

An archaeobacterial cell-free transcription system. The expression of tRNA genes from *Methanococcus vannielii* is mediated by a transcription factor

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ABSTRACT

Our understanding of the mechanism of RNA biosynthesis in archaeobacteria is limited, due in part to the inability of purified RNA polymerases to transcribe purified genes accurately *in vitro*. In the present study, we show that cell extracts of *Methanococcus vannielii* and *Methanococcus thermolithotrophicus* purified by gradient centrifugation synthesize a distinct transcript from templates harboring a cloned homologous tRNA^{Val} and tRNA^{Arg} gene. The *in vitro* transcripts initiate with GTP at the same sites as in *Methanococcus* cells. About 60% of the sequence of the *in vitro* RNA products was analyzed by dideoxycyterminated primer extension and found to be identical with that of the precursors of tRNA^{Val} and tRNA^{Arg}. This findings indicate that this RNA polymerase fraction both initiates and terminates transcription faithfully *in vitro*.

After purification of a cell-free extract (S-100) of *M. thermolithotrophicus* by phosphocellulose chromatography, the endogenous RNA polymerase has lost its ability to transcribe the tRNA^{Val} gene accurately. The activity directing specific expression of this template was reconstituted by the addition of a protein-fraction devoid of RNA polymerase activity. Thus, a transcription factor appears to be required for accurate cell-free expression of tRNA genes from *M. vannielii*.

INTRODUCTION

The discovery of archaeobacteria as a second prokaryotic line of descent (1) posed the question as to the mechanism regulating gene expression in archaeobacterial cells. To provide a means of studying these processes, attempts were made to express purified archaeobacterial genes *in vitro*. However, the RNA polymerases isolated from genera of both phylogenetic branches of archaeobacteria (2) are unable to transcribe homologous genes faithfully *in vitro* (reviewed by Zillig et al., 1985 (3)). Recently, the purified enzyme of the methanogen *Methanococcus vannielii* has been shown to bind specifically to the upstream DNA region of cloned homologous tRNA, rRNA and protein-encoding genes (4,5,6). On the basis of these footprinting experiments an

octanucleotide similar in location and sequence to the TATA-box of eukaryotic polymerase II promoters (7) has been proposed as an archaeobacterial consensus promoter sequence (8). The finding that the purified enzyme of *M. vannielii* recognizes the promoters of both genes encoding stable RNA's and proteins suggests that a single type of RNA polymerase directs the synthesis of tRNA, rRNA and mRNA in archaeobacterial cells. To identify the components required in addition to the RNA polymerase for faithful transcription of archaeobacterial genes, we investigated the ability of soluble extracts from two *Methanococcus*-species to transcribe distinct RNA products from cloned homologous tRNA templates.

In the present study, we show that a RNA polymerase fraction purified by density gradient centrifugation directs faithful *in vitro* transcription of tRNA genes from *M. vannielii*. Moreover, reconstitution experiments provide evidence that the expression of these templates is mediated by an archaeobacterial transcription factor.

MATERIALS AND METHODS

Strains and culture conditions

Cells of *M. thermolithotrophicus* were grown in medium 3 of Balch et al. (9), as described previously (10). *M. vannielii* was cultivated in a medium containing (g/l): KH₂PO₄ (0.5), MgSO₄×7H₂O (0.4), NaCl (0.4), NH₄Cl (0.4), CaCl₂×2H₂O (0.05), FeSO₄×7H₂O (0.1), NaHCO₃ (6.5), Na₂S×9H₂O (0.5), L-cysteine hydrochloride×H₂O (0.5), resazurin (1 mg) and 6 ml of trace minerals (9). The growth temperature was 37°C.

Templates

A DNA restriction fragment from plasmid pMT31 (11) harboring the tRNA^{Val} gene was inserted into the cloning vector pIC19H (12). The resulting plasmid pIC31/2 (Fig. 1A) contains the DNA region from -58 to +131 with respect to the tRNA^{Val} gene.

To construct plasmid pIC21/1 (Fig. 1B), a *DdeI/AvaII* restriction fragment of plasmid pMT21 (11) containing the DNA region from -72 to +98 of the tRNA^{Arg} gene was ligated to an *AvaII/SacI* fragment harboring the sequence from +103 to

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+131 of the tRNA^{Val} gene and inserted into pIC19H cleaved with *SmaI/SacI*.

Plasmid DNA was purified by repeated centrifugation in CsCl density gradients as described previously (4).

Partial purification of the endogenous RNA polymerase by ultracentrifugation

All operations were performed at 4 °C and under exclusion of oxygen (13). 1 g cells (wet weight) were suspended in 2 ml TMK buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 50 mM KCl, 50 μM ZnSO₄) and lysed with a French pressure cell at 1000 psi. The crude extract was cleared by ultracentrifugation (39000 rpm, 1 h, rotor 50 Ti, Beckman L5-50 ultracentrifuge). 200 μl of the supernatant (S-100) were applied to a linear glycerol gradient (8%–24%) and centrifuged for 3 h at 50000 rpm in a Beckman SW 50.1 rotor. Fractions of 300 μl were taken from the top of the gradient.

