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Deletions in the t_L structure upstream to the rRNA genes in the *E.coli* rrnB operon cause transcription polarity

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ABSTRACT

A number of deletions have been constructed within the leader region of the rrnB operon from E. coli. The deletions remove a potential transcription terminator structure downstream from an antitermination recognition sequence (Box A), which precedes the structural gene for the 16S RNA. Cells harbouring plasmids, where the terminator structure was deleted, partially or totaly, showed a reduction in growth rate under minimal growth conditions. Measurement of the ribosomal RNA synthesis rates of such cells determined by pulslabeling and hybridisation to appropriate DNA probes, showed that the amount of the more distally located 23S RNA was reduced compared to the promoter-proximal 16S RNA. This polarity in transcription, resulting in a non-stoichiometric synthesis of the ribosomal RNAs, is most likely the result of a defective antitermination. The reduction in the amount of 23S RNA in such cells is compensated for by an increase in the overall ribosomal RNA synthesis, in concordance with the ribosomal RNA feedback regulation model. The accumulation of transcripts of the $tRNA^{Glu}_2$ gene, coded in the spacer region between the 16S and 23S RNA genes, in cells with an altered rRNA stoichiometry supports this interpretation.

INTRODUCTION

The regulation of ribosomal RNA transcription in E. coli is a carefully controlled process and many of the regulatory features are not completely understood at present (for details see 1-3). Transcription of the seven ribosomal transcription units is started from tandem promoters which are differentially regulated (4,5). Between the promoter region and the structural gene for the 16S RNA, sequences known as antitermination recognition sites (Box A, Box B, Box C) have been identified (6). In conjunction with E. coli antitermination factors (NusA, NusB, NusE) these elements trigger the RNA polymerase to read through termination signals, which are known to occur within the ribosomal RNA genes

(7). Some years ago, Kingston and Chamberlin showed that RNA polymerase pauses at several sites within the leader region of the rrnB operon, before transcription of the rRNAs proceeds (8). As one of the attenuation sites they identified a DNA structure with strong homology to the rho-dependent trp t' terminator (9). This site, designated t_L, is localized 260 bases downstream from the P1 promoter and is highly conserved in all rRNA operons (10). It is located only 100 bases downstream from the antitermination recognition sequence Box A. Termination or pausing of transcription at this site occurs in vitro, and is strongly dependent on the presence of the NusA protein and also on the stringent control effector ppGpp. The activity, however, is not dependent on the rho factor (8). It was suggested that the t_L terminator acts as an attenuator to regulate ribosomal RNA transcription. Terminators , on the other hand, have been localized in the upstream regions of eukaryotic rRNA transcription units, where they have an important function in transcription regulation (11,12). To better understand the functional implications of the t_L structure, which seems to be related somehow to the E. coli antitermination system of rRNA transcription, we have constructed a set of mutants with deletions in the t_L region. The cell growth of such mutants was characterized and the effects on the transcription of the ribosomal RNA genes investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strains HB 101 (13) and JM 107 (14) and the plasmid pKK 3535 which harbours the complete rrnB operon (15) were used. The phage vectors M13 mp18/19 and mp11 were obtained from PL Biochemicals.

Restriction enzymes, DNA ligase and the large fragment of the DNA polymerase (Klenow) were obtained from New England Biolabs. The exonucleases III and Bal 31 were supplied by Boehringer. The radiochemicals [^{32}P] orthophosphate, α[^{32}P]dATP and [^{3}H] uridine were purchased from Amersham Buchler.

The oligonucleotide 5' CAA GTC TCG AGA GTG AAC 3', which corresponds to the pKK 3535 sequence positions 1456 to 1473 with a G to A mismatch at position 1464 (underlined), was synthesized manually according to the phosphite triester method (16).

DNA sequencing was performed according to the chain termination method (17) using M13 single stranded DNA templates.

Plasmid copy number determination

The relative plasmid copy numbers were determined as described by Stueber and Bujard (18) or, alternatively, for the clones pKK 3535Xho, pΔ32, pΔ40 and pΔ49 by growing cells to 0.5 A_{560} units. Equal amount of cells were mixed with an equal amount of cells harbouring the wild type plasmid pKK 3535. Plasmid DNA was isolated from such cultures (19) and digested completely with the restriction enzyme Dra I. The resulting fragments were separated electrophoretically on agarose gels in the presence of ethidiumbromide and the relative concentration of the bands corresponding to the promoter region, with and without deletions, were compared and quantified using a Zeineh Soft Laser Scanning Densitometer SL-504-XL.

