The t_L structure within the leader region of *Escherichia coli* ribosomal RNA operons has post-transcriptional functions

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ABSTRACT

We have investigated a series of mutations within a plasmid encoded *E. coli* ribosomal RNA leader region. The mutations are localized within a structure known as t_L, which has been shown to mediate RNA polymerase pausing *in vitro*, and which is assumed to have a control function in rRNA transcription antitermination. The effects of the mutated plasmids were analyzed by *in vivo* and *in vitro* experiments. Some of the base change mutations led to severely reduced cell growth. As opposed to previous results obtained with mutants where the t_L structure has been deleted in part or totally, the t_L base change mutations did not result in polar transcription *in vivo*, rather they revealed a general reduction in the amount of the promoter proximal 16S versus the distal 23S RNA. The deficiency of the 16S RNA, which was most pronounced for some of the slowly growing transformants, can only be explained by a post-transcriptional degradation. In addition, many mutants showed a defective processing after the initial RNase III cut. In line with these results a quantitative analysis of the ratio of ribosomal subunits and 70S tight couple ribosomes showed a reduced capacity to form stable 70S particles for the slowly growing mutants. Together, these findings indicate an important function of the t_L structure in post-transcriptional events like processing of rRNA precursors and correct assembly of 30S subunits.

INTRODUCTION

Ribosomal RNAs in *E. coli* are encoded in 7 transcription units (*rrn* operons) (1). The highly conserved structural genes are preceded in each case by a less well conserved leader region. However, within these leader sequences a number of equally well conserved control elements are localized, some of which are implicated in regulation, and others are believed to be important for transcription antitermination (2–4). In addition to the lambda *nut*-like sequences (box A, B, C), assumed to function in antitermination, RNA polymerase pausing sites have been identified within the rRNA leader. One of these sites, designated t_L, mediates a Nus A- or ppGpp-dependent RNA polymerase pausing *in vitro* (5), and therefore had been suspected to function in a kind of attenuation mechanism.

In a recent study (6) we have measured rRNA synthesis rates of mutants with deletions in the t_L region. We could show that larger deletions comprising the t_L structure resulted in rRNA transcription polarity. Based on these results we have concluded that the t_L structure might act as a discriminator for rRNA transcription antitermination.

Here we have extended our studies employing a systematic collection of all possible C to T point- and some double mutations within the t_L structural region. Transformation of the mutated plasmids into different strains showed that base changes at some of the t_L positions lead to a drastic reduction of the cell growth rates. We analyzed rRNA transcription products from the mutated plasmids *in vitro*, and *in vivo* by use of the maxicell labeling procedure, which allows a selective labeling of plasmid encoded genes. These studies as well as quantitative determination of the ribosomal subunit stoichiometry from exponentially growing cells clearly point to the fact that the function of the t_L structure is important for post-transcriptional events and not restricted to antitermination or pausing of transcription.

MATERIAL AND METHODS

Bacterial strains and plasmids

HB101 (7), DH1 (8), CSR603 (9), JM109 (10), DG 156 (11) and CP78 (12) were used throughout this study.

The plasmid pKK3535 (13) was used as a source of the *rrn B* operon. The nucleotide positions of plasmids used in this study are numbered in the following way: The transcription start point of the rRNA promoter P1, according to pKK3535 position 1226, is counted as 1. pT0, which was used in most of our assays as a reference, is identical with the plasmid pKK3535 XhoI (6). It deviates from pKK3535 by only one base change (A239 to G) which creates a new unique restriction site for Xho I.

Construction of t_L point mutations

C to T transition mutations were created within the t_L region by bisulfite catalyzed desamination reactions (14). A pT0 DNA

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template was used into which a single stranded gap around the t_L region had been created according to published procedures (15). Mutations downstream from the t_L region within the 16S RNA gene were separated by recloning the 4889 bp Pvu I-Bcl I fragments to a 6975 bp Pvu I-Bcl I fragment, obtained from unreacted pKK3535. The presence of the correct fragments was verified by the existence of a Xho I site. To exclude additional base changes further upstream of the Xho I site the mutant DNA was subcloned using the 1780 bp Xho I-Xba I fragment from mutant plasmids, which was ligated to a 10084 bp vector fragment originating from pKK3535. The base changes were verified by dideoxy sequencing (16) of M13 ssDNA containing the Xho I-Hind III fragments isolated from the host JM 109.

t_L deletion mutants

The construction of the mutant plasmids pΔ4, pΔ32, pΔ40, and pΔ49 has been described by Zacharias and Wagner (6). Within these plasmids the following sequences are deleted: pΔ4: 237–240; pΔ32: 218–249; pΔ40: 232–271; pΔ49: 213–261.

Determination of growth rates

Doubling times were determined in YT- medium (17) containing ampicillin (50 μg/ml) by monitoring the optical densities of the cultures at 560 nm (OD_{560}).

Plasmid copy number determination

Relative plasmid copy numbers were determined at mid-log phase by mixing equal amounts of cells carrying reference and mutant plasmids prior to isolation. Plasmid mixtures were then restricted with appropriate enzymes to yield DNA fragments of different sizes for the two plasmids. The intensity of the different bands obtained after agarose gel separation was determined using a laser densitometer. Alternatively, the relative copy numbers were determined taking advantage of the plasmid encoded β-lactamase activity, following the procedure described by Lupski et al. (18) with cephalosporin as substrate. Within the experimental error the results obtained by the two different methods were identical.

in vitro transcription assay

in vitro transcription was performed with supercoiled plasmid templates by preformation of a specific radioactively labeled G34 ternary complex, initiated with the synthetic trinucleotide ApCpU exclusively at the promoter P1. The conditions developed by Levin et al. (19) for the formation of the ternary complex and its isolation on a Sepharose 6B column were exactly followed. Single round transcription elongation reactions were performed in 'transcription buffer' (19) in the following way: All components were prewarmed to 30°C for 5 min at which point either purified Nus A (final concentration 2 μg/ml), or a crude protein extract (FI extract), isolated from DG 156 cells (20), was added. After one minute incubation the elongation reaction was started by the addition of all four NTPs to a concentration of 400 μM. Samples were taken at different times and prepared for electrophoresis as described (19), except that a phenol extraction was performed before precipitation. Transcription products were separated on 5% polyacrylamide gels in the presence of 8M urea. Transcripts were made visible by autoradiography.

