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Transcription in methanogens

Evidence for specific *in vitro* transcription of the purified DNA-dependent RNA polymerase of *Methanococcus thermolithotrophicus*

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The purification of the DNA-dependent RNA polymerase of *Methanococcus thermolithotrophicus* is described. As the first step of purification the endogenous template was removed from the enzyme by hydrophobic interaction chromatography. The purified enzyme consists of seven components with different molecular masses.

Transcription studies on T7 DNA and the recombinant plasmid pMV15, containing rRNA genes of *Methanococcus vannielii*, revealed that only the methanogen DNA is transcribed specifically, indicating a principal structural difference between archaebacterial and eubacterial promoters. This could be shown both by analysis of ternary transcription complexes and Southern hybridization. The site of initiation was found within a restriction fragment harbouring the first 390 nucleotides of the sequence coding for mature 16S rRNA and 1100 base pairs of upstream sequences. The specific initiation on this fragment strongly suggests that the enzyme can start *in vitro* transcription from the promoter(s) of rRNA synthesis.

Within the archaebacteria the DNA-dependent RNA polymerases of different thermoacidophiles (for a review see Zillig et al. [1]), two extreme halophiles [2, 3] and of one methanogen [4] have been isolated. They all show significant structural differences from the eubacterial DNA-dependent RNA polymerase [5]. Specific *in vitro* transcription of archaebacterial templates by these enzymes has not been realized. Reports on selectivity are so far limited to a paper describing asymmetric transcription of the heterologous T7 DNA by the RNA polymerase of *Desulfurococcus mucosus* probably not from the eubacterial promoters [6]. On the same template, however, unspecific binding of the *Sulfolobus acidocaldarius* RNA polymerase was found [7]. Within methanogens, specificity of transcription *in vitro* has not been investigated.

In this study we have purified the highly stable DNA-dependent RNA polymerase of *Methanococcus thermolithotrophicus*. With this enzyme we obtained evidence for selective *in vitro* initiation within a small segment of the methanogen DNA on the recombinant plasmid pMV15 [8]. This plasmid contains the rRNA genes of *Methanococcus vannielii*, which is closely related to *Mc. thermolithotrophicus* [9, 10].

MATERIALS AND METHODS

Chemicals and reagents

Restriction enzymes, DNA ligase from T4-infected *E. coli* cells, *E. coli* RNA polymerase, unlabelled nucleoside triphosphates, poly[d(A-T)], α-amanitin and dithiothreitol were obtained from Boehringer (Mannheim, FRG); acrylamide, *N*′,*N*′-methylenebisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylenediamine, calf thymus DNA and stains were from Serva (Heidelberg, FRG); Brij 58, cordycepin 5′-triphosphate and ethyleneglycol were from Sigma. [^{14}C]ATP (40–60 