Determination of ribosomal RNA synthesis rates

HB 101 cells containing the different plasmids were grown in the presence of 100 μg/ml ampicillin and 0.5 MBq [^{32}P] orthophosphate to an optical density of 0.6 A_{560}. 4 MBq/ml of a prewarmed [^{3}H] uridine solution were added to the logarithmic growing cell culture. Cell growth was stopped after 30, 60, 90, or 120 seconds by the addition of 10% ethanol followed by immediate hot lysis. Total RNA was prepared from these cultures as described (20). The radioactive RNA preparations were hybridized to nitrocellulose filters (BA 85/22, Schleicher and Schüll). Each filter contained 1 μg of immobilized single stranded M13 DNA with different inserts. Hybridisation was performed at 42°C for 12 hours followed by a treatment with RNase A (5 μg/ml) for 1 hour at 20°C. The radioactivity on the filters was determined by scintillation counting and the relative synthesis rates were calculated from the ratios of [^{3}H] to [^{32}P] counts at different labeling times. The data were corrected for differences in the hybridisation efficiencies, and blanks obtained using M13 probes without inserts were substracted. The synthesis rates increased linearily with time for all labeling times measured.

Determination of the tRNAGlu2 accumulation

Steady state concentrations of $tRNA^{Glu}_{2}$ were measured in cells containing different plasmids by growing the cultures for three generations in the presence of 0.5 MBq [^{32}P] orthophos-

phate/ml. Total RNA was isolated, and the tRNA fraction was separated on two-dimensional gels as described by Ikemura and Nomura (21). The amount of $tRNA^{Glu}_2$ was assessed densitometrically from the autoradiograms of the two-dimensional separations or by counting the radioactivity of the corresponding gel bands. The $tRNA^{Glu}_2$ accumulation was normalized to the $tRNA^{Leu}_1$ which is well resolved on the two- dimensional gels and not encoded in rRNA operons (22).

RESULTS

Construction of a unique XhoI restriction site in the leader region of the rrnB operon

The mutagenic oligodeoxynucleotide CAA GTC TCG AGA GTG AAC was designed to change the nucleotide position 1464 of the plasmid pKK 3535 from A to G, thereby creating a new unique restriction site (XhoI) within the leader sequence of the rrnB transcription unit. The mutagenesis protocol of Dalbadie-Mc Farland et al. (23) was followed with some major modifications.

Nicked circular DNA from the plasmid pKK 3535 was prepared by incubating 20 µg supercoiled plasmid DNA with 50 units HindIII in the presence of 6 µg ethidium bromide in 50 µl reaction buffer for 1 hour at 37°C. The resulting nicked circular DNA (35%) was isolated and the nicks were extended to gaps of about 300 nucleotides by the use of exonuclease III. The gap size was determined by nuclease S1 mapping (data not shown). The 5' phosphorylated mutagenic oligonucleotide was hybridized to the gapped duplex DNA, and after *in vitro* filling of the remaining gaps by a polymerase reaction and ligation, the resultant plasmid was transformed into competent HB 101 cells (24). The transformants were grown in liquid culture. Plasmid DNA from such cells was isolated and digested with XhoI. This digest creates linear DNA molecules from plasmids where the mutation was successfull. The linear and the undigested plasmid forms were separated by agarose gel electrophoresis. At this stage only a very small fraction of the plasmid population was linearized. The linear DNA fraction was excised, eluted, religated and used for a second transformation. After the procedure had been repeated, single colonies were grown on agar plates. When tested with XhoI, 9 out of 10 such colonies had acquired a unique XhoI restriction site. Figure

