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Promoter of the *Mycoplasma pneumoniae* rRNA Operon

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RNA transcripts starting from the 5′ end of the single *Mycoplasma pneumoniae* rRNA operon were analyzed by several methods. By primer extension analysis a start site was found 62 nucleotides upstream from the start site of the 16S rRNA. This site was preceded by a putative Pribnow box; however, a defined −35 recognition region was absent. The cloned rRNA operon was transcribed in vitro by using purified RNA polymerase of *Escherichia coli*. A single start site could be demonstrated within a few nucleotides of the start site found by primer extension analysis of *M. pneumoniae* transcripts. When fragments from the cloned operon were used as hybridization probes, S1 nuclease mapping yielded a single transcript extending approximately 193 nucleotides upstream from the 16S rRNA start site. The region surrounding this endpoint did not resemble any known promoter sequence. Dot blot hybridization of *M. pneumoniae* RNA to three oligonucleotides consisting of nucleotides −5 to −21, −38 to −54, and −112 to −132 (from the start of the 16S rRNA gene) indicated that most rRNA transcripts were processed at the stem site preceding the 16S rRNA gene. The majority of the longer precursor transcripts, extending beyond this point, did not extend further upstream to an oligonucleotide consisting of nucleotides −112 to −132. It was concluded that transcription of the rRNA operon of *M. pneumoniae* is initiated by a single promoter. The nucleotide sequence of the region is presented.

The study of the molecular biology of mycoplasmas (class *Mollicutes*) has revealed them to possess several unique and interesting properties (18). While the group is very diverse, the mycoplasmas share several basic properties, including the lack of a cell wall, the smallest genome among self-replicating procaryotes (500 to 1,000 megadaltons in size), and an unusually low G+C content in their DNA, usually ranging between 24 and 35 mol% G+C, with *Mycoplasma pneumoniae* and some anaerobic mycoplasmas being exceptions in having about 40 mol% G+C (19). All the *Mollicutes* species tested so far have also been found to carry only one or two rRNA operons, whereas other eubacteria usually have a multiplicity of rRNA operons, often more than five (18).

The synthesis of rRNA is a very essential process in the growth of any organism. It is not surprising, therefore, that genes for rRNA have been highly conserved among procaryotes (28). Transcription of rRNA operons in *Escherichia coli* (4) as well as in *Mycoplasma capricolum* (7) is under negative stringent control. Although much has been learned about this mechanism in *E. coli* and in several other eubacteria (4), there are many aspects of this complex control system that are still unclear. The study of the structure and function of elements controlling the expression of rRNA operons in mycoplasmas has a special appeal, as the small copy number of these operons in mycoplasmas is expected to provide a simpler system for study than that provided by bacteria having a multiplicity of rRNA operons. In addition, the basic structural similarity of the mycoplasmal rRNA genes to the rRNA genes of other procaryotes (29) enables the use of procaryotic rRNA probes in the cloning and mapping of mycoplasmal rRNA genes (1). On the other hand, structural and functional differences in the rRNA genes, when found, may uncover important variations within the procaryotic world and provide valuable information on the phylogenetic status of *Mollicutes* species.

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As a preliminary step, the single rRNA operon of *M. pneumoniae* (1, 31) was cloned into the lambda vector EMBL3 and subsequently subcloned on two *Eco*RI-*Bam*HI restriction fragments into the *Eco*RI-*Bam*HI sites of the plasmid vector pBR322. A clone containing the 5′ end of the 23S rRNA, the 16S rRNA, and more than 1.2 kilobases of upstream sequences was called pPR10 (9). The structural and transcriptional analyses of the 5′ region of the *M. pneumoniae* rRNA operon are reported here.

MATERIALS AND METHODS

Organisms and growth conditions. *M. pneumoniae* FH was obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, Md. It was grown in 100-ml volumes of modified SP-4 medium containing 10% (vol/vol) horse serum in place of fetal bovine serum and lacking the CMRL component of the original SP-4 medium (26). After 3 to 4 days of static incubation at 37°C, the medium was replaced with fresh medium, and the flasks were incubated for another day. The cell layer sticking to the flask surface was then harvested by scraping it off into cold 0.25 M NaCl containing 0.1 M EDTA (pH 7.0). The cells were sedimented by centrifugation at 12,000 × *g* for 30 min at 4°C, and the pellet was washed once in the same solution and kept at −20°C for DNA extraction.

DNA extraction, cleavage, and ligation. Mycoplasmal DNA was extracted as described by Marmur (13). Standard restriction cleavage, electrophoresis, and cloning techniques were carried out as described by Maniatis et al. (12).

