FEMS Microbiology Letters 98 (1992) 95–102

© 1992 Federation of European Microbiological Societies 0378-1097/92/$05.00
Published by Elsevier

FEMSLE 05118

Transcription in vitro and in vivo of the 7S RNA gene associated with the ribosomal RNA operon in the hyperthermophilic archaeon *Methanothermus fervidus*

Gabriele Koller [a], John N. Reeve [b], Gerhard Frey [a] and Michael Thomm [a,1]

[a] *Lehrstuhl für Mikrobiologie, Universität Regensburg, Regensburg, FRG, and* [b] *Department of Microbiology, The Ohio State University, Columbus, Ohio, USA*

Received 3 August 1992
Accepted 5 August 1992

Key words: Methanogen promoter; 7S RNA; Archaea; Thermophile; *Methanothermus fervidus*

1. SUMMARY

The gene encoding the archaeal 7S RNA in the hyperthermophile *Methanothermus fervidus* is linked to a tRNA^{Ser} and rRNA operon in the arrangement 5′-7S RNA-14nt-tRNA^{Ser}-196nt-16S rRNA and the promoter directing transcription of this 7S RNA gene has now been identified. Initiation of transcription of the 7S RNA gene has been shown to occur both in vivo in *M. fervidus* and in vitro, using a *Methanococcus thermolithotrophicus* derived cell-free transcription system, at the first G residue within the initiator sequence ATGG, located 6 bp upstream of the 5′ end of the 7S RNA coding region. Cotranscription of the 7S RNA and tRNA^{Ser} has been demonstrated in vitro.

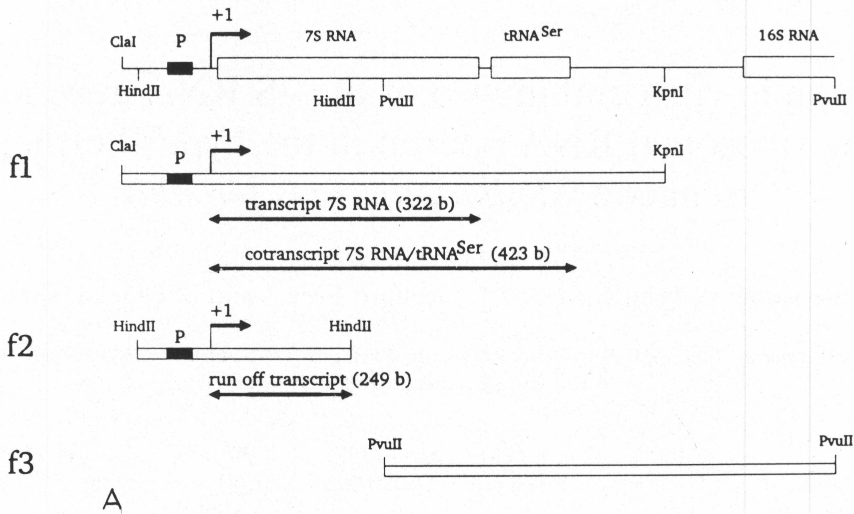
Correspondence to: J.N. Reeve, Department of Microbiology, The Ohio State University, 484 W. 12th Avenue, Columbus, Ohio 43210-1292, USA.

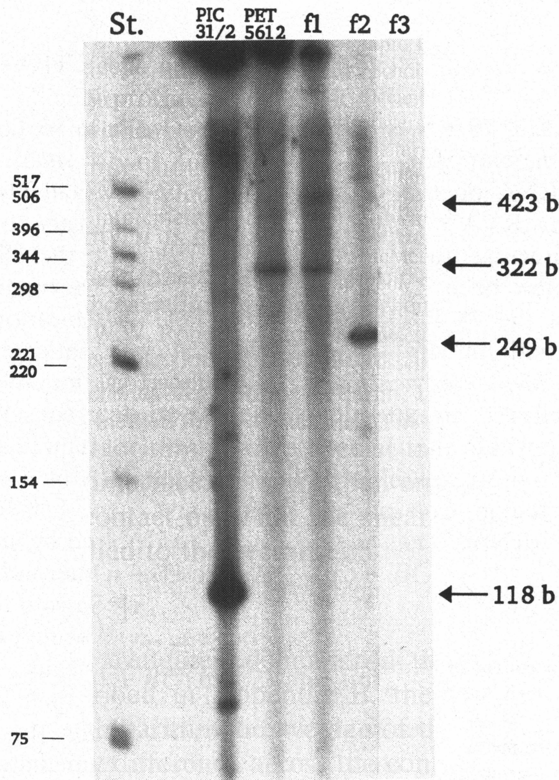
[1] Present address: Institut für Allgemeine Mikrobiologie, Universität Kiel, Am Botanischen Garten 1-9, D-2300 Kiel, FRG.

