Kinetic Analysis of tRNA-Directed Transcription Antitermination of the *Bacillus subtilis* glyQS Gene In Vitro

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Received 30 March 2004/Accepted 5 May 2004

Binding of uncharged tRNA to the nascent transcript promotes readthrough of a leader region transcription termination signal in genes regulated by the T box transcription antitermination mechanism. Each gene in the T box family responds independently to its cognate tRNA, with specificity determined by base pairing of the tRNA to the leader at the anticodon and acceptor ends of the tRNA. tRNA binding stabilizes an antiterminator element in the transcript that sequesters sequences that participate in formation of the terminator helix. tRNA-dependent antitermination of the *Bacillus subtilis* glyQS leader was previously demonstrated in a purified in vitro assay system. This assay system was used to investigate the kinetics of transcription through the glyQS leader and the effect of tRNA and transcription elongation factors NusA and NusG on transcriptional pausing and antitermination. Several pause sites, including a major site in the loop of stem III of the leader, were identified, and the effect of modulation of pausing on antitermination efficiency was analyzed. We found that addition of tRNAgly can promote antitermination as long as the tRNA is added before the majority of the transcription complexes reach the termination site, and variations in pausing affect the requirements for timing of tRNA addition.

The rate of transcription elongation plays a crucial role in many genetic regulatory systems that operate at the level of premature termination of transcription. Pausing of RNA polymerase (RNAP) allows interaction of the transcription elongation complex (TEC) with cis-acting elements within the nascent transcript or with trans-acting factors that modulate the structure of the RNA or the properties of the TEC. The rate of transcription elongation in vivo has been estimated at 40 to 50 nucleotides (nt)/s (17, 23). The decision between termination and antitermination is therefore made within a very short time-frame. This is especially important in systems in which the nascent transcript folds into alternate terminator and antiterminator structures (13). In these cases, termination can be prevented by sequestration of the 5’ portion of the terminator helix by pairing with upstream sequences to form the antiterminator element. It is likely that formation or stabilization of the antiterminator element must occur before synthesis of the 3’ portion of the terminator helix; once the terminator helix is established in the appropriate position within the TEC, the residues participating in this helix are unlikely to be available for formation of the antiterminator element. Pausing of the TEC prior to synthesis of the complete terminator helix may therefore be required to allow an opportunity to monitor the relevant regulatory signal. A role for pausing has been established or postulated in a number of termination control systems, including the *Escherichia coli trp* operon attenuation system (14), L4-mediated attenuation in the *E. coli* S10 operon (22), the *Bacillus subtilis* TRAP (24) and PyrR (25) systems, and the *E. coli* tryptophanase operon (5).

The T box transcription termination control system, which is utilized in gram-positive bacteria for regulation of many aminoacyl-tRNA synthetase, amino acid biosynthesis, and transporter genes of a variety of amino acid classes, represents an example of a regulatory mechanism involving mutually exclusive antiterminator and terminator elements in the nascent transcript (6, 7). Genes in the T box family contain a complex array of conserved primary sequence and secondary structural elements within the leader RNA, preceding an intrinsic terminator element (Fig. 1) (6, 20). Readthrough of the terminator in each transcriptional unit is induced in response to decreased charging of the cognate tRNA so that, for example, the gene encoding tyrosyl-tRNA synthetase is induced when charging of tRNA Tyr is reduced (10). In the absence of interaction with the cognate uncharged tRNA, transcription terminates very efficiently at the leader region terminator. Specific tRNA recognition requires pairing of the anticodon of the tRNA with a triplet sequence, designated the “specifier sequence,” positioned in a bulge within the stem I portion of the leader (Fig. 1) (6). A second interaction between the acceptor end of the tRNA and a bulge in the antiterminator element is also required and provides discrimination between uncharged and charged tRNA (10). These two positions of interaction are necessary, but not always sufficient, for efficient tRNA-dependent antitermination (8).

Recent progress in the demonstration of tRNA-dependent antitermination in vitro has provided new tools for analysis of this system. tRNA gly-mediated antitermination of the *B. subtilis* glyQS leader occurs in a minimal system containing only purified *B. subtilis* RNAP, nucleoside triphosphates (NTPs), tRNA gly (generated with a T7 RNAP in vitro transcription system), and DNA template (11). In contrast, tRNA Thr-dependent antitermination of the *B. subtilis* thrS gene requires either cellular extracts or high levels of spermidine and employs tRNA purified from the cell (18). In both systems, antitermination is dependent on the cognate tRNA; tRNAs that do not
match the leader at the specifier or antiterminator regions fail to promote readthrough.

