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Base-pairing of 23 S rRNA Ends is Essential for Ribosomal Large Subunit Assembly

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In ribosomal RNA precursors the spacer sequences bracketing mature 16 S and 23 S rRNA are base-paired to form long helices (processing stems). In pre-23 S rRNA, the processing stem is continued by eight base-pairs of mature 23 S rRNA known as helix 1. Recently, we have found that any part of 23 S rRNA between positions 40 and 2773 could be deleted without the loss of ribosome-like particle formation, while both end regions were indispensable. In this paper we have analyzed the role of the 5′ and 3′ end regions of 23 S rRNA during ribosomal 50 S assembly *in vivo* by using mutants of the 23 S rRNA gene. Deletions and substitutions in both strands of the helix 1 lead to the loss of plasmid derived 50 S formation. Compensatory mutations restoring helix 1 were assembled into functional 50 S subunits. We conclude that the helix 1 of 23 S rRNA is the main RNA determinant for ribosomal large-subunit assembly. Deletions in both the 5′ and 3′ strand of the processing stem reduced the ability of the 23 S rRNA to form ribosomal 50 S subunits. However, even the complete removal of either the 5′ or the 3′ strand of the processing stem did not abolish the 50 S assembly completely. Thus, processing stem facilitates, but is not essential for assembly.

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Introduction

Ribosome formation in *Escherichia coli* is a fast and efficient process. Prokaryotic ribosomes consist of three rRNA and 53 different proteins. Coordinated synthesis of nearly 60 different molecules is achieved by a variety of regulatory mechanisms (Nomura *et al.*, 1984, Zengel & Lindahl, 1994, Condon *et al.*, 1995). Ribosome assembly involves processing, modification and folding of the rRNA and association of the ribosomal proteins with the rRNA into functional ribosomal subunits. Ribosomal components are assembled into functional subunits within two to three minutes (Lindhal, 1975). Efficiency of the ribosome assembly is evident by the negligible turnover of ribosomal components during exponential growth at moderate to fast rates in wild-type *E. coli* (Bremer & Dennis, 1987).

The role of the different ribosomal proteins during ribosome assembly has been identified mostly by *in vitro* experiments using the reconstitution technique (Held *et al.*, 1974; for review see Nierhaus, 1991). During reconstitution of the 50 S subunit, three intermediate particles were found. These particles exhibited similar protein composition as compared to the ribosome precursor particles found *in vivo* showing that the reconstitution and *in vivo* assembly proceed in similar ways (for review see Nierhaus, 1991). Two assembly initiator proteins were found for the large subunit of the *E. coli* ribosome, L3 and L24 (Nowotny & Nierhaus, 1982). Assembly initiator proteins, upon binding to their cognate sites, are believed to create the functional structure of the rRNA and thereby direct the folding of nascent rRNA during transcription (Brimacombe, 1991; Noller, 1991).

rRNA was found to be the most important ribosomal component both in a structural and functional sense (Noller, 1991). The functional structure of rRNA is formed during ribosome assembly. Formation of the functional ribosomal subunits is RNA polymerase dependent, indicating an importance of the co-transcriptional events during ribosome assembly (Lewicki *et al.*, 1993, Cowgill de Narvaez & Schaup 1979). In *E. coli* rRNA is transcribed under the control of two tandem promoters as a single transcription unit which includes, in addition to the mature rRNA sequences, leader, spacer and trailer sequences. Conserved leader sequences of rrn operons are known to be involved in rRNA transcription and ribosomal small subunit assembly. Importance of the leader and spacer sequences in ribosome assembly is indicated by several lines of evidence. Base substitutions in the

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leader terminator (t_L element) sequence were found to affect the structure and function of the 30 S ribosome subunit (Theissen *et al.*, 1993, Pardon & Wagner, 1995). Interaction between leader rRNA and mature 16 S rRNA was proposed to take place during the co-transcriptional folding of 16 S rRNA (Dammel & Noller, 1993; Pardon & Wagner, 1995). The processing stem bracketing 16 S rRNA was shown to stimulate 30 S subunit assembly (Mangiarotti *et al.*, 1975). The intergenic spacer sequence is involved in the ribosomal 50 S subunit formation as shown by the inability of some deletion mutants to form ribosomal subunits (Stark *et al.*, 1985, Szymkowiak & Wagner, 1987). Mature 16 S, 23 S and 5 S rRNAs are formed after a series of coordinated processing events (Srivastava & Schlessinger, 1990). The main processing enzyme of rRNA is RNase III. RNase III cleavage sites are in the processing stems which are long helices formed by base-pairing of the sequences flanking mature 16 S and 23 S rRNA (Bram *et al.*, 1980). In the strains lacking RNase III activity, mature 16 S, but not mature 23 S rRNA, is formed (King *et al.*, 1984). The stem bracketing 23 S rRNA contains 114 nucleotides on the 5′ side and 71 nucleotides 3′ to the 23 S rRNA (Bram *et al.*, 1980). This stem also contains eight base-pairs of the mature 23 S rRNA, known as helix 1 (Garrett *et al*, 1984). Deletions between positions 40 and 2773 of 23 S rRNA affected ribosomal large-subunit formation to different degrees (Liiv *et al.*, 1996). However, all 23 S rRNA mutants were found to form ribosome-like particles. In contrast, if one end of the 23 S rRNA was deleted, the mutant rRNA was unstable and did not form ribosomal particles (Liiv *et al.*, 1996). Therefore, in addition to the ribosomal proteins, the sequences in both end regions appear to be essential elements in stabilizing 23 S rRNA. Here we have analyzed the involvement of the both ends and the spacer sequences of 23 S rRNA during ribosomal large-subunit assembly in more detail by using the mutagenesis approach.

