Transcriptional Analysis of Bacillus subtilis rRNA-tRNA Operons

II. UNIQUE PROPERTIES OF AN OPERON CONTAINING A MINOR 5 S rRNA GENE

Barbara S. Vold‡, Christopher J. Green†, Nalini Narasimhan¶, Mary Stremlf, and J. Norman Hansen‡

From the 1Department of Molecular Biology, SRI International, Menlo Park, California 94025, and the 2Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742

This is part of a series of two papers on gene regulation in Bacillus subtilis rRNA-tRNA operons that contain large clusters of tRNA genes. The preceding paper (Vold, B. S., Okamoto, K., Murphy, B. J., and Green, C. J. (1988) J. Biol. Chem. 263, 14480-14484) investigates the rrnB operon containing 21 tRNA genes, and this paper investigates a Bacillus subtilis rRNA-tRNA operon containing 16 tRNA genes and a minor 5 S rRNA. Hybridization studies suggest this minor 5 S rRNA occurs as a single copy in the B. subtilis 168 genome. S1 nuclease mapping indicates that this minor 5 S rRNA gene has its own promoter. No promoters have been found immediately 5' to any of the major 5 S rRNA species in B. subtilis rRNA operons. S1 mapping of the spacer region between the 23 S and minor 5 S rRNA revealed that the maturation of the 23 S rRNA in this operon may arise from an unusual processing mechanism.

S1 nuclease mapping experiments suggest the existence of a promoter element immediately upstream of the last gene, for tRNAAS, in the operon. A precursor leucine tRNA resulting from transcription of this last tRNA gene was observed in Northern hybridizations, and the amounts of this precursor increased during sporulation. A single terminator-like element is located just upstream of this last tRNA gene; however, S1 nuclease mapping experiments suggest that some read-through transcription occurs. Thus, all 16 tRNA genes are under control of the upstream 16 S rRNA promoters and the minor 5 S rRNA promoter. However, the last tRNA gene is primarily under the control of its own unique promoter.

The present study is an analysis of a Bacillus subtilis rRNA-tRNA gene set containing a minor 5 S rRNA gene and 16 downstream tRNA genes. The sequence of the 5 S rRNA and 16 tRNA genes has been previously published (1), but the position of this region on the genomic map and thus the designation for this rRNA gene set is not known. Also, the sequence of regions upstream of the 3' terminus of the 23 S rRNA gene is not known. The 3' end of this operon has a dual terminator element with two stem-loop structures followed by a stretch of Ts, separated by about 10 bp. This dual terminator occurs 6 bp downstream of the last tRNA gene (1).

Two distinct 5 S rRNA species have been observed in B. subtilis 168 (2). They are quite disparate in their abundances in vegetative cells and so are referred to as major and minor 5 S rRNAs. It has been established that there are 10 copies of rRNA operons in B. subtilis 168 (3-5). Sequence analyses of 5 S rRNA genes have so far shown three identical major genes and one minor gene. In this study, hybridization experiments were used to determine whether any of the remaining unsequenced 5 S rRNA genes have sequences similar to those of the known minor 5 S rRNA genes. Also, processing sites observed in the 23-5 S rRNA gene spacer region for this minor 5 S rRNA gene and the major 5 S rRNA gene in the rrnB operon are compared.

EXPERIMENTAL PROCEDURES

Subcloning into M13mp19 and Production of Probes for SI Mapping—The DNA region from B. subtilis, including part of the 23 S rRNA, 5 S rRNA, and 16 tRNA genes, was subcloned from a construction in pBR322 (1) by cleavage with PstI and EcoRI and ligation into PstI, EcoRI cut M13mp19. The M13mp19 phage with the B. subtilis insert, designated M13-H, was propagated in Escherichia coli JM109. The sequence of the insert was verified by dideoxynucleotide sequencing (6). A diagram showing the organization of genes in the fragment under investigation is given in Fig. 1.

Two 15-mer synthetic primers were used. Both were complementary to regions within the structural gene, the 5' flanking sequence of which was under investigation for a transcriptional start site: 5' CCCCCGACTACCATC 3' for the 5 S rRNA and 5' CGAACCGACCTCCA 5' for tRNAAS. Primers were extended with Klenow as previously described (7), and the resulting double-stranded DNA was cut with either PvuI (for 5 S rRNA) or HaeIII (for tRNAAS) to terminate the probe at the 5' end. The length of the probe for the 5 S rRNA gene regions was 208 nucleotides, and the probe for the leucine tRNA gene region was 194 nucleotides. Regions covered by the probes are shown in Fig. 1.