Fractionation of cellular extracts by phosphocellulose chromatography

A S-100 from 1 g cells was applied to a phosphocellulose column (1 × 10 cm) equilibrated with TMK buffer pH 7.5 containing 20% glycerol and 100 mM KCl. After washing of the column with 3 volumes of buffer, bound proteins were eluted in steps with TMK-buffer containing 350 mM, 600 mM or 1 M KCl, respectively.

In vitro transcription and electrophoretic analyses of RNA products

20 μl of glycerolgradient fractions were incubated with 3.5 μg plasmid DNA (harboring the tRNA^{Val} or tRNA^{Arg} gene of *M. vannielii*) in a total reaction volume of 100 μl containing 20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 50 mM KCl, and 0.1 mM EDTA. After 5 min of preincubation at 55 °C (*M. thermolithotrophicus*) or 50 °C (*M. vannielii*), the reactions were started by the addition each of 0.33 mM ATP, CTP, GTP, 16.5 μM UTP (Boehringer, Mannheim), and 10 μCi α-³²P-UTP (800 Ci/mMol, NEN). After 30 min at 55 °C (*M. th.*) or 50 °C (*M. van.*) transcription was stopped by adding 100 μl 0.6 M Na acetate pH 7.0, immediately followed by 200 μl phenol/chloroform. The aqueous phase was then extracted with 200 μl chloroform and precipitated with ethanol. The labelled RNA products were suspended in 4 μl formamide, containing 0.1% xylene-cyanol, heated for 2 min at 85 °C, chilled on ice-water and applied to polyacrylamide/urea gels (8% polyacrylamide, 0.4% bisacrylamide and 8.3 M urea). Electrophoretic separation of RNA products was performed at 350 V for 2 h.

To detect primary transcripts, γ-³²P-labelled ATP or GTP (100 μCi; 6000 Ci/mmol) were added to transcription reactions. After an incubation time of 5 min transcription was started by the addition of unlabelled nucleotides.

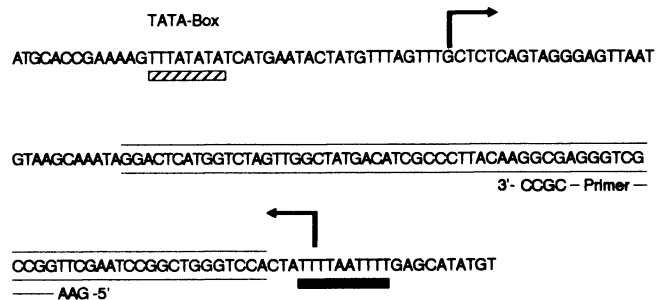
For reconstitution experiments a total of 30 μl of the various fractions from the phosphocellulose was incubated in 100 μl transcription assays as indicated in the legend of Fig. 7B.

Primer extension and sequence analysis of in vitro RNA products

Synthetic oligonucleotides were end-labelled with γ-³²P-ATP and polynucleotide kinase. The labelled primers were purified by polyacrylamide gel electrophoresis (14% acrylamide, 0.7% N,N'-Methylene bisacrylamide, 8.3 M urea) and isolated as described by Dingermann and Nerke (14).

A

M. vannielii tRNA^{Val} (pIC31/2)



B

M. vannielii tRNA^{Arg} (pIC21/1)

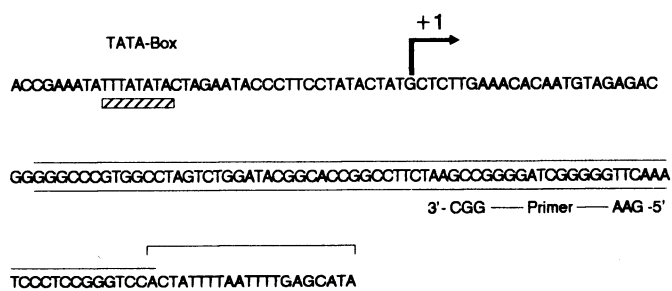


Fig. 1. Genetic map and DNA sequence of the archaeobacterial tRNA genes used as templates. The TATA box (4) is indicated by a hatched box, the possible terminator signal (15) by a black bar below the sequence. The arrows label the *in vivo* initiator and terminator nucleotides. The parallel bars above and below the sequence indicate the sequences coding for mature tRNAs. The synthetic oligonucleotides used for primer extension experiments are shown below their complementary DNA sequences. The tRNA^{Arg} gene is part of an operon consisting of two tRNA genes (11). The 3' end of the tRNA^{Arg} gene was ligated with the downstream region of tRNA^{Val}, containing the putative terminator. The bracket above the sequence indicates the nucleotides originating from the tRNA^{Val} gene.

For primer extension, *in vitro* transcription reactions were performed as described above but without radioactive precursors. After transcription, the assays were incubated with 5 units RNase-free DNase (Promega Biotec) for 30 min at 37 °C, followed by phenol extraction. End-labelled primer (0.1 pmol, 30000 cpm) was coprecipitated with RNA obtained from one *in vitro* transcription reaction. After washing with 1 ml 70% ethanol, the dry pellet was redissolved in 8 μl annealing buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM KCl, and 2.5 mM EDTA. RNA was denatured for 10 min at 85 °C and the primer was annealed 10 min at 65 °C. After 1 min at 37 °C, 6 μl of this annealing mixture were added to 4 μl of extension buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 2 mM DTT, 0.66 mM each of dATP, dCTP, dGTP, dTTP and 1000 units/ml AMV reverse transcriptase) tempered for 1 min at 37 °C. After 30 min incubation at 37 °C, the extension reactions were stopped by adding 10 μl 0.6 M Na-acetate pH 7.0 and 60 μl ethanol. Reverse transcriptase products were analyzed on 8% sequencing gels.