RNA analysis using maxicells

Specific labeling of plasmid encoded RNA was achieved after transformation of the mutated plasmids into the maxicell strain CSR 603. The labeling procedure and the RNA separation was performed as described (21). Radiolabeled bands were detected by autoradiography, and the band intensities were assessed quantitatively using a Zeineh Soft Laser Scanning Densitometer SL-504-XL.

Preparation and analysis of ribosomes

Ribosomes were isolated from cells grown to mid-log phase, similar to the procedure described previously (22). Crude preparations were layered on a 1.1M sucrose cushion in 500 mM NH_4Cl, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol and centrifuged for 30 hours at 40 000 rpm (Beckman L8–22 centrifuge, Ti 55.2 rotor, 4°C). Analysis of the fraction of tight couple 70S and free ribosomal subunits was performed by centrifugation of 3.5 A_{260} units of ribosomes, prepared as described above, through a 5% to 30% linear sucrose gradient in 50 mM Tris-HCl, pH 7.6, 50 mM NH_4Cl, 6 mM $MgCl_2$, 3 mM 2-mercaptoethanol (Beckman SW 28 rotor, 21 000 rpm, 4°C,). After centrifugation the A_{260} of the gradient was recorded using a UV monitor. The resulting peaks were quantitatively evaluated by integration procedures.

RESULTS

To better understand the functional importance of the t_L structure within the rRNA leader region we constructed a set of single and double base transitions (C to T) starting from the plasmid pT0, which contains the entire *rrn B* operon. Figure 1 gives a schematic representation of the different point mutations, and includes some recently described deletion mutations in the same region for comparison.

Effects of mutated plasmids on cell growth

The effects of the different point mutations were first determined by measuring the doubling times of HB101 or DH1 cells containing the respective plasmids under standard conditions (YT-medium, 37°C) . Independent from the strains the doubling times increased severely for pT4, pT7 and pT11. However, no obvious correlation between the sites of the base changes and the reduction in the growth rates is apparent. For example, nucleotide position 262 is the only common altered position within the slowly growing t_L mutants (pT4, pT7, pT11). However, in combination with another base transition the effect is compensated and normal cell growth is restored (pT9: C to T changes at positions 262 and 257).

Table 1 summarizes the doubling times obtained for all the mutants measured in the strain HB 101. The numbers are given relative to the reference strain HB101/pT0. Values for the clones transformed with pΔ4, pΔ32, pΔ40 and pΔ49, which have already been published before (6) are included for comparison. However, we have to correct the doubling times for cells harboring pΔ4 and pΔ32. We have to assume that in these cases erroneously revertant clones had been measured (see below).

The slow growing phenotype of cells transformed with pT4, pT7, pT11, pΔ4 and pΔ32 could be easily followed during growth on agar plates. Slow growers can all be characterized by a significantly reduced colony size after overnight incubation, compared to strains transformed with pT0.

The relative doubling times were very similar when measured in DH 1 cells (data not shown), and the small growing phenotype was also observed after transformation in CSR 603 or CP78 cells. One has to conclude, therefore, that the effect of the different mutations on the growth rate is not restricted to a specific strain,

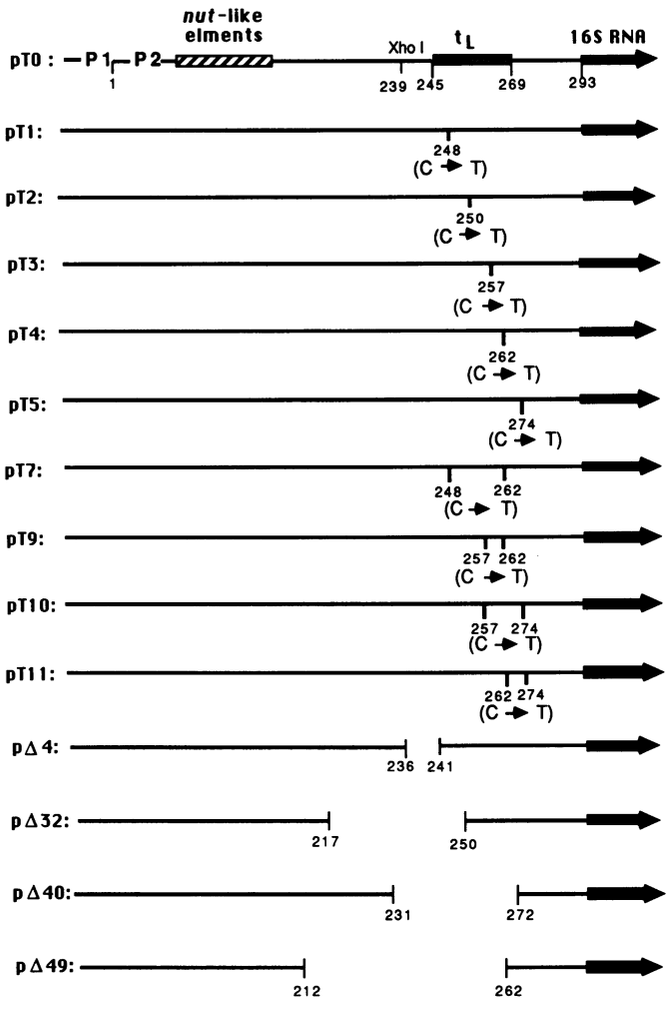

Figure 1: Scheme of t_L mutants. P1 and P2 indicate the rRNA promoters. Numbers represent nucleotide positions relative to the transcription start at promoter P1. The *nut* -like elements (box A, B and C) and the t_L structure are shown as boxes. Xho indicates a single base transition creating a unique Xho I restriction site, which is present in all the t_L transition mutants. The t_L deletion mutations with the respective deletion endpoints are indicated for comparison. The 16S RNA gene is presented as a dark arrow. The schematic arrangement is not drawn to scale.