2. INTRODUCTION

All Archaea so far investigated contain large amounts of a small, novel RNA approximately 300 nucleotides in length, designated the 7S RNA [1]. The secondary structure of this RNA is similar to that of the 7SL RNA component of eukaryal signal recognition particles that are involved in translocation of secreted proteins across the endoplasmatic reticulum [2–4]. The primary sequences of the archaeal and eukaryal 7S RNA molecules are conserved within a 16 nucleotide structural domain [2,3] which is also conserved in the 4.5S RNA of *Escherichia coli*. The essential function of the 4.5S RNA in translation in *E. coli* can be replaced by an archaeal 7S RNA [6–8]. It seems likely, therefore, that the 7S RNAs in the Archaea play a role in translation and a 7S RNA has, in fact, been isolated from *Halobacterium halobium* physically associated with polysomes [9]. The discovery that, in the genome of *Methanothermus fervidus*, the gene encoding the 7S RNA is located immediately upstream of a tRNA^{Ser} gene and rRNA operon ([3]; see Fig. 1A) adds

further circumstantial evidence for the involvement of the 7S RNA in translation. Upstream of this 7S RNA gene are sequences that conform to the consensus for methanogen promoters established by studies of tRNA genes and protein-encoding genes [10–12]. Here we demonstrate the





function of these sequences as promoter elements for transcription of the *M. fervidus* 7S RNA gene both in vivo and in vitro. We have also addressed the question, posed by the gene organization [3], of cotranscription of the 7S RNA gene with the adjacent $tRNA^{Ser}$ and rRNA genes.

3. MATERIALS AND METHODS

3.1. Cultivation of methanogens

Methanococcus thermolithotrophicus and *M. fervidus* cultures were grown anaerobically at 65°C and 85°C, respectively, as described previously [13,14]. Large scale cultures were grown in a 100l enamel-coated fermentor (Bioengineering, Wald, Switzerland).

3.2. Preparation of template DNA

Plasmid DNA was purified by CsCl density gradient centrifugation as described previously [15]. DNA restriction fragments were purified after their separation by electrophoresis through agarose gels, by using a gel eluter (Biometra, Göttingen, FRG).

3.3. Purification of the components of the M. thermolithotrophicus cell-free transcription system

RNA polymerase (RNAP) and transcript ion factors were purified by phosphocellulose (PC) chromatography using KCl elution as described previously [11]. The RNAP (0.5 mg/ml) eluted in the 0.35 M KCl fraction and the transcription factor was eluted by 0.6 M KCl.

3.4. Cell-free transcription reactions

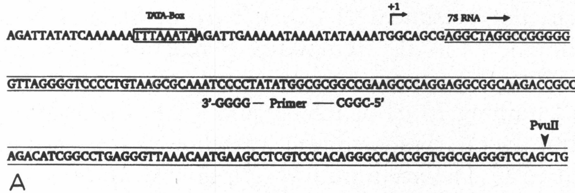
Transcription in vitro used 3 μg of intact, circular plasmid DNA or 0.2 μg of linear DNA fragments as the template, in reaction mixtures (100 μl) composed as previously described [11], containing 20 μl of RNAP and 2 μl of transcription factor. Transcription was allowed for 30 min at 55°C. The accurate transcription of *M. fervidus* genes in vitro by this *M. thermolithotrophicus* derived system has been documented [16].

