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Analysis of the Ribosome Large Subunit Assembly and 23 S rRNA Stability *in Vivo*

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The ability of mutant 23 S ribosomal RNA to form particles with proteins of the large ribosomal subunit *in vivo* was studied. A series of overlapping deletions covering the entire 23 S rRNA, were constructed in the plasmid copy of an *E. coli* 23 S rRNA gene. The mutant genes were expressed *in vivo* using an inducible *tac* promoter. Mutant species of 23 S rRNA, containing deletions between positions 40 and 2773, were incorporated into stable ribonucleoprotein particles. In contrast, if one end of the 23 S rRNA was deleted, the mutant rRNA was unstable and did not form ribosomal particles. Protein composition of the mutant particles was specific; the presence of the primary rRNA-binding proteins corresponded to their known binding sites. Furthermore, several previously unknown ribosomal protein binding sites in 23 S rRNA were identified. Implications of the results on ribosome assembly are discussed.

Keywords: ribosome; 23S rRNA; L-proteins; ribosome assembly; ribosomal protein binding sites

Introduction

Ribosome formation in *Escherichia coli* involves coordinated synthesis of three ribosomal RNAs and 54 ribosomal proteins, processing and modification of the rRNAs, and assembly of both rRNA and r-proteins into functional subunits. Processing and modification of the rRNA starts during its transcription and is fully completed in the ribosome. In the cell, assembly of the ribosomes is coupled to rRNA synthesis (Cowgill de Narvaez & Schaup, 1979) and takes one to two minutes (Lindahl, 1975), time comparable to the time used by RNA polymerase to transcribe rRNA genes. It was shown that both the small and the large subunit of the *E. coli* ribosome can be reconstituted into functionally active particles using isolated rRNA and r-proteins (Traub & Nomura, 1968; Nierhaus & Dohme, 1974). The reconstitution technique has led to the establishment of assembly maps depicting the order of association of each protein with the rRNA. Assembly maps have been defined for both ribosomal subunits (Held *et al*., 1974; Herold & Nierhaus, 1987). During reconstitution of the 50 S subunit, three intermediate particles were found. These particles exhibited similar protein composition as compared to ribosome precursor particles formed *in vivo* (for a review see Nierhaus, 1991). Based on the reconstitution data, r-proteins that can individually bind to the rRNA and are essential for the formation of an active ribosomal subunit were defined as assembly initiator proteins (Nowotny & Nierhaus, 1982). 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).

It is evident that the formation of ribosomes in living cells is considerably different compared with *in vitro* reconstitution. First, *in vivo* rRNA precursors are assembled rather than mature, fully modified rRNA molecules used *in vitro*. Secondly, the assembly process *in vivo* is coupled to the synthesis of rRNA and is thus dependent on rRNA polymerase activity (Lewicki *et al*., 1993). Thirdly, ribosome biogenesis occurs in minutes at 37°C, while the reconstitution of 50 S subunits requires an incubation period of 1.5 hours at 50°C under non-physiological ionic conditions. In addition, there are several reports in the literature describing non-ribosomal proteins that are involved in the cellular assembly of both ribosomal subunits. Among these proteins are the ''DEAD'' box RNA

Abbreviations used: ITS, intergenic spacer sequence; r-proteins, ribosomal proteins; rRNA, ribosomal RNA.

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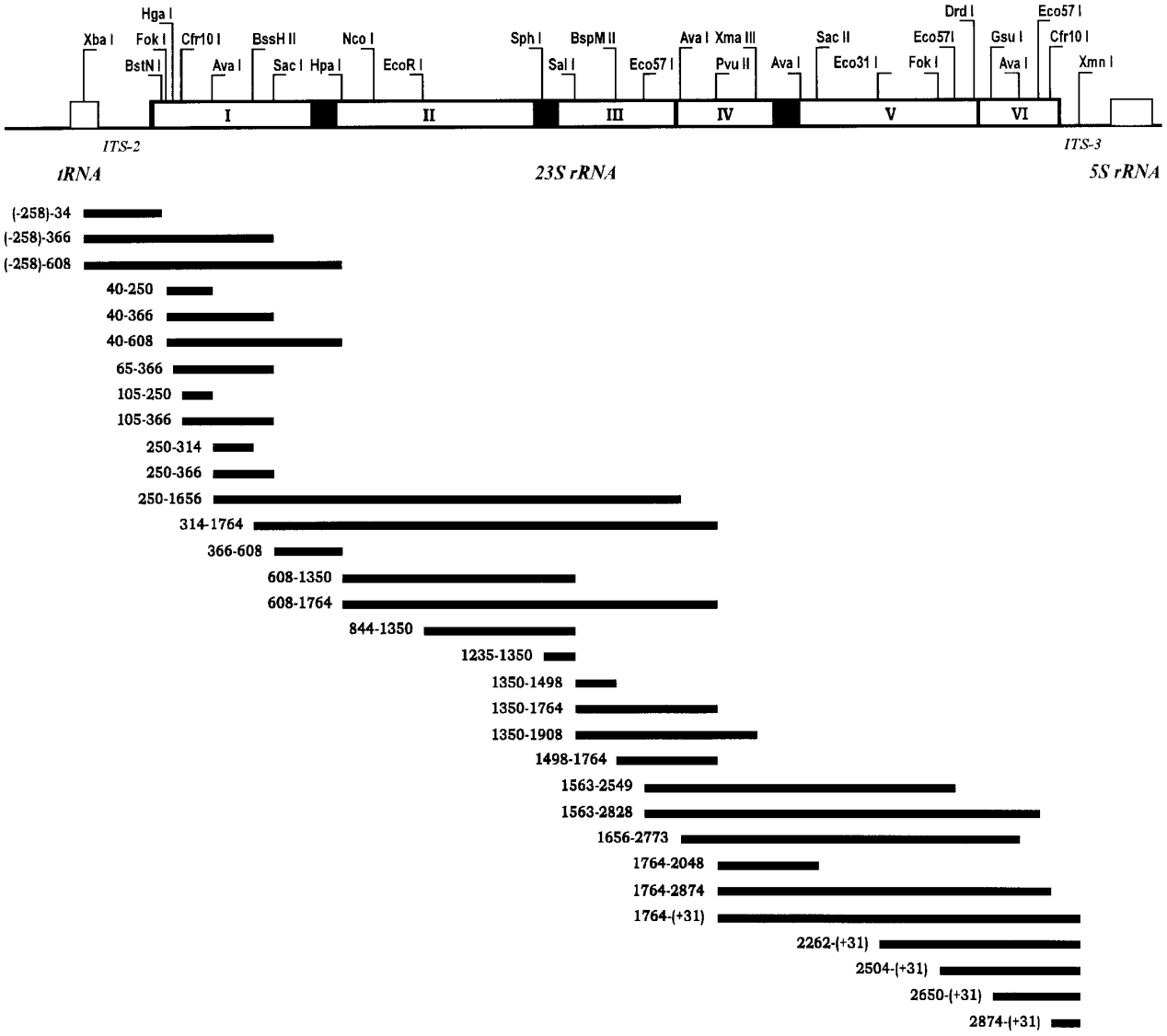

Figure 1. Deletion mutants of 23 S rRNA. The transcription unit under control of the *tac* promoter is shown above and restriction sites used to construct the deletions are indicated. Secondary structure domains of 23 S rRNA are marked by roman numerals. Deletions are shown as black bars. Numbering is according to the mature 23 S rRNA; the first and the last nucleotide deleted in the 23 S rRNA gene are used to identify the mutant. Deletions extending into the upstream spacer are denoted by negative numbers, i.e. −1 indicates the first nucleotide upstream from the mature 23 S rRNA. Deletions extending into the downstream spacer are indicated by positive numbers.

helicases SrmB, DeaD and DbpA (Nishi *et al*., 1988; Toone *et al*., 1991; Fuller-Pace *et al*., 1993; Nicol & Fuller-Pace, 1995). Likewise, the chaperonin DnaK was shown to affect ribosome assembly (Alix & Guerin, 1993). The mechanisms of action of these proteins are not known, but their involvement already indicates a further difference between cellular ribosome assembly and cell-free reconstitution.

