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Transcription-frequency-dependent modulation of an attenuator in a ribosomal protein–RNA polymerase operon requires an upstream site

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Although the attenuator located between the ribosomal protein and RNA polymerase gene domains of the *Escherichia coli rplKAJLrpoBC* operon has a maximum termination efficiency of 80%, the level of termination is diminished with decreasing transcription frequency. In this report, the use of transcriptional fusions to further investigate the mechanism of transcription-frequency-dependent regulation is described. The termination efficiency of two other weak terminators was assayed over a wide range of transcription frequencies programmed by different strength promoters. The results indicated that a decrease in termination efficiency with decreasing transcription frequency is not an inherent property of weak terminators. Deletion of the 165 bp segment located 439–274 bp upstream of the attenuator abrogated the difference in termination efficiency normally seen between high and low levels of transcription. This suggests that a *cis*-acting site located in this upstream region is necessary for transcription-frequency-dependent modulation of the attenuator's function. However, this site apparently works only in combination with the attenuator, since it did not cause transcription-frequency-dependent modulation when placed upstream of two other weak terminators. Analysis of the readthrough frequencies of single or tandem copies of the attenuator indicated that the transcription complexes which pass through the attenuator have not been converted to termination-resistant complexes in a manner analogous to the N-mediated antitermination system of lambda. Finally, an examination of termination efficiency in three *nusA* mutants suggested that although NusA increases readthrough at the attenuator it is not directly involved in transcription-frequency-dependent modulation.

Keywords: transcription termination, attenuator, RNA polymerase, *Escherichia coli*

INTRODUCTION

The synthesis of RNA polymerase in *Escherichia coli* is not constitutive, but is autogenously regulated and correlated with the growth requirements of the cell. The minimal core RNA polymerase ($\beta\beta'\alpha_2$), which is competent for elongating RNA transcripts, can associate with one of at least six sigma subunits during transcription initiation (Ishihama, 1993). Consistent with this transient interaction, the sigma factors are present in the cell at lower levels relative to core enzyme. The primary sigma factor utilized during exponential growth, σ^{70}, is present at approximately 40% of the level of core enzyme (Iwakura *et al*., 1974; Engbaek *et al*., 1976; Hayward & Fyfe, 1978; Kawakami *et al*., 1979). The α subunit however is synthesized in excess of that required to assemble core enzyme (Iwakura *et al*., 1974; Engbaek *et al*., 1976; Pedersen *et al*., 1976; Hayward & Fyfe, 1978; Kawakami *et al*., 1979). Therefore, the assembly of core enzyme, and thus the global transcription capacity of the cell, appears to be limited by the synthesis of the β and β' subunits.

The β and β' subunits are encoded by the *rpoB* and *rpoC* genes, respectively, which are located at the distal end of

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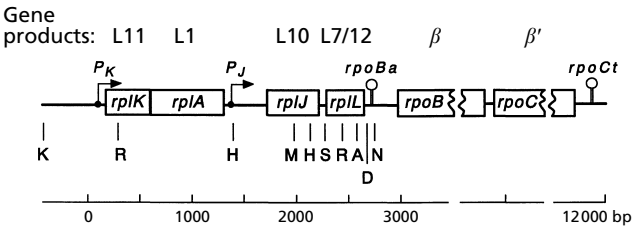


Fig. 1. Organization of the *rplKAJLrpoBC* ribosomal protein and RNA polymerase gene cluster. The *rplKAJL* region is drawn to scale. The *rplKp* (P_K) and *rplJp* (P_J) promoters and the direction of transcription are indicated by the bent arrows. Hairpins indicate the locations of the *rpoBa* attenuator and *rpoCt* terminator. The positions of restriction sites used in the construction of the *rplrpo–lacZ* fusions are drawn below the map. A, *Alu*I (nt 2623); D, *Dde*I (2672); H, *Hin*dIII (2154 and engineered at 1339); K, *Kpn*I (−428); M, *Sma*I (1986); N, *Nar*I (2730); R, *Eco*RI (280 and 2444); and S, *Swa*I (2279). The nucleotide numbering is according to Post *et al.* (1979).

the 12 kb *rplKAJLrpoBC* gene cluster (Fig. 1). The four upstream genes encode ribosomal proteins and all six genes are cotranscribed in a complex pattern (Steward & Linn, 1991). Promoters of approximately equal strength are located upstream of *rplK* (*rplKp*) and *rplJ* (*rplJp*) (Fig. 1), and since there is no terminator between them, both promoters are responsible for transcription of the *rplJLrpoBC* genes. However, during exponential growth on rich media, the *rpoBC* genes are transcribed at about one-fifth the frequency of the upstream *rplJL* genes due to termination at an attenuator (*rpoBa*) located within the *rplL–rpoB* intercistronic region.

A number of studies have indicated that there are both transcriptional and translational control mechanisms involved in determining the intracellular level of RNA polymerase (Steward & Linn, 1992; Dykxhoorn *et al.*, 1996; and references cited therein). Recently, we showed that the translation of *rpoB* and *rpoC* mRNA is reduced in response to increased concentrations of either β or β′ (Dykxhoorn *et al.*, 1996). However, when the concentration of all the holoenzyme subunits is increased, the transcription of *rpoBC* is reduced, primarily by lowering initiation at *rplKp* and *rplJp* (Dykxhoorn *et al.*, 1996). These results suggest that excess production of the β and β′ subunits can autogenously regulate *rpoBC* expression at either the transcriptional or translational level depending on whether they are assembled into holoenzyme or remain as individual polypeptides.

