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rRNA Antitermination Functions with Heat Shock Promoters

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Transcription antitermination in the rRNA operons of *Escherichia coli* requires a unique nucleic acid sequence that serves as a signal for modification of the elongating RNA polymerase, making it resistant to Rho-dependent termination. We examined the antitermination ability of RNA polymerase elongation complexes that had initiated at three different heat shock promoters, *dnaK*, *groE*, and *clpB*, and then transcribed the antitermination sequence to read through a Rho-dependent terminator. Terminator bypass comparable to that seen with σ^{70} promoters was obtained. Lack of or inversion of the sequence abolished terminator readthrough. We conclude that RNA polymerase that uses σ^{32} to initiate transcription can adopt a conformation similar to that of σ^{70}-containing RNA polymerase, enabling it to interact with auxiliary modifying proteins and bypass Rho-dependent terminators.

Regulatory studies of gene expression have traditionally concentrated on transcription initiation, but the processes of elongation, termination, and antitermination have become increasingly important aspects of gene regulation to address (2, 3, 15, 17). In particular, transcription antitermination (modification of the transcription apparatus so that terminators can be bypassed) presents an interesting regulatory mechanism. Transcription and translation are coupled for most operons of *Escherichia coli.* However, when translation is arrested or untranslated regions of RNA are synthesized early termination of transcription often results, causing a dramatic decrease in downstream gene expression, a phenomenon known as polarity (17). Untranslated regions of RNA frequently permit access by the transcription termination protein, Rho, leading to premature termination of transcription and release of RNA polymerase from the transcript (for a review, see reference 17). The transcripts of rRNA operons are not translated and contain Rho-dependent termination sites but are not subject to polarity (1, 5, 14). The absence of polarity and the synthesis of stoichiometric amounts of all three rRNA subunits are accounted for by a specific type of transcription antitermination (4, 10).

Rho-dependent terminator suppression in the rRNA operons is mediated by special antiterminator sequences that occur once in the leader region and again in the spacer region between the 16S and 23S genes (4, 8, 10, 18). These sequences can effectively suppress a variety of Rho-dependent terminators both in vivo and in vitro (10, 20). An important part of the rRNA antiterminator is the sequence GCTCTTTAACAA, called boxA (4). Host proteins NusA, NusB, NusE (ribosomal protein S10), and NusG are thought to be involved, and additional cellular proteins such as ribosomal protein S4 are also required for efficient terminator readthrough (20, 22). Several of these host factors interact directly with RNA polymerase, rendering it resistant to Rho-dependent termination (11–13, 22). Exactly how they accomplish this task is not known.

A further unanswered question is whether the nature of the σ subunit associated with RNA polymerase affects antitermination. For example, if a particular σ factor did not cycle off after initiation, RNA polymerase might not be able to recruit other factors necessary for the alteration of its transcription properties. When cells are subjected to stresses such as rapid heat or osmotic changes, selective groups of proteins are rapidly and transiently induced to protect the cell or help it adapt to the new environment. The heat shock response results when RNA polymerase associates with an alternative σ factor, σ^{32}, which directs core RNA polymerase to distinct promoters (7, 23). The consensus sequences of heat shock gene promoters differ considerably from those of σ^{70}-dependent promoters. There is no evidence that these promoters undergo cross-recognition (a mechanism which provides heat shock genes with regulation distinct from that of most of cellular proteins) (7, 25) either in vivo and in vitro. In the present study, we tested whether the antitermination properties of RNA polymerase are altered as a result of initiation at heat shock promoters. It is particularly interesting that all rRNA operon P1 promoters have interdigitated heat shock promoters. Both the σ^{70}-dependent and σ^{32}-dependent promoters initiated RNA transcription at the same nucleotide (16).