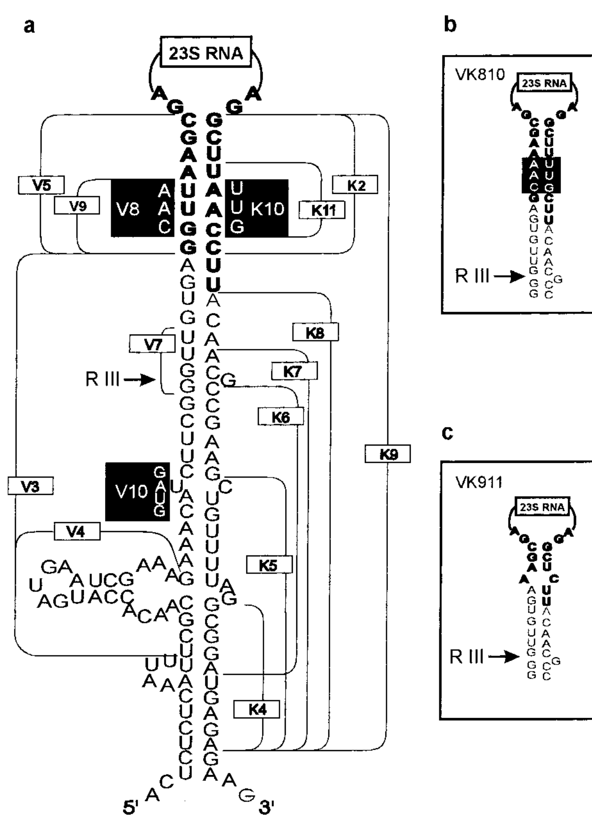

Figure 1. Secondary structure of pre-23 S rRNA according to Bram *et al.* (1980) (a). Mature 23 S rRNA is in bold. Deletion mutations are shown by lines and substitutions are indicated in black boxes. Putative secondary structure of the mutant 23 S rRNA VK810 (b) and VK911 (c).

Results

The secondary structure of the processing stem and the helix 1 of 23 S rRNA is shown in Figure 1a. Mutations (shown in Figure 1a) were constructed in the plasmid copy of 23 S rRNA gene. Mutated genes were expressed using an inducible *tac* promoter leading to the mixed population of plasmid-encoded and chromosome-encoded 23 S rRNA. A point mutation A1067 to T was introduced into the 23 S rRNA gene in order to be able to distinguish between plasmid borne and chromosomal rRNA. Plasmid ptBsB1067U, containing *E. coli* $tRNA^{Glu2}$, 23 S rRNA and 5 S rRNA genes, was used to construct deletion mutants in the gene for 23 S rRNA. This plasmid was earlier shown to code a functional 23 S rRNA which is incorporated into active ribosomes (Saarma & Remme, 1992; Lewicki *et al.*, 1993). The A1067U mutation in 23 S rRNA confers resistance to the antibiotic thiostrepton during *in vitro* translation (Thompson *et al.*, 1988). Functional activity of the plasmid encoded ribosomes was analyzed by cell-free translation in the presence of thiostrepton. Three to five independent ribosome preparations were isolated for each mutant. Each ribosome preparation was analyzed in double. Representative examples of double-determinations are shown in Table 1. The fraction of plasmid 23 S rRNA in the ribosome preparations was determined by RNA sequencing around the 1067 region for one to two preparations (column M in Table 1). Wild-type plasmid-encoded 23 S rRNA (1067U) was found to constitute around 30% of total 23 S rRNA pool in the ribosomes and exhibited thiostrepton resistance in poly(U) translation by the same degree (Table 1), in agreement with the earlier observation (Lewicki *et al.*, 1993). Sucrose gradient and RNA sequencing analysis demonstrated that the plasmid borne wild-type 23 S rRNA was present in nearly equal amounts in the polysomes and 70 S ribosomes (Figure 2). Ribosomes extracted from both strains without plasmid containing only chromosomal 23 S rRNA gene

Table 1. Translational activity of the plasmid-encoded ribosomes *in vitro*

	Strain XL1-Blue (RNase III$^+$)				Strain CAG1632 (RNase III$^-$)			
Mutant	% thiostrepton resistance	*M* (%)	Average thiostrepton resistance	*s*	% thiostrepton resistance	*M* (%)	Average thiostrepton resistance	*s*
—	2	5	1.6	0.7	4	8	3.0	1.0
1067U	34	28	34.4	4.6	26	28	25.5	0.5
V3	19	20	19.0	0.2	27	23	22.8	3.8
V4	27	33	27.6	2.3	23	25	22.2	1.1
V7	9	14	9.3	2.4	14	16	14.0	0.3
V10	20	21	20.4	0.4	15	21	16.9	2.1
VK38	19	18	18.6	1.1	23	20	21.2	2.3
K1	26	34	24.4	2.2	33	26	36.0	3.0
K4	27	29	26.6	1.2	18	20	18.3	1.1
K5	19	25	15.8	2.1	19	22	18.1	0.9
K6	14	16	14.2	0.5	31	32	29.4	2.5
K7	13	13	15.0	1.6	22	18	22.1	1.2
K8	11	13	11.9	1.2	26	20	22.5	5.1
K2	2	7	1.5	0.6	6	12	4.1	1.5
K9	2	11	1.6	0.7	6	15	4.9	1.0
K10	7	9	5.9	1.4	21	26	21.6	1.6
K11	3	9	5.6	2.4	8	11	5.7	1.9
V5	6	4	2.8	2.1	6	7	4.9	0.3
V8	3	7	4.8	1.4	15	19	14.8	0.7
V9	2	6	2.4	1.2	11	10	10.6	1.1
VK52	5	9	5.3	2.4	9	11	7.7	1.6
VK810	29	29	26.5	2.5	26	34	26.0	0.8
VK911	6	7	5.9	0.8	9	10	8.2	0.9