RNA purification. RNA was purified from *E. coli* by the procedure described by Salser et al. (20). The organisms were grown at 37°C in 350 ml of LB medium (12) to an optical density at 650 nm of 0.7. The cells were poured onto ice, collected by centrifugation, and suspended in 10 ml of Tris-KCl-$MgCl_2$ solution (10 mM Tris [pH 7.3], 10 mM KCl, 5 mM $MgCl_2$) containing 3 mg of lysozyme. The cells were lysed by freezing (−70°C) and thawing at 64°C. Following the addition of 0.7 ml of a 10% sodium dodecyl sulfate (SDS) solution, the suspension was incubated for 2 min at 64°C.

Sodium acetate (pH 5.2) was added to a final concentration of 0.1 M, followed by two extractions with water-saturated phenol (pH 4.0) at 64°C. The RNA in the aqueous phase was precipitated with ethanol, and the moist pellet was suspended in 5 ml of deionized water and dialyzed extensively at 4°C against 0.1 mM EDTA. For the isolation of *M. pneumoniae* RNA, the organisms were grown in 1 liter of modified SP-4 medium and harvested as described above. The cell pellet was suspended in a 0.1 M sodium acetate solution (pH 5.2) containing 0.1 M EDTA and frozen at −70°C. RNA extraction was carried out as described above for *E. coli*, except that sodium acetate was not added before the phenol extractions.

S1 nuclease mapping. RNA-DNA hybridization and digestion with S1 nuclease were carried out as described by Berk and Sharp (2) with 5′-end-labeled DNA probes prepared as described by Maniatis et al. (12). Hybridization was carried out for 3 h at 48 to 49°C in 80% (vol/vol) formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.4)–1 mM EDTA (pH 8.0)–0.4 M NaCl. Following hybridization, digestion with S1 nuclease (10 U per reaction mixture; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was carried out for 30 min at 37°C. DNA fragments protected from S1 nuclease digestion were resolved under denaturing conditions by electrophoresis on an 8 M urea–4.5% (wt/vol) polyacrylamide gel (acrylamide-bisacrylamide, 30:0.8).

Radioactive marker fragments for use in S1 nuclease mapping or in in vitro transcription experiments were prepared by digesting pBR322 DNA with *Msp*I or lambda DNA with *Cla*I. Following digestion, 30 μCi of [α-^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.), 1 μl of 2-mercaptoethanol, and 1 μl of DNA polymerase I (large Klenow fragment; Bethesda Research Laboratories) were added, and the reaction mixture was incubated for 20 min at room temperature. The reaction was stopped with 10 mM EDTA, and the DNA markers were purified from free nucleotides in a Sephadex G-50 column.

Primer extension analysis. *M. pneumoniae* or *E. coli* RNA (100 μg) was mixed with 4 ng of 5′-end-labeled oligonucleotide primer synthesized as described by Caruthers (3) in 45 μl of 0.1 M Tris hydrochloride (pH 8.3)–0.14 M KCl–0.01 M $MgCl_2$. This solution was incubated at 65°C for 10 min and cooled at room temperature for 5 min. Dithiothreitol was added to a final concentration of 0.01 M, and the four deoxynucleotide triphosphates were each added to a final concentration of 1 mM. Avian myeloblastosis virus reverse transcriptase (18 U; International Biotechnologies, Inc., New Haven, Conn.) was added, and the mixture was incubated at 42°C for 1 h. The reaction was stopped by heat inactivation at 75°C for 10 min. DNase-free RNase (1 μg) was added, and incubation was continued for 30 min at 37°C. Ammonium acetate (4 M; 0.1 volume) was added with 2.5 volumes of ethanol, and the mixture was precipitated overnight at −20°C. The precipitate was washed in 70% ethanol, dried, suspended in sequencing mixture (98% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 2 mM EDTA), and separated on an acrylamide sequencing gel.

Dot blot hybridization. Total *M. pneumoniae* RNA was pretreated with RQI RNase-free DNase I (0.1 U/μg of RNA; Promega) in a solution consisting of 40 mM Tris hydrochloride (pH 7.9), 10 mM NaCl, and 6 mM $MgCl_2$ at 37°C for 30 min. The DNase was then phenol extracted, and the purified RNA was ethanol precipitated. Oligonucleotide probes were 5′ end labeled, and 20 ng was used for each hybridization. Plasmid pPR10, containing the 5′ end of the rRNA operon (described above), was used as a positive hybridization control, and pPR3, a plasmid carrying the 3′ end of the *M. pneumoniae* rRNA operon (9), served as a negative control.

RNA and DNA samples tested for hybridization were diluted in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) in 7.5% formaldehyde and heated for 15 min at 60°C to denature the nucleic acids. A solution of 11× SSC (0.875 volume) was added, and samples were loaded onto nitrocellulose filters presoaked in 10× SSC by using a dot blot apparatus. The filters were baked at 80°C for 2 h in a vacuum oven. Prehybridization and hybridization were carried out at 27°C in a solution of 5× SSC–1% SDS–10× Denhardt solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin in 3× SSC)–0.02 M Na_2HPO_4 (pH 7.0)–100 μg of denatured salmon sperm DNA per ml. After hybridization (12 to 16 h) the filters were washed in 5× SSC–0.5% SDS at 27°C for 2 h with three changes of wash solution.