Another aspect of autogenous control of RNA polymerase synthesis may involve regulation of termination at the *rpoBa* attenuator. The *rpoBa* sequence resembles a simple terminator in that it produces an RNA transcript which can fold into a 11 bp GC-rich hairpin followed by five consecutive U residues. However, we have shown previously that readthrough of *rpoBa* increases from a minimum of 20% to greater than 60% as the frequency of transcription of the region containing *rpoBa* decreases (Steward & Linn, 1992). The finding that the frequency of termination at *rpoBa* is inversely modulated by transcription frequency suggests an autogenous control mechanism that senses the level of functional polymerase, and in particular the level of transcription of *rplKAJLrpoBC*. Accordingly, as transcription into *rpoBa* is reduced, there is a partial compensatory response by increasing readthrough of *rpoBa* and thus transcription of the *rpoBC* genes.

In the current study we have further examined the mechanism of the transcription-frequency-dependent modulation of *rpoBa* termination efficiency. Previously, we observed that the level of transcription of *rpoB* relative to the upstream *rplJL* genes was decreased in a *nusA1* mutant strain (Ralling & Linn, 1987). Subsequently, we found that the addition of either NusA or NusG to a purified *in vitro* transcription system increased the readthrough of *rpoBa* (Linn & Greenblatt, 1992). Since these results suggested NusA and NusG can affect termination of *rpoBa*, and because they were first identified as components of the N-mediated antitermination system in phage λ (Friedman, 1988; Das, 1992; Greenblatt *et al.*, 1993), we examined whether aspects of the mechanism operating at *rpoBa* were analogous to those in λ. We found that, analogous to the λ system, modulation of termination at *rpoBa* appears to require upstream sequences. However, in contrast to λ, this upstream region does not function with other terminators and the polymerase molecules that read through *rpoBa* have not been converted into stable termination-resistant complexes.

METHODS

***E. coli* strains.** MG2 is a Δ(*argF–lac*)*U169* derivative of MG1655, MG4 is a *recA56* derivative of MG2 (Ralling *et al.*, 1985). C600 was described by Appleyard (1954). DH5α [F^-(φ80d*lacZ*ΔM15) Δ*lacU169 endAI recA*1 *hsd*R17 (r_K^- m_K^+) *deoR supE44 thi-1* λ^- *gyrA96 relA1*] was obtained from BRL. MG1457 [*nusA1 argG::*Tn*5* Δ(*argF–lac*)*U169*] was constructed by P1 cotransduction of the kanamycin resistance and *nusA1* markers from K1457 to MG2. MG1914 [*nusA10*(Cs) *argG*::Tn*5* Δ(*argF–lac*)*U169*] was constructed by P1 cotransduction of the kanamycin resistance and *nusA10*(Cs) markers from K1914 to MG2. MG3903 [*nusA11*(Ts) Tn*10* Δ(*argF–lac*)*U169*] was constructed by P1 cotransduction of the tetracycline resistance and *nusA11*(Ts) markers from K3909 to MG2. K1457, K1914 and K3903 were kindly supplied by D. Friedman (University of Michigan).

Recombinant DNA. Transcriptional or operon fusions were constructed in the lambda TL61 transcriptional fusion vector (Linn & St Pierre, 1990). Restriction endonucleases, DNA modifying enzymes and linkers were obtained from Boehringer Mannheim, Pharmacia or New England Biolabs. Insert DNA fragments and vector phage arms were produced by restriction endonuclease digestion of source DNA and resolved by agarose gel electrophoresis. Appropriate bands were excised from the standard agarose or Nusieve GTG (FMC BioProducts) agarose gels and purified by the GeneClean process (Bio101) with final resuspension of the DNA in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7·5). When required, staggered ends were filled in using the Klenow enzyme. Recombinant phage were recovered from ligation

Table 1. Details of *rplrpo–lacZ* transcriptional fusions

Fusion	Features*	5′ end†	3′ end†	Source
KS166	*Pant244–rplJLrpoBa*	*Hin*dIII (1339)	*Nar*I (2730)	Steward & Linn (1992)
KS169	*Pant285–rplJL*	*Hin*dIII (1339)	*Dde*I (2672)	Steward & Linn (1992)
KS170	*Pant285–rplJLrpoBa*	*Hin*dIII (1339)	*Nar*I (2730)	Steward & Linn (1992)
KS195	*Pant224–rplJL*	*Hin*dIII (1339)	*Dde*I (2672)	Steward & Linn (1992)
KS253	*Pant285–rplL*	*Swa*I (2279)	*Dde*I (2672)	This study
KS257	*Pant285–rplLrpoBa*	*Swa*I (2279)	*Nar*I (2730)	This study
KS264	*Pant285–rpl′L*	*Eco*RI (2444)	*Dde*I (2672)	This study
KS265	*Pant285–rpl′LrpoBa*	*Eco*RI (2444)	*Nar*I (2730)	This study
KS266	*Pant1045–rplJLrpoBa*	*Hin*dIII (1339)	*Nar*I (2730)	This study
KS267	*Pant1045–rplJL*	*Hin*dIII (1339)	*Dde*I (2672)	This study
KS269	*Pant224–rplJLrpoBa–rpoBa*	*Hin*dIII (1339)	*Nar*I (2730) + *Dde*I (2672) − *Nar*I (2730)	This study
KS270	*Pant1045–rpl′L*	*Eco*RI (2444)	*Dde*I (2672)	This study
KS271	*Pant1045–rpl′LrpoBa*	*Eco*RI (2444)	*Nar*I (2730)	This study
KS285	*Pant1045–rplL*	*Swa*I (2279)	*Dde*I (2672)	This study
KS286	*Pant1045–rplLrpoBa*	*Swa*I (2279)	*Nar*I (2730)	This study
KS287	*Pant285–rplJLrpoBa–rpoBa*	*Hin*dIII (1339)	*Nar*I (2730) + *Dde*I (2672) − *Nar*I (2730)	This study
KS305	*Pant285–rpl′LrpoBa*	*Alu*I (2623)	*Nar*I (2730)	This study
KS311	*Pant285–rpl′L*	*Alu*I (2623)	*Dde*I (2672)	This study
KS312	*Pant1045–rpl′L*	*Alu*I (2623)	*Dde*I (2672)	This study
KS313	*Pant1045–rpl′LrpoBa*	*Alu*I (2623)	*Nar*I (2730)	This study

* Primes before a gene designation indicate truncation of the 5′ end of the gene; primes after a gene designation indicate truncation of the 3′ end of the gene.

