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Mutations affecting transcription pausing in the *Bacillus subtilis* *pyr* operon

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Abstract

Pausing during transcription of the *Bacillus subtilis* *pyr* operon has been proposed to play a role in its regulation by attenuation. Substitution mutations in the *B. subtilis* *pyr* DNA specifying the 3'-terminal nucleotides of previously identified transcription pause sites substantially reduced pausing at these sites in vitro. This result confirms the general utility of this mutagenic strategy for studying transcriptional pausing. Pyrimidine-mediated repression in vivo of *pyr-lacZ* fusions containing some of these substitution mutations was substantially lower than observed with the wild-type *pyr-lacZ* fusions. However, these defects in regulation were correlated with alterations in the stability of the terminator stem-loop specified by the attenuator, rather than with their effects on transcriptional pausing in vitro.

Keywords

pyrimidine biosynthesis; transcription pausing; attenuation; mutations; *Bacillus subtilis*

Introduction

The *Bacillus subtilis* pyrimidine biosynthetic (*pyr*) operon is regulated by a transcription attenuation mechanism in which the PyrR regulatory protein acts at three potential transcription termination sites in untranslated regions of the operon (reviewed in Switzer et al. 1999). At each site PyrR binds to a specific sequence in *pyr* mRNA and prevents formation of an antiterminator RNA stem-loop; this action allows a distal terminator stem-loop to form and reduces the expression of downstream genes. Since UMP and UTP promote binding of PyrR to the RNA (Lu and Switzer 1996, Bonner et al. 2001), this mechanism brings about repression of *pyr* genes by elevated intracellular uridine nucleotide levels. We suggested in a previous publication (Zhang and Switzer 2003) that the optimal functioning of this attenuation mechanism might require pausing during the transcription of *pyr* mRNA. Pausing of transcription at appropriate sites would allow time for the RNA secondary structure needed for PyrR binding (Bonner et al. 2001) to fold and for activated PyrR to bind before the relatively more stable full-length antiterminator stem-loop is transcribed. We demonstrated that such paused transcripts were formed during transcription in vitro of DNA templates specifying each of the three *pyr* attenuation regions (Zhang and Switzer 2003). For each template a single major NusA-stimulated pause site was identified. In each case this pause site was located at a position consistent with the proposed role for pausing in regulation. That is, the pause sites were located at points in the transcripts after the PyrR-binding sequences were fully transcribed, but before the complete antiterminator stem-loop was synthesized.

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In the present study we sought to determine by genetic means whether the pausing we observed with transcription experiments in vitro plays a role in the regulation of *pyr* genes in vivo. Base substitution mutations that were expected to substantially reduce or abolish pausing were constructed in template DNA from *pyr* attenuation regions. These substitutions were chosen so as to have only small effects on the secondary structure of the *pyr* RNA. The thorough characterization of the pause site in the 5' leader region of the *E. coli his* operon by Chan and Landick (1993) provided a valuable guide to doing this. Chan and Landick found that replacement of the base specifying the 3'-terminal U residue of the *his* pause transcript so that A or G became the 3' terminus greatly reduced accumulation of the paused transcript. In this work we demonstrate that similar mutations at the previously identified *pyr* pause sites also markedly reduced transcription pausing at these sites in vitro. The same mutations were then introduced into *pyr-lacZ* fusions, which were integrated into the *B. subtilis* chromosome so their effects on the regulation of *lacZ* expression by pyrimidines in vivo could be studied. However, transcription pausing in vitro and altered regulation of the *pyr-lacZ* fusions were not clearly correlated in our experiments.

Materials and methods

Construction of mutated *pyr* templates for in vitro transcription assays

Template 2, a 239-bp DNA fragment containing the native *B. subtilis pyr* promoter fused to nt +670 to +848 from the *pyrR-pyrP* intercistronic region was prepared by PCR amplification from pLS622 as previously described (Zhang and Switzer 2003). Substitution mutations in template 2 were constructed by PCR amplification of the entire pLS622 plasmid with mutagenic primers containing the desired base substitutions. Methylated template DNA was digested with *DpnI* endonuclease and the amplified linear mutated DNA was ligated into circles and used to transform *E. coli* DH5 α . The isolation of the desired plasmids was confirmed by sequencing, and the mutated DNA fragments corresponding to template 2 were amplified by PCR for use as templates for transcription in vitro.

