JOURNAL OF BACTERIOLOGY, July 1985, p. 155–166
0021-9193/85/070155-12$02.00/0

Vol. 163, No. 1

Organization and Nucleotide Sequence Analysis of an rRNA and tRNA Gene Cluster from *Caulobacter crescentus*

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Received 19 February 1985/Accepted 16 April 1985

rRNA genes of *Caulobacter crescentus* CB13 were isolated and shown to be present in two gene clusters in the genome. The organization of each rRNA gene cluster was found to be 5′-16S-tRNA spacer-23S-5S-3′. The DNA sequence of 40% of the 16S rRNA gene, the entire 16S/23S intergenic spacer region, and portions of the 23S rRNA gene were determined. Analysis of the nucleotide sequence in the 16S-23S intergenic spacer region revealed the presence of $tRNA^{Ile}$ and $tRNA^{Ala}$ genes. Large invert repeat sequences were found surrounding the 16S rRNA gene. These inverted repeat sequences are analogous to the RNase III-processing sites in the *E. coli* rRNA precursor. Small invert repeat sequences were also found flanking the individual tRNA genes. RNA polymerase-binding studies with restriction fragments of the rRNA gene cluster revealed three regions which bound enzyme, and these regions were shown to contain transcription initiation sites. One of these sites was located within the 16S gene near its 3′ end, and the other two were found at the 5′ end of the 23S gene.

Caulobacter crescentus is a gram-negative eubacterium that undergoes a series of obligate differentiation events (13). During each cell cycle a motile swarmer cell differentiates into a sessile stalked cell. The stalked cell begins to elongate, and at a precise time in the cell cycle a flagellum and pili are assembled at the pole opposite the stalk. The cell then divides, yielding the stalked cell and a motile swarmer cell. Specific biochemical events, such as DNA replication and flagellin synthesis, are confined to certain cell types. A study was initiated to isolate and characterize rRNA genes in *C. crescentus*. The rRNA genes have been most extensively studied in *Escherichia coli*, in which they have been shown to be organized in operons, each of which is transcribed in the order 5′-16S-23S-5S-3′ (27, 29). The transcription of this polycistronic message appears to be influenced by the rate of growth in *E. coli* (19, 29). *C. crescentus* provides an excellent system in which to study the regulation of rRNA and tRNA gene expression because (i) there appear to be only two rRNA gene clusters per chromosome (30; this paper) and (ii) it has a defined cell cycle with biochemically distinct cell types (13).

We report here the isolation of the *C. crescentus* CB13 rRNA genes and show that they reside in only two rRNA gene clusters. The nucleotide sequence of 40% of the 16S gene, the region 5′ to the start of the gene, and portions of the 23S gene have also been determined. In addition, the spacer region between the 16S and 23S genes was sequenced and found to encode $tRNA^{Ile}$ and $tRNA^{Ala}$. Examination of the nucleotide sequence revealed the presence of potential RNase III-processing sites flanking the 16S rRNA gene. Furthermore, transcription initiation sites were detected within the cloned rRNA gene cluster by RNA polymerase binding assays and in vitro transcription.

MATERIALS AND METHODS

Bacterial strains. *C. crescentus* CB13 was grown at 30°C in either PYE (peptone-yeast extract) broth (32) or in minimal broth (PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]) with 0.2% glucose as a carbon source (11).

Restriction enzyme digestion of DNA and DNA sequencing. Restriction digests were carried out under conditions described by Davis et al. (12). Supercoiled plasmid DNA (2 to 3 U/μg; Boehringer Mannheim), 1 U of restriction enzyme per μg of λ DNA, and 5 to 10 U of restriction enzyme per μg of chromosomal DNA were used. DNA was sequenced by the method of Maxam and Gilbert (26), with the following modification. For the G+A reaction, 25 μl of formic acid was used for 4 min, and the reaction was stopped with the hydrazine stop buffer and continued as for the other reactions.

Isolation of rRNA. *C. crescentus* CB13 was grown in 100 ml of either PYE or PIPES medium to an optical density at 660 nm of 0.4 to 0.75. The cells were collected by centrifugation and suspended in 3 ml of 25% sucrose (10 mM Tris-hydrochloride, pH 8). To this suspension, 0.3 ml of 10 mg of lysozyme per ml (0.25 M Tris-hydrochloride, pH 8) and 0.3 ml of 0.05 M EDTA (pH 8) were added and incubated on ice for 5 min. The cell suspension was then mixed with 0.3 ml of 7.5% Brij 35 in 10 mM Tris-hydrochloride (pH 7.2), 0.3 ml of 100 mM $MgCl_2$, 0.3 ml of 2% sodium deoxycholate in 100 mM Tris-hydrochloride (pH 8), and 0.06 ml of DNase I (5 mg/ml) and incubated for 10 min on ice. After this, 60 μl of diethylpyrocarbonate was added, and the disrupted cells were removed by centrifugation. The supernatant was collected and centrifuged in a type 40 rotor (Beckman Instruments) at 38,000 rpm for 2.5 h to pellet the ribosomes. The ribosomal pellet was suspended in 20 mM sodium acetate (pH 5.5)–0.5% sodium dodecyl sulfate–1 mM EDTA and then extracted twice with phenol at 65°C for 10 min (1). The rRNA was then precipitated with three volumes of cold 100% ethanol and 0.3 M sodium acetate. After the pellet was dried under vacuum, the rRNA was suspended in water and labeled with [γ-^{32}P]ATP as described by Maxam and Gilbert (26). ^{32}P-labeled 23S and 16S rRNA were separated on a preparative 2% acrylamide–0.5% agarose gel prepared and run as described by Peacock and Dingman (31). Labeled 5S and 4S RNA were separated on 6% acrylamide gels. The gels were exposed to Kodak X-Omat film to identify the RNA

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bands. Each RNA band was then cut out of the gel and eluted into 20 mM sodium acetate (pH 5.2)–1 mM EDTA for 18 to 25 h at room temperature. The eluted RNA samples were then extracted twice with phenol saturated with 20 mM sodium acetate (pH 6.2)–1 mM EDTA and precipitated in 3 to 4 volumes of 100% ethanol containing 0.3 M sodium acetate. The purified RNA species were then collected by centrifugation in a SW41 rotor (Beckman Instruments) at 4°C for 30 min at 35,000 rpm and dried under vacuum. The RNA was labeled in vitro with [γ-^{32}P]ATP as described by Maxam and Gilbert (26).

Isolation of *C. crescentus* CB13 rDNA clones. The λWES · B/CB13 clone bank was prepared by L. Shapiro and A. Skalka at the Roche Institute of Molecular Biology by the method of Maniatis et al. (24). *C. crescentus* CB13 DNA was prepared as described previously (34), partially restricted with *Eco*RI endonuclease (Bethesda Research Laboratories), and ligated to λWES · B arms which had been treated with *Eco*RI and purified by sucrose gradient centrifugation. The recombinant phage were prepared using the in vitro packaging technique (8). ^{32}P-labeled *C. crescentus* CB13 RNA was used to identify rRNA sequences by the method of Benton and Davis (6); plaques containing DNA which hybridized to the RNA probe were isolated, and the phage were eluted with dilution buffer (12). The various rDNA fragments were then individually subcloned into pBR325 with T4 DNA ligase (Bethesda Research Laboratories), and the hybrid plasmids were then transformed into *E. coli* HB101 as previously described (25). Transformants were selected for tetracycline resistance (Tc^r) and screened for chloramphenicol sensitivity (Cm^s) at drug concentrations of 25 μg/ml. A total of 15 hybrid plasmids containing *C. crescentus* rDNA sequences were isolated, and plasmid DNA was prepared as described by Clewell and Helinski (10).

Southern blots and hybridization. Agarose gels containing plasmid DNA and chromosomal DNA that had been treated with restriction enzymes (Boehringer Mannheim) were transferred to nitrocellulose filters as described by Southern (37). The filters were incubated in a buffer containing 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 50% formamide, 0.02 M Tris-hydrochloride buffer (pH 7.4), and 0.5% sodium dodecyl sulfate and then hybridized with the probe for 36 to 48 h at 37°C in the same buffer. The probe, in vitro-labeled [^{32}P]rRNA (specific activity, ~10^5 cpm/μg), or nick-translated plasmid (specific activity, ~10^7 cpm/μg) was in a solution of 50% formamide–5 μg of yeast tRNA carrier per ml. After hybridization, the filters were washed in 0.2× SSC–0.2% sodium dodecyl sulfate for 2 to 4 h at 37°C with shaking, rinsed extensively with 0.2× SSC, incubated with pancreatic RNase A (Worthington) in 0.2× SSC at 37°C with shaking for 30 min in the case of RNA probe, and washed in 0.2× SSC for 1 h. The filters were then dried and exposed to Kodak X-Omat film with intensifying screen at −70°C for 1 to 7 days.