Mutant 23 S rRNA genes were expressed and the ribosomes were isolated from both RNase III$^+$ (XL1-Blue) and RNase III$^-$ (CAG1632) strains. Mutants are shown in Figure 1. Ribosomes were analyzed in a poly(U) directed translation system in the presence or absence of thiostrepton. Thiostrepton resistance (% thiostrepton resistance) indicates the functional activity of the plasmid-encoded ribosomes from a representative preparation. The fraction of plasmid encoded 23 S rRNA in the same ribosome preparations (*M*) is indicated. Mean values over three to five independent determinations of thiostrepton resistance (Average thiostrepton resistance) and the average deviation (*s*) values are shown. 1067U is full-length 23 S rRNA gene with single-point transversion A1067 to U, (—) denotes the ribosomes extracted from strains without the plasmid.

(A1067) were inactive in a poly(U) translation assay in the presence of the drug (2% and 4% in Table 1). Background values for RNA sequencing were 5% and 8% for XL1-Blue and CAG1632, respectively (Table 1).

Mutations in the helix 1 of 23 S rRNA

Ribosomes isolated from the strain XL1-Blue containing plasmids with deletion mutations in either the 5′ or 3′ strand of the helix 1 of 23 S rRNA (V5, V9 and K2, K11, respectively) were not able to translate poly(U) in the presence of thiostrepton (Table 1). In agreement with this plasmid borne 23 S rRNA was present in the ribosomes in trace amounts (Table 1). Mutation V5 was analyzed in more detail. 23 S rRNA V5 was incorporated into polysomes, monosomes, and 50 S subunits in reduced amounts (Figure 2). In the 40 S and 30 S fractions corresponding to precursor particles, the ratio plasmid/chromosome 23 S rRNA was over two times higher as compared with the corresponding value in 70 S and polysome fractions (Figure 2). The optical sucrose gradient pattern of the V5 was similar to the wild-type one and did not exhibit additional particles. Thus, the 30 S and 40 S V5 particles constituted only a minor amount of total 23 S rRNA. Two substitution mutations were constructed in the helix 1 by replacing three nucleotides in the 5′ end by the corresponding three nucleotides in the 3′ end and *vice versa* (V8 and K10, respectively, in Figure 1a). Mutant ribosomes expressed in the wild-type strain, derived from both plasmids were inactive in poly(U) translation and were not incorporated into total ribosomes. The sucrose gradient pattern and the distribution of plasmid borne 23 S rRNA was similar to that of the mutant ribosomes V5 (Figure 2).

When the mutant 23 S rRNA genes were expressed in the strain CAG1632 (RNase III$^-$), 23 S rRNA lacking the entire 5′ (V5) or 3′ (K2) half of the helix 1 was incorporated into ribosomes at low amounts and the corresponding translational activity was close to the background value (Table 1). In contrast, partial deletion of the helix 1 (mutants V9, K11) did not inactivate the 23 S rRNA completely in the RNase III$^-$ strain as it was observed in the RNase III$^+$ strain (Table 1). In the RNase III-deficient strain (CAG1632), expression of the substitution mutant genes (V8, K10) conferred high-level thiostrepton resistance during *in vitro* translation and the mutant 23 S rRNA species were present in corresponding amounts (Table 1).

Taken together, both deletion and substitution mutations of the helix 1 of 23 S rRNA severely affect ribosomal large-subunit formation in the