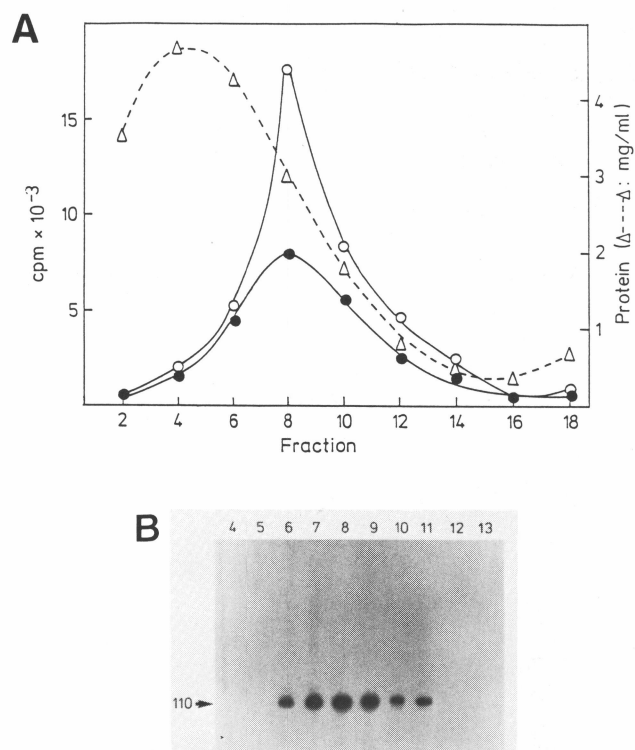


Fig. 2. Purification of a specific RNA polymerase activity by glycerol-gradient centrifugation. The ability of the various fractions of the gradient to synthesize distinct RNA products was analyzed by polyacrylamide/urea gel electrophoresis. The radioactivity incorporated into the 110 nucleotide transcript (●—●) and into acid-insoluble RNA (○—○) was determined. To quantitate the specific transcript, the labelled band was excised and the efficiency of RNA synthesis analyzed by Cerenkov counting. The protein content of the glycerol-gradient fractions is shown at the top of the figure (△—△). After 30 min, the amount of UMP incorporated into pre-tRNA was 0.14 pmol corresponding to 1 pre-tRNA per 13 genes present.

For sequence analysis, RNA from four *in vitro* transcription reactions was coprecipitated with 3 pmol (1×10^6 cpm) end-labelled primer. Pellets were suspended in 30 μ l annealing buffer. The extension buffer contained in addition dideoxynucleotides at 0.1 mM (ddATP or ddTTP) or 0.04 mM (ddCTP or ddGTP).

RESULTS

Evidence for faithful *in vitro* transcription

As an approach to investigate the requirements for the *in vitro* expression of archaeobacterial genes, we analyzed the RNA products transcribed by a soluble extract of *M. vannielii* from plasmid pIC31/2. This recombinant plasmid harbors a tRNA^{Val} gene of *M. vannielii* (Fig. 1). When both initiation and termination of transcription occurs *in vitro* at the proposed *in vivo* start and stop signals (15), an RNA product of 110 nucleotides should be expected. A cell-free extract (S-100) was unable to direct specific transcription of this template (data not shown). To separate components possibly inhibiting faithful *in vitro* transcription from the endogenous RNA polymerase, the S-100 was fractionated by centrifugation in glycerol-density gradients. Analysis of the RNA products revealed that the fractions containing the endogenous RNA polymerase are able to transcribe this template specifically (Fig. 2). The *in vitro* RNA product corresponds in size to 110 nucleotides. This extract also

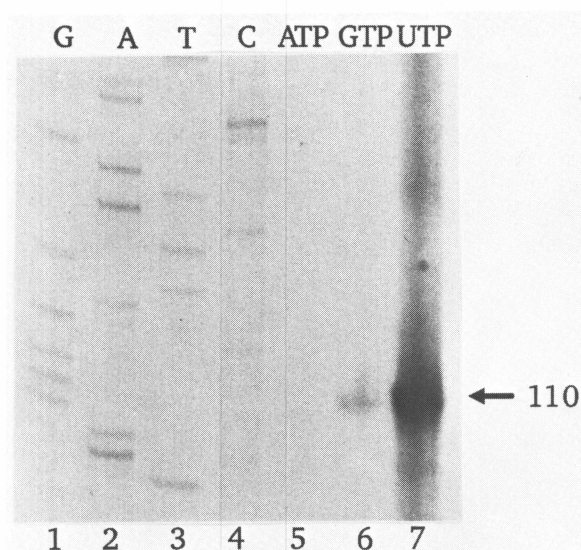


Fig. 3. The *in vitro* RNA product represents a primary transcript. *In vitro* transcription reactions contained γ -³²P-ATP (lane 5), γ -³²P-GTP (lane 6) or α -³²P-UTP (lane 7) as labelled nucleotide. Lanes 1–4 show a DNA-sequence reaction used as standard. G,A,T and C indicate the sequence specificity in the corresponding reactions. The arrow marks labelled RNA of 110 nucleotides. The RNA products were separated on a 6% DNA sequencing gel.

directs the synthesis of distinct transcripts from templates harboring further tRNA genes from *M. vannielii*, but not from the vector DNA and eubacterial tRNA genes (data not shown).