Determination of the 3′ end of the *C. crescentus* 16S rRNA gene by nuclease S1 mapping with in vivo 16S rRNA. S1 mapping (7) was used to determine the 3′ end of the 16S rRNA, with 16S rRNA isolated as described above. A 3-μg amount of 16S rRNA was ethanol precipitated in the presence of 0.03 μg of labeled DNA probe (see legend to Fig. 4) and 25 μg of yeast tRNA. The pellet was suspended in 30 μl of hybridization buffer (80% formamide, 400 mM NaCl, 40 mM PIPES [pH 6.4] 1 mM EDTA), heated to 72°C for 10 min, and then incubated at 60°C for 3 h. The reaction mixture was then diluted 10-fold with S1 digestion buffer (50 mM NaAc [pH 4.6], 280 mM NaCl, 4.5 mM $ZnSO_4$, 20 μg of denatured calf thymus DNA) containing 2,500 U of S1 nuclease (Boehringer Mannheim) and incubated at 37°C for 30 min. Carrier tRNA (10 μg) was added, and the nucleic acid was precipitated with ethanol. The precipitate was suspended in 95% formamide–1 mM EDTA and tracking dyes, heated at 90°C for 90 s, quick-cooled at 0°C, and electrophoresed through a 7 M urea–6% polyacrylamide gel.

Transcription assays. The *C. crescentus* and *E. coli* RNA polymerases were prepared as previously described (3). The transcription reaction mixture (50 μl) contained, unless stated otherwise, 40 mM Tris-hydrochloride (pH 8.0), 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 2 mM spermidine, 0.5 mM ATP, GTP, and UTP, 0.1 mM [α-^{32}P]CTP (800 to 5,000 cpm/pmol), and DNA and enzyme as indicated. The enzyme and DNA was incubated for 10 min at 37°C before the addition of ribonucleotide triphosphates and heparin (50 μg/ml). The reaction mixture was then incubated for 15 min at 37°C before the reaction was stopped with 10 μl of 10% sodium dodecyl sulfate. After addition of 50 μl of TE buffer (10 mM Tris-hydrochloride (pH 8) with 0.1 mM EDTA), the reaction mixtures were extracted twice with phenol, and the RNA was precipitated with ethanol in the presence of 20 μg of yeast tRNA as carrier. The RNA was rinsed once with 100% ethanol, reprecipitated with 0.5 ml of 70% ethanol, and dried for 15 min under vacuum. The final RNA pellet was suspended in 25 to 50 μl of TE and 5 to 10 μl of RNA stop mix (2). The amount of acid-precipitable counts incorporated into RNA was determined for a 2.5- or 5-μl sample. Equal amounts of acid-precipitable counts were then applied to a 2% polyacrylamide–0.5% agarose composite slab gel (13 by 25 cm) containing 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA. Conditions for electrophoresis and autoradiography were as described by Amemiya and Shapiro (2).

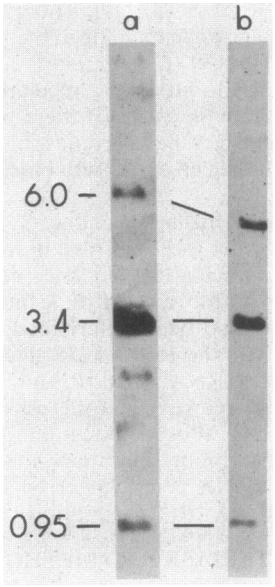


FIG. 1. Southern blots of the *C. crescentus* CB13 genome digested with *Eco*RI and probed with total cellular RNA or rRNA. The DNA was electrophoresed through a 0.7% agarose horizontal gel. The sizes of the DNA fragments were determined by comparison with the migration of λ DNA digested with *Hin*dIII. Total cellular RNA (lane a) and rRNA (lane b) were labeled in vitro as described in the text.

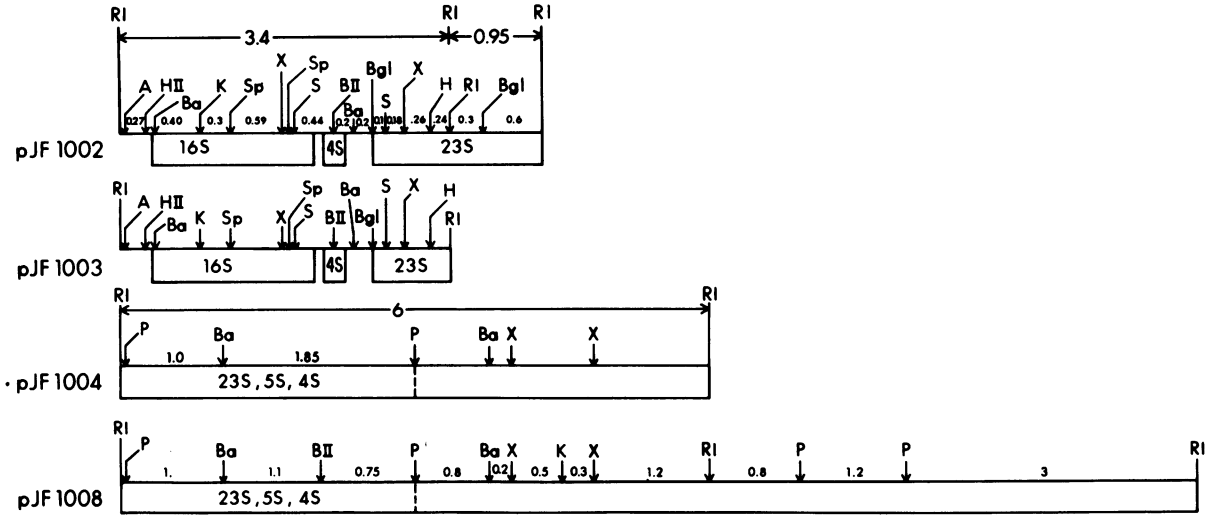


FIG. 2. Restriction maps of ribosomal DNA fragments in the subclones pJF1002, pJF1003, pJF1004, and pJF1008. The regions that encode the various rRNA and tRNA species are identified and are discussed in later figure legends. A, *Ava*I; Ba, *Bam*HI; *Bgl*, *Bgl*I; BII, *Bgl*II; RI, *Eco*RI; HII, *Hinc*II; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sma*I; Sp, *Sph*I; X, *Xho*I. pJF1002 and pJF1003 contain 3.4-kb *Eco*RI fragments that are identical, as far as can be determined by the restriction enzymes used here. The 6-kb fragment of pJF1008 has a *Kpn*I site and a *Bgl*II site not observed on the 6-kb fragment of pJF1004.

RESULTS

Isolation of the *C. crescentus* CB13 rRNA genes. Restriction fragments of *C. crescentus* CB13 chromosomal DNA containing rRNA genes were identified by hybridization of in vitro-labeled RNA to Southern blots of *Eco*RI digests of the genome. Four bands of homology at 0.95, 2.8, 3.4, and 6 kilobases (kb) were observed when total cellular RNA was used as probe (Fig. 1, lane a). rRNA isolated from purified ribosomes was found to hybridize to bands of 0.95, 3.4, and 6 kb (Fig. 1, lane b). The relatively abundant RNA encoded by the 2.8-kb *Eco*RI fragment has not been identified.

The *Eco*RI fragments carrying portions of the rRNA genes were isolated from a λWES · B/CB13 clone bank and subcloned into pBR325, as described above. The three *Eco*RI fragments of rRNA genes identified in chromosomal Southern blots, 0.95, 3.4, and 6 kb, were found grouped in the pBR325 subclones pJF1002, pJF1003, pJF1004 and pJF1008. Restriction maps of these subclones are shown in Fig. 2.

pJF1002 and pJF1003 each contain a 3.4-kb *Eco*RI fragment which have identical restriction maps. pJF1002 also contains a 0.95-kb *Eco*RI fragment. pJF1004 and pJF1008 each contain a 6-kb *Eco*RI fragment which have some differences in their restriction maps (Fig. 2). The pJF1008 fragment contains a *Bgl*II site and a *Kpn*I site not seen on the 6-kb fragment in pJF1004. Additionally, pJF1008 has a 5-kb *Eco*RI fragment which may have been randomly ligated to the 6-kb fragment during cloning or subcloning or which may be adjacent to the 6-kb fragment in vivo. This fragment did not hybridize to either the total cellular RNA or the rRNA probes.