but is clearly caused by the single or double base changes of the mutated plasmids.

For the slow growers with point mutations in t_L (pT4, pT7, pT11), doubling times were also determined at 30°C and 42°C. Again, cells with the mutated plasmids were seriously retarded in growth at both temperatures (data not shown). At 30°C the mutants have to be described as 'semi-lethal', and cultures were regularly overgrown by revertant clones. We could show that in all cases the reversions leading to normal growth were not linked to the plasmids but rather must be due to chromosomal alterations. The normally growing phenotype was restored in each case by a reduction in the copy number down to one or two copies per cell (plasmid mini-preparations appeared to be 'plasmid-free'!). This reduction occurs also for all other Col E1 type plasmids tested. Similar mutants with decreased copy numbers of Col E1 derivatives have already been reported (23, 24). As in our case, these mutants were able to suppress deleterious effects of high gene dosages. They were characterized by a mutation

Table 1: Doubling times and plasmid copy numbers for mutant clones

mutant plasmids	relative a) doubling times	relative b) copy numbers
pBR322	0.54	n.d.
pKK3535	0.98	1.3
pT0	**1**	**1**
pT1	1.00	1.2
pT2	0.87	1.2
pT3	0.78	1.5
pT4	>1.47	1.5
pT5	0.86	1.3
pT7	>1.50	1.6
pT9	0.86	1.4
pT10	0.91	1.2
pT11	>1.50	1.9
pΔ4	>1.36	1.8
pΔ32	>1.37	0.5
pΔ40	0.90	1.3
pΔ49	0.90	0.9

a) Doubling times were determined with the strain HB 101 at 37°C in YT medium. The numbers were normalized to pT0=1, for which the doubling times varied between 39 and 41 minutes.
b) Relative plasmid copy numbers were determined at mid-log phase in HB 101 cells and were normalized to pT0=1. The error between several independent determinations did not exceed 10%.
n.d. : not determined

in the *pcn B* locus, encoding a 47.3 kDa protein. It remains to be seen if in our case the same kind of mutation has occurred.

As a consequence of the appearance of the revertants we have to admit that doubling times for the slow growing mutants could not be determined exactly. The values for these clones given in Table 1 are therefore subject to a large error and must be taken as minimal doubling times.

Plasmid copy number determination

As was evident from the analysis of the revertants, which suppressed the slow growth by reducing the mutant plasmid copy numbers, all the clones had to be controlled for differences in their plasmid copy numbers. The phenomenon of plasmid copy number variation due to mutations outside from the genes responsible for plasmid replication is well known (25, 26), and has already been demonstrated for plasmids containing a ribosomal RNA operon with or without several deletions (27).

The relative copy numbers were determined in the strain HB 101 and are listed in Table 1, second column. With the exception of pΔ32 and pΔ49 all clones have slightly enhanced copy numbers. Although in case of the revertants the slow growing phenotype was lost due to a reduction in the plasmid copy number, there is no direct correlation between growth rates and copy numbers for the different mutations. The slowly growing mutants pT4, pT7, pT11 and pΔ4 have elevated copy numbers, while the copy number is reduced for clones with pΔ32. Strains harboring the plasmids pT3, pT5, pT9 or pΔ40, on the other hand, show relatively high copy numbers but are growing normally. Therefore, the detrimental effects of some of the plasmids are not simply based on an altered gene dosage, although they can be compensated by drastically lowering the plasmid copy number, as is evidently the case with the revertants. Obviously, at least two different parameters in combination, namely the mutation itself and the gene dosage, determine the detrimental effects on the cell.

