AN ARCHAEBACTERIAL IN VITRO TRANSCRIPTION SYSTEM

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ABSTRACT

An RNA polymerase fraction of Methanococcus vannielii purified by gradient centrifugation synthesizes an RNA product of 110 nucleotides in the presence of a template harbouring a homologous $tRNA^{Val}$ gene. The length of this transcript corresponds exactly to that of the tRNA precursor molecule synthesized in vivo. After internal deletions of 5 and 11 basepairs from the DNA region encoding the tRNA gene, in vitro transcripts of 105 and 99 nucleotides were observed. This finding confirms our conclusion that the RNA products originate from the tRNA gene. Optimal transcription of the tRNA gene occurs at a incubation temperature of 50 °C in the presence of 10 mM $MgCl_2$ and 20 mM KCl. Synthesis of the 110 nucleotide RNA product is maximal at a DNA-concentration of 100 µg/ml and is inhibited at higher DNA-concentrations. By mutagenesis of the DNA region upstream of the tRNA gene, the DNA sequence promoting in vitro transcription was located between -58 and -22. Therefore, the TATA-box at -25 which has been proposed as an archaebacterial consensus promoter sequence (Thomm and Wich, 1988), appears to be indispensable for initiation of transcription.

INTRODUCTION

In contrast to the RNA polymerases of eubacteria, the RNA polymerases purified from archaebacterial cells are unable to initiate transcription accurately in vitro (Zillig et al., 1988). However, recently specific binding of the purified enzyme from the methanogen Methanococcus vannielii to the promoter region of both protein-encoding (Thomm et al., 1988a; Brown et al., 1988) and tRNA/rRNA genes (Thomm and Wich, 1988) has been demonstrated. From these footprinting experiments a TATA-box at -25 has been inferred as an archaebacterial consensus promoter sequence (Thomm et al., 1989). However, also the purified RNA polymerase of M. vannielii is unable to accurately transcribe purified genes. To investigate the requirements for cell-free transcription of archaebacterial genes, the

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expression of a tRNA^{Val} gene of M. vannielii by less purified RNA polymerase fractions was studied. We describe here the purification and some properties of an RNA polymerase fraction, directing correct in vitro transcription of this template.

MATERIAL AND METHODS

Purification of the endogenous RNA polymerase from the crude extract

A soluble extract of M. vannielii cells (S-100) was prepared as described previously (Thomm et al., 1988b). The endogenous RNA polymerase was separated from the bulk of cellular proteins by glycerol-gradient centrifugation (Wingender et al., 1984).

DNA isolation and construction of mutated plasmids

The plasmids for the in vitro transcription reactions were purified by repeated centrifugation in CsCl density gradients as described previously (Thomm and Wich, 1988). Plasmid pIC31/1 contains the ClaI fragment of plasmid pMT31 (Wich et al., 1986b) inserted into the ClaI site of the cloning vector pIC-19H (Marsh et al., 1984). The different 5' deletion clones of the tRNA^{Val} gene were constructed by unidirectional digestion with exonuclease III using the protocol of Henikoff (1984). The clones pIC31/4 and pIC31/6 which contain deletions of internal sequences of the tRNA gene were constructed by the ligation of

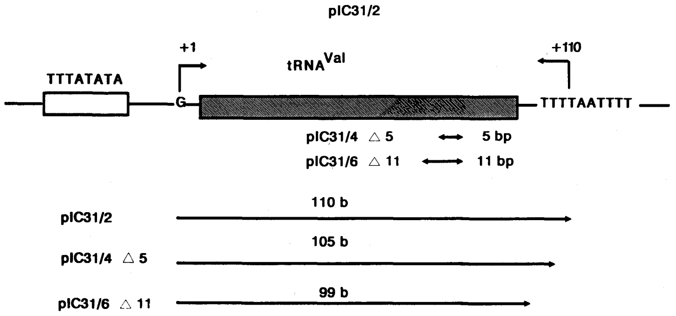

Fig.1. Genetic map of the tRNA^{Val} gene used as a template for the in vitro transcription reactions. Plasmid pIC31/2 contains the 5' flanking DNA sequence of the tRNA gene including the nucleotide at position -58. The plasmids pIC31/4 and pIC31/6 are subclones with internal deletions of 5 and 11 bp, respectively. The TATA-element upstream from the transcription start site is boxed, the region encoding the tRNA indicated by thick dark bars. The arrows indicate the in vivo initiation and termination sites of transcription determined by S1 mapping (Wich et al., 1986) and primer extension experiments (data not shown). The length of the in vitro transcripts from the different templates is indicated in the lower part of the figure.

appropriate DNA restriction fragments. The DNA sequences of all mutated templates were verified by dideoxy sequencing (Sanger et al., 1977).

