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Putative promoter elements for the ribosomal RNA genes of the thermoacidophilic archaebacterium *Sulfolobus* sp. strain B12

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ABSTRACT

In Sulfolobus sp. strain B12, single-copy genes encode the three ribosomal RNAs. The genes for the 16S rRNA and for the 23S rRNA are closely linked but separated from the 5S rRNA gene. Transcription of the 16S/23S rRNA gene cluster starts 139 nucleotides upstream of the 5'-end of mature 16S rRNA. For the 5S rRNA gene the point of transcription initiation coincides with the 5'-end of mature 5S rRNA. The comparison of the upstream regions for these transcriptional start sites shows the presence of a completely conserved trinucleotide sequence around the point of transcription initiation and a completely conserved octanucleotide sequence about 22 nucleotides upstream of it. These sequences are only moderately homologous to putative promoter elements for stable RNA genes in the closely related archaebacterium Thermoproteus tenax (1), but they are very similar to corresponding sequences in the distantly related archaebacterium Methanococcus vannielii (2). The consensus sequence found for Sulfolobus and Methanococcus could therefore constitute the archetype of an archaebacterial promoter for stable RNA genes.

INTRODUCTION

Archaebacteria constitute a third group of organisms besides eubacteria and eukaryotes (3). Two major archaebacterial branches can be distinguished, one of them comprising methanogens and extreme halophiles, the other one comprising extremely thermophilic, sulfur-dependent archaebacteria (4). In order to determine promoter structures in archaebacteria, genes encoding stable RNAs have been cloned from a variety of archaebacterial species and sequences upstream of these genes have been analyzed for the presence of conserved sequences that could be involved in transcription initiation (for review see ref. 5). These studies revealed a high conservation of putative promoter sequences within a given organism but no general

archaebacterial promoter consensus sequence has been derived. In Methanococcus vannielii two highly conserved promoter elements have been identified: a box "B" containing the transcriptional start site and a box "A" located 18-19 nucleotides upstream of box "B" (2). For extremely halophilic archaebacteria, precise mapping data concerning the 5'-ends of pre-rRNAs have only been obtained in the case of Halobacterium cutirubrum (6). A box "B" motif similar to Methanococcus was found around the point of transcription initiation but there was only a weak conservation of the box "A" motif between Methanococcus vannielii and Halobacterium cutirubrum. In case of extremely thermophilic, sulfur-dependent archaebacteria, regions upstream of stable RNA genes have been analyzed for Thermoproteus tenax (1). Within this group of genes no striking sequence conservation has been found around the point of transcription initiation but there are extremely AT-rich regions about 18 nucleotides upstream of it. Since this situation differed from that in methanogens and extreme halophiles, it appeared that promoter sequences in archaebacteria are only conserved over short phylogenetic distances. In order to obtain more data about the signal structures for transcription initiation in extremely thermophilic archaebacteria, we studied the transcription of the ribosomal RNA genes in Sulfolobus. The Sulfolobus isolate B12 (7) was chosen for this study because the gene expression from a virus-like particle of this strain is already under investigation (8,9). It will therefore be possible to compare within the same organism the promoter sequences for genes encoding viral proteins and genes encoding stable RNAs.

Here we report the organization of the ribosomal RNA genes in Sulfolobus sp. strain B12 and the putative promoter structures governing the expression of these genes. We also present the sequence of the 5S rRNA from Sulfolobus B12 which is different from the 5S rRNA sequences of Sulfolobus acidocaldarius (10) and Sulfolobus solfataricus (11).

MATERIALS AND METHODS

Restriction endonucleases were obtained from BRL or from Boehringer, Mannheim. S1 endonuclease, T4 polynucleotide kinase

and the Klenow fragment of E. coli DNA polymerase I were purchased from Pharmacia and vaccinia virus guanylyltransferase and cloned M-MLV reverse transcriptase were from BRL. All radiochemicals were obtained from Amersham.

Total chromosomal DNA was purified as described by Yeats et al. (7). For the construction of a genomic library this DNA was partially cleaved with MboI and fragments were fractionated by sucrose gradient centrifugation (12). DNA fragments of a size between 15 kb and 20 kb were cloned into λEMBL4 (13) cleaved with BamHI. The λ arms required for cloning were purified by centrifugation through a potassium acetate gradient (12).