1 Experiments in this and the preceding paper (18) were undertaken to investigate transcriptional regulation for the genes in B. subtilis rRNA-tRNA operons containing large clusters of tRNA genes and to gain information on processing intermediates. The previous paper (18) concerns the B. subtilis rrnB operon, which contains 21 tRNA genes. Included in that paper are a general introduction, an evaluation of various methods for investigating tRNA precursors, and a mini-print section describing methods used in both papers.

2 The abbreviation used is: bp, base pair(s).
FIG. 1. Schematic drawing showing the organization of the minor 5 S rRNA and tRNA genes in the 16 tRNA gene region and the sequence and start sites for the promoter region preceding the minor 5 S rRNA and the leucine tRNA genes. Solid boxes represent tRNA genes that do not encode the CCA 3' terminus. P, promoter; T, terminator; [T], terminator-like structure; start, start site for transcription determined by S1 nuclease mapping. Brackets under the sequence indicate the -35 and -10 regions of the promoters and the consensus sequence (coincident with the start site of transcription) for stable RNA genes of B. subtilis. Regions covered by probes are shown by arrows at the top of the figure. The sequence corresponding to the 20-mer for Southern hybridization to the minor 5 S rRNA is given under "Experimental Procedures." The sequence for the probe for Northern hybridization of the leucine tRNA gene is given in Table I.

Analysis of the Minor 5 S rRNA by Southern Hybridizations—
Hybridization and wash conditions were optimized to give good specificity with the 20-mer probe that corresponded to the first 20 residues of the 5' end of the structural gene for the minor 5 S rRNA. Separate hybridizations were carried out at 25, 37, and 50 °C for 16 h. The nitrocellulose strips were then washed by soaking in three changes of 6 × SSC (1 × SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.6) at room temperature for 30 min to remove unbound radioactivity. The strips were covered with plastic film and exposed against x-ray film. After exposure, the filters were washed again, using successively more stringent conditions. This was done by washing the filters in 0.1 M HCl, 100 mM glucose, and 100 mM morpholinopropane sulfonate, pH 7.0. When the Asp reached 1.0, sporulation was induced by adding deoxycholate to a final concentration of 0.2 mg/ml. RNA was extracted from cells grown without deoxycholate or 0.5, 5.5, 5.5, and 7.5 h after deoxycholate addition. For RNA isolation, cells were chilled on ice, centrifuged at 5,000 × g at 4 °C for 10 min, and resuspended in 2 ml of cold TE buffer (50 mM Tris-HCl and 20 mM EDTA, pH 7.6). centrifuged in 1.5-ml Eppendorf tubes for 30 s, and resuspended in 0.1 ml of TE buffer. Cells were broken by shaking with glass beads in a dental amalgamator for 60 s in a capsule containing 0.52 g of 0.11-mm, acid-washed glass beads and 0.1 ml of phenol. The lysate was centrifuged in a 1.5-ml Eppendorf tube, and the supernatant was extracted with phenol and precipitated with ethanol. The RNA was electrophoresed on a denaturing 5% polyacrylamide, 7 M urea gel. After electrophoresis, RNAs were electroblotted onto Du Pont-New England Nuclear Gene Screen Plus using the protocol provided by the manufacturer (Du Pont-New England Nuclear). The HindIII-YaII DNA fragment from the 16 tRNA gene cluster was end-labeled with 32P using polynucleotide kinase and 32P-ATP. Hybridization was done in 50% formamide, 10% dextran sulfate, 0.1% sodium dodecyl sulfate, and 1 M NaCl for 24 h at 42 °C. The blots were washed in two successive washes with 2 × SSC and 0.1% sodium dodecyl sulfate at 80 °C for 30 min, followed by two washes in 0.1 × SSC at room temperature. The blots were dried and autoradiographed against Kodak XAR-5-x-ray film.