Characterization of the *in vitro* RNA products

To investigate whether the RNA product of 110 nucleotides represents a primary transcript, the *in vitro* RNA was synthesized in the presence of γ -³²P labelled ribonucleoside-5'-triphosphates. When γ -³²P-GTP was added to the transcription reactions, a distinct transcript of 110 nucleotides was synthesized (Fig. 3, lane 6). When γ -³²P-GTP was replaced by γ -³²P-ATP, no labelled RNA product could be detected (Fig. 3, lane 5). These data clearly demonstrate that the RNA product of 110 nucleotides initiates with GTP.

To provide conclusive evidence that the RNA products shown in Figures 2 and 3 do indeed originate from the tRNA gene in question, the *in vitro* transcripts from pIC31/2 were analyzed by dideoxy terminated sequencing (16). A synthetic oligonucleotide complementary to positions 40–58 of tRNA^{Val} was annealed with *in vitro* RNA and extended with reverse transcriptase. Using this method, the 5' end of the *in vitro* RNA was located at a guanosine residue 25 bp upstream of the first nucleotide of the mature tRNA^{Val} (Fig. 4A). From this data, we conclude that the RNA product initiates with GTP within the sequence ATGC which has been determined as initiation site for tRNA synthesis in *Methanococcus vannielii* (15). The sequence of the cDNA from position +45 to –25 with respect to tRNA^{Val} was complementary to the the known sequence of the precursor of this tRNA species (Fig. 4 A).

To investigate the fidelity of transcription of an additional tRNA gene, the sequence of the RNA product synthesized from plasmid pIC21/1 was analyzed in addition. Using the same method but employing a primer homologous to positions 41–58 of the tRNA^{Arg}, the 5' end of the transcript from the tRNA^{Arg} gene was located 26 nucleotides upstream from the initiator nucleotide

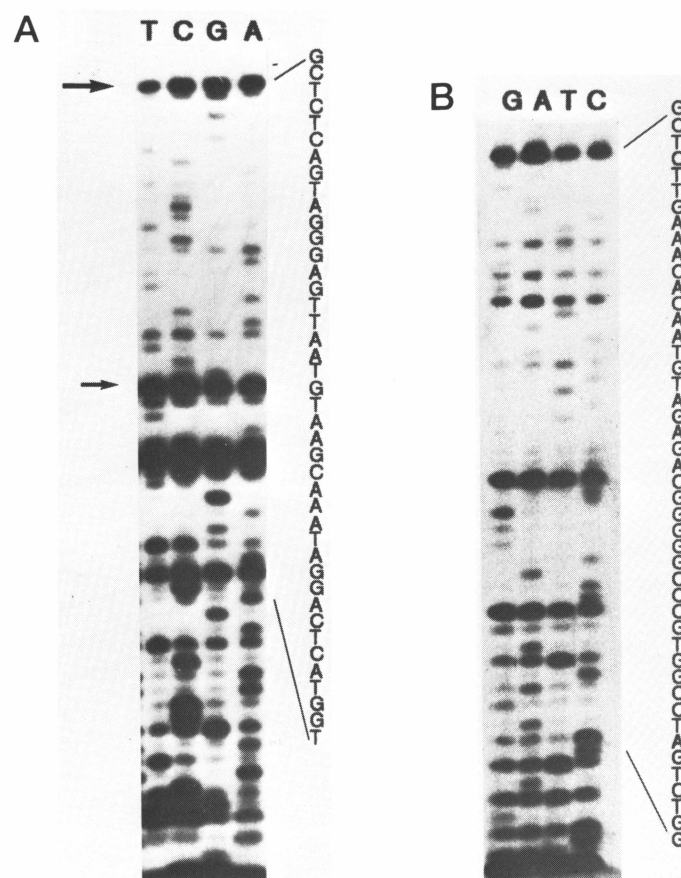


Fig. 4. RNA sequence of the *in vitro* transcripts from two tRNA genes. The RNA sequences were analyzed by the dideoxylated primer extension procedure (16). The synthetic oligonucleotides employed as primers are shown in Fig. 1. T, C, G, and A on top of the figure indicate the presence of the chain terminating dideoxynucleotides in the primer extension reactions. The sequences complementary to the cDNA sequences are shown to the right of the Figure.

Panel A: Sequence of the precursor of tRNA^{Val}; the 5' end of the transcripts is labelled by a large arrow. The small arrow marks a site where unspecific chain-termination of the reverse transcriptase reaction occurs. This nucleotide corresponds to a putative 5'-processing site (see also Fig. 7C).