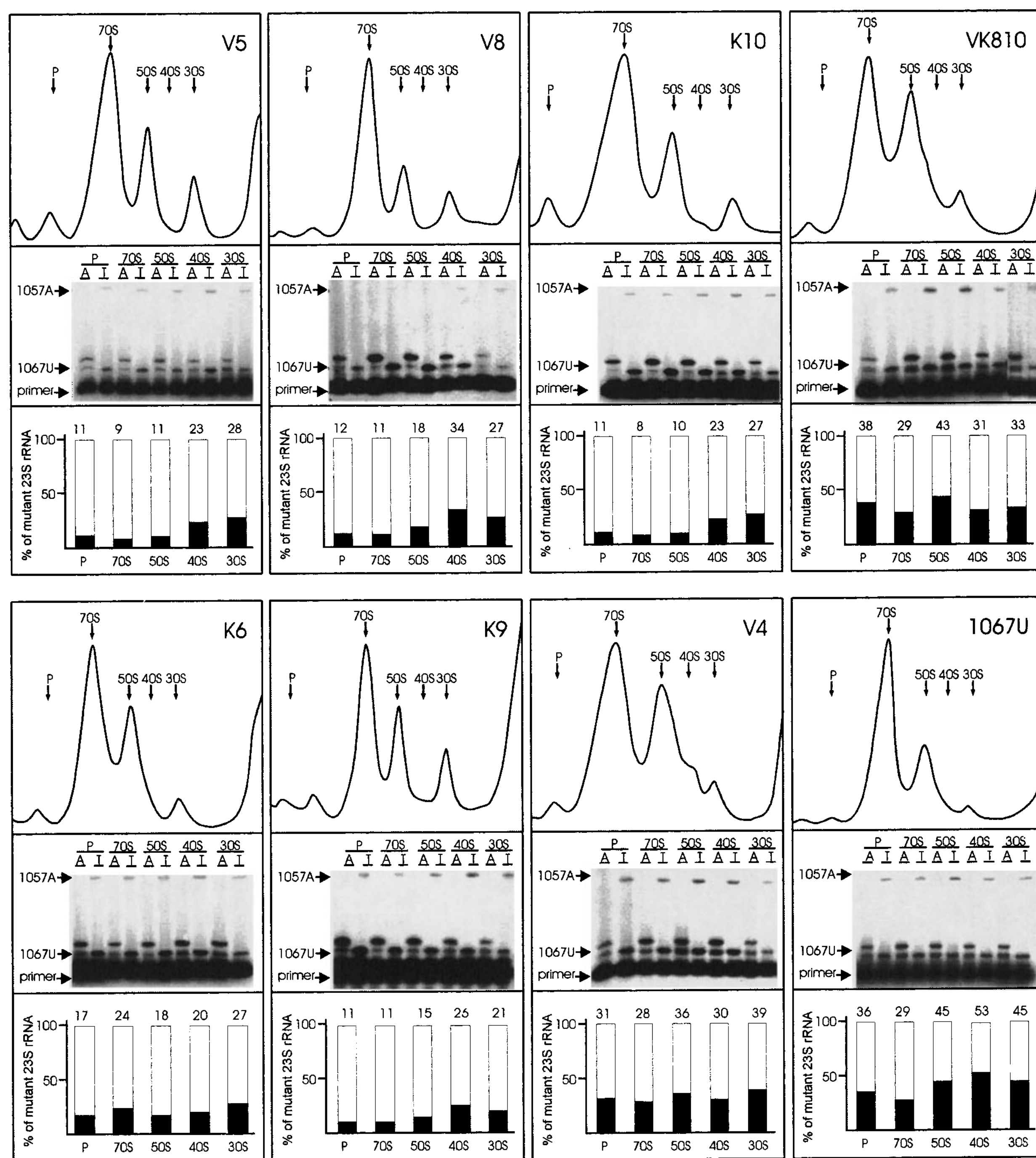

Figure 2. Distribution of plasmid encoded 23 S rRNA. Mutant plasmids were expressed in the strain XL1-Blue (RNase III^{+}), cells were lysed and ribosomes were fractionated by sucrose gradient centrifugation. The portion of plasmid encoded 23 S rRNA in the ribosomal fractions was determined by sequencing. Each mutant is shown in three panels: upper panel, sucrose gradient pattern; middle panel, minisequence around the 1067 region of 23 S rRNA; lower panel, percentage of the plasmid encoded 23 S rRNA in the corresponding fraction (P, polysomes).

RNase III positive strain XL1-Blue. In the RNase III negative strain the 23 S rRNA species containing substitutions in the helix 1 were assembled into functional ribosomes by a similar amount to wild-type 23 S rRNA. The nucleotides in the helix 1 are consequently important for the formation of the functional 50 S ribosomal subunit. On the other hand, these effects can be attributed to the secondary structure.

Mutations V8 and K10 were combined in VK810 where base-pairing in the helix 1 is restored (see Figure 1b). Ribosomes encoded by the double mutant 23 S rRNA gene (VK810) were able to translate poly(U) by the same level as 1067U ribosomes in both rnc^{+} and rnc^{-} strains (Table 1). Sequencing results of both total ribosomes and ribosomal subpopulations showed similar distribution of the mutant 23 S rRNA as compared to

the U1067 ribosomes (Table 1, Figure 2). This result confirms the importance of the helix 1 as a secondary structure element during the incorporation of 23 S rRNA into ribosomes.

Deletion mutants V9 and K11 were combined to form mutant VK911. The 23 S rRNA encoded by this mutant gene contains three base-pairs of the native helix 1, an A-C mismatch, and is continued by the processing stem (Figure 1c) which is not removed by the processing enzymes (see Figure 3). Ribosomes VK911 were present in the ribosomal fractions at a low level in both strains and accordingly, thiostrepton resistance was low but clearly detectable (Table 1). When both strands of the helix 1 were deleted (VK52), functional activity of the mutant ribosomes was very low and the plasmid 23 S rRNA was found in the ribosomes at a reduced level (Table 1). These results demonstrate the importance of the nucleotides in the helix 1 region. The processing stem can only partially replace the helix 1 during ribosomal 50 S subunit assembly.

Mutations in the processing stem

We have constructed a series of deletion mutations in the processing stem of pre-23 S rRNA in order to analyze its role during 50 S assembly

