JOURNAL OF BACTERIOLOGY, June 1989, p. 3479–3485
0021-9193/89/063479-07$02.00/0

Vol. 171, No. 6

Isolation and Characterization of the rRNA Gene Clusters of *Halobacterium marismortui*

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Received 8 December 1988/Accepted 21 March 1989

Two rRNA operons of *Halobacterium marismortui* were identified and cloned into plasmid pBR322 as 10- and 20-kilobase-pair (kbp) *Hin*dIII fragments, respectively. Restriction maps of the 10-kbp clone (pHH10) and an 8-kbp *Hin*dIII-*Cla*I subclone (pHC8) of the other operon were established. Southern hybridization of 16S, 23S, and 5S rRNA probes to the clones demonstrated that both operons code for the three rRNA species. By S1 nuclease analysis, the transcription initiation sites, some of the processing sites within the primary transcripts, and the boundaries of the mature 16S and 23S rRNA molecules were determined. Both operons are transcribed in vivo. Comparison of the two operons indicated that they are not identical. The most striking difference between the operons is the existence of three putative transcription initiation sites in one operon (HC8) and only one such site in the other operon (HH10). The regions surrounding these 5′ transcript end sites share a high level of sequence similarity to each other and to the rRNA promoter regions of other halophilic archaebacteria. Analysis of the proximal 130 nucleotides of the two 16S rRNA genes indicated greater-than-expected sequence heterogeneity. There are a 2-base-pair insertion in the HC8 16S gene and 10 additional sites of nucleotide sequence heterogeneity.

Ribosomes are an indispensable component of the protein synthesis apparatus. Because of its universality and structural conservation, the RNA component of the small ribosome subunit has proven to be an important and useful molecular chronometer for quantitating evolutionary relationships between organisms. Measurements based on these rRNA sequence comparisons have led Woese and Olsen to suggest that organisms can be arranged into three phylogenetic groups: eubacteria, archaebacteria, and eucaryotes. Each group represents a separate line of evolutionary descent from a common primordial ancestor (16, 17).

Within the archaebacteria, the genomic copy numbers of rRNA genes in different species range from one to four. The rRNA gene order is 5′-16S-23S-5S-3′, except in sulfur-dependent thermoacidophiles, in which the 5S gene is not linked to the 16S and 23S genes (2, 7, 8, 10, 11, 14; for a review, see reference 4). In several halophilic and methanogenic organisms, an alanine tRNA is present in the 16S-23S intergenic space, and in the halophiles, a cysteine tRNA gene is present distal to the 5S gene (2, 4, 7–9). When there is more than one operon, the tRNA genes are not always present in all copies.

In *Halobacterium cutirubrum*, there is a single rRNA operon that contains the spacer alanine tRNA and the distal cysteine tRNA genes (7). Expression of this operon is driven by a series of five tandemly arranged promoters that operate with different efficiencies and exhibit growth rate-dependent regulation (3). A second species, *H. marismortui*, contains two separate rRNA operons. In this communication, we describe the cloning and initial characterization of these two operons. In many features, the two operons appear to differ both from each other and from the well-characterized *H. cutirubrum* operon.

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MATERIALS AND METHODS

Bacterial strains. *H. marismortui* (6) was obtained from B. Z. Ginzburg (Hebrew University, Jerusalem). *Escherichia coli* HB101 and 71/18 were used for recombinant DNA work.

Culture conditions for *H. marismortui*. The culture conditions used for *H. marismortui* are described by Mevarech and Werczberger (12).

Fractionation of nucleic acids. Bacterial culture (1 liter) was grown for 3 days to a cell density of 3×10^9 cells per ml. The cells were harvested in a Sorvall GSA rotor at 10,000 rpm for 10 min at 4°C. The supernatant was removed carefully, and the pellet was washed in a solution containing 204 g of NaCl and 39.6 g of $MgSO_4 7H_2O$ per liter. The washed bacteria were suspended in 100 ml of 10 mM $MgCl_2$–10 mM Tris hydrochloride (pH 7.5), and 100 ml of buffer-saturated phenol was added. The mixture was agitated for 30 min at 37°C, and the phases were separated by centrifugation. The aqueous phase was re-extracted with phenol, NaCl was added to a final concentration of 0.5 M, and the solution was cooled on ice. One hundred milliliters of cold ethanol was added carefully, and the DNA was collected by being spooled on a glass rod. The DNA was washed twice with absolute ethanol and twice with ether, air-dried, and redissolved in TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA) at 4°C. Bulk RNA was prepared by adding 100 ml of cold ethanol to the solution left from the DNA preparation, and after 2 h at −20°C, the suspension was centrifuged in a Sorvall SS-34 rotor at 7,000 rpm for 10 min in the cold. To separate the large RNA molecules from the small ones, the pellet was suspended in 1 M NaCl and then left overnight at 4°C. The suspension was centrifuged, and the large RNA molecules were collected in the pellet. Two and one-half volumes of ethanol was added to the supernatant at −20°C to precipitate the 5S rRNA and tRNA species. Following centrifugation, these RNA species were redissolved and

