the crosslink product observed with tRNA\textsubscript{gly} migrates at about 100 kDa which is consistent with the molecular weight of a GlyRS monomer (72kDa) plus the RNA (28 kDa) (Fig. 2A). The \textit{ADH2} (Fig. 2B) and \textit{CYC1} (Fig. 2C) RNAs migrate at 53 kDa and 50 kDa respectively and the resulting crosslink products migrate above the 120 kDa marker which is also consistent with a 72 kDa increase. The crosslink products are specific as they are not observed with a version of the \textit{ADH2} 3’ end containing three point mutations or with the substrate \textit{cyc-512} (Fig. 2B and C). GlyRS was also unable to crosslink to the \textit{ADH2} complement sequence (data not shown).

**Sequences in the ADH2 3’ end are important for the GlyRS interaction.** A previous study identified tRNA-like structures at the 3’ end of several yeast mRNAs (11). Fig. 3A illustrates this potential structure in the \textit{ADH2} 3’ end. Although the validity of these structures has not been tested, this region was found to interact directly with GlyRS (Fig. 2). Site-directed
mutagenesis was performed to further explore the importance of this 3’ end region to the GlyRS interaction.

The mutated versions of the ADH2 3’ end were cloned into a reporter construct (Fig. 3B) (18) that measures the efficiency of a particular sequence to direct transcription termination. Normal termination results in a truncated transcript with no β-galactosidase expression while defective termination leads to readthrough into the lacZ gene and expression of β-galactosidase. The effect of each of the ADH2 mutations on termination efficiency was measured by comparing the β-galactosidase activity for each construct and expressed as percent readthrough as compared to the reporter lacking a terminator sequence (100% readthrough). The C101G mutation increases readthrough by more than 6 fold as compared to the wild type ADH2 3’ end (22). Additional mutations in the first half of this region (G18A and G18A,G19A) did not increase transcriptional readthrough significantly. However, a construct containing all three mutations increased the level of readthrough to almost twice that of the C101G mutation alone, indicating that this region of the ADH2 3’ end is important for normal transcription termination (Fig. 3B).

To determine if the decrease in termination efficiency seen in the in vivo assay correlated with a decrease in GlyRS binding, the ADH2 triple mutant was tested for its ability to crosslink to GlyRS as well as compete with wild type ADH2 for binding. As seen in Fig. 2B, the mutant ADH2 3’ end is unable to crosslink to GlyRS. Labeled wild type ADH2 was then incubated with 10, 100, and 1000-fold unlabeled wild type (Fig. 3C, left) or mutant (Fig. 3C, right) ADH2 before crosslinking to GlyRS. Even at 1000-fold excess, the mutant ADH2 3’ end is unable to compete with wild type RNA for binding, indicating that this region is necessary for binding GlyRS as well as for promoting efficient termination.

GlyRS substrates can compete with each other for binding. To determine if the different GlyRS substrates bind to the same site on the enzyme, a cross competition assay was performed. Increasing amounts of each of the unlabeled substrates were pre-incubated with 1 nM GlyRS before adding labeled RNA. As seen in Fig. 4A, increasing amounts of unlabeled tRNA10 are
able to compete with labeled tRNA\textsubscript{gly} for binding (lanes 2-4). In addition, unlabeled ADH2 and CYC1 RNA were also able to compete with labeled tRNA\textsubscript{gly} for binding (lanes 5-7 and 8-10, respectively), indicating that all three substrates bind at the same site on GlyRS. As expected, unlabeled cyc-512 was unable to compete with tRNA\textsubscript{gly} (lanes 11-13).

These results were confirmed through cross competition assays performed with labeled CYC1 RNA. As seen in Fig. 4B, unlabeled CYC1, ADH2, and tRNA\textsubscript{gly} could compete with labeled CYC1 RNA for binding to GlyRS while cyc-512 was unable to compete. Interestingly, unlabeled tRNA\textsubscript{gly} was able to compete with labeled CYC1 for binding at a lower concentration than unlabeled CYC1 (compare lanes 3 and 4 to lanes 9 and 10). Similar results were also seen with labeled ADH2 RNA (data not shown).