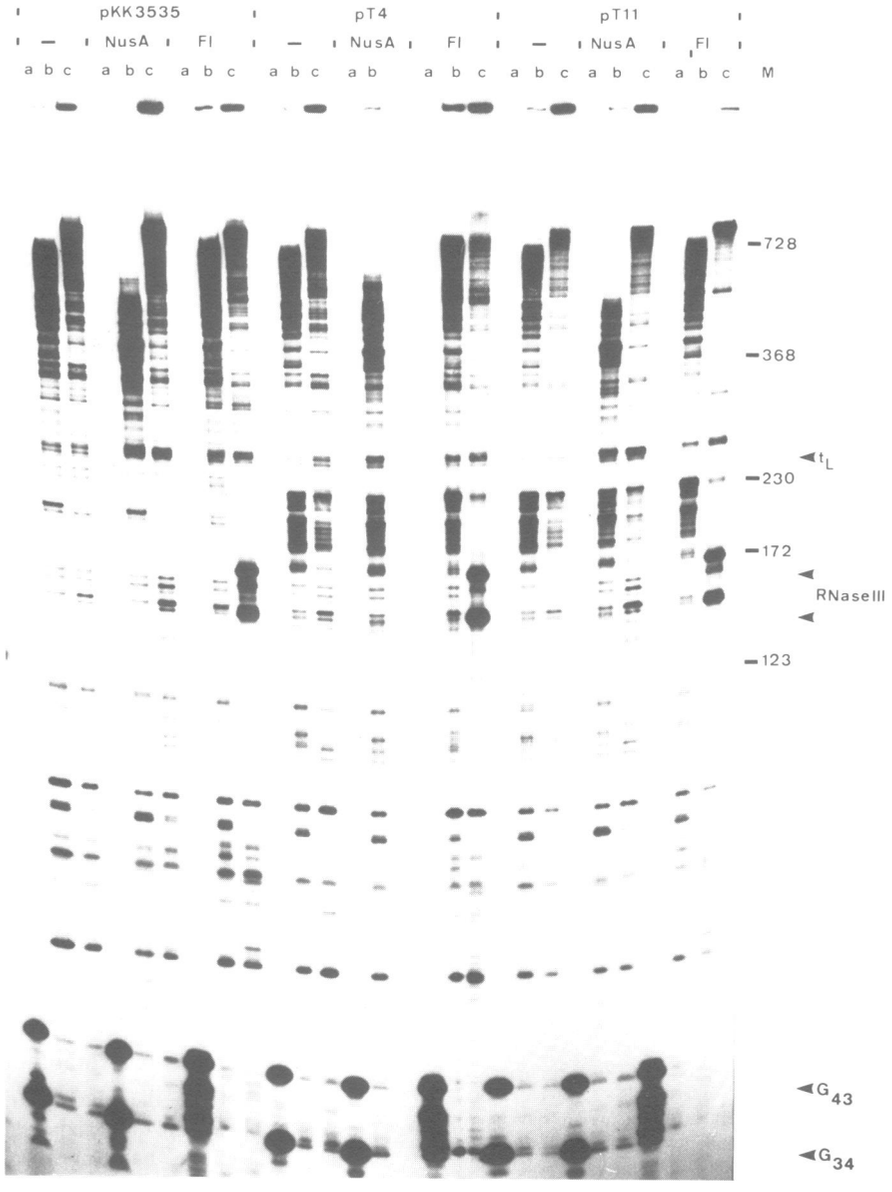

Figure 2: Analysis of *in vitro* transcription products. The gel electrophoretic separation of transcription products from the plasmids pKK3535, pT4 and pT11 is shown. Elongation reactions were performed in the absence or presence of Nus A protein or F1 extract as indicated. In a, b and c elongation reactions of a preformed ternary complex were carried out for 0, 1 and 10 minutes, respectively. The position of RNA markers are indicated at the margin. Arrows denote the positions of bands corresponding to the t_L structure, the *in vitro* RNase III cleavage products and the transcripts obtained after ternary complex formation.

in vitro transcription from plasmids with base change mutations in the t_L structure

The t_L region, 260 nucleotides downstream from the ribosomal RNA promoter P1, has first been shown to be a Nus A and ppGpp dependent pause or termination site in *in vitro* transcription (5). Therefore, we tested some of our point mutations within t_L for differences in this pausing/termination event. We changed the *in vitro* transcription conditions described in the original manuscript by using i) supercoiled plasmids to better match the template topology within the cell and ii) a preformed ternary complex, called G34 (19), to start transcription exclusively from the promoter P1. Additionally, in some experiments reactions were carried out in the presence of a crude *E. coli* DG 156 cell extract (FI extract) to monitor differences in the processing of the transcripts by RNase III (20). An example of these studies is shown in Figure 2. It can be seen that the point mutations within t_L, which cause extremely small growth, do not result in a significantly changed pausing or termination at t_L *in vitro* , when compared with the respective reactions of the wild type plasmid pKK3535. No difference in pausing or termination at t_L was obtained between reference and mutant plasmids, when experiments were performed with or without Nus A or FI extract. One has to conclude, therefore, that the characteristic factor dependent enhancement of the pause/termination at t_L, and consequently any potential attenuator function is fully retained in the mutant plasmids. Even under conditions described to match more closely the *in vivo* situation (28) or at 10 μM NTPs no differences in pausing/termination at t_L were apparent between pT0 and pKK3535, and also not between pT0, pT4 and pT11(data not shown). Furthermore, from the experiments carried out in the presence of F1 extracts it follows that the processing of the