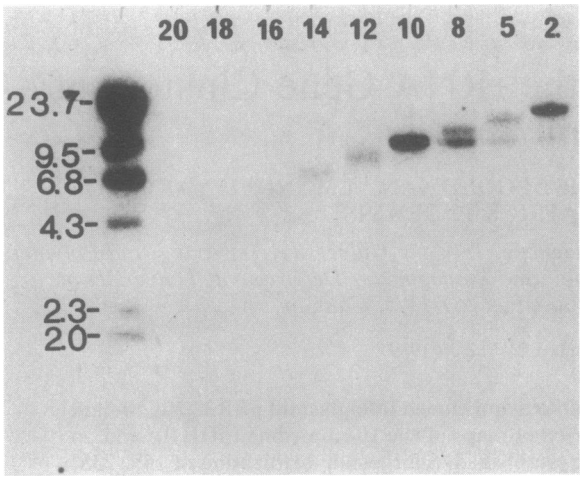

FIG. 1. Autoradiogram of Southern hybridization of ^{32}P-labeled rRNA to a gel containing sucrose gradient fractions of *Hin*dIII-digested genomic DNA. The gradient fraction numbers are indicated at the top. Bacteriophage λ DNA *Hin*dIII fragments were used as molecular size markers, which are identified in kilobase pairs on the left.

fractionated by chromatography through a Sephadex G-100 column equilibrated with 1 M NaCl–10 mM Tris hydrochloride (pH 7.5). The large 16S and 23S rRNA molecules were separated by centrifugation through a linear 5 to 20% sucrose gradient (in 150 mM NaCl–10 mM Tris hydrochloride [pH 7.5]) in a Beckman SW65 rotor at 25,000 rpm for 18 h at 4°C. Fractions were collected and analyzed by agarose gel electrophoresis.

In vitro labeling of RNA. Fractionated 16S and 23S rRNAs were partially cleaved as follows. A 10-μg sample of RNA was dissolved in 50 mM Tris hydrochloride (pH 9.5) and transferred to a siliconized 1.5-ml polypropylene test tube. The test tube was tightly closed and incubated at 90°C. The incubation times were 20 and 40 min for the 16S and 23S rRNAs, respectively. The 5S rRNA and tRNA were dephosphorylated by treatment with calf intestinal alkaline phosphatase. Phosphorylation of these RNAs was performed in 10 mM $MgCl_2$–5 mM dithiothreitol–1 mM spermidine–50 mM Tris hydrochloride (pH 9.5) by using 50 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and 2 U of polynucleotide kinase. The reaction was incubated at 37°C and terminated after 1 h by addition of 100 μl of 2.5 M ammonium acetate and 2.5 volumes of cold ethanol.

Southern hybridization. Genomic DNA was digested with various restriction enzymes and transferred to nitrocellulose filters as described by Southern. The filters were hybridized to the radioactively labeled RNA probes in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C.

S1 nuclease mapping. Nuclease S1 mapping of rRNA was performed as described previously (1, 3, 5), with restriction fragments either 3′ end labeled with Klenow polymerase and [α-^{32}P]dNTP or 5′ labeled with [γ-^{32}P]ATP and polynucleotide kinase (1, 3). For these experiments, RNA was isolated from logarithmically growing cultures (A_{600}, 0.2 to 0.3) as previously described (3).

DNA sequence analysis. Restriction fragments from the cloned genes were subcloned into vector M13 for dideoxy-DNA sequencing as described by Sanger et al. (13). When necessary, deletions were created with exonuclease III digestions followed by mung bean nuclease digestions. All sequences were determined from both strands.

RESULTS

Genomic DNA from *H. marismortui* was digested with restriction endonuclease *Hin*dIII and then fractionated on a linear sucrose gradient. Portions of alternating fractions were analyzed on a 0.7% agarose gel, transferred to nitrocellulose, and probed with a mixture of ^{32}P-labeled 16S and 23S rRNAs (Fig. 1). The radioactivity hybridized strongly to fractions 2 and 10 containing, respectively, 20- and 10-kilobase-pair (kbp) fragments of genomic DNA. This indicates that the genome of *H. marismortui* contains two separate rRNA gene clusters. This was confirmed by Southern hybridization to a *Hin*dIII digest of total genomic DNA (data not shown).