Another important aspect of ribosome assembly is the formation of the functional structure of the rRNA. The rRNA precursor folds co-transcriptionally starting from the 5' end. Therefore, it is reasonable to assume that the sequence elements which initiate 50 S subunit assembly and determine its stability reside in the spacer separating tRNA and 23 S RNA (ITS-2, see Figure 1) and in the 5' end region of the mature 23 S rRNA. Accordingly, deletions in ITS-2 abolish 50 S subunit formation (Stark *et al*., 1985; Szymkowiak & Wagner, 1987), and deletions in domain I of 23 S rRNA affect protein L24 binding and 50 S subunit assembly (Skinner, *et al*., 1985). Mutant rRNA with deletions in other parts of the mature 23 S rRNA were shown to be processed and assembled into 50 S subunits (Stark *et al*., 1982). Although several deletion mutants were analysed, the importance of different parts of the 23 S rRNA precursor for ribosome assembly is largely unknown.

We have used systematic deletion analysis of the

23 S rRNA gene to study the importance of different RNA regions in ribosomal particle formation.

Results

Accumulation of particles containing mutant 23 S 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 had been shown to code a functional 23 S rRNA which is incorporated into active ribosomes (Saarma & Remme, 1992; Lewicki *et al*., 1993). A single point mutation A1067T was present in the 23 S rRNA gene for identification of the plasmid-derived rRNA. Overlapping deletions covering the entire 23 S rRNA were introduced into the 23 S rRNA gene by using various restriction enzymes (Figure 1). Transcription of plasmid-encoded 23 S rRNA was induced at the early exponential growth phase, when bacterial ribosome biogenesis is highly active (Gausing, 1977). Induction of plasmid-borne genes had very little effect on bacterial growth with two exceptions; the deletion mutants 250 to 1656 and 314 to 1764 reduced the growth rate significantly (data not shown).

Expression of the mutant 23 S rRNA was first analysed by sucrose gradient centrifugation. Preliminary experiments indicated that the mutated rRNA accumulate during the exponential growth phase. Maximal amounts of mutant rRNA were observed two to three hours after induction (data not shown). Three to five independent experiments were run for each mutant. Only small differences in the area of the mutant peak were found between the gradient patterns of parallel experiments. In Figure 2, representative sucrose gradient patterns are compiled. Nearly all mutants generated a new peak on the gradient with sedimentation coefficients between 20 S and 50 S (filled arrows in Figure 2). The gradient pattern of cells carrying wild-type ptBsB1067T was identical to that of minus plasmid control (not shown).

To test whether the new peak contained mutant rRNA, the rRNA was sequenced using a primer directed against the 1067 region of 23 S rRNA. Sequencing results confirmed the presence of plasmid-borne rRNA in the mutant particles (Table 1). Note that the wild-type ptBsB1067T encoded 23 S rRNA constitutes approximately 40% of the total 23 S rRNA pool, as measured by sequencing of the position 1067 from the total cellular 23 S rRNA (not shown). Plasmid-derived wild-type 23 S rRNA was found to the same extent in the 70 S ribosomes and in the 50 S subunits (Table 1). Deletion mutant 23 S rRNA species were found exclusively in smaller particles. All mutant rRNAs were associated with proteins forming ribonucleoprotein (RNP) complexes (see below).

When the 5' end region of the 23 S rRNA was deleted, together with the sequence upstream of the 23 S rRNA structural gene, neither abnormal peaks could be detected in the sucrose gradients nor was mutant rRNA found by sequencing analysis. The gradient patterns of the corresponding deletion mutants (–258) to 34, (–258) to 366 and (–258) to 608 were identical to the wild-type. When the 23 S rRNA contained short deletions at the 3' end region, as in the mutants 2650 to (+31) and 2874 to (+31), the mutant rRNA was found in fractions sedimenting at 20 S to 30 S; however, these mutant rRNAs were not of sufficient quantities to be detected optically. All the other mutants formed particles which could be traced in the sucrose-gradient profiles (Figure 2), and were confirmed by sequencing when the 1067 region was present (Table 1). With a single exception, all mutant rRNAs were found in a single particle on the sucrose gradient; only the deletion mutant 250 to 1656 showed two new peaks sedimenting at 20 S and 40 S, respectively. Since the 1067 region was deleted in this mutant, we did not determine which of them contained plasmid-encoded rRNA. Protein analysis suggested that the 20 S particles were derived from the plasmid and that the 40 S particles were a product of assembly deficient wild-type 23 S rRNA (see below). Notably, the same mutation exhibited a deleterious effect on cell growth (data not shown). Mutant 23 S rRNAs, with small deletions in domain I, were migrating as 30 S to 40 S particles and the corresponding peak areas were small. In contrast, mutants with more extensive deletions in the central region (608 to 1350, 844 to 1350, and 1350 to 1908) produced faster sedimenting particles in larger amounts, i.e. the size of the deletion did not correlate with the size of the mutant particle.

Accumulation of the mutant 23 S rRNA

The accumulation of mutant 23 S rRNA was analysed using *in vivo* labeling, followed by hybridization of rRNA to single-stranded DNA probes. Two probes, complementary to 23 S rRNA, were used in order to analyse the mutants which lack one of the probed regions. Since the plasmids used throughout this study did not contain a 16 S rRNA gene, we have used a 16 S rRNA probe complementary for chromosomal rRNA.

In Table 2, the mutants are arranged in order of decreasing accumulation. The accumulation of plasmid-encoded 23 S rRNA was assayed in both induced and uninduced states. Induction led to a 1.4-fold overproduction of 23 S rRNA with respect to 16 S rRNA. In general, the extent of hybridization correlated with the size of the peak detected on the sucrose gradient profile. The hybridization method used reflects the ratio between 23 S rRNA transcription and degradation. Therefore, the gradient profiles and hybridization data are not quantitatively comparable. The most probable reason for differences in accumulation is differences in stability of the mutant rRNA transcripts.

