Transcriptional Polarity in rRNA Operons of *Escherichia coli nusA* and *nusB* Mutant Strains

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Synthesis of ribosomes in *Escherichia coli* requires an antitermination system that modifies RNA polymerase to achieve efficient transcription of the genes specifying 16S, 23S, and 5S rRNA. This modification requires nucleotide signals in the RNA and specific transcription factors, such as NusA and NusB. Transcription of *rrn* operons in strains lacking the ability to produce either NusA or NusB was examined by electron microscopy. The distribution and numbers of RNA polymerase molecules on *rrn* operons were determined for each mutant. Compared to the wild type, the 16S gene in the *nusB* mutant strain had an equivalent number of RNA polymerase molecules, but the number of RNA polymerase molecules was reduced 1.4-fold for the *nusA* mutant. For both mutant strains, there were twofold-fewer RNA polymerase molecules on the 23S RNA gene than for the wild type. Overall, the mutant strains each had 1.6-fold-fewer RNA polymerase molecules on their *rrn* operons than did the wild type. To determine if decreased transcription of the 23S gene observed by electron microscopy also affected the 30S/50S ribosomal subunit ratio, ribosome profiles were examined by sucrose gradient analysis. The 30S/50S ratio increased 2.5- to 3-fold for the *nus* mutant strains over that for wild-type cells. Thus, strains carrying either a *nusA* mutation or a *nusB* mutation have defects in transcription of 23S rRNA.

In the synthesis of rRNA in *Escherichia coli*, RNA polymerase undergoes several *rrn* operon-specific modifications that double the transcription elongation rate and allow read-through of Rho-dependent terminators (1, 7, 29, 30, 32). The modification events require specific nucleotide sequences in the leader and spacer regions of the *rrn* operons as well as interacting proteins that alter the properties of the transcribing polymerase. Thus, profound changes in the basic capabilities of RNA polymerase occur when *rrn* operons are transcribed, but these changes and their consequences have yet to be clearly described. The nucleotide sequence signals leading to the changes in RNA polymerase activity in *rrn* operon transcription are called BoxB, BoxA, and BoxC in the leader sequence and BoxB and BoxA in the spacer region between the 16S and 23S genes. BoxA is a conserved 12-nucleotide sequence (Fig. 1), BoxB is a conserved stem-loop structure, and BoxC is a GT-rich region (3, 19). These sequences are related to those used in the bacteriophage lambda N protein antitermination system (8, 12). Protein factors involved in *rrn* antitermination (rRNA-AT) are less well defined, but they likely include the N utilization *s*ubstances (Nus) NusA, NusB, NusE (ribosomal protein S10), NusG, and ribosomal protein S4 (15, 23, 24, 27, 29, 30, 32). In vivo studies have documented the importance of NusB and NusG for rRNA-AT and, in conjunction with BoxA, for increasing the rate of elongation by RNA polymerase (28, 32). NusG, NusE (S10), and S4 are all essential proteins. It was assumed that NusB would also be an essential cellular protein, but in studies with transport protein mutants, two groups iso-