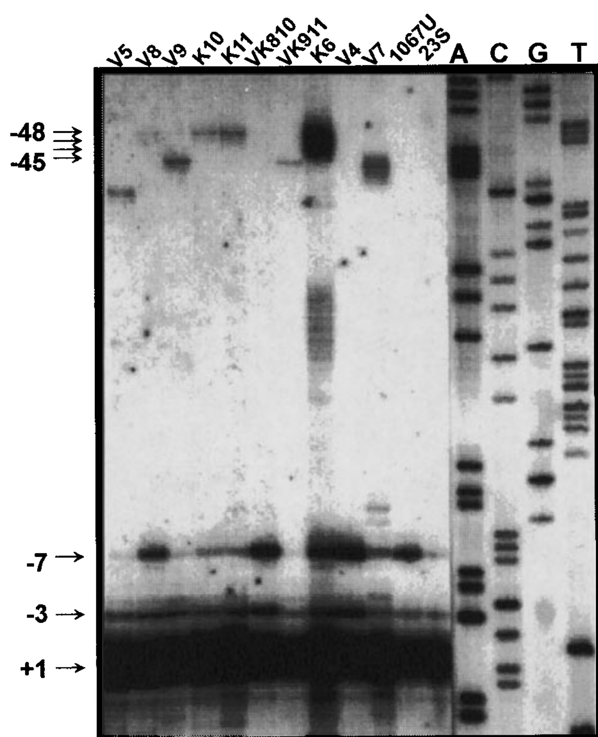

Figure 3. Mapping of the 5′ end of 23 S rRNA. Total rRNA was extracted from 70 S ribosomes and the 5′ ends of 23 S rRNA were detected using reverse transcriptase directed primer extension. The sequence of the corresponding region is shown by the lanes A, C, G and T. The mature 5′ end of the 23 S rRNA (+1), RNase III cleavage sites (−3 and −7) and position −46 are indicated.

in vivo (see Figure 1a). Mutations in the 3′ side of 23 S rRNA were constructed in a way that the region necessary for the processing of the 5 S rRNA (Cormack & Mackie, 1993) were left intact. In the mutant K1, nucleotides 38 to 57 downstream of the mature 3′ end of 23 S rRNA are deleted (not shown in Figure 1). 23 S rRNA encoded by the mutant plasmids K1 and K4 were incorporated into functionally active ribosomes by the same degree as control 1067U 23 S rRNA, i.e. wild-type. Mutations K4 to K8 are successive deletions in the 3′ strand of the processing stem (see Figure 1A). Both the amount of plasmid encoded 23 S rRNA and the thiostrepton resistance (functional activity of the mutant ribosomes) decreased proportionally with the growing length of deletion in the strain XL1-Blue (Table 1). 23 S rRNA derived from mutant gene K6 was found at the reduced level in all ribosomal fractions (Figure 2). The mutant 23 S rRNA K8 lacking the complete 3′ strand of the processing stem was clearly active albeit at a reduced level in ribosome formation both according to poly(U) translation and rRNA sequencing (Table 1). Thus, the processing stem of 23 S rRNA facilitates incorporation of the 23 S rRNA into 50 S subunits. However, it is not an essential requirement for the ribosomal large-subunit formation. The mutant 23 S rRNA K9 lacking both the 3′ strand of the processing stem and the 3′ strand of the helix 1 was found in the ribosomes in both strains at a reduced level. However, the ribosomes did not show up functional activity (Table 1). Therefore, the mutant 23 S rRNA K9 was assembled into unfunctional subunits. This result was confirmed by sucrose gradient analysis. Mutant 23 S was found mostly in the 50 S and in the precursor particles (Figure 2).

A transcribed spacer in front of 16 S rRNA was proposed to be involved during folding of 16 S rRNA and in the assembly of ribosomal 30 S subunits (Theissen *et al.*, 1993, Dammel & Noller, 1993). We have analyzed the importance of the 5′ spacer sequence of the 23 S rRNA gene during 50 S subunit assembly. The mutations V3 and V7 (see Figure 1) were found in the functional ribosomes at a reduced level (Table 1). This effect can be attributed to the loss of the processing stem according to the results obtained with the deletions in the 3′ strand of the processing stem. Destabilization of the processing stem by the four nucleotide substitution (V10) had only a small effect on the ribosome formation (Table 1). Deletion of the both strands of the processing stem (mutant VK38) lead to the 43% reduction of the plasmid borne 23 S rRNA in the ribosomes in comparison to the 1067U control (Table 1).

Deletion of the 5′ side-arm of the processing stem (mutant V4) does not affect assembly of the 23 S rRNA into functional ribosome (Table 1). Sucrose gradient analysis data are in agreement with this conclusion (Figure 2). Evidently, this structure does not have an important role during ribosome assembly.

Processing of the plasmid encoded 23 S rRNA

Mutations affecting the processing stem of pre-23 S rRNA are expected to abolish RNase III directed rRNA cleavage and consequently, one would expect the plasmid 23 S rRNA to be prolonged. Therefore, it was important to analyze the 5′ end of the 23 S rRNA. Control 23 S rRNA extracted from strains without the plasmid has mostly mature 5′ end (23 S in Figure 3). Expression of full-length 23 S rRNA from plasmid ptBsB1067U leads to the accumulation of two additional 5′ end products, −3 and −7 (1067U in Figure 3). These two positions have been identified as RNase III cleavage sites (King *et al*., 1984). Over-expression of the wild-type 23 S rRNA lead to the incomplete processing at the 5′ end possibly by saturating processing enzymes. Deletion of the 3′ half of the processing stem leads to the accumulation of the unprocessed 23 S rRNA with the main 5′ ends between positions −45 and −48 with respect to the mature 23 S rRNA (K6 in Figure 3). It should be taken into account that the mutant 23 S rRNA variants are present in different amounts in comparison to the chromosome encoded wild-type 23 S rRNA (see Table 1). The amount of the 5′ termini around position −46 correlates with the amount of the plasmid-encoded 23 S rRNA (as determined by RNA sequencing, see Table 1) and, therefore, these rRNA species can be attributed to the plasmid encoded 23 S rRNA. Note that the deletions in the 5′ region alter the positions of the upstream bands on the gel (Figure 3) by the number of deleted nucleotides. It is evident that the appearance of the signal around −46 indicates a failure of RNase III processing on the plasmid encoded 23 S rRNA (e.g. V7 in Figure 3). In the mutant K4 a part of the processing stem is deleted at the 3′ strand (Figure 1). In spite of the abundant plasmid encoded 23 S rRNA in the ribosomes (Table 1), there are no additional bands upstream of position −7 (not shown). Consequently, this part of the stem is not necessary for the RNase III cleavage. A similar result was obtained with the 23 S rRNA gene with the side-arm of the processing stem deleted (V4 in Figure 3). In contrast, deletions adjacent to the 23 S rRNA (K5, K6, K7, and K8) were unprocessed by the RNase III (K6 is shown in Figure 3). These data indicate that the processing stem of 23 S rRNA is indeed the substrate for RNase III, as was found to be the case with pre-16 S rRNA (Srivastava & Schlessinger, 1989). 23 S rRNA variants with mutated helix 1 have extended 5′ ends (V5, V8, V9, K10, K11, and VK911 in Figure 3). Thus, in addition to the processing stem, helix 1 appears to be important for the RNase III cleavage. Precursor 23 S rRNA containing double mutation VK810 was cleaved by RNase III (Figure 3).