Order of the rRNA and tRNA genes. Stable RNA species of 23S, 16S, and combined 5S and 4S were isolated and labeled at the 5′ end as described above. Each was then hybridized to separate Southern blots of pJF1002, pJF1003, pJF1004, and pJF1008 DNA which had been digested with various restriction enzyme combinations (Fig. 3). The 16S rRNA probe hybridized to the 2.2-kb *Eco*RI-*Bgl*II fragment but not to the 1.2-kb *Bgl*II-*Eco*RI fragment nor to the 0.95-kb *Eco*RI fragment of pJF1002 (Fig. 3A, panel a). The 16S rRNA probe also hybridized to the 1.65-kb *Eco*RI-*Xho*I fragment and to the 0.5-kb *Xho*I-*Bgl*II fragment of both pJF1002 and pJF1003, but it did not hybridize to the 6-kb *Eco*RI fragments of pJF1004 and pJF1008 (data not shown). The 23S rRNA probe hybridized to the 1.2-kb *Bgl*II-*Eco*RI fragment of pJF1002 and pJF1003, the 0.95-kb *Eco*RI fragment of pJF1002, and the 2.9-kb *Pst*I fragment of pJF1004 and pJF1008 (Fig. 3A, panel b). The 5S rRNA + 4S tRNA probe hybridized the 0.9-kb *Xho*I-*Bgl*I fragment of pJF1002 (Fig. 3A, panel c) and pJF1003 (data not shown) but not to the adjoining 0.3-kb *Bgl*I-*Xho*I fragment. This probe also hybridized to the 2.9-kb *Pst*I fragment of pJF1004 and pJF1008 (Fig. 3A, panel c). A block diagram of the restriction fragments that hybridized to each RNA species is shown in Fig. 3B. DNA sequence analysis described below allowed the precise assignment of the genes to the different regions of the cloned DNA (Fig. 4B). The restriction maps and the gene organization suggest that there are two types of rRNA gene sets. Both contain the same 3.4- and 0.95-kb *Eco*RI fragments which encode the 16S rRNA gene, the intergenic spacer, and part of the 23S rRNA gene. The remainder of the 23S rRNA gene can be accounted for on either of two 6-kb *Eco*RI fragments which have different *Bgl*II and *Kpn*I restriction sites (Fig. 2 and 4B).

Number of rRNA gene sets in the *C. crescentus* CB13 genome. The number of rRNA gene sets in the genome can be determined by hybridizing an rRNA probe to chromosomal DNA which had been digested with restriction enzymes with known cleavage sites within the gene set. The restriction enzyme cleavage sites that are contiguous with, but outside of, the gene set will likely be different for each gene set. Therefore, the number and size of chromosomal restriction fragments which hybridize to an rRNA probe should reflect the number of gene sets in the genome.

Southern blots of *C. crescentus* CB13 chromosomal DNA digested with various restriction enzymes and probed with purified rRNA are shown in Fig. 4A. Lane 1 is an *Eco*RI digest of chromosomal DNA that shows the rRNA probe hybridizing to 6-, 3.4-, and 0.95-kb restriction fragments.

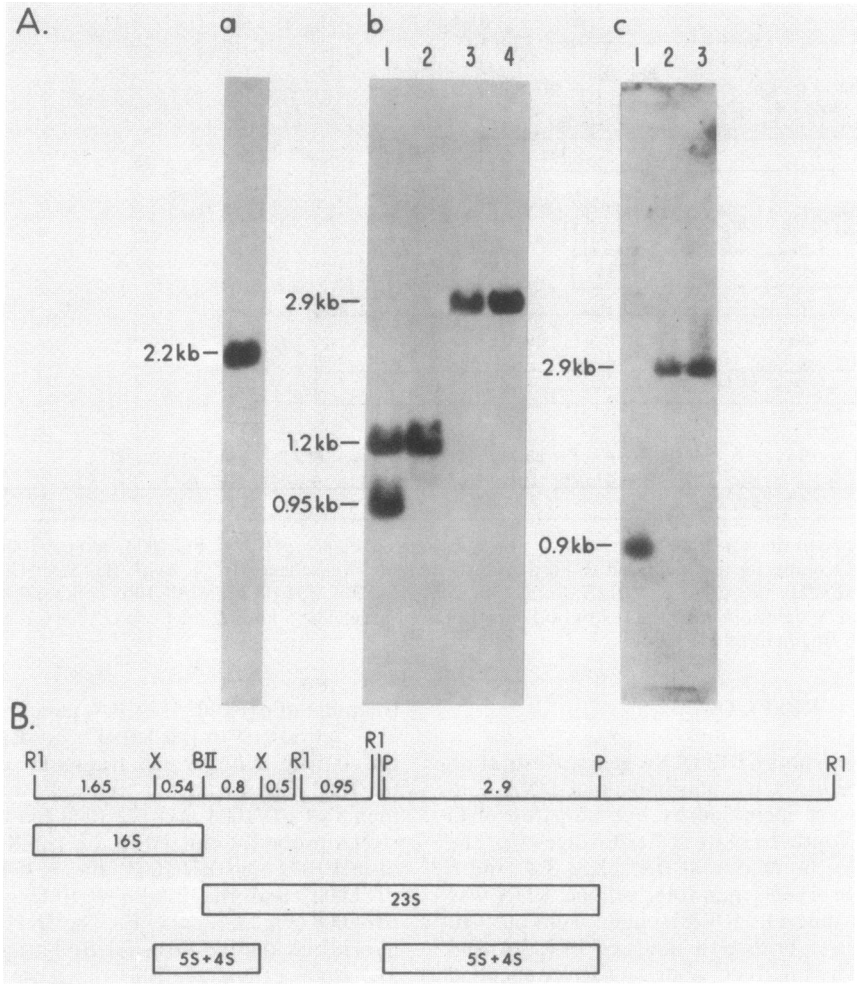


FIG. 3. Hybridization of rRNA and tRNA probes to restriction digests of ribosomal DNA subclones. (A) Southern blots of the rRNA subclones digested with various restriction enzymes and hybridized with rRNAs labeled in vitro with (a) 16S rRNA, (b) 23S rRNA, and (c) 4S + 5S rRNA. Panel a contains pJF1002 DNA digested with *Bgl*II and *Eco*RI. Panel b contains (lanes): 1, pJF1002 digested with *Bgl*II and *Eco*RI; 2, pJF1003 digested with *Bgl*II and *Eco*RI; 3, pJF1004 digested with *Pst*I; 4, pJF1008 digested with *Pst*I. Panel c contains (lanes): 1, pJF1002 digested with *Bgl*I and *Xho*I; 2, pJF1004 digested with *Pst*I; 3, pJF1008 digested with *Pst*I. The blots were exposed to X-ray film with an intensifying screen for 2 h at −70°C. (B) Partial restriction map of the 3.4-, 0.95-, and 6-kb *Eco*RI fragments aligned as if they are contiguous. The regions that hybridized to the probes, as identified in the first part of this figure, are shown schematically as blocks below the map.

There was only one *Hin*dIII site in either type of operon so that two chromosomal *Hin*dIII fragments would be expected to hybridize to the probe for each operon present on the genome (Fig. 4B). Hybridization of rRNA to *Hin*dIII-digested chromosome showed three size fragments at 8, 10, and 20 kb (Fig. 4A, lane 2). These fragments are all larger than the 3.1- and the 7.2-kb *Hin*dIII-*Eco*RI fragments which represent the smallest size of the possible *Hin*dIII fragments contiguous with the operon. If two of the *Hin*dIII sites outside the operon happen to be approximately the same distance from a *Hin*dIII site within an operon, one of the bands, most probably the 20-kb band, would be a doublet. This doublet together with the two additional fragments suggest the presence of two rRNA gene sets on the genome. To confirm this, rRNA was hybridized to chromosomal DNA digested with *Bgl*II (Fig. 4A, lane 3). Hybridization to a *Bgl*II restriction fragment of 4.2 kb which corresponds to the internal *Bgl*II fragment only present in the second gene set (Fig. 4B) was seen. Because the rRNA genes do not appear to extend beyond the second *Bgl*II site in the second gene set (boxed in Fig. 4B), we would expect to see hybridization to three additional restriction fragments, all larger than the 2.2-kb *Eco*RI-*Bgl*II fragment and the 8.1-kb *Bgl*II-*Eco*RI fragment, if the genome contained two rRNA gene sets. Three additional fragments at 5.3, 6, and 20 kb were in fact detected (Fig. 4A, lane 3), arguing that the genome contains two gene sets. Based on the restriction map of the two putative gene sets, rRNA would be expected to hybridize to *Bgl*II-*Eco*RI restriction fragments of *C. crescentus* DNA of 6, 0.95, and 1.2 kb for one operon and 2.1, 0.95, 1.2, and 2.2 kb for the other (Fig. 4B). The hybridization pattern shown in Fig. 4A, lane 4 agrees with this prediction. The fact that there was no hybridization to the 3.9-kb *Bgl*II-*Eco*RI fragment (comprising the remainder of the 6-kb fragment of pJF1008) confirms that the remainder of the 23S rRNA gene, as well as the 5S rRNA and 4S tRNA genes, are encoded in the 2.1-kb *Eco*RI-*Bgl*II fragment of the second gene set. These data indicate that there are two rRNA gene sets on the *C. crescentus* CB13 genome.

