Transcriptional Analysis of Bacillus subtilis rRNA-tRNA Operons

I. THE tRNA GENE CLUSTER OF rrnB HAS AN INTERNAL PROMOTER*

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Barbara S. Vold, Kathleen Okamoto, Brian J. Murphy, and Christopher J. Green
From the Molecular Biology Department, SRI International, Menlo Park, California 94025

Although the sequence and organization of many Bacillus subtilis tRNA genes are known, primary transcripts from these regions have not been previously analyzed. In this paper, S1 nuclease mapping, S1-type mapping, and Northern analyses were applied to the end of the 23 S rRNA, the 5 S rRNA, and the 21 tRNA genes of B. subtilis operon rrnB. Primary transcripts from the 5 S rRNA and tRNA genes up to approximately 600-800 nucleotides long were observed with S1-type mapping. The presence of discrete bands of processing intermediates indicated preferred processing points within the initial transcript. S1 nuclease mapping delineated a start point for transcription between the second and third tRNA genes. The -10 sequence was within the 37-base pair spacer region between tRNA genes, and the -35 sequence was within the structural gene for the upstream tRNA. Precursors from this region were evident during midexponential growth and two sporulation stages. Thus, in addition to promotion from the rRNA promoters, 19 of the 21 downstream tRNA genes are also under the control of an internal tRNA gene promoter. The accompanying paper (Vold, B. S., Green, C. J., Narasimhan, N., Strem, M., and Hansen, J. N. (1988) J. Biol. Chem. 263, 14485-14490) investigates the minor 5 S rRNA and 16 tRNA genes of another rRNA-tRNA gene set and emphasizes unique promoter elements in that system as well as a potentially unique rRNA processing scheme.

Studies involving precursors for stable RNA species in Escherichia coli have been aided by the availability of mutants lacking certain processing enzymes. Similar mutants are not presently available in Bacillus subtilis. Nevertheless, valuable information on transcription of stable RNA species can be obtained from strains with normal processing functions. Promoter regions upstream of the 16 S rRNA for the B. subtilis operons rrnB (1), rrnO, and rrnA (2) have been observed by S1 mapping techniques. The rrnB operon studied in this paper was shown to have dual promoters, the -10 regions being 180 and 272 bp upstream of the start site for mature 16 S rRNA. Both rRNA promoters were transcriptionally active in vegetative cells (1). Extracts from sporulating cells were not examined.

Similar investigations for B. subtilis tRNA genes have not been done. Using a combination of pulse-labeling and blocking of transcription with antibiotics, Bleyman et al. (9) suggested the existence of large polycistronic transcripts containing tRNA sequences, although until now no precursor tRNAs from B. subtilis have been seen. Several tRNA gene regions of B. subtilis have been sequenced. Indeed, the majority of tRNA genes in B. subtilis occur in either of two large clusters, one containing 16 tRNA genes and the other, 21 tRNA genes. This is in contrast to the E. coli situation, in which tRNA genes are dispersed throughout the chromosome in groups of one to seven. One or two tRNA genes occur downstream of three of the E. coli rRNA operons (4). To help understand the mechanisms by which gene transcription within rRNA-tRNA operons are regulated in B. subtilis, the experiments in this and the accompanying paper (32) were performed. The rrnB operon is particularly valuable for such investigations because it has been completely sequenced (5).