In vitro runoff transcription. A reaction mixture containing 40% glycerol, 80 mM Tris (pH 8.0), 100 mM KCl, 20 mM $MgCl_2$, 80 mM 2-mercaptoethanol, 0.2 mM EDTA, and 0.5 mM each ATP, GTP, CTP, and UTP was prepared. To this mixture were added 3 μCi of [α-^{32}P]ATP (Amersham), 3 U of RNA polymerase (New England BioLabs, Inc., Beverly, Mass., or Pharmacia, Uppsala, Sweden), and 0.5 μg of template DNA previously digested with an appropriate restriction enzyme. The transcription reaction, in a final volume of 50 μl, was carried out at 37°C for 30 min. An equal volume of a solution of 50 mM EDTA and 200 μg of tRNA per ml was then added to each sample, and the RNA was precipitated with 2.5 volumes of ethanol. The transcripts were separated on a 0.5% agarose–5% acrylamide–0.14% bisacrylamide gel run in buffer containing 50 mM Na_2HPO_4 (pH 6.8), 0.1% SDS, and 2 mM EDTA.

DNA sequencing. DNA sequencing was performed by the dideoxy chain termination method (21) with ordered deletions generated by exonuclease III digestion (8). Computer analysis of the DNA sequences was performed with the Genetic Computer Group Sequence Analysis Software Package (5).

RESULTS

Sequence analysis. The 5′ end of the rRNA operon of *M. pneumoniae* was sequenced from both strands extending from approximately 300 base pairs upstream of the start of the 16S rRNA to approximately 270 base pairs within the 16S rRNA. The noncoding, RNA-like strand is shown in Fig. 1. Within this region, 72% of the *M. pneumoniae* 16S rRNA nucleotides are homologous to those of the same region in the *M. capricolum* rRNA operons (10). The upstream regions are totally divergent, except for the 16S rRNA processing sequence (designated "stem" in Fig. 1), which was found here, in *Bacillus subtilis* (16), in *Mycoplasma* sp. strain PG50 (17), in *M. hyopneumoniae* (24), and in *M. capricolum* (10). In these other organisms this sequence was shown to be able to form a stem with a complementary sequence located between the 16S and 23S rRNA genes.

Primer extension mapping of *M. pneumoniae* transcripts. To identify possible precursor rRNA transcripts, we synthesized three different primers, marked on the sequence in Fig. 1, and used them in primer extension experiments. Two of these primers, consisting of nucleotides −38 to −54 and −112 to −132, did not detect any transcripts when total RNA of *M. pneumoniae* was used. An experiment with total *M. pneumoniae* RNA and the third primer, extending from