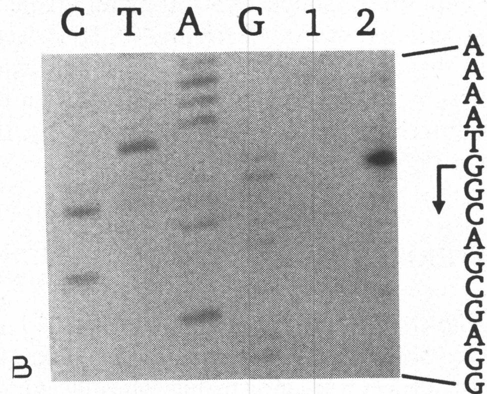
3.5. S1-nuclease mapping

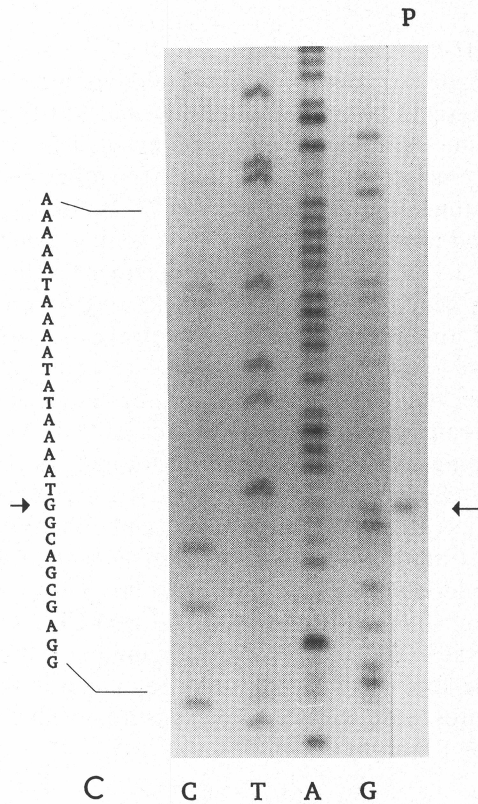
Single-stranded, ^{32}P-end-labeled probes were synthesized by primer extension and purified by alkaline agarose gel electrophoresis. The probe (131 nucleotides (nt) in length) synthesized to determine the 5′ end of the 7S transcript extended from within the 7S RNA coding sequence (Fig. 2A) to a position 60 bp upstream of the 7S RNA gene. The probe (727 nt in length) synthesized to detect cotranscripts of the 7S RNA, $tRNA^{Ser}$ and 16S rRNA genes extended from within the 16S rRNA coding region to 36 bp upstream of the 7S RNA gene (Fig. 3A). S1 mapping experiments were performed as previously described [10,17]. Briefly, RNA molecules purified from *M. fervidus* [17] cells or from in vitro transcription reactions were annealed with ^{32}P-end-labeled probe (10^5 cpm) in a buffer containing 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl and 80% v/v formamide at 57°C. Single-stranded molecules that remained in these mixtures were digested by exposure to nuclease S1 for 1 h at 30°C.

Fig. 1. Transcription of the 7S RNA and $tRNA^{Ser}$ genes in vitro. **(A)** Map of the DNA region upstream of the rRNA operon in *M. fervidus* [3] and the linear templates used in cell-free transcription. The 7S RNA, $tRNA^{Ser}$ and 16S RNA genes are shown as open boxes, +1 indicates the transcription initiator nucleotide with the arrow indicating the direction of transcription. The black box labeled P represents the promoter. Restriction sites are shown which were used to obtain the linear templates, designated as fragments f1, f2 and f3 from plasmid pET5612 [3]. The arrows below fragments f1 and f2 indicate the location and lengths of the transcripts synthesized in vitro from these templates (see **B**). **(B)** Electrophoretic separation of transcripts synthesized in vitro. Radioactively labeled transcripts synthesized in vitro were separated by electrophoresis through a 6% (w/v) polyacrylamide/urea gel and visualized by autoradiography. The lane designated St. contained *Hin*fI generated ^{32}P-end-labeled restriction fragments of plasmid pBR322 used as size standards. Transcripts synthesized as controls from a circular template [10] containing a $tRNA^{Val}$ gene from *M. vannielii* are shown in the lane designated pIC31/2. Lane pET5612 contained the transcripts synthesized from the circular pET5612 template [3] and lanes f1, f2 and f3 contained the transcripts synthesized from the templates identified by these letters in **(A)**. The sizes of standards are indicated to the left, and the sizes of prominent transcripts to the right of the figure.