RESULTS

S1 Nuclease Mapping for the Region Upstream of the Leucine tRNA
Gene—A schematic diagram showing the organization of 5 S rRNA and 16 tRNA genes is given in Fig. 1. Results of S1 nuclease mapping of the region upstream of the last tRNA gene, for leucine tRNA5CA, in the 16 tRNA cluster are given in Fig. 2. The primer for synthesis of the 32P-labeled probe was complementary to a region within the 3' half of the structural gene for leucine tRNA5CA. Thus, the probe would not protect any of the sequence flanking the 3' end of the leucine tRNA gene, and processing intermediates involving the 3' end of this precursor would not be observed. The position of the 5' end of precursor leucine tRNA locates the start site(s) for transcription relative to the sequencing ladders to within three nucleotides. It is unlikely that all three nucleotides are used as start sites. The C residue labeled with an arrow (Fig. 1) is probably the start site for the following reasons. 1) Although the nucleotide, which is the start site for transcription in E. coli, is usually 5-8 bp from the Prinbo box (8), efficiently utilized B. subtilis promoters are usually 7-8 bp from the initiation sites (9). 2) Start sites for known promoters of stable RNA genes in B. subtilis have a consensus sequence beginning with the G following the start site (10).

Mature leucine tRNA would be located at the bottom of the gel shown in Fig. 2. Examinations of the full sequence of the original autoradiographs do not show a fragment indicative of the mature leucine tRNA. We presume some modification in the mature tRNA prevents complete hybridization to the S1 probe, resulting in susceptability of the mature leucine tRNA to attack by the S1 nuclease.

The position labeled Read Through marks the location of a fragment protected by the full length of the probe. This would indicate a promoter upstream of the promoter for the leucine tRNA gene and is consistent with transcription from either the promoter immediately 5' to the 5 S rRNA gene (described below) or from the dual promoters at the beginning of the operon. Transcription from the internal tRNA gene promoter and upstream rRNA promoters is evident at all growth stages investigated. The existence of this read-through transcript implies that transcription can continue to some degree through the terminator element found upstream of the leucine tRNA gene. The amount of transcript generated from the leucine tRNA gene promoter is much greater than the read-
Minor 5 S rRNA and Unique Leucine tRNA Gene

The results of S1 nuclease mapping of the region preceding through transcription, so this last gene seems predominantly under the control of its own promoter.

S1 Nuclease Mapping of the Region Upstream of the Minor 5 S rRNA Gene—The primer used to generate a probe upstream of the 5 S rRNA gene was complementary to a region in the 3′ half of the structural gene for the 5 S rRNA. Therefore, the resultant probe would not protect any of the sequence 3′ to the structural gene for the 5 S rRNA. The 5′ terminus of the probe ends 14 nucleotides within the 3′ terminus of the 23 S rRNA gene. Thus, no protection by the 23 S rRNA would be evident since the protected 14-mer would run off the gel used to fractionate protected DNAs. All of the spacer sequence between the 23 and 5 S rRNA genes is included in the probe.

The results of S1 nuclease mapping of the region preceding the minor 5 S rRNA gene are presented in Fig. 3. The mature of 5 S rRNA is located at the bottom of the region shown in the figure. Fragments labeled with a P result from processing intermediates detected by the S1 mapping technique. The spacer region between the 23 S rRNA gene and this minor 5
Minor 5 S rRNA and Unique Leucine tRNA Gene

S rRNA gene has a 56-bp insert not found in the spacer regions between 23 S rRNA genes and any of the known major 5 S rRNA genes. The processing sites indicated on this figure are different from those upstream of the major 5 S rRNA gene regions and this difference is discussed below.

The promoter regions preceding the start sites for the 5 S rRNA and leucine tRNA are summarized in the diagram shown in Fig. 1. These start sites are compatible with recognition by aA containing RNA polymerase from B. subtilis. Approximately 1 bp after the start site is a consensus sequence common to stable RNA genes of B. subtilis (10, 12).

Southern Hybridizations with a Probe for the Minor 5 S rRNA Gene—To design a hybridization probe for this study, we took advantage of the fact that four of the eight nucleotide differences that exist between the genes for the major and minor 5 S rRNA species occur in the first 15 nucleotides of the 5' end. One can therefore expect that an appropriate hybridization probe could readily distinguish between the major and minor gene. An EcoRI digest was used since restriction fragments digested with EcoRI have already been characterized with respect to hybridization to 16 S rRNA, 23 S rRNA, and tRNAs (5, 11).

The results of hybridization of the synthetic 20-mer primer (used to make the minor 5 S RNA gene probe for S1 nuclease mapping) to total B. subtilis 168 genomic DNA are shown on the right-hand side of Fig. 4. This is a composite of the results of hybridization under the three different temperatures shown at the top. For comparison, hybridization of 32P-labeled 23 S rRNA, 16 S rRNA, or tRNA against EcoRI digests of total genomic DNA is shown in the left-hand side of Fig. 4. These results suggest that there is only one EcoRI fragment from genomic DNA that specifically hybridizes to the minor 5 S rRNA sequence. The length of this fragment was 4.2 kilobases, exactly the length of the fragment containing the previously sequenced minor 5 S rRNA gene and the trrnD tRNA gene cluster (1). This evidence strongly suggests that B. subtilis 168 has only one copy of the minor 5 S rRNA gene in its genome.