In vitro transcription assays

The reaction mixture for the synthesis of the tRNA precursor contained 40 mM Tris-HCl, pH 8.0, 10 mM KCl, 8 mM $MgCl_2$, 0.1 mM EDTA, 0.05 mM $ZnSO_4$ and plasmid pIC31/2 (see Fig. 1) at a final DNA concentration of 50 µg/ml. Aliquots of 20 µl from the glycerol gradient fractions were added to the reaction mixtures to give a final volume of 100 µl. After 5 min preincubation at 50 ^{o}C, the transcription was started by the addition of 0.33 mM each of ATP, GTP, CTP and 0.0165 mM and 10 µCi α-^{32}P UTP (600 Ci/mmol, NEN). The transcription reaction was allowed to proceed for 30 min at 50^{o}C. The reaction was stopped and the RNA products purified and separated by electrophoresis on 6% polyacrylamide/urea gels as described by Jahn et al. (1987).
To determine unspecific RNA synthesis the same conditions were employed exept that the plasmid DNA was replaced by poly d(A-T) (0.1 mg/ml) in the transcription reactions. The incorporation of radioactivity into acid-insoluble RNA was measured as described previously (Thomm and Stetter, 1985).

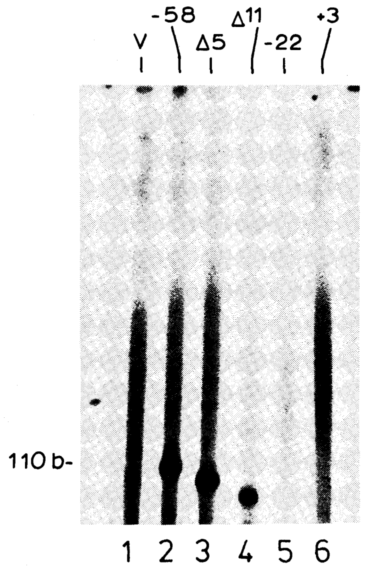

Fig. 2. Analysis of the <u>in vitro</u> RNA products transcribed from the templates shown in Fig. 1 and 3. The position of the RNA product with 110 nucleotides is indicated on the left side of the figure. The various templates used for the <u>in vitro</u> transcription reactions were: Lane 1, vector DNA (indicated by V on top); lanes 2, 5 and 6, deletion clones of the upstream DNA region generated by exonuclease III mutagenesis. The 5' boundaries of the corresponding upstream deletions are indicated on top. Lanes 3 and 4, internal deletion clones pIC31/4 and pIC31/6 (see Fig. 1); the number of nucleotides which have been deleted from the tRNA encoding region are marked by Δ5 and Δ11 on top.

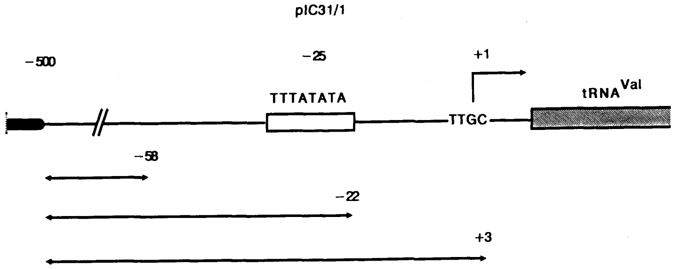

Fig. 3. Map of the upstream region of the tRNA^{Val} gene of M. vannielii. The arrows indicate the extent of the various 5' deletions. The TATA-element at -25 is boxed, the region encoding the mature tRNA marked by thick dark bars. The nucleotides at the transcription start site (+1; labelled in addition by an arrow) are shown. Plasmid pIC31/1 contains 500 bp of the wildtype upstream DNA sequence.