The mutants were divided into three groups

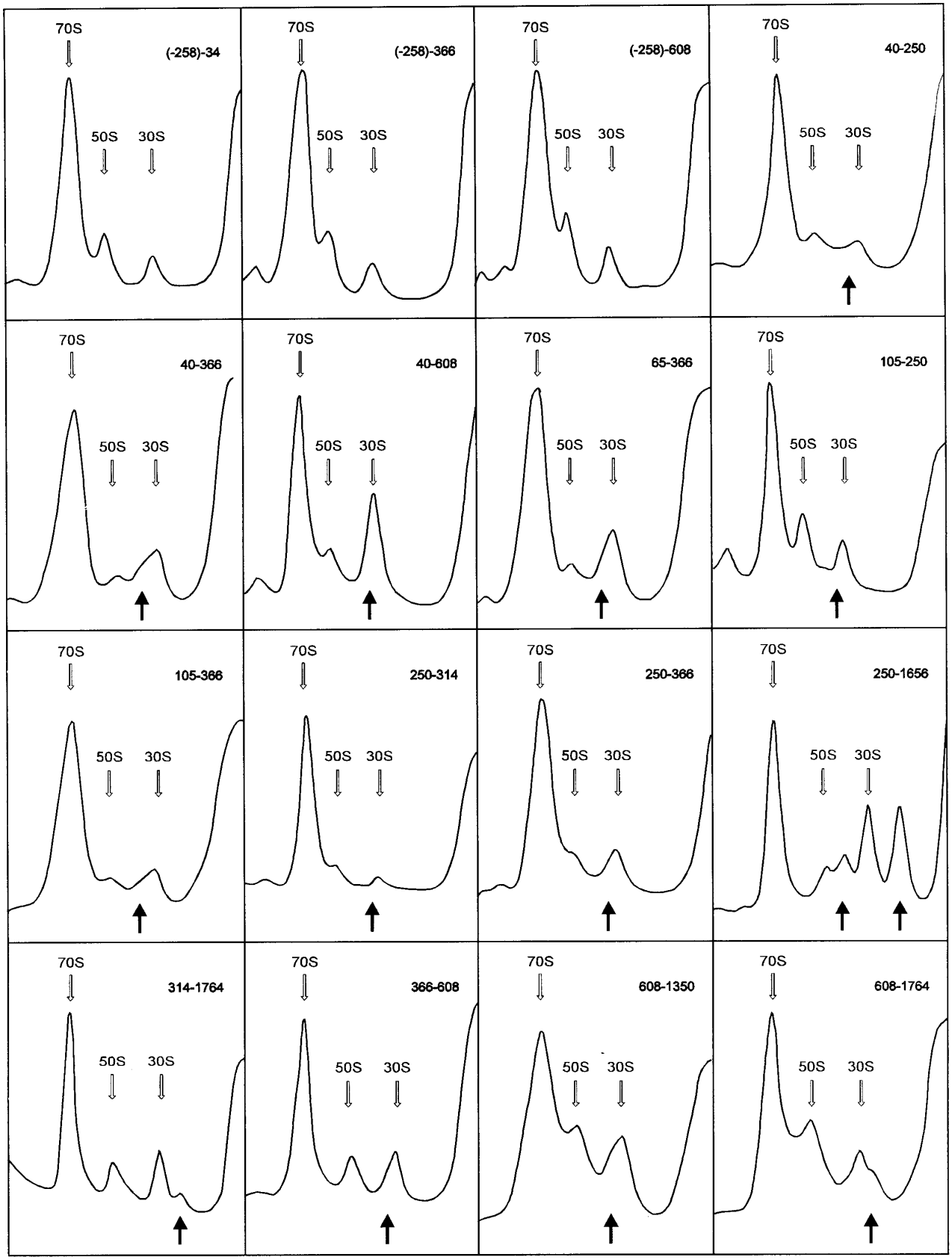

Figure 2A *(legend on page 400)*

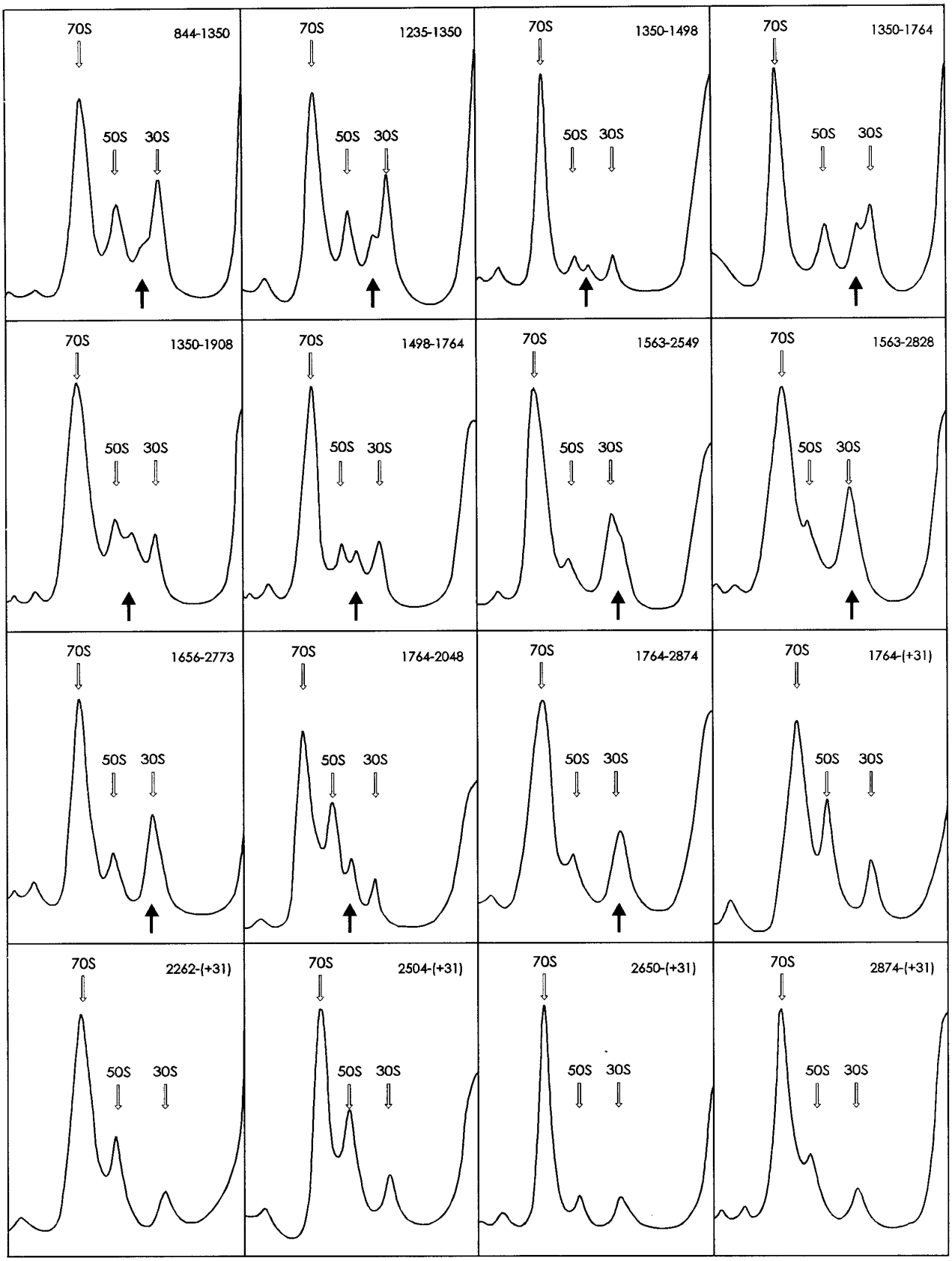

Figure 2B

Figure 2. Identification of the mutant 23 S rRNA by sucrose gradient centrifugation. Cells were lysed after induction and the S30 extract was fractionated on a 10 to 30% sucrose gradient by centrifugation. Absorption was monitored at 260 nm. Mutations are marked by the numbers of the deleted nucleotides (see legend to Figure 1). Positions of 30 S, 50 S, and 70 S ribosomes are indicated by open arrowheads. Plasmid-derived mutant particles are shown by filled arrows.