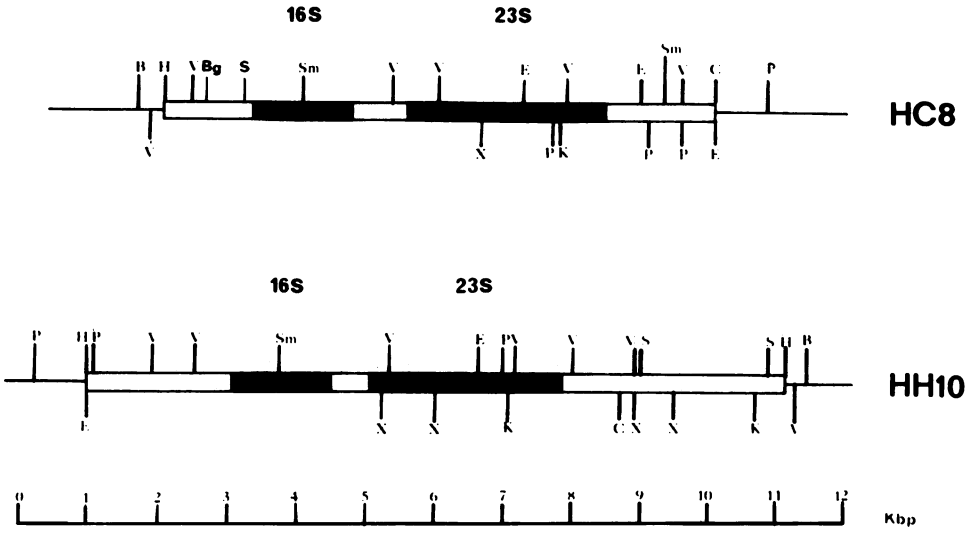

FIG. 2. Restriction maps of recombinant plasmids pHC8 (A) and pHH10 (B). The boxes indicate inserted *H. marismortui* DNA. The darkened areas indicate the approximate positions of the coding regions of 16S and 23S rRNAs. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; V, *Eco*RV; X, *Xho*I.

The 10- and 20-kbp *Hin*dIII genomic fragments were ligated into the *Hin*dIII site of pBR322, and Amp^r Tet^s transformants were obtained. Colonies containing recombinant plasmids were transferred to nitrocellulose and probed with ^{32}P-labeled rRNA. Plasmid DNAs from several positive clones from each *Hin*dIII size class were extracted, subjected to restriction enzyme analysis, and reprobed with purified 16S and 23S rRNAs. Both the 10- and 20-kbp clones hybridized to both species of rRNA.

The 10-kbp clone was designated pHH10. The 20-kbp clone was digested with *Cla*I and religated to produce a plasmid containing an 8-kbp *Hin*dIII-*Cla*I fragment inserted between the *Hin*dIII and *Cla*I sites of pBR322. This plasmid, designated pHC8, retained positive hybridization with 16S and 23S rRNAs. The restriction maps of the two plasmids are presented in Fig. 2. Restriction enzyme sites within the region encoding the 16S and 23S rRNAs were similar but not identical in the two plasmids, and sites in the 5′- and 3′-flanking regions were totally different. Plasmid pHH10 contains an extra *Xho*I site early in the 23S gene and lacks the *Eco*RV site from the 16S-23S intergenic space compared