Sequence analysis of the rRNA and tRNA gene cluster. The

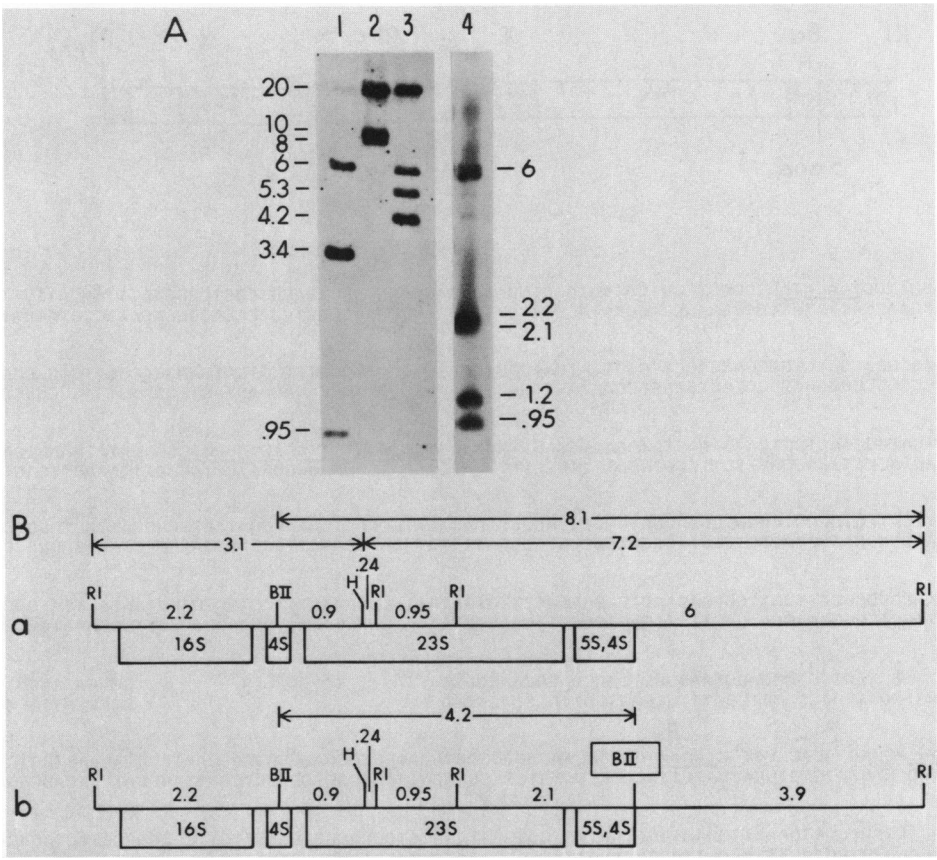
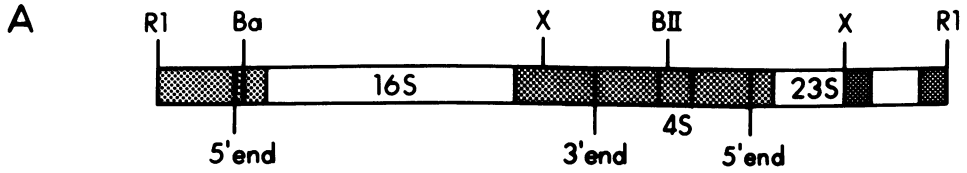


FIG. 4. Hybridization analysis of the *C. crescentus* genome to determine the number of rRNA genes. The *C. crescentus* DNA was digested with various restriction enzymes and probed with rRNA. (A) The *C. crescentus* genome was restricted with *Eco*RI and electrophoresed through a 0.8% horizontal agarose gel and transferred to nitrocellulose. The blots were probed with rRNA that had been labeled in vitro at the 5′ end as described in the text. (B) Schematic diagram of the two proposed rRNA gene sets on the *C. crescentus* genome. The regions that hybridized 16S, 23S, 5S rRNA, and 4S RNA are indicated. The 3.4- and 0.95-kb *Eco*RI fragments appear to be the same in both operons, but the 6-kb *Eco*RI fragments are not identical in that the region distal to the rRNA and tRNA genes contains a *Bgl*II site (boxed) in one of the gene sets.

nucleotide sequence of approximately 60% of the 3.4-kb *Eco*RI fragment of pJF1003 was determined by the chemical modification method of Maxam and Gilbert (26). The nucleotide sequence shown in Fig. 5 was determined by sequencing both strands of the appropriate restriction fragments and by sequencing overlapping restriction fragments. The sequenced regions, shown diagrammatically in Fig. 5A, begin with the *Eco*RI boundary of the cloned segment and include 296 nucleotides preceding the start of the 16S rRNA gene and 254 nucleotides within the 5′ region of the 16S rRNA gene. The sequenced region in the middle of the 3.4-kb fragment encompasses 358 nucleotides within the 3′ end of the 16S rRNA gene, the entire 16S/23S intergenic spacer region (653 nucleotides) and 56 nucleotides within the 5′ end of the 23S rRNA gene. The *Eco*RI site at the right boundary of the cloned 3.4-kb *Eco*RI fragment is approximately 830 nucleotides downstream from the 5′ end of the 23S gene. Two additional nucleotide sequences within this region of the 23S rRNA structural gene were also determined.

The 5′ and 3′ ends of the 16S rRNA gene and the 5′ end of the 23S rRNA gene were identified by comparison with the comparable regions of the *E. coli* rRNA genes (9). The position of the 3′ end of the 16S rRNA gene was also confirmed by S1 nuclease mapping as described by Berk and Sharp (7). Hybridization of various restriction fragments of pJF1003 to rRNA (Fig. 3) suggested that the 3′ end of the 16S rRNA structural gene was within the 550-base-pair *Xho*I-*Bgl*II fragment. Accordingly, this *Xho*I-*Bgl*II DNA restriction fragment of pJF1003 (Fig. 2) was isolated and labeled on one strand at the 3′ end of the *Xho*I site, as described in the legend to Fig. 6. Hybrid formation between purified 16S rRNA and the *Xho*I-*Bgl*II DNA probe protected a 210 to 220 base fragment of the labeled DNA from nuclease S1 digestion (Fig. 6, lane 1). Thus, the 16S rRNA appears to end 210 to 220 bases downstream from the *Xho*I site. Furthermore, protection of the 3′ end-labeled *Xho*I site by the 16S rRNA indicated that the labeled strand is the sense strand for 16S rRNA transcription. Thus, the 16S rRNA gene is transcribed from left to right (as diagrammed in Fig. 2) within the 3.4-kb rDNA of pJF1003.

Further analysis of the nucleotide sequence revealed that a portion of the intragenic spacer region encoded two tRNAs. These were identified as $tRNA^{Ile}$ and $tRNA^{Ala}$ by virtue of their anticodons. Invert repeats were found to flank both tRNA genes (underlined arrows shown in Fig. 5; see Fig. 10) and may represent processing sites for the production of mature tRNA species (15). In addition to these invert repeat sequences surrounding the tRNAs, invert repeat sequences were found flanking the 16S rRNA gene (Fig. 5, underlined arrows, regions A and B). They are located approximately 25 and 28 nucleotides, respectively, from the 16S rRNA gene. If these two regions base paired to form a



B

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1    GAATTCGAGGTTGGTAAGATAGCCGCCTCCGTCGATATGGGTGCAAATGATGGGGTCGCTGAGGTGGCTCGGGTCTTTGACATTGTTGAATGGAAAGGGA
     CTTAAGCTCCAACCATTCTATCGGCGGAGGCAGCTATACCCACGTTTACTACCCCAGCGACTCCACCGAGCCCAGAAACTGTAACAACTTACCTTTCCCT

101  AACCAGGCGGCGGGCGCTCTGGCGATGACCTTCGGTCATCAACTGACGCTGACGATACGGTCTCTTGATGAAGCACCATTCGGCCGGTTGGCCTTCGGGT
     TTGGTCCGCCGCCCGCGAGACCGCTACTGGAAGCCAGTAGTTGACTGCGACTGCTATGCCAGAGAACTACTTCGTGGTAAGCCGGCCAACCGGAAGCCCA

                                                                                              5'end 16S
201  CGATCGAGTGATGGGAACTCGTCAAGAAACTATGCAAACCAGATACCTAGTCCTAGGTTTTCCCCTAGGATCGGAAGTCTGAAGTCAATGTCAACTCAAC
     GCTAGCTCACTACCCTTGAGCAGTTCTTTGATACGTTTGGTCTATGGATCAGGATCCAAAAGGGGATCCTAGCCTTCAGACTTCAGTTACAGTTGAGTTG

301  CTGAGAGTTTGATCCTAGCCTCAGAGCGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGATCCTTCGGGATTAGTGGCGGACGGGTGAGTAAC
     GACTCTCAAACTAGGATCGGAGTCTCGCTTGCGACCGCCGTCCGGATTGTGTACGTTCAGCTTGCCTAGGAAGCCCTAATCACCGCCTGCCCACTCATTG

401  ACGTGGGAAACGTGCCCTTTGGTTCGGAACAACTCAGGGAAACTTGAGCTAATACCGGATGTGCCCTTCGGGGGAAAGATTTATCGCCATTGAGCGGCCC
     TGCACCCTTTGCACGGGAAACCAAGCCTTGTTGAGTCCCTTTGAACTCGATTATGGCCTACACGGGAAGCCCCCTTTCTAAATAGCGGTAACTCGCCGGG

501  GCGTCTGATTAGCTAGTTGGTGGAGGTAAAGGCTCACCAAGGCGACGGTC...      (~930 bp)      ...GGGGATAACGTCAAGTCCTCATGG
     CGCAGACTAATCGATCAACCACCTCCATTTCCGAGTGGTTCCGCTGCCAG                              CCCCTATTGCAGTTCAGGAGTACC