To measure the terminator readthrough properties of RNA polymerase molecules initiated at these promoters, we used gene fusion plasmids to construct an antitermination assay system (Fig. 1A). Each promoter sequence and its position relative to the boxA feature of the antiterminator sequence are shown in Fig. 1B, and their relevant structures with respect to promoters, antiterminators, and terminators are listed in Table 1. Plasmid pSL100 was used as the parental plasmid for all constructs. Its structure and those of pSL102 and pSL103 (containing $rrnGP_2$ [the σ^{70} promoter]) have been detailed by Li et al. (10). A fragment containing the *groE*P heat shock promoter was obtained from plasmid pDC440 (7) by digestion with *Taq*I and *Hpa*I. The isolated fragment was ligated into pSL102 and pSL103 digested with *Cla*I to yield pSGE102 and pSGE103,

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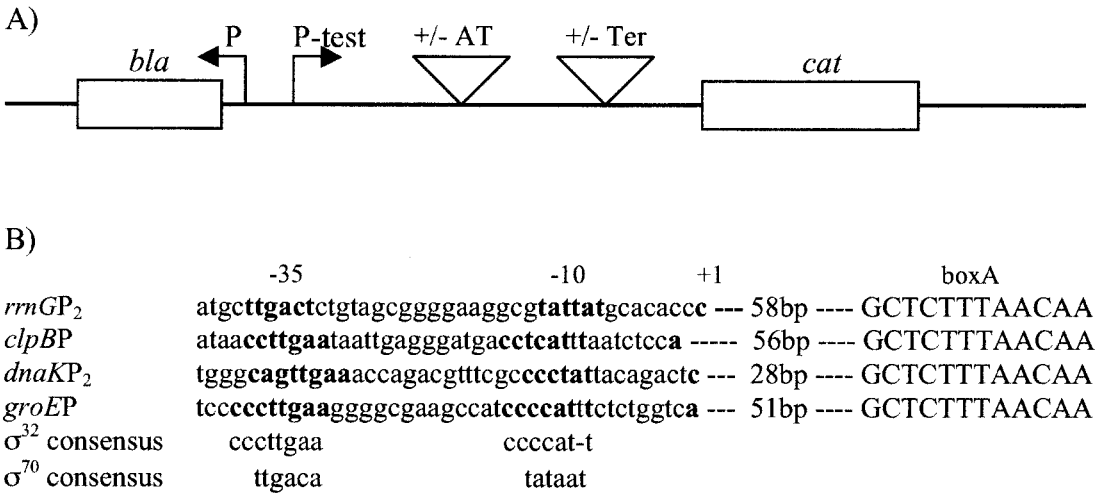

FIG. 1. Gene fusion plasmid antitermination assay system and sequences of test promoters. (A) A reporter gene, CM acetyltransferase (*cat*), was placed down stream of a Rho-dependent terminator, Ter. Open boxes represent the *cat* gene and the *bla* gene (encoding β-lactamase). Large inverted triangles show the insertion points for the antiterminator (AT) and a Rho-dependent terminator (Ter) sequences. P-test, insertion point of the test promoter transcribing the *cat* gene. Terminator readthrough was determined by analysis of the level of the *cat* gene mRNA transcript normalized to the level of *bla* gene transcript. (B) Sequences of the $rrnGP_2$ and heat shock promoters *clpBP*, $dnaKP_2$, and *groEP* and their relative distances to the *rrnG* boxA antiterminator. Promoter recognition sites and the start of transcription are indicated by −35, −10, and +1 (sequences in boldface characters). Numbers (in base pairs) indicate the distances between the start of transcription and the boxA sequence in nucleotides.

respectively. The heat shock promoter from the *clpB* gene was amplified from plasmid pClpB (21) by PCR with a 5′ *Bgl*II site and a 3′ *Cla*I site and used to replace the $rrnGP_2$ fragment of pSL102 and pSL103, resulting in pSCB102 and pSCB103, respectively. The heat shock promoter from the *dnaK* gene was cloned from pDC403 (7).