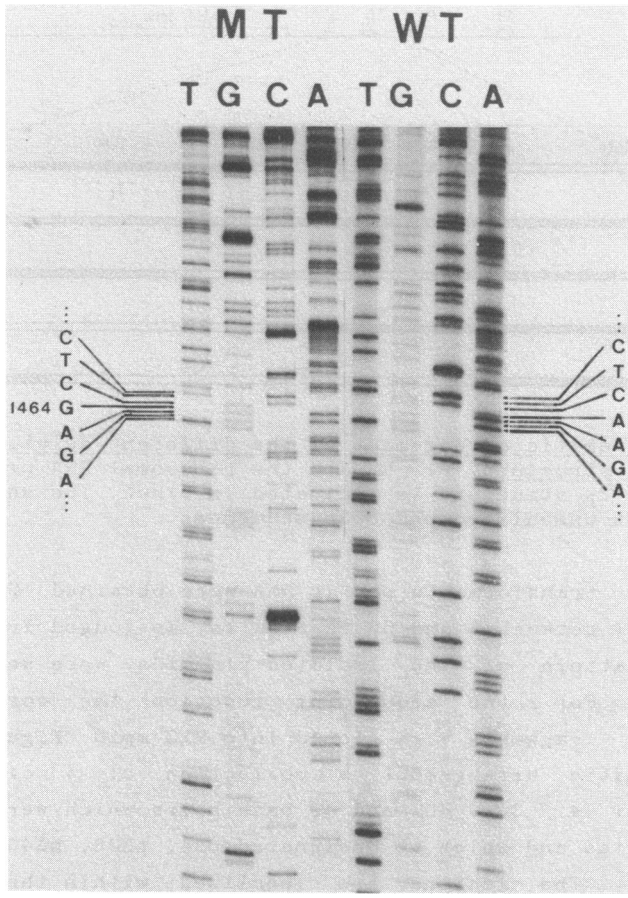


Fig. 1: DNA sequence of the point mutation A1464 to G. MT and WT denote sequencing tracks for the mutant and the wild type, respectively. The base change G1464 is indicated at the left margin.

1 shows the sequencing result of one positive transformant, designated pKK 3535XhoI.

Construction of deletion mutants in the rrnB leader region

pKK 3535XhoI plasmid DNA was linearized with XhoI and subjected to a limited exonuclease Bal 31 digestion (15 m units exonuclease Bal 31/µg DNA in 50 µl 0.6 M NaCl, 20 mM Tris-HCl, pH 7.2, 12.5 mM $CaCl_2$, 12.5 mM $MgCl_2$, 1 mM EDTA for 3 minutes at 37°C). The reaction was stopped by phenol extraction and the isolated DNA was ligated and used to transform competent HB 101

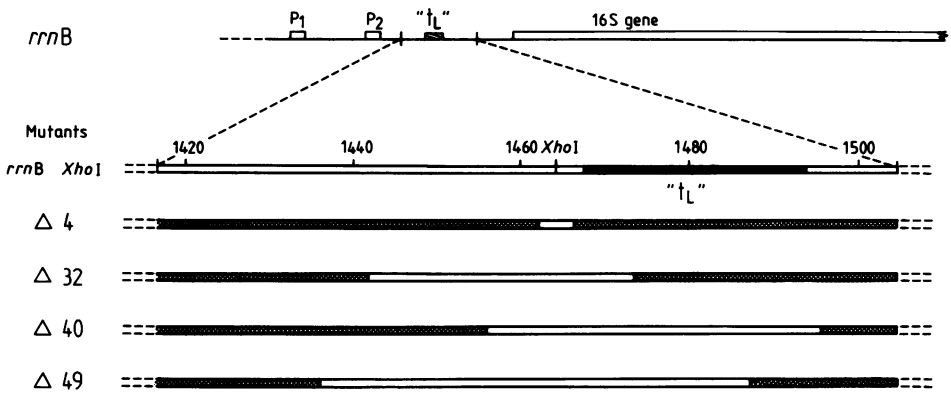


Fig. 2: Schematic arrangement of the different deletions affecting the t_L structure. P1, P2 are the ribosomal RNA promoters P1 and P2. The t_L structure is indicated in black. The shaded areas are represent unmodified pKK 3535 sequences.

cells. 1200 transformants per µg DNA were obtained. Clones with deletions of between 5 and 50 base pairs, as judged from a Dra I digestion pattern of the isolated plasmids, were selected for sequencing. For the sequencing reaction the corresponding PstI–HindIII fragments were cloned into M13 mp18. Figure 2 shows in a schematic arrangement a collection of 4 clones with deletions of 4, 32, 40 and 49 base pairs which were used for further studies and which we designated pΔ4, pΔ32, pΔ40 and pΔ49, respectively. The lesions are localized within the potential terminator structure t_L resulting in its partial or total deletion (see Fig. 2).