RESULTS AND DISCUSSION

The endogenous RNA polymerase of M. vannielii purified by gradient centrifugation of a crude extract was incubated with a supercoiled plasmid harbouring an homologous tRNA^{Val} gene and 58 nucleotides of the 5' flanking DNA region (pIC31/2; Fig. 1). When initiation and termination of transcription occurs in vitro at the same sites as in Methanococcus cells (Wich et al., 1986a), a transcript of 110 nucleotides should be expected as major RNA product. Analysis of the labelled in vitro RNA by electrophoresis in calibrated polyacrylamide/urea gels revealed that a transcript of this size was synthesized (Fig. 2, lane 2). When the vector DNA without a tRNA gene was

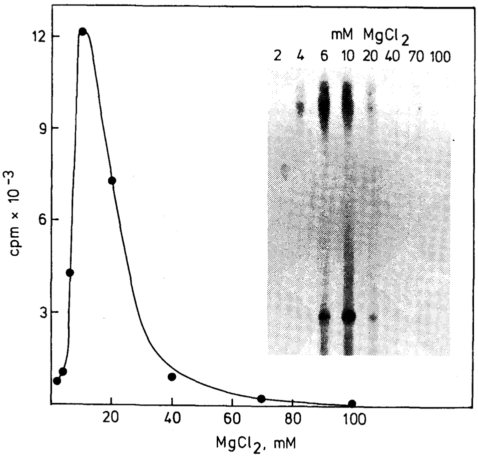

Fig. 4. MgCl$_2$-dependence of tRNA transcription. The amounts of pre-tRNA synthesized in the presence of varying amounts of MgCl$_2$ was determined after electrophoresis of the reaction products. The labelled RNA bands were excised from 6% polyacrylamide/urea gels and quantified by Cerenkov counting.

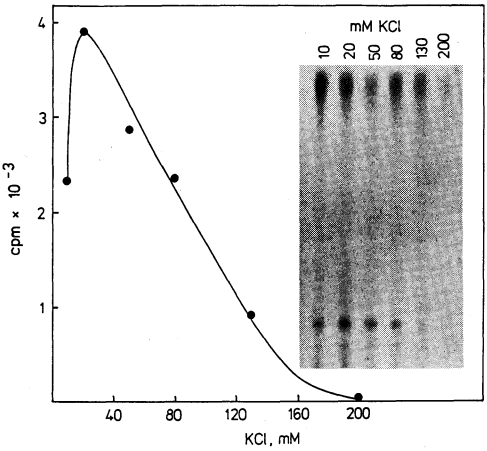

Fig. 5. Influence of KCl concentration on the *in vitro* expression of the $tRNA^{Val}$ gene. Pre-tRNA synthesis was measured at different concentrations of KCl. The $MgCl_2$ concentration was 10 mM. Product analysis and quantitation was as described in the legend of Fig. 4.

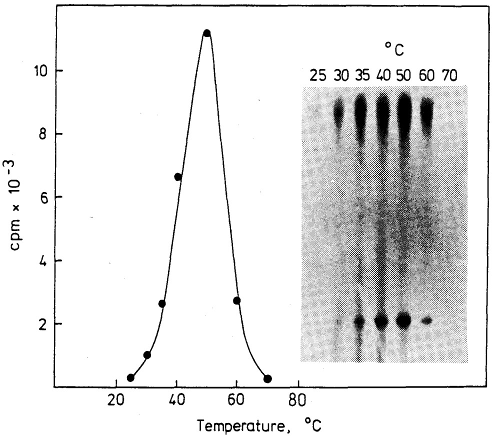

Fig. 6. Effect of the incubation temperature on transcription of the $tRNA^{Val}$ gene. The radioactivity incorporated into the RNA product of 110 nucleotides in response to different incubation temperatures of the transcription reactions was measured as described in the legend of Fig. 4. The $MgCl_2$- and KCl concentrations were 10 and 20 mM, respectively.

employed as a template no distinct RNA product could be detected (Fig. 2, lane 1). To provide additional evidence that the *in vitro* transcripts originate indeed from the $tRNA^{Val}$ gene, internal deletions of 5 and 11 basepairs were introduced into the tRNA template (Fig. 1). The transcripts from the deletion clones pIC31/4 and pIC31/6 should therefore be reduced in their size by 5 and 11 nucleotides, respectively. Analysis of the corresponding *in vitro* transcripts revealed that RNA products of 105 and 99 nucleotides were synthesized (Fig. 2, lanes 3 and

4). These results support the conclusion, that this RNA polymerase fraction of M. vannielii is able to faithfully transcribe homologous tRNA genes.