Cleavage at positions −45 to −48) is probably a result of an unspecific nucleolytic attack since there are four bands with the same density (Figure 3).

Discussion

Ribosome assembly is a complex process involving both rRNA folding and association of the r-proteins with the rRNA. Formation of the functional structure of the rRNA should proceed in two steps: co-transcriptional folding of the rRNA precursors and conformational rearrangements of the rRNA which are dependent on the r-proteins. *In vitro* reconstitution studies reflect only the latter since the rRNA extracted from mature ribosomes is already folded, as is evident from the severe effect of RNA denaturation upon active 50 S subunit formation by reconstitution (Green & Noller, 1996). In contrast, the formation of the ribosomal subunits *in vivo* is sensitive to effects both in co-transcriptional folding and in ribosomal protein directed events. In paper found that both end regions of 23 S rRNA gene are important for stable 50 S particle formation (Liiv *et al*., 1996). In this work we have analyzed the importance of both 5′ and 3′ ends of 23 S rRNA and the possible role of the processing stem during ribosomal 50 S subunit formation. The results obtained with the deletion mutants in the helix 1 and in the processing stem clearly indicate that the nucleotides in the helix 1 are critical for ribosomal large subunit formation (Table 1). However, the deletion experiments do not show whether the primary sequence or the secondary structure is the main requirement for 50 S formation. To answer this question, we have constructed two substitution mutations in the helix 1 by replacing three nucleotides in the 5′ side of the helix with the corresponding nucleotides in the 3′ side and *vice versa* (mutants V8 and K10, respectively). Combination of these mutations leads to the restoration of the helix 1 with the altered sequence (mutant VK810). Both single-side mutants were found to be inactive (Table 1). In contrast, a double-side mutant was able to form functional subunits at a level comparable to the wild-type 23 S rRNA gene (Table 1). Consequently, helix 1 is essential for the 50 S subunit formation as a secondary structure element. Thus, we can conclude that the deleterious effects of mutations in the both end regions of the 23 S rRNA gene on the ribosome assembly (Liiv *et al*., 1996) can be attributed to disruption of the helix 1.

In variance with the results obtained in the wild-type strain XL1-Blue, the substitution mutations in the helix 1 (K10 and V8) showed only a small effect upon the 50 S subunit assembly as determined both by sequencing and cell-free translation in the RNase III-deficient strain CAG1632 (Table 1). Evidently, the stable processing stem present in the mature 23 S rRNA derived from the rnc^- strain compensates for the instability of the helix 1. In addition, efficient translation of the ribosomes mutant in the helix 1 of 23 S rRNA clearly shows that this stem is not functionally important during translation. Thus, helix 1 of 23 S rRNA is mainly important in ribosome assembly and is not essential for ribosomal functions.

23 S rRNA genes containing deletions in the processing stem were found to code for 23 S rRNA which is incorporated into functional ribosomes, albeit in reduced amounts. The resulting ribosomes were active during *in vitro* translation and the plasmid 23 S rRNA showed up in the ribosomes by the corresponding amount. Consequently, the processing stem of 23 S rRNA is a stimulating element in ribosomal large-subunit assembly.

23 S rRNA mutant in the helix 1 is rapidly degraded as is evident from sucrose gradient analysis (Figure 2). Degradation can occur during assembly and in the level of mature 50 S subunits. The 23 S rRNA variants (V8 and K10) defective in the helix 1 are stable in the RNase III negative strain suggesting that in the presence of the processing stem the intact helix 1 is not essential for the stability of 50 S subunits. In the wild-type strain XL1-Blue, the 23 S rRNA encoded by helix 1 mutant genes (V5, V8 and K10) showed up in the 50 S, 70 S and in the polysomal fractions by the same degree, around 10% of total 23 S rRNA *versus* 35% with the wild-type 1067U and double mutant 23 S rRNA (VK810, see Figure 2). Thus, once assembled, the mutant ribosomes are stable enough to carry out normal translation. Increased level of precursor particles with these mutants indicates an assembly deficiency of the 23 S rRNA defective in helix 1. Notably, the RNA sequencing results are in general agreement with the functional activity of the ribosomes, i.e. the specific activity of the plasmid borne ribosomes is in most cases equal. However, exceptions to this rule are the ribosomes containing substitutions or deletions in the helix 1 of 23 S rRNA (K2, K9, K10, K11, V8 and V9). The amount of the mutant 23 S rRNA in the ribosomes was significantly higher as compared to the functional activity shown by thiostrepton resistance indicating the accumulation of misassembled ribosomal subunits (compare thiostrepton resistance with the fraction of plasmid 23 S rRNA). These results can be readily explained by the important role of the helix 1 during 23 S rRNA folding. Consequently, formation of the helix 1 of 23 S rRNA is an important event during 50 S assembly. It is most likely that the formation of the helix 1 is a key event in co-transcriptional folding of the 23 S rRNA. However, degradation of the mature 50 S subunits defective in helix 1 cannot be ruled out.

