AN ARCHAEBACTERIAL IN VITRO TRANSCRIPTION SYSTEM

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ABSTRACT

An RNA polymerase fraction of <u>Methanococcus vannielii</u>
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 corres-
ponds exactly to that of the tRNA precursor molecule synthe-
sized <u>in vivo</u>. After internal deletions of 5 and 11 basepairs
from the DNA region encoding the tRNA gene, <u>in vitro</u> tran-
scripts 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 ^{O}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 promo-
ting <u>in vitro</u> 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 tran-
scription.

INTRODUCTION

In contrast to the RNA polymerases of eubacteria, the RNA
polymerases purified from archaebacterial cells are unable to
initiate transcription accurately <u>in vitro</u> (Zillig et al.,
1988). However, recently specific binding of the purified en-
zyme from the methanogen <u>Methanococcus vannielii</u> to the promo-
ter 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 <u>M. vannielii</u> is unable to accura-
tely 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 <u>M. vannielii</u> by less purified RNA polymerase fractions was studied. We describe here the purification and some properties of an RNA polymerase fraction, directing correct <u>in vitro</u> transcription of this template.

MATERIAL AND METHODS

<u>Purification of the endogenous RNA polymerase from the crude extract</u>

A soluble extract of <u>M. vannielii</u> 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).

<u>DNA isolation and construction of mutated plasmids</u>

The plasmids for the <u>in vitro</u> transcription reactions were purified by repeated centrifugation in CsCl density gradients as described previously (Thomm and Wich, 1988). Plasmid pIC31/1 contains the <u>ClaI</u> fragment of plasmid pMT31 (Wich et al., 1986b) inserted into the <u>ClaI</u> 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
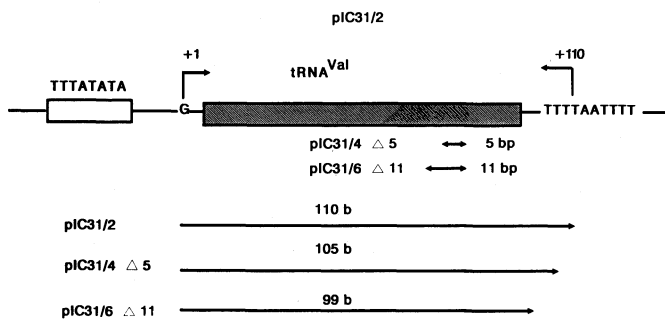

Fig.1. Genetic map of the tRNA^{Val} gene used as a template for the <u>in vitro</u> transcription reactions. Plasmid pIC31/2 contains the 5' flanking DNA sequence of the tRNA gene including the nucleotide at position -58. The plasmids pIC31/4 and pIC31/6 are subclones with internal deletions of 5 and 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 <u>in vivo</u> 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 <u>in vitro</u> 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 $^{\circ}$C, the transcription was started by the addition of 0.33 mM each of ATP, GTP, CTP and 0.0165 mM and 10 µCi $\propto -^{32}$P UTP (600 Ci/mmol, NEN). The transcription reaction was allowed to proceed for 30 min at 50°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).
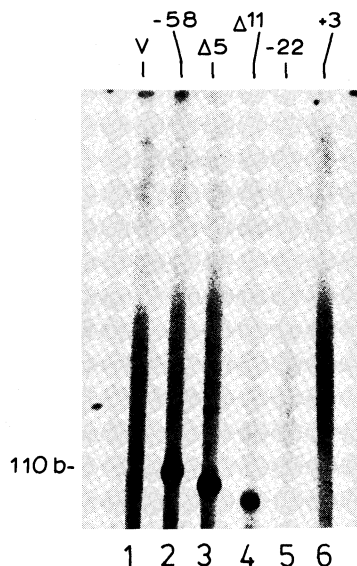

Fig. 2. Analysis of the in vitro RNA products transcribed from the templates shown in Fig. 1 and 3. The position of the RNA product with 110 nucleotides is indicated on the left side of the figure. The various templates used for the in vitro transcription reactions were: Lane 1, vector DNA (indicated 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.
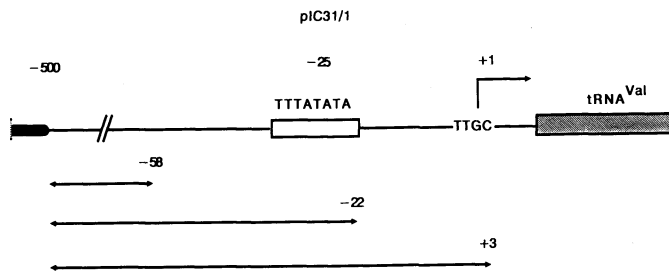
pIC31/1

