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Transcript hairpin structures are not required for RNA polymerase pausing in the gene encoding the *E. coli* RNase P RNA, M1 RNA

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Strong pauses at nucleotides +118 and +121 relative to the transcriptional start occur during in vitro transcription of the *E. coli rnpB* gene encoding the catalytic M1 RNA subunit of Ribonuclease P. These pauses are immediately downstream of 2 phylogenetically conserved stem-loop structures in the RNA. In the present work, single-base changes which disrupted Watson-Crick base-pairing in the hairpins were introduced into *rnpB*. Transcription studies in vitro with these modified templates revealed that none of the nucleotide changes predicted to increase or decrease the stability of the first hairpin significantly affected the pause half-lives. A mutation which disrupted the second hairpin increased the pause half-life 2-fold. The data suggest that the upstream stem and loop structures in the transcript are not involved in the pausing event.

RNA structure; Transcription

1. INTRODUCTION

Transcription in vitro of many bacterial genes is discontinuous, i.e. is interrupted by 'pauses' [1,5,13,16]. Release of the ternary complex from pausing results in further elongation to full-length transcripts. In vivo, transcriptional pausing may allow the coupling of translation with transcription as occurs during attenuation [19].

Pause sites that have been mapped so far have been placed in 2 groups based on the features thought to be involved in the basic event. The first group includes pauses that occur about 8–12 nucleotides downstream from a 'hairpin' stem-loop structure in the transcript. The best-studied example is in the *trp* attenuator region, *trpL* [7]. Recent evidence suggests that DNA sequences downstream of the *trpL* pause site also determine pause half-life [10,12]. The second group includes examples for which a specific RNA structure immediately upstream of the 3' end of the paused transcript is not apparent.

We described previously the presence of strong transcriptional pause sites within the *E. coli rnpB* gene encoding M1 RNA, the catalytic subunit of ribonuclease P [11]. These pauses at positions 118 and 121 of the M1 RNA structural sequence have a half-life of 27 s at NTP concentrations of 400 μM. Potential transcript hairpin structures exist at appropriate locations upstream of these pause sites (Fig. 1). The present study was undertaken to determine whether one or both of these features are required to elicit the pausing response. We found that the hairpin structures are dispensable arguing that transcriptional pausing in *rnpB* is dependent on other features of the template and/or transcript.

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2. MATERIALS AND METHODS

2.1. *Materials*

Restriction enzymes and T4 DNA ligase (for routine manipulations) were obtained from Bethesda Research Laboratories and New England Biolabs. [γ-^{32}P]GTP was synthesized by the procedure of Johnson and Walseth [4]. *E. coli* strains were DH5α [2], JM101 [15] and CJ236 [6]. Plasmid pRRP1–3 has been described [11]. Standard methods [14] were used for plasmid purification, restriction digestion and ligation.

2.2. *Site-directed mutagenesis*

The *Kpn*I–*Sma*I fragment from pRRP1–3 [11] carrying all 3 promoter elements and the first 290 nucleotides of the M1 RNA structural gene was subcloned into M13mp18. The subclone was used for the creation of site-specific mutations in the M1 RNA gene. Site-directed mutagenesis was performed according to the manufacturer's instructions (Muta-Gene M13 in vitro mutagenesis kit: Bio-Rad, Richmond, California) with 2 modifications. First, *E. coli* strain CJ236 was transfected with a lesser multiplicity of infection (<0.01) than the value of 0.2 suggested by the manufacturer. Secondly, twice the suggested amount of T4 DNA polymerase and T4 DNA ligase were used during the in vitro synthesis of the second DNA strand.

The sequences of the oligonucleotides (complementary to the M1 RNA sequence) used in the mutagenesis procedure were as follows: for nucleotide 102 [5'pCCCCCA(C)GCGTTACC3'], nucleotide 107 [5'pGGTTTCCCC(A/G)CCAGG3'] and nucleotide 116 [5'pGG TCGTG(C)GTTTCCC3']. The altered nucleotides are shown in parentheses.

Individual mutations (Fig. 1) in the M1 RNA gene were recovered from the recombinant M13mp18 plasmid DNAs in *Kpn*I–*Sma*I fragments. These fragments were then subcloned back into the parent plasmid, pRRP1–3 by replacing the existing wild-type *Kpn*I–*Sma*I fragment. The various pRRP1–3 derivatives were subsequently propagated in *E. coli* DH5α (recA$^-$).

DNA sequencing reactions were performed using modified T7

DNA polymerase (Sequenase) according to the manufacturers protocol (United States Biochemical Corporation, Cleveland, OH). The universal forward primer (New England Biolabs, Beverly, MA) was used in initial screening of mutations in single-stranded M13mp18. The presence of the mutations in the pRRP1–3 derivatives used for in vitro transcription reactions was confirmed by sequencing the respective double-stranded plasmid DNAs with an oligonucleotide primer specific for the M1RNA sequence.

2.3. *Single-round transcription reactions*

DNA templates for in vitro transcription reactions were prepared by digesting the plasmids carrying mutations in the M1 RNA sequence with *Eco*RI and *Hin*dIII. Subsequent purification of fragments and single-round transcription reactions were carried out as previously described [11]. The efficiency of pausing at nucleotides 118–121 for each template was calculated by integrating the progress curve for the reaction [9].