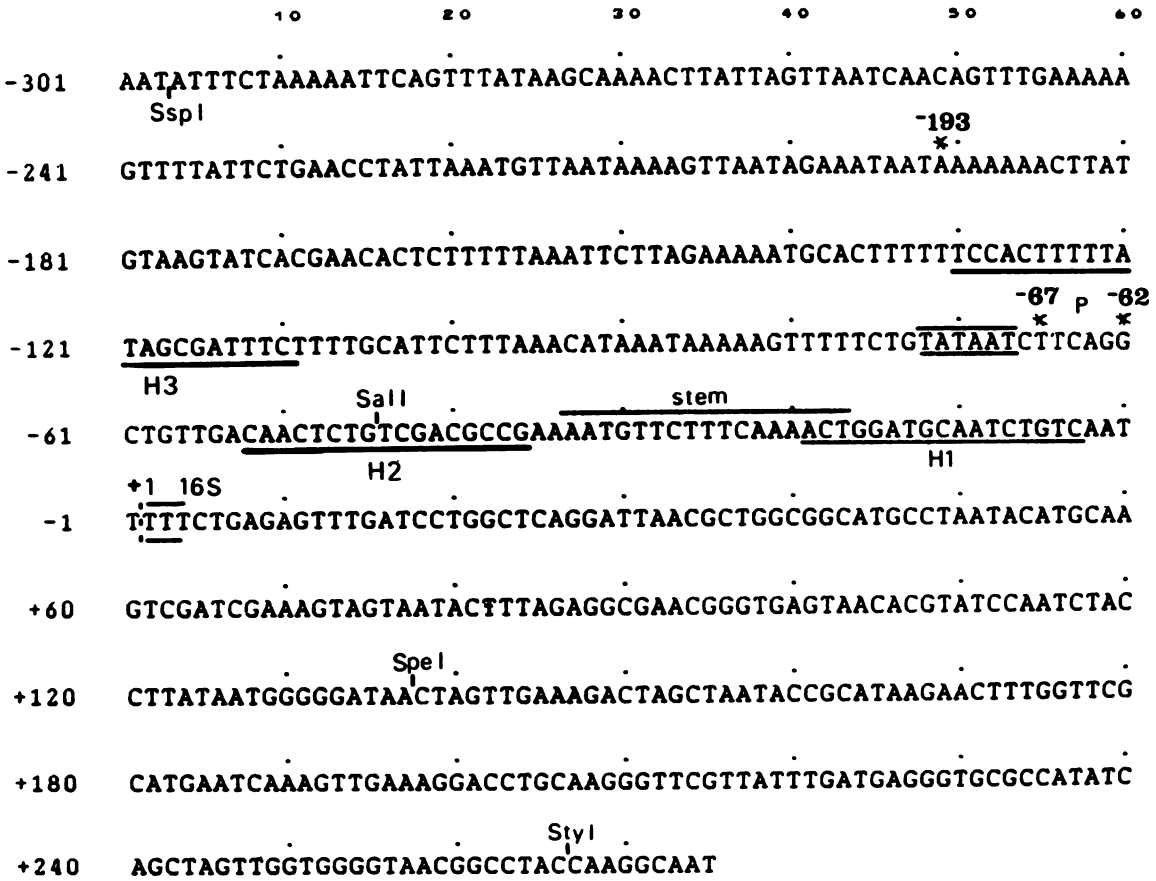

FIG. 1. Nucleotide sequence of the 5′ end of the *M. pneumoniae* rRNA operon. The noncoding, RNA-like strand is shown. The nucleotides are numbered from the start site of the 16S rRNA gene designated +1. Mapped start sites are marked by asterisks and by nucleotide numbers. A putative Pribnow box is marked by a line above and below the sequence (TATAAT). H1, H2, and H3 are the complementary strands of primers synthesized for primer extension analysis. The sequence designated as "stem" is part of the 16S rRNA processing sequence discussed in the text. The start site at −193 is the 5′ end of the transcript detected by S1 nuclease (see Fig. 4); the start site at −67 is the 5′ end of the transcript produced by *E. coli* RNA polymerase in vitro (see Fig. 5); the start site at −62 is the 5′ end of the transcript determined by primer extension (see Fig. 2).

nucleotides −5 to −21, produced one prominent band (Fig. 2, lane 1). This cDNA fragment was run next to the sequence determined with the same primer so that the location of the band on the sequence could be easily identified. The endpoint of this fragment falls 62 nucleotides upstream from the start of the 16S rRNA (Fig. 1). No other transcripts extending further upstream could be detected in these experiments. Repeated primer extension experiments with RNA isolated from *E. coli* cells carrying the pPR10 plasmid failed to reveal any specific transcripts.

Dot blot hybridization. To verify the amounts of RNA transcribed from each DNA region, we used the three oligonucleotide primers described above (Fig. 1) as probes in dot blot filter hybridizations with total *M. pneumoniae* RNA. Hybridization of each of the three primers to pPR10 DNA and to pPR3 DNA (see Materials and Methods) served as positive and negative controls, respectively. The intensity of hybridization of primer H1 to total *M. pneumoniae* RNA, assessed by the size and intensity of the spots obtained with different concentrations of RNA, exceeded that obtained with primer H2 by more than 10-fold, while the intensity of hybridization of primer H3 to *M. pneumoniae* RNA was less than 10% that of primer H2 (Fig. 3).

S1 nuclease mapping. Products of S1 nuclease mapping experiments with total *M. pneumoniae* RNA hybridized to the 5′-end-labeled DNA probe shown in Fig. 4 were run next to a known sequence to accurately map the resulting protected DNA fragments. These experiments, one of which is shown in Fig. 4, produced a protected DNA fragment extending 193 nucleotides upstream from the 16S rRNA start site (marked with an asterisk on the sequence in Fig. 1). A longer probe whose 5′ end extended into the 16S rRNA gene was also protected by a transcript extending upstream to this site.

In vitro transcription. The promoter region of the rRNA operon contained on an *Eco*RI-*Hin*dIII restriction fragment (Fig. 5) was cloned into the plasmid vector pGC14 upstream of a promoterless *galK* gene (14). This resulted in a Gal$^+$ phenotype and allowed the analysis of runoff transcripts produced by *E. coli* RNA polymerase in the presence of α-^{32}P-labeled ribonucleotides, with no interference from vector transcripts. The resulting clone (pGRP5) was digested with restriction enzymes known from previous mapping (9) to cut within the desired region; the transcripts that were produced were separated on gels with marker fragments or an M13 sequence (Fig. 5) to map the transcript start site. No transcripts could be detected when vector pGC14 by itself was uncut or digested with *Hin*dIII. Additional controls, including undigested pGRP5 and pGRP5 digested with *Eco*RI, which cuts upstream of the promoter region, yielded