Template 1, a 290-bp DNA fragment containing the native *B. subtilis pyr* promoter and 5' untranslated leader from nt -60 to +230 was obtained by PCR amplification from pUC19-290 as previously described (Zhang and Switzer 2003). Template 1 was mutated using methods analogous to those described above for mutation of template 2 to introduce an A nucleotide in place of the native T99 residue that specifies the 3' terminus of the major NusA-stimulated pause transcript formed from this template in vitro.

Transcription in vitro

Template 1 and 2 DNA and DNA fragments derived from these templates that contained base replacement mutations were used to examine the kinetics of transcription and pausing with *B. subtilis* RNA polymerase and 0.6 μ M *B. subtilis* NusA using the two step, single round method described in detail previously (Zhang and Switzer 2003). In this method an 11-nt halted transcript (indicated by H in Fig. 1) was formed from template 2 in the first step because transcription was initiated with GCUG, [α -³²P]ATP, UTP, and CTP, but without GTP. For template 1 a 12-nt halted transcript was formed after initiation with GCUG, UTP, ATP and [α -³²P]GTP in the absence of CTP. After addition of heparin to prevent initiation of new transcripts, all four rNTPs were added and the products of transcription after elongation times of 30 to 300 s were analyzed by electrophoresis. Construction and integration of *pyr-lacZ* fusion-integrand strains and assays of *lacZ* regulation by pyrimidines

Excision of the *pyr* DNA fragments with *EcoRI* and *BamHI* endonucleases from the plasmids described above in which the mutations were constructed, followed by ligation of the *EcoRI*-*BamHI pyr* fragments into the pDH32 plasmid (Grandoni et al. 1993) yielded plasmids in

which the *pyr* promoter and native or mutated attenuation regions were placed upstream of a *lacZ* reporter gene as transcriptional fusions. The corresponding plasmids were integrated into the *amyE* locus of three *B. subtilis* strains: a wild type strain (MO199 *trpC2 pheA amyE::erm*, Grandoni et al. 1993), a constitutive Δ *pyrR* strain (DB104 *his, nprR2 nprE18 ΔnprA3 ΔpyrR*, Turner et al. 1994), and a pyrimidine auxotroph HC11 (*his, nprR2 nprE18 ΔnprA3 pyrB::Spec^r*, Hu and Switzer 1995) as previously described (Lu et al. 1995).

Expression of the *pyr-lacZ* fusions was examined in the MO199 integrants grown on minimal medium containing 50 μg Trp and Phe per ml (Lu et al. 1995) and on the same medium containing 50 μg uracil and 50 μg uridine per ml, conditions corresponding to moderate and complete repression, respectively. The same conditions were used to study the DB104 Δ *pyrR* integrants, but because of the deletion of *pyrR*, expression of the *pyr* operon in these cells was derepressed and was not repressible by exogenous pyrimidines. The HC11 (*pyrB*) integrants were grown with 50 μg uracil and uridine per ml or with 50 μg orotate per ml, a condition leading to slow growth of this pyrimidine auxotroph and strong derepression (Lu et al. 1995). Approximately two to three fold of the total fold derepression in HC11 cells grown with orotate has been shown to result from a nonspecific effect of slow growth and is not specifically the result of *pyr* derepression (Lu et al. 1995). Strains DB104 β *pyrR* and HC11 were grown with 50 μg His per ml instead of Trp and Phe. All cultures were harvested in the late exponential phase of growth for β -galactosidase assays by previously described procedures (Lu et al. 1995).

