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Ribosomal RNA precursors of *Bacillus subtilis*

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ABSTRACT

The DNA sequence of the region corresponding to the 5'-end of a 16S rRNA gene of B. subtilis 168 was determined. Comparison of this sequence with the sequences flanking other 16S and 23S rRNA coding regions (1-4) indicated that large RNA stem structures, surrounding the mature 16S and 23S rRNAs, could form in a precursor rRNA. The 5'-ends of the precursors of 16S and 23S rRNAs (p16S and p23S) were mapped to the middles of these potential RNA stem structures. We propose that the initial cleavages of the primary rRNA transcript occur near the "opposed G's" which interrupt the basepairing of each of these stem structures. This model is supported by the finding that the 5'-end of the 5S rRNA precursor, p5A (5), maps to the region of the "opposed G's" in the 23S rRNA stem structure.

INTRODUCTION

The structural organization and genetic localization of the ribosomal RNA (rRNA) genes of Bacillus subtilis have been the subject of much recent work and a consistent picture has begun to emerge from these studies. There are ten rRNA gene sets in B. subtilis, each with a 16S, a 23S and a 5S rRNA gene arranged in that order (1,6-9). Most of these gene sets are located at (10,11) or near (12) the chromosomal origin of replication but a few may be located elsewhere on the chromosome (13).

Little is known about the transcription and processing of the ribosomal RNAs of B. subtilis. Although 16S, 23S and 5S rRNAs are co-transcribed (14), no large precursor rRNA has been observed. Precursors of the individual rRNAs have been identified among the RNAs from pulse labeled cells and these precursor rRNAs also accumulate following treatment of B. subtilis cells with chloramphenicol (15). The precursor to 23S rRNA (p23S) is 75-150 nucleotides larger than the mature rRNA (m23S); p16S contains about 200 nucleotides that are not found in m16S (15). There are several species of 5S rRNA precursors. At their 5'-ends they contain either 21 or 60 nucleotides which are not found in the mature 5S rRNA and they vary

in the number of additional nucleotides found at their 3'-ends (5,16).

If rRNA processing in B. subtilis is similar to that of E. coli (17) a primary rRNA transcript would be cleaved to yield p16S, p23S and p5S rRNAs. These cleavage sites would fall within two long RNA stem structures surrounding the sequences of m16S and m23S rRNAs (18).

We present here the DNA sequence flanking the 5'-end of a B. subtilis 16S rRNA gene and compare this sequence with the sequences flanking other 16S and 23S rRNA genes (1,2,4). We identify regions which, following transcription, could form stem structures surrounding the 16S and 23S rRNAs and show that the 5'-ends of the precursor 16S and 23S rRNAs are located within these stem sequences.

MATERIALS AND METHODS

Strains

Bacillus subtilis 168 was obtained from Dr. G. Chamblis, University of Wisconsin-Madison. E. coli HB101 was used for transformations.

Isolation of pR315

In order to clone promoter containing rDNA fragments we constructed a derivative of pBR322 which contained the terminator region of an rRNA operon of E. coli. The rationale for using such a plasmid vector was based on two observations: B. subtilis promoters are often recognized by E. coli RNA polymerase (19,20) and plasmids containing E. coli rRNA promoters are unstable in the absence of a strong terminator (21,22). In order to generate this plasmid, the terminator region of the rrnA gene of E. coli was isolated as a BamHI fragment (23) and inserted into the BamHI site of pBR322. The orientation of this fragment was such that transcription proceeding clockwise from other inserts in the EcoRI site would be terminated. EcoRI fragments of B. subtilis DNA were ligated into the EcoRI site of this terminator plasmid and a recombinant plasmid with a 3.1 kilobase (kb) EcoRI insert was further characterized. This plasmid, pR315 (EcoRI, 3.1 kb, 5'-end), contained the 5'-end of an rRNA gene set (see Figure 1A).

Two regions of the 3.1 kb EcoRI fragment of pR315 were subcloned into pBR322. The 0.8kb EcoRI-PstI fragment (see Figure 1A) was subcloned for use as a hybridization probe and the 0.9kb HindIII-EcoRI fragment was subcloned for DNA sequence analyses. In addition, this latter subclone was used to generate DNA probes used in the S1 nuclease mapping experiments.