\textit{GlyRS binds substrates with similar affinity.} The discovery that all three GlyRS substrates compete with each other indicates that the enzyme may have a similar affinity for the different RNA substrates. Using a nitrocellulose filter-binding assay, 1 nM GlyRS was incubated with increasing amounts of labeled RNA to determine the affinity of GlyRS for each of its substrates. The concentration of bound RNA was calculated from the amount of radioactivity bound to the filter and the specific activity of the RNA substrate. The dependence of the concentration of bound RNA substrate on the concentration of total RNA showed a hyperbolic binding curve (Fig. 5). The affinity or equilibrium dissociation constant (K\textsubscript{d}) for each RNA was determined from each curve as the concentration of RNA substrate at which there is 50\% binding. The values of K\textsubscript{d} are summarized in Table 2. GlyRS had the highest affinity for its cognate tRNA followed by the CYC1 3’ end, then the ADH2 3’ end. cyc-512 RNA failed to produce a significant increase in bound RNA.

\textit{The grs1-1 mutation affects the interaction of the enzyme with mRNA 3’ ends.} The grs1-1 mutation was shown to affect the aminoacylation function of the enzyme (Fig. 1C) but not the glycine binding activity (Fig 1B) indicating that the mutant may have an RNA binding defect. In order to determine the effect on the other RNA substrates, the filter binding experiment was performed with GlyRS-P552F (Fig. 5). As seen in Table 2, the value of K\textsubscript{d} for each substrate
increased at least 10-fold over wild type values. This decrease in affinity seen with the GlyRS-P552F was confirmed by crosslinking studies. GlyRS-P552F was crosslinked to 20 nM RNA, which was the concentration sufficient to produce a product with wild type GlyRS. At this concentration, no product is detected (Fig. 6, lane 2). However, increasing the amount of RNA by 10-fold results in the formation of a crosslinked product (Fig. 6, lane 4). These data indicate that the phenotype of the grs1-1 strain cannot be fully attributed to a decrease in aminoacylation function as the transcription termination defect is most likely due to a loss of the interaction between GlyRS and the 3’ end of mRNA.
DISCUSSION

The identification of a tRNA synthetase that binds substrates in addition to its cognate tRNA is not unprecedented (31, 32) and the specificity of the interaction uncovered between GlyRS and the 3’ end of ADH2 and CYC1 suggests that GlyRS may play a direct role in the transcription termination process in vivo. Our conclusion is based on the following evidence. First, crosslinking analysis showed that GlyRS was able to bind directly to the 3’ end of these yeast genes. The specificity of the interaction was tested by ensuring that the synthetase was unable to bind to a nonspecific RNA as well as to non-functional versions of both mRNA 3’ ends. Next, it was determined that GlyRS likely binds all three substrates at the same site as they are all able to compete with each other for binding. We demonstrate that the mutant versions of the RNA 3’ ends were unable to compete with their wild type counterparts for binding which reconfirmed the specificity of the interaction. Finally, the discovery that GlyRS has a similar affinity for all three substrates in a filter-binding assay agrees with the results from the crosslinking and competition assays. It is unlikely that the interaction between GlyRS and the 3’ end of mRNA in vivo would interfere with the role of GlyRS in protein synthesis. Therefore it is encouraging to note that not only does GlyRS have the strongest affinity for tRNA\(^{\text{gly}}\) in vitro, but that tRNA\(^{\text{gly}}\) is also able to compete with the mRNA substrates for binding at lower concentrations.

The GlyRS-P552F gene, grs1-1 results in a proline to phenylalanine change at amino acid position 552 (11). This region corresponds to the RNA binding domain of the synthetase as determined by the crystal structure of the T.thermophilus GlyRS which shares 26% identity and 46% similarity to the yeast GlyRS (33). This proline residue is also conserved among the other yeast synthetases with a similar class II C-terminal domain structure (34). Thus, it is likely that a mutation in this region would affect the ability of GlyRS to bind substrate. Indeed, a decrease in the ability of GlyRS-P552F to charge tRNA was observed as well as a decrease in binding affinity for all three GlyRS substrates. It was determined that GlyRS-P552F was active and otherwise functional by testing its ability to bind glycine. Because no difference was seen in the
ability of the GlyRS-P552F to bind glycine as compared to the wild type, it is reasonable to assume that any observed decrease in GlyRS-P552F function is the result of a localized disorder in the C-terminal domain and not to general misfolding or instability of the protein.

The affinity of GlyRS-P552F for its various substrates decreased over 10-fold in the filter binding assays. The reduced affinity measured in these assays was confirmed through the crosslinking assay. The amount of RNA needed to produce a crosslink band with GlyRS was insufficient for GlyRS-P552F. A 10-fold increase of RNA, however, was sufficient to produce a visible crosslink product, which directly correlates with the observed decrease in affinity. Together, these data indicate that the transcription termination defect of the grs1-1 strain is presumably due to the inability of the GlyRS-P552F to interact efficiently with the 3’ end of mRNA.