Panel B: Sequence of pre-tRNA^{Arg}.

of mature tRNA^{Arg} (Fig. 4B). The 5' end of this *in vitro* transcript maps at the same nucleotide as the *in vivo* transcript from the tRNA^{Arg} gene (15). The sequence of the cDNA from + 40 to -26 was identical with the corresponding sequence of the noncoding DNA strand of pIC21/1. The same results were obtained with extracts prepared from *M. thermolithotrophicus*. From this data, we conclude that the glycerol gradient fractions of these RNA polymerases direct faithful expression of tRNA genes from *M. vannielii*.

Characterization of some properties of the glycerol-gradient fraction of the RNA polymerase

To characterize further the specific RNA polymerase activity, the effects of divalent cations, KCl and temperature on pre-tRNA synthesis have been analyzed. The glycerol gradient fractions of the RNA polymerases from *M. vannielii* and *M. thermolithotrophicus* show maximal activity at 10 mM MgCl₂ and 20 mM KCl. Mn²⁺ does not support pre-tRNA synthesis (data not shown). The temperature optimum was observed at 50 (*M. vannielii*) and 55 °C (*M. thermolithotrophicus*). These enzymatic properties of the activity directing faithful expression of tRNA templates are identical with those of the purified RNA polymerases (17).

Analysis of the kinetics of specific RNA synthesis revealed

that a lag period of 20 min precedes pre-tRNA synthesis (Fig. 5). The primary transcript was visible after an incubation time of 30 min. Then, RNA synthesis was linear for 20 min.

RNA synthesis in the archaebacterial system strictly depends upon the addition of exogenous DNA to the transcription assay (Fig. 6). The rate of pre-tRNA synthesis is optimal at DNA concentrations between 12 and 50 µg/ml. At higher DNA concentrations, specific transcription is inhibited whereas total RNA synthesis reaches a plateau.

Evidence for an archaebacterial transcription factor

In an attempt to define the components required for accurate *in vitro* transcription, a purification scheme developed for the separation of the transcription factors involved in the expression of eukaryotic RNA polymerase III genes (18) was employed to detect archaebacterial transcription factors in cells of *M. thermolithotrophicus*. Similar experiments were performed with *M. vannielii*. Since the various activities affecting *in vitro* transcription were more stable in extracts from *M. thermolithotrophicus*, we only report the results obtained with the thermophilic *Methanococcus*.

A S-100 was separated into four distinct protein fractions by phosphocellulose chromatography. The ability of the individual fractions to transcribe the tRNA^{Val} gene faithfully was analyzed.

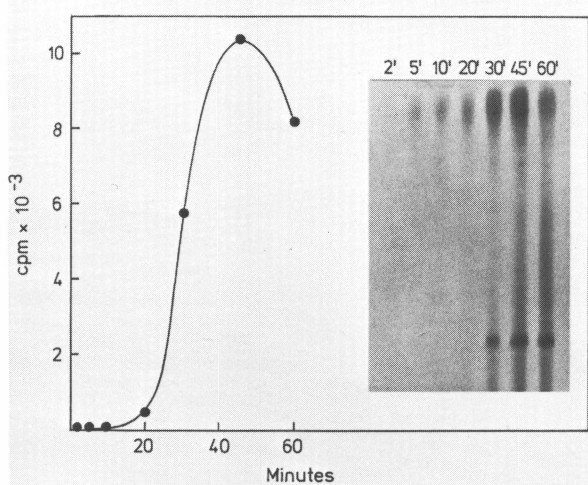


Fig. 5. Time-dependence of pre-tRNA synthesis. The RNA products were analyzed by polyacrylamide/urea gel electrophoresis. The radioactivity incorporated into the precursor of tRNA^{Val} was measured as indicated in Fig. 2.

Using an unspecific assay measuring the incorporation of radioactivity into acid-insoluble RNA, the endogenous RNA polymerase activity was found to elute almost entirely with the 0.35 M KCl fraction (Fig. 7A; fraction B). Neither this fraction nor the break-through of the column (fraction A) and the proteins eluted with 0.6 M (fraction C) and 1 M KCl (fraction D) alone direct accurate transcription of the tRNA gene (Fig. 7B, lanes 1–4). However, using a combination of fractions B and D, the activity synthesizing a RNA product of 110 nucleotides was reconstituted (Fig. 7B, lane 7). When pIC31/2 was replaced by the vector pIC19/H in the transcription reaction, no distinct transcript could be detected (Fig. 7B, lane 8). A primer extension experiment revealed that the 5' end of the RNA synthesized by the reconstituted fractions from the phosphocellulose maps at the proposed *in vivo* transcription start site (Fig. 7C, lane 2). To investigate whether fraction D is able to form distinct RNA bands by processing of unspecific transcripts, the RNA synthesized by fraction B was purified and incubated with fraction D. Analysis of the RNA by polyacrylamide gel electrophoresis revealed that no distinct RNA bands are formed under these conditions (Fig. 7B, lane 2). Therefore, the possibility can be ruled out that the RNA product induced by the addition of fraction D (Fig. 7B, lane 7) results from a processing event. From this data, we conclude that fraction D contains a transcription factor conferring upon the RNA polymerase the ability to transcribe the tRNA^{Val} gene faithfully.