Table 1. Identification of mutant 23 S rRNA

Deletion	70 S	50 S to 60 S	35 S to 45 S	30 S to 35 S	20 S to 30 S
1067U	+ +	+ +	+	+	–
(–258) to 34	–	–	+	+	+
(–258) to 366	–	–	+	+	+
–(258) to 608	–	–	+	+	+
40 to 250	–	–	+	+ + +	+ + +
40 to 366	–	–	+	+ + +	+ + +
40 to 608	–	–	+	+ + +	+ + +
65 to 366	–	–	+	+ + +	+ + +
105 to 250	–	–	+	+ + +	+ + +
105 to 366	–	–	+	+ + +	+ + +
250 to 314	–	–	+	+ + +	+ + +
250 to 366	–	–	+	+ + +	+ + +
366 to 608	–	–	+	+ + +	+ + +
1235 to 1350	–	+	+ + +	+	+
1350 to 1498	–	+	+ + +	+	+
1350 to 1764	–	+	+ + +	+	+
1350 to 1908	–	+	+ + +	+	+
1498 to 1764	–	+	+ + +	+	+
1656 to 2773	–	–	+	+ + +	+
1764 to 2048	–	+	+ + +	+	+
1764 to 2874	–	–	+	+ + +	+
1764 to (+31)	–	–	+	+	+
2262 to (+31)	–	–	+	+	+
2504 to (+31)	–	–	+	+	+
2650 to (+31)	–	–	+	+ + +	+ + +
2874 to (+31)	–	+	+	+ + +	+ + +

Cell lysates were fractionated by sucrose gradient centrifugation and the rRNA was analysed by sequencing the region containing position 1067 of 23 S rRNA. Data in line 1067U correspond to the full-length 23 S rRNA. The amounts of mutant rRNA are indicated as major (over 50%, + + +), intermediate (10 to 50%, + +) or small (<10%, +) in comparison to the total amount of 23 S rRNA.

according to the accumulation of the corresponding rRNA transcripts. The first class comprises stable mutants which accumulated 23 S rRNA 3.5 to 6.5 times in excess over 16 S rRNA. The deletions varied in size from 396 nucleotides to 1406 nucleotides and were located between positions 40 and 2873 of 23 S rRNA, thus covering almost the entire molecule. A common feature of the stable mutants is the presence of both 5' and 3' end regions.

The second and most numerous group contains the mutants of moderate stability. 23 S rRNA accumulated 1.2 to 2.5 times over 16 S rRNA. The complete 23 S rRNA is also a member of the moderate group if expressed in the system used. The mutations in this group have deletions 115 to 506 nucleotides in length, making the average length of deletions considerably shorter than that of the first group. Common features of both groups are the presence of 5' and 3' end regions as well as the intergenic spacer sequence (ITS-2) with the exception of the (– 258) to 34 deletion. The deleted regions were located in domains I to V of 23 S rRNA; the mutant rRNAs with deletions in domain I tended to be less stable than those with deletions in domains II to V.

The last group consists of mutants which did not show a significant accumulation of rRNA within the limits of experimental error (12%). Uninduced plasmids fall into the same group. Five out of six members of the group have a deletion in either the 5' or 3' end of 23 S rRNA. This strikingly demonstrates the importance of both end regions for stabilizing the 23 S rRNA. The low stability of 23 S rRNA with deletion 250 to 314 is in line with the labile nature of 23 rRNAs with deletions in domain I.

The intergenic spacer of *rrn* operons between the tRNA and 23 S rRNA genes (ITS-2) contains boxB to boxA sequences which were suggested to be involved in an antitermination event of transcription (Berg *et al*., 1989). Deletion of this structure may have influenced rRNA production in our experiments. Therefore, the rate of 23 S rRNA synthesis was measured for mutants with deletions in the upstream spacer. Induced cells were labeled for one minute with [^{3}H]uridine and the total rRNA was hybridized to single-stranded DNA probes specific for 16 S and 23 S rRNA (Table 3). Transcription of the 23 S rRNA genes was 6 to 15 times higher than that of the 16 S rRNA genes in induced cells. In contrast, transcription rates were the same for both rRNAs in uninduced cells. Hybridization to the 3' probe was reproducibly reduced by about 20% indicating that the processivity of transcription was slightly reduced.

Ribosomal proteins in the mutant particles

Sedimentation behavior of mutant 23 S rRNA indicated that the rRNA was complexed with proteins. We have isolated the mutant particles and

Table 2. Accumulation of mutant rRNA

	23 S rRNA probes		
Mutant	3861 to 4343	5701 to 6272	Average
40 to 608	1.7[a]	6.4	6.4
1764 to 2874	5.3	1.5[a]	5.3
1350 to 1764	4.4	5.9	5.1
250 to 1656	1.7[a]	4.3	4.3
1656 to 2773	3.6	0.5[a]	3.6
608 to 1764	0.7[a]	3.5	3.5
1764 to 2048	2.2	2.8	2.5
366 to 608	0.7[a]	2.5	2.5
1498 to 1764	1.6	2.1	1.8
1235 to 1350	1.74	1.9	1.6
(–258) to 34	1.3	1.9	1.6
65 to 366	1.4	1.8	1.6
1350 to 1498	1.7	1.5	1.6
844 to 1350	1.7	1.4	1.5
250 to 366	1.4	1.6	1.5
40 to 250	1.3	1.6	1.4
wt + IPTG	1.4	1.4	1.3
40 to 366	1.1	1.4	1.2
105 to 250	1.5	1.0	1.1
1764 to (+31)	1.1	0.4[a]	1.1
wt – IPTG	1.1	1.1	1.0
2650 to (+31)	0.7	1.2	1.0
(–258) to 608	0.4[a]	1.0	1.0
2874 to (+31)	0.8	1.1	0.9
250 to 314	0.8	1.0	0.9
2504 to (+31)	0.6	1.2	0.8

After inducing plasmid-encoded 23 S rRNA synthesis, transcribed rRNA was labeled with [^{3}H]uridine for 3.5 hours and the total RNA hybridized to ssDNA probes specific for two regions in 23 S rRNA and one region in 16 S rRNA (positions 1599 to 2130). Numbering is according to Brosius *et al*., (1981). The amounts of 23 S rRNA are normalized to the level of chromosomally derived 16 S rRNA; the corresponding numbers therefore indicate the ratios of plasmid-endoded rRNA to that of chromosome-derived rRNA. Mutants are divided into three groups according to the stabilty of corresponding rRNA.

[a] The sequence complementary to the probe was either completely or partially deleted in the 23 S rRNA gene of the plasmid.

identified the ribosomal proteins by two-dimensional gel electrophoresis. Note that proteins L7/12 were lost and L16 was partially lost during isolation. Proteins L12, L31, L34, L35 and L36 were not detected and L32, L33 were not separated owing to the electrophoresis system used. Protein L2 was found in all of the mutant particles. The examples of two-dimensional protein gels are shown in Figure 3. Results of the protein analysis are presented in Table 4.