The use of halted complex transcription conditions for glyQS antitermination permits more detailed analyses of the kinetics of transcription. Since antitermination is dependent on a complex RNA-RNA interaction, which in vivo also requires selection of the appropriate tRNA from the cellular pool, pausing of the TEC during transcription could play an important role in the antitermination mechanism. In the present study, we identify multiple pause positions within the glyQS leader during transcription in vitro and investigate the effect of manipulation of pausing by variations in nucleotide sequence and NTP concentration on the efficiency of antitermination and the requirements for the timing of tRNA addition.

MATERIALS AND METHODS

Materials. The DNA template was a 440-bp PCR fragment including the B. subtilis glyQS promoter, leader, and terminator regions, with 85 bp past the termination site. His-tagged B. subtilis RNAP was isolated as described by Qi and Hulett (19). E. coli RNAP, prepared as described elsewhere (12), was provided.
RESULTS

tRNA\textsuperscript{Gly}-dependent \textit{glyQS} antitermination in vitro requires low NTP concentrations. Single-round transcription of a \textit{glyQS} template by \textit{B. subtilis} RNAP was initiated in the presence of 2 μM GTP, ATP, and UTP and halted at position +17 by omission of CTP. Reinitiation was blocked by addition of heparin, and synchronous restart of the halted complexes was triggered by addition of CTP, in the presence or absence of tRNA\textsuperscript{Gly} (11). NTP concentrations during the restart reaction were varied from 5 to 400 μM. Efficient tRNA\textsuperscript{Gly}-dependent antitermination occurred at 5 or 10 μM NTPs, with a 10-fold stimulation in readthrough in response to addition of the tRNA (Fig. 2). As the NTP concentration increased, both an increase in the level of readthrough in the absence of tRNA and a decrease in the tRNA response were observed, so that at the highest concentration all of the observed readthrough was tRNA independent. These results indicate that both termination and tRNA-dependent antitermination are sensitive to NTP concentration.

The decrease in termination at high NTP concentrations could be due to direct interference with terminator function because of alterations in transcription kinetics, as previously described (16). Alternatively, transcription in the presence of high concentrations of NTPs could cause nonspecific stabilization of the competing antiterminator conformation. This was tested using a \textit{glyQS} template containing only the promoter and terminator regions (AG19-G168) (Fig. 1); termination in this construct was also inefficient at high NTP concentrations (data not shown), indicating that this effect is a feature of the terminator itself and is independent of the presence of the competing antiterminator structure in the nascent transcript. Since the \textit{glyQS} leader region terminator is efficient in vivo (11), reduction of NTP concentration appears to be important for similar efficiency in vitro.

Low NTP concentration also appears to be important for tRNA-dependent antitermination, which was reduced to 4-fold at 30 μM NTPs and to 1.5-fold at 100 μM NTPs (Fig. 2). Loss of tRNA-dependent antitermination at high NTP concentrations could be due to an increased transcription rate, which could affect folding of the nascent transcript into the proper conformation for interaction with the tRNA. Rapid transcription through the leader could also result in a reduced opportunity for the tRNA to interact, since progression of the TEC to the termination site occurs rapidly. Alternatively, a high NTP concentration could affect pausing of RNAP at one or more required discrete pause sites.

Pausing of RNAP during \textit{glyQS} transcription in vitro. Time course assays were used to examine the processivity of transcription of the \textit{glyQS} leader during transcription with \textit{B. subtilis} RNAP (Fig. 3A). Samples were removed at 20-s intervals after restart of the halted complexes, and the resulting products were resolved on a denaturing polyacrylamide gel and compared to an RNA sequencing ladder. At 20 and 40 s, the majority of the products observed ended at several positions near the specifier loop and the 3' side of the base of stem I.
At 60 s, the majority of the product was at position G138, in the loop of stem III. The pause at this position was prolonged ($t_{1/2}$), with product gradually appearing at positions corresponding to the 3' region of the antiterminator (position U177) and the terminator (G220, the first position following the U run). Position G138 represents a pause rather than an arrest site, since no product remained at this position after prolonged incubation (data not shown).

The pausing profile and rate of appearance of the terminated product were unaffected by the addition of tRNA$^{Gly}$, although tRNA$^{Gly}$ addition resulted in the appearance of two readthrough bands, one corresponding to position U240, 20 nt downstream of the terminator, and the second, more prominent band representing the runoff product. It therefore appears that the tRNA-nascent RNA interaction does not affect the processivity of transcription until the TEC reaches the terminator-antiterminator decision point.