Determination of doubling times of HB 101 cells harbouring different plasmids

Any severe disruption of cell metabolism caused by the various rDNA mutations should be apparent in the growth rates of cells harbouring the recombinant plasmids. The doubling times of the mutants pKK 3535Xho, pΔ4, pΔ32, pΔ40 and pΔ49 were therefore determined in different media and compared with the strain containing the wild type plasmid (pKK 3535). The following media were used: YT (25), M9CA (26), M9mCA (M9CA, 0.5g/l casamino-acids). Table I shows the different doubling times obtained for the various mutants. No significant differences for strains containing the deletion plasmids can be noted for YT and M9CA

Table I: Growth rates for t_L deletion mutants
(doubling times in minutes)

strain	YT	M9CA	M9mCA
pKK 3535	49	75	100
pKK 3535 XhoI	50	74	105
pΔ 4	45	68	125
pΔ 32	54	75	130
pΔ 40	45	80	105
pΔ 49	45	75	130

The doubling times are averaged from three measurements with standard deviations ≦ 5, 7 and 12 for YT-, M9CA- and M9mCA media, respectively.

media. In the minimal media M9mCA, however, a noticable increase in the doubling times can be observed for the clones with deletions of 4, 32 and 49 base pairs. The Xho I point mutation and, strikingly, the pΔ40 mutant do not show reduced growth compared to the wild type pKK 3535.

When the strains containing the mutant plasmids were grown in mixed culture together with the wild type, and the amounts of the deletion-containing Dra I restriction fragments were compared with the corresponding wild type fragment, at different times of cell growth, the same differences in the doubling times as given in Table I were reproducibly obtained.

Determination of the relative copy number of mutant plasmids

The copy numbers relative to the wild type plasmid pKK 3535 which is known to exist in 14 copies (27) were determined for the plasmids pKK 3535Xho, pΔ4, pΔ32, pΔ40 and pΔ49 as described in Methods. No difference in the copy number could be observed within the limits of accuracy for all the mutants with the exception of pΔ32, which has a copy number of only 52% relative to pKK 3535. The reasons for this difference in the plasmid copy number are not known, but they had to be considered for the measurements of the ribosomal RNA synthesis rates.

Determination of ribosomal RNA synthesis rates

To study whether modifications of the potential transcription terminator structure t_L has an effect on the expression of the distal ribosomal RNA genes, we determined the individual ribosomal RNA synthesis rates for the 16S and the 23S RNAs after

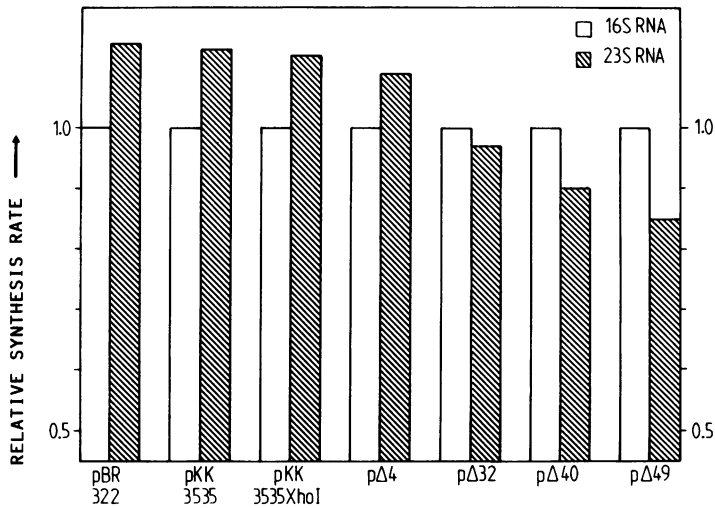
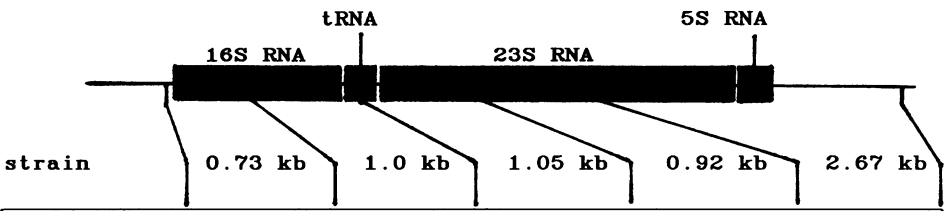


Fig. 3: Diagram representing the ratio of 16S to 23S RNA synthesis rates. The different plasmid systems are indicated at the bottom of the bars.

pulse labeling. Fig. 3 gives the results of 3 independent measurements. It is apparent that for the plasmid pBR 322 control, the wild type pKK 3535, the point mutant pKK 3535Xho and the smallest deletion mutant (pΔ4), the ratio for the 16S and 23S rRNA synthesis rates are the same, while for clones with larger deletions (pΔ32, pΔ40, pΔ49) a small but significant reduction in the 23S RNA synthesis is observed.