When pIC31/2 was replaced in the in vitro transcription reactions by plasmid pIC31/1 which contains 500 basepairs of the 5' flanking region instead of 58, the same rate of tRNA expression was observed (data not shown). This finding indicates that the DNA region upstream of -58 is not essential for in vitro transcription of the $tRNA^{Val}$ gene. To define the DNA sequences promoting in vitro transcription of the $tRNA^{Val}$ gene, two additional plasmids with deletions extending to the DNA region downstream of -58 were constructed (Fig. 3). After deletion of the nucleotides of the TATA-box including position -22, the efficiency of transcription was dramatically reduced (Fig. 2, lane 5). When the deletion extends to position +3 of the tRNA gene (Fig. 3) no distinct in vitro transcripts from this template could be detected (Fig. 2, lane 6). Thus, the DNA sequence required for specific transcription of this tRNA gene is located in the DNA region between -58 and -22. These data strongly suggest that the TATA-box represents the main signal promoting the expression of this tRNA gene. Since this sequence is conserved at the same location in most archaebacterial genes (Thomm and Wich, 1988) the TATA-box might be regarded as a major promoter signal directing the transcription of constitutive genes in archaebacteria.

To facilitate a further characterization of the RNA products and the factors involved in expression of the tRNA gene, some properties of the extract directing the cell-free transcription were determined. Mg^{2+} is absolutely required for the expression of the tRNA gene. Synthesis of the tRNA precursor occurs between 6 and 20 mM $MgCl_2$, with an optimum at 10 mM (Fig. 4). The rate of transcription of the $tRNA^{Val}$ gene is optimal at 20 mM KCl. A significant expression of this template

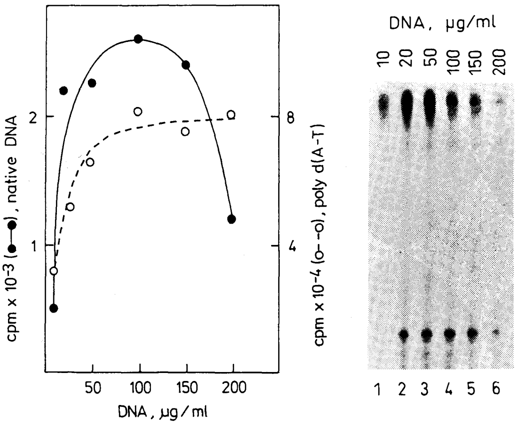

Fig. 7. Effect of DNA concentration on the synthesis of unspecific RNA (O--O) and pre-tRNA (●—●). The templates employed for the reactions were poly d(A-T) and plasmid pIC31/2 (Fig. 1), respectively. The pre-tRNA product was quantified as described in Fig. 4, the transcripts from poly d(A-T) by liquid scintillation counting of acid insoluble labelled RNA.

was observed up to a KCl concentration of 130 mM (Fig 5). Optimal transcription of the tRNA gene occured at a temperature of 50 °C (Fig. 6) although M. vannielii is a mesophilic strain which shows its temperature optimum for growth at 37 °C. In general, the activation profiles for the specific synthesis of the tRNA precursor resemble those obtained when the synthetic template poly d(A-T) was transcribed with the purified RNA polymerase (Frey, 1987). When the DNA-dependence of transcription was determined a striking difference between specific and unspecific RNA synthesis was observed. With polyd(A-T) as template, the rate of RNA synthesis is higher at increased DNA-concentrations in the transcription reactions until a plateau is reached (Fig. 7). However, the rate of pre-tRNA synthesis is decreased when the DNA concentration in the transcription reactions is higher than 100 µg/ml (Fig. 7). This inhibition of specific RNA synthesis suggests that a cooperative interaction of both a DNA-binding factor and the RNA polymerase with the promoter is a prerequisite for correct initiation of transcription. Assuming that a DNA-binding factor exists, at high DNA-concentration the probability is lower that the transcription factor and the RNA polymerase can form a preinitiation complex at the same promoter. The inhibition of pre-tRNA synthesis at high DNA concentrations thus might be explained by a distribution of this factor and the RNA polymerase onto different DNA molecules.

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