**Effect of NusA and NusG on pausing in vitro.** NusA and NusG are transcription elongation factors that affect the processivity of RNAP. NusA has been reported to promote pausing at hairpin pause sites by both *B. subtilis* (24, 25) and *E. coli* (2) RNAP, while NusG promotes release of *E. coli* RNAP from backtrack pause sites (2). Addition of *B. subtilis* NusA protein during the glyQS transcription elongation reaction increased termination efficiency but had no effect on tRNA-dependent readthrough, as previously reported (11, 21). NusA also had no effect on the pausing pattern or pause half-life (data not shown), consistent with the absence of hairpin structures in appropriate positions relative to the observed major pause sites. In contrast, addition of *E. coli* NusG resulted in a two- to fourfold reduction of pause half-life at all sites (data not shown). The efficiency of tRNA$^{Gly}$-dependent antitermination was not affected by addition of NusG, indicating that if the pause sites in the nascent transcript are important for readthrough, the residual pausing in the presence of NusG is sufficient.

**Kinetics of glyQS transcription by *E. coli* RNAP.** tRNA$^{Gly}$-dependent antitermination in vitro occurred with similar efficiency using either *B. subtilis* or *E. coli* RNAP (11). Transcription reactions with *E. coli* RNAP exhibited a pausing pattern similar to those with *B. subtilis* RNAP, although pausing was more pronounced, additional pause sites were detectable, and the overall rate of transcription through the leader was reduced (Fig. 3B). The pause at position G138 found for *B. subtilis* RNAP was also apparent in the *E. coli* RNAP reactions but was accompanied by a more prominent band at position U140. An additional major pause at A170, in the terminal loop of the antiterminator, was also detected. As was observed with *B. subtilis* RNAP, addition of tRNA$^{Gly}$ had no effect on the pausing pattern but resulted in efficient readthrough of the

![FIG. 3. Pausing during glyQS transcription in vitro. Transcription was halted by omission of CTP, and elongation was carried out in the presence of 10 μM NTPs and in the presence or absence of tRNA$^{Gly}$, as indicated. Samples were removed at the indicated time points after restart, and the reactions were terminated by phenol extraction. The positions of the major pause sites and the terminated (T) and readthrough (RT) products were identified using an RNA sequencing ladder and are labeled. Lanes 1 and 10, 0 s; lanes 2 and 11, 20 s; lanes 3 and 12, 40 s; lanes 4 and 13, 60 s; lanes 5 and 14, 80 s; lanes 6 and 15, 100 s; lanes 7 and 16, 120 s; lanes 8 and 15, 5 min; lanes 9 and 16, 10 min. (A) *B. subtilis* RNAP, wild-type template; (B) *E. coli* RNAP, wild-type template; (C) *B. subtilis* RNAP, Eco1 mutant template.](image-url)
leader region terminator. It therefore appears that while there are differences in the pausing profiles for the two forms of RNAP, both exhibit significant pauses in approximately the same positions of the leader.

**Mutation of the pause site in stem III.** The significance of the pause at G138 was further investigated by alteration of three positions in this region (Eco1 mutant, U136G, U137A, and G138A) by site-directed mutagenesis. The Eco1 mutant exhibited greatly reduced pausing within the loop of stem III, with increased representation of products at positions 136, 137, and 139 in addition to 138 (Fig. 3C). In addition, transcription through the leader region was faster, with accumulation of the terminated and readthrough products at earlier time points compared to those with the wild-type construct. The efficiency of tRNA-directed antitermination was unaffected, indicating that the precise sequence at the pause site is not required for antitermination. The Eco1 mutant also exhibited normal expression in vivo (data not shown).