          -35 .                .Pin
1504 CCCTTAGAAGGTGGGCTACACACGTGCTACAATGGCGACTACAGAGGGCTGCAACCCGCGAGGGGGAGCCAATCCCTAAAAGTCGTCTCAGTTCGGATTG
     GGGAATCTTCCACCCGATGTGTGCACGATGTTACCGCTGATGTCTCCCGACGTTGGGCGCTCCCCCTCGGTTAGGGATTTTCAGCAGAGTCAAGCCTAAC
                     |----->

1604 CTCTCTGCACTCGAGAGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACA
     GAGAGACGTGAGCTCTCGTACTTCAACCTTAGCGATCATTAGCGCCTAGTCGTACGGCGCCACTTATGCAAGGGCCCGGAACATGTGTGGCGGGCAGTGT

1704 CCATGGGAGTTGGACTTTACCCGAGCGCTAGCTCCTAACCTGCTAAGGGGGCTAGGCTGATTCTGGTAGGGCCGACTGACTGGGGTTGAAGCGTAACAAG
     GGTACCCTCAACCTGAAATGGGCTCGCGATCGAGGATTGGACGATTCCCCCGATCCGACTAAGACCATCCCGGCTGACTGACCCCAACTTCGCATTGTTC

                3'end of 16S                                  B
1804 GTAACCGAGGGGAACCGCGGTTGGACACCTCTTCTTTTGCATGATTCACCGGACGTTAGCTAGATCCTAGGGAAAAACCTAGCACCTTCAGAGCCCAGCT
     CATTGGCTCCCCTTGGCGCCAACCTGTGGAGAAGAAAACGTACTAAGTGGCCTGCAATCGATCTAGGATCCCTTTTTGGATCGTGGAAGTCTCGGGTCGA

1904 CCCGAAGCATTGAAGCCCAGACACGCGCTAGAGGACCAAAAATGACGAACGCCGCCGACTTCGTTCCTTCCACCACTTGGTCGTAGGAACAAGCGACGCG
     GGGCTTCGTAACTTCGGGTCTGTGCGCGATCTCCTGGTTTTTACTGCTTGCGGCGGCTGAAGCAAGGAAGGTGGTGAACCAGCATCCTTGTTCGCTGCGC

                                                  ←tRNAile→
2004 AGCCGGAGGCAAACCCTGCGAGACCGCATAGGCCTGTAGCTCAGGTGGTTAGAGCGTACGCCTGATAAGCGTAAGGTCGGCAGTTCGAGTCTGCCTAGGC
     TCGGCCTCCGTTTGGGACGCTCTGGCGTATCCGGACATCGAGTCCACCAATCTCGCATGCGGACTATTCGCATTCCAGCCGTCAAGCTCAGACGGATCCG

                                                                ←tRNAala→
2104 CTACCACTCTTTCCTCTGATGCTTCGGACACGACCAGCTCTCTTGGCAGATCTACAGAGGGGGCCATAGCTCAGTTGGTAGAGCGCCTGCTTTGCAAGC
     GATGGTGAGAAAGGAGACTACGAAGCCTGTGCTGGTCGAGAGAACCGTCTAGATGTCTCCCCCGGTATCGAGTCAACCATCTCGCGGACGAAACGTTCG

2204 AGGTGTCGTCGGTTCGAATCCGTCTGGCTCCACCACTCTCCTCCCGATGAGGACCGAGATTGAATCTCGCGATTGGAATGACAAGTTTGCGCCGGCTCT
     TCCACAGCAGCCAAGCTTAGGCAGACCGAGGTGGTAGAGGAGGGCTACTCCTGGCTCTAACTTAGAGCGCTAACCTTACTGTTCAAACGCGGCCGAGA

                                                  C
2304 TTGAGCTGGTTGCGAAATGACATTGTGAAGGCAGGGTTCTCCCGCCGACCCCAGCTCACCCCTAGGATGCGGATCCGTTTAAGGGCGGACTTAAGAAGAC
     AACTCGACCAACGCTTTACTGTAACACTTCCGTCCCAAGAGGGCGGCTGGGGTCGAGTGGGGATCCTACGCCTAGGCAAATTCCCGCCTGAATTCTTCTG

                                                                5'end of 23S
2404 ATCATTGTCTGACTAAAGGTAGAGCCCAAGCTCAGGCCTCCCCGCGCGTGCGGCGAGGGCTGAAACAGCATGGGTTTTGCTGAGAACGATCAAGCGCATA
     TAGTAACAGACTGATTTCCATCTCGGGTTCGAGTCCGGAGGGGCGCGCACGCCGCTCCCGACTTTGTCGTACCCAAAACGACTCTTGCTAGTTCGCGTAT

2504 AGGGCTTCTGACGGATGCCTTGGCATTGAGAGGCGATGAAAGG... (~300) ...GGGCGGGACACGTGAAATCCTGTCTGAACATGGGGGGACCACC
     TCCCGAAGACTGCCTACGGAACCGTAACTCTCCGCTACTTTCC                CCCGCCCTGTGCACTTTAGGACAGACTTGTACCCCCCTGGTGG

2889 CTCCAAGCCTAAGTACTCCTCAATGACCGTGCGACAAGTCCGTGAGGGAAGGTGAA... (~270) ...AGGTAAGGTAAGGTAACACTTACTGAGGAC
     GAGGTTCGGATTCATGAGGAGTTACTGGCACGCTGTTCAGGCACTCCCTTCCACTT                TCCATTCCATTCCATTGTGAATGACTCCTG

3244 CGAACCGGTGAATGTTGAAAAATTCTCGGATGACTTGTGGATAGGGGTGAAAGGCCAATCAAACCTGGACATAGCTGGTTCTCCGCGAAAACTATTTAG...
     GCTTGGCCACTTACAACTTTTTAAGAGCCTACTGAACACCTATCCCCACTTTCCGGTTAGTTTGGACCTGTATCGACCAAGAGGCGCTTTTGATAAATC
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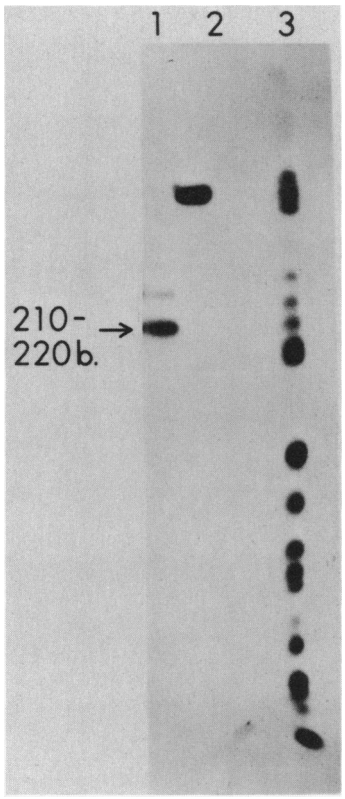


FIG. 6. Nuclease S1 mapping of the 3′ end of the 16S rRNA gene. pJFJ1003 DNA was cut with *Bgl*II and *Xho*I and the 3′ end of the *Xho*I sites were labeled with [α-^{32}P]TTP and the Klenow fragment of DNA polymerase I. The fragments were separated on a 6% acrylamide gel, and the 550-bp *Bgl*II-*Xho*I fragment from the internal region of the *C. crescentus* rDNA insert of pJF1003 was electroeluted from a gel slice, passed through an NACS-52 column (under the conditions described by Bethesda Research Laboratories, Inc.), and used as probe, as described in the text. The DNA-RNA hybrid and the two DNA controls were electrophoresed through a 7 M urea–6% polyacrylamide gel, and an autoradiogram of the gel is shown. Lane 1 contains DNA probe hybridized to in vivo 16S *C. crescentus* rRNA and digested with 1,500 U of S1 nuclease. Lane 2 contains the 550-bp DNA probe alone. Lane 3 contains labeled pBR322 *Hae*II restriction fragments used as molecular weight standards.