Purification of total RNA from early logarithmic cells of Sulfolobus B12 was done as described previously (8). Ribosomal RNAs from Sulfolobus B12 were purified as described by Neumann et al. (14).

5S rRNA was labelled with polynucleotide kinase after dephosphorylation of its 5'-end (14). 16S rRNA and 23S rRNA were fragmented by limited alkaline hydrolysis prior to labelling by polynucleotide kinase (15). Radioactive labelling of restriction fragments by random priming was carried out as described by Feinberg and Vogelstein (16).

The identification of restriction fragments by Southern analysis and the screening of a genomic library by plaque hybridization were done according to standard procedures (12).

DNA sequences were determined by the dideoxy chain termination method (17) using the M13 cloning and sequencing technique (18). Cloning of restriction fragments and sequencing of M13 clones were done according to the protocol of Amersham International plc. The sequences were partly determined from defined restriction fragments and partly from exonuclease III-generated deletion clones (19,20). In one case a 17mer synthetic oligonucleotide hybridizing to the insert DNA was used as a primer for the sequencing reaction. The programs of Devereux et al. (21) and Staden (22) were used for computer-aided editing and alignment of sequences.

For in vitro capping, 4 μg of 5S rRNA were dissolved in 36 μl of water, heated to 95°C for 30 sec and chilled on ice.

4 µl of a solution containing 0.5 M Tris/HCl pH 7.9, 12.5 mM $MgCl_2$, 60 mM KCl and 25 mM DTT were added and this mixture was transferred to a tube containing 500 µCi of dried [α-^{32}P]GTP (410 Ci/mMol). The mixture was incubated with 8 units of vaccinia virus guanylyltransferase for 30 min at 37°C. The reaction was stopped by addition of EDTA (2 mM final concentration) and twice extracted with phenol/chloroform. Fifteen micrograms of E. coli tRNA were added as carrier and after addition of ammonium acetate to 2 molar final concentration the RNA was precipitated with 2.5 volumes of ethanol. This precipitation step was repeated four times to remove most of the labelled GTP. The amount of [α-^{32}P]GTP incorporated into RNA was determined as acid-insoluble counts.

S1 nuclease analysis of the transcriptional start in front of the 16S/23S rRNA gene cluster was carried out by the method described in ref. 9. A 700-base single-stranded DNA probe was obtained by extension of the 17mer 5'-[^{32}P]-labelled oligonucleotide d(TGGGGGTAGGAAGCTAA) (oligo RRN) by Klenow enzyme and subsequent cleavage by HinfI. The map position of oligo RRN was immediately adjacent to the coding region for 16S rRNA. As a template for primer extension an M13 clone was used that contained a 3.4 kb BglII-fragment comprising 0.7 kb of the 16S rRNA gene and 2.7 kb of upstream sequences.

For an S1 nuclease protection experiment using labelled RNA and non-labelled DNA, 1.3 µg of 5S rRNA labelled by in vitro capping were hybridized to 50 µg of single-stranded M13 (+)-DNA containing a 2.0 kb BglII-fragment carrying the Sulfolobus B12 5S rRNA gene. The conditions for hybridization and S1 nuclease digestion were as in ref. 9, except that the mixture was heated to 95°C for 60 sec before hybridization.

cDNA analysis of pre-rRNA was carried out with 5'-[^{32}P]-labelled oligo RRN as primer. 0.1 picomoles (4 µl) of primer were mixed with 10 µg (5 µl) of total Sulfolobus RNA and 1 µl of a solution containing 100 mM Tris/HCl pH 7.5 and 400 mM KCl. This mixture was heated to 95°C for 60 sec, transferred to a 55°C water bath and allowed to cool to 45°C over a period of 45 min. cDNA synthesis was carried out in 30 µl of 45 mM Tris/HCl pH 7.5, 8 mM $MgCl_2$, 13 mM KCl, 1.7 mM DTT, 50 µg/ml

actinomycin D and 0.2 mM of each of the four dNTPs by incubation with 150 U of reverse transcriptase at 37°C for 30 min. After extraction with phenol/chloroform, the nucleic acid was precipitated with ethanol and analyzed on a 6% polyacrylamide sequencing gel.

RESULTS AND DISCUSSION

Organization of the ribosomal RNA genes in Sulfolobus sp. strain B12.