A promoter containing *Hpa*II and *Hin*PI fragments was inserted into the *Cla*I sites of pSL100 and pSL101 (10) to yield pSDK102 and pSDK103, respectively. *rrn* antitermination sequences were obtained (using 5′ and 3′ *Bam*HI primers) from the pRATT1 plasmid (20). The amplified fragment was digested with *Bam*HI and purified. This antiterminator-containing fragment was then ligated into previously constructed plasmids (except pSDK102 and pSDK103) digested with *Bam*HI. The antiterminator sequence was inserted in either the correct or an inverse orientation. The source of antitermination *rrnG* leader sequences for pSDK114 and pSDK115 was pSL104 (10), which was cleaved with *Bgl*II and *Taq*I and inserted into pSL102 and pSL103 (10). The Rho-dependent terminator sequence (16S←) used was a *Hin*dIII 16S fragment from the *rrnB* operon inserted into pSL100 in the backwards orientation to yield pSL103. In this orientation, the fragment fortuitously contains a strong Rho-dependent terminator (10).

TABLE 1. Plasmids used in this study

Plasmid designation	Elements in multicloning site			Reference or source
	Promoter	Antiterminator	Terminator	
pSL102	$rrnGP_2$			Li et al. (10)
pSL103	$rrnGP_2$		16S←	Li et al. (10)
pSLW114	$rrnGP_2$	AT		This work
pSLW115	$rrnGP_2$	AT	16S←	This work
pSLW126	$rrnGP_2$	AT_{Inv}		This work
pSLW127	$rrnGP_2$	AT_{Inv}	16S←	This work
pSGE102	*groEP*			This work
pSGE103	*groEP*		16S←	This work
pSGE114	*groEP*	AT		This work
pSGE115	*groEP*	AT	16S←	This work
pSGE126	*groEP*	AT_{Inv}		This work
pSGE127	*groEP*	AT_{Inv}	16S←	This work
pSCB102	*clpBP*			This work
pSCB103	*clpBP*		16S←	This work
pSCB114	*clpBP*	AT		This work
pSCB115	*clpBP*	AT	16S←	This work
pSCB126	*clpBP*	AT_{Inv}		This work
pSCB127	*clpBP*	AT_{Inv}	16S←	This work
pSDK102	$dnaKP_2$			This work
pSDK103	$dnaKP_2$		16S←	This work
pSDK114	$dnaKP_2$	AT		This work
pSDK115	$dnaKP_2$	AT	16S←	This work

The sequences of promoters and the orientation of the antiterminator sequence were verified by DNA sequencing using a Perkin-Elmer Prism sequencer (Perkin-Elmer, Boston, Mass). *E. coli* strain MC1009 [Δ(*lacIPOZY*) *galU galK* Δ(*ara-leu*) *rpsL srl*::Tn*10 recA spoT relA*] (20) was the host strain for all plasmids used in this study. Strains harboring test plasmids were used to inoculate 6 ml of Luria broth supplemented with 1% glucose and 100 μg of ampicillin/ml from overnight cultures in the same medium and incubated with shaking at 37°C. When the culture density reached an optical density at 600 nm of 1.0, cells were harvested by centrifugation in a microcentrifuge. The cell pellets were then frozen in a dry ice-ethanol bath and kept at −80°C. RNA isolation was done using an RNeasy RNA isolation kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The concentration of total RNA was measured using absorbance at 260 nm and kept at −80°C until further analysis was performed. Two end-labeled oligonucleotide probes were used to quantitate mRNA levels: (i) a chloramphenicol (CM) acetyltransferase (*cat*) probe (5′-TGCCAT TGGGATATATCAACGGTGG-3′) (located at nucleotides 26 to 50 of the *cat* gene encoding sequence and used to mea-