lated suppressors of secretion mutations (*secY*[ts] and *secA5*[ts]) that mapped to the *nusB* gene. One mutational change, *ssyB*, was an insertion of IS*10* into the *nusB* gene (*nusB*::IS*10*), while another, *ssaD1*, introduced a nonsense codon early in the gene (17, 21, 25). The fact that cells containing a disrupted or dysfunctional *nusB* gene are viable suggests that NusB is not an essential protein. Cell physiology studies revealed that cells containing the *nusB*::IS*10* mutation have a greatly increased doubling time and are cold sensitive. They also have a decreased peptide elongation rate at low temperatures, suggesting a potentially intriguing relationship between NusB and peptide synthesis (21). A point mutation in *nusB*, *nusB5*, has previously been examined for its effects on rRNA synthesis. A Y18D mutational change affecting a surface residue in the NusB5 protein results in defective lambda phage replication and defective rRNA transcription (2, 20). Compared to synthesis of the *rrn* leader region, 1.45-fold and 1.6-fold decreases in the amounts of 16S and 23S rRNA, respectively, have been documented. This drop-off in expression of 16S and 23S rRNA in *nusB5* was interpreted as a loss of the rRNA-AT system leading to transcription termination within the *rrn* operon (20). Sucrose gradient profiles of the *nusB5* mutant strain suggest that its 70S ribosomes are unstable and that it has a higher proportion of free ribosomal subunits than do wild-type strains when grown at 42°C (26). Another study of how antitermination features of *rrn* operons affect rRNA transcription focused on mutant BoxA sequences in the leader and spacer regions carried on plasmids encoding an *rrn* operon. The total ribosome content from strains carrying such plasmids was examined, and the proportion encoded by the mutant operon was determined. Quantitation of plasmid-encoded 16S and 23S rRNA in 30S and 50S subunits and 70S ribosomes showed that the leader BoxA mutation causes a 25% decrease in both 16S

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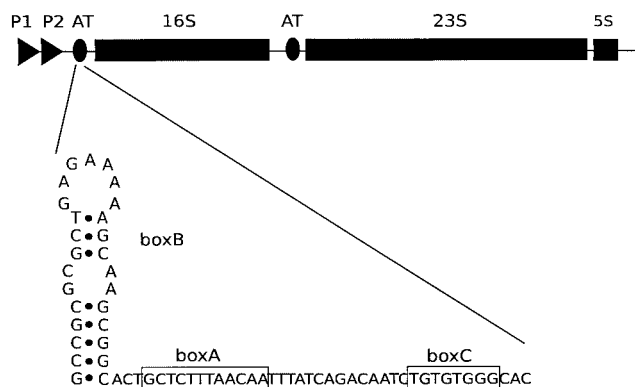
FIG. 1. Schematic diagram of the rrnB operon showing the regulatory BoxBAC region and its position relative to the 16S gene in the leader and the 23S gene in the spacer region.

and 23S rRNA and a further 15% decrease in plasmid-encoded 23S in 50S subunits when a spacer BoxA mutation is added (11, 16). These in vivo results with *nusB* and BoxA mutants demonstrate the importance of each for efficient rRNA synthesis.

Although NusA seems by some tests to be an essential protein, a particular transposon insertion in *nusA* permits continued viability (4). In addition, Zheng and Friedman (33) were able to delete *nusA* in a strain that also carries a mutation in the Rho termination factor gene that causes Rho activity to be reduced eight- to ninefold (Δ*nusA*::*cat rhoE134D*). They were unable to delete *nusA* alone and hence postulated that the transcription pausing caused by binding of NusA to RNA polymerase is necessary to keep RNA polymerase from outpacing translation and revealing lethal Rho-binding and termination sites normally covered by translating ribosomes. The Δ*nusA*::*cat rhoE134D* mutant strain, like the *nusB*::IS*10* mutant, has a slow-growth phenotype and is cold sensitive.

Because strains with mutations in *nusA* or *nusB* are viable but debilitated, we set out to determine directly what effect these mutations have on transcription of the *rrn* operons. Using electron microscopy, we visualized the number and position of transcribing RNA polymerase molecules on *rrn* operons in mutants that lack a fully functional antitermination system. Based on previous measurements of *rrn* expression in *nusB5* and rRNA-AT mutants, we expected to find regions in the *rrn* operons where RNA polymerase molecules were falling off the DNA. We found that the number and spacing of RNA polymerase molecules on the 16S gene of the *nusB*::IS*10* mutant were similar to those found on the *rrn* operons in a wild-type strain, but there was a twofold drop in the number of RNA polymerase molecules on the 23S gene. In the Δ*nusA*::*cat rhoE134D* strain, there was a 1.4-fold decrease in RNA polymerase molecules on the 16S gene as well as a twofold drop on the 23S gene. Sucrose gradient profiles of ribosomes from both of the *nus* mutant strains showed a 50S subunit peak that was decreased relative to the 30S peak, a result consistent with the decreased expression of 23S rRNA. Our data suggest that in the absence of Nus factors A and B, transcription of *rrn* operons is incomplete because RNA polymerase molecules leave the DNA near the 16S–23S junction. Apparently, there is a transcription block or terminator in this region that releases a

portion of transcription complexes when a Nus factor is lacking.