† The restriction sites used to generate the 5′ and 3′ endpoints of the *rplrpo* region are listed. Restriction site coordinates (in parentheses) are according to the numbering of Post *et al.* (1979).

reactions by packaging *in vitro* and plating the reactions on fresh lawns of C600 as previously described (Maniatis *et al.*, 1982). Recombinant plasmids were recovered from ligation reactions by transformation of frozen competent DH5α cells prepared as described by Hanahan (1985). All phage and plasmid constructs were screened by extensive restriction endonuclease digestion analyses. Critical regions of some constructs were confirmed by dideoxy sequencing. Phage DNA was prepared from plate lysates by the small-scale method (Sambrook *et al.*, 1989) or a modification of the $ZnCl_2$ method (Santos, 1991). Plasmid DNA for screening was prepared by a modification of the method of Holmes & Quigley (1981). Preparative amounts of plasmid were purified by the Qiagen method.

Transcriptional fusions. Transcriptional fusions of *rplrpo* regions to *lacZ* were constructed in the lambda TL61 fusion vector (Linn & St Pierre, 1990). Details of genetic and regulatory features contained in the various fusions are presented in Table 1. Fusions KS166, KS169, KS170, KS176 and KS195 were described previously (Steward & Linn, 1992).

(i) Source of promoters for driving transcription of *rplrpo* segments and *tR2* terminators. The original recombinant phage constructs containing the *tac* (TAC1), *Pant285* (Pa285), *Pant224* (KS200) and *Pant219* (Pa219) promoters were described previously (Steward & Linn, 1992). The additional constructs used in this study containing the *Pant222* and *Pant1045* promoters were made in the same way as the *Pant*-containing phage listed above (Steward & Linn, 1992).

(ii) Fusions containing *tR2*^{2} and *tR2*^{9} downstream of different promoters. In a series of constructs, the *tR2*^{2} and *tR2*^{9} terminators isolated as 440 bp *Sma*I–*Xba*I fragments from pKL600tR2^2 and pKL600tR2^9 (Cheng *et al.*, 1991) were inserted between various promoters and the *lacZ* gene. A summary of the features contained in the fusions is presented in Table 2. tR35 and tR29 were constructed by inserting the *tR2*^{2}- and *tR2*^{9}-containing fragments between the *Xba*I-generated left arm of TL61 and the *Sac*I-generated right arm of KS211. KS211 was constructed from the *Eco*RI-generated left arm of TL61 and *Eco*RI-generated right arm of Pa285. This construction places a *Sac*I site downstream of the *Eco*RI site in Pa285. tR33 and tR34 were constructed from the *Eco*RI-generated left arm of tR35 and the *Eco*RI-generated right arm of Pa222 or Pa1045, respectively. tR44, tR47, tR48 and tR49 were constructed from the *Eco*RI-generated left arm of tR29 and the *Eco*RI-generated right arm of Pa1045, Pa222, Pa219 or TAC1, respectively.

(iii) Fusions used in deletion analysis to delineate an upstream modulation site. For each of the *Pant285* and *Pant1045* promoters, fusions were constructed with or without the 58 bp *rpoBa*-containing *Dde*I–*Nar*I region such that they

contained the *rpl* DNA extending 1375 bp (KS169 and KS170, and KS267 and KS266, respectively), 439 bp (KS253 and KS257, and KS285 and KS286, respectively), 274 bp (KS264 and KS265, and KS270 and KS271, respectively) or 95 bp (KS311 and KS305, and KS312 and KS313, respectively) upstream of the *rpoBa* termination site. The 3′ end of the *rpl* segment in those fusions that lacked the attenuator was at the *Dde*I site at nt 2672 (nucleotide numbering according to Post *et al*., 1979), while the 3′ end in those fusions that contained *rpoBa* was at the *Nar*I site at nt 2729. The 5′ ends of the *rpl* segments were provided by the *Hin*dIII (engineered at nt 1339), *Swa*I (2279), *Eco*RI (2444) or *Alu*I (2623) sites (Table 1). KS169 and KS170 were described previously (Steward & Linn, 1992). KS267 was constructed from the *Xho*I-generated left arm of KS169 and the *Xho*I-generated right arm of Pa1045. KS266 was constructed from the *Xho*I-generated left arm of KS170 and the *Xho*I-generated right arm of Pa1045. KS253 was constructed from the *Swa*I-generated left arm of KS167, 1692 bp *Sma*I–*Bam*HI fragment of KS203 (which is identical to the *Sma*I–*Bam*HI fragment of Pa285) and *Bam*HI-generated right arm of TL61. KS257 was constructed from the *Swa*I-generated left arm of KS170, 1692 bp *Sma*I–*Bam*HI fragment of Pa285 and *Bam*HI-generated right arm of TL61. KS285 was constructed from the *Swa*I-generated left arm of KS169, 1692 bp *Sma*I–*Bam*HI fragment of Pa1045 and *Bam*HI-generated left arm of TL61. KS286 was constructed from the *Swa*I-generated left arm of KS170, 1692 bp *Sma*I–*Bam*HI fragment of Pa1045 and *Bam*HI-generated fragment of TL61. KS264 and KS265 were both constructed with the *Eco*RI-generated right arm of Pa285, and the *Eco*RI-generated left arm of KS167 or KS170, respectively. KS270 and KS271 were both constructed with the *Eco*RI-generated right arm of Pa1045, and the *Eco*RI-generated right arm of KS169 or KS170, respectively. KS311 was constructed from the *Xba*I-generated right arm of TL61, 97 bp *Xba*I–*Xho*I fragment of pKS309 and *Xho*I-generated right arm of Pa285. pKS309 contains the 60 bp *Xho*I–*Dde*I (blunted) fragment of pKS290 inserted between the *Xho*I and *Sma*I sites of pTL61T (Linn & St Pierre, 1990). pKS290 contains the 121 bp *Alu*I (blunted)–*Hin*dIII fragment of pKS284 inserted between the *Sma*I and *Hin*dIII sites of pTL61T. pKS284 contains the 303 bp *Eco*RI–*Hin*dIII fragment of KS265 that contains *rpoBa*, inserted between the *Eco*RI and *Hin*dIII sites of pGEM-11Zf(-). KS305 was constructed from the *Xba*I-generated left arm of TL61, 154 bp *Xba*I–*Xho*I fragment of pKS290 and *Xho*I-generated right arm of Pa285. KS312 was constructed from the *Xba*I-generated left arm of TL61, 97 bp *Xba*I–*Xho*I fragment of pKS309 and *Xho*I-generated right arm of Pa1045. KS313 was constructed from the *Eco*RI-generated left arm of KS305 and *Eco*RI-generated right arm of Pa1045.