Distinct transcripts were also observed when fraction B was combined with fraction C (Fig. 7B, lane 6). These transcripts show a lower apparent molecular weight than the pre-tRNA^{Val}. The smallest RNA band comigrates with mature tRNA^{Val}. The most plausible explanation for these RNA bands is that fraction C contains the same transcription factor as fraction D and tRNA processing enzymes in addition. To investigate the origin of the smaller RNA bands, *in vitro* RNA synthesized in the presence of fractions B and D was purified and treated with fraction C. After an incubation time of 30 min, the 110 nucleotide transcript was no longer detectable and two transcripts of 85 and 75 nucleotides were observed instead (Fig. 7D, lane 5). Assuming that processing at the 5' end occurs 25 nucleotides downstream from the initiator nucleotide as proposed by Wich et al. (11), the

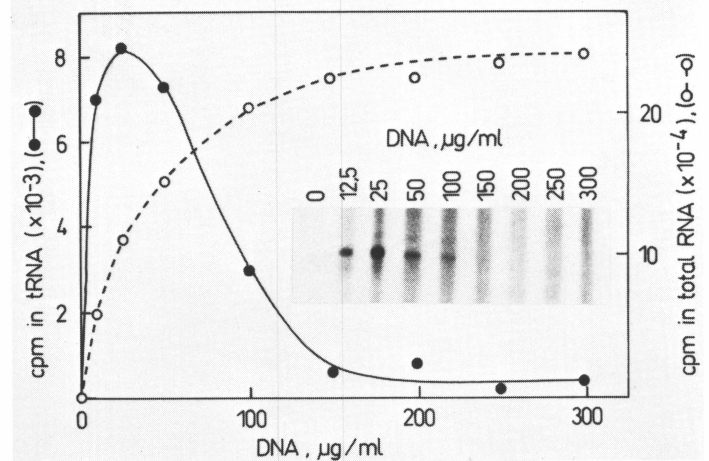


Fig. 6. DNA-dependence of pre-tRNA and total RNA synthesis. The tRNA^{Val} precursor was quantitated as indicated in Fig. 2. To determine total RNA synthesis aliquots of 10 μ l were removed from the transcription reactions, added to 90 μ l H₂O containing 1 μ g pBR322-DNA as carrier and radioactivity insoluble in trichloroacetic acid determined as usual.

transcript of 85 nucleotides might be caused by nucleolytic cleavage of a phosphodiester bond at this site. The additional bands in Fig. 7B, lane 6 might correspond to further processing intermediates which were not further analyzed in this study. The finding that the incubation of primary transcripts with fraction C leads to the formation of mature tRNA molecules suggests that this phosphocellulose step fraction contains the processing enzymes which tailor the tRNA precursors.

When the purified RNA polymerase was combined with fraction D, no distinct RNA products could be detected (Fig. 7B, lane 10). This finding indicates that the factor(s) present in fraction D and the RNA polymerase are not sufficient to direct faithful initiation of transcription. Therefore, beside the RNA polymerase a second component required for accurate expression of tRNA genes seems to be present in the phosphocellulose B fraction.

DISCUSSION

Soluble extracts of two *Methanococcus* species purified by gradient centrifugation and reconstituted fractions eluted from a phosphocellulose column direct the synthesis of discrete RNA species from templates harbouring cloned tRNA genes of *M. vannielii*. The following observations provide evidence that these RNA products initiate and terminate transcription faithfully *in vitro*. First, the transcripts from the tRNA^{Val} gene correspond in size to the tRNA precursor found *in vivo*. Furthermore, they carry a triphosphate at their 5' end and initiate at the same sites as in *Methanococcus* cells (15). Finally, the *in vitro* transcripts from the tRNA^{Val} and tRNA^{Arg} gene are identical in their sequence to the known sequences of the corresponding tRNA genes.

Some properties of the cell-free extracts containing the specific activity are similar to those of the purified RNA polymerases. For example, both the glycerol gradient fraction and the system reconstituted from fractions of the phosphocellulose show the same salt, MgCl₂ and temperature optimum as the RNA polymerases purified from *M. thermolithotrophicus* (17), and *M. vannielii*. In contrast to the purified enzyme, the activity