Precursor Leucine tRNA Detected by Northern Hybridization—Among known tRNA genes in B. subtilis, the tRNA\textsubscript{Leu} has been found only once, and most tRNA genes from that organism have been sequenced (12). Thus, having a probe identical to a sequence at another genomic locus is unlikely. A comparison of the sequence of the probe for this leucine tRNA gene with sequences of other known B. subtilis leucine tRNA genes, given in Table I, indicates sufficient nonhomology to expect specific hybridization using appropriate hybridization conditions. The results of a Northern analysis utilizing this probe are given in Fig. 5. The major band hybridizing at all growth stages is the mature leucine tRNA. A fragment in this position would have a length of approximately 100 nucleotides. The mature leucine tRNA is 85 nucleotides long. S1 mapping experiments indicated that the 5' flanking sequence for the precursor was 25–27 nucleotides long. The 3' end of this precursor would be expected to end in the adjacent terminator stem-loop structure and would be approximately 22 nucleotides in length. Thus, the entire precursor would be about 128 nucleotides in length and the 5' processed precursor with the 3' flanking sequences would about 103 nucleotides long. The length of the fragment specifically hybridizing to the leucine tRNA gene probe corresponds to the expected length of the primary transcript or 5' processed precursor. A probe for the tRNA\textsubscript{Leu} gene, a gene for which occurs in both the 21 and 16 tRNA gene clusters, was also used with RNA from the same growth stages as shown in Fig. 5. With overexposure, faint bands that could represent precursors were seen, but there were no differences in migration or amounts of these bands during growth (19). This suggests that the leucine tRNA\textsubscript{Leu} gene is probably subject to regulatory influences distinct from the tRNA genes upstream of it. In addition, inspection of the sequence shows a putative terminator signal after the 15th tRNA gene and the last tRNA gene, again indicating that this last leucine tRNA gene may be under independent control from the upstream genes, although the S1 nuclease mapping experiments show some read through from upstream.

A tRNA\textsubscript{Leu} precursor with 5' flanking sequences was detectable by S1 nuclease mapping of RNAs from cells in midexponential growth and two early sporulation stages (Fig. 2). In this Northern analysis, it is difficult to see whether a leucine precursor is present during midexponential growth but, clearly, it was not detected at the early sporulation stage. Our results comparing S1 nuclease mapping and Northern hybridizations for a lysine tRNA gene indicated that we were able to detect precursor tRNAs by S1 nuclease mapping that we were not able to detect by Northern hybridizations (18). We presume that the S1 nuclease mapping technique was more sensitive than the Northern hybridization technique. Because this leucine precursor was detectable by Northern analysis, it may be present in relatively large amounts in late sporulation stages. Profiles of elution of leucine isoacceptors done using RPC-5 chromatography do show changes in the amounts of various isoacceptors during sporulation (13). However, since we do not know the sequences of each isoaccepting species represented in the elution profile, we do not know whether one of the isoacceptors that increases during sporulation relative to other leucine tRNA isoacceptors might be leucine tRNA\textsubscript{Leu}.

![Fig. 4. Hybridization of probes for the minor 5 S rRNA or 23 S rRNA, 16 S rRNA, and tRNA to genomic DNA from B. subtilis 168. The right-hand side of this figure shows the results obtained by hybridization of EcoRI cut B. subtilis 168 DNA with a probe specific for the minor 5 S RNA. The left-hand side shows the results obtained by hybridization of similar DNA fragments with probes specific for either 23 S rRNA (A), 16 S rRNA (B), or tRNA (C) (kindly provided by Eric Wawrousek). Probes were the appropriate 32P-labeled species hybridized in the presence of excess nonradioactive 16 S rRNA (for 23 S), 23 S rRNA (for 16 S), and mixed rRNAs (for tRNA). Hybridizations were done at 70 °C for 16 h as previously described (11).](image-url)
Minor 5 S rRNA and Unique Leucine tRNA Gene

TABLE I
A comparison of the sequence of the HinfI/TaqI restriction fragment used as a leucine tRNA<sub>CAA</sub> probe with the corresponding regions in other leucine tRNA genes