**Effect of varying NTP concentration on pausing and readthrough.** Since tRNA-dependent antitermination was sensitive to NTP concentration, we examined the effect of limiting NTPs on both pausing and readthrough. Decreasing the NTP concentration during the elongation reaction from 10 to 3 μM resulted in a twofold increase in the duration of the pause at stem III and conferred a small increase in readthrough efficiency (Table 1). A further reduction of NTP concentration to 2 μM resulted in a sevenfold increase in the half-life of the pause but did not increase readthrough. When the GTP concentration was raised to 150 μM (with ATP, CTP, and UTP at 10 μM), the half-life of the pause in the stem III loop region decreased twofold, with no decrease in antitermination. Under these conditions, the position of the pause product at stem III shifted from G138 to U136, U137, and U139, suggesting that the pause at G138 is dependent on low GTP and that neither the prolonged pause nor the precise position of the pause at G138 is essential for antitermination. Raising the CTP or ATP concentration to 150 μM had no effect on either pausing or readthrough, while an increase in UTP concentration resulted in high tRNA-independent readthrough (data not shown). Limitation of UTP, in the presence of high GTP, ATP, and CTP, resulted in a fivefold decrease in the duration of the stem III pause, but tRNA-directed antitermination was still efficient. Limitation of any one of the other NTPs (in the presence of high concentrations of the other three) resulted in elevated readthrough in the absence of tRNA Gly (data not shown), as was observed when all four NTPs were used at a high concentration (Fig. 2). It therefore appears that low UTP is required for efficient termination and that the duration of the pause at stem III can be reduced without loss of tRNA-directed antitermination in vitro.

**Variation in the time of addition of tRNA Gly.** The position of the major pause at stem III, after synthesis of the stem I domain that interacts with the anticodon region of the tRNA, suggested the possibility that the pause could be important for the initial interaction of the leader with the tRNA. We therefore added tRNA Gly at intervals during the elongation reaction and compared the antitermination efficiencies. Addition of tRNA Gly at any point prior to the time when the majority of the elongation complexes had left the pause site in stem III resulted in efficient antitermination; addition of tRNA after that time resulted in a significant reduction in readthrough (Fig. 4). When the duration of the pause at stem III was reduced by increasing the GTP, ATP, and CTP concentrations, the timing for addition of tRNA was also altered, so that tRNA addition only at the earliest time points promoted antitermination. In contrast, increasing the duration of the pause by reduction of NTP concentrations permitted efficient antitermination when tRNA was added later in the transcription reaction. These results indicate that the window for tRNA addition is influenced by the kinetics of transcription elongation and suggest that tRNA must access the leader before the TEC reaches the termination site.

### DISCUSSION

Current models for transcription termination at intrinsic terminators invoke an interaction of the terminator helix with RNAP, coupled with destabilization of the DNA-RNA hybrid contributed by the run of U residues at the termination site (1). Termination at the glyQS leader region terminator in vitro was inefficient at high NTP concentrations. This could be due to rapid transcription through the terminator region, so that the terminator helix fails to form in the nascent transcript at the required time. Alternatively, the fact that limitation of UTP was sufficient to allow efficient termination suggests that slow addition of U residues to the 3′ end of the transcript may be a key parameter for termination at this site in vitro. In support of this idea, extension of the run of Us in the B. subtilis tyrS T box family leader caused increased termination in vivo (9). Furthermore, T box leader terminators generally contain an uninterrupted U stretch 5 to 7 nt in length, usually followed by a single purine and then additional U residues, resulting in a U stretch considerably longer than those usually observed in E. coli (4). While few terminators have been characterized in detail in B. subtilis and other gram-positive bacteria, the extended U stretch, coupled with a longer terminator helix, may prove to be a general feature of transcriptional terminators from low-G+C gram-positive organisms.

Time course analysis of glyQS transcription with B. subtilis RNAP revealed several discrete products that appeared at characteristic times during the transcription reaction. These included short-lived products representing several positions in the 3′ portion of stem I, a prominent product representing a
prolonged pause at G138 in the loop of stem III, and a product representing position U177 in the 3' region of the antiterminator opposite the bulge. Each of these products eventually disappeared after extended incubation, and the final products represent the terminated and readthrough transcripts (if tRNA was present), with an additional product 20 nt downstream of the termination site; this product appears to represent an additional pause site that is found only in reactions containing tRNA. Transcription in the absence of tRNA resulted in a small amount of the readthrough product, with a 10-fold increase in the presence of tRNA.