Results

Mutation of *pyr* DNA templates at the site of the 3' terminus of paused transcripts reduces pausing of transcription in vitro

For most studies we mutated a DNA segment, called template 2 in our previous publication (Zhang and Switzer 2003), which contains a fusion of the *B. subtilis pyr* promoter to the *pyrR-pyrP* intercistronic attenuation region. Template 2 was chosen because it specifies only a single pause site *in vitro* that is strongly stabilized by NusA. The *pyr* RNA transcribed from template 2 is shown in Fig. 1 in two predicted functional secondary structures: the binding loop to which PyrR binds plus the terminator structure (Fig. 1A) and an alternative antiterminator structure (Fig. 1B). Substitution of the nucleotide at the 3' terminus of the NusA-stimulated pause transcript, U781, has no effect on the structure or stability of the PyrR binding stem-loop or the terminator formed by the RNA and only minimal effect on the stability of the antiterminator conformation of the RNA (Fig. 1). The free energies of formation of the wild type antiterminator stem-loop were calculated with MFOLD version 3.1 (Zuker et al 1999) as -24.2 kcal/mol and for the U781G and U781A mutants as -24.1 kcal/mol and -25.2 kcal/mol, respectively.

Transcription from the native (WT) template was compared to transcripts from seven mutated templates (Fig. 2). The results with native template 2 were essentially the same as those reported previously (Zhang and Switzer 2003). A major paused transcript ending at U781 accumulated transiently; its half-life in the presence of 0.6 μM NusA was 92 s. A secondary, NusA-independent pause transcript ending at C791 was also observed, as in our previous experiments (Zhang and Switzer 2003). This transcript decayed more slowly (half-life of 265 s) than that ending at U781, but a previously published chase experiment (Zhang and Switzer 2003) demonstrated that it is a paused transcript. Two of the mutated templates, T781A and T781G, replaced the 3'-terminal U of the major paused transcript formed from template 2 with purine nucleotides. These changes were predicted to reduce or eliminate pausing at U781, and, indeed, much less of this transcript was accumulated when the templates specifying A or G at the 3' terminus of the pause transcript were used (Fig. 2, upper panel). These findings support our previous identification of U781 as the 3' terminus of this paused transcript (Zhang and Switzer

2003). With the T781A and T781G mutated templates an increased amount of the secondary paused transcript ending at C791 was formed, especially with the T781G template. This transcript accumulated faster than with the native template and decayed with a time course typical of a paused transcript (half-life of 65 s and 150 s in T781A and T781G, respectively), suggesting that mutation of the NusA-stimulated pause site increased pausing at the alternative site. A template containing a single mutation, C791A, in the nucleotide specifying the 3' terminus of the secondary pause transcript was constructed and tested in the *in vitro* transcription system. As expected, pausing at the C791 site was substantially reduced with this template, while pausing at the U781 site was similar to that seen with the native template (Fig. 2, lower panel).

Since the most important objective of mutating template 2 was to generate a template in which little or no pausing could occur, a double T781A, C791A mutant template was constructed and tested. As seen in Fig. 2 (upper panel), very little of either paused transcript was detected with this template in the *in vitro* transcription system. Pausing at both sites was also significantly reduced in a related double mutant, T781A, C791G (Fig. 2, lower panel), in which G instead of A replaced C791.

However, replacement of C791 by a purine is predicted to destabilize the terminator stem-loop by disrupting a C-G base pair (Fig. 1A). To analyze the effects of this destabilization in the *in vivo* expression experiments described below, we constructed a triple mutant T781A, C791G, G800C, in which pausing at both sites should be sharply reduced, but the stability of the terminator stem-loop would be restored by replacing the C-G base pair with a G-C base pair. As shown in Fig. 2 (lower panel), pausing during transcription *in vitro* of the triple mutant template was reduced as with the U781A, C791A and U781A, C791G double mutants.

As a control experiment, a base pair immediately adjacent to T781 was mutated to create the A780G template. As expected, the rates of formation and disappearance of the products of transcription from this template were very similar to those seen with the native template (Fig. 2, upper panel).

To check the generality of our findings, a single mutational substitution in template 1 (Zhang and Switzer 2003), which specifies the *pyr* promoter and 5' leader RNA was constructed, and the products of its transcription *in vitro* were compared to native template 1 (Fig. 3). The mutated template introduced an A nucleotide in place of the native U99 residue at the 3' terminus of the major NusA-stimulated pause transcript formed from template 1 *in vitro*. This mutation substantially reduced the accumulation of this pause transcript without significantly affecting the accumulation of three other minor pause transcripts (Fig. 3). This observation indicates that U99 was correctly identified as the 3' terminus of this pause transcript. Furthermore, the findings with both the template 2 and template 1 mutations indicate that the finding of Chan and Landick (1993) that substitution of pyrimidines at the 3' termini of pause transcripts with purines strongly reduces pausing applies not only to *E. coli*, but also to *B. subtilis*. Such substitution mutations may be useful as a general method for examining sites and physiological consequences of transcriptional pausing.