Total chromosomal DNA from Sulfolobus B12 was cleaved with restriction enzymes recognizing hexanucleotide sequences and the resulting restriction patterns were analyzed by Southern hybridization using the individual ribosomal RNAs as probes. A single band hybridizing to both 16S rRNA and 23S rRNA was found for EcoRV, HindIII and XbaI (Fig. 1). This result indicated that the genes for 16S rRNA and 23S rRNA are present in one copy and that they are closely linked. The nucleotide sequence of the spacer between the two genes was determined from a BamHI site within the 23S rRNA gene close to its 5'-end. This sequence analysis indicated that the spacer is about 185 nucleotides long and does not contain a tRNA gene (data not shown). Challenging Southern blots with labelled 5S rRNA resulted in a single hybridizing band for 15 different restriction enzymes tested (shown for three enzymes in Fig. 1). These bands differed in size from the bands obtained with 16S rRNA and 23S rRNA. Therefore we conclude that Sulfolobus B12 contains a single 5S rRNA gene without close linkage to the 16S/23S rRNA gene cluster. A similar organization of rRNA genes was found in the sulfur-dependent archaebacterium Desulfurococcus mobilis (23) whereas the 5S rRNA gene is closely linked to the genes for 16S rRNA and 23S rRNA in methanogenic and extremely halophilic archaebacteria (24-26). The minimum distance between the 23S rRNA gene and the 5S rRNA gene of Sulfolobus B12 was determined by hybridization analysis of a BamHI digest. This enzyme cleaves the gene for 23S rRNA about 0.1 kb from its 5'-end and it cleaves the gene for 5S rRNA about 30 bases from its 3'-end (Fig. 2). Challenging a Southern blot of BamHI-cleaved total chromosomal DNA with an internal 23S rDNA

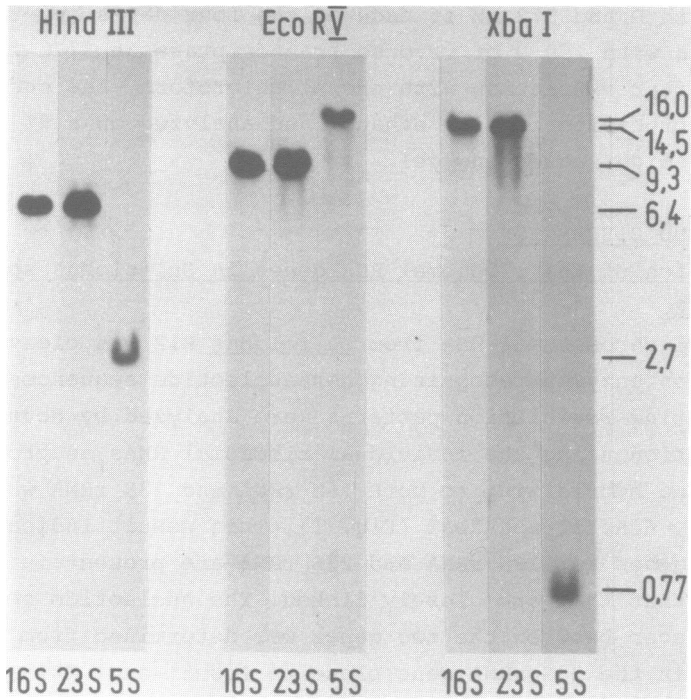

Fig. 1. Southern analysis of the rRNA genes of Sulfolobus B12. Total chromosomal DNA was digested with the restriction enzymes indicated, separated on a 1% agarose gel and transferred to nitrocellulose. The blot was challenged with 16S, 23S and 5S rRNA labelled with ^{32}P in vitro. The numbers on the right margin give the sizes of hybridizing restriction fragments.

probe resulted in the hybridization to a 15 kb BamHI-fragment whereas a probe comprising the whole 5S rRNA gene hybridized to BamHI-fragments of 3.3 kb and 3.6 kb (data not shown). It can be inferred from these results that the distance between the 23S rRNA gene and the 5S rRNA gene is at least 15 kb. It could also be ruled out that the 5S rRNA gene is in an upstream position close to the gene for 16S rRNA since 5S rRNA did not hybridize to 7.5 kb of DNA 5' to the 16S rRNA gene. Our results confirm the conclusion of Neumann et al. (14) that the 16S rRNA and the 23S rRNA of Sulfolobus B12 are encoded by single copy genes and that there is an isolated gene for 5S rRNA. In contrast to this work, however, we did not find an additional