Mt. fervidus 7S RNA Gene







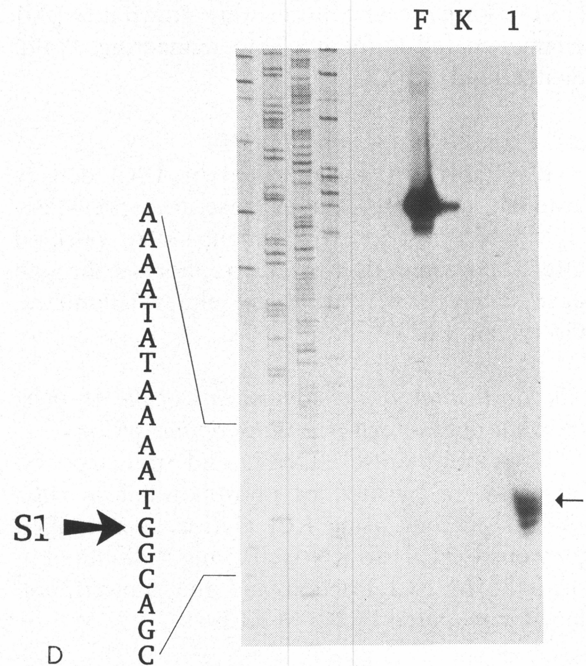


Fig. 2. Location of the site of transcription initiation of 7S RNA in vitro and in vivo. **(A)** The DNA sequence of part of the 7S RNA gene and of the upstream region are shown. The TATA box element of the promoter is boxed, +1 indicates the site of transcription initiation and the 7S RNA coding region is indicated by lines above and below the sequence. The oligonucleotide used as the primer in primer extension experiments (**B**, **C**) and for synthesis of the S1 probe (**D**) is indicated. (**B**) Primer extension experiment locating the site of transcription initiation in vitro. The primer shown in (**A**) was 5′ end-labeled (3×10^5 cpm), annealed with RNA synthesized in vitro, extended with reverse transcriptase and the products of this reaction were separated by electrophoresis through a DNA sequencing gel. The same primer was also used to obtain the sequence of part of the 7S RNA gene which is shown in the tracks labeled C, T, A, G. The sequence shown is complementary to the sequence of the reverse transcriptase product. Lane 1 shows the product of a control primer extension reaction which did not contain RNA synthesized in vitro and lane 2 shows the product synthesized in the presence of in vitro RNA. The position identified as the initiator nucleotide is indicated by the arrow in the sequence shown to the right of the figure. (**C**) Primer extension experiment locating the site of transcription initiation in vivo. The end-labeled primer (10^5 cpm) was annealed with 18 μg of RNA purified from *M. fervidus* cells [17] and extended with reverse transcriptase. The sequencing reactions shown are the same as in (**B**). The lane labeled P contained the primer extension product. Transcription in vivo initiated at exactly the same site as in vitro.(**D**) S1 mapping experiment locating the site of transcription initiation in vivo. The S1 probe (1.5×10^5 cpm) was annealed with 12 μg of RNA purified from *M. fervidus* cells and the molecules which survived subsequent digestion with S1 nuclease were identified following their electrophoresis through a 6% sequencing gel. The sequencing reactions used as size standards were the same as in (**B**) and (**C**). Lane F contained the intact probe, lane K a control reaction in which *M. fervidus* RNA was not added, and lane 1 shows the molecules that survived S1 nuclease digestion. The 5′ end of the in vivo transcript identified by these procedures, and indicated in the sequence, is the same G residue as identified by the primer extension experiments in (**B**) and (**C**).

3.6. Primer extension analyses

Single stranded ^{32}P-end-labeled primer (10^5 cpm; Fig. 2A) was annealed with unlabeled RNA from in vitro transcription reactions, or with RNA extracted from *M. fervidus* cells, and extended with reverse transcriptase as described previously [11].