Expression and regulation *in vivo* of *pyr-lacZ* fusions containing mutations that abolish pausing *in vitro*

The mutations in templates 1 and 2 were introduced into *pyr-lacZ* fusions so that the effects of the mutations on regulation of β -galactosidase expression by pyrimidines could be compared to fusions containing the native *pyr* sequences. The regulation of the native and mutated *pyr-lacZ* fusions was examined under a wide range of repressing and derepressing conditions as detailed in Materials and methods and summarized in Table 1. It should be noted that the repressed level of β -galactosidase expression varied by 2 to 3 fold from fusion to fusion, as

did the derepressed level of β -galactosidase expression in the $\Delta pyrR$ background. Since these variations could result from differences in the efficiency of translation or message stability and were seen in the $\Delta pyrR$ background, we have not interpreted them in terms of differences in *pyr* regulation. Instead, we have relied only on the ratio of β -galactosidase expression under derepressing versus repressing conditions to analyze the regulatory effects of the various mutations in vivo.

The regulation of expression of the T781A, T781G and A780G mutations in the *pyr* attenuation region of the *pyrR-pyrP* attenuation region was essentially the same as observed with the fusion containing the native sequence. Likewise, the regulation of the fusion from template 1 containing the T99A mutation was the same as with the native *pyr* 5'-leader fusion. Thus, elimination of pausing at only the NusA-sensitive sites did not affect repression in the corresponding *pyr-lacZ* fusion strains. On the other hand, the T781A, C791A double mutant, in which transcriptional pausing at both sites in vitro was reduced, demonstrated significant defects in the PyrR-dependent repression of *pyr-lacZ* expression by pyrimidines (Table 1). This seemed to support a role for transcriptional pausing in the normal repression of *pyr* genes, as previously proposed (Zhang and Switzer 2003). However, similar defects in regulation were observed in the C791A mutant strain, in which pausing was blocked only at the NusA-insensitive secondary site and was normal at the U781 site. This indicates that the defects in regulation in these two strains resulted specifically from the C791A mutation, not from defects in pausing. Only the C791A mutation would be expected to affect the stability of the terminator hairpin (Fig. 1). Analysis with the MFOLD computer program (Zuker et al 1999) indicated that the C791A mutation reduces the stability of the terminator hairpin by about 5 kcal/mol, which is almost 40% of its calculated free energy of formation. None of the other mutations studied in this work were predicted to alter the free energy of formation of secondary structures significantly.

To test the hypothesis that the defects in regulation observed with the C791A and T781A, C791A mutants resulted from destabilization of the terminator stem-loop, two additional mutants were constructed. One was T781A, C791G, in which replacement of the 3' pyrimidine nucleotides at both pause sites by purines strongly reduced pausing in vitro (Fig. 2, lower panel), as seen with the T781A, C791A double mutant. The second was a triple mutant, T781A, C791G, G800C, in which pausing was also reduced (Fig. 2, lower panel), but the stability of the terminator stem-loop was restored by replacement of the native C-G base pair with a G-C base pair. The T781A, C791G double mutant had defects in regulation of the corresponding *pyr-lacZ* fusion in vivo that were similar to those observed with the C791A and T781A, C791A pausing mutants (Table 1). However, restoration of the G-C base pair in the triple mutant restored normal regulation (Table 1). We note that the termination efficiency of the C791A and C791G mutants during transcription in vitro was not significantly decreased, as would have been predicted from decreased stability of the terminator stem-loop (Fig. 2). The reason for this is not known. Nonetheless, it is difficult to interpret the results of the *pyr-lacZ* expression experiments except in terms of decreased termination in the C791 mutants. We conclude that the defects in regulation of the mutated *pyr-lacZ* fusions by pyrimidines resulted from reduced stability of the attenuator (terminator) stem-loop, not from the reduction of transcriptional pausing, at least as it was observed in our in vitro assays.