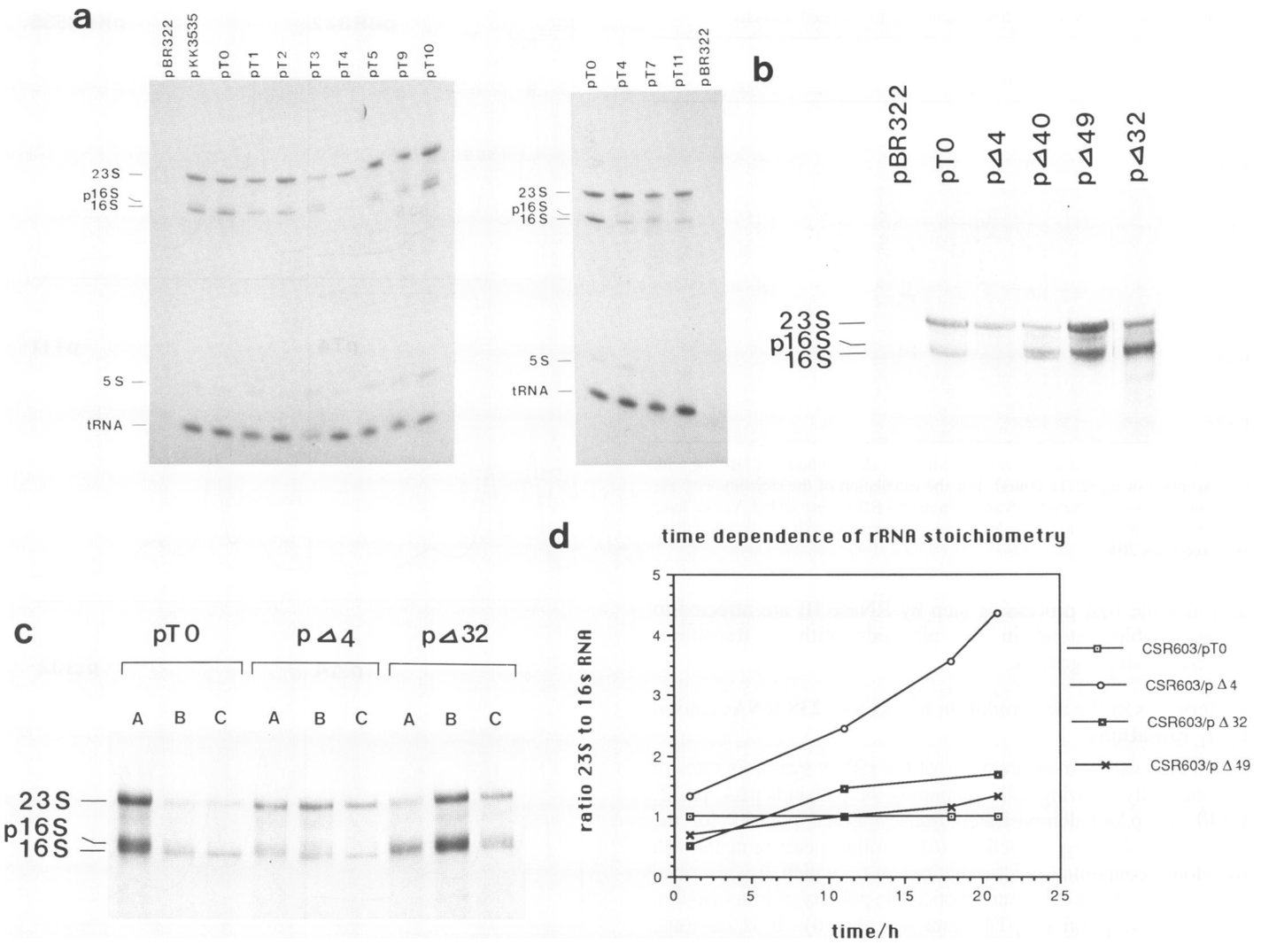

Figure 3: Analysis of RNA isolated from maxicells. a) RNA extracted from the t_L transition mutants. pBR322, which does not code for any ribosomal RNA is shown as a control. pKK3535 and pT0 are reference clones. The positions for mature 16S, 23S, 5S, tRNA as well as for the precursor 16S RNA (p16S) are indicated. The labeling time was 18 hours. b) RNA extracted from the t_L deletion mutants. pBR322 and pT0 are included as controls. The labeling time was 18 hours, except for pΔ32 (11 hours). c) RNA isolated after increasing labeling times. Examples are shown for pΔ4 and pΔ32. In lanes A, B and C material is separated after 1, 11 or 21 hours of labeling time, respectively. d) Graph showing the dependence of the 23S to 16S RNA ratio with increasing labeling periods for the t_L deletion mutants. The values for the reference clone pT0 are normalized to 1.

transcripts by RNase III is unchanged in the t_L mutants, at least within the *rrn B* leader. Due to the end-labeling of the G34 complex we cannot observe the corresponding RNase III cut in the spacer region, but since we know that under the conditions used the complete *rrn B* sequence is transcribed, we assume that the RNase III cut within the rRNA leader is accompanied simultaneously by the corresponding second cut in the spacer region, as it is known to occur *in vivo* (29). This conjecture is in accordance with the time needed for the appearance of the processing bands and the *in vitro* transcription rate (see Figure 2).

There is, however, one obvious difference between plasmids mutated in t_L and the reference plasmid. In the region between nucleotides 180 and 230 relative to the P1 start site a new series of pause sites is apparent. The effect is very likely a pausing, not a termination event. This can be inferred from the time-dependent decrease of the corresponding bands (see Figure 2). These pauses must depend on sequence changes 30 to 80 nucleotides downstream from the RNA growing point (the first mutation is at position 262!). We assume that a long range effect of the template conformation, induced by the mutations, might be responsible for the observed result. Since the base changes are located downstream from the observed pausing sites, we can exclude different secondary structure stabilities of the nascent transcripts. Since the transition mutations in the respective plasmids change GC to AT base pairs, known to be thermodynamically less stable, one might speculate that melting-up events, facilitated by the superhelicity of the templates, are responsible for the altered transcription pattern. Further work to explore this phenomenon in more detail is currently in progress.

From the *in vitro* transcription experiments the following conclusions can be summarized : Neither the pausing/termination

Table 2: Ratio of 23S to 16S RNA according to maxicell analysis

mutant plasmids	ratio 23S/16S
pKK3535	1.2
pT0	1
pT1	1.8
pT2	1.5
pT3	0.8
pT4	4.3
pT5	2.3
pT7	1.7
pT9	1.6
pT10	1.2
pT11	3.0
pΔ4	3.6
pΔ32	1.5
pΔ40	0.7
pΔ49	1.2

Numbers were obtained as described in Methods after 18 hours of labeling with the exception of pΔ32 (11 hours). For the calculation of the stoichiometry the band intensities of precursor 16S and mature 16S RNA were added. Values have been obtained from two to four independent measurements with standard deviations not exceeding 20%.

at t_L nor the first processing step by RNase III are affected to a measurable extend in the plasmids with t_L transitions conferring small growth.