RESULTS

Visualization of rRNA transcription in *nusA* and *nusB* mutant strains. To observe *rrn* operon transcription directly, we used electron microscopy to examine chromosomal spreads of *E. coli* strains with mutations in the genes encoding NusA or NusB. From the image data collected, we counted the RNA polymerase molecules on the 16S and 23S genes, measured the distance between adjacent RNA polymerase molecules, and determined RNA polymerase distribution across the operon.

Electron microscopy provides a unique perspective into the transcriptional activity of individual genes that is difficult to obtain using conventional biochemical methods. The *rrn* operons are identified on the chromosome as sites of high transcriptional activity with a characteristic spatial arrangement of RNA polymerase molecules and nascent rRNA transcripts, suggestive of a double Christmas tree, that is easy to recognize (9).

Figure 2 shows electron micrographs of an rRNA operon from each of the two *nus* mutant strains as well as a wild-type control for comparison. On each operon there are two gradients of increasing transcript length resulting from left-to-right transcription and RNase III cleavage of the nascent transcripts between the 16S and 23S cistrons. Based on the relative scarcity of RNA polymerases and nascent rRNA transcripts on the second gradient, it is evident that the mutant strains were deficient in transcription of the 23S gene. Total RNA polymerase numbers and their individual positions along each operon that could be unambiguously traced from the 5′ end to the 3′ end were recorded for the Δ*nusA*::*cat rhoE134D* and *nusB*::IS*10* mutant strains and compared to those for a wild-type control (Table 1). The 3′ position of the 16S gene was
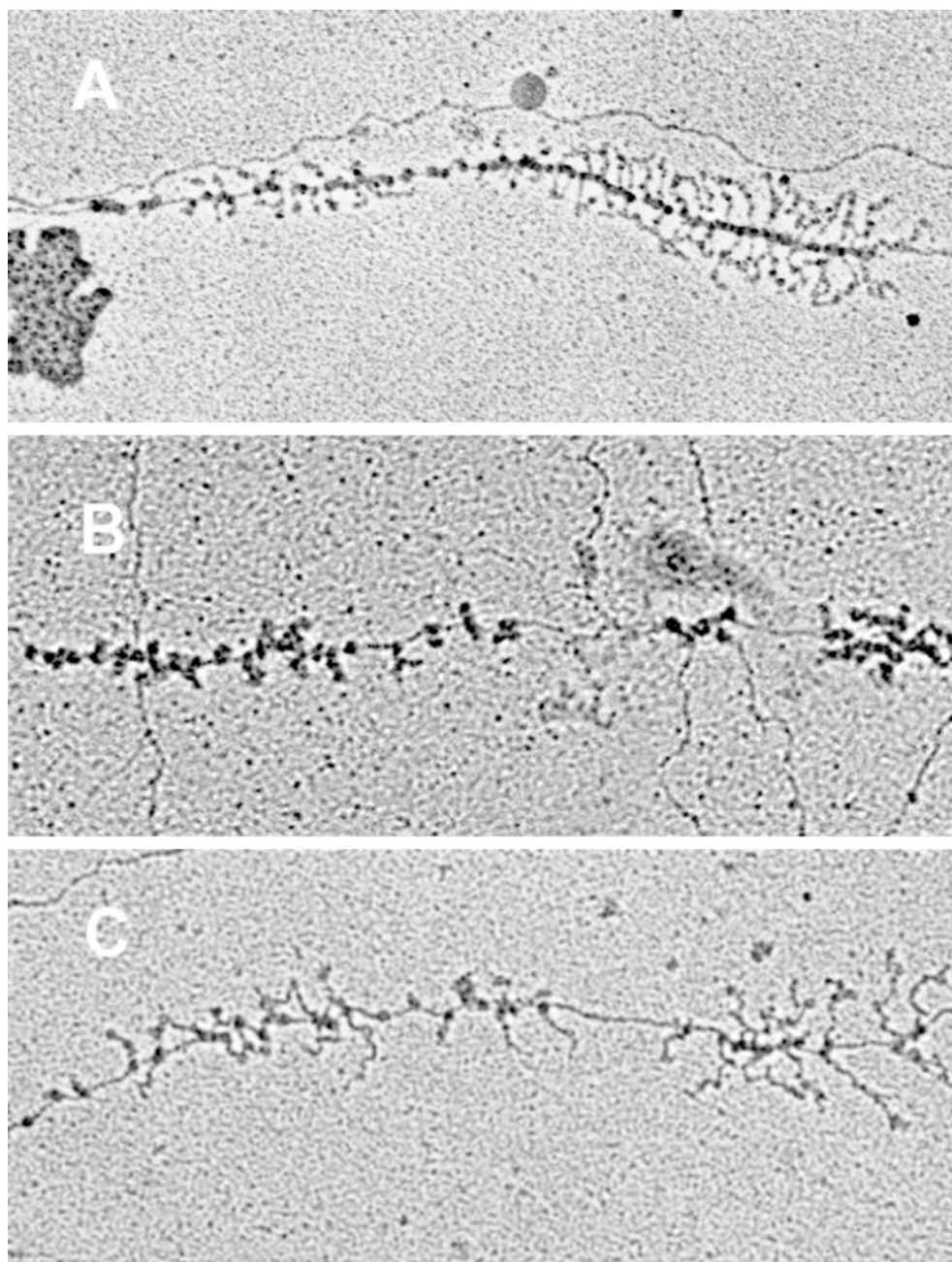
FIG. 2. Electron micrographs of *rrn* operons. A. Chromosomal *rrn* operon from a wild-type strain. B. *rrn* operon from a *nusA*::*cat rhoE134D* mutant strain. C. *rrn* operon from a *nusB*::IS*10* mutant strain. *rrn* operons are all 5.5 kb in length.