The methods used for the construction of the plasmids, isolation of

plasmid DNAs, restriction mapping, preparation of labeled DNA fragments, DNA sequencing and RNA isolation have been described (1). γ^{32}P-ATP was synthesized using the GammaPrep-A kit from Promega Biotec, Inc., Madison WI.

S1 Mapping

rRNA was isolated from cells growing exponentially in MOPS minimal medium or from cells treated with chloramphenicol (150 μg/ml) for twenty minutes prior to isolation of the rRNA. S1 nuclease mapping was performed according to the method of Berk and Sharp (24) as modified by Dr. E.A. Craig. The rRNA and the DNA probe were co-precipitated with 80 μg of carrier yeast tRNA and resuspended in 0.05 ml of 80% deionized (25) formamide (Eastman-Kodak), 1.6mM EDTA, 10mM PIPES-pH6.8, 0.4M NaCl. The mixtures were heated at 80°C for 3 minutes and then incubated at 50°C for 16 hours. These hybridizations were done under conditions of rRNA excess. The hybridizations were terminated by addition of 0.45 ml of 0.275 M NaCl and 1 ml of ethanol. The precipitates were resuspended in 0.2 ml of S1 buffer (50mM NaOAc, pH5.5; 0.3M NaCl; 1 mM $ZnCl_2$) containing 80 μg of carrier RNA and 100 units of S1 nuclease (P-L Biochemicals) and then incubated at 30°C for 30 minutes. The S1 digestions were terminated by the addition of 5 μl of 2M Tris pH 9.6 and 5 μl of 0.2 M EDTA, then phenol extracted and ethanol precipitated. Following resuspension of the precipitates in 10 μl of deionized formamide (containing 50 μg/ml each of bromphenol blue and xylene cyanol ff) the samples were heated to 90°C for one minute and then analyzed by gel electrophoresis and autoradiography. The gels were either 6% (60:1) polyacrylamide, 8M urea gels or 10% (19:1) polyacrylamide, 8.3M urea sequencing gels. 1 x TEB buffer (89mM Tris base, 89mM boric acid, and 2.8mM EDTA pH 8.3) was used for all gels.

RESULTS

Isolation and Characterization of pR315

Cloning of the 5'-end of an rRNA Gene Set. The plasmid pR315 contained a 3.1 kb EcoRI rDNA fragment which included the 5'-end of an rRNA gene set. Figure 1A shows a restriction map of the insert DNA. Hybridization analyses revealed that it contained about 0.8 kb of a 16S rRNA gene and about 2.3 kb of 5'-flanking region sequences.

Sequencing the 5'-flanking Region. The region near the 5'-end of the 16S rRNA gene was further characterized by determination of the DNA sequence for a portion of the cloned fragment. The scheme for the DNA sequencing is

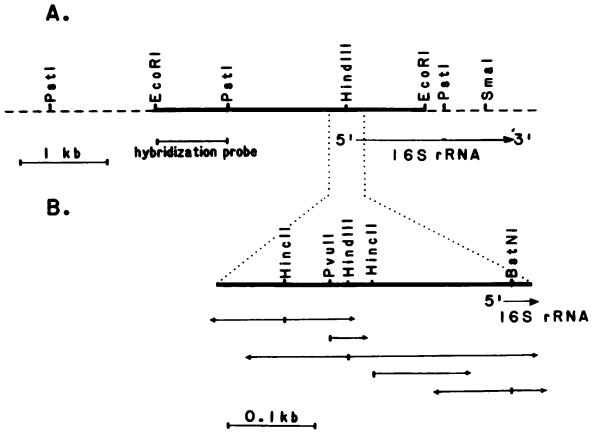

Figure 1. A. Restriction Map of the 3.1 kb EcoRI insert of pR315. The solid dark line indicates the 3.1 kb B. subtilis rDNA fragment. The adjacent dashed lines represent DNA which is contiguous to the 3.1 kb fragment in B. subtilis genomic DNA. The region corresponding to mature 16S rRNA is shown. The EcoRI-PstI fragment labeled "hybridization probe" was used to investigate 5'-flanking region homologies (see text).
B. DNA Sequencing Strategy for a Portion of pR315. The DNA sequence of the region shown was determined. The dotted lines indicate the portion of the map in Figure 1A which corresponds to the sequenced region. The arrows under the map delineate the extent of each sequencing reaction, the vertical dashes across the lines indicate the location of the labeled 5'-ends. The sequence was determined for both strands of the DNA or was determined twice for one strand. The region corresponding to the 5'-portion of mature 16S rRNA is shown.

shown in Figure 1B and the DNA sequence is shown in Figure 2. The 5'-end of the mature 16S rRNA (26) is indicated as are two DNA sequences which showed homology to the consensus sigma^{55} promoter sequences of B. subtilis (27). These regions were identified as promoters only by DNA homology since no transcriptional studies were performed.