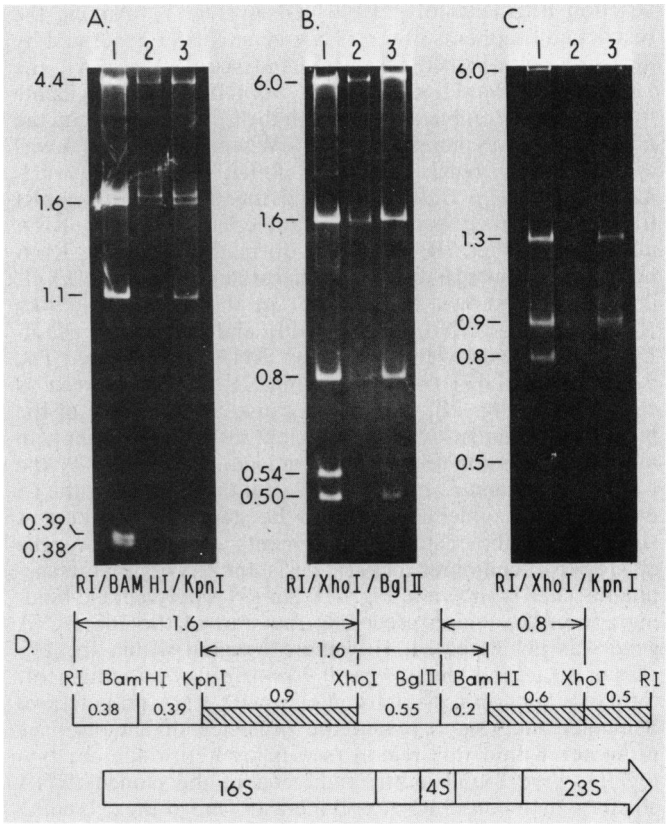


FIG. 7. Complex formation between pJF1003 restriction fragments and *C. crescentus* or *E. coli* RNA polymerase. Heparin-resistant complexes between RNA polymerase and restriction fragments of pJF1003 were examined as described by Amemiya and Shapiro (3). In each panel either *C. crescentus* (4.8 μg), lane 2, or *E. coli* (4.2 μg), lane 3, RNA polymerase were mixed with 1.0 μg of restricted pJF1003 DNA (enzyme/DNA ratio, 32 and 30, respectively). Lane 1 in all panels represents the digested fragments alone. (A) Enzyme complexed with *Eco*RI-*Bam*HI-*Kpn*I-digested restriction fragments. (B) Enzyme complexed with *Eco*RI-*Xho*I-*Bgl*II-digested restriction fragments. (C) Enzyme complexed with *Eco*RI-*Xho*I-*Kpn*I-digested restriction fragments. The complexed fragments were analyzed on a 1% agarose gel and visualized with a UV illuminator after soaking the gel in 1 μg of ethidium bromide per ml. (D) A schematic drawing of a partial restriction map of pJF1003 with the position of the rRNA genes shown below. The hatched areas indicate the fragments that formed complexes with RNA polymerase.

stem structure enclosing the entire 16S rRNA gene (see Fig. 10), the structure would have a ΔG of −42 Kcal (39). Interestingly, another invert repeat sequence was found between the tRNA^{Ala} gene and the 5′ end of the 23S rRNA gene. There was some homology between this smaller invert repeat sequence (Fig. 5, underlined arrow, region C) and the invert repeat at the 3′ end of the 16S rRNA gene (region B).

Complex formation between RNA polymerase and the rRNA gene cluster. To determine whether any RNA polymerase-binding sites were present in the cloned rRNA gene cluster, especially in the region 5′ to the 16S rRNA gene, we examined the ability of both *C. crescentus* and *E. coli* RNA polymerase to form heparin-resistant complexes with re-

FIG. 5. (A) Schematic diagram of the 3.4-kb *Eco*RI fragment of pJF1003. The shaded areas indicate the regions that have been sequenced. (B) The nucleotide sequence of the regions that are shaded in (A). The sequence of the nonsense strand is on top. The number one designates the first nucleotide at the *Eco*RI site located upstream from the 5′ end of the 16S gene. The 16S gene ends at nucleotide 1838. The 5′ end and 3′ end of the 16S gene and the 5′ end of the 23S gene were assigned by analogy with the *E. coli* rRNA nucleotide sequences (9). The underlined sequence 5′ to the 16S gene identifies a possible consensus Pribnow box sequence, as does the internal P_{in}. −35 identifies a region that resembles the sequence found upstream from many *E. coli* promoters. The dashed lines with an arrowhead represents a transcript that initiates from this region. Ⓐ and Ⓑ are the sequences of the two halves of a 23-nucleotide inverted repeat. Ⓒ is a nine-base sequence that is an inverted repeat of part of Ⓑ. tRNA^{Ile} (tRNA$_{ile}$) and tRNA^{Ala} (tRNA$_{ala}$) are sequences that can be folded into the cloverleaf structure representative of tRNA species accepting isoleucine and alanine. The identification of tRNA^{Ile} and tRNA^{Ala} was based on their anticodon sequence. The lines with arrows identify short inverted repeat sequences enclosing the tRNA^{Ile} gene and the tRNA^{Ala} gene.

striction fragments of pJF1003 DNA (Fig. 7). Among the restriction fragments of the rRNA gene cluster generated by digestion with *Eco*RI, *Bam*HI, and *Kpn*I (Fig. 7A), the 1.6-kb *Kpn*I-*Bam*HI and 1.1-kb *Bam*HI-*Eco*RI fragments formed stable complexes with both the *C. crescentus* and the *E. coli* RNA polymerase (Fig. 7A). When pJF1003 DNA was digested with *Eco*RI, *Xho*I, and *Bgl*II, the 1.6-kb *Eco*RI-*Xho*I, the 0.8-kb *Bgl*II-*Xho*I, and the 0.5-kb *Xho*I-*Eco*RI fragments formed heparin-resistant complexes with RNA polymerase (Fig. 7B). Complex formation between RNA polymerase and pJF1003 DNA digested with *Eco*RI, *Kpn*I, and *Xho*I is shown in Fig. 7C. In this case the 0.9-kb *Kpn*I-*Xho*I, the 1.3-kb *Xho*I-*Xho*I, and the 0.5-kb *Xho*I-*Eco*RI fragments were bound by RNA polymerase. The 0.5-kb *Xho*I-*Eco*RI fragment in Fig. 7C cannot be seen as clearly as in Fig. 7B, possibly because the recovery of the restriction fragment was less efficient in this experiment. In all cases, the same restriction fragments were bound by the *C. crescentus* and *E. coli* enzymes, but the binding by the *C. crescentus* enzyme appeared to be generally weaker. A summary of the restriction fragments of the rRNA gene cluster that contained heparin-resistant RNA polymerase-binding sites is shown in Fig. 7D. An RNA polymerase-binding site in the region preceding the start of the 16S rRNA gene was not detected. However, a region within the 16S rRNA structural gene (the 0.9-kb *Kpn*I-*Xho*I fragment) formed a heparin-resistant complex with RNA polymerase. Sequence analysis revealed the presence of a consensus promoter within this region (see below). In addition, two regions were found at the right end of the cloned rRNA cluster which bound RNA polymerase. One of these binding regions was found on the 0.6-kb *Bam*HI-*Xho*I fragment, and the other was located downstream in the 0.5-kb *Xho*I-*Eco*RI fragment. The tRNA spacer region up to the *Bam*HI site did not contain RNA polymerase-binding sites (data not shown).

In vitro transcription of the cloned rRNA gene cluster. Because specific regions within the rRNA gene cluster contained heparin-resistant RNA polymerase-binding sites, we carried out in vitro transcription studies of the isolated 3.4-kb fragment with *C. crescentus* and *E. coli* RNA polymerase to determine whether transcripts initiated from within the gene cluster (Fig. 8). Transcripts synthesized by the *E. coli* and *C. crescentus* RNA polymerase in the absence of heparin are shown in Fig. 8, lanes 1 and 2, respectively. The *E. coli* enzyme produced at least four transcripts, a 1.8-kb transcript, a 1.7-kb transcript, a 0.7-kb transcript, and a 0.26-kb transcript. When heparin was added to the reaction at the same time as the nucleoside triphosphates to prevent reinitiation by the enzyme, the *E. coli* RNA polymerase produced essentially the same number of transcripts as it did in the absence of heparin (Fig. 8, lane 3). A small amount of a 3.3-kb transcript could be seen which starts from the left end of the 3.4-kb *Eco*RI fragment (5). In contrast to the *E. coli* enzyme, the *C. crescentus* RNA polymerase produced a heterogeneous array of transcripts in the absence of heparin. However, in the presence of heparin