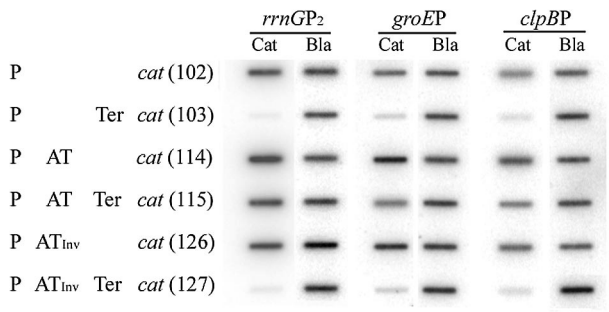

FIG. 2. Slot blot analysis of *cat* and *bla* mRNA levels. The specific transcripts were measured from total RNA extracted from cells harboring the indicated plasmids. Slot blot membranes hybridized with radiolabeled *cat* and *bla* probes were exposed on a Phosphorimager and scanned with a Storm Scanner (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.) for quantitation. P, promoter; Ter, Rho-dependent terminator; AT, *rrnG* antiterminator sequence; ATInv, *rrnG* antiterminator sequence in reverse orientation; *cat*, gene encoding CM acetyltransferase. Numbers in parentheses indicate designated plasmid numbers (Table 1).

sure *cat* gene expression) and (ii) β-lactamase (*bla*) probe (5′-GGGAATAAGGCGACACGGAAATG-3′) (located at nucleotides 13 to 36 of the *bla* gene encoding sequence and used to quantitate the level of *bla* gene expression). This measurement serves as an internal control to correct for variations in sample preparation and plasmid copy number (9). Slot blot analyses were carried out in triplicate for each sample of 5 μg of denatured total RNA by the method described by Zellars and Squires (24).

The rRNA antiterminator sequence can promote terminator readthrough by RNA polymerase initiated from heat shock promoters. Previous studies showed that the *E. coli* rRNA leader region antiterminator sequence is able to promote transcription readthrough of Rho-dependent terminators when transcription is initiated from unrelated σ^{70} promoters such as $rrnGP_2$, P*tac*, and P*lac* (10). Similarly, the lambda *nutR* antitermination sequence is functional with galactose operon promoters (8). Alternative σ factors, such as those used in responding to heat shock or stationary-phase conditions, differ dramatically in size from σ^{70} and have widely differing DNA sequence recognition properties (7). It is thus possible that the overall architecture and properties of RNA polymerase initiated at such promoters differ substantially from RNA polymerase molecules that initiate with σ^{70}. If the elongating polymerase configuration were changed or the alternative σ factor were not to cycle off the polymerase, such differences could lead to alterations in response to, or interaction with, other cellular factors during transcription. We tested the well-defined *dnaK*, *groE*, and *clpB* heat shock promoters (7, 21) to see whether RNA polymerase that initiates at σ^{32} promoters can recognize the rRNA antiterminator.

Preliminary experiments revealed that CM resistance levels of the strains carrying the assay plasmids were increased in the presence of the antiterminator. Quantitative slot blot analyses were then carried out to measure terminator readthrough activity in conjunction with the different promoters and the antiterminator sequence. The measured *cat* mRNA levels were normalized to the *bla* (β-lactamase gene) mRNA (also carried on the plasmid) to compensate for any difference in plasmid copy numbers and total RNA amounts recovered (9). The results showed that when the rRNA antiterminator sequence was placed between the promoter and the Rho-dependent terminator, terminator readthrough was observed with all promoters tested (Fig. 2 and Table 2). The presence of the antiterminator sequence resulted in 61, 42, 29, and 58% terminator readthrough (P+AT+T mRNA level; Table 2) compared to that seen with constructs without the terminator (P+AT) for the *rrnG*, *groE*, *clpB*, and *dnaK* promoters, respectively.