3. RESULTS

The role of the upstream pause determinants in the M1 RNA sequence was evaluated by the effect of base alterations in this region on the pause half-lives. Four different individual point changes affecting 3 different nucleotide positions were introduced into the *rnpB* gene (Fig. 1). Single-round transcription reactions were then performed in vitro using wild-type and mutant templates [11].

None of the nucleotide alterations affected the overall distribution of the RNA bands after autoradiography of the separated products of the transcription reactions (data not shown). Additionally, in all the templates examined, strong pause RNAs were seen at nucleotide positions 118 and 121 corresponding to the M1 RNA sequence, as characterized previously [11].

The C→G transversion at position 103 of M1 RNA (designated 103G in Table I) is predicted to disrupt base pairing in the putative 5-base pair stem and loop structure preceding the pause site, reducing the calculated stability from −4.9 kcal·mol^{-1} to −0.5 kcal·mol^{-1} [17]. Therefore, this mutation is an excellent candidate to probe the requirement for transcript hairpin structure. No significant change in the pause half-life was observed when this mutant DNA served as a template for

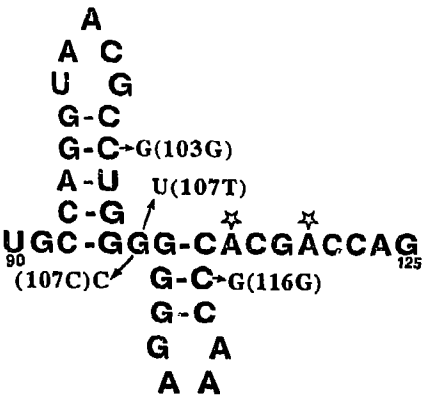

Fig. 1. Sites of mutations in the *rnpB* gene. Templates used in the transcription reactions are shown in brackets. The nucleotides underneath the stars correspond to the 3' ends of the 2 most prominent paused transcripts [11].

transcription (Table I). Similarly, the G→C and G→T transversions at position 107 (mutations 107C and 107T, respectively) between the hairpins did not affect the pause half-life significantly. The former mutation increases the predicted hairpin stability by allowing an extra base pair. If the hairpin structure were responsible for pausing increasing its stability should have increased the efficiency of pausing; this was not observed. We conclude that the stability of the first hairpin does not affect pausing.

In contrast, a C→G transversion at nucleotide 116 (116G) within the second hairpin enhanced the pause by 2-fold. This mutation is predicted to abolish the second stem-loop structure but does not alter overall base composition of the template. The increase in the pause half-life was judged to be statistically significant ($P<0.05$ by Student's paired t-test). Finally, we calculated the efficiency of pausing, that is, the fraction of polymerase molecules that were paused on each template [9]. This parameter varied with the observed half-life such that the 116G mutation resulted in an increase in the fraction of polymerase molecules that paused from 0.27 in the wild-type template to 0.40 in the mutant.

Table I

Pause half-lives of mutant *rnpB* templates

DNA template	Predicted stabilities of hairpins*		Pause half-life
	93–106	108–117	
Wild-type	−4.9	−1.6	16.4±5.7
103G	−0.5	−1.6	19.0±5.7
107C	−8.3	−1.6	13.0±2.0
107T	−6.8	−1.6	13.3±4.7
116G	−4.9	**	28.0±7.7

*$\Delta G'$ values in units of kcal·mol^{-1} at 37°C in 1 M NaCl.
**No stable structure predicted.

4. DISCUSSION

Structural features predicted to elicit a transcriptional pause exist upstream of the sites of pausing in the M1 RNA gene. These include a phylogenetically conserved dual stem and loop structure and a GC-rich sequence. Therefore, it was of interest to assess the individual contributions of these features to the M1 RNA pauses. Base changes affecting the M1 RNA sequence immediately upstream of the pause site were introduced into M1 RNA.

Mutation 103G is predicted to eliminate the forma-

tion of the stable 5-base pair stem and loop structure from nucleotides 92–106. The native structure is phylogenetically conserved in RNAse P RNAs [3] and is predicted to be thermodynamically stable ($\Delta G' = -4.9$ kcal·mol^{-1}). The finding that this change failed to affect the pause half-life suggests that formation of this structure is not required in the pausing event. (Other known stem and loop structures in the M1 RNA transcript likewise failed to cause the elongating RNA polymerase to pause). Previously we found that the *E. coli* elongation factor NusA had no effect on pausing in *rnpB* [11]. NusA has been proposed to enhance pausing in the *trp* operon by modifying the stability of the hairpin structure in the paused complex [8]. The present data indicate that a functional hairpin structure is not involved in M1 RNA pausing; therefore this proposal for NusA action is consistent with our results.

The second group of mutations included 2 independent base changes at nucleotide 107. These changes either decreased or increased the predicted hairpin stability but did not affect transcriptional pausing. It is unlikely from these data that the upstream hairpin is essential for the pause. In this regard we have noted that pausing does not occur at other GC-rich hairpins present in the M1 RNA sequence [11].

It is intriguing that a C→G transversion at nucleotide position 116 caused a 2-fold increase in the pause half-life as this nucleotide would presumably be a part of the RNA:DNA hybrid [18] in the ternary complex. The base change did not greatly affect the predicted strength of a potential RNA:DNA hybrid. Therefore we conclude that hybrid stability does not affect pausing in *rnpB*. Rather, we suggest that other features of the template or nascent transcript directly contribute to the pausing event.

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