4. RESULTS

4.1. Transcription of the 7S RNA gene in vitro

The restriction fragments used as template DNAs (Fig. 1A) for in vitro transcription were obtained from plasmid pET5612 [3]. Transcription of fragment f1 generated two transcripts, 322 nt and 423 nt in length (Fig. 1B). Transcription of the shorter fragment f2, generated only one transcript, 249 nt in length. These results are consistent with transcription initiating at the predicted initiator sequence, upstream of 7S RNA gene, and terminating at two sites, one between the 7S RNA gene and $tRNA^{Ser}$ gene and the second downstream of the $tRNA^{Ser}$ gene. As transcription of fragment f3 did not generate discrete transcripts (Fig. 1B), the region between the $tRNA^{Ser}$ gene and 16S rRNA apparently does not contain a promoter that functions in vitro with the *M. thermolithotrophicus* derived system.

Plasmid pET5612 contains the *M. fervidus* genomic DNA shown in Fig. 1A plus 2.5 kbp of upstream region cloned in pUC18 [3]. When this intact plasmid was provided as a negatively-supercoiled, circular template DNA, only the 322 nt transcript was clearly detectable as a discrete product (Fig. 1B). Large amounts of much higher molecular mass RNAs were synthesized but these could not enter the polyacrylamide gels. These molecules presumably resulted from the methanogen RNAP proceeding around the circular template DNA and frequently reading through the transcription terminators that functioned more efficiently on the linear templates.

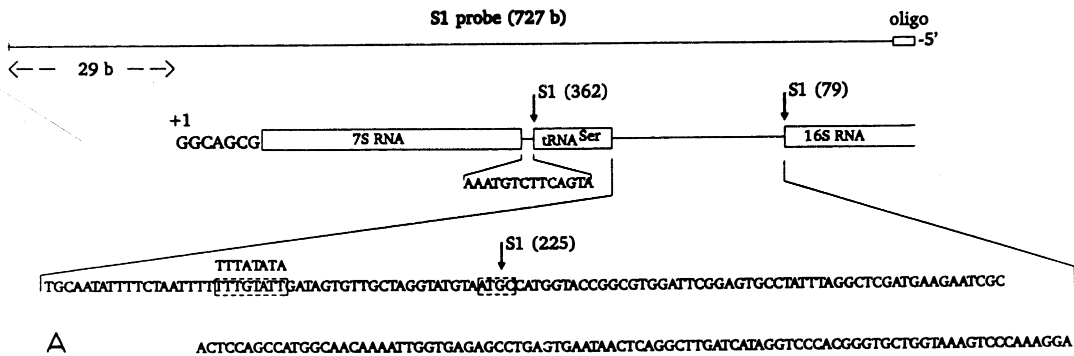
4.2. Determination of the site of 7S RNA transcription initiation in vitro and in vivo

Primer extension was used to determine the precise site at which the RNA molecules synthesized in vitro and in vivo were initiated upstream of the 7S RNA gene. An oligonucleotide primer complementary to an internal region of the 7S RNA (see Fig. 2A) was ^{32}P-end-labeled, annealed with the RNA synthesized in vitro and in vivo and extended with reverse transcriptase. Electrophoresis of the extension products demonstrated that transcription of the 7S RNA gene initiated at a G residue, 7 bp upstream of the 7S RNA coding sequence, both in vitro and in vivo (Fig. 2B and 2C). This is exactly the site predicted from the consensus to be the initiator sequence [10,12,15] and also the site calculated from the run-off transcription experiments using fragments f1 and f2 (Fig. 1). S1 nuclease protection experiments confirmed that, in vivo, transcription initiated at the first G residue in the ATGG initiator sequence [10] located 7 bp upstream of the 7S RNA gene (Fig. 2D).