Discussion

The most striking result from Table 1 is that mutations which substantially reduced transcription pausing at the major NusA-stimulated sites in two *pyr* attenuation regions, as observed with in vitro transcription experiments, did not lead to alterations in the regulatory behavior of *pyr-lacZ* fusions containing these mutations unless those mutations also altered the stability of the attenuator stem-loop. Thus, our previous proposal (Zhang and Switzer

2003) that transcription pausing plays a role in the regulation of the *pyr* operon in vivo should be called into question, even though it cannot be conclusively ruled out by these experiments. It is very unlikely that any of the mutations studied affected PyrR binding because they were all located so far from the experimentally determined PyrR binding sequences on RNA from template 2 (Bonner et al 2001) (Fig. 1) and were not predicted by MFOLD to alter folding of that region. It is possible that the paused transcripts observed in the two-step, single round in vitro transcription procedure used to examine kinetics of transcription are not formed to a significant extent in vivo. It is also possible that the small amount of residual transcription pausing with the mutant templates that was detected by our in vitro assay is sufficient to permit normal regulation of *pyr* attenuation under the conditions studied. Nonetheless, the physiological significance of transcription pausing in the regulation of the *B. subtilis pyr* operon, while an attractive hypothesis, should be regarded as unsubstantiated.

Transcription pausing has been implicated in the regulation by attenuation of the *E. coli trp* and *pyrBI* operons (Landick et al. 1996). In these cases mutational evidence supported the physiological importance of pausing. The use of genetic methods to test whether pausing is important for regulation *in vivo* of *B. subtilis* operons has yielded mixed results. In vitro transcription analysis similar to that used in our previous studies (Zhang and Switzer 2003) was used to identify transcription pausing in the *B. subtilis trpEDCFBA* operon (Yakhnin and Babitzke 2002). Uridine residues were also identified as the 3' termini of the paused transcripts in that system. Mutational methods similar to those described in this work have provided part of the evidence that pausing in the *trp* leader regulates expression of the *B. subtilis trp* operon in vivo (P. Babitzke, personal communication). Grundy and Henkin (2004) have identified a number of pause sites, including one strong NusA-independent pause site, in studies of the transcription in vitro of the *B. subtilis glyQS* leader, a system that is subject to regulation by the T box transcription antitermination mechanism. Mutation of three positions at the 3' terminus of the corresponding pause intermediate largely eliminated pausing at this site in vitro, an observation that accords well with our results. However, no effect of these mutations on the regulation of *glyQS* in vivo was detectable, which leaves the importance of pausing for normal regulation of this antitermination system as an unresolved question.

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Fig. 1. Secondary structure of the *pyr* RNA specified by template 2 in the predicted PyrR-bound terminator (A) and antiterminator (B) conformations showing the location of transcription pause sites (P and bold arrows) and mutations (dashed arrows to base substitutions) characterized in this study. MFOLD version 3.1(Zuker et al 1999) was used to predict these structures. The nucleotide sequences are numbered from the start of transcription of the *pyr* operon as +1. The boxed sequence indicates alterations in the native transcript that result from fusion of the *B. subtilis pyr* promoter plus nt +1 to +8 to the *pyrR-pyrP* intercistronic attenuation sequence. Circled nucleotides in A denote sequences in the terminator secondary structure that form the stem-loop in the alternative antiterminator secondary structure in B. “H” denotes the

3' nucleotide of the halted transcripts that were formed in the first step of the single round transcription experiments described in the text. The experimentally defined PyrR binding site for this RNA (Bonner et al. 2001) is enclosed in a large oval.

Fig. 2.

Transcriptional pausing *in vitro* with native and mutated *pyrR-pyrP* (template 2) DNA. Single round, two step transcription reactions were conducted and the transcripts were analyzed for the transcription elongation times shown. Arrows indicate the positions of run-off transcripts (RO), transcripts terminated at the attenuator terminator (T), a secondary pause transcript with its 3' terminus at C791, and the major, NusA-stimulated pause transcript with its 3' terminus at U781 (Zhang and Switzer 2003). WT, template 2 with the native sequence was used; in the other lanes templates with the base substitutions shown were used. In the lower panel the run-off transcripts are contaminated with an unrelated polynucleotide that is formed during the first step of the reaction.