Initial cleavage by RNase III is indispensable for the maturation of 23 S rRNA; maturation fails completely in the absence of the prior RNase III cleavage (King *et al.*, 1984). Therefore, the 23 S rRNA species which are not cleaved by RNase III have extended sequences in both ends (Figure 3). On the other hand, RNase III processing can be used as an indicator of the presence of the processing stem in the pre-23 S rRNA. Deletions in the processing stem abolish RNase III directed processing event (V7, K6 in Figure 3). Interestingly, mutations in the helix 1 exhibited similar effect (V5, V8, V9, K10, K11 and VK911 in Figure 3). The helix 1 mutants were processed by RNase III when transcribed *in vitro* (Liiv *et al.*, unpublished data). Consequently, helix 1 by itself is not essential for the RNase III cleavage but seems to be obligatory for the formation of the processing stem. This observation reinforces an importance of the helix 1 during co-transcriptional folding of the precursor 23 S rRNA.

The helix 1 is conserved element in eubacterial and most archeal 23 S rRNA like molecules (Garrett *et al.*, 1984). However, a regular helix cannot form in two archeal 23 S rRNAs. Notably, in both organisms lacking helix 1 of 23 S rRNA, long and stable processing stems are present (Leffers *et al.*, 1987). Thus, stable base-pairing between the both ends of precursor 23 S rRNA appears to be a universal requirement for the 23 S rRNA folding and ribosomal large-subunit assembly.

Materials and Methods

Strains and plasmids

The host strain for plasmids and phages was *E. coli* strain XL1-Blue (*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac$^-$ F'[proAB$^+$ lacI^q lacZΔM15 Tn10(tet^r)*). CAG1632 (*F$^-$ his bio RNase I$^-$ RNase III$^-$ relA recA56 srl::Tn10*) is an RNase III-deficient strain derived from BL107, kindly provided by Dr A. Dahlberg. Plasmid ptBsB1067 T (Saarma & Remme, 1992; Lewicki *et al.*, 1993) containing the *Bst*EII-*Bam*HI fragment of the *rrnB* operon (tRNA^{Glu2}-23 S rRNA and the 5 S rRNA genes) under the control of the inducible *tac* promoter was used to construct the mutations. A single point mutation in position 1067 A to T confers the thiostrepton resistance of plasmid borne ribosomes. *E. coli* strain CJ236 (dut1, ung1, thi-1, relA1) was used for the preparation of uracil containing single-stranded DNA template for site directed mutagenesis, and strain XL1-Blue was used for propagating the M13 derivates. Plasmid pHSI (a kind gift from Dr M. O'Connor) encoding the *lacI* repressor and a chloramphenicol resistance marker was used for generating the IPTG inducible system in strain CAG1632 (further CAG1632/pHSI).

Construction of mutants

The mutations K1, K2, K6, K10, K11, V3, V4, V5, V7, V8, V9, and V10 were constructed by oligonucleotide directed mutagenesis (Kunkel, 1985) using the *XbaI*-*Sal*I (5' part of 23 S rRNA) or *Sal*I-*Bam*H I (3' part of 23 S rRNA) fragments of *rrnB* ribosomal rRNA operon cloned into the M13mp18/19. Following the mutagenesis, the *Xba*I-*Sac*I or *Eco*52I-*Bam*HI fragments were cloned into the expression vector ptBsB1067 T. Mutations K4, K5, K7, K8 and K9 were introduced by one step PCR mutagenesis using the M13mp19*Sal* I-*Bam*HI as a template, 5' mutagenic primers which contained the *Xmn*I restriction site in its 5' end, and the M13mp19 reverse primer. PCR generated mutagenic product was further digested with the *Eco*52I and *Xmn*I and cloned into the ptBsB1067 T expression vector. The *Xba*I-*Eco*52I fragments of the mutants V3, V5, V8 and V9 were further cloned accordingly into the mutants K8, K2, K10 and K11, generating the double mutants VK38, VK52, VK810 and VK911.

All mutations were verified by DNA sequencing of the M13mp19/18 inserts and the reconstructed expression vectors. The DNA manipulations followed standard procedures (Sambrook *et al.*, 1989) and competent cells for plasmid transfection were prepared according to the SEM protocol (Inoue *et al.*, 1990). Restriction and other DNA enzymes were from New England Biolabs, Boehringer Mannheim, Pharmacia and Fermentas.