We previously postulated that the potential tRNA-like sequences at the 3’ end of mRNAs were the site of interaction with GlyRS (11). Our study supports this hypothesis, although it does not address the validity of the potential tRNA-like structure. A combination of point mutations in two regions of the ADH2 sequence produces an additive effect and increases transcriptional readthrough as compared with the single mutations alone. In addition, the ADH2 triple mutant is no longer able to compete with wild type ADH2 for binding and is unable to crosslink directly to GlyRS. While this result is also seen with the CYC1 mutant, it is less surprising given that the cyc-512 mutation removes a large region in the substrate and would, therefore, have a much greater effect on any RNA secondary or tertiary structure. The ADH2 mutation is much more subtle yet yields the same end result lending support to the notion that these regions of RNA are folding into a structure recognized and bound by GlyRS.

Destabilization of this structure, and subsequent loss of binding, could occur as a result of these point mutations given that two of the mutated nucleotides (G19 and C101) would interact as part of the tRNA tertiary structure (35).

Transcription termination is a complex process and the inclusion of a tRNA synthetase into the system only increases the number of unanswered questions. Synthetases are known to be
both present and active in the nucleus (13, 36), which could bring them in close proximity to actively transcribing complexes. However, to date, these enzymes have not been identified as a part of any complex involved in 3’ end formation, leading to the hypothesis that the interaction between GlyRS and pre-mRNA could be transient or that the enzyme may not directly interact with known complexes. Interestingly, an examination of *S. cerevisiae* protein interactions through mass spectrometry identified an interaction between an RNA splicing factor, Prp6p, and several tRNA synthetases including GlyRS (37). Prp6p was also found to interact with the poly (A) binding protein, Pab1p. It is possible that Prp6p could act as a bridge and bring the synthetase into the transcriptional complex and allow the interaction with mRNA. Once the synthetase is localized near the transcriptional complex, it could bind to the secondary structure within the nascent RNA strand upstream of the poly (A) site and cause destabilization of the downstream elongating polymerase. Transcription termination in bacteria is influenced by the formation of an RNA hairpin upstream of the termination site that acts to destabilize the downstream elongation complex and this hairpin structure can also destabilize yeast RNAP II *in vitro* (7, 38). Therefore, it is tempting to propose that a similar structure forms upstream of the poly (A) site in yeast, and that this structure may be bound by additional protein factors such as GlyRS which in turn would destabilize the RNAP II complex and lead to termination.
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REFERENCES


FOOTNOTES

a RNA polymerase II

b Glycyl tRNA synthetase

c No binding

d Not determined
FIGURE LEGENDS

Figure 1. Activity of GlyRS and GlyRS-P552F isolated from yeast.  GlyRS was expressed and purified from yeast cells.  A) A coomasie stained gel displaying the purified fraction from cells expressing empty vector (lane 1), His6-GlyRS (lane 2) or His6-GlyRS-P552F (lane 3). Both His6-GlyRS and His6-GlyRS-P552F have the same molecular weight, approximately 72 kDa. B) His6-GlyRS is able to bind [14C] glycine, shown here as µmol glycine bound calculated from cpm bound. Equal amounts of His6-GlyRS and His6-GlyRS-P552F, [14C] glycine, and MgATP were incubated for 20 minutes at 30°C and the resulting complexes filtered through a nitrocellulose filter before scintillation counting. All reactions were done in triplicate and values show an average of two independent experiments. Reactions performed with the background co-eluting proteins, without enzyme (BSA +ATP) and without ATP do not demonstrate glycine-binding activity. C) His6-GlyRS and His6-GlyRS-P552F do not have the same charging efficiency. His6-GlyRS and His6-GlyRS-P552F were incubated with excess MgATP, [14C] glycine and total yeast tRNA for 20 minutes. Cpm bound was converted to µmol tRNA charged. Results were normalized by dividing the µmol tRNA charged in each reaction by the µmol GlyRS added (as determined from the active site assay). Each graph represents the average of four reactions done in triplicate.

Figure 2. GlyRS crosslinks to the 3’ end of mRNA. His6-GlyRS was incubated with labeled A) tRNAgly, or the 3’ ends of B) ADH2 and C) CYC1. The reactions were subjected to UV crosslinking and resolved on a 10% SDS-PAGE gel. Each reaction contains 1 nM protein and 20 nM labeled RNA. Crosslinking reactions were performed in the absence (-) or presence (+) of GlyRS. Protein-RNA complexes are visible only in the presence of GlyRS and are indicated by the top arrow (*). Unbound RNA is indicated by the bottom arrow. A mutated ADH2 3’ end (B) as well as a deletion mutant of CYC1, cyc-512(C) are unable to produce a crosslink product.