Two major classes of pause signals have been described for bacterial RNAPs. Hairpin-dependent pause sites involve formation of a stem-loop region in the nascent transcript at an appropriate distance from the pause position, while backtrack pauses are sequence dependent and result from slippage of RNAP relative to the template DNA (2). For the short-lived products paused in the 3' region of stem I, it is likely that the nascent transcript contains extensive structure, which could contribute to pausing in this region. In contrast, no hairpin structure can be found in the region upstream of the major pause in the loop of stem III, and the helical region of the antiterminator upstream of the pause at U177 is too close to the pause site to be likely to act as a hairpin-dependent pause site. NusA had no effect on the pausing pattern, consistent with the idea that the major pause sites do not represent hairpin-dependent pauses. Addition of NusG reduced pausing at all of the observed sites, including the site downstream of the terminator, consistent with the idea that these pause sites are likely to represent backtrack events. B. subtilis RNAP was previously shown to respond to backtrack pause sites but not hairpin-dependent pause sites from E. coli, indicating that there are some differences in the sensitivity of these enzymes to different pausing signals (3). The B. subtilis trp and pyr termination control systems, in contrast, exhibit pause sites that have the characteristics of hairpin-dependent pauses, including a strong stimulation by NusA (24, 25). In the glyQS leader, it appears that most of the pausing signals recognized by B. subtilis RNAP are also recognized by E. coli RNAP, with additional sites recognized by the E. coli enzyme, consistent with the results of Artsimovitch et al. (3).

The observation of an extended pause in the stem III loop region of the glyQS leader during transcription in the presence of low NTP concentrations raised the question of whether this pause plays a functional role in tRNA-dependent antitermination. We found that it was possible to add the tRNA at any time prior to or during the pause, but once the majority of the TEC left the pause site, termination occurred quickly and tRNA addition had little effect. Changing the duration of the pause by variation of the NTP concentration resulted in cor-
responding changes in the window for tRNA addition. However, the distribution of TECs within the leader and the rapid progression to the termination site after departure from the G138 pause make it difficult to determine if the pause site represents a crucial opportunity for tRNA binding.

Transcription through the major pause sites was unaffected by the presence of tRNA. This suggests that if pausing facilitates the interaction of tRNA with the nascent transcript, then this interaction does not affect the pause itself, i.e., tRNA binding does not stimulate the TEC to continue elongation. Again, this differs from the B. subtilis trp system, where binding of TRAP results in release of the NusA-dependent pause (24).

Similarly, the translating ribosome stimulates release from the pause site in the E. coli trp and trn operons (5, 14), whereas addition of PyrR has no effect on pausing in the B. subtilis pyr operon (25).

The position of the major pause site in the loop of stem III is intriguing. The specific loop, which is located at the 3' side of the base of stem I, contains the primary determinant for pairing with the anticodon loop of the tRNA. Pausing at stem III could permit this first major structural element to be completely synthesized and to fold into the appropriate structure. The glyQS leader is missing two large structural elements, stem II and the IIA/B pseudoknot, that are found in the majority of T box leaders (20). Stem III is therefore the next available element. A stem-loop is found in this region, just upstream of the antiterminator, in nearly all T box family leaders, although there is little conservation of sequence or structure (F. J. Grundy, S. M. Rollins and T. M. Henkin, unpublished results).

A mutation in the glyQS stem III loop had no effect on expression in vivo or antitermination in vitro, supporting the idea that the primary sequence in this region is not important for antitermination activity. Mutations in the stem III loop of the tyrS leader also had no effect on antitermination in vivo, and extension of stem III by insertion of an additional helical element allowed normal expression (F. J. Grundy and T. M. Henkin, unpublished results). It will be interesting to determine the effect of major alterations in stem III on glyQS antitermination in vitro and whether other leaders with different structural arrangements (e.g., the presence of stem II and IIA/B) exhibit a similar pausing pattern.

Variation in the NTP concentration, the use of E. coli RNAP, or altering the sequence in the stem III loop resulted in minor shifts in the position of the major pause site. Since tRNA-dependent antitermination occurred efficiently under all of these conditions, the precise position of the pause within this loop does not appear to be crucial for antitermination, consistent with the sequence variability in this region of T box leaders and its lack of sensitivity to mutation. However, reduction of the stem III pause half-life allowed antitermination only if the tRNA was added early in the reaction, while extension of the pause permitted later addition of the tRNA. These data are consistent with a model in which pausing of RNAP provides an opportunity for interaction of the tRNA with the nascent transcript. No condition was found that completely blocked pausing at stem III, precluding a direct test of whether the pause site is dispensable. The pause at U177 in the 3' side of the antiterminator could also play a role in tRNA interaction, but the short duration of this pause, and the rapid progression from this position to the termination site, complicates assessment of its possible function. Further analysis will be required to determine if either the stem III or antiterminator region pause sites play a role in the antitermination mechanism.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM47823 from the National Institutes of Health.

We thank F. M. Hulett for providing the strain for production of B. subtilis RNAP. M. Haldeman for preparation of B. subtilis NusA protein, I. Artsimovitch for providing E. coli RNAP, NusG, and advice on time course experiments, and S. Tigert for testing glyQS expression in vivo.

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