Differences in the accumulation of 16S and 23S RNAs caused by t_L mutations

In a recent study, measurements of the rRNA synthesis rates of exponentially growing cells containing the plasmids pΔ4, pΔ32, pΔ40 and pΔ49 demonstrated transcriptional polarity for the clones with the larger deletions (6). Similar measurements with the clones containing point mutations in t_L, which are described in this study, did not indicate a noticeable polarity of transcription, with the exception of pT4 (data not shown). It is obvious, therefore, that the detrimental effects on cell growth of some of the t_L mutants cannot simply be explained by the loss of a putative control function for antitermination. Furthermore, from the *in vitro* transcription analysis one has to conclude that the base change mutations did not alter the RNA polymerase pausing or attenuation at the t_L sequence. Instead, we had to assume that so far unrecognized post-transcriptional functions of the t_L region were affected by the point mutations we created. Consequently, we looked for differences in processing and accumulation of ribosomal RNAs *in vivo*, taking advantage of the maxicell procedure, where only plasmid encoded rRNAs are labeled (21). The *in vivo* transcription products from maxicell experiments were analyzed after 1 to 21 hours of labeling time. Examples of a number of experiments are shown in Figure 3, and the results for the rRNA stoichiometries are summarized in Table 2. The ratio of the band intensities for 16S and 23S RNA were normalized in each individual experiment to the reference strain pT0. (Note that within different maxicell experiments the amounts of 16S and 23S RNAs were not constant for the control strain, and therefore an authentic reference for normalization had to be included in every experiment). Figure 3 a shows the analysis of the RNA fraction after 18 hours of labeling for the t_L point mutants together with the reference clones pBR322, pKK3535 and pT0. The experiments lead to the surprising result, that for nearly all the transformants with point mutations in the t_L structure the band characteristic for the 16S RNA is clearly under-

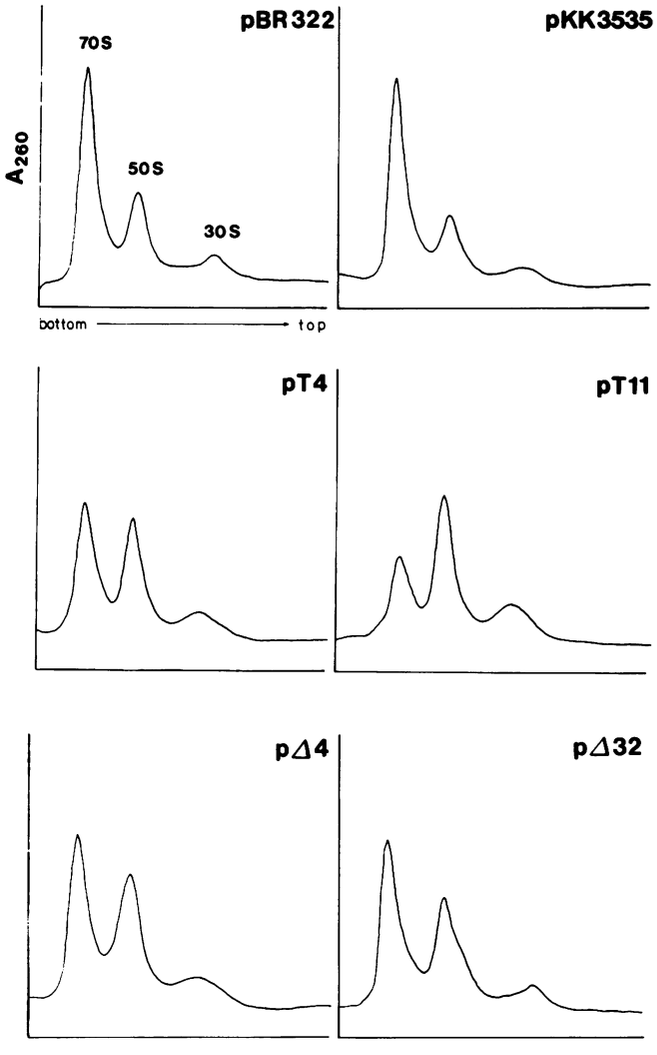

Figure 4: Analysis of the 70S, 50S and 30S subunit population in exponentially growing cells. Examples of the absorption profiles for the ribosomal subunit stoichiometry determinations are shown after sucrose gradient separation. Separations were performed at 6 mM Mg^{2+} under tight couple conditions (38).

represented. If the antitermination function would be the only target of the base change mutations one would expect transcriptional polarity resulting in a reduction of the promoter-distal 23S RNA. However, this is not the case, and most notably, the deficiency of 16S RNA is most pronounced for the slow growth conferring plasmids pT4, and pT11, while clones with pT7, despite their slow growth, have only a moderate 16S RNA deficiency (see Table 2). Qualitatively the same effect (less mature 16S RNA) was found for most of the t_L deletion mutants. However, clones with pΔ32, pΔ40 and pΔ49 show an initial surplus of 16S RNA, in line with the polar transcription effect reported recently (6). This effect is inverted after longer labeling times for pΔ32 and pΔ49, but not for pΔ40 (see Figure 3b, c, d). Obviously, in almost all the mutants a great part of the synthesized 16S RNA never enters the stable 30S ribosome population but must somehow be degraded before assembly is completed.