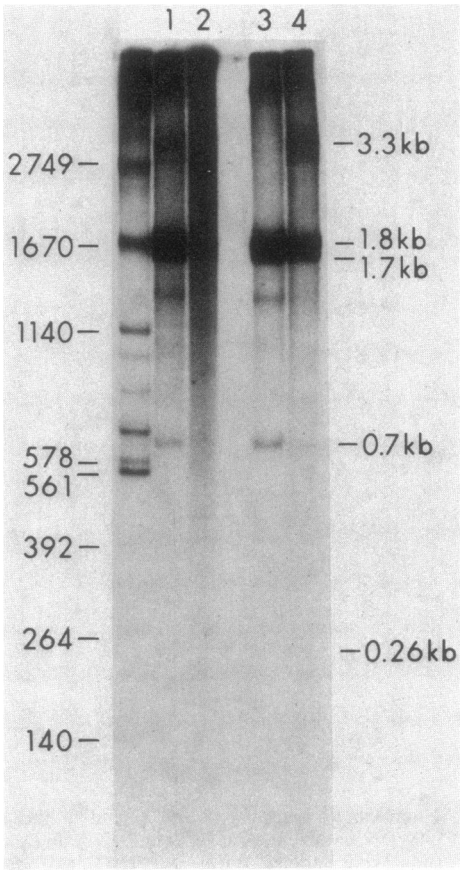
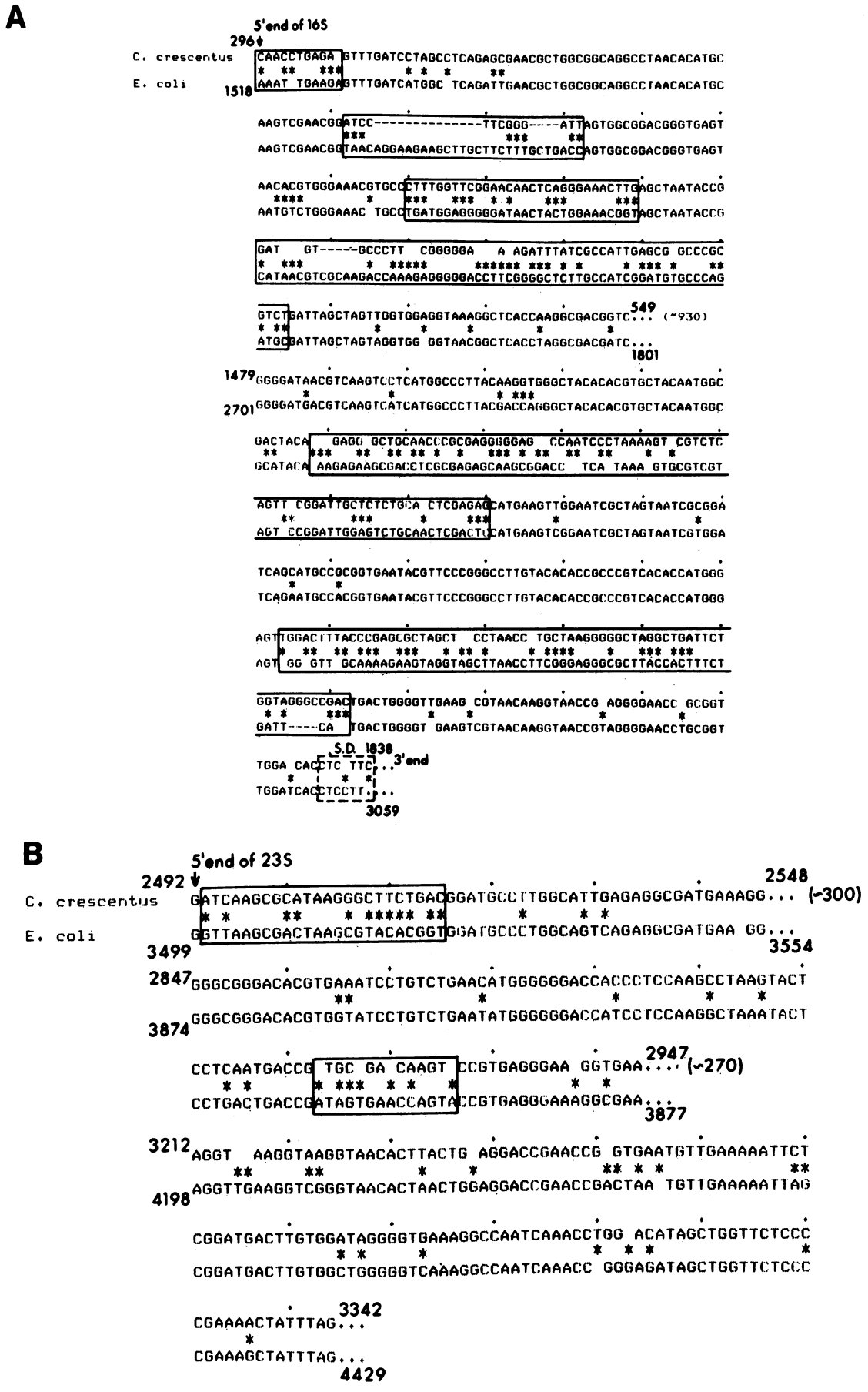


FIG. 8. Electrophoretic analysis of RNA transcripts synthesized by *C. crescentus* and *E. coli* RNA polymerase from the 3.4-kb *Eco*RI fragment of pJF1003. Transcription assay conditions were as described in the text. The reaction mixture contained 0.12 μg of the DNA and either 0.4 μg of *E. coli* RNA polymerase (enzyme/DNA ratio, 15; lanes 1 and 3) or 1.6 μg of *C. crescentus* RNA polymerase (enzyme/DNA ratio, 59; lanes 2 and 4). The reactions shown in lanes 1 and 2 contained no heparin. Rifampin (2 μg) was added after 10 min at 37°C and incubation was continued for another 10 min before termination. The reactions shown in lanes 3 and 4 had 50 μg of heparin per ml added at the same time as the nucleoside triphosphates. Equal amounts of acid-precipitable counts were added to each lane. The total amount of [α-^{32}P]CMP incorporated into RNA in each reaction was (lane 1) 57, (lane 2) 165, (lane 3) 22, and (lane 4) 27 pmol. The lane on the left contained T7 early RNA digested with RNase III as molecular weight standards.

the *C. crescentus* enzyme synthesized the same transcripts as the *E. coli* enzyme (Fig. 8, lane 4). The transcription initiation sites of these transcripts have been further characterized and all but one have been found to be located on the restriction fragment which contained RNA polymerase-binding sites (K. Amemiya, V. Bellofatto, L. Shapiro, and J. Feingold, in press). The sequence of one of these transcrip-

FIG. 9. Comparison of *C. crescentus* and *E. coli* 16S and 23S rRNA nucleotide sequences. The numbering system for the *E. coli* sequences was taken from Brosius et al. (9). Regions of nonhomology are enclosed in boxes. Mismatched nucleotides are identical with an asterisk. Regions of four nucleotides or more that are deleted from one or the other species are represented with dashed lines. (A) Comparison of the *C. crescentus* CB13 16S sequence with that of the *E. coli* rrnB 16S rRNA gene. S.D. indicates the consensus Shine-Delgarno sequence (36). (B) Comparison of three regions of the 23S rDNA from *C. crescentus* CB13 and *E. coli*. The *E. coli* rrnB 23S sequence and numbering system was taken from Brosius (9). A total of 56 nucleotides at the 5′ end as well as regions of 99 nucleotides and 129 nucleotides from within the first one-third of the *C. crescentus* CB13 23S gene have been determined. Regions of nonhomology are boxed.


A
5'end of 16S
296
C. crescentus
E. coli
1518
S.D.
1838
3'end
3059
B
5'end of 23S
2492
2548
C. crescentus
E. coli
3499
3554
2847
3874
2947
3877
3212
4198
3342
4429

tion initiation sites (for the 1.8-kb transcript), located within the 3′ end of the 16S rRNA gene, is shown in Fig. 5.

DISCUSSION

The number of copies of rRNA gene clusters per genome varies widely among different microorganisms. *E. coli* (27, 29) and *Bacillus subtilis* (22) have 7 and 9 or 10 rRNA operons, respectively. On the other hand, *Anacystis nidulans* (40) and *Mycoplasma capricolum* (16) have two rRNA gene clusters per genome, whereas *Halobacterium halobium* was reported to have only one rRNA gene cluster per genome (18). We have shown here that there are two copies of rRNA gene clusters in *C. crescentus* CB13. The same number of copies of rRNA genes was previously reported for *C. crescentus* CB15 (30). However, the rRNA gene clusters in CB13 and CB15 are not identical because at least one *Hin*dIII restriction site in the *C. crescentus* CB13 rRNA gene cluster is not present in CB15. The number of rRNA gene clusters in a given organism may be one factor which limits their maximum growth rate, because generation time appears to be directly correlated with the number of rRNA gene clusters.

In general, the homology between 16S and 23S rRNA genes of various microorganisms is extensive (9, 14, 42). Comparison of the partial sequences of the *C. crescentus* genes encoding 16S rRNA and 23S rRNA with the 16S and 23S rRNA genes of the *E. coli* rrnB operon show significant homology (Fig. 9). However, no sequence homology was observed in the regions preceding the 16S rRNA gene or in the 16S/23S intergenic spacer surrounding the tRNA species. Within the 16S and 23S rRNA structural genes, regions of nearly perfect homology are interspersed with nonconserved regions (boxed sequences in Fig. 9). Approximately 82% homology with *E. coli* sequences was detected in the 10% of the 23S gene that has been sequenced (Fig. 9B). Comparison of the 40% of the *C. crescentus* 16S rRNA gene sequenced with the *E. coli* 16S rRNA sequence revealed approximately 71% homology (Fig. 9A). One interesting region of the 16S rRNA gene is a nucleotide sequence which forms a helix extending from nucleotide 1591 to nucleotide 1615 in the *E. coli* sequence shown in Fig. 9A. This region, which is also present in *Proteus vulgaris* (42), is missing from the *C. crescentus* rRNA gene. The small rRNA species from chloroplasts, archaebacteria, and eucaryotes appear to also delete this region (42). Analysis of the 16S rRNA nucleotide sequence from many bacterial species has been used to establish a phylogenetic classification of procaryotes (14). Analysis of the partial sequence of the *C. crescentus* 16S rRNA gene has shown that *C. crescentus* is a member of the α group of the purple photosynthetic bacteria (G. Fox, personal communication). This group includes the majority of the *Rhodomicrobium* species and the *Rhodopseudomonas* species (14).