The specificity of isolated RNP complexes can be estimated using primary 23 S rRNA binding proteins with known binding sites (L1, L2, L3, L4, L10, L11, L12, L23 and L24). Two large deletions (250 to 1656 and 1656 to 2773) divide the 23 S rRNA roughly into two halves. Both mutants formed stable ribonucleoprotein particles according to gradient pattern and hybridization data (Figure 2 and Table 2). The mutant with the deletion 1656 to 2773 contained the 5' half of 23 S rRNA with domains I, II and III intact. The corresponding particle contained proteins L2, L4, L10, L11, L13, L17, L20, L21, L22, L23, L24, L28, L29 and L30 (Figure 3B). The mutant 250 to 1656 contained the 3' half of 23 S rRNA with domains IV, V and VI intact; the respective rRNA was complexed with proteins L1, (L2), L3, L5, L6, L14, L15, L17, L18, L19, L24, L25, L27, L28, L30 and L32/L33 (Figure 3A). The protein set of both particles complemented each other (see Table 4). Proteins L2, L17, L24, and L30 were present, while proteins L9 and L16 were absent, in both particles. The protein content of both particles correlated well with the *in vitro* mapped ribosomal protein binding sites in the 23 S rRNA. For example, L10, L11, L13 and L23 were found in the particle with the 5' half of 23 S rRNA; the binding sites of these proteins are located in domain II (Schmidt *et al*., 1981; Beauclerk *et al*., 1984; Egebjerg *et al*., 1990). In contrast, L1, L5, L6, L18 and L25 are related to domain V (Egebjerg *et al*., 1990, 1991) and were found on the particle containing the 3' half of 23 S rRNA. A more detailed picture is obtained when the protein contents of the various particles compiled in Table 4 are correlated with the corresponding deletions of the mutant rRNA. For many proteins, an unequivocal correlation to a region of 23 S rRNA could be established (Figure 4). When deletions fell in this region, the ribosomal protein under consideration was not found in the corresponding particles. For example, domain I is almost completely deleted in the mutant 40 to 608, and the corresponding particle lacks proteins L4, L14 to L16, L20, L28 to L33 and shows only traces of L22 and L24 (Table 4). The proteins L14 to L16 are also absent in the particle of the mutant 1563 to 2828; they therefore cannot be exclusively ascribed to domain I. The same is true for proteins L28 to L33 in the mutant 608 to 1350. Proteins L4, L20, L22 and L24 remain, they are

Table 3. Transcription rate of ITS-2 deletion mutants

	16 S rRNA probes		23 S rRNA probes	
Mutant	1599 to 2130	2192 to 2900	3861 to 4343	5701 to 6272
(–258) to 608	1.0	0.9	7.7[a]	13.7
(–258) to 366	1.0	1.1	7.1	5.9
(–258) to 34	1.0	0.8	16.7	14.0
None	1.0	1.0	1.0	1.0

Induced cells were labeled with [^{3}H]uridine for one minute, total RNA was isolated and hybridized to the ssDNA probes. Regions complementary to the probe are indicated according to Brosius *et al*. (1981). The results are normalized to the amounts of the labeled 5' region of 16 S rRNA.

[a] Part of the probe region was deleted in the plasmid.

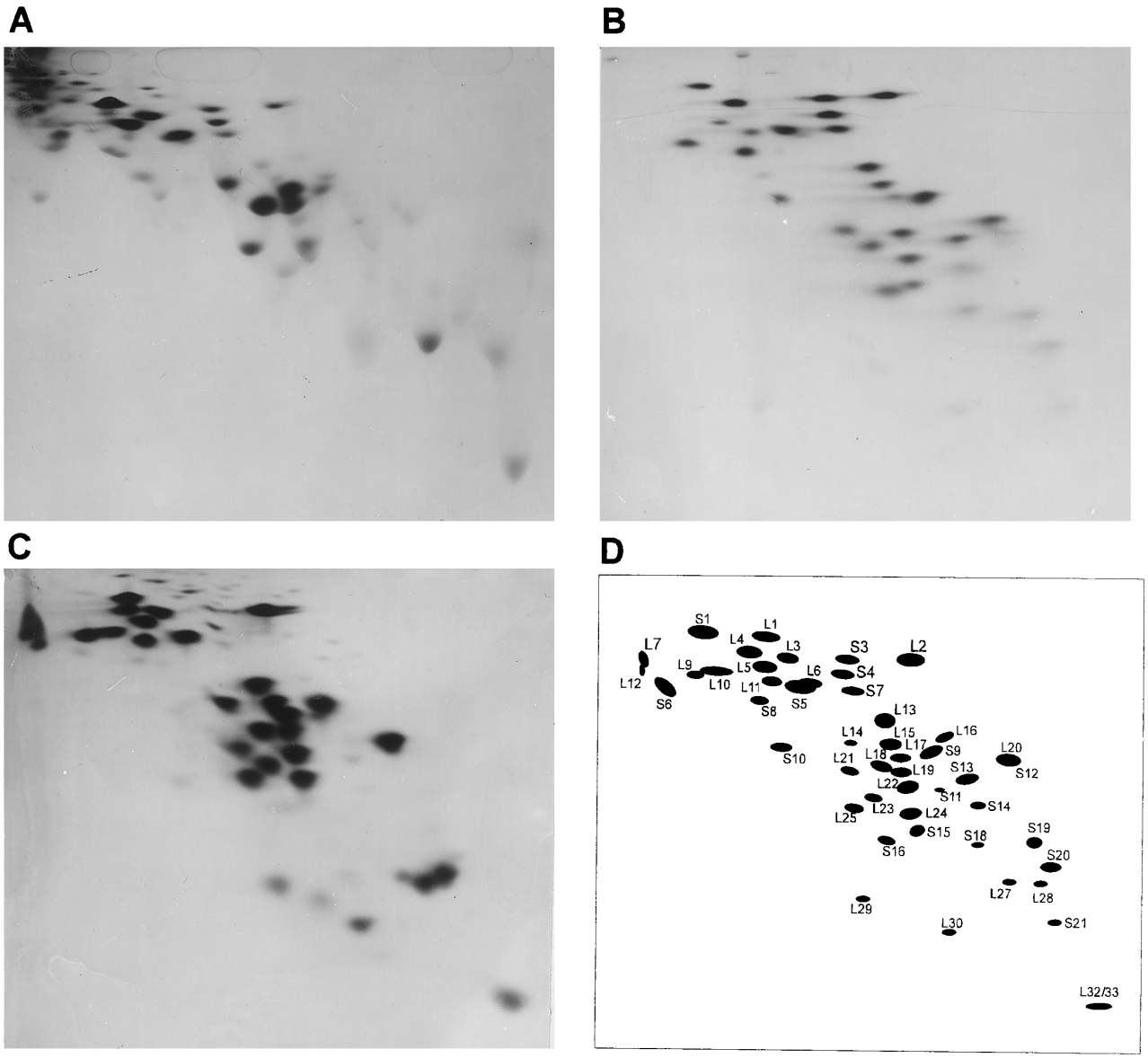

Figure 3. Identification of ribosomal large subunit proteins by two-dimensional gel-electrophoresis. Proteins were extracted from 20 S particles containing mutant rRNA with deletion 250 to 1656 (A), 30 S particles, 1656 to 2773 (B), and wild-type 50 S subunits (C). For designation of mutants see legend for Figure 1. Schematic of the two-dimensional electrophoregram of the *E. coli* 70 S ribosomal proteins (D) according to Geyl (*et al*., 1981).

therefore clearly related to domain I, being in excellent agreement with their known binding sites (Egebjerg *et al*., 1987; Leffers *et al*., 1988). Using similar reasonings, proteins L10, L11, and L13 can be attributed to domain II (mutant 608 to 1746 in Table 4) being in agreement with the known binding sites for L10 and L11 (helices 42 and 43 of domain II; Schmidt *et al*., 1981; Beauclerk *et al*., 1984; Egebjerg *et al*., 1990). However, when domain II was deleted (mutant 844 to 1350), L10 and L11 were found in the mutant particles. The most likely explanation is that proteins L10 and L11 exhibit additional binding sites to proteins and/or other regions of 23 S rRNA. Additional binding sites allow tight binding of these proteins even in the absence of their primary binding sites. The presence of L9 and L23 correlates with domain III. An rRNA to protein crosslink has been reported between L9 and helix 58 in domain III of 23 S rRNA (Osswald *et al*., 1990), and the binding site for L23 has been identified by chemical and enzymatic footprinting in this domain (Egebjerg *et al*., 1991). Another example is the mutant particles that contain domain V intact; these particles always contained proteins L1, L5, L6, L18 and L25 as expected from protection (Egebjerg *et al*., 1991) and crosslinking experiments (Osswald *et al*., 1990). Finally, association of protein L3 with the mutant particles depended on the presence of domain VI, which is in perfect agreement with footprinting analysis (Leffers *et al*., 1988) and with the fact that L3 could be crosslinked to helix 100 (Osswald *et al*., 1990).