EXPERIMENTAL PROCEDURES*

RESULTS

S1-type Mapping—To investigate the lengths of precursor tRNAs that might be observed in wild-type B. subtilis, S1-type mapping with a probe covering a 3.3-kb region (see Fig. 1) was employed. The S1-type mapping procedure depends on the formation of RNA-RNA hybrids and digestion of hybridized regions with RNase A and RNase T1 (18). This was found to be a much more sensitive technique than the Northern analyses discussed later. Fig. 2 shows the results of S1-type mapping using a 3.3-kb antisense probe with products separated on an 8% polyacrylamide, 7 M urea gel. RNAs used as molecular weight markers are shown on the right of the figure and explained in the figure legend. As expected, a number of bands appeared in the region representative of mature tRNAs. In addition, partially processed precursor RNAs of discrete, higher molecular weights were detected. RNAs approximately 600, 260, 160, 130, 110, and 84 nucleotides in length were observed in lanes 1-3; these RNAs represent fragments protected by endogenous RNA from B. subtilis cells in midexponential growth or stationary phase. To separate large fragments better, the same RNAs were electrophoresed on a 3% polyacrylamide, 7 M urea gel, but no fragments larger than 600 nucleotides could be detected (data not shown).

Lanes 4 and 8 show the results of hybridizations of the B. subtilis [32P]RNA probe to endogenous RNA from E. coli carrying pUC8 without a foreign DNA insert. Somewhat surprisingly, since B. subtilis and E. coli tRNAs share sequence similarities, there is very little nonspecific hybridization.
Promoter within tRNA Genes of rrnB

Probe for S1-type mapping

Fig. 1. Schematic drawing showing the organization of 23 S rRNA, 5 S rRNA, and 21 tRNA genes under study. Solid boxes represent tRNA genes that do not encode the 3'CCA terminus. P, promoter; T, terminator; start, start site for transcription determined by S1 nuclease mapping. Brackets under the sequence indicate the -35 and -10 consensus sequence of the putative promoter and the consensus sequence (coincident with the start site of transcription) for stable RNA genes of B. subtilis. The region covered by the probe for S1-type mapping and the probes for S1 nuclease mapping are indicated by arrows at the top of the figure. Probes used for Southern or Northern analyses are given under "Experimental Procedures," in the Miniprint.

Fig. 2. Protected RNA fragments from S1-type mapping. 32P-Labeled RNA fragments protected by endogenous RNA extracted from B. subtilis or E. coli were fractionated on an 8% polyacrylamide, 7 M urea gel. Lanes 1–3 show results of experiments using B. subtilis RNA extracted by Procedure 3 (see "Experimental Procedures," in the Miniprint). Lanes 5–8 show results of experiments using RNA extracted by the procedure of Alba et al. (6) from E. coli cells carrying pUC8 or pUCTG. All cells were extracted in midexponential growth except for those used in the experiment shown in lane 1, which were in stationary phase. Lanes 4 and 8 show results using RNA from E. coli, with pUC8 carrying no B. subtilis DNA insert. Lanes 5–7 show results using RNA from E. coli with pUCTG, which carries an insert with the major 5 S RNA and 21 tRNA genes from B. subtilis. Lane 9 is a control showing the undigested antisense [32P]RNA probe. RNA markers on the right of the figure represent migrations of the 3.3-kb antisense [32P]RNA; 32P-labeled RNAs transcribed from the control templates provided with the Riboprobe Kit (Promega Biotech) which are 1.386, 0.557, and 0.125 kb in length; 5 S rRNA was unlabeled and visualized by UV shadowing.

The results of the S1 nuclease mapping experiments using a probe covering the region upstream of the leucine tRNA CAG are shown in Fig. 3 (Fig. 1 shows the region covered by the probe). A precursor RNA transcribed under the control of the putative internal promoter can be detected by S1 nuclease mapping from cells in exponential growth or sporulation stages t1 and t2 (fragment marked S in Fig. 3). There is also a larger precursor protecting the total length of the probe. We assume that this longer transcript originates from the dual promoters located before the rRNA genes, because S1 mapping for the region of the major 5 S rRNA gene (described below) revealed no promoter elements between the gene for 23 and 5 S rRNA, and no putative promoter element is evident from inspection of the spacer region between the 16 and 23 S rRNAs. The partially processed lysine tRNA precursor, marked P in Fig. 3, has a length consistent with a 5' processed lysine tRNA plus unprocessed 3' flanking sequence. This is similar to the sequence of processing events for precursor tRNAs in E. coli in which the 5' processing of several tRNA precursors appears to occur prior to 3' processing (22).