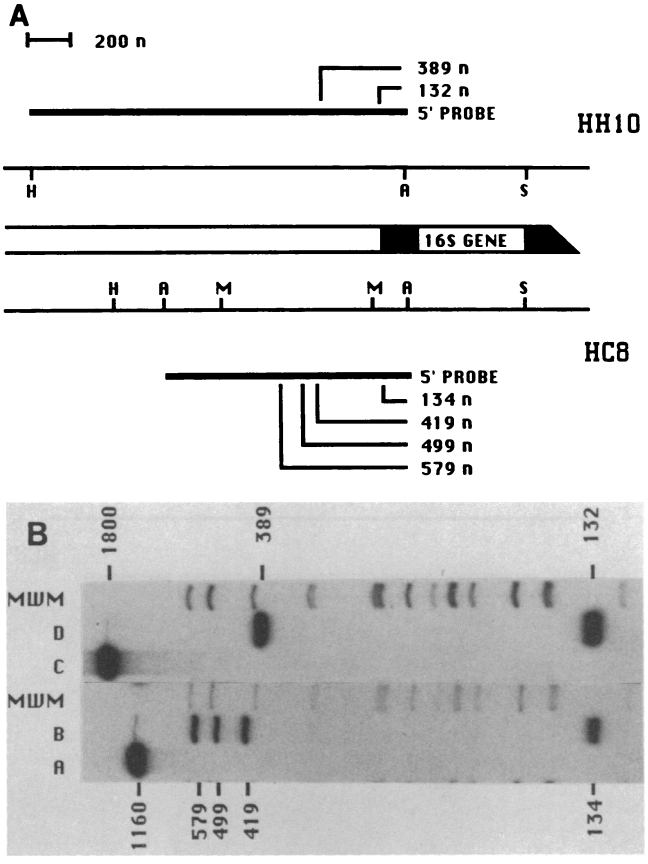

FIG. 3. Protection of 5′-end-labeled DNA fragments by precursor and mature rRNA transcripts derived from the 5′-flanking and 16S gene sequences of the two operons. (A) Schematic representation of the protected fragments. In the HH10 operon, a 1,800-nucleotide (n) *Hin*dIII-*Ava*I fragment was used, and in HC8, a 1,160-nucleotide *Ava*I-*Ava*I fragment was used. (B) Autoradiogram of the protected DNA fragments. Lanes: MWM, molecular size markers; A and C, HC8 and HH10 control DNA fragments without RNA or S1 nuclease; B and D, HC8 and HH10 fragments protected with total RNA and digested with S1 nuclease. Molecular sizes are given in nucleotides.

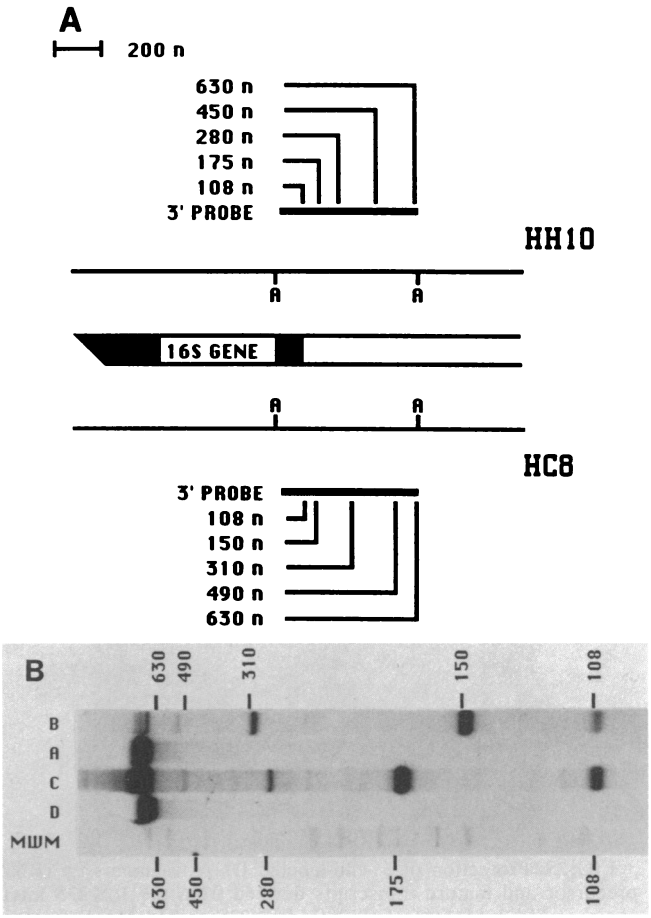

FIG. 4. Protection of 3′-end-labeled DNA fragments by rRNA precursor and mature transcripts derived from the distal end of the 16S gene and extending into the 16S-23S intergenic region. (A) Schematic representation of the protected fragments of the 630-nucleotide (n) *Ava*I-*Ava*I fragments derived from the two operons. (B) Autoradiogram of the protected DNA fragments. Lanes: MWM, molecular size markers; A and D, HC8 and HH10 control DNA fragments without RNA and S1 nuclease; B and D, HC8 and HH10 fragments protected with total RNA and digested with S1 nuclease. Molecular sizes are given in nucleotides.

with plasmid pHC8; numerous other restriction site polymorphisms were also observed within the rRNA-coding regions (data not shown).

The presence of 5S and tRNA genes associated with the two rRNA transcription units was demonstrated by probing restriction enzyme digests of pHH10 and pHC8 with ^{32}P-labeled 5S rRNA or tRNA. The results (Table 1) suggest that both 5S and tRNA sequences are located distal to the 23S genes in both operons. In addition, apparent tRNA hybridizing sequences were also detected on restriction fragments that contain the 16S-23S intergenic region. These regions are being sequenced.

RNA transcript analysis. Protection from S1 nuclease digestion of end-labeled DNA fragments by precursor and product rRNA transcripts was used to identify the positions of transcription initiation sites, processing sites, and mature 16S and 23S end sites within the two rRNA operons. Analysis of the 5′-flanking region of the 16S rRNA genes was performed by using restriction fragments labeled at the common *Ava*I site at position 134 within the 16S gene (Fig.