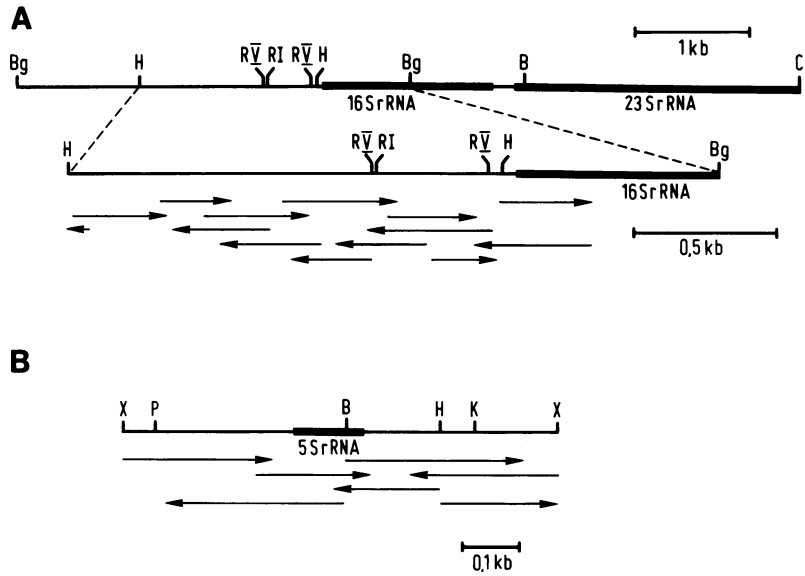

Fig. 2. Restriction map of the rRNA genes of Sulfolobus B12 and the strategy used for DNA sequencing.
A. Top: Physical map of the 16S/23S rRNA gene cluster from a ClaI-site within the 23S rRNA gene up to a BglII-site 2.7 kb 5' to the 16S rRNA gene. Bottom: Strategy used for sequencing.
B. Physical map and sequencing strategy for a 0.77 kb XbaI-fragment containing the 5S rRNA gene of Sulfolobus B12.
Abbreviations used for restriction enzymes: RI: EcoRI, RV: EcoRV, B: BamHI, Bg: BglII, C: ClaI, H: HindIII, K: KpnI, P: PstI, X: XbaI.

5S rRNA gene with close linkage to the 16S/23S rRNA gene cluster.

Cloning and sequence analysis of the genes for ribosomal RNAs.

Two thousand λ-plaques of a genomic library of Sulfolobus B12 were screened for hybridization with ribosomal RNAs. Five clones hybridizing to both 16S rRNA and 23S rRNA and two clones hybridizing to 5S rRNA were identified and further characterized by restriction mapping. From a λ-clone hybridizing to 16S rRNA and 23S rRNA, a 12 kb ClaI-fragment containing almost the whole 16S/23S rRNA gene cluster and 7.5 kb of upstream sequences was subcloned into pBR322. From a λ-clone hybridizing to 5S rRNA, a 2.0 kb BglII-fragment and a 0.77 kb XbaI-fragment both containing the 5S rRNA gene were subcloned into M13

Fig. 3. Nucleotide sequence of the region upstream of the Sulfolobus 16S rRNA gene.
The HinfI site indicated at the beginning of the sequence was used for the generation of a hybridization probe for S1 mapping (see text). The 5'-terminal sequence of 16S rRNA is boxed. Putative promoter elements (including the CTTATAT motif; see also Fig. 6) are underlined. A 13 bp direct sequence repeat (containing one mismatch) which partly overlaps the CTTATAT motif is indicated by a broken line above the sequence.

vectors. Restriction maps of the regions encoding the ribosomal RNAs are shown in Fig. 2.

The nucleotide sequence was determined for a contiguous stretch of DNA comprising the 5'-end of the 16S rRNA gene and 1.5 kb upstream of it (see Fig. 2 for the sequencing strategy and Fig. 3 for the nucleotide sequence). The gene for the 16S rRNA was located within this sequence by alignment with the sequence of the 16S rRNA from Sulfolobus solfataricus (27). The nucleotide sequence of the 0.77 kb XbaI fragment containing the 5S rRNA gene was also determined (see Fig 2 for the sequencing strategy and Fig. 4 for the nucleotide sequence) and the gene was identified within this region by alignment with the sequence of 5S rRNA from Sulfolobus acidocaldarius (10). At three positions there were differences between the sequence of the 5S rRNA gene of Sulfolobus B12 and the sequence of 5S rRNA from S. acidocaldarius (10) (identical to that from S.