Fig. 3.

Transcriptional pausing *in vitro* with native and mutated *pyr* 5' leader (template 1) DNA. Arrows indicate the positions of run-off transcripts (RO), transcripts terminated at the attenuator terminator (T), the major, NusA-stimulated pause transcript with its 3' terminus at U99 (Zhang and Switzer 2003), and three minor pause transcripts with 3' termini as shown. WT, template 1 with the native sequence was used. In other experiments not shown it was demonstrated that the percent termination at the attenuator with this template was not affected by the T99A mutation.

Table 1Regulation of expression of *pyr-lacZ* fusion integrants with mutations in transcription pause sites

Source of <i>pyr</i> DNA for <i>pyr-lacZ</i> fusion	Host strain for <i>pyr-lacZ</i> integrant	Relevant genotype	β-galactosidase activity (Miller units) ^a		
			No pyrimidines or +orotate ^b	+Uracil and uridine	Ratio
Template 2 (<i>pyrR-pyrP</i>)					
Native	MO199	wt	162 ± 43	75 ± 11	2
	DB104Δ <i>pyrR</i>	Δ <i>pyrR</i>	398 ± 100	431 ± 95	1
	HC11	<i>pyrB</i>	2600 ± 277	96 ± 21	27
T781A	MO199	wt	214 ± 55	87 ± 28	2.5
	DB104Δ <i>pyrR</i>	Δ <i>pyrR</i>	313 ± 24	334 ± 45	1
	HC11	<i>pyrB</i>	2416 ± 162	113 ± 11	21
T781G	MO199	wt	268 ± 45	112 ± 13	2.5
	DB104Δ <i>pyrR</i>	Δ <i>pyrR</i>	760 ± 47	723 ± 23	1
	HC11	<i>pyrB</i>	2566 ± 165	129 ± 6	19
A780G	MO199	wt	224 ± 26	93 ± 33	2.5
	DB104Δ <i>pyrR</i>	Δ <i>pyrR</i>	587 ± 14	628 ± 56	1
	HC11	<i>pyrB</i>	2295 ± 42	113 ± 18	20
T781A, C791A	MO199	wt	584 ± 14	378 ± 19	1.5
	DB104Δ <i>pyrR</i>	Δ <i>pyrR</i>	659 ± 12	651 ± 6	1
	HC11	<i>pyrB</i>	1673 ± 55	417 ± 3	4
C791A	MO199	wt	476 ± 11	317 ± 14	1.5
	DB104Δ <i>pyrR</i>	Δ <i>pyrR</i>	799 ± 39	779 ± 24	1
	HC11	<i>pyrB</i>	1659 ± 130	297 ± 12	6
T781A, C791G	MO199	wt	204 ± 5	184 ± 11	1
	DB104Δ <i>pyrR</i>	Δ <i>pyrR</i>	264 ± 30	289 ± 27	1
	HC11	<i>pyrB</i>	2003 ± 117	210 ± 12	10
T781A, C791G, G800C	MO199	wt	85 ± 39	46 ± 7	2
	DB104Δ <i>pyrR</i>	Δ <i>pyrR</i>	125 ± 22	135 ± 23	1
	HC11	<i>pyrB</i>	1334 ± 88	57 ± 11	23
Template 1 (<i>pyr</i> 5' leader)					
Native	MO199	wt	300 ± 5	119 ± 7	2.5
	DB104Δ <i>pyrR</i>	Δ <i>pyrR</i>	1011 ± 24	978 ± 12	1
	HC11	<i>pyrB</i>	2582 ± 51	218 ± 8	12
T99A	MO199	wt	276 ± 4	126 ± 9	2
	DB104Δ <i>pyrR</i>	Δ <i>pyrR</i>	610 ± 19	671 ± 13	1
	HC11	<i>pyrB</i>	2822 ± 84	211 ± 5	14

^aCultures were grown in minimal medium (Lu et al. 1995) containing 50 μg uracil and 50 μg uridine per ml where indicated. Values indicate average ± standard deviations of at least three determinations.

^bCultures of integrants into strain HC11 were grown in minimal medium containing 50 μg orotate per ml.