Two tRNA genes were identified in the 16S/23S intergenic spacer region and shown to be $tRNA^{Ile}$ and $tRNA^{Ala}$ by virtue of their anticodon sequences. The *C. crescentus* tRNAs can be folded into the consensus secondary structure described for typical tRNAs (Fig. 10). Conservation of the restriction fragment size between the two types of *C. crescentus* rRNA gene sets suggest that the $rRNA^{Ile}$ and $tRNA^{Ala}$ genes sequenced in the 3.4-kb clone reside in both rRNA gene sets. The same two tRNA genes have been identified in the 16S/23S intergenic spacer of three *E. coli* rRNA operons (27) and two *B. subtilis* rRNA gene sets (22). Bacterial species and eucaryotic plastids that have been shown to encode tRNA genes in the 16S/23S spacer, including *A. nidulans* (41), *Euglena gracilis* chloroplasts (20), *Zea mays* chloroplasts (21), and *Nicotinum tobacum* chloroplasts (38), encode $tRNA^{Ile}$ and $tRNA^{Ala}$. In addition to the conservation of the position of these tRNA species through evolution, their nucleotide sequence has been well conserved. The *C. crescentus* $tRNA^{Ile}$ and $tRNA^{Ala}$ nucleotide sequence is similar but not identical to their *E. coli* counterparts (28). Small inverted repeat sequences were found to flank both tRNA genes (arrows in Fig. 5 and 10) and may represent processing sites for the production of mature tRNA species, as is the case in *E. coli* (4).

Examination of the nucleotide sequences surrounding the 16S rRNA gene revealed the presence of large invert repeat sequences. We have previously shown that a large 3.3-kb transcript produced in vitro from the 3.4-kb *Eco*RI fragment containing the rRNA gene cluster could be cleaved in vitro with *C. crescentus* RNase III (5). A schematic of the RNase III digestion products from the 3.3-kb transcript and their location is shown in Fig. 10A. The 3.3-kb transcript was processed to fragments of 0.4, 1.3, and 1.6 kb. A stem structure with $\Delta G = -42$ Kcal can be formed with the inverted repeat sequence (A and B) that would include the entire 16S gene in the loop (Fig. 10B). If RNase III cleaved within this stem, as has been shown to be the case in *E. coli* (43) and proposed for *B. subtilis* (23), products of 0.26, 1.7, and 1.4 kb would be expected. These products are in close agreement with those actually obtained (Fig. 10A, open arrows). However, another RNase III cleavage site was detected within the 5′ end of the 23S rRNA gene. This cleavage site was not in the same region as the invert repeat sequence (region C) found by nucleotide sequencing. It is not known if this cleavage site could be recognized in vivo; however, we have found that the transcript which initiates within the 3′ end of the 16S rRNA gene can be cleaved by RNase III at this site (Amemiya et al., in press).

The results from the RNA polymerase-binding studies and in vitro transcription assays suggest that the major promoter(s) of the rRNA gene cluster are not present on the 3.4-kb *Eco*RI fragment cloned from *C. crescentus* CB13. Examination of the nucleotide sequence at the 5′ end of the 3.4-kb *Eco*RI fragment did not reveal the presence of a consensus promoter (17, 35), although 15 to 20 nucleotides from the 5′ end of the fragment a possible "Pribnow-like" sequence (33) was present. If this sequence is part of a promoter, the −35 region would be located outside of the cloned region 5′ to the *Eco*RI site. In fact, we found that RNA polymerase binding to this region was heparin sensitive, and transcription initiation from this site was very inefficient. Significantly, RNA polymerase-binding sites and transcription initiation sites were detected within the rRNA gene cluster. It may be that the internal promoters are expressed because the major promoter(s) preceding the 16S rRNA gene is not present on the cloned template. Transcripts originating from the internal sites have been further characterized, and all but one of them (the 1.7-kb transcript) initiate from within the restriction fragments complexed by RNA polymerase (Amemiya et al., in press). The transcription start site of the 1.8-kb transcript was determined by S1 nuclease mapping, and the initiating nucleotide was shown to be located eight nucleotides from a promoter sequence 300 nucleotides upstream from the 3′ end of the 16S rRNA gene. This 16S internal rRNA promoter appears to be highly conserved and was found in all 16S rRNA genes examined from both procaryotes and plastids. In addition, the 1.8-kb transcript could be cleaved by *C. crescentus* RNase III. Further studies are in progress to identify the major

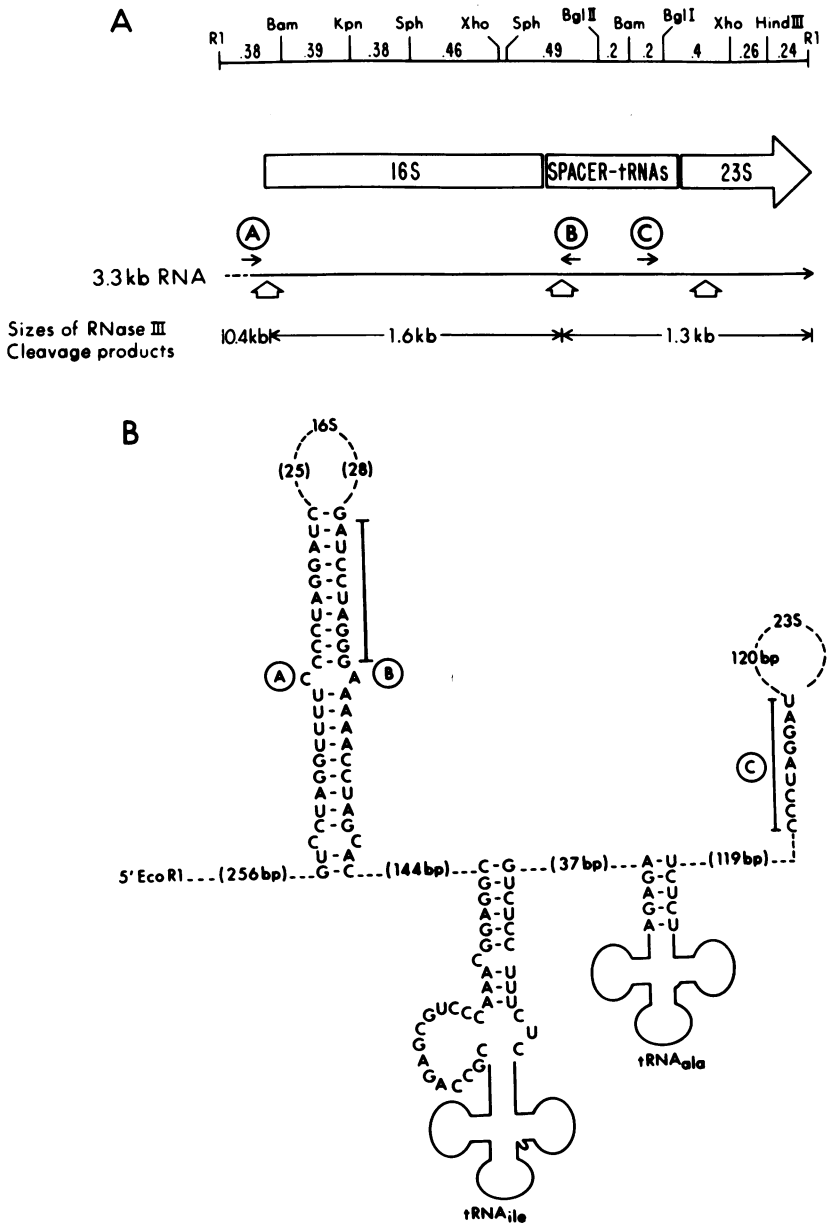


FIG. 10. Potential RNA processing sites in an rRNA gene cluster from *C. crescentus*. (A) A restriction map of the cloned 3.4-kb *Eco*RI fragment in pJF1003 containing part of a rRNA gene cluster from *C. crescentus* CB13 is shown at the top. The boxed regions in the middle show the location of the rRNA genes. The bottom of the diagram shows a 3.3-kb transcript synthesized in vitro from the 3.4-kb *Eco*RI fragment, and the RNase III cleavage sites in this transcript are shown below by vertical arrows (5). The short horizontal arrows above the transcript labeled A, B, and C represent the invert repeat sequence determined by nucleotide sequence analysis. (B) Possible stem structures formed by the invert repeat sequences surrounding the 16S rRNA and tRNA genes. The regions labeled A and B are invert repeat sequences found flanking the 16S rRNA gene. The region labeled C is an invert repeat sequence of B as shown by the vertical lines located in front of the 23S rRNA gene.

promoter(s) of the rRNA gene cluster and to examine in vivo and in vitro expression from the major promoter(s) and internal promoter(s) during the *Caulobacter* cell cycle.

ACKNOWLEDGMENTS

We thank A. Skalka for preparation of the λWES · B clone bank, M. Purucker for the restriction enzyme *Pst*I and for pBR325 DNA. We also acknowledge G. Fox for the preliminary phylogenetic analysis of the *C. crescentus* 16S rRNA DNA sequence and B. Raboy for initial detection of the ribosomal genes in chromosome digests. Also, we thank Ruth Bryan for suggestions during the preparation of this manuscript.

This investigation was supported by Public Health Service grants GM 32506 and GM 11301 from the National Institutes of Health and partially by grant NCI P30-CA1-13330).

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