<table>
<thead>
<tr>
<th>tRNA gene region</th>
<th>Anticodon</th>
<th>31</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 tRNA</td>
<td>CAA</td>
<td>ACT</td>
<td>AAA ATT CG TGG TTT C TT GAG TGG TGG TT</td>
<td></td>
</tr>
<tr>
<td>16 tRNA</td>
<td>UAA</td>
<td>- T -</td>
<td>C - C - C - C G T A G G - A C T A C C G T - C C</td>
<td></td>
</tr>
<tr>
<td>21 tRNA</td>
<td>UAA</td>
<td>- T -</td>
<td>C - C G T A G G - A C T A C C G T - C C</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Dashes represent nucleotides that are the same as the tRNA<sub>P</sub>. Boxed sequences represent the anticodon region of the mature tRNA.

**FIG. 5.** Northern analysis of RNA from *B. subtilis* to a probe specific to leucine tRNA<sub>CAA</sub>. The sequence of the probe compared to other *B. subtilis* leucine tRNA genes is shown in Table I. Hybridization conditions are given under "Experimental Procedures." Migration position for mature leucine tRNA<sub>CAA</sub> is marked on the right. The putative precursor is indicated as tRNA<sup>*</sup>. Growth stages at which RNA was extracted are shown at the top of the figure and are explained under "Experimental Procedures," before decoyinine (lane 1) or after the addition of decoyinine for 0.5 h (lane 2), 3.5 h (lane 3), 5.5 h (lane 4), and 7.5 h (lane 5).

**DISCUSSION**

The promoter upstream of the last tRNA gene in the 16 tRNA gene cluster is particularly interesting. Northern analyses suggest that the transcription from this promoter may increase during sporulation even though the consensus for this and all of the *B. subtilis* promoters so far examined for stable RNAs is consistent with recognition by an RNA polymerase containing ρA (formerly designated ρ43). Another unique feature about this tRNA promoter is the existence of an upstream terminator-like element. S1 nuclease mapping experiments, however, show that there is some read through, so this last tRNA gene is part of the entire rRNA-tRNA transcriptional unit. Another interesting promoter is the one upstream of the minor 5 S rRNA gene. Corresponding promoters have not been observed in any of the known major species of *B. subtilis* 5 S rRNA genes. Because there is no terminator between the minor 5 S rRNA gene and 15 of the 16 downstream tRNA genes, the expression of these 15 tRNA genes is coordinated with the expression of the minor 5 S rRNA. The leucine tRNA<sub>CAA</sub> gene has its own promoter and is thus released from coordinate expression with the minor 5 S rRNA gene.

The major and minor 5 S rRNAs in *B. subtilis* 168 appear to provide an opportunity to study questions of transcriptional activity and functionality. The large number of major 5 S rRNA genes that appear to be identical suggests the involvement of a gene correction mechanism that maintains their
sequence similarities. In contrast to the consistency among the major species genes, the minor gene contains eight nucleotide substitutions, which must be the result of an accumulation of mutations. All eight are accommodated by the usual 5 S rRNA folding scheme (2), which suggests that the minor 5 S rRNA is functional (although the function is unclear) and that its gene has been subjected to selective pressure. In addition to changes in the structural genes for the 5 S rRNAs, the spacer regions between the 5 S rRNAs and their upstream 23 S rRNA genes are different. The minor 5 S rRNA gene has a 56-bp insertion in the otherwise highly conserved 54-bp 5–23 S rRNA intergenic space (1, 3, 10, 11, 14). This increase could be to accommodate the promoter for the minor 5 S rRNA gene; however, an alteration in processing may also be necessitated by the sequence and extra base pairs in this spacer region. In at least two signals formed by other nucleotide substitutions, which must be the result of an accumulation of mutations. All eight are accommodated by the usual 5 S rRNA folding scheme (1), is consistent with the formation of an extended stem-loop structure of the major species genes, the minor gene contains eight nucleotide substitutions except that the 23 S rRNA precursor is entirely upstream of this putative processing site. The conserved part of the double-stranded sequence is also virtually identical to the mature sequence. It will be interesting to determine the sequence upstream of the 23 S rRNA gene to help elucidate this possible alternative processing scheme.

Acknowledgments—We wish to thank Kathleen Okamoto for technical assistance on parts of this research. We are grateful to Eric Wawrousek for use of data from his Ph.D. thesis (left-hand side of Fig. 4). Computer resources were provided by BIONET National Computer Resource for Molecular Biology, whose funding is provided by the Biomedical Research Technology Program, Division of Research Resources, National Institutes of Health, Grant P41RR01685.

REFERENCES