Siehnel & Morgan (1985) reported the presence of L1, L2, L3, L17 and L22 in a particle lacking the nucleotides 608 to 1764. We isolated a particle with the same deletion and found it to contain this set of proteins in addition to some other proteins (Table 4). Possible reasons are different expression

Table 4. Identification of L-proteins in the mutant particles

LSU prot	1498-1764	844-1350	1235-1350	65-366	1350-1498	250-314	250-366	105-366	40-250	105-250	2650-(+31)
L1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
L2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
L3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
L4	+++	+++	+++	+	+++	+	-	-	+++	++	+++
L5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+
L6	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
L9	+++	++	++	++	+++	++	+	+++	-	+	++
L10	+++	+++	+++	+++	+++	+++	++	+++	+	+	++
L11	+++	+++	+++	+++	+++	+++	++	+++	++	+	++
L13	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
L14	+++	+++	+++	+++	+++	+++	++	-	-	-	-
L15	+++	++	+++	+++	+++	++	++	+	-	++	+++
L16	+	-	-	-	+	-	-	-	-	-	-
L17	+++	+++	+	+++	+++	+++	++	++	+++	+++	+
L18	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
L19	+	+	+	+++	+++	++	++	++	+++	++	-
L20	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++
L21	+++	+++	++	++	+++	+++	+++	+++	+++	-	+++
L22	+++	+++	+++	++	+++	++	++	++	+++	-	+++
L23	-	+	+	++	+	++	+++	+++	+++	+++	++
L24	+++	++	+++	++	+++	+++	++	+	+++	+++	+++
L25	+	-	+	-	+	+	++	-	-	-	-
L27	+++	-	+++	-	+++	+++	+++	+++	+++	-	++
L28	+++	-	++	-	+++	+++	+++	-	-	-	+++
L29	-	-	+	-	-	+++	-	-	-	-	+++
L30	+++	-	+++	-	+++	+++	+++	-	+++	-	++
L32/L33	+++	-	+++	-	+++	+++	+++	+++	+++	-	-

continued opposite

Table 4. *Continued*

LSU prot	40-608	1350-1764	1350-1908	250-1656	1656-2773	608-1764	1563-2549	1563-2828	1764-2048	366-608	608-1350
L1	+++	+++	+++	+++	-	+++	-	-	+++	+++	+++
L2	+++	+++	+++	+	+++	+++	+	+++	+++	+++	+++
L3	+++	+++	+++	+++	-	+++	+++	-	+++	+++	+++
L4	-	+++	+++	-	+++	+++	+++	+++	+++	+++	+++
L5	+++	+++	+++	+++	-	+++	-	-	+++	+++	+++
L6	+++	+++	+++	+++	-	+++	+++	+++	+++	+++	+++
L9	+++	+++	+++	-	-	-	-	-	+++	-	++
L10	+++	+	+++	-	+++	-	+++	+++	+++	++	++
L11	+++	+++	+++	-	+++	-	+++	+++	+++	+++	-
L13	+++	+++	+++	-	+++	-	+++	+++	+++	+++	-
L14	-	+++	+	++	+	+	+++	-	+++	+++	+++
L15	-	-	+++	+++	-	+	+++	+	+++	+	+++
L16	-	++	-	-	+	-	-	-	-	-	-
L17	+++	+++	+++	+++	+++	+++	+++	-	+++	+++	+++
L18	+++	+++	+++	+++	-	+++	-	-	+++	+++	+++
L19	++	+++	++	+++	-	+++	+++	-	+++	+++	+++
L20	-	+++	+++	-	+++	-	+++	+++	+++	+++	+++
L21	+++	+++	+++	-	+++	-	+++	+++	+++	+	-
L22	+	+++	+++	-	+++	+++	+++	+++	+++	-	+++
L23	++	+	-	-	+++	-	+	-	+++	+++	-
L24	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
L25	++	+++	++	+++	-	-	-	+	-	-	-
L27	+++	+++	+++	+++	-	+++	-	-	+++	-	-
L28	+++	+++	+++	+	+++	+	-	+++	-	+	-
L29	-	+	-	-	+++	-	-	-	+++	-	-
L30	-	+++	+++	+++	+++	+++	+	+++	+++	-	-
L32/L33	-	+++	+++	+++	-	+++	+++	-	+++	+++	-

L-proteins are proteins present in the large ribosomal subunit. Ribonucleoprotein particles were isolated and their protein composition determined by two-dimensional gel electrophoresis. The amount of a protein in a particle is indicated as a large (+ + +), intermediate (+ +), or small (+) spot. Mutants are listed according to decreasing accumulation of the corresponding rRNA.

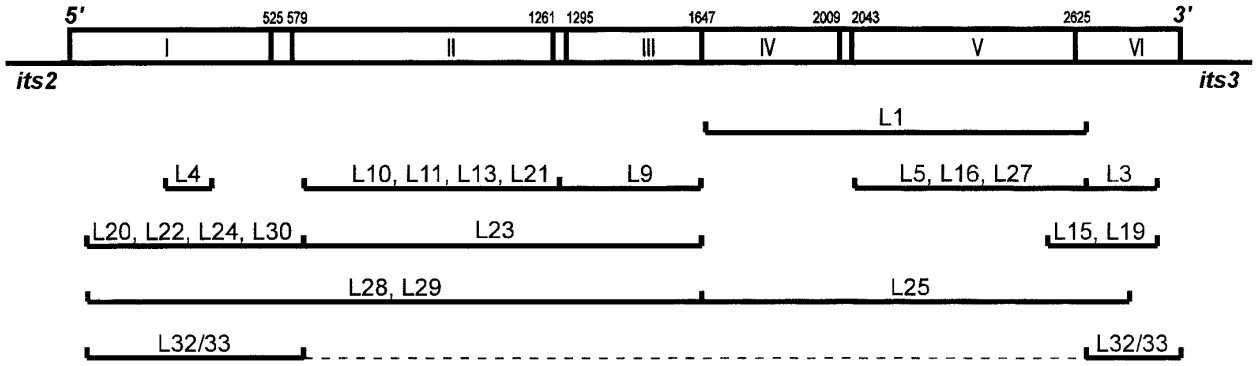

Figure 4. Correlations between the absence of ribosomal proteins in mutant particles with the corresponding deletions in their 23 S rRNA. Secondary structure domains of the 23 S RNA are indicated in the upper line.