By the use of several hybridisation probes, which span different sequences of the rrnB transcription unit, we tried to find out, whether the observed transcription polarity was the result of a premature transcription termination event occuring at a single site. To identify such a site the following DNA fragments were used as hybridisation probes: A 0.73 kb XhoI–EcoRI fragment comprising the 5' half of the 16S RNA, a 1.0 kb EcoRI–XbaI fragment with the 3' half of the 16S RNA, a 1.05 kb XbaI–EcoRI fragment with the 3' third of the 23S RNA, a 0.92 kb EcoRI–PvuII fragment with the middle part of the 23S RNA and a 2.67 kb PvuII–EcoRI fragment with the 3' third of the 23S RNA as well as a distal portion of the rrnB transcription unit. The results are given in Table II. From the comparison of the

Table II: Relative RNA synthesis rates for different portions of the rrnB operon



pKK 3535	1	1	1	1	1
pKK 3535 XhoI	1	0.99±0.1	0.93±0.03	0.93±0.09	1.05±0.1
pΔ 4	1	1.07±0.17	0.95±0.1	1.07±0.17	1.10±0.18
pΔ 32	1	0.97±0.02	0.94±0.007	0.89±0.11	0.88±0.07
pΔ 40	1	0.97±0.09	0.85±0.04	0.86±0.03	0.90±0.06
pΔ 49	1	0.91±0.04	0.81±0.08	0.76±0.07	0.83±0.05

Numbers have been normalized to the wild type plasmid pKK 3535. The relative synthesis rates of the promoter distal fragments are compared to the promoter proximal 0.73 kb fragment, which is normalized to 1. The scheme indicates the location of the different fragments within the rrnB operon.

relative synthesis rates of the different portions of rRNA we conclude, that rather than a sudden stop a gradual termination of transcription occurs between the 16S and the start of the 23S RNA gene for the larger deletion mutants. No further decrease in the transcription rates can be noted for transcripts distal to the 1.05 kb XbaI-EcoRI hybridisation probe. Therefore, premature transcripton termination seems to reach a plateau of approximately 15-20 %, and RNA polymerases which have successfully transcribed within the 23S RNA gene do not undergo further premature termination events. Furthermore, from the data pesented in Table II it is evident, that only the larger deletions Δ32, Δ40 and Δ49 result in a noticable reduction in promoter distal transcription, while the XhoI point mutant and the Δ4 deletion behave as the wild type.

Due to the high background (approximately 50%) of ribosomal RNA transcription from chromosomal rRNA genes in our experiments the measured differences in rates of synthesis are relatively small (15 to 20%). They are, however, significantly higher than the standard error and were reproducibly obtained in all the experiments.

Table III: Ratio of tRNA accumulation. The ratio of the rRNA operon encoded $tRNA^{Glu}_2$ to the non rRNA operon encoded $tRNA^{Leu}_1$ is given

	strains						
tRNA ratio	pBR 322	pKK 3535	pKK 3535XhoI	pΔ 4	pΔ 32	pΔ 40	pΔ 49
Glu_2/ Leu_1	1.2 (0.66)	2.0 (0.78)	1.8 (n.d.)	1.1 (1.5)	3.2 (n.d)	5.0 (3.6)	2.5 (2.1)

numbers were obtained in M9CA minimal media or YT (in brackets)
n.d.: not determined

Determination of the tRNAGlu2 accumulation

Because the tRNA fraction of a cell represents a rather stable set of molecules, the assessement of the tRNA accumulation offers a relatively easy insight into the different tRNA synthesis rates. Measurements of the accumulation of different tRNA species were performed as described in Methods after [^{32}P] labeling of the cells and isolation of the tRNA fraction on two-dimensional gels. Table III gives the results of 4 independent measurements, normalized to $tRNA^{Leu}_1$. Measurements performed in YT- or in minimal media gave virtually identical ratios. The accumulation of $tRNA^{Glu}_2$ in the mutants with deletions of 32, 40 or 49 nucleotides from the terminator structure is significantly increased, compared to $tRNA^{Leu}_1$ for instance. It is important to note that $tRNA^{Glu}_2$ is only coded on the rRNA operons (rrnB, rrnC, rrnE, rrnG), while $tRNA^{Leu}_1$ is coded outside ribosomal RNA transcription units on the chromosome. The increase in the tRNA Glu accumulation therefore indicates a increased transcription of the rRNA operons. The higher transcription frequency presumably compensates for the decrease in 23S RNA synthesis rates caused by premature transcription termination. This in turn is consistent with the model of ribosomal RNA feed back regulation (3), whereafter the amount of ribosomal RNA is kept constant in a cell under constant growth conditions. From the determination of the ribosomal RNA synthesis rates it is apparent that a significant fraction of the rRNA transcripts was prematurely terminated before the synthesis of the 23S RNA was