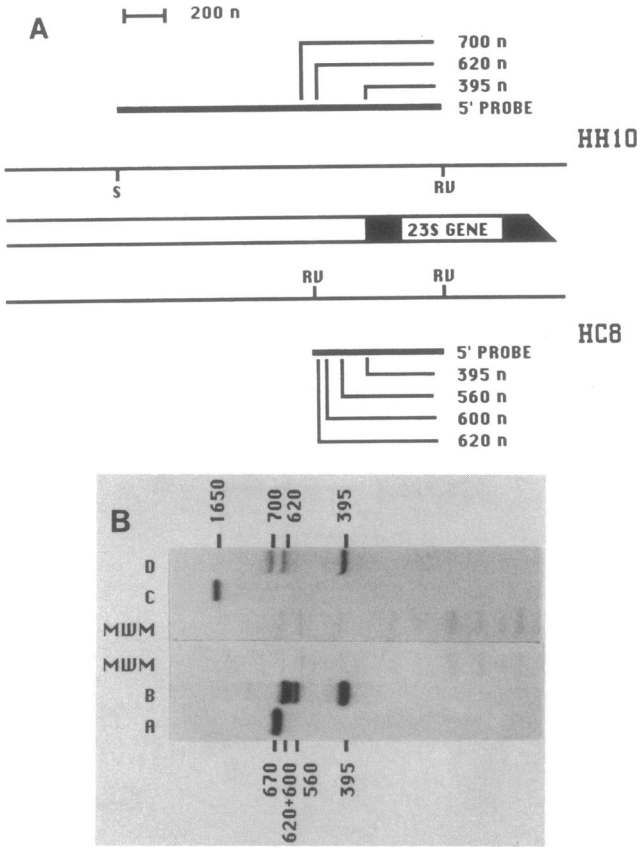

FIG. 5. Protection of 5'-end-labeled DNA fragments by rRNA precursor and mature transcripts derived from the 16S-23S intergenic region and extending into the 23S gene. (A) Schematic representation of the protected fragments. In the HH10 operon, a 1,650-nucleotide (n) *Sma*I-*Eco*RV fragment was used, and in the HC8 operon, a 620-nucleotide *Eco*RV-*Eco*RV fragment was used. (B) Autoradiogram of the protected DNA fragments. The lanes are labeled as in Fig. 3.

3). The protected products from the HH10 operon were about 389 and 132 nucleotides long and correspond to the probable transcription start site and the 5' end of 16S rRNA gene. The more numerous major protection products from the HC8 operon were about 579, 499, 419, and 134 nucleotides long and correspond to three probable tandem transcription start sites and the 5' end of the 16S rRNA gene. A fifth protection product of the HC8 operon (evident upon longer exposure of the autoradiogram in Fig. 3) was approximately 234 nucleotides long and most probably represents an RNase III-like processing site used to excise precursor 16S rRNA from the primary RNA transcript. Potential processing end sites have yet to be observed in the HH10 rRNA operon. The DNA sequences of the 5'-flanking regions of the two rRNA operons extending to the *Ava*I sites at position 134 in the 16S genes are described below, and the positions of transcript end sites are indicated (see Fig. 7).

Analysis of the 3'-flanking regions of the 16S rRNA genes was performed by using a 3'-end-labeled 630-base-pair (bp) *Ava*I fragment. The protection products from the HH10 operons were about 108, 175, 280, 450, and 630 nucleotides long, whereas the products of the HC8 operon were 108, 150, 310, 490, and 630 nucleotides long (Fig. 4). The shortest product (108 nucleotides) for each operon represents the 3'-end site of the mature 16S rRNA. The longer products represent intermediates derived from processing of the primary transcripts in the 16S-23S intergenic space in the two operons; the sites of transcript processing within these two spaces are clearly different.

The 5'-flanking region of the 23S rRNA genes was analyzed by using restriction fragments labeled at the common *Eco*RV site early in the 23S rRNA genes (Fig. 5). The protection products from the HH10 operons were about 700, 620, and 395 nucleotides long, whereas the products from the HC8 operon were 620, 600, 560, and 395 nucleotides long. The shortest product from each operon (395 nucleotides) represents the 5'-end site of 23S rRNA. Other products

TABLE 1. Sizes of restriction fragments from plasmids pHH10 and pHC8 that hybridized to 5S rRNA and tRNA[a]

Plasmid and probe	Size(s) (kbp) of fragment(s)		
	*Eco*RI	*Xho*I-*Bam*HI	*Pst*I
pHH10			
5S rRNA	9.0[b]	3.0[b]	8.0[b]
tRNA	9.0,[b] 5.6[c]	3.0,[b] 8.2[c]	8.0,[b] 5.6[c]
pHC8			
5S rRNA	1.8[b]	7.4[b]	1.5[b]
tRNA	1.8,[b] 9.6[c]	7.4,[b] 4.8[c]	1.5,[b] 9.5[c]

[a] Restriction maps of the inserts in pHH10 and pHC8 are illustrated in Fig. 2.
[b] Fragment with one end in the 23S gene and extending distally.
[c] Fragment with one end in the 23S gene and extending proximally.