4.3. Investigation of cotranscription of the 7S RNA, $tRNA^{Ser}$ and 16S rRNA genes

A ^{32}P-end-labeled molecule 727 nt in length, that extended from 79 bp within the 16S rRNA coding region to a site 29 bp upstream of the start site for 7S RNA transcription, was synthesized by primer extension (Fig. 3A) and annealed with RNA isolated from *M. fervidus* cells. Hybridization resulted in three molecules that were resistant to subsequent digestion with S1 nuclease (Fig. 3B). The longest, 362 nt in length (Fig. 3B), is consistent with the presence of a transcript in vivo that extends from the 5′ terminus of the $tRNA^{Ser}$ gene to the end of the probe within the 16S rRNA gene. The shortest, 79 nt in length, was expected as a fragment of the probe with this length would be protected by the mature 16S rRNA genes. The 225 nt molecule indicates the presence in vivo of a transcript with a 5′ terminus located at the site, identified in Fig. 3A, between the $tRNA^{Ser}$ and 16S rRNA genes. This is within a sequence that conforms to the consensus for methanogen transcription initiator elements and is located at an appropriate distance downstream from a sequence that resembles a methanogen TATA-box. This could therefore be the 5′ end of a 16S rRNA transcript, transcribed from a promoter that functions in vivo but not in vitro (Fig.

1B). If so, the TATA-box of this promoter has a G residue at position no. 4 (Fig. 3A) which would be unusual, especially as previous studies which deliberately introduced a G at this position, into a consensus TATA box, resulted in an almost complete loss of promoter activity [10,11]. S1 nu-



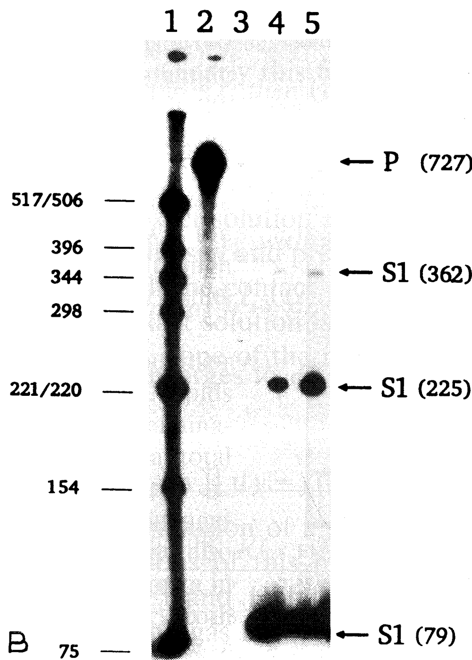


Fig. 3. Mapping of transcripts containing 16S rRNA sequences in RNA preparations isolated from cells of *M. fervidus*. (**A**) The probe synthesized and used for S1-nuclease mapping (**B**) and the intergenic sequences between the tRNA^{Ser} and 16S rRNA genes are shown. The predicted locations of the 5′ termini of the transcripts identified in (**B**) are indicated by vertical arrows. The putative TATA box and transcription initiator sequence, located between the tRNA^{Ser} and 16S rRNA genes, are indicated. (**B**) Electrophoretic separation of S1-nuclease resistant molecules. RNA (12 μg) isolated from *M. fervidus* cells was annealed with the ^{32}P-end-labeled S1 probe shown in (**A**) and the mixture then exposed to S1 nuclease. Lane 1 contained size standards (see **B**), lane 2 contained the undigested 727 nt probe, lane 3 the products of a control reaction which lacked *M. fervidus* RNA and lanes 4 and 5 contained the molecules that survived digestion with S1 nuclease. In tracks 4 and 5, the hybridization mixtures initially contained 5×10^4 and 1×10^5 cpm of the radioactively labeled probe DNA, respectively. The lengths of the S1-nuclease-resistant fragments of the probe molecule are indicated to the right of the figure.

clease resistant fragments of the probe longer than 362 nt, which would have indicated the presence of contiguous transcripts containing 7S RNA, $tRNA^{Ser}$ and 16S rRNA sequences, were not detected.

5. DISCUSSION

Transcription of the *M. fervidus* 7S RNA gene has been shown to be initiated 7 bp upstream of the 7S coding region at the first G within a 5′ATGG sequence. This sequence conforms well to the consensus for initiator sequences established for methanogen promoters of tRNA and protein genes [10–12] and transcription initiates, as mandated by the consensus, at a pyrimidine/purine dinucleotide. The consensus methanogen promoter contains a TATA-box located 21–25 bp upstream of the site of transcription initiation [10–12]. The sequence 5′TTTAAATA, located 24 bp upstream of the site of 7S RNA initiation, conforms precisely to the consensus for methanogen TATA-boxes. It appears therefore that the *M. fervidus* 7S RNA gene is transcribed from a 'typical' methanogen promoter. The sequences initially proposed as the consensus for promoter elements in methanogens on the basis of their conservation upstream of many genes [12,15,18], and subsequently shown to function in vitro as promoters for tRNA and protein genes [10,11], have now therefore been extended to include a promoter for a 7S RNA gene.