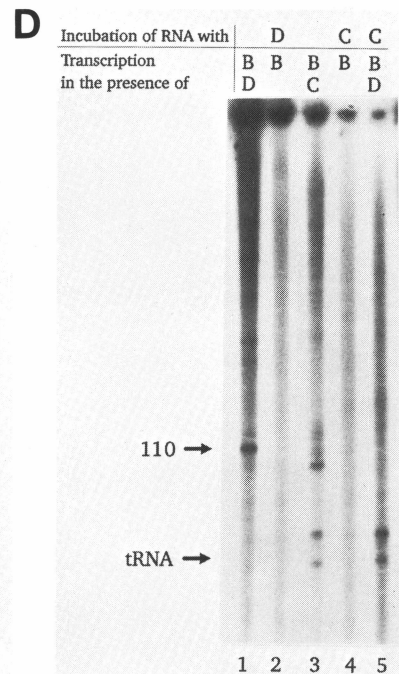
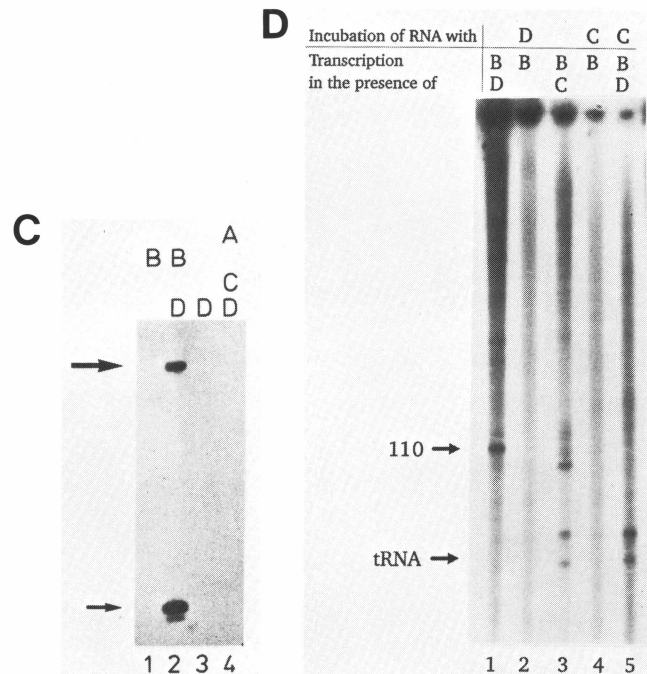
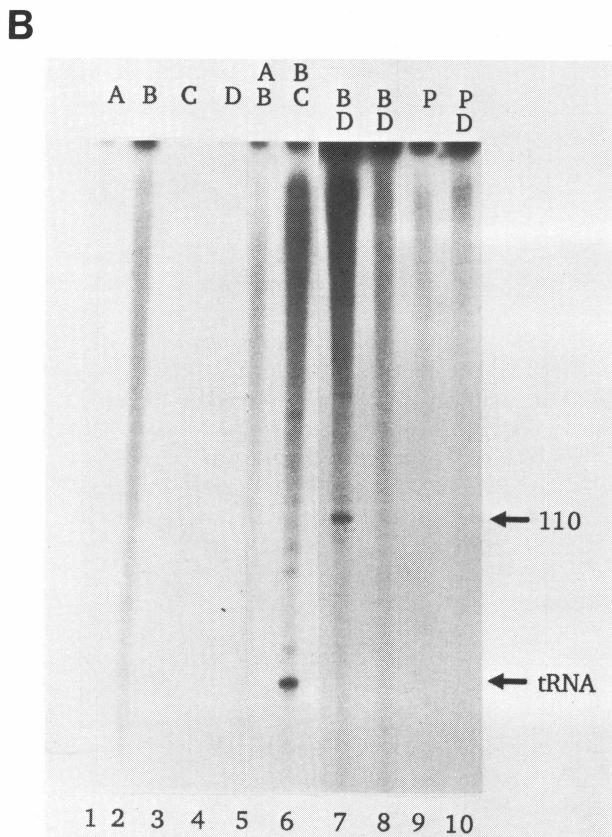
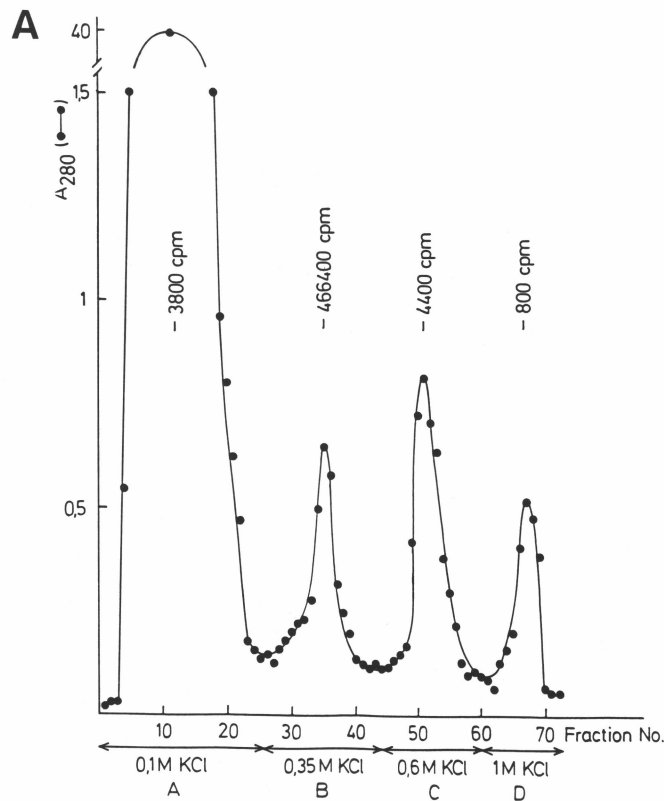


Fig. 7. Specific transcription of the tRNA^{Val} gene is mediated by a transcription factor.

Panel A: Fractionation of a crude extract of *M. thermolithotrophicus* by phosphocellulose chromatography. The absorbance profile (A_{280}) of the fractions separated by step elution is shown. The fractions A (break through) and B, C, and D containing the components eluted with 0.35, 0.6 and 1 M KCl are indicated below the abscissa. The unspecific RNA polymerase activity of each peak fraction determined in an RNA polymerase standard assay is indicated on top.