determined as the point of transcript cleavage. RNA polymerase numbers were consistent between the wild type and the *nusB*::IS*10* mutant on the 16S gene but were down 1.4-fold for the Δ*nusA*::*cat rhoE134D* strain. A twofold decrease in the number of RNA polymerase molecules engaged in 23S rRNA synthesis was found for both *nus* mutant strains, accounting for most of the 1.6-fold decrease in RNA polymerase numbers between the wild-type strain and two mutant strains over the entire operon (Table 1).

The distribution of RNA polymerase molecules on the *rrn* operons for the wild-type and *nusB*::IS*10* strains used for Table

TABLE 1. Electron-microscopic analysis of *rrn* operons in *E. coli* wild-type and *nus* mutant strains

Genotype (*n*[a])	No. of polymerase molecules observed on *rrn* DNA[b]			Doubling time (min)
	16S	23S	Total	
Wild type (10)	20.5 ± 5	31.0 ± 10	51.5 ± 12	24
nusB::IS*10* (70)	16.4 ± 3	16.2 ± 4	32.6 ± 5	54
Δ*nusA*::*cat rhoE134D* (14)	14.6 ± 3	16.9 ± 3	31.5 ± 4	38

[a] *n*, no. of operons.
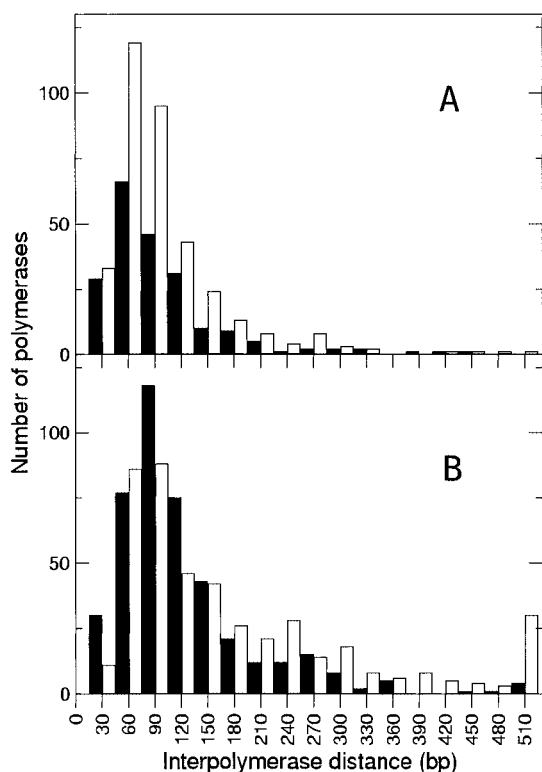[b] Results are expressed as means ± standard deviations.

FIG. 3. Determination of interpolymerase distance. The distance from polymerase center to polymerase center of all adjacent poly-merases was measured. A. Interpolymerase spacing in the wild-type strain ($n = 217$ for 16S and $n = 356$ for 23S from 11 operons). B. Interpolymerase spacing in the *nusB*::IS*10* mutant strain ($n = 459$ for 16S and $n = 444$ for 23S from 27 operons). Closed columns indicate polymerase molecules on the 16S genetic region, and open columns represent polymerase molecules on the 23S genetic region.

FIG. 4. Distribution of RNA polymerase molecules on *rrn* operons normalized to total polymerase numbers. A. Measurements of RNA polymerase numbers on *rrn* operons for the wild-type strain ($n = 11$). B. Measurements of RNA polymerase numbers for the *nusB*::IS*10* mutant strain ($n = 27$).

1 was analyzed by determining the center-to-center distance between adjacent RNA polymerases (Fig. 3). A subset ($n = 27$) of the *nusB*::IS*10 rrn* operons where RNA polymerases were clearly visible on a linear DNA contour were selected for further analysis. The average interpolymerase distance on the 16S gene was 85 and 111 bp for wild-type and *nusB* strains, respectively. In the wild-type strain, the interpolymerase distance on the 23S gene was 88 bp, similar to that along its 16S gene. In the *nusB* strain, however, the average interpolymerase distance on the 23S cistron increased to 176 bp. While the interpolymerase distance between the majority of RNA poly-merases remained the same along the entire length of the *rrn* operons in *nusB*::IS*10* (the mode of interpolymerase distances was the same for the 16S and 23S cistrons), the average dis-tance between individual polymerases on the 23S cistron in-creased because of fewer polymerases. In the *nusB* mutant strain, 29% of polymerase molecules had interpolymerase gaps of more than 175 bp, while only 12% of polymerase molecules in the wild-type strain were separated by similarly sized gaps.