A second result is apparent from the maxicell analysis. Processing from precursor 16S RNA (p16S) to mature 16S after the initial RNase III cut seems to be retarded for many of the

Table 3: Ribosomal subunit stoichiometry in exponentially growing cells

mutant plasmids	30S free 30S total [%]	50S free 50S total [%]	50S total 30S total
pBR322	41.1 (±1.4)	45.0 (±0.3)	1.07 (±0.02)
pKK3535	40.2 (±2.8)	41.2 (±0.9)	1.02 (±0.03)
pT0	54.6 (±8.6)	51.9 (±0.1)	0.94 (±0.18)
pT1	26.0 (±7.1)	44.2 (±2.6)	1.34 (±0.19)
pT2	41.1 (±3.1)	46.0 (±0.1)	1.09 (±0.06)
pT3	40.6 (±3.0)	42.5 (±2.8)	1.04 (±0.01)
pT4	51.8 (±2.1)	57.1 (±1.7)	1.13 (±0.01)
pT5	43.2 (±1.0)	44.8 (±4.2)	1.04 (±0.07)
pT7	51.3 (±1.0)	55.4 (±2.7)	1.10 (±0.09)
pT9	52.5 (±5.3)	49.7 (±1.5)	0.94 (±0.08)
pT10	44.2 (±4.9)	44.4 (±3.5)	1.01 (±0.04)
pT11	65.3 (±2.9)	66.0 (±6.5)	1.02 (±0.04)
pΔ4	56.0 (±1.1)	56.7 (±2.9)	1.02 (±0.04)
pΔ32	46.7 (±2.3)	56.0 (±3.8)	1.26 (±0.02)
pΔ40	38.5 (±8.6)	44.9 (±4.9)	1.11 (±0.06)
pΔ49	21.7 (±4.8)	40.1 (±2.9)	1.31 (±0.02)

Subunit stoichiometries were determined from exponentially growing HB101 cells containing the different plasmids. Numbers are obtained from 2 to 4 independent experiments. Numbers in parenthesis are standard deviations.

t_L mutants. However, this processing reaction is slowed down for different mutants to a different extend (slight retardation for pT11, where p16S RNA can be detected if labeling periods were shorter than 18 hours; significant retardation for pT3, pT7, pT10, pΔ32, pΔ40 and pΔ49, visible even after 18 to 21 hours of labeling (see Figure 3).

The results of the maxicell analysis can be summarized by two major findings: First, the maturation from p16S to mature 16S RNA is slowed down for many of the t_L mutants. Second, a deficiency of 16S compared to 23S RNA, clearly the result of a post- transcriptional degradation of the 16S RNA, can be noticed for all the mutants after long labeling periods with the exception of pT3, pT10, pΔ40 and pΔ49.

Analysis of the ribosomal subunit composition of exponentially growing cells containing t_L mutations

It should be noticed that the results obtained using the maxicell procedure do not necessarily reflect the overall stoichiometry of ribosomal RNAs in cells under normal growth conditions, because only transcription from the plasmid genes is detected. Dependent on the plasmid copy number and the relative transcription efficiencies of the plasmid operons the amount of 16S and 23S RNA transcribed in cells containing the different mutated plasmids can be different. Furthermore, because there is no cell division and no exponential growth, important parts of the regulatory mechanisms may be disturbed in maxicells. To avoid these possible disadvantages, and to test the mutations under alternative conditions, we analyzed the ratio of free ribosomal subunits and 70S tight couple ribosomes from exponentially growing cells. The analysis was performed by sucrose gradient centrifugation and quantitative assessment of the different sedimentation peaks. The results of a series of such determinations are summarized in Table 3, and some examples of typical gradient profiles are shown in Figure 4. A clear reduction in the amount of 70S tight couple ribosomes, and accordingly an increase in the amount of free subunits is apparent for clones containing the plasmids with the t_L point mutations pT4, pT7, pT11, and the deletion mutations pΔ4 and pΔ32, indicative of a reduced stability of the 70S particles for these clones under the analysis conditions. The ratio of the total 50S to 30S subunits (free and bound to 70S particles under tight couple conditions) is, however, not significantly enhanced for the slowly growing strains, except pΔ32. This indicates an efficient mechanism for maintaining the overall balance of ribosomes under normal growth conditions. The only significant deviation in the stoichiometry of total 50S to total 30S subunits is apparent for cells containing pΔ32 and pΔ49, in accordance with the results obtained after long time maxicell labeling.

DISCUSSION

The role of the t_L structure in pausing or termination

The t_L structure, which shows the same high degree of conservation as the *nut* sites within the leader regions of different rRNA operons, has first been shown to be a Nus A dependent RNA polymerase pausing or low termination site *in vitro* (5). In this study we show that the same Nus A dependent RNA polymerase pausing can be detected when the small growth conferring plasmids with base transitions in the t_L region are transcribed *in vitro*. We have to conclude, therefore, that the reduction in growth rate of the t_L transition mutants is not caused by a change in the observed Nus A dependent pause or termination.

However, a new series of factor independent pausing sites of the ternary transcription complexes was observed during *in vitro* transcription of the slow growth conferring plasmids with transition mutations in the t_L structure. The pausing sites (a pattern of several adjacent bands, see Figure 2) are located 30 to 80 nucleotides upstream from the points of the mutations! The occurrence of pausing sites at such a long distance upstream from the site of modification is rather unusual, and very likely has to be attributed to long range effects of the template stability, which might be influenced by the superhelical density of the plasmids we used. The dependence of pausing from sequence

elements downstream from the RNA growing point has been reported before, both from superhelical and linear templates (28, 30). Work is currently in progress to see whether the observed effect has any significance *in vivo*.