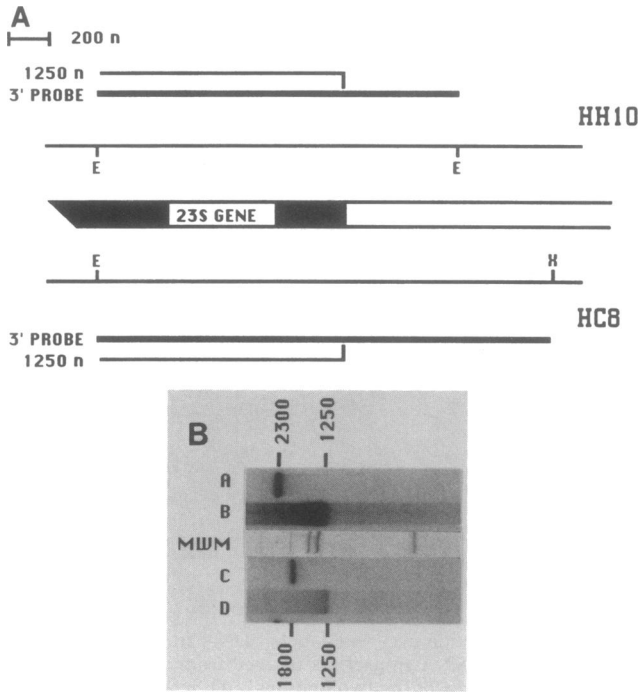

FIG. 6. Protection of 3'-end-labeled DNA fragments by rRNA precursor and mature transcripts derived from the distal end of the 23S gene and extending into the 3'-flanking region of the operon. (A) Schematic representation of the protected fragments. In the HH10 operon, a 1,800-nucleotide (n) *Eco*RI-*Eco*RI fragment was used, and in the HC8 operon, a 2,300-nucleotide *Eco*RI-*Xho*I fragment was used. (B) Autoradiogram of the protected DNA fragments. The lanes are labeled as in Fig. 3.