(iv) Fusions used to test readthrough of tandem copies of *rpoBa*. These fusions contained either *Pant285* (KS169, KS170, KS287) or *Pant224* (KS195, KS166, KS269) fused upstream of an *rplrpo* segment extending from the engineered *Hin*dIII site at nt 1339 to the *Dde*I site located 43 bp before the *rpoBa* termination site, and contained in addition no (KS169, KS195), one (KS170, KS166), or two (KS287, KS269) tandem copies of the *Dde*I–*Nar*I *rpoBa*-containing region (Table 1). KS166, KS169, KS170 and KS195 were described previously (Steward & Linn, 1992). KS287 was constructed from the *Xho*I-generated left arm of KS269 and *Xho*I-generated right arm of Pa285. KS269 was constructed from the *Xho*I-generated left arm of KS176, 480 bp *Sal*I–*Swa*I fragment of KS170 and *Swa*I-generated right arm of KS166.

(v) Fusions containing the 393 bp *rplL* fragment. A summary of the features contained in the fusions is presented in Table 3. tR46 was constructed from the *Xba*I-generated right arm of KS285 and *Xba*I-generated left arm of TL61. tR40 and tR43 were constructed from the *Sma*I-generated left arm of tR35 and *Bam*HI-generated right arm of Pa222 surrounding, respectively, the *Sal*I(blunted)–*Bam*HI *Pant1045*-containing fragment of KS285 or the *Sal*I(blunted)–*Bam*HI *Pant285*-containing fragment of KS253. tR45 was constructed from the *Sma*I-generated left arm of tR29, *Bam*HI-generated right arm of Pa222 and *Sal*I(blunted)–*Bam*HI *Pant1045*-containing fragment of KS285.

Lysogenization and β-galactosidase assays. Monolysogens of each recombinant *lacZ* transcriptional fusion phage were recovered in MG4 as described previously (Linn & St Pierre, 1990) with the exception of the lysogens described in Table 4. Lysogens were confirmed as monolysogens using the TER excision test (Linn & St Pierre, 1990; Mousset & Thomas, 1969). β-Galactosidase assays were performed essentially as described by Miller (1972), except that cultures were grown in AB medium (Clark & Maaloe, 1967) supplemented with thiamin (2 mg ml^{-1}), 0·4% (w/v) glucose and 0·4% (w/v) Casamino acids. Samples of the culture were taken at an optical density of 15 Klett units (Klett–Summerson colorimeter), then 30–500 µl cells were permeabilized by the addition of 20 µl chloroform and 10 µl 0·1% (w/v) SDS in a final volume of 800 µl. All β-galactosidase values reported in the tables and figures represent the means of six to ten assays performed on three or more independent cultures and had standard deviations that ranged from 1 to 5%.

RESULTS

Weak terminators do not exhibit modulation of termination

Previously, we showed that the attenuator preceding the *rpoB* gene (*rpoBa*) is not fixed in its termination efficiency, but is modulated by the frequency with which transcription complexes enter the attenuator (Steward & Linn, 1992). In contrast, two other well-characterized terminators, *rrnBT2* (Brosius *et al*., 1981) and *λtR2* (Schmidt & Chamberlin, 1987), were found to meet the expectation of constant termination efficiency independent of transcription frequency (Steward & Linn, 1992). One concern, however, was that these two terminators are very efficient, with termination frequencies of greater than 95%, whilst the maximum frequency of termination at *rpoBa* only approaches 80%. Perhaps termination efficiency at weaker terminators would be modulated by transcription frequency in the same manner as *rpoBa*. To test this directly we examined termination at two mutated versions of *λtR2*. The $tR2^2$ terminator has a CG base pair in the stem replaced by a GC base pair, while the $tR2^9$ terminator has two adjacent GC base pairs replaced by two CG base pairs with an additional AU base pair added to the stem (Cheng *et al*., 1991). Although the stability of the stem in these variants should be as great ($tR2^2$) or greater ($tR2^9$) than wild-type *tR2*, both have reduced termination efficiency. We tested their response to varying transcription frequency by constructing a series of transcrip-

Table 2. Termination efficiency of weak terminators as transcription frequency is varied

	Promoter alone		$+tR2^2$			$+tR2^9$		
Promoter	Fusion	β-Gal (units)*	Fusion	β-Gal (units)*	Term (%)†	Fusion	β-Gal (units)*	Term (%)†
Ptac	TAC1	13588	–	–	–	tR49	4333	68
Pant285	Pa285	7199	tR35	1436	80	tR29	2329	68
Pant219	Pa219	2891	–	–	–	tR48	952	67
Pant222	Pa222	1260	tR33	128	90	tR47	429	66
Pant1045	Pa1045	511	tR34	52	90	tR44	161	68

* β-Galactosidase activities (β-gal) were determined as described in Methods.