Figure 3. Mutations in the tRNA like structure of ADH2 inhibit GlyRS binding. A) A graphic
representation of the potential tRNA like structure of the ADH2 3’ end as modified from (11). Regions modified through site directed mutagenesis are indicated by the circles (G18, G19 and C101). B) Mutations in the ADH2 3’ end increase transcriptional readthrough as measured by a reporter assay. Wild type and mutant ADH2 3’ ends were cloned into the D16 plasmid and transformed into W303. Site directed mutagenesis was used to generate the following mutants: G18A, G18A,G19A, and G18A,G19A,C101G. Presence of a functional termination signal prevents transcription of the lacZ gene and downstream β-galactosidase expression. After growth in galactose containing media, β-galactosidase levels were measured to determine percent readthrough as compared to empty vector (100%). The triple mutant (G18A,G19A,C101G) demonstrated the highest percent readthrough as compared to wild type ADH2. C) The ADH2 triple mutant is unable to compete with wild type ADH2 for GlyRS binding.

Figure 4. GlyRS substrates can compete with each other for binding. 1 nM GlyRS was pre-incubated with 0.200 µM (lanes 1 and 4), 2.0 µM (lanes 2 and 5) and 20 µM (lanes 3 and 6) unlabeled ADH2 (left) or ADH2(G18A,G19A,C101G) (right) for 10 minutes at 30ÚC before adding 20 nM labeled ADH2. Reactions were then UV crosslinked and resolved on a 10% SDS-PAGE gel. The top arrow indicates crosslinked complexes; the bottom arrow indicates unbound, labeled ADH2. At the highest concentration of competitor, wild type ADH2 is able to compete for binding while the triple mutant is not (compare lanes 3 and 6, top arrow).

A) Reactions in lanes 2- 4 were incubated with increasing concentrations of unlabeled tRNAgly, lanes 5- 7 contained unlabeled ADH, lanes 8- 10 unlabeled CYC, and 11-13 unlabeled cyc-512. In contrast to the other RNAs, cyc-512 is unable to compete with tRNAgly for binding. B) Reactions in lanes 2- 4 were incubated with increasing concentrations of
unlabeled *CYC1*, lanes 5-7 contained unlabeled *cyc-512*, lanes 8-10 unlabeled tRNA$_{\text{gly}}$, and lanes 11-13 unlabeled *ADH2*. *Cyc-512* is unable to compete for binding.

**Figure 5. Binding curves for GlyRS and GlyRS-P552F.** A $K_d$ value (Table 2) for the interaction between GlyRS and its RNA substrates was determined through filter binding assays. Varying concentrations of $^{32}$P-labeled A) tRNA$_{\text{gly}}$, B) the 3’ end of *ADH2*, and C) the 3’ end of *CYC1* were incubated with 1nM GlyRS or GlyRS-P552F. Reactions were isolated on nitrocellulose filters and counted. The amount of bound RNA was determined from percent bound by comparing filtered and unfiltered reactions. Nonspecific binding was less than 0.02%. The concentration of bound RNA was calculated from the amount of radioactivity bound to the filter and the specific activity of the RNA substrate. The concentration of bound RNA was plotted against the total concentration added to each reaction using Sigma Plot (Jandel Scientific). All reactions were done in triplicate, and values shown are an average of three independent experiments.

**Figure 6. GlyRS-P552F is unable to crosslink at wild type RNA concentrations.** Reactions were performed as with wild type enzyme. Lanes 1 and 2 show crosslinking in the absence and presence of 1 nM protein with 20 nM labeled RNA. GlyRS-P552F does not crosslink at these concentrations as no product appears in lane 2. A crosslink product is visible with a 10-fold increase in RNA concentration (lane 4). Lanes 1a-4a show input RNA to verify equal loading.
Table 1. Primer sequences used in this study (5’ – 3’)

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Table 2. Equilibrium dissociation constants (K_d) for GlyRS and GlyRS-P552F.

<table>
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<th>K_d (nM)</th>
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K_d values were calculated from nitrocellulose filter binding assays. Binding curves were generated by incubating 1 nM enzyme with increasing amounts of labeled RNA and are available as supplemental information. Nonspecific binding was less than 0.02%. All values have less than 70% error.
A.  

B. (I) Glycine + ATP = Glycine-AMP

C. (II) tRNA + Glycine = Glycine-tRNA