complete. In order to maintain the supply 23S RNA needed for ribosome assembly, the complete operon has to be transcribed more often. While this can be demonstrated by measuring the accumulation of the metabolicaly stable tRNA products, we were unable to show a similar increase for the 16S RNA accumulation. This is not unexpected, because it is known that excess free rRNA is rapidly degraded (28).

DISCUSSION

In this study we describe the construction of a set of plasmids with deletions in a part of a ribosomal RNA operon known to act as a transcription terminator *in vitro*. We could show that cells transformed with plasmids deleted for most of the terminator structure, exhibit increased doubling times under minimal growth conditions. Surprisingly, one of the mutants (pΔ40) did not show an increase in growth rate, although the t_L structure is completely deleted. We do not know the reason for this but it might be taken as an indication, that sequences flanking the t_L structure, and not only the potential terminator hairpin structure, are important for function. It is noteworthy, that in the pΔ40 mutation the deletion extends further in the 3' direction than do any of the other deletions.

The effect of the various deletions on the transcription of the rRNA genes was investigated by measuring the synthesis rates of the individual rRNA products. From Fig. 3 it is apparent, that the relative synthesis rates for the 23S RNA decreases, compared to the 16S RNA, for mutants with larger deletions in the t_L structure. The somewhat higher synthesis rate for the 23S RNA compared to the 16S RNA for the control and the wild type plasmid (Fig. 3) most likely reflects different stabilities of the different RNAs and differences in the hybridisation efficiencies. Since we compare only the ratios of the synthesis rates this does not affect our conclusion.

If the synthesis rates for different RNA portions of the rrnB operon were compared, a gradual reduction for the promoter distal fractions is apparent for the larger deletions (pΔ32, pΔ40, pΔ49). This reduction is not dramatic and it seems to reach a maximal value of 15 to 20%. One has to keep in mind, however,

that these measurements reflect the sum of the synthesis rates from all rRNA operons. Approximately 50% of the rRNA transcription originates from the 7 chromosomal transcription units and therefore will show a normal ratio of synthesis rates (29).

One of the plasmids with a 32 base deletion (pΔ32) shows, for unknown reasons, a 50% reduction in the copy number. We were unable to detect any other structural alterations to the other plasmids used, apart from the t_L deletion. To circumvent variations in the rRNA synthesis rates, due to copy number effects, we compared the relative synthesis rates and ratios thereof which we normalized either to 16S RNA synthesis rates (Fig. 3) or to the hybridisation probe, comprising the 5' half of the 16S RNA (0.73 kb fragment, Table II).

The results obtained by measuring the different synthesis rates were supported when we determined the accumulation of different tRNAs from cells harbouring the deletion plasmids. The accumulation of the $tRNA^{Glu}_2$, which is encoded in the 16S - 23S RNA spacer region, increases in the same manner as the synthesis rates for the 23S RNA decrease. One has to conclude, that the operon is transcribed more frequently to compensate for the reduction in 23S RNA synthesis. As a result, the metabolicaly stable tRNA molecules accumulate.

A similar polarity in the transcription of the rRNA genes has been demonstrated recently by deleting the antitermination recognition sequence, Box A, close to the 23S RNA gene. Interestingly, the Box A sequence is followed by a potential terminator structure (29). Since the arrangement of structural elements is identical in the 16S RNA leader region (Box A sequence followed by a terminator structure) we propose that the terminator t_L functions as a discriminator of rRNA antitermination. It is assumed, that RNA polymerase will be rendered resistant to termination signals by interaction with one or more antitermination factors (NusA, NusB, NusE). This event is triggered at the Box A sequence. The closely following t_L structure will select antitermination active from unmodified RNA polymerases. Deletion of the t_L terminator results in all polymerase molecules entering the transcription unit. A considerable fraction of the RNA polymerases may not be anti-

termination active and therefore cause premature termination during transcription of the long rRNA genes.

Further studies with Box A mutations and *in vitro* investigations with individual antitermination factors will show, whether this hypothesis is correct.

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