systems used (Siehnel & Morgan used constitutive expression) and / or differences in the procedure for the isolation of the particles. Correlations between proteins L1, L3, L4, L23 and L24 and relatively short sequences of the 23 S rRNA were found. For example, the presence of L4 depended on the presence of the sequence 250 to 366 (helices 18 to 20). In contrast, L2 was found in all of the mutant particles, as mentioned already, although particles from mutants 250 to 1656 and 1563 to 2549 exhibited reduced amounts of this protein. It follows that the assembly of L2 with the ribosomal particle is independent of the presence of its binding site in domain IV (Beauclerk & Cundliffe, 1988; Egebjerg *et al*., 1991); protein to protein interactions might play an important role for the incorporation of L2 into the 50 S subunit. On the other hand, two distinct RNA cross-link sites have been recently identified in protein L2 (Urlaub *et al*., 1995). The two RNA binding sites in proteins L2 may correspond to the two separate protein recognition sites in 23 S rRNA. Protein L24 is an assembly initiator during 50 S subunit formation *in vitro* (Nowotny & Nierhaus, 1982) and is essential for ribosome assembly *in vivo* (Spillmann & Nierhaus, 1978). All isolated particles contained protein L24, in agreement with its prominent role during 50 S subunit assembly. Only one mutant (40 to 608) produced stable particles with reduced amount of L24.

The functional activity of the mutant particles was determined according to their ability to catalyze peptide bond formation. All mutant particles were inactive (data not shown).

Discussion

In wild-type *E. coli*, turnover of ribosomal components appears to be negligible during exponential growth at moderate to fast rates (Bremer & Dennis, 1987). In the system used in this study, 23 S rRNA is overproduced in respect of 16 S rRNA. The transcription rate of plasmid-encoded 23 S rRNA was around 80% of total rRNA synthesis and plasmid-borne full-length 23 S rRNA constituted 40% of the total ribosome population when expressed from the plasmid ptBsB1067U. In spite of the high transcription rate of the plasmid-encoded 23 S rRNA, the stoichiometry of 30 S and 50 S ribosomal subunits remains close to unity according to sucrose gradient analysis (Figure 2). This data indicates that a considerable amount of the plasmid-borne 23 S rRNA was degraded, and therefore, did not show up in assembled particles. Some of the mutant 23 S rRNA species were accumulating in three to fivefold excess over chromosomally encoded rRNA (Table 2); in other words, the deletion made the mutant 23 S rRNA resistant to degradation. Figure 5 demonstrates that the extent of deletion is correlated with the stability of the mutant rRNA: the longer the deletion, the more stable the resulting particle. According to Figure 2 and Table 2, any region between positions 40 to 2874 of 23 S rRNA can be deleted without losing the capability of forming particles. However, upon closer inspection of the stability of particles and the borders of deletion in the corresponding rRNA, one general rule emerges: mutants, in which one of the structural domains was only partially deleted, tended to form less stable particles than mutants lacking a complete domain. For example, out of three deletions starting at the same position, 40 to 250, 40 to 366 and 40 to 608, only the last, which lacked domain I completely, was stable. The likely explanation is that the domains of the 23 S rRNA represent both folding and assembly domains, which may act more or less independently of each other. A partial loss of one of these folding and assembly modules could lead to misassembled structures that can be disastrous for the overall formation of a particle. It could be that complete loss of a domain is tolerated better than a partial loss because of the relative independence of structural domains.

In contrast with the inner regions, both ends of the 23 S rRNA are important for the formation of stable particles. Mutant 23 S rRNAs, lacking either the 5' or the 3' end, are completely degraded. Ends of the 23 S rRNA are base-paired, forming helix I. In the primary transcript, helix I is preceded by a processing stem, formed by base pairing between sequences in ITS-2 and ITS-3. Thus, the likely

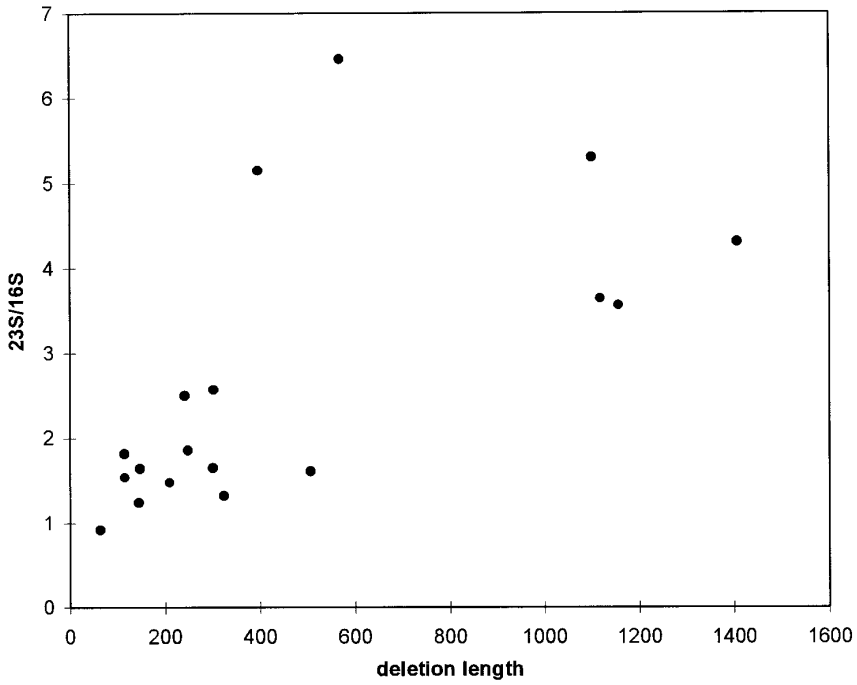

Figure 5. Correlation between the length of a deletion and the accumulation of the mutant rRNA. Accumulation of the plasmid-encoded mutant rRNA is expressed as the ratio of 23 S rRNA to chromosomally encoded 16 S rRNA.

reason for assembly defects observed is an alteration of the secondary structure of pre-23 S rRNA. The data presented in this paper do not allow a distinction between the importance of the processing stem and helix I. We are currently analysing this point in more detail. It appears that an alteration of the helix I is responsible for most of the assembly effects (Liiv & Remme, unpublished results). Therefore, in addition to ribosomal proteins, helix I appears to be an essential element in stabilizing 23 S rRNA.

Several single point mutations in the *E. coli* 23 S rRNA gene have been shown to inhibit bacterial growth (Porse & Garrett, 1995). Most of the deletion mutations analysed in this study did not affect bacterial growth. The mutant particles were non-functional. It has been shown that overexpression of non-functional rRNA does not affect transcription of the chromosomal rRNA (Jinks-Robertson *et al*., 1983). Thus, the growth effect observed with point mutations is probably due to abnormal translation. Expression of only deletion mutant 250 to 1656 led to accumulation of two different particles (see Figure 2). Therefore, this rRNA species affected assembly of the chromosome-encoded 23 S rRNA to 50 S subunit significantly. Deleterious effect on cell growth of this mutation can be attributed to disturbance of the normal assembly process, owing to trapping of a specific set of assembly proteins.

Ribosomal peptidyltransferase activity appears as a final step during 50 S subunit assembly, as was shown by reconstitution studies (Nierhaus, 1991). The fact that all mutant particles were unable to catalyze peptide bond formation can be interpreted as a defect in the late steps of 50 S subunit assembly. However, mutant 23 S rRNAs were found to form discrete particles (Figure 2) and therefore competed efficiently with full-length rRNA for association of nearly all of the proteins of the large subunit *in vivo*. Assembly of the proteins was obviously specific since the presence of the so-called primary 23 S rRNA binding proteins (Brimacombe, 1991) depended on the presence of their corresponding *in vitro* mapped binding sites in the mutant 23 S rRNA. On the other hand, the inactivity of the mutant particles can be caused by misfolding of rRNA and/or r-proteins.