Additional Characterization of the 5'-flanking Sequences. The DNA sequences found 5'-proximal to the rRNA gene set represented by pR315 were further characterized with respect to sequence organization. First, the 5'-flanking region was assayed for the presence of tRNA genes since it was known that tRNA genes and rRNA genes were clustered in B. subtilis (28). This involved using plasmid DNA immobilized on nitrocellulose filters for hybridization to ^{32}P-labeled small RNAs of B. subtilis. Although 5S rRNA hybridized to pR315 plasmid DNA (presumably to the E. coli rrnA 5S rRNA gene sequences) no tRNAs hybridized. Thus, we concluded that the 2.3 kb region flanking the

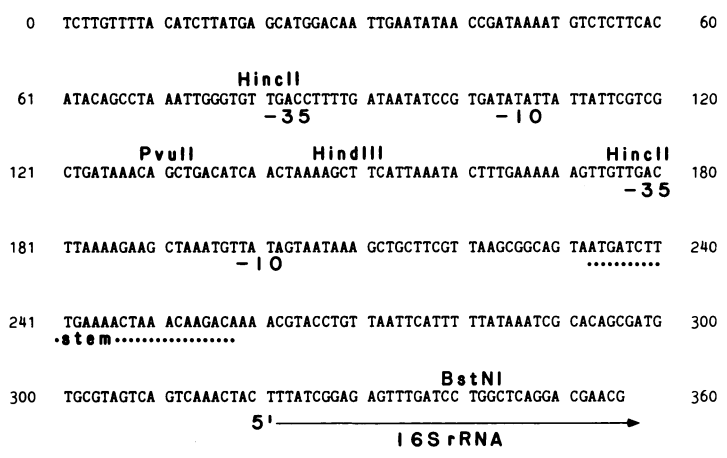

Figure 2. DNA Sequence for a Region of pR315. The DNA sequence for the portion of pR315 included in Figure 1B is shown. Restriction enzyme cleavage sites used in the DNA sequencing studies are marked. The 5'-end of mature 16S rRNA is indicated as are the two sequences which resemble the consensus sigma^{55} promoter sequences (27); for these two regions the -35 and the -10 regions of the promoter sequences are shown. The dotted line under the sequence labeled "stem" corresponds to the bottom portion of the stem structure shown in Figure 3.

5'-end of this rRNA gene set did not contain tRNA genes.

Secondly, since there are four 3.1 kb EcoRI rDNA fragments which contain the 5'-portion of a 16S rRNA gene (1) we analyzed the homology between the far upstream regions of these fragments. The region of pR315 labeled "hybridization probe" in Figure 1A was used to probe a Southern blot of *B. subtilis* genomic DNA digested with various restriction enzymes. In each case only a single hybrid band was obtained (data not shown) indicating that this region of pR315 did not share enough homology with other rDNA fragments to cross-hybridize. Thus it seems likely that the similarity in location of the upstream EcoRI sites closest to these four rRNA gene sets was coincidental.

Thirdly, the fragment containing the *rrnA* terminator was deleted from pR315. The resultant plasmid appeared to have no deleterious effects on *E. coli* cells. Thus it is not clear whether the terminator plasmid in fact facilitated the cloning of the insert in pR315. Nor is it clear in what way this 5'-end rDNA fragment differs from another *B. subtilis* 5'-end rDNA fragment which was recently cloned and found to be detrimental to *E. coli* cells (29,30).