Preparation of crude ribosomes

E. coli strains XL1-Blue and CAG1632/pHSI transformed with the plasmids were grown at 37°C in 2 × YT medium (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) supplemented with ampicillin (100 μg/ml), tetracycline (10 μg/ml) and chloramphenicol (30 μg/ml, in the case of CAG1632/pHSI). Ribosomes were isolated from cells after induction with IPTG (1 mM) at $A_{600} = 0.12$ to 0.2 for two hours. Bacteria were collected by low-speed centrifugation and resuspended in buffer LP (16% sucrose (w/v) in 6 mM $MgCl_2$, 60 mM NH_4Cl, 60 mM KCl, 50 mM Tris-HCl pH 8.0, 6 mM β-mercaptoethanol). After addition of lysozyme (0.5 mg/ml final concentration) the suspension was lyzed by freezing and thawing three times. S-30 lysate was prepared by centrifugation at 12,000 *g* for 30 minutes in an SS34 rotor (Sorvall). The volume of the lysate was enlarged fourfold with the buffer LLP (12 mM $MgCl_2$, 60 mM NH_4Cl, 60 mM KCl, 20 mM Tris-HCl pH 8.0, 6 mM β-mercaptoethanol), loaded onto a 5 ml sucrose cushion (20% sucrose in 12 mM $MgCl_2$, 500 mM NH_4Cl, 50 mM Tris-HCl pH 8.0, 6 mM β-mercaptoethanol) followed by the centrifugation for $\omega^2 t = 5.0 \times 10^{11}$ using a Beckman SW-41 rotor. The crude ribosomes were redissolved in buffer LLP and stored in aliquots at −100°C.

Cell-free protein synthesis

Poly(U) translation was performed essentially as described in Saarma & Remme (1992). Thiostrepton (Calbiochem) was dissolved in dimethylsulfoxide (DMSO) at concentration 1 mM and used to inhibit wild-type ribosomes. A 0.5 A_{260} units of ribosomes were preincubated at 37°C for ten minutes in the presence or absence of 7.5 μM thiostrepton and 0.02 mg poly(U) in 50 μl buffer LLP followed by the addition of 50 μl of factor mix containing 0.02 mg bulk tRNA (Boehringer Mannheim), 2 mM ATP, 0.5 mM GTP, 8 mM phosphoenolpyruvate (PEP), 2 μM pyruvate kinase, 0.01 mM [^{14}C]Phe (40 cpm/pmol, UVVVR, Czechoslovakia) and 0.2 mg S-100 enzyme. After 30 minutes incubation at 37°C, reactions were stopped by addition of 1 ml 5% (w/v) trichloroacetic acid (TCA), heated for 20 minutes at 95°C. Precipitates were collected onto GF/A filters (Whatman) and counted for radioactivity in the Rackbeta 1219 scintillation spectrometer (LKB). The thiostrepton resistance of the ribosomes was calculated by dividing TCA insoluble radioactivity obtained in the presence of the drug to that obtained in the absence of thiostrepton. The error in poly(U) assay was ±10% of the radioactivity level.

Sucrose gradient analysis and quantification of plasmid borne 23 S rRNA

For preparation of 70 S and 50 S ribosomes the cell lysates were diluted two times with LLP buffer and loaded onto a 15% to 40% sucrose gradient in LLP buffer and centrifuged for $\omega^2 t = 3.5 \times 10^{11}$ in a Beckman SW-28 rotor. Polysomal, 70 S, 50 S, 40 S and 30 S gradient fractions were collected and precipitated with 2.5 volumes of ice-cold ethanol. To prepare rRNA, ribosomes were extracted once with phenol (pH 6.5) containing 1% SDS, twice with phenol (pH 6.5), and precipitated with ethanol. After washing the precipitate by 70% ethanol (v/v), RNA was dissolved in water. The relative amount of plasmid-encoded 23 S rRNA was determined by the primer extension method (Sigmund *et al.*, 1988) using the 1067 T as a marker of plasmid borne 23 S rRNA. The 5′-[^{32}P]-end-labeled primer was complementary to bases 1069 to 1087 of 23 S rRNA. In the annealing step 2 to 4 pmol of RNA was annealed with the 0.5 pmol of 5′-[^{32}P]-end-labeled primer in 100 mM KCl, 20 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.6) in final volume of 6.5 μl for two minutes at 65°C, followed by quick cooling on ice. A total of 2.5 μl of the annealing mixture, individual dATP/dGTP/dCTP/ddTTP (T mix) or dTTP/dGTP/dCTP/ddATP (A mix; each final concentration 50 μM), 1 U of AMV reverse transcriptase (avian myelobastosis virus reverse transcriptase; Boehringer Mannheim) in a RT buffer (30 mM KCl, 8 mM $MgCl_2$, 1 mM DTT, 50 mM Tris-HCl pH 8.5) in final volume of 10 μl was incubated for 30 minutes at 42°C. Reactions were stopped by adding 7 μl of sample buffer (98% (v/v) formamide, 2 mM EDTA, 0.1% (w/v) of bromophenol blue and xylene cyanol), heated to 95°C for two minutes and loaded onto 20% polyacrylamide-8 M urea-1 × TBE gel. Autoradiograms were digitalized using PhosphoImager (Molecular Dynamics) and the A and U lanes of each sample were scanned and quantified using the ImageQuant software (Molecular Dynamics). The error of quantification of a distinct band with respect to a neighboring band is ±10%.

Primer extension analysis

The 5′ ends of RNA were determined by a primer extension reaction using AMV reverse transcriptase (Boerhinger Mannheim) according to (Sambrook *et al.*, 1989). The labeling reaction (in final volume10 μl) contained 1 to 2 pmol of 23 S RNA, oligo-DNA primer complementary to the 23 S RNA (positions 3530 to 3547 of *rrnB* (20)), and [α-^{32}P]dCTP in RT buffer was incubated 15 minutes at 42°C. For primer extension the chase solution (120 mM dNTP in final concentration and 1unit AMV reverse transcriptase) was added and the reaction was continued for 30 minutes at 42°C. Reaction products were separated on 12% urea-PAAG-TBE.

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