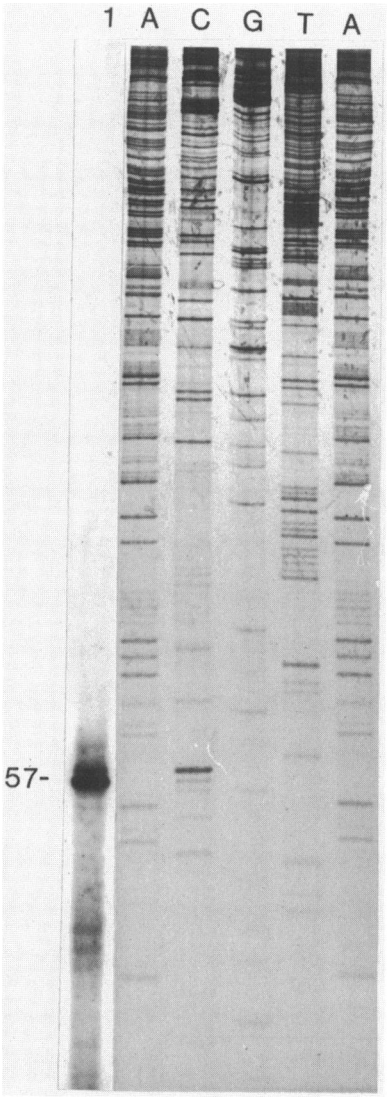

FIG. 2. Primer extension analysis of *M. pneumoniae* transcripts synthesized in vivo. Analysis was carried out with the H1 primer (marked in Fig. 1) hybridized to total *M. pneumoniae* RNA. The DNA fragments resulting from reverse transcription (as described in Materials and Methods) were run next to the sequence (ACGTA) of the same region (cloned on an M13 vector) produced with the same primer. This allowed the accurate location of the 5′ end of the RNA transcript. The number 57 denotes the nucleotide length of the cDNA. The gel was 12% polyacrylamide (acrylamide-bisacrylamide, 20:1). A similar experiment carried out in the absence of RNA produced no detectable bands in this region.

no transcripts (results not shown). However, transcripts were produced when pGRP5 was digested with *Sty*I, *Spe*I, and *Hin*dIII. The shorter transcripts produced by digestion with *Spe*I and *Sty*I (Fig. 5, lanes 1 and 2, respectively) are shown next to the known sequence of M13 mp18. This allowed the accurate assignment (within a few nucleotides) of a single *E. coli* in vitro transcript start site at the point marked "P" in Fig. 5 and on the sequence in Fig. 1. It is 202 nucleotides from *Spe*I and 331 nucleotides from *Sty*I and is within a few nucleotides of the start site detected by primer extension analysis.

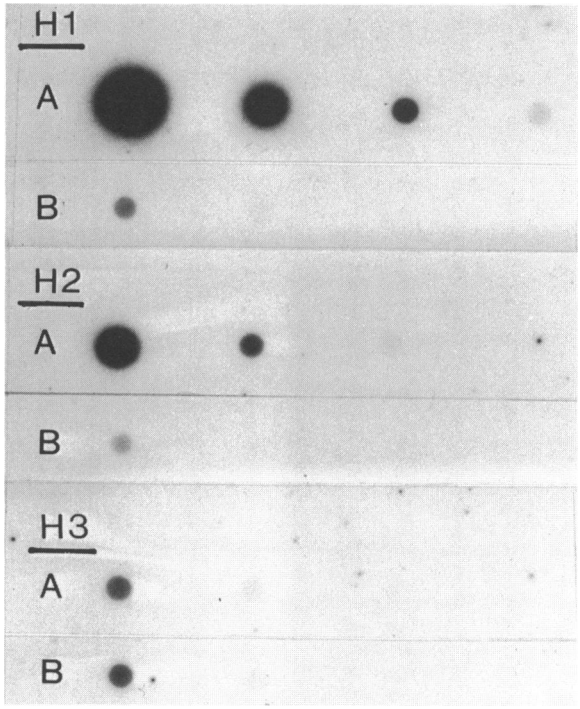

FIG. 3. Hybridization of total *M. pneumoniae* RNA with the three different oligonucleotide probes (H1, H2, and H3) shown in Fig. 1 (series A) as compared with hybridization of the same probes with plasmid pPR10, which includes the entire DNA sequence shown in Fig. 1 (series B). The RNA concentrations (from left to right) in series A were 50, 5, 0.5, and 0.05 μg, and those of plasmid DNA (from left to right) in series B were 10, 1, and 0.1 ng.