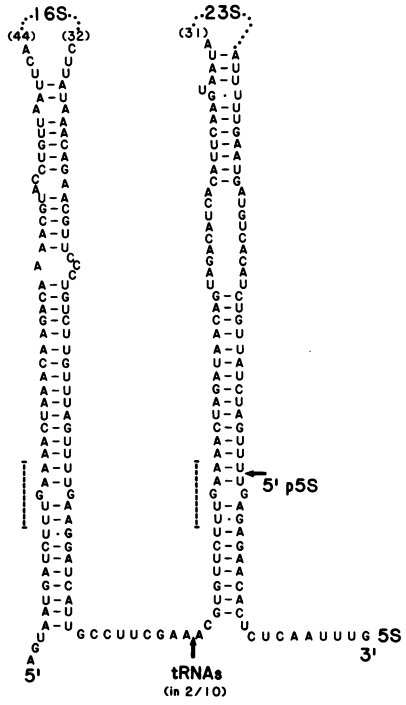

Figure 3. Possible RNA Stem Structures Surrounding 16S and 23S rRNAs. Two stem structures which could form in the rRNA precursor are shown. Numbers in parentheses refer to nucleotides present in the rRNA precursor but not shown in the figure and dotted lines represent mature 16S and 23S rRNA sequences. In two of the ten 16S/23S rRNA spacer regions, the $tRNA^{Ile}$ and $tRNA^{Ala}$ sequences would be found in the location indicated (1). The 5'-end of the 5S rRNA precursor, p5A, is marked (5). The 5'-ends of p16S and p23S rRNAs fall within the range of sequences indicated by the vertical dashed lines to the left of each stem (see text and Figures 4 and 5). The 5'-ends of m16S and m23S rRNAs have been determined (26) but the 3'-end of m23S rRNA was localized by analogy to that of E. coli, as described (4). The 3'-end of m16S rRNA in B. subtilis has not been reported; it is placed here by analogy to that of B. brevis (see ref. 1). These specific RNA stem structure sequences correspond to the DNA sequences described in this work and elsewhere (1,4).

Intermediates in the Processing of Ribosomal RNAs

Stem Structures. Following transcription, sequences located between the putative rRNA promoters (see Figure 2) and the mature 16S rRNA could pair with sequences found 3'-distal to the 16S rRNA (in the 16S-23S rRNA spacer region) to form a stem structure surrounding the 16S rRNA as shown in Figure 3. Also shown is a similar stem structure which could form around the 23S rRNA. The location of the 5'-end of the 5S rRNA precursor, p5A, of

B. subtilis (5) is marked by an arrow in the stem surrounding the 23S rRNA.

If rRNA processing in B. subtilis follows a scheme similar to that of E. coli (17) the 5'-ends of B. subtilis p16S and p23S would be expected to map within these stem structures. To test this model the DNA sequences corresponding to the 5'-ends of these rRNA precursors were localized by S1 nuclease mapping experiments.

S1 Nuclease Mapping. The DNA probes used to determine the ends of the p16S and p23S rRNAs were labeled at a site within the 16S or 23S rRNA gene and extended beyond the 5'-end of the stem structure described above. RNAs isolated from chloramphenicol-treated and exponentially growing cells were used.

5'-ends of p16S and m16S rRNAs. The 5'-ends of p16S and m16S rRNAs were determined by the S1 nuclease mapping experiment shown in Figure 4A. A DNA fragment about 190 nucleotides long was protected, particularly when RNA from chloramphenicol treated cells was used (indicated by an arrow, lanes 3 and 4). The size of this fragment indicated that the 5'-end of p16S was located at or near the "opposed G's" in the stem structure shown in Figure 3. A protected DNA fragment about 110 nucleotides long was also seen (lanes 3 and 4). The size of this fragment was that predicted for hybrids to the mature 16S rRNA since the nucleotide corresponding to the 5'-end of 16S rRNA is 109 nucleotides from the restriction site used to generate the labeled DNA end. The remaining lanes in Figure 4A show controls which indicate that the appearance of the protected DNA fragments require the addition of both S1 nuclease (lane 1) and rRNA (lane 2). The presence of full length DNA probe fragments in lanes 3 and 4 must be due either to reannealing of the DNA probe to itself or due to protection of the entire DNA probe by a larger rRNA precursor.