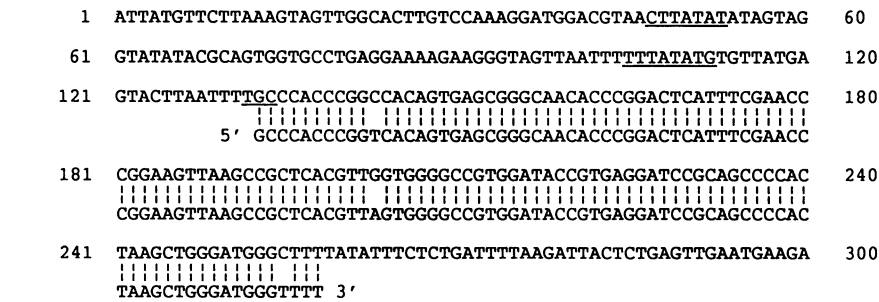

Fig. 4. Nucleotide sequence of the region encoding the 5S rRNA of Sulfolobus B12.
The sequence is aligned with the primary structure of the 5S rRNA of S. acidocaldarius (10). Putative promoter elements are underlined.

solfataricus, ref. 11). The residues U_{11} and U_{123} which are involved in G-U base pairs in S. acidocaldarius are changed to C residues in Sulfolobus B12 allowing the formation of G-C pairs. Residue A_{71} of S. acidocaldarius which forms an A-U base pair within an extensive secondary structure is changed to a G residue in Sulfolobus B12. A computer analysis of the sequence of the whole XbaI fragment using the program of Staden (22) indicated that the 5S rRNA gene is not linked to tRNA genes as in Methanococcus vannielii (28).

Determination of the transcriptional starts for the rRNA genes.

The transcriptional start site upstream of the 16S/23S rRNA gene cluster was determined by S1 nuclease mapping and by cDNA analysis. The 5'-end of the probe used for S1 mapping as well as of the primer used for cDNA analysis mapped immediately adjacent to the coding region for mature 16S rRNA. S1 nuclease mapping revealed two major fragments of 140 and 141 nucleotides which were taken as the fragments corresponding to the primary transcript since no larger species were found even upon overexposure of the gel (Fig. 5A). Just below these major fragments the autoradiograph showed a short ladder of smaller species which might be due to "nibbling" of the S1 endo-nuclease. A ladder of very weak bands with sizes between 82 and 95 nucleotides was also observed (Fig. 5A). We assume that these short fragments were due to processing intermediates.