A guanosine residue, 7 bp from the Pribnow box, is the start site for transcription initiation. In E. coli, a purine
Fig. 3. S1 nuclease mapping of the threonine-lysine tRNA gene region. A 5% polyacrylamide, 7 M urea gel was used to determine the position of fragments protected from S1 nuclease digestion relative to the sequence of the putative promoter in the threonine-lysine tRNA gene region. Lane 1 represents the 32P-labeled antisense DNA probe before S1 nuclease digestion. Lanes 2 and 3 were control lanes and contained no protected DNA fragments: hybridizations used for lane 2 had no added RNA, lane 3 had E. coli RNA. Lanes 4-7 contained DNA fragments protected from S1 nuclease digestion: hybridizations used for lane 4 had RNA from E. coli cells containing pUCTG. Hybridizations used to generate lanes 5-7 had RNA from B. subtilis extracted by procedure 2 at midexponential growth (lane 5), t1 (lane 6), or t2 (lane 7). Lanes GATC represent dideoxy sequencing lanes produced from the same primer and template as those used to generate the probe. The expanded sequence on the left represents the sequence complementary to the RNA transcript. S indicates the start site for transcription; P indicates a precursor lysine tRNA with the 5' end processed but still carrying the 3' end; Lys indicates the position of mature lysine tRNA.

Fig. 4. Analysis of fragments protected from S1 nuclease by a probe for the region upstream of the major 5 S RNA sequence. A 5% polyacrylamide, 7 M urea gel was used to determine the position of fragments protected from S1 nuclease digestion relative to the sequence of the putative promoter in the region upstream of the major 5 S rRNA gene. Lane 1 represents the 32P-labeled antisense DNA probe before S1 nuclease digestion. Lanes 2 and 3 were control lanes and contained no protected DNA fragments: hybridizations used for lane 2 had no added RNA, lane 3 had E. coli RNA. Lanes 4-7 contained DNA fragments protected from S1 nuclease digestion by RNA extracted from B. subtilis cells by Procedure 1 (see "Experimental Procedures," in the Miniprint), hybridizations used for lane 4 had RNA from midexponentially growing cells; lanes 5 and 6 had RNA from sporulation stages t1 and t3, respectively. Lanes GATC represent dideoxy sequencing produced from the same primer and template as those used to generate the probe. The expanded sequence on the right represents the sequence complementary to the RNA transcript. P, processing site; Read Through indicates a fragment, presumably from transcription from the 16 S rRNA promoter region, equivalent in length to the entire probe (lane 1). The boxed sequence at the bottom indicates the 5' end of the mature 5 S RNA.

23 S rRNA precursor associated with the minor 5 S rRNA gene. For comparison, S1 nuclease mapping was performed on the major 5 S rRNA in the rrrB operon. The results are shown in Fig. 4. The fragment labeled P is interpreted to be a processing site and not a start site because 1) there is no identifiable promoter the anticipated distance before the site and 2) this is a site characteristic for processing of B. subtilis rRNAs (26). Read Through indicates transcripts initiated from a promoter upstream of the 5 S rRNA gene, presumably the dual promoters at the beginning of the rrrB operon. Processing sites in the spacer region between the 23 S rRNA and either the major or minor 5 S rRNA are compared in the accompanying paper (32).

Residue located 5-8 bp from the Pribnow box is the preferred site of initiation (23). Although fewer B. subtilis promoters have been sequenced, it appears that a nucleotide 7-8 bp downstream from the Pribnow box is common for efficiently utilized promoters (24). The B. subtilis promoter described here has a sequence that is characteristic of stable RNA genes from B. subtilis, underlined in Fig. 1, starting at the initiation site (2). The addition of chloramphenicol to the culture medium prior to RNA extraction was reported to enhance the buildup of ribosomal RNA precursors (25); however, higher levels of precursors were not obvious after chloramphenicol addition (data not shown).