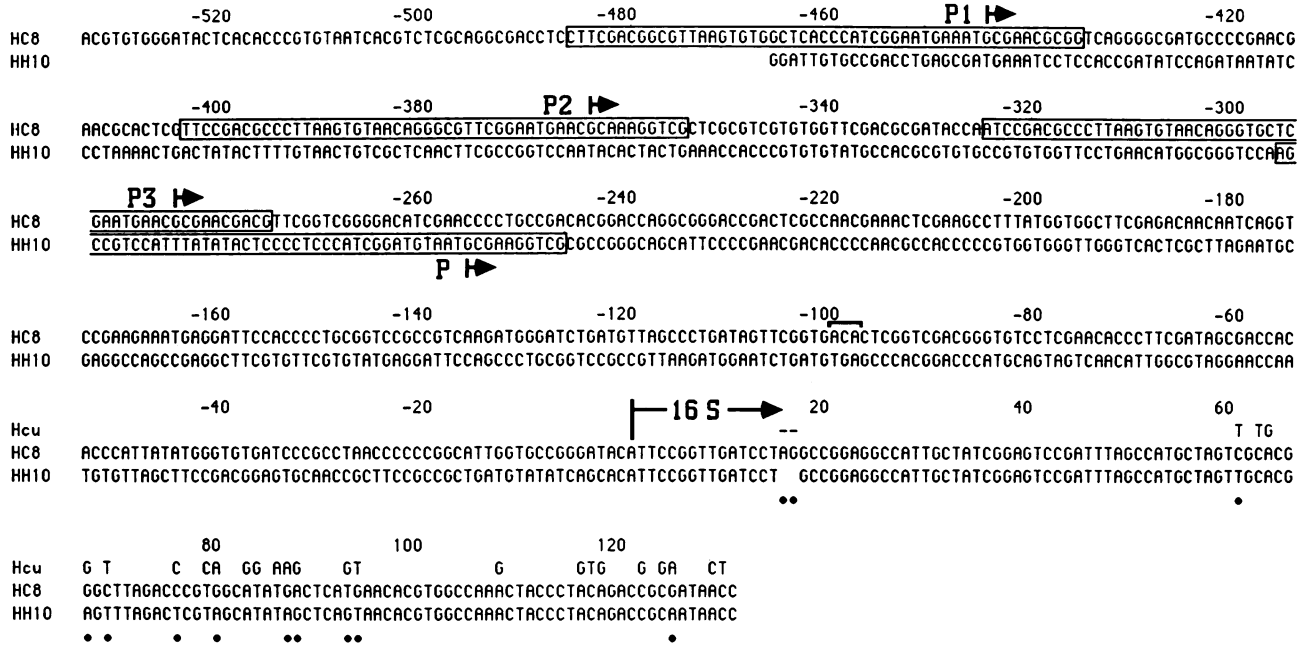

FIG. 7. Nucleotide sequence of the 5'-flanking and 5' ends of the 16S rRNA gene from the HH10 and HC8 operons. The conserved sequences surrounding the transcription start sites are boxed. The G residue at or near the start site of each boxed sequence is indicated (→). The ACA sequence (positions −100 to −98) is an RNA-processing site in the HC8 primary transcript. The 16S gene sequences begin at position +1. Positions of heterogeneity between the two 16S coding sequences are indicated below the sequences (●). The HC8 16S gene has a 2-bp insertion at positions 16 and 17. The conserved *Ava*I site used for S1 mapping is at position +130. The nucleotide sequence of the single *H. cutirubrum* 16S rRNA gene is also depicted at positions +1 to +130, where it differs from either one or both of the *H. marismortui* sequences; nucleotides 16 and 17 are absent from this sequence.

result from protection by the primary transcript or its processed intermediates. As with the 16S 3'-end sites, the 23S 5'-end sites originating within the 16S-23S intergenic space are different in the two operons.

Analysis of the 3'-flanking regions of the 23S rRNA gene was performed by using restriction fragments labeled at the common *Eco*RI site within the 23S gene (Fig. 6). Protection products of about 1,250 nucleotides were observed for each operon and represent the 3'-end site of 23S rRNA. Longer products representing processing intermediates were also apparent but were not resolved.

DNA sequence analysis. The nucleotide sequences of the 5'-flanking regions of the HH10 and HC8 rRNA operons extending to the common *Ava*I site (position 130) within the 16S genes were determined (Fig. 7). The two operons are not identical in sequence within the 16S coding region, the HC8 operon contains a 2-bp insertion at positions 16 and 17, and there are 10 base substitution differences located between positions 61 and 131 in the two operons. In contrast to the 16S rRNA-coding regions, the 5'-flanking regions immediately upstream (positions −1 to −95) exhibit no nucleotide sequence homology.

The three putative transcription initiation sites for the HC8 operon have been mapped to near G residues at positions −445, −365, and −285. The single putative initiation site for the HH10 operon is near the G residue at −256. The sequences surrounding these 5' transcript end sites are highly similar to each other and to the promoters for other halophilic and, to a lesser extent, methanogenic rRNA operons (Fig. 8A; 3, 4, 10, 11, 15). The putative RNA-processing site within the 5' leader of HC8 operon transcripts was mapped to about position −100. The GTGACA sequence at this site is similar to the recognition sites used to excise precursor 16S rRNA from the primary transcripts from other halophilic rRNA operons (1, 10). The HH10 operon does not contain this hexanucleotide, and we have observed no processing within the leader region of the HH10 primary transcript. Upstream of the processing signal in the HC8 operon is an extended sequence (positions −114 to −166 in HC8) that is nearly identical to a similarly located sequence in the HC10 operon (positions −94 to −146 in HH10; Fig. 8B). These two sequences are identical at 49 of 53 positions and are as highly conserved as the 5' end of the 16S rRNA gene sequences. The role for this conserved sequence was not determined.

DISCUSSION

Agarose gel and sucrose density fractionations of *Hin*dIII-digested *H. marismortui* genomic DNA revealed two fragments of 10 and 20 kbp capable of hybridizing to rRNA. These two fragments were cloned into pBR322, and each was shown to contain a 16S-23S-5S rRNA operon. Radioactive tRNA was also shown to hybridize to these clones; in other halophilic archaebacteria, an alanine tRNA has been located in the 16S-23S intergenic space and cysteine tRNA has been located distal to the 5S gene (2, 7, 9).

The boundaries of the 16S and 23S genes within the HH10 and HC8 clones were mapped by S1 nuclease protection analysis. The genes in the two operons are nearly identical in size, whereas the 16S-23S intergenic spaces are different, being about 510 and 370 bp long in HH10 and HC8, respectively.