Functional ambiguity of the t_L structure

Apart from its putative function in RNA polymerase pausing or attenuation the t_L structure has been proposed to be a control element in transcription antitermination (6). Due to this recently proposed functionality we anticipated transcriptional polarity as the major effect of the t_L mutations. In some cases (pT4, pT7, pT11, pΔ4, pΔ32) the mutations lead to severe retardation of cell growth. However, this effect is not linked to transcriptional polarity, which can only be detected in the slow growers with pT4 and pΔ32. Evidently, the sequence region altered in this study is not restricted to mediate antitermination, but must be involved in some other important function with strong implications on the cell growth. For instance, deletions removing most or all of the t_L structure (pΔ32, pΔ40, pΔ49) cause transcription polarity, and thereby support the function of t_L in antitermination, while the two double base change mutations in the same region (pT7, pT11), leading to reduced cell growth, do not result in polar transcription, and therefore strongly suggest that a different function must be encoded in the same region. The higher 16S RNA content of the t_L deletion mutants observed after short maxicell labeling times could partly be explained as compensation of 23S RNA deficiencies by polar transcription, which has been reported for these mutants. Interestingly, most of the 16S RNA in these mutants is present in the form of the precursor 17S RNA. It seems that the point mutations lead to degradation with processing being slow or normal, whereas the deletion of the same region inhibits further processing after the RNase III cut, but the transcripts are not degraded at the same speed. The different results obtained with t_L deletion- versus t_L base change mutations may, therefore, point to a functional significance of the t_L structure in discriminating between correct processing and degradation of 16S precursor molecules, independent from a possible involvement of the t_L sequence in antitermination. It remains to be seen whether two (or more) independent control elements for different functions are overlapping by chance in the same structural region (t_L), or whether the opposing effects caused by the t_L mutations are just different facets of one and the same complex regulatory circuit for the correct and coordinated biogenesis of 30S subunits.

The rRNA leader sequence affects 16S RNA stability

As a striking result most of the t_L mutants showed a strong deviation from 16S to 23S RNA stoichiometry, with the 16S RNA being largely under-represented. This is exactly opposite to what one would expect from polar transcription. Since the order of transcription is from 16S to 23S RNA, and an efficient internal transcription start site can decisively be excluded (31−33), the imbalance in rRNA stoichiometry has to be attributed to an instability of the 16S RNA during or after synthesis.

Although we do not presently know at what stage of the synthesis or maturation the degradation of the 16S starts, and what intermediates and enzymes are involved, the results of the maxicell experiments and *in vitro* transcription assays allow a number of conclusions to be drawn: i)The processing by RNase III seems not to be affected. This is evident from the lack of accumulation of a precursor 30S transcript under maxicell labeling conditions and from the correct RNase III cuts, visible for instance for the t_L point mutants during the *in vitro* transcription experiments. ii)The precursor 16S RNA (p16S or 17S) is accumulating to a variable degree for the different t_L mutants. This indicates that a late processing step known to depend on a pre 30S ribonucleoprotein particle containing 17S RNA (34) is affected. iii) The ratio of 23S to 16S RNA increases in general for all the t_L mutants during several hours of maxicell labeling, again indicating that a late maturation step, possibly involving a preassembled 30S particle is disturbed. The assumption that not the free precursor 16S RNA but preassembled 30S particles are attacked and degraded is supported by the result, that many of the slow growth conferring transition mutations lead to a decreased stability of the functional 70S ribosome fraction. It is surprising, on the other hand, that a single endonucleolytic cleavage seems to be sufficient to generate mature 16S RNA (35). Very likely such a cut can only occur, if a correctly preassembled particle has been formed. The transcribed t_L sequence within the rRNA leader may either be part of such a pre 30S particle or influence its composition or structure by long range secondary or tertiary structural interactions. Degradation or further maturation may, therefore, depend on processing activities, known to act on a 16.3S precursor RNA (36). This is in line with processing cuts, which have been localized very close or within the t_L structure (37), and the fact that processing and assembly are simultaneous and coordinated events (29,34). Whether or not these enzymatic activities initiate the 16S degradation if a correct precursor particle has not been formed remains to be seen.

Effects of the t_L mutations on the 70S stability and subunit stoichiometry

Sucrose gradient analysis of the ribosome fractions from exponentially growing mutant cells allowed us to observe whether the stoichiometric aberrations of the rRNAs, detected during maxicell labeling, would also be manifested on a later stage of biosynthesis, when the rRNAs are stably incorporated into ribosomal particles. Two kinds of information were derived from these experiments. First, and very obvious from the examples shown in Figure 4, the stability of the tight couple 70S fraction is strongly decreased under the analysis conditions for cells with pT4, pT7, pT11, pΔ4 and pΔ32. This is apparent by the increase of free subunits (Table 3). The observed effects cannot simply be explained as a consequence of the slow growth of the mutants for two reasons: i) there is no difference in the ribosomal subunit distribution for HB101/pKK3535 and HB101/pBR322, although both strains differ in growth rate by approximately 100%. ii) mutations in other parts of the rRNA leader, created in our laboratory, which severely affect growth have an unchanged ribosomal subunit pattern (data not shown). We conclude therefore that the observed effects are t_L-specific. Second, with the exception of pΔ32 and pΔ49 the 16S RNA deficiency observed under maxicell conditions is not reflected in the overall subunit stoichiometry (we do not consider strains transformed with pT1 as 'out of stoichiometry' because of the relative high standard deviation in this case; see Table 3).

Very likely, the detected imbalance in rRNA stoichiometry is not reflected in the pool of ribosomal subunits due to an efficient degradation of excess ribosomal RNA or ribosomal particles which are out of proportion. The reduced 70S stability, however, indicates that the mature ribosomal particles due to the t_L

mutation on a plasmid encoded operon are defective in either structure or composition. This implies that the t_L structure affects 30S ribosomal biogenesis.

Taken together, our results provide evidence that the t_L structure within the rRNA leader plays an important role in the process of rRNA maturation with consequences on the subunit assembly and 70S ribosome stability. Despite their possible involvement in antitermination, sequences within the t_L structure must be important to direct the correct 16S RNA maturation and 30S ribosome assembly.

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