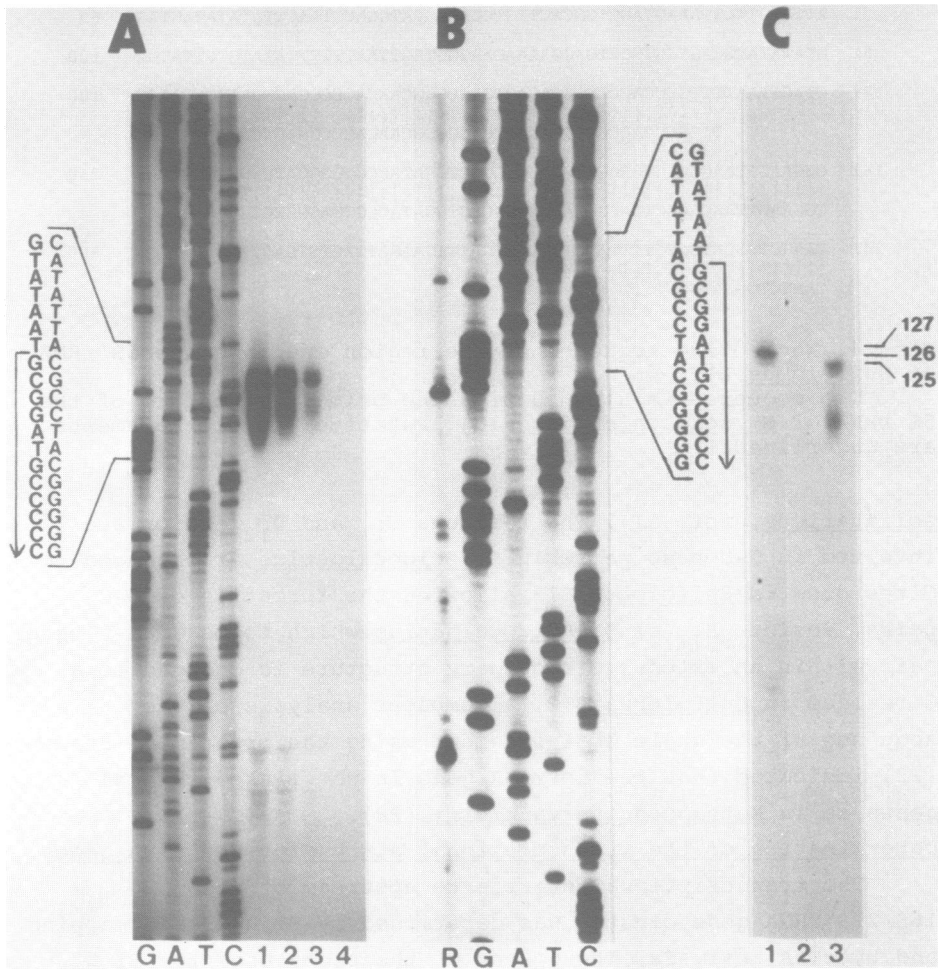

Fig. 5. Analysis of the transcriptional initiation sites for the rRNA genes of Sulfolobus B12.

The sequencing ladders (lanes marked G,A,T,C) were obtained using oligo RRN as primer (see text). The transcriptional start sites are marked by vertical arrows.

A. S1 endonuclease analysis for the 16S/23S rRNA gene cluster. The autoradiograph shows the sizes of protected fragments using 30000 cpm of labelled DNA probe and different amounts of Sulfolobus RNA: lane 1: 32 µg, lane 2: 8 µg, lane 3: 1.4 µg, lane 4: no Sulfolobus RNA added.

B. cDNA analysis of the pre-16S/23S rRNA. Products formed by reverse transcription of Sulfolobus B12 RNA using 5'-labelled oligo RRN as primer are shown in the lane marked "R".

C. Analysis of 5S rRNA labelled by vaccinia virus guanylyltransferase. The sizes (in nucleotides) given on the

right margin were determined from a sequencing ladder run in parallel. Lane 1: 0.1 μg of labelled RNA directly applied to the gel. Lane 2: S1 endonuclease digest of 1.2 μg of labelled RNA hybridized to M13 (+)-DNA containing the non-coding strand of the 5S rRNA gene. Lane 3: same as lane 2, but using M13 (+)-DNA containing the strand complementary to 5S rRNA.

Since a mapping uncertainty of about two nucleotides was expected for S1 nuclease analysis, we also used cDNA analysis to determine the exact 5'-end of the pre-rRNA. Several cDNA species were seen in this experiment (Fig. 5B), the largest one with a length of 139 nucleotides corresponding to the transcriptional start already mapped with S1 endonuclease. Two cDNA species of 91 and 125 nucleotides had no clear equivalent in S1 nuclease mapping and we therefore assume that they were due to premature termination of cDNA synthesis possibly caused by a high degree of secondary structure within the RNA. From the results of S1 nuclease mapping and cDNA analysis we conclude that the transcriptional start for a 16S/23S rRNA precursor is the G residue within the sequence ..AATGC.. located 139 nucleotides upstream of the 5'-end of mature 16S rRNA.

The sequence around this transcriptional start was compared to the sequence data obtained for the 5S rRNA gene. As will be discussed below, this comparison strongly suggested that in Sulfolobus B12 the 5'-end of mature 5S rRNA coincides with the point of transcription initiation. In order to demonstrate the presence of a triphosphate group at the 5'-end of this RNA, we incubated 5S rRNA purified from ribosomes with [α-^{32}P]GTP and vaccinia virus guanylyltransferase. The electrophoretic analysis of this labelling mixture on a denaturing polyacrylamide gel showed that label had been incorporated mainly into an RNA species of 126 nucleotides and to a lesser extent into RNA species of 125 and 127 nucleotides length (Fig. 5C). These sizes are in agreement with the size predicted for Sulfolobus 5S rRNA (10,11). The observed length heterogeneity is a known feature of Sulfolobus 5S rRNA; it is due to a ragged 3'-end (10,11). The determination of acid-precipitable counts indicated that only 1.8% of the 5S rRNA molecules had been labelled though an efficiency of capping of