A comparison of the first 131 nucleotides of the two 16S genes revealed significant sequence heterogeneity. There are eight transitions, two transversions, and an insertion of 2 bp

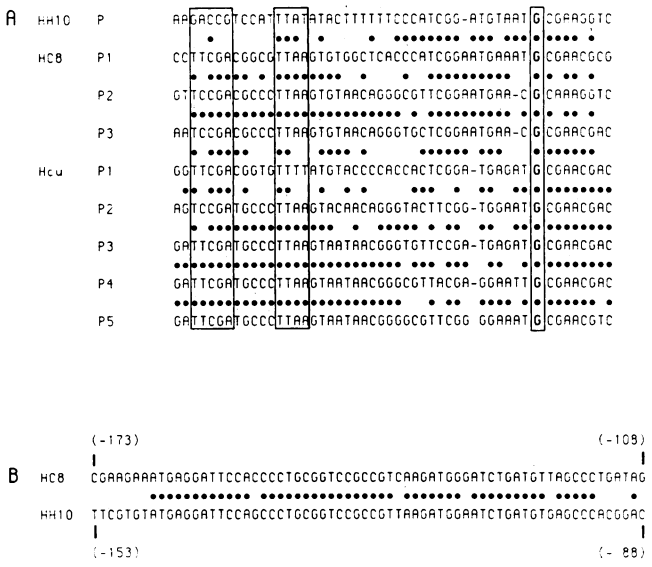

FIG. 8. Conserved sequence in the 5'-flanking region between the HC8 and HH10 operons. Nucleotide identities in pairwise comparisons are indicated (●). (A) Sequences surrounding the putative transcription start sites of the HH10 and HC8 operon and boxed in Fig. 7 are aligned to each other and to the five tandem promoters in front of the single rRNA operon of *H. cutirubrum*. The *H. cutirubrum* transcripts have been shown to start at or adjacent to the conserved boxed G residue and, for the P1 promoter, to contain a triphosphate 5' end (3; P. Joshi and P. P. Dennis, unpublished data). Transcription initiation occurs at an analogous position within the rRNA promoter of *Methanococcus vannielii* (15). The upstream boxed elements are found in many but not all archaebacterial promoters (4, 18). (B) Sequence homology of unknown function that is conserved within the 5'-flanking regions of the HH10 and HC8 operons; nucleotide positions are indicated at the beginning and end of each sequence.

at positions 16 and 17 of the HC8 16S gene. The two transitions occurring at position 67 and 74 are compensatory in that they conserve 1 bp within a helical region of the 16S rRNA. Most of the other differences either occur in single-stranded regions or disrupt base pairs at the beginnings or ends of helical regions.

In most organisms, sequence variation within rRNA genes is believed to be small, probably because of periodic homogenization by gene conversion type mechanisms. On the basis of the limited sequence available, it appears as if the two *H. marismortui* genes are only moderately more similar to each other than either is to the single 16S rRNA sequence from the distantly related halophile *H. cutirubrum* (Fig. 7). The two *H. marismortui* 16S sequences differ at 12 of 131 positions. The *H. cutirubrum* 16S sequence lacks the two nucleotides at positions 16 and 17 and differs from HC8 and HH10 at 24 and 16 positions, respectively.

Recently, a partial nucleotide sequence of the 16S rRNA of *H. marismortui* was determined by a modified chain termination method in which rRNA was used as the template and synthetic oligonucleotides were used to prime reverse transcription (A. Oren, P. P. Lau, and G. E. Fox, Syst. Appl. Microbiol., in press). In this study, the existence of sequence heterogeneity within the 16S rRNA template was not considered, although at many positions the bases could not be determined unequivocally. Several of these equivocal positions correspond to the mismatch position we identified by direct gene sequencing. The number of restriction enzyme site polymorphisms that we have observed between the two operons is consistent with the high degree of sequence heterogeneity (unpublished data). The role of this sequence heterogeneity in the structure and function of the *H. marismortui* ribosome has yet to be investigated.

Another striking difference between the two rRNA operons is present in the 5'-flanking regions. Nuclease S1 and DNA sequence analyses revealed three tandem promoterlike elements and a potential RNA-processing signal in the 5'-flanking region of the HC8 operon. A single promoterlike element but no processing signal was identified in the HH10 5'-flanking region.

All four *H. marismortui* promoterlike elements share substantial sequence similarity to the rRNA promoter elements of *H. cutirubrum* (Fig. 8A) and other methanogenic and halophilic archaebacteria (3, 4, 7, 10, 11, 15). Transcript initiation appears to occur at the conserved G residue, and most of these promoters conserve the TTCGA and TTAA motifs between positions −40 and −25 (18). The HH10 element apparently lacks the TTCGA-like component.

What is the significance of this remarkable conservation within the rRNA promoters of halophilic archaebacteria? It is possible that the conserved regions are the targets for transcription factors that regulate expression of rRNA genes. On the basis of the intensities of the protection products in the transcript-mapping experiment, it appears as though all four promoters are utilized with about equal frequency to produce rRNA during exponential growth.

ACKNOWLEDGMENTS

This work was supported by grants from the U.S. Department of the Navy, Office of Naval Research. P.P.D. was a Jacob Michael Visiting Professor at the Weizmann Institute of Science and is a fellow of the Canadian Institute of Advanced Research.

We thank both T. Shimon for technical assistance and Y. Lamed, who participated in the initial phases of this work.

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