† Percentage termination frequencies (term) were calculated as $100 \times [1 - (\beta\text{-gal units from the fusion containing both the promoter and terminator} \div \beta\text{-gal units from the fusion containing only the promoter})]$.

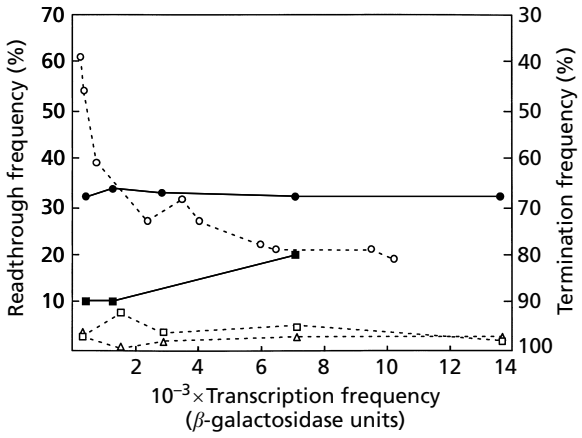
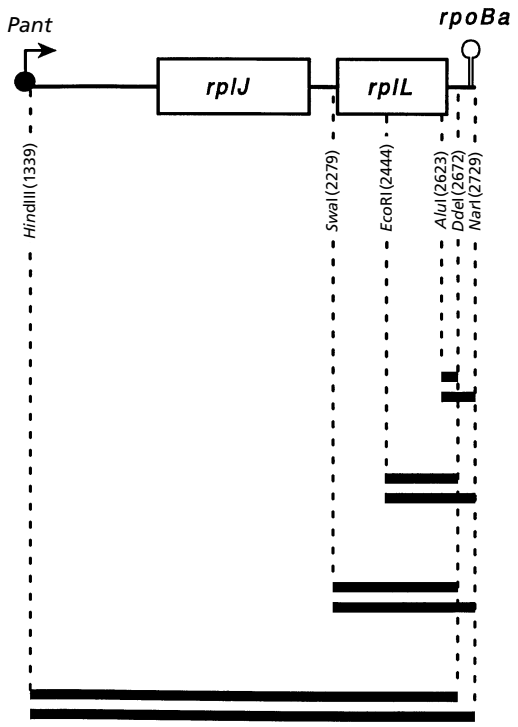


Fig. 2. Termination efficiency of $tR2^2$ and $tR2^9$ as a function of transcription frequency. The frequency of transcription entering the terminators is plotted versus the readthrough or termination frequencies presented in Table 2. The curve for $tR2^2$ is indicated by filled squares and for $tR2^9$ by filled circles. For comparison, the previously determined curves (Steward & Linn, 1992) for *rpoBa* (open circles), *tR2* (open squares) and *rrnBT2* (open triangles) are also shown.

tional fusions in which each terminator, carried on a 440 bp fragment, was inserted between different strength promoters and the *lacZ* gene. The promoters used included *Ptac* and four variants of the P22 *ant* promoter. The *Pant* variants differ from one another by single bp changes in the −10 and −35 regions (Moyle *et al.*, 1991). Although promoter strength is affected, all *Pant* variants initiate at the same position and produce identical RNA transcripts. These fusions were assembled on λ vectors so a single copy could be stably integrated into the bacterial chromosome (see Methods). The termination efficiencies were then calculated from the β-galactosidase levels produced in lysogens containing fusions with or without the terminator. As seen in Table 2 and Fig. 2, the frequency of readthrough of $tR2^2$ or $tR2^9$ did not increase with decreasing transcription frequency. The weakest terminator, $tR2^9$, showed a constant termination efficiency of approximately 68% as transcription frequency was varied over a 27-fold span, indicating that at least these weaker terminators do not show transcription-frequency-dependent modulation.

Identification of an upstream region required for modulation of *rpoBa* efficiency

If the termination decision at *rpoBa* was regulated by a mechanism similar to the N-mediated antitermination system of λ, then one might predict that sequences located upstream of *rpoBa* may be required for modulation of *rpoBa* efficiency. To test this model decreasing amounts of DNA upstream of *rpoBa* were used to construct two sets of fusions, with and without *rpoBa* fused upstream of *lacZ*. In those constructs lacking *rpoBa*, the *rplJL* sequences present in the fusion ended at the *Dde*I site 43 bp before the *rpoBa* termination site. The set of fusions that contained *rpoBa* extended to the *Nar*I site 15 bp beyond the termination site. β-Galactosidase activity measurements for the first set of fusions revealed the transcription frequencies just prior to the attenuator, while measurements for the second set revealed readthrough frequencies. The transcription-frequency-dependent modulation was tested by driving transcription of the constructs with either the strong *Pant285* or weak *Pant1045* promoters. These fusions were assembled on lambda vectors and the β-galactosidase levels were measured in monolysogens of the recombinant fusion phage as described in Methods. As observed earlier (Steward & Linn, 1992), fusions containing virtually the complete upstream region extending from the normal position of *rplJp*, but which had this endogenous promoter replaced, showed an approximately twofold higher readthrough frequency at *rpoBa* when transcription was driven by the weak *Pant1045* promoter (42% readthrough) as compared to the strong *Pant285* (23%) (Fig. 3). A similar difference in readthrough frequency was observed when the DNA upstream of *rpoBa* was shortened to 439 bp. However, when the region was reduced to 274 bp the difference between low (25%) and high (24%) frequency tran-



Size of upstream region (bp)	Pant285 β-Gal (units) −rpoBa (DdeI)	Pant285 β-Gal (units) +rpoBa (NarI)	Pant285 RT (%)	Pant1045 β-Gal (units) −rpoBa (DdeI)	Pant1045 β-Gal (units) +rpoBa (NarI)	Pant1045 RT (%)
95	5694	1815	32	371	102	27
274	5754	1392	24	443	112	25
439	6134	1502	24	402	165	41
1379	6388	1458	21	427	181	42