In order to determine the 5'-end of p16S rRNA more accurately, the products from another S1 nuclease mapping experiment were analyzed by high resolution gel electrophoresis. These products were electrophoresed next to the products of a "G"-specific (31) DNA sequencing reaction which had been performed on the DNA probe. An autoradiogram of this gel is shown in Figure 4B and the DNA probe used is indicated. Again, two groups of fragments were generated by S1 nuclease treatment. One group of protected DNA fragments was the size expected if p16S rRNA extended to within a few nucleotides of the "opposed G's" (see arrow in Figure 4B). A second group migrated as predicted for protection by m16S rRNA but also included several fragments which were a few nucleotides longer than expected. These larger fragments

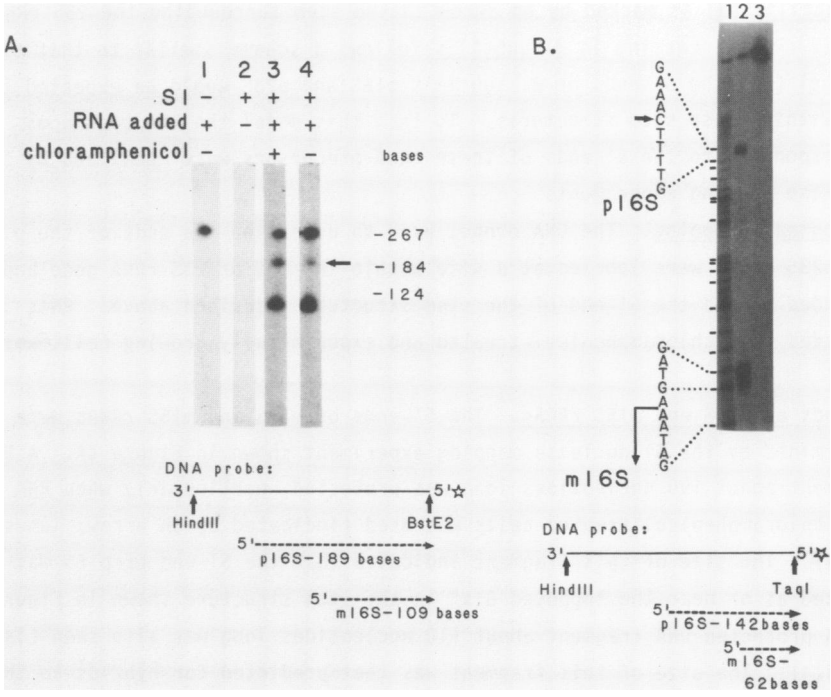

Figure 4. S1 Nuclease Mapping of the 5'-ends of p16S and m16S rRNAs.
A. RNA from exponentially growing (- chloramphenicol) and chloramphenicol treated cells was used. RNA and S1 nuclease were added to or omitted from the reactions as indicated. The 5'-labeled DNA probe used is shown at the bottom of the figure. Only the regions of p16S and m16S rRNAs which would hybridize to this probe are shown and the size of the hybrids which could form are indicated (assuming p16S rRNA extended to the "opposed G's" of Figure 3). An autoradiograph of a 6% polyacrylamide gel is shown. The arrow indicates the hybrid band resulting from protection by p16S rRNA (see text). pBR322 DNA cut with Hae III was used for the size markers shown to the right of the autoradiograph. All four lanes are from the same gel; however, the exposures of lanes 3 and 4 were for a longer time.
B. An autoradiograph of a 10% polyacrylamide gel is shown. Lane 1 contains the products of a "G"-specific DNA sequencing reaction (30) and the products of S1 nuclease reactions are shown in lane 2 (+S1 nuclease) and lane 3 (-S1 nuclease). The labeled DNA fragment is shown at the bottom of the figure as are the regions of p16S and m16S which could hybridize to it. rRNA from chloramphenicol treated cells was used in the S1 nuclease reactions. The "G" bands are marked by dashes to the left of the autoradiograph and some of the DNA sequence is shown. The position in the DNA sequence which corresponds to the "opposed G's" in the RNA stem structure (see Figure 3) is shown by an arrow as is the region which corresponds to the 5'-end sequences of m16S rRNA.