S1 Nuclease Mapping of the Region Upstream of the 5 S rRNA Gene—Wawrousek et al. (21) sequenced the 3' end of an rRNA operon containing a 5 S rRNA sequence different from that of other 5 S rRNAs previously examined. They suggested that this minor 5 S rRNA species might have its own promoter element, identified in the accompanying paper by S1 nuclease mapping. The sequence of the spacer region between the 23 S and minor 5 S rRNA genes differed from that of the spacer between 23 S and major 5 S rRNA genes. S1 mapping revealed potential, unique processing sites for the
**Southern and Northern Hybridizations**—For Northern hybridizations, probes were designed complementary mainly to the spacer region between tRNA genes and not the structural gene, since structural genes in one cluster often occur in another as well (27). Southern analyses were performed before Northern analyses to determine whether the probes were specific for only one genomic locus. Fig. 5 (in the Miniprint) shows the results of a Southern hybridization with a probe for the threonine-lysine tRNA gene region (left side) and with another probe for the serine-glutamic acid tRNA gene region (right side). Because both probes have only five nucleotides of the structural gene and 15 nucleotides of 5’ flanking sequence, we would not expect to pick up a similar structural gene unless the 5’ flanking sequences were very similar. As expected, the spacer region for serine and glutamic acid tRNA genes hybridized to one band in each lane of restriction fragments and the EcoRI band had a length of approximately 4.4 kb characteristic of the rrnB operon (21). On the other hand, the spacer region between the threonine and lysine tRNA genes hybridized with two bands in each restriction lane. Thus 15 bp of this spacer region, possibly including the promoter region, is represented at another as yet undiscovered genomic locus. The unknown locus is reflected in the faster moving HindIII band and the slower moving Pst1 band. As expected, both probes hybridize to the 4.4-kb EcoRI fragment (21). The second band expected in the EcoRI lanes of the Thr-Lys tRNA gene spacer region either ran off the gel or comigrated with the 4.4-kb band.

Probes for Northern hybridizations are described under "Experimental Procedures," in the Miniprint. Although both probes hybridized to the Southern filters, the 20-mer for the glutamic acid tRNA gene spacer did not hybridize to the Northern filters at 40°C. The 20-mer for the lysine tRNA gene spacer hybridized to two fragments approximately the same size as the 23 and 16 S rRNA at 40°C and showed no hybridization at 45°C. From these experiments, we conclude that specific hybridization to precursor regions, not including structural genes, could not be detected by this technique for the threonine-lysine and serine-glutamic acid tRNA genes. In the accompanying paper (32), a precursor from the last tRNA gene in the 16 tRNA gene cluster was observed in Northern hybridizations at late sporulation stages. Thus, depending on the levels of precursor, Northern hybridizations may be useful in specific cases.

**DISCUSSION**

In this paper we investigated several techniques for obtaining information about transcription of tRNA genes that follow an rRNA gene set in *B. subtilis*. Northern analyses were not sensitive enough to detect tRNA precursors that were detectable by S1 nuclease mapping. As an alternative to Northern analyses, S1-type mapping was useful in discriminating discrete size classes for processing intermediates for stable RNAs, which may indicate preferred or ordered processing sites. S1-type mapping demonstrated the existence of precursors up to 600–800 nucleotides in length even in a strain with normal processing nucleases. In *E. coli*, tRNA precursors ranging from 135 to 690 nucleotides in length were observed utilizing a mutant with a temperature-sensitive RNase P, the enzyme responsible for the 5’ cleavage of precursor tRNAs (28). It is somewhat surprising that such large precursors could be observed in a normal *B. subtilis* strain.