DISCUSSION

The analysis presented here has mapped the 5′ end of a precursor rRNA species of *M. pneumoniae*. This species, whose 5′ end is labeled "P" on the sequence in Fig. 1, was detected by primer extension analysis of *M. pneumoniae* RNA as well as by in vitro transcription with a purified plasmid clone and RNA polymerase of *E. coli*. The dot blot filter hybridization experiments provided additional information regarding transcription from this operon. The intensity of hybridization of *M. pneumoniae* RNA to oligonucleotide H1 as compared with that to oligonucleotide H2 indicated that most of the RNA transcripts reaching H1 do not extend upstream to H2. The presence of the processing stem between these two oligonucleotides may explain this observation. As in *B. subtilis* and other mycoplasmas (10, 16, 17, 24), the *M. pneumoniae* rRNA precursor may be processed at this site. We found that the quantity of transcripts hybridized to H2 was at least 10 times greater than that hybridized to H3, an oligonucleotide located 80 nucleotides further upstream. The most likely explanation is that the promoter for rRNA synthesis is located between H2 and H3, supporting the location of the promoter (P) indicated by primer extension analysis (Fig. 1). The very low level of hybridization of total RNA to the H3 oligonucleotide may represent upstream transcription which is either terminated or processed at a site preceding H2. However, one cannot eliminate the possibility that there are remnants of *M. pneumoniae* DNA in the RNA preparation, even after DNase I treatment, which are responsible for the weak hybridization observed with H3. In any case, primer extension experiments and in vitro transcription with *E. coli* RNA