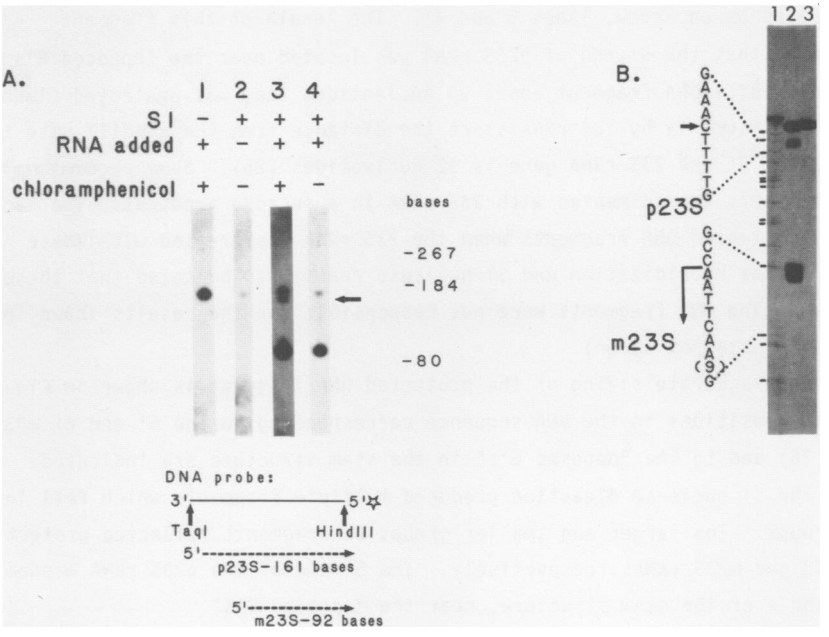

Figure 5. S1 nuclease mapping of the 5'-ends of p23S and m23S rRNAs. A. An autoradiograph of 6% polyacrylamide gel is shown. Details of the experiment are as in Figure 4A. All the lanes are from the same gel; however, a longer exposure of lane 3 is presented. The arrow indicates the hybrid band resulting from protection by p23S rRNA (see text). B. An autoradiograph of a 10% polyacrylamide gel is shown. Details of the experiment are as in Figure 4B. Numbers in parentheses within the DNA sequence represent nucleotides which are not specifically shown. The DNA fragment used in the S1 nuclease reaction as a probe is the same as that shown in Figure 5A and is 176 nucleotides in length. The fragment used in the DNA sequencing reaction was 187 nucleotides in length and was also labeled at the same HindIII site but extended to a Sau3A site at the 3'-end (1).

probably resulted from the protection of the DNA probe by precursor rRNAs which had been transcribed from another class of rRNA gene sets. This other class contained a 64 basepair insertion three bases upstream of the 5'-end of the 16S rRNA coding region (3) when compared to the rRNA gene set used for the generation of the probes. S1 nuclease presumably cleaved opposite the RNA loop in a hybrid formed with a transcript from this other class of rRNA gene sets.

5'-end of p23S and m23S rRNAs. Similar experiments were performed to map the 5'-ends of p23S and m23S rRNAs as shown in Figure 5A. A fragment about 160 nucleotides long resulting from protection by p23S rRNA was seen

(indicated by an arrow, lanes 3 and 4). The length of this fragment indicated that the 5'-end of p23S rRNA was located near the "opposed G's". In addition, a DNA fragment about 90 nucleotides long was protected (lane 3 and 4), presumably by 23S rRNA since the distance from the HindIII site to the 5'-end of the 23S rRNA gene is 92 nucleotides (26). Some chromosomal DNA fragments co-sedimented with 23S rRNA in a sucrose gradient. The lack of any protected DNA fragments when the 23S rRNA was treated with RNase A prior to the hybridization and S1 nuclease reactions indicated that these contaminating DNA fragments were not responsible for the results shown in Figure 5 (data not shown).

A more accurate sizing of the protected DNA fragment is shown in Figure 5B. The positions in the DNA sequence corresponding to the 5'-end of m23S rRNA (26) and to the "opposed G's" in the stem structure are indicated. Again the S1 nuclease digestion produced multiple fragments which fell into two groups. The larger and smaller groups of fragments reflected protection by p23S and m23S rRNAs, respectively. The 5'-end of the p23S rRNA mapped to the middle of the stem structure, near the "opposed G's".

DISCUSSION

DNA sequence analyses revealed regions which could potentially form two long RNA stem structures in the primary transcript from the rRNA genes of *B. subtilis*. These stem structures would surround the 16S and 23S rRNAs (Figure 3), creating two nearly identical cleavage sites for an enzyme involved in processing p16S, p23S and p5S rRNAs from the initial transcript.