about 15% can be expected for mRNAs (ref. 29 and literature cited therein). The poor efficiency of capping of Sulfolobus 5S rRNA is not unexpected, however, since the 5'-end of this RNA is involved in a highly stable secondary structure (10,11). In order to rule out the possibility that the label had been incorporated into a contaminating RNA species that fortuitiously coincided in length with 5S rRNA, we hybridized the labelled RNA with the 5S rRNA gene cloned into M13 and digested this mixture with S1 endonuclease. As expected, the labelled RNA was protected from S1 nuclease digestion by M13 (+)-DNA containing the coding strand of the 5S rRNA gene but it was not protected by a clone containing the opposite strand (Fig. 5C). Since mature 5S rRNA could thus be labelled by "capping enzyme", we conclude that its 5'-end coincides with the transcriptional initiation site. Termination of 5S rDNA transcription probably occurs directly at the 3'-end of the gene. At this position there is a run of four T residues followed by two additional T clusters (Fig. 4). Similar sequences have already been shown to effect termination of transcription in Sulfolobus B12 (ref. 8 and unpublished work from this laboratory).

In order to detect putative promoter elements in Sulfolobus B12, we aligned the sequences for the 16S/23S rRNA gene cluster and for the 5S rRNA gene around the transcriptional initiation sites. From this comparison the following results were obtained (Fig. 6):

i) In both cases the transcriptional initiation site is the G residue within the sequence TGC.

ii) Aligning positions -32 to -13 for the 16S/23S rRNA primary transcript with positions -28 to -9 for the 5S rRNA gene a homology of 75% is found. Within this region an octanucleotide sequence (TTTATATG) is completely conserved.

iii) The most striking sequence conservation further upstream is the presence of the heptanucleotide CTTATAT centered around position -88 for the 16S/23S rRNA primary transcript and position -83 for the 5S rRNA gene.

We also compared the putative promoter sequences for the rRNA genes in Sulfolobus B12 with corresponding sequences from

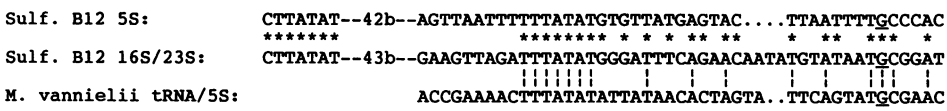

Fig. 6. Comparison of putative promoter sequences for the rRNA genes of Sulfolobus B12 and for the tRNA/5S rRNA gene cluster of Methanococcus vannielii (2).
Nucleotides which are identical in the two Sulfolobus sequences are marked by asterisks. Vertical lines indicate nucleotides which are identical in Sulfolobus and Methanococcus. The transcriptional initiation sites are underlined.

other archaebacteria. In case of the genes encoding stable RNAs in the related archaebacterium Thermoproteus tenax, there is an extremely AT-rich region about 18 nucleotides upstream of the transcriptional initiation site (1). A similar situation is found for the rRNA genes of Sulfolobus B12, but the only contiguous stretch of sequence identity between Sulfolobus and Thermoproteus is the tetranucleotide TTTA. In contrast to Sulfolobus B12, no striking sequence conservation was found around the transcriptional initiation site in Thermoproteus (1). A very high degree of sequence homology exists between the putative promoter elements for the rRNA genes in Sulfolobus B12 and corresponding sequences characterized in Methanococcus vannielii (2). For this methanogenic archaebacterium, a box "B" has been described containing the transcriptional start on the G residue within the conserved sequence motif TGC. About 18-19 nucleotides upstream of box "B" there is a highly conserved box "A" motif containing the sequence element TTTATAT (2). As shown above, these motifs are also completely conserved between the putative promoter regions for the rRNA genes in Sulfolobus B12 with a spacing similar to that in Methanococcus (Fig. 6). This high conservation of promoter sequences between two phylogenetically distant archaebacteria is quite unexpected, since no archaebacterial promoter consensus sequence has been derived from the data available for Halobacterium, Methanococcus and Thermoproteus. It appears that the promoter sequences found in Methanococcus and Sulfolobus have remained essentially unchanged since the divergence of the major archaebacterial

branches whereas the corresponding sequences in Thermoproteus and Halobacterium have evolved more rapidly. In order to gain more insight into the evolution of trancription signals, the characterization of promoter sequences from other archaebacteria is highly desirable.

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