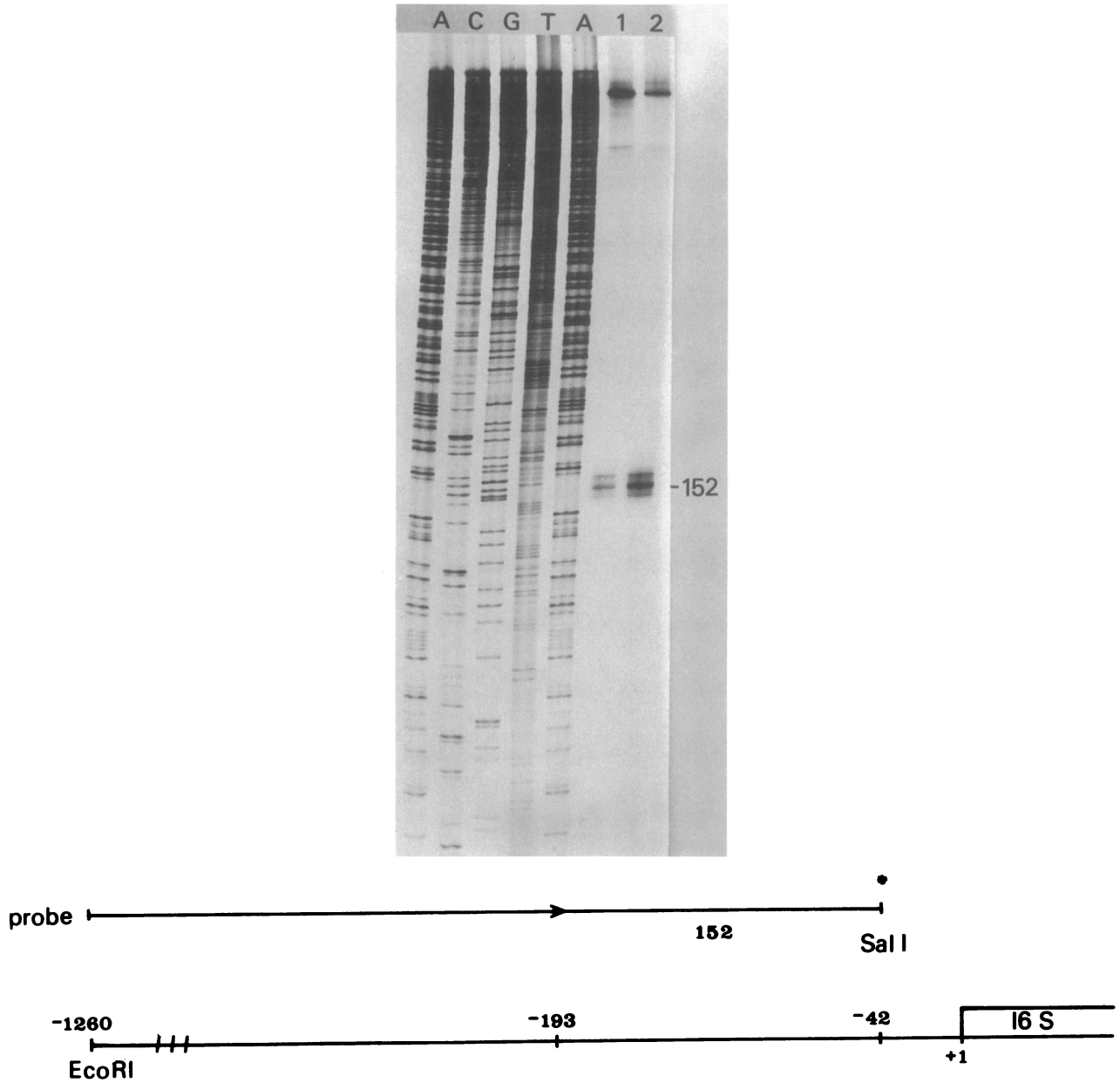

FIG. 4. S1 nuclease mapping of the 5′ ends of transcripts originating from the 5′ end of the *M. pneumoniae* rRNA operon. S1 nuclease mapping was carried out with the *Eco*RI-*Sal*I probe (shown at the bottom of the figure) 5′ end labeled at the *Sal*I site (*) and hybridized to total RNA of *M. pneumoniae*. The DNA fragments protected from S1 nuclease were run on a sequencing gel (lanes 1 and 2) next to a known sequence (ACGTA) for accurate size measurement (152 nucleotides from the labeled end). A similar experiment carried out in the absence of RNA produced no detectable bands.

polymerase did not reveal any RNA species whose 5′ ends extend beyond P.

S1 nuclease mapping experiments with pPR10 DNA fragments protected by *M. pneumoniae* RNA revealed the presence of a transcript whose 5′ end extended to nucleotide −193 (Fig. 1 and 4). Hence, the possibility that there is another promoter whose transcript is either initiated or processed at a site −193 nucleotides from the 16S rRNA gene should be considered. However, transcript initiation from this site appears unlikely because there is no resemblance to a −10 consensus sequence in the required location. Promoters identified in mycoplasmas so far have shown typical procaryotic −10 consensus sequences (6, 17, 18, 23, 24). This fact and the fact that the other three methods all failed to reveal a transcript starting at −193 lead us to interpret the S1 nuclease results as an artifact.

In the sequence analysis carried out by Rasmussen et al. (17) on *Mycoplasma* sp. strain PG50, each of the two operons of this organism was preceded by a single putative promoter sequence, detected by computer analysis, which largely fitted the consensus sequences for *E. coli* and *B. subtilis* promoters. However, in *B. subtilis* and *E. coli* there are dual promoters for each of the rRNA operons (4, 22). The two rRNA operons of *M. capricolum* were recently found to correspond closely to the rRNA operons of *Mycoplasma* sp. strain PG50 (6; C. Taschke and R. Herrmann, personal communication). S1 nuclease and primer extension analyses of RNA transcripts of the *rrnA* operon of *M.*