S1 nuclease mapping was useful in characterizing transcripts from a promoter within the tRNA gene region of *B. subtilis*. An internal promoter has been described in the thrU operon of *E. coli*, in which the promoter occurs between two upstream and three downstream tRNA genes (4, 29). A particularly interesting observation is that the order of 1) promoter, 2) lysine tRNA gene, and 3) leucine tRNA gene found in *B. subtilis* has been preserved in a rather distantly related organism, *Mycoplasma* gP50 (30). Rasmussen et al. (30) suggest that this promoter is part of an independent transcriptional unit not associated with tRNA genes and that the large tRNA gene clusters found in *B. subtilis* tend to be separated into smaller units in *Mycoplasma*. The *B. subtilis* internal tRNA gene promoter has a consensus sequence typical for recognition by an RNA polymerase containing αα, formerly α43 (31) and a sequence characteristic of stable RNA genes of *B. subtilis* (2). S1 nuclease mapping of the regions upstream of the internal promoter and the 5 S rRNA gene indicates that the tRNA promoters and the internal promoter are active during vegetative growth and early sporulation stages. Pero et al. (25) suggested that total RNA synthesis decreases significantly during sporulation. However since S1 nuclease mapping experiments were all done using the same amount of RNA, not necessarily with the probe in excess for each species under investigation, our experiments may not reflect the relative activity of these promoters during development. We are presently addressing that question using promoter elements cloned into promoter probe vectors.

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**REFERENCES**

Promoter within tRNA Genes of rRNA


SUPPLEMENTAL MATERIAL TO

EXPERIMENTAL PROCEDURES

Southern Hybridization Analysis—DNA was obtained from exponentially growing A. niger 168 by phenol extraction, ethanol precipitation, and purifications by gel electrophoresis. The DNA preparation was cut with restriction enzymes AccI, EcoRI, or HindIII under conditions recommended by the manufacturer, and the restriction digests were fractionated on 0.5% agarose gel (20 cm, 0.5-3 mm thick) using 1 μg of restricted DNA per well. After fractionation, the DNA was transferred to nitrocellulose paper (Schleicher & Schuell BA-85) by capillarity. The paper was divided into sections and hybridized with 32P-labeled probes and 7% polyethylene glycol (according to the Bethesda Research Laboratories Catalogue and Reference Guide) and purified by gel electrophoresis through 1% agarose (New England Nuclear). Filters were hybridized and washed as described by Staehelin (1981) except that prehybridizations were done for 24 h. The 32P-labeled DNA probes were hybridized in a 100 ml polymerase hybridization solution without formamide at a temperature of 42°C and washed at 65°C in 0.1 x SSC.

Northern Hybridization Analysis—DNA extracted for 3 S RNA mapping was separated on a denaturing formaldehyde-agarose (0.6%) gel with a conforming Formaldehyde-halt buffer. Samples were heated at 65°C for 5 min, and 0.5 μg/ml of formaldehyde-formamide buffer was loaded per well and run on a 30-cm gels for 3-4 h. The DNA was transferred overnight to nitrocellulose according to the "Northern blot" protocol (Kitchener & Soule, inc. pamphlet "Methods for Transfer of DNA, RNA and protein to nitrocellulose and diazobenzidine paper solid supports," 1981). After transfer, the membranes were rinsed in 6 x SSC, air-dried and baked for 2 h at 80°C in a vacuum. Northern hybridizations were done as described for Southern hybridizations except that formamide was hybridized at 65°C instead of 42°C.

Fig. 5. Southern hybridizations to probes complementary to spacer regions between URR genes in the 25-S RNA gene cluster. Hybridizations and probes are described in Experimental Procedures. The left side of the figure shows hybridizations with a 32P-labeled probe covering a sequence in the spacer region between the formamides and 16 S RNA genes. The right side of the figure shows hybridizations with a probe for the spacer region between the 3 S RNA and 25 S RNA genes. The lane at the top of each lane indicates the restriction enzymes used to excise genomic DNA from A. niger 168, 8 × EcoRI; 8 × HindIII; 8 × AccI. Fragments marked on the left, lane A, show migration of HindIII cut lambda DNA markers of the following lengths: 23160, 21460, 19597, 18431, 23242, and 47014 nitrocellulose.