The antitermination activity was dependent on the antiterminator sequence characteristics. Lack of (P+T) or inversion of ($P+AT_{Inv}+T$) the sequence decreased terminator read-

TABLE 2. Terminator readthrough analysis of σ^{32} promoters

	cat/*bla* ratio[a]					
	Promoter (P)	Termination (P+T)	AT effect (P+AT)	AT RT (P+AT+T)	AT_{Inv} effect ($P+AT_{Inv}$)	AT_{Inv} RT ($P+AT_{Inv}+T$)
$rrnGP_2$						
mRNA	100	1	209	127 (61)	118	1 (1)
CM^r	100	3	100	67 (67)	100	3 (3)
*groE*P						
mRNA	100	4	307	129 (42)	191	2 (1)
CM^r	100	12	200	80 (40)	394	12 (3)
*clpB*P						
mRNA	100	4	146	42 (29)	121	4 (3)
CM^r	100	10	100	40 (40)	140	0 (0)
$dnaKP_2$						
mRNA	100	6	173	100 (58)	ND[b]	ND

[a] Levels of *cat* expression are shown as ratios of percentages relative to each of the promoter-only (P) constructs. The numbers in parentheses indicate AT or AT_{Inv} sequence terminator readthrough activities (in percentages) relative to an AT effect (P+AT) or to an AT_{Inv} effect ($P+AT_{Inv}$), respectively. The mRNA transcript data were obtained from scanned slot blot analysis (Fig. 2). CM^r data were obtained by plating cells harboring test plasmids on Luria agar media with various concentrations of CM. The level of CM resistance was determined as the maximum concentration of CM at which cells could grow. Data shown are the averages of the results of at least three independent experiments. P, promoter; AT, antiterminator sequence; AT_{Inv}, AT in the reverse orientation; T, Rho-dependent terminator.

[b] ND, not done.

through to the basal level (1 to 4% of readthrough without terminator) (Table 2). The antiterminator sequence, whether in the forward or reverse orientation, also increased the measured *cat* expression of both σ^{70} and σ^{32} promoters by as much as threefold in mRNA level and fourfold in CM^r level (*groE*; Table 2). This result was obtained with constructs lacking the terminator. Increased message stability or facilitation of translation (thus increasing mRNA lifetime) could account for the increased level of messages in the presence of the antiterminator sequence. The overall promoter activity and terminator readthrough were higher with the *rrnG* promoter than with the heat shock promoters we tested. The *cat* mRNA level (expressed as a *cat*/*bla* ratio) from the $rrnGP_2$ promoter was 10- to 12-fold higher than those from heat shock promoters (before normalization to 100% for each promoter). This result is in agreement with previous measurements of the relative strength of rRNA operon versus those of other promoters (particularly heat shock promoters) (6, 19).

Our results show that the *rrn* operon antiterminator sequence can promote transcriptional antitermination of RNA polymerase molecules initiated from σ^{32}-dependent promoters. Because the models for modification of RNA polymerase to a terminator-resistant state all involve the addition of new proteins factors, it is of interest to examine under which circumstances these modifications can take place (22). Why might RNA polymerase molecules initiated at σ^{32} promoters be refractive to such modifications? A smaller sigma factor may result in subtle conformation changes in RNA polymerase that in turn are unfavorable for adding modification proteins. Information as to when or even whether or not σ^{32} cycles off of RNA polymerase is not available. If σ^{32} were to change the conformation of or interfere with proper binding sites for the modification proteins, then terminator readthrough would not occur. Our findings suggest that the ability of RNA polymerase to interact with host antitermination factors is not affected by putative conformational changes in its structure that might result from an altered initiation status or association with alternative σ factors. We conclude that the initiation status of RNA polymerase is not a crucial parameter for transcriptional antitermination occurring 30 to 60 nucleotides downstream of the initiation region.

S. Kustu posed the question that prompted this study: does the rRNA-AT system function with other sigma factors? We appreciate her input and interest. We are grateful to C. Cerami and K. Eisinger for the construction of plasmids pSDK102, pSDK103, pSDK114, and pSDK115. We also thank C. A. Gross for the gift of pDC440 and pDC403 and M. J. Casadaban for strain MC1009. We thank A. L. Sonenshein for critically reading the manuscript. We thank the Tufts Core Facility in the Physiology Department for oligonucleotide preparation and DNA sequencing.

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