Two proteins, L24 and L3, were shown to be necessary for initiation of 50 S subunit assembly *in vitro* (Nowotny & Nierhaus, 1982). At lower temperatures, protein L20 can replace assembly initiator protein L24, although with lower efficiency (Franceschi & Nierhaus, 1988). Three stable mutants, lacking either the 3' half of the 23 S rRNA (1563 to 2828 and 1656 to 2773) or the 3' end (2650 to (+ 31)), did not bind L3; whereas only one stable mutant (40 to 608) contained a reduced amount of L24. These data imply that L24 is also an assembly initiator *in vivo*, equivalent to its prominent role during the reconstitution of 50 S subunits *in vitro*.

Association of the proteins L4, L20, L22, and L24 with the mutant rRNA was found to depend upon the presence of an intact domain I of 23 S rRNA. Proteins L4 and L24 have been shown to bind to domain I of 23 S rRNA *in vitro* (Egebjerg *et al*., 1987; Zengel & Lindahl, 1993). The proteins L4, L20, and L22 were localized in the center of the back side of the 50 S subunit by immuno-electron microscopy, protein to protein crosslinking (Walleczek *et al*., 1988) and by inter-protein distance measurements using neutron scattering (May *et al*., 1992). In addition, both ends of 23 S rRNA, which are base-paired as already mentioned, have been

shown to be located in the same region by immuno-electron microscopy (Shatsky *et al*., 1980). It follows that domain I of 23 S rRNA is located mostly in the central part of the back side of the 50 S subunit and probably forms a structural unit in the ribosome. The proteins L4, L13, L20, L22, and L24 are necessary and sufficient for the formation of the first assembly intermediate particle *in vitro* (Spillmann *et al*., 1977). Thus, the presence of four out of five critical early assembly proteins is dependent on domain I of 23 S rRNA.

Data presented in this work show which 23 S rRNA species can form stable and specific particles *in vivo*. This information can be used for structural studies of subribosomal particles, e.g. by RNA footprinting and electron microscopy.

Materials and Methods

Construction and expression of mutants

Plasmid ptBsB1067T (Saarma & Remme, 1992; Lewicki *et al*., 1993), containing $tRNA^{Glu2}$-23 S rRNA-5 S rRNA genes under *tac* promoter, was used to construct deletion mutations in the 23 S rRNA gene using methods by Sambrook *et al*. (1989). Plasmid was digested with appropriate restriction enzymes and the 5' overhanging ends were filled by Klenow fragment of DNA polymerase. The 3' overhanging ends were removed using T4 DNA polymerase. Fragments were ligated and transformed using the standard procedures. Enzymes were from New England Biolabs and Fermentas (Lithuania). Host strain was XL-1 (Sambrook *et al*., 1989). Transformed bacteria were grown in 2 × YT medium at 37°C, and plasmid-encoded rRNA genes were induced at cell density A_{600} = 0.4 by addition of 0.75 mM IPTG.

Isolation and analysis of the mutant ribosomal particles and rRNA

The cells were collected by centrifugation and resuspended in lysis buffer (60 mM NH_4Cl, 60 mM KCl, 20 mM Tris-HCl (pH 8), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 16% (w / v) sucrose) and lysed by lysozyme (0.5 mg / ml) with three freeze–thaw cycles. Cell debris was removed by centrifugation at 16,000 rpm for 15 minutes using a Sorvall SS-34 rotor. Lysate was loaded onto a 10 to 30% (w / w) sucrose gradient in buffer A (60 mM NH_4Cl, 60 mM KCl, 12 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 8)) and centrifuged in a Sw 41 rotor for $\omega^2 t = 3.7 \times 10^{11}$ rad^2 s^{-1} (analytical probe) or in a Sw 28 rotor for $\omega^2 t = 3.5 \times 10^{11}$ rad^2 s^{-1} (for protein analysis). Fractions containing ribosomal particles were combined and precipitated with two volumes of ethanol in the presence of 50 mM $MgCl_2$. Precipitate was collected, dissolved in buffer A and centrifuged under the same conditions again. To avoid contamination with the native 50 S subunits, only the fractions clearly separated from 50 S subunits were taken for r-protein analysis. Ribosomal proteins were extracted with acetic acid and separated by two-dimensional PAAG according to the method of Geyl *et al.* (1981). In case of contamination with the native 50 S subunits (less than 10%) the r-proteins in the mutant particles were identified on a comparative difference basis. Peptidyltransferase activity of the ribosomal particles was detected as described by Saarma & Remme (1992) using f[^{35}S]Met-tRNA as a donor substrate and puromycin as acceptor.

Ribosomal RNA was prepared from ribosomal fractions by extraction with phenol (pH 6.5) followed by extraction with phenol / chloroform / isoamyl alcohol (25:24:1 by vol.) and then with chloroform / isoamyl alcohol, and subsequent precipitation with ethanol. The 1067 region in 23 S rRNA was sequenced by the reverse transcriptase method (Sigmund *et al*., 1988), using primer complementary to bases 1068 to 1086 in 23 S rRNA.

rRNA transcription and stability

The transcription rate of the rRNA genes was analysed according to Siehnel & Morgan (1985). Cells were grown in 2 ml of MOPS medium (Neidhardt *et al*., 1974) supplemented with 0.4% (w / v) glucose, 50 mg / ml amino acids, and 6×10^{-4}% (w / v) thiamin. Plasmid-encoded rRNA genes were induced by adding IPTG (1 mM final concentration) at cell density A_{600} = 0.15. RNA was labeled with [^{3}H]uridine, 0.2 mCi / ml for one minute. Growth was terminated by the addition of three volumes of 100°C mixture (2 / 3 phenol, 1 / 3 1% (w / v) SDS, 0.1 M NaCl, 8 mM EDTA, 20 mM Tris-HCl (pH 7.4)) and mixed for ten minutes at 100°C. Probes were cooled and re-extracted twice with phenol. RNA was precipitated with two volumes of ethanol and dissolved in water. For determination of plasmid-encoded rRNA accumulation, cells were grown in the same medium containing 10 μCi / ml [^{3}H]uridine for 3.5 hours. RNA was extracted as described above. ^{3}H-labeled RNA was mixed with uniformly labeled [^{32}P]RNA and hybridized to filter-bound ssDNA probes. DNA probes used were *Eco*RI-*Xma*I fragment (positions 2192 to 2900) in mp19 and *Hin*dIII-*Xma*I (positions 1599 to 2130), *Sac*I-*Eco*RI (positions 3861 to 4343) and *Hin*dII-*Ava*I (positions 5701 to 6272) fragments in mp18. Numbering is according to pKK3535 (Brosius *et al*., 1981). Hybridization of labeled rRNA was performed in triplicate by the method of Jinks-Robertson *et al*. (1983). Each hybridization reaction contained 100,000 cpm of each isotope and two nitrocellulose filters bearing M13 mp18 ssDNA probes. Filters were treated with RNaseA, washed and radioactivity retained on the filters was counted. The values for the relative abundance of rRNA regions are calculated by dividing the ^{3}H / ^{32}P ratios by the ^{3}H / ^{32}P ratio of 16 S RNA specific probe.

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