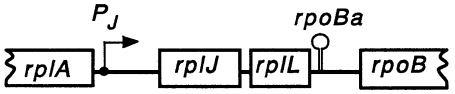
Fig. 3. Deletion analysis of the upstream region required for the transcription-frequency-dependent modulation of *rpoBa* termination. *lacZ* transcriptional fusions were constructed with and without the *rpoBa* attenuator and varying amounts of proximal DNA (filled bars), each with either the strong *Pant285* or the weak *Pant1045* promoters. For each fusion, the size of the region upstream of the *rpoBa* termination site, the β-galactosidase activity measurements (β-gal) for 3′ fusion sites located 43 bp before (*Dde*I) and 15 bp beyond (*Nar*I) *rpoBa*, and the percentage readthrough (RT) are shown. Restriction sites used as endpoints for the fusions are indicated.

scription disappeared. A further deletion, leaving only 95 bp upstream of *rpoBa* also showed no greater readthrough when transcription was driven by a weak promoter, but the mean of the readthrough levels at high and low transcription frequency increased somewhat to 29·5%. This higher level of readthrough may be due to the closer proximity of the *Pant* sequences to *rpoBa*, which partially inhibits function of the terminator. These results suggest that a *cis*-acting site located in the 165 bp *Swa*I–*Eco*RI interval is required for *rpoBa* modulation.

Modulation of *rpoBa* does not involve a transcription 'juggernaut'

In λ the upstream *cis*-acting site (*nut*) serves as a nucleation site for assembly of an antitermination complex or transcription juggernaut (Greenblatt *et al*., 1993). This termination-resistant elongation complex is capable of transcribing through multiple downstream terminators. Accordingly, one possible explanation for increased readthrough of *rpoBa* when transcription occurs from weak promoters is that at lower transcription frequencies a higher fraction of transcription complexes become modified during elongation through the *rplJL* region in a manner analogous to that of λ. These modified juggernauts would be resistant to termination and would show increased readthrough at *rpoBa*. Consequently, this model predicts that the population of transcription complexes which finally emerge downstream of *rpoBa* should be enriched for modified termination-resistant transcription complexes, and that subsequent readthrough of a second tandem copy of *rpoBa* should be more efficient than the first.

We tested this by joining an additional copy of a 58 bp *Dde*I–*Nar*I fragment containing *rpoBa* onto the end of existing constructs that contained the complete *rplJLrpoBa* region (*Hin*dIII–*Nar*I) in a transcriptional fusion to *lacZ*. β-Galactosidase activities of these constructs were then compared with fusions either ending before *rpoBa* or containing a single copy of *rpoBa*. This allowed us to determine the readthrough of each of the tandem copies of *rpoBa* (Fig. 4). The results showed that with the strong *Pant285* promoter, readthrough of a single copy of *rpoBa* in its normal configuration is 23%, but when an additional copy of *rpoBa* is fused onto the end, readthrough of the second copy drops to 14%. Similarly, with the weaker *Pant224* promoter, transcription across the first copy of *rpoBa* is 35%, while that of a second copy drops to 22%. Thus, in contrast to the model that some fraction of the transcription complexes traversing the *rplrpoBa* region become juggernauts, the results showed that the efficiency of



	β-Gal (units)	Total RT (%)	RT of 2nd *rpoBa* (%)
Pant285	6388	–	–
Pant285	1458	23	–
Pant285	204	3	14
Pant224	741	–	–
Pant224	259	35	–
Pant224	56	8	22

Fig. 4. Readthrough of single or tandem copies of *rpoBa*. Two sets of *lacZ* transcriptional fusions were constructed with *rplJp* (P_J) replaced by either *Pant285* or *Pant224* attached at the *Hin*dIII site located 1379 bp upstream of *rpoBa*. Each set of fusions contained either no (*Dde*I, D), one (*Nar*I, N) or two (extra 58 bp *Nar*I–*Dde*I fragment) copies of *rpoBa* (represented as a hairpin) at the 3′ end fused to *lacZ*. Total readthrough frequency (RT) was calculated as the percentage β-galactosidase activity (β-gal) for constructs with one or two copies of *rpoBa* relative to the fusions without *rpoBa*. RT of the second copy of *rpoBa* was calculated as the percentage β-gal for constructs with two copies of *rpoBa* relative to the fusions with one copy.

readthrough of the second tandem copy of *rpoBa* is not increased, in fact somewhat decreased, when transcribed from either the strong or weak promoter.

The *cis*-acting site upstream of *rpoBa* does not modulate the efficiency of other terminators

The *nut* sites of λ also reduce termination when placed upstream of terminators other than the endogenous λ terminators. To address the question of whether the region between the *Swa*I and *Dde*I sites preceding *rpoBa* would affect other terminators in the same fashion as *rpoBa*, we placed this 393 bp segment upstream of the $tR2^2$ and $tR2^9$ terminators. Two sets of transcriptional fusions to *lacZ* were constructed with $tR2^2$, one driven by the strong *Pant285* promoter and the second by the weaker *Pant1045*. With $tR2^9$ a single set of fusions transcribed by *Pant1045* was assembled. As summarized in Table 3, in no case did the insertion of this region upstream of $tR2^2$ or $tR2^9$ relieve termination. Even when the 393 bp region was placed between a weak promoter and these terminators, conditions that would relieve termination at *rpoBa*, no decrease in termination was observed. This suggests that this site works only in combination with *rpoBa* to modulate termination.