A plot of the relative positions of RNA polymerase mole-cules on the *rrn* operons in the *nusB*::IS*10* strain showed no noticeable decrease in RNA polymerase molecules in the 16S–23S boundary region. This decreased density persisted throughout the 23S gene (Fig. 4). The wild-type operons also
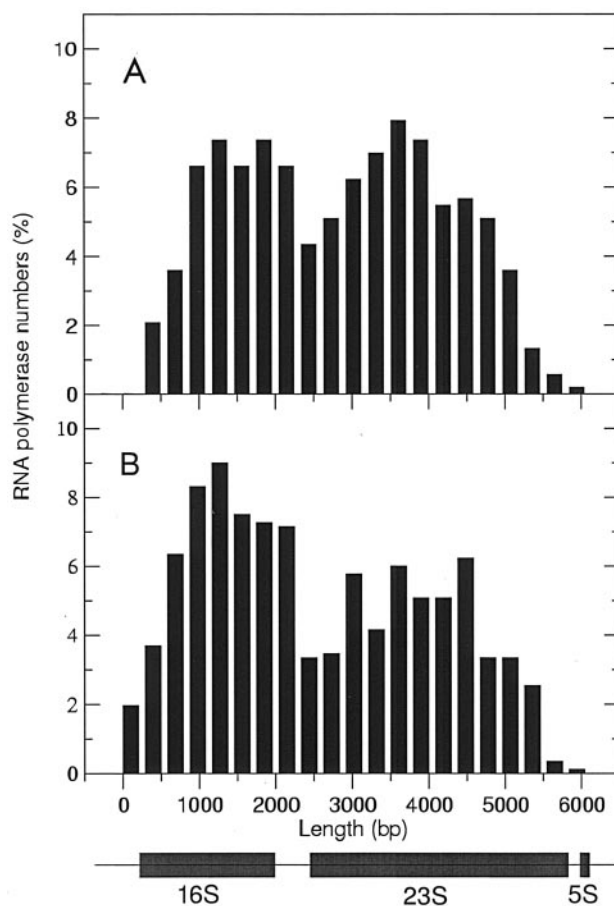
showed a slight decrease of the RNA polymerase population in the 16S–23S boundary, but the numbers once more increased throughout the rest of the 23S gene, suggesting that polymer-ase molecules were not lost but for some reason were more sparse in the boundary region.

Sucrose gradient analysis of ribosomes and ribosomal sub-units in *nusA* and *nusB* mutant strains. Since electron-micro-scopic analysis indicated a significant reduction in 23S rRNA transcription for both the *nusA* and *nusB* mutants, we exam-ined whether this difference was also reflected in the relative amounts of 30S and 50S ribosomal subunits. Sucrose gradient profiles of ribosomes from the wild-type strain and two mutant strains were prepared, using a magnesium ion concentration that maintained the subunit association of 70S ribosomes (Fig. 5).

The Δ*nusA*::*cat rhoE134D* and *nusB*::IS*10* mutant strains showed a significant change in the relative proportion of free 30S to 50S subunits compared to that for the wild-type strain (Table 2). Analysis of the *rhoE134D* mutant strain showed a pattern similar to that of wild-type cells, indicating that the *rho* mutation itself did not alter the proportion of 30S and 50S subunits in the Δ*nusA*::*cat rhoE134D* strain (data not shown).
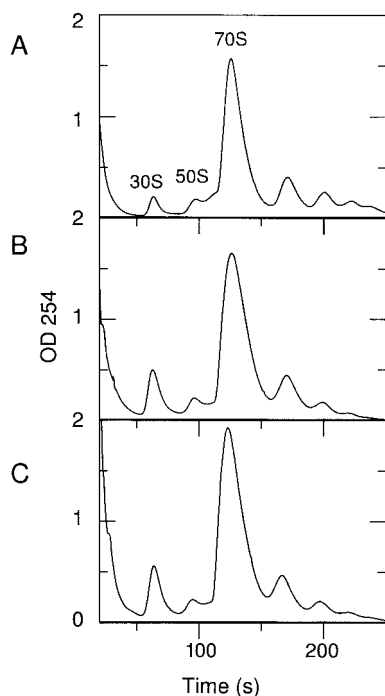
FIG. 5. Sucrose gradient analysis of ribosomes prepared from the following: A. wild-type strain, MC4100; B. *nusB*::IS*10* mutant strain; and C. *nusA*::*cat rhoE134D* mutant strain.

Thus, results obtained with both *nus* mutant strains supported the electron microscope analysis and confirmed that diminished transcription of the 23S gene resulted in a relative decrease in the 50S subunit pool (Table 2 and Fig. 5).