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
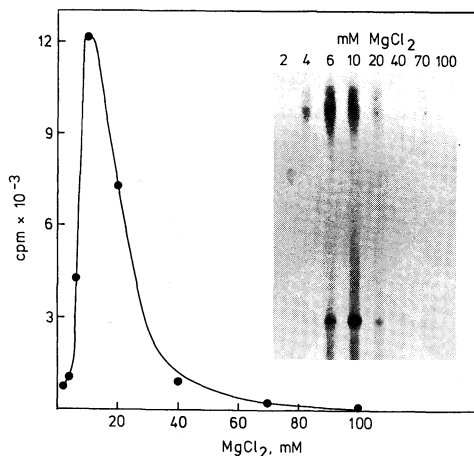

Fig. 4. MgCl$_2$-dependence of tRNA transcription. The amounts of pre-tRNA synthesized in the presence of varying amounts of MgCl$_2$ was determined after electrophoresis of the reaction products. The labelled RNA bands were excised from 6% polyacrylamide/urea gels and quantified by Cerenkov counting.
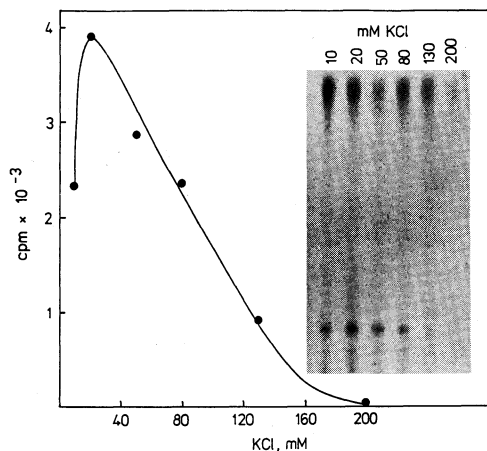
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Fig. 5. Influence of KCl concentration on the in vitro expression of the tRNA^{Val} gene. Pre-tRNA synthesis was measured at different concentrations of KCl. The MgCl$_2$ concentration was 10 mM. Product analysis and quantitation was as described in the legend of Fig. 4.
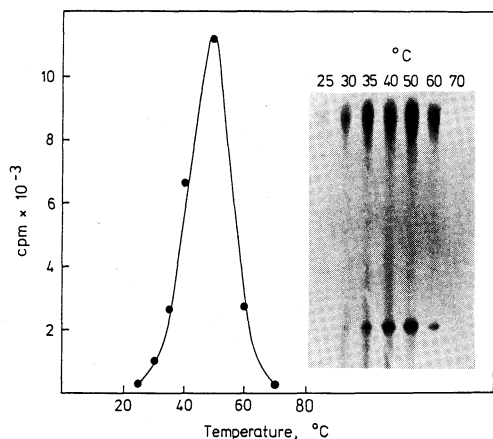

Fig. 6. Effect of the incubation temperature on transcription of the tRNA^{Val} gene. The radioactivity incorporated into the RNA product of 110 nucleotides in response to different incubation temperatures of the transcription reactions was measured as described in the legend of Fig. 4. The MgCl$_2$- 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

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

When pIC31/2 was replaced in the in vitro transcription reac-
tions 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 tran-
scription 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 consti-
tutive 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 pre-
cursor 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
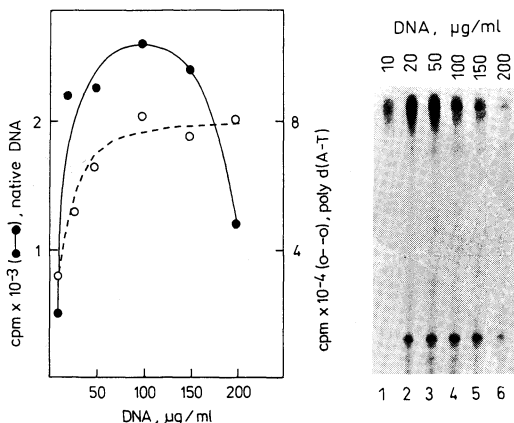optimal at 20 mM KCl. A significant expression of this template

Fig. 7. Effect of DNA concentration on the synthesis of un-
specific 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 de-
scribed 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 ^{O}C (Fig. 6) although <u>M. vannielii</u> is a mesophilic strain which shows its temperature optimum for growth at 37 ^{O}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 pre-initiation 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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