Since the RNA sequences of the two stem structures are very similar, the formation of alternate stem structures might be envisioned. Such alternate structures would result from the pairing of the two stem sequences between the 16S and 23S rRNAs with each other and/or the pairing of the sequence upstream from the 16S rRNA with the sequence downstream from the 23S rRNA. Although the predicted stabilities of these latter stem structures (each about -13 kcals/mole [32]) are much less than the predicted stabilities for the stem structures which were shown in Figure 3 (each about -40 kcals/mole), the existence of alternate stem structures *in vivo* cannot be ruled out. If only one pair of stem structures is active, the formation of this pair might well be favored by conformational changes introduced by the binding of ribosomal proteins and/or the coupling of transcription, stem formation and RNA cleavage.

The DNA sequences used to generate the stem structures proposed in

Figure 3 were determined from two independently derived cloned rDNA fragments. Thus, we do not know that these sequences actually flank the same rRNA gene set in B. subtilis genomic DNA. However, we do know that the sequences represented by pR315 are followed in the genome by a 1.2 kb EcoRI 16S/23S rDNA spacer region; at least three of the eight 16S/23S rDNA spacer regions of this size have been sequenced (1,2) and all contained identical stem sequences downstream from the 16S rRNA coding region. If the stem structures were involved in the processing of rRNA precursors one would expect to find them in every rRNA gene set. Sequences potentially able to form these structures have been found surrounding the rRNA genes in each rRNA gene set which has been examined. (1-4; this work; P. Zuber, Ph.D. Thesis, University of Virginia, Charlottesville, Virginia, 1982; G. Stewart and K. Bott, personal communication).

In one case, both halves of a stem structure surrounding a single 16S rRNA gene have been analyzed (1,3). This rRNA gene set, rrnO, (10) contains a 1.4 kb EcoRI 16S/23S rDNA spacer region. The stem sequences upstream and downstream from the 16S rRNA coding region each differ at two positions from those shown flanking the 16S rRNA in Figure 3. These sequence changes are compensatory and do not disrupt the overall stem structure.

Only one of the three species of 5S rRNA precursors of B. subtilis has been completely sequenced. When compared to m5S rRNA, this species (p5A) contained 21 extra bases at its 5'-end (5). A second species, p5B, also contained 21 nucleotides at its 5'-end which were not found in the mature 5S rRNA. This latter sequence appeared identical to the 5'-precursor sequence of p5A when analyzed by RNase T1 fingerprinting (16). Thus, the sequence analyses were consistent with each species of 5S rRNA precursors containing RNA sequences at their 5'-ends which could have been part of the stem structure shown in Figure 3.

It is interesting to note that the RNA sequences found at the 5'-ends of the 5S rRNA precursors from Bacillus licheniformis (33) and Bacillus Q (34) are very similar to the sequence of the 5'-end of p5A. Thus the 5S rRNA precursors from these other species of Bacilli would be able to form the same portion of the stem structure as could be formed by B. subtilis p5A.

The S1 mapping experiments localized the 5'-ends of p16S and p23S to within two nucleotides on either side of the "opposed G's" in the RNA stem structures (see vertical dashed lines of Figure 3). Sequence analysis of the 5'-ends of the precursor rRNAs is required to determine precisely the 5'-end nucleotides since S1 nuclease treatment of the rRNA/DNA hybrids

always generated a slightly heterogeneous population of protected DNA fragments. The "opposed G's" may serve as part of a recognition site for a rRNA processing activity similar to the RNase III of E. coli. The sites for this E. coli enzyme are localized in stem structures surrounding the E. coli 16S and 23S rRNAs and staggered cuts are made in each stem, presumably as the transcript is being made (17,18).

An enzyme which can cleave an rRNA precursor in vitro has recently been isolated from B. subtilis cells (35). Unfortunately the exact position(s) in the precursor rRNA where cleavage(s) took place were not determined. This enzyme cuts phage SP82 mRNAs in RNA loop structures (rather than in stem structures) (36). It is currently unclear whether this enzyme plays a role in the processing of rRNA precursors in vivo.

The sequences of the stem structure and of the regions flanking the 5'-ends of rRNA gene sets in B. subtilis are very different from those of E. coli. However, the general scheme for early events in rRNA processing in B. subtilis, a sporulating, gram-positive bacterium, appears to be very similar to that of E. coli, a gram-negative bacterium. Thus it seems likely that the early events in rRNA processing occur in an analogous fashion in many bacteria, but that for each bacterium the specificity of the RNA processing enzymes and the sequences of their RNA substrates are unique.

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