Although the gene organization indicates that cotranscription of the 7S RNA, $tRNA^{Ser}$ and rRNA operon is possible [3], we have been unable to obtain definitive proof for such a transcript. The intergenic region between the 7S RNA and $tRNA^{Ser}$ genes is too short to accommodate a promoter and these genes are cotranscribed in vitro (Fig. 1). The evidence extending this to cotranscription of the $tRNA^{Ser}$ and 16S rRNA genes, obtained by S1-nuclease protection studies is, however, only tenuous (Fig. 3B). The S1 mapping experiments indicate that the 16S rRNA could also be transcribed in vivo from a novel promoter located between the $tRNA^{Ser}$ and 16S rRNA genes (Fig. 3B). If so, this promoter with a G residue at position no. 4 of the TATA-box [10–12] would be very unusual and, as observed (Fig. 1B), would not be expected to function in vitro with the *M. thermolithotrophicus* derived system [10,11].

ACKNOWLEDGEMENTS

We thank K.M. Sandman for advice. This work was supported by a grant from the Deutsche Forschungsgemeinschaft, by contract N00014-86-K-0211 from the Office of Naval Research, the Fonds der Chemischen Industrie and by NATO Scientific Affairs collaborative grant No. 0148/85. We thank K.O. Stetter for supporting this study with funds from the Leibniz-Preis.

REFERENCES

[1] Luehrsen, K.R., Nicholsen, D.E. and Fox, G.E. (1985) Curr. Microbiol. 12, 69–72.
[2] Kaine, B.P. (1990) Mol. Gen. Genet. 221, 315–321.
[3] Haas, E., Brown, J.W., Daniels, C.J. and Reeve, J.N. (1990) Gene 90, 51–58.
[4] Walter, P. and Blobel, G. (1982) Nature 299, 691–698.
[5] Hsu, L.M., Zagorski, J. and Fourneir, N.J. (1984). J. Mol. Biol. 178, 509–531.
[6] Bourgaize, D.B. and Fourneir, M.J. (1987) Nature 325, 281–284.
[7] Brown, S. (1987) Cell 49, 825–833.
[8] Brown, S. (1991) J. Bacteriol. 173, 1835–1837.
[9] Gropp, R., Gropp, F. and Betlach, N.C. (1992) Proc. Natl. Acad. Sci. USA 89, 1204–1208.
[10] Hausner, W., Frey, G. and Thomm, M. (1991) J. Mol. Biol. 222, 495–508.
[11] Frey, G., Thomm, M., Brüdigam, B., Gohl, H.P. and Hausner, W. (1990) Nucleic Acids Res. 18, 1361–1367.
[12] Brown, J.W., Daniels, C. and Reeve, J.N. (1989) Crit. Rev. Microbiol. 16, 287–338.
[13] Huber, H., Thomm, M., König, H., Thies, G. and Stetter, K.O. (1982) Arch. Microbiol. 132, 47–50.
[14] Stetter, K.O., Thomm, M., Winter, J., Wildgruber, G., Huber, H., Zillig, W., Janecovic, D., König, H., Palm, P. and Wunderl, S. (1981) Zbl. Bakt. Hyg., I Abt. Orig. C2, 166–178.
[15] Thomm, M. and Wich, G. (1988) Nucleic Acids Res. 16, 151–163.
[16] Thomm, M., Sandman, K., Frey, G., Koller, G. and Reeve, J.N. (1992) J. Bacteriol. 174, 3508–3513.
[17] Weil, C.F., Cran, D.S., Sherf, B.A. and Reeve, J.N. (1988) J. Bacteriol. 170, 4718–4726.
[18] Wich, G., Hummel, H., Jarsch, M., Bär, U. and Böck, A. (1986) Nucleic Acids Res. 14, 2459–2479.