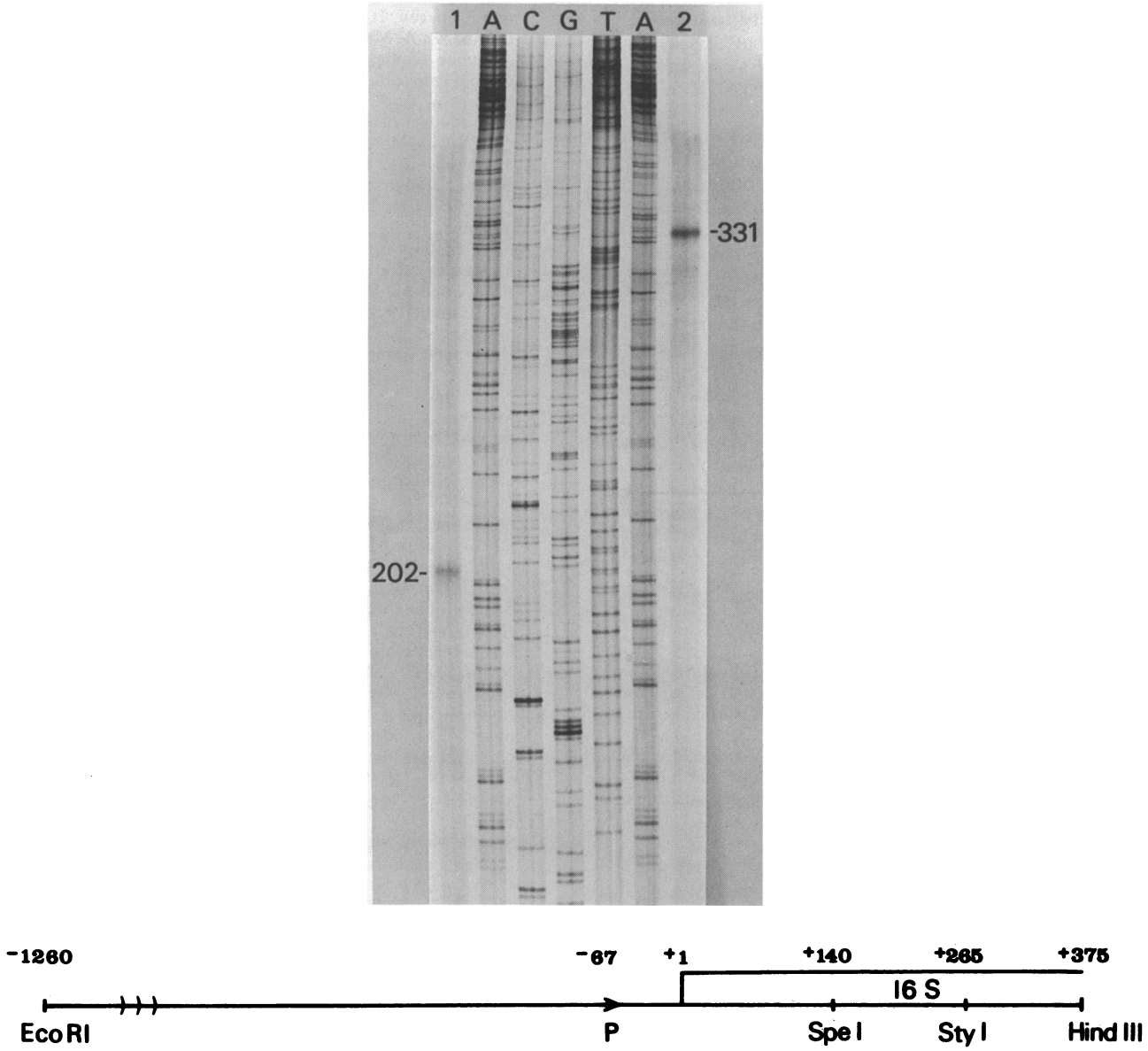

FIG. 5. Mapping transcripts of the 5′ end of the *M. pneumoniae* rRNA operon with *E. coli* RNA polymerase. Transcripts produced by the RNA polymerase on the templates shown at the bottom of the figure were run next to the sequence of M13 mp18 (ACGTA) for size comparison. *Spe*I digestion of the template before transcription resulted in the 202-nucleotide product seen in lane 1, and the *Sty*I-digested template produced the 331-nucleotide transcript seen in lane 2. The start site deduced from these two runoff transcripts is marked "P" on the diagram and on the sequence shown in Fig. 1.

capricolum indicated the presence of a promoter about 140 nucleotides upstream from the 16S rRNA 5′ end, followed upstream by two tRNA genes preceded by another promoter about 450 nucleotides upstream from the 16S rRNA gene. This structure closely resembles that found for the *rrnA* operon of *Mycoplasma* sp. strain PG50 (17). In vivo experiments indicated that the *rrnB* operon is transcribed only from one promoter, although a second promoter could be identified with the *M. capricolum* cell extract (C. Taschke and R. Herrmann, personal communication). All of the above promoters have the typical −10 and −35 boxes, in agreement with the consensus sequences of *E. coli* promoters.

The single rRNA operon of *M. hyopneumoniae* was found by Taschke and Herrmann (24) to be transcribed from two promoters 87 and 187 nucleotides upstream from the 16S rRNA start site (24). S1 nuclease and primer extension mapping of the RNA transcripts initiated at the 5′ end of this operon indicated that the promoter situated nearer the structural genes (P2) is the predominant one. Both promoters have Pribnow −10 consensus sequences; however, their −35 regions bear no resemblance to consensus −35 recognition sequences. Instead, the −35 regions are very A+T rich. In the *M. pneumoniae* rRNA operon studied here, the promoter is only 62 to 67 nucleotides upstream from the 16S rRNA start site. This location is similar to that of the P2 promoter in *M. hyopneumoniae*. In addition, the sequences of these two promoters are similar in that both have a −10 consensus sequence with an A+T-rich upstream region. In our study, the *M. pneumoniae* initiation site was also shown to function as a promoter for *E. coli* RNA polymerase in vitro. However, the absence of a defined −35 recognition

region may be responsible for our inability to detect transcripts in *E. coli* in vivo. Suggestions that at least some mycoplasmal promoters can function in *E. coli* have been made (6, 11, 15, 27). In sum, it appears impossible to deduce generalizations from the available data, possibly reflecting the diversity of *Mollicutes* species.

An interesting illustration of diversity among the mycoplasmas is obtained on comparing the nucleotide sequence of part of the *M. pneumoniae* 16S rRNA gene sequenced by us with the sequences of the homologous parts of the 16S rRNA genes of *M. capricolum* (10) and *M. hyopneumoniae* (25). Within this region 72% of the *M. pneumoniae* sequence is homologous to the *M. capricolum* sequence and 71% of the *M. pneumoniae* sequence is homologous to the *M. hyopneumoniae* sequence. For comparison, the results of Iwami et al. (10) show that the 16S rRNA sequence of *M. capricolum* is 68% homologous with the 16S rRNA sequence of *E. coli* and 64% homologous with that of *Anacystis nidulans*. Thus, it appears that the difference between the sequences of the 5′ parts of the 16S rRNA genes of *M. pneumoniae* and *M. capricolum* or *M. pneumoniae* and *M. hyopneumoniae* is only slightly smaller than that between the *M. capricolum* and *E. coli* sequences, demonstrating, perhaps, the evolutionary breadth of the class *Mollicutes* attributed to this class being in a state of rapid evolution (30).

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