NusA increases readthrough of *rpoBa*, but is not involved in transcription-frequency-dependent modulation

We have previously found evidence, both *in vivo* (Ralling & Linn, 1987) and *in vitro* (Linn & Greenblatt, 1992), that NusA decreases termination frequency at *rpoBa*. To test whether NusA played a role in the transcription-frequency-dependent modulation of *rpoBa*, two pairs of *lacZ* transcriptional fusions were used to assay *rpoBa* readthrough in wild-type cells and three *nusA* mutants, *nusA10*(Cs) (Schauer *et al*., 1987), *nusA1* (Friedman, 1971) and *nusA11*(Ts) (Nakamura *et al*., 1986). One pair of fusions replaced *rplJp* with the strong *Pant285* promoter, while the second pair used the weaker *Pant1045*. The promoters were attached via an engineered *Hin*dIII site located 1379 bp upstream of the *rpoBa* termination site. Since the normal transcription

Table 3. Effect of 393 bp *Swa*I–*Dde*I fragment on other terminators

Fusion	Features	β-Gal (units)*	RT (%)†
Pa285	*Pant285*	7199	–
tR35	*Pant285* + *tR2*[2]	1436	20
KS253	*Pant285* + 393 bp‡	6069	–
tR43	*Pant285* + 393 bp + *tR2*[2]	1387	23
Pa1045	*Pant1045*	511	–
tR34	*Pant1045* + *tR2*[2]	52	10
tR46	*Pant1045* + 393 bp	545	–
tR40	*Pant1045* + 393 bp + *tR2*[2]	44	8
Pa1045	*Pant1045*	511	–
tR44	*Pant1045* + *tR2*[9]	161	32
tR46	*Pant1045* + 393 bp	545	–
tR45	*Pant1045* + 393 bp + *tR2*[9]	143	27

* β-Galactosidase activities (β-gal) were determined as described in Methods.

† Percentage readthrough frequencies (RT) were calculated as 100 × (β-gal units for constructs with terminator ÷ β-gal units for constructs without terminator).

‡ The 393 bp *Swa*I–*Dde*I fragment encompasses the region from 436 bp to 43 bp upstream of the *rpoBa* termination site.

start site of *rplJp* is 8 bp downstream of this *Hin*dIII site, these fusions position the promoters so the complete *rplJ* leader is present. The first construct of each pair contained the complete *rplJL* region but ended 43 bp before (*Dde*I) the *rpoBa* termination site, while the second construct extended an additional 58 bp to end 15 bp downstream (*Nar*I) of *rpoBa*. β-Galactosidase activity measurements for the first fusion revealed transcription frequency into the attenuator, whereas measurements for the second fusion revealed readthrough frequency. In *nusA*$^+$ cells the frequency of readthrough of *rpoBa* was approximately twofold higher with the lower levels of transcription driven by *Pant1045* (43%) as compared to the stronger *Pant285* (23%) (Table 4). In the *nusA10*(Cs) and *nusA1* backgrounds, the level of readthrough decreased modestly, while in the *nusA11*(Ts) strain, readthrough decreased to 26% and 12%, respectively, for the fusions transcribed by the strong and weak promoters. Thus the *nusA11*(Ts) allele reduced readthrough by approximately half, but none of the *nusA* mutations significantly altered the twofold difference in readthrough efficiency seen between the high and low levels of transcription.

DISCUSSION

The efficiency of termination at the *rpoBa* attenuator was modulated by the frequency with which transcription complexes enter this site. Current models of simple terminator function imply that terminator efficiency should be constant regardless of transcription frequency (Yager & von Hippel, 1987; von Hippel & Yager, 1992). We have now directly tested this with four

Table 4. Effect of NusA on termination at *rpoBa*

Fusion	Features	Genotype*	β-Gal (units)†	RT (%)‡
KS169	*Pant285–rpJL*	*nusA*$^+$	6454	–
KS170	*Pant285–rplJLrpoBa*	*nusA*$^+$	1468	23
KS267	*Pant1045–rplJL*	*nusA*$^+$	598	–
KS266	*Pant1045–rplJLrpoBa*	*nusA*$^+$	258	43
KS169	*Pant285–rplJL*	*nusA10*(Cs)	7004	–
KS170	*Pant285–rplJLrpoBa*	*nusA10*(Cs)	1409	20
KS267	*Pant1045–rplJL*	*nusA10*(Cs)	737	–
KS266	*Pant1045–rplJLrpoBa*	*nusA10*(Cs)	284	37
KS169	*Pant285–rplJL*	*nusA1*	9066	–
KS170	*Pant285–rplJLrpoBa*	*nusA1*	1524	17
KS267	*Pant1045–rplJL*	*nusA1*	958	–
KS266	*Pant1045–rplJLrpoBa*	*nusA1*	349	36
KS169	*Pant285–rplJL*	*nusA11*(Ts)	10973	–
KS170	*Pant285–rplJLrpoBa*	*nusA11*(Ts)	1321	12
KS267	*Pant1045–rplJL*	*nusA11*(Ts)	1406	–
KS266	*Pant1045–rplJLrpoBa*	*nusA11*(Ts)	367	26

* The *nusA*$^+$ strain was MG2. The mutated *nusA* alleles were transferred to MG2 by P1 transduction as described in Methods. Cultures were grown at 37 °C. Additional measurements were conducted with the *nusA10*(Cs) lysogens grown at 33 °C and the *nusA1* and *nusA11*(Ts) lysogens grown at 40 °C, but the readthrough frequencies did not change significantly (data not shown).

† β-Galactosidase activities (β-gal) were determined as described in Methods.

‡ Percentage readthrough frequencies (RT) were calculated as 100 × (β-gal units for constructs with terminator ÷ β-gal units for constructs without terminator).

terminators which have termination efficiencies of approximately 98–68%. All of these terminators had fairly constant termination efficiencies over a wide range of transcription frequency. Since the efficiency of $tR2^9$ in particular is in the same range as that of *rpoBa*, it argues that a decrease in termination efficiency with decreasing transcription frequency is not an inherent property of weaker terminators and may be unique to *rpoBa*.