Panel B: The activity directing faithful transcription of the pre-tRNA^{Val} can be reconstituted. The RNA products synthesized by the phosphocellulose fractions alone and in combination were analyzed by polyacrylamide/urea gel electrophoresis. The presence of the various phosphocellulose fractions (A–D, see panel A) in the transcription reactions is indicated above each lane. Twenty microliters of fraction B and ten microliters of the other fractions were added to the transcription reactions. The DNA employed as template was the vector pIC19H (lane 8) and pIC31/2 (other lanes). Lane 9, 10 transcription reactions conducted with the purified RNA polymerase (P); equal amounts of unspecific RNA polymerase activity (200 000 cpm) were added to the transcription reactions analyzed in lanes 2, 5–8 and 9, 10. The minor distinct signals larger in size than the major RNA product of 110 nucleotides were not analyzed in this study. They presumably are caused by readthrough at the primary terminator, as observed in other cell-free transcription systems (20). Under the conditions employed, one pre-tRNA was synthesized per 30 DNA molecules.

Panel C: The transcripts synthesized by the reconstituted fractions originate from the tRNA^{Val} gene. Unlabelled RNA transcribed *in vitro* from pIC31/2 by the phosphocellulose fractions indicated above each lane was annealed to a 5' endlabelled primer (Fig. 1) and extended with reverse transcriptase. The cDNA was analyzed on a sequencing gel. The large arrow labels the 5' end of the primary transcript, the small arrow a putative 5' processing site, which are also indicated in Fig. 4A.

Panel D: The phosphocellulose D fraction exerts its effect at the level of initiation of transcription. Labelled RNA synthesized by the phosphocellulose fractions indicated above each lane was analyzed as described in panel B. To detect RNA bands caused by processing of tRNA precursors, the RNA synthesized in the presence of the phosphocellulose fractions B (lanes 2 and 4), B and D (lanes 1 and 5), B and C (lane 3) was purified by phenol-extraction, precipitated with ethanol and incubated in transcription buffer (in the absence of ribonucleoside-5'-triphosphates) with fraction D (lane 2) and fraction C (lanes 4 and 5) The absence of discrete RNA products in lane 2 clearly demonstrates that fraction D directs specific initiation of transcription but not processing of random transcripts.

synthesizing the tRNA^{Val} precursor is inhibited at high DNA concentrations and requires a period of preincubation before RNA synthesis starts. Assuming that binding of both the RNA polymerase and a second component at the same DNA molecule is a prerequisite for correct initiation of transcription, the efficiency of specific transcription should be reduced in the excess of DNA. Similar DNA-template dosage effects and kinetics have been observed in RNA polymerase III transcription systems directing the expression of eukaryotic 5S rRNA (19,20). Here, specific initiation of transcription indeed depends upon binding of two components, the transcription factors TFIIA and TFIIC, to distinct sites at the same DNA molecule (reviewed by Geiduschek and Tocchini-Valentini, 1988 (21)). When the primary interaction of a single DNA binding polypeptide with the promoter is sufficient to mediate faithful transcription (e.g. that of TFIIC in the case of eukaryotic tRNA genes), the synthesis of distinct RNA products is not inhibited in the excess of the template (22). Thus, the failure of the archaeobacterial system to direct pre-tRNA synthesis at high DNA concentrations might be considered to be an indication for the presence of a DNA-binding transcription factor in the glycerol gradient fraction of the RNA polymerase.

Direct evidence for the existence of an archaeobacterial transcription factor has been provided by reconstitution experiments. Addition of the 1 M KCl phosphocellulose step fraction (fraction D ; Fig. 7) to the 0.35 M KCl step fraction (fraction B) which is only capable of unspecific transcription, induces the synthesis of the primary transcript. Since fraction D does not show any detectable RNA polymerase activity it seems to contain at least one component directing faithful expression of homologous tRNA genes. Although fraction C from the phosphocellulose is contaminated with this component, the purification scheme described here may prove useful for further studies since it allows separation of processing enzymes present in fraction C from the activity mediating specific transcription in a one step procedure.

Similar to the endogenous RNA polymerase in the phosphocellulose B fraction, the enzyme of *M. thermolithotrophicus* purified from the crude extract by DEAE-cellulose chromatography (17) also fails to transcribe tRNA genes specifically (data not shown). Therefore, the factors mediating correct initiation of transcription apparently do not copurify with the RNA polymerase during ion-exchange chromatography. This is reminiscent of eukaryotic transcription systems and might explain the failure of purified RNA polymerases from archaeobacteria to transcribe homologous templates faithfully.

The factor(s) present in fraction D effects the expression of tRNA genes by the phosphocellulose fraction of the RNA polymerase but not by the purified enzyme. This observation suggests that the phosphocellulose fraction contains an additional component necessary for initiation of transcription. Thus, beside the RNA polymerase at least two transcription factors appear to be involved in the expression of tRNA genes in *Methanococcus*. This is a similarity to eukaryotic transcription of tRNA genes by RNA polymerase III where two distinct components (the transcription factors TFIIB and TFIIC) are necessary for initiation of transcription (18,21). In contrast to eukaryotic tRNA genes, which are expressed mainly from internal promoters (21), the archaeobacterial system appears to use upstream sequences as a major promoter signal (W. Hausner, G. Frey, M. Thomm, manuscript in preparation). The further purification and characterization of the factors directing the expression of tRNA

genes in *Methanococcus* may provide a basis for understanding the mechanism of transcription in archaeobacterial cells.

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