DISCUSSION

The results of our electron microscope analysis of *rrn* transcription in strains lacking Nus factor A or B provided direct visual evidence that these proteins are needed for efficient expression of *rrn* operons. Because of their association with the transcription machinery, we assume that the two proteins act by direct or indirect interaction with RNA polymerase, RNA, and other proteins involved in the modifications of RNA polymerase required for *rrn* transcription. However, the specifics of these modifications of the *rrn* operon transcription machinery are not understood.

Both NusA and NusB have been previously implicated in rRNA transcription antitermination, but their precise role has not been determined. A study of rRNA-AT function in a *nusA*

TABLE 2. Sucrose gradient analysis of 70S ribosomes and their subunits from wild-type and *nus* mutant strains

Strain or genotype	Peak area[a]			% Relative to 70S		30S/50S ratio
	30S	50S	70S	30S	50S	
MC4100	1.7	1.8	30.7	5.7	5.9	1
nusB::IS*10*	4.8	1.8	37.7	12.7	4.8	2.5
Δ*nusA*::*cat rhoE134D*	5.9	2.1	38.8	14.4	5.1	3

[a] Relative molar ratio.

cold-sensitive strain showed that the increased rate of transcription elongation associated with BoxA-modified RNA polymerase complexes is abolished at the nonpermissive temperature, implicating NusA in this process (30). Likewise, in vivo studies of transcription rate and antitermination in the *nusB*::IS*10* strain and in a strain depleted of NusG show that there is no elongation rate increase and no antitermination in these strains (28, 32). In vitro gel shift studies demonstrate that a complex of NusB and NusE binds to the *rrn* BoxA feature but that ribosomal protein S1 can compete for binding at this site (13, 14). Also, previous experiments suggest rRNA transcriptional polarity in a *nusB5* mutant strain and BoxA substitution mutants (16, 20). Our finding of a relatively reduced number of RNA polymerase molecules transcribing the 23S gene in the *nus* strains (Table 1) supports the idea of a transcription defect and suggests that the mechanism of this defect in the absence of either *cis* or *trans* components of the antitermination complex is premature termination. Whether or not premature termination at the 16S leader is similarly increased is not apparent from the electron microscopy data. Sharrock et al. (20) suggested that because of premature termination at the rRNA operon in a *nusB5* mutant, feedback control resulted in increased transcription initiation, compensating for reduced rRNA levels. Premature termination at the 16S leader region might be counteracted by increased transcription initiation, resulting in near-normal levels of 16S. Levels of 23S, however, are not maintained, since transcription at the 23S leader region is also subject to an increased termination frequency.

Previous studies looking at the effect of BoxA mutations on transcription have described a decrease in the stability of rRNA. Replacement of the leader BoxA leads to a decrease in 16S rRNA stability and to a relative reduction in 30S to 50S subunit ratio (26). Similarly, measurements of the relative proportion of plasmid-encoded rRNA in the 30S and 50S subunits from plasmids with a leader region BoxA mutation show a decrease of both plasmid-encoded 16S and 23S rRNA and a further drop in 23S rRNA when both leader and spacer BoxA mutations are present (11, 16). This is consistent with previous data that show that in the case of an unbalanced production of either 16S or 23S rRNA, excess RNA is rapidly degraded (22, 31). Combining these observations with the results we present here suggests that perturbation of the rRNA-AT system, whether at the level of the nucleotide sequence signaling the modifications (BoxA) or in the modifying proteins themselves (NusA and NusB), leads to a decrease in the stability or quantity of the rRNA transcript and to a relative decrease in the amount of 23S rRNA, probably as a result of premature termination at the 23S gene.