In the lytic cycle of λ, the phage-encoded N protein acts with four *E. coli* proteins, NusA, NusB, NusG and S10, to modify RNA polymerase such that it fails to respond to termination signals. This modification requires transcription through a *cis*-acting site (*nut*) located upstream of the terminators. The nascent *nut* RNA is an essential component in the ribonucleoprotein complex that assembles on the surface of the transcribing RNA polymerase. This modified complex, which contains N, NusA, NusB, NusG, S10 and *nut* RNA, becomes a transcriptional juggernaut with an enhanced ability to processively read through multiple terminators (Greenblatt *et al*., 1993). Since termination efficiency at *rpoBa* can be modulated and because our previous results indicated that both NusA and NusG appear to play a role in termination at this site, we tested whether the mechanism operating at *rpoBa* was analogous to the λ antitermination system.

Analysis of deletions upstream of *rpoBa* suggested that at least part of the sequences required for discriminating between high and low frequency transcription, and adjusting *rpoBa* readthrough accordingly, may map within the 165 bp interval between the *Swa*I site at the start of *rplL* and the *Eco*RI site within *rplL*. The observation that two different upstream endpoints (*Hin*dIII and *Swa*I) show significant differences in *rpoBa* readthrough for high and low transcription frequency, while two other endpoints (*Eco*RI and *Alu*I) no longer show this difference, argues that the apparent loss of *rpoBa* modulation is not likely to be due to a novel fusion junction between the *Pant* and *rplJL* sequences that interferes in some way with modulation.

The deletion analysis did not distinguish whether the functional form of this putative region was RNA or DNA. In N-mediated antitermination it is the *nut* site RNA that functions as part of the termination resistant elongation complex. The Q-mediated antitermination system of λ, which is responsible for expression of the late genes, also requires a *cis*-acting site (*qut*), but this site functions in the form of DNA. Shortly after initiation, the polymerase pauses at the downstream section of the *qut* site, but when Q, in the presence of NusA, interacts with the upstream sequence of *qut*, polymerase is accelerated out of this site and is transformed into a termination-resistant complex (Yarnell & Roberts, 1992; Greenblatt *et al*., 1993). Further work will be required to more precisely map the site upstream of *rpoBa* and determine if it functions as DNA or RNA.

The *nut* sites of λ can also decrease termination at heterologous terminators when placed upstream. However when the 393 bp *Swa*I–*Dde*I fragment upstream of *rpoBa* was inserted in front of the $tR2^2$ and $tR2^9$ terminators there was no reduction in termination even at low levels of transcription. This suggests that the *cis*-acting site found in this region works only in combination with *rpoBa* to modulate termination in response to transcription frequency and is not a general antitermination site as are the *nut* sites. Also, the result that a second copy of *rpoBa* placed downstream of the original had a termination efficiency no lower than the first copy indicates those transcription complexes that read through *rpoBa* do not appear to be a select class of termination-resistant complexes. This result does not rule out the possibility that RNA polymerase passing through the *Swa*I–*Eco*RI region becomes modified in some fashion that reduces termination at *rpoBa*. It simply argues that this putative modification only enhances readthrough at the first copy of *rpoBa* and is not a persistent modification that allows the processive antitermination seen with the N-modified transcriptional juggernauts. If polymerase was to pause at the *cis*-acting site upstream of *rpoBa* and supposing some step in the modification was rate-limiting, then a higher fraction of transcription complexes may become modified at lower transcription frequencies. This could explain the increased percentage of polymerase molecules seen reading through *rpoBa* at lower transcription frequencies.

If NusA was essential for regulating the transcription-frequency-dependent efficiency of *rpoBa* then in a *nusA* mutant the difference in readthrough at high and low levels of transcription should have disappeared. The *nusA10*(Cs) and *nusA1* mutants showed only a modest effect, while in the *nusA11*(Ts) mutant readthrough of *rpoBa* decreased to approximately half that seen in the wild-type strain, confirming that NusA normally increases readthrough at this site. However, in the mutants the ratio of readthrough at high and low levels of transcription did not vary significantly from the wild-type strain, arguing that NusA is not directly involved in transcription-frequency-dependent modulation. The *nusA11*(Ts) mutation changes a single nucleotide at position 542 of the coding sequence, while *nusA1* has a single change at nucleotide 548 and *nusA10*(Cs) has two changes at nucleotides 311 and 634 (Craven & Friedman, 1991). Each of these alterations causes a single amino acid change in the protein. The fact that *nusA11*(Ts) has a greater effect at *rpoBa* suggests that the amino acid residue altered by this mutation is directly involved in the interactions that decrease termination at *rpoBa*. Alternatively, this change may have a greater detrimental effect on the overall structure or stability of NusA under the conditions tested.

The regulation of termination efficiency at *rpoBa* has some parallels with the N and Q antitermination systems of λ in that NusA increases readthrough and also there appears to be the involvement of a *cis*-acting upstream element. However, in contrast to λ, the upstream element functions only in combination with *rpoBa*, the

polymerase molecules traversing this region do not become transcriptional juggernauts and NusA does not appear to be essential for transcription-frequency-dependent modulation. The apparent terminator specificity of the upstream site and the lack of processivity might suggest a direct mRNA antiterminator–terminator interaction. Such a mechanism would be similar in principle to that seen in the *trp* operon and other amino acid biosynthetic operons (Landick & Yanofsky, 1987), but here the antiterminator–terminator interaction would be long-range, with more than 270 nucleotides separating these sites. However, sequence analysis has not shown any compelling complementarity between *rpoBa* and the upstream region. Moreover, since the 165 bp *Swa*I–*Eco*RI interval lies within the *rplL* gene, one would expect that translation of this ribosomal protein sequence would inhibit formation of a long-range mRNA secondary structure. Also, it is not obvious how such an antiterminator–terminator interaction would be responsive to transcription levels. The transcription frequency, and thus the spacing of the RNA polymerase molecules elongating through this region, might be expected to affect the transient local superhelicity, which in turn may play some role in modulating *rpoBa* function. Additional studies will be required to further elucidate the mechanism that senses the frequency with which transcription complexes approach *rpoBa* and then adjusts termination efficiency accordingly.

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