The excess of 30S free subunits over 50S free subunits in the *nus* mutant cells (Fig. 5) correlates with the electron microscopy results measuring RNA polymerase numbers on the 16S gene. Yamagishi and Nomura (31) show that a plasmid-encoded excess synthesis of either 16S or 23S rRNA does indeed lead to a corresponding imbalance in the relative molar ratios of ribosomal subunits. As with the *nus* mutants, a relative increase in 16S rRNA results in a corresponding relative increase in the 30S ribosome subunit because of the lack of partnering 50S subunits or because these "dead-end" 30S subunits are incompletely assembled and cannot proceed to form functional 70S ribosomes (31).
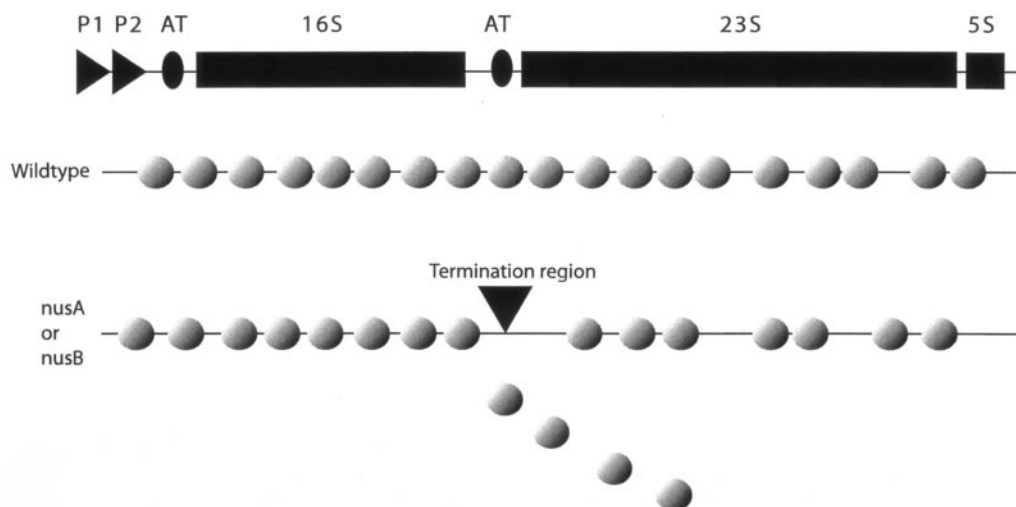
FIG. 6. Diminished transcription of the 23S genetic region in a *nusA* or *nusB* mutant strain due to premature termination.

Our observation that the Δ*nusA*::*cat rhoE134D* strain was defective in *rrn* transcription was somewhat surprising (Fig. 2 and Tables 1 and 2). If overcoming of Rho-dependent terminators in the *rrn* operons were the only reason for an rRNA-AT system, the greatly diminished Rho activity in this strain would have prevented the 23S gene transcription polarity evident with the *nusB*::IS*10* mutant strain. Instead, 23S gene transcription was decreased by at least twofold (Fig. 2 and Table 1). Because little is known about specific Rho-dependent terminators and their function, it is possible that such terminators in *rrn* operons have a high affinity for Rho protein and, while the mutant Rho protein's activity may be greatly diminished on some terminators, its activity on others might not be affected to the same extent (5). Alternatively, the antitermination system might play another role necessary for the maximal rate of rRNA synthesis.

Because the majority of RNA polymerase molecules on the 23S gene have the same relative spacing as on the 16S gene, the frequent gaps in RNA polymerase density present throughout the 23S gene in both *nus* mutant strains likely represent a significant malfunction of the transcription apparatus that occurs sporadically after transcription of the 16S gene. It may also be possible that whatever is releasing the hold of RNA polymerase on the DNA at the 16S-23S boundary is more readily overcome by groups of molecules than by single polymerases.

In summary, electron-microscopic examination of *rrn* operon transcription showed decreased transcription of the 23S rRNA gene and RNA polymerase molecules that were in more widely separated clusters in *nusA* and *nusB* mutant strains than in the wild type. Consistent with the electron microscopy data, the ratio of the concentration of free 50S subunit to that of free 30S subunits was decreased in the mutant strains. Our results suggest a mechanism for triggering transcription termination in *rrn* operons that works particularly effectively in the 16S–23S boundary region when factors necessary for increased transcription elongation rates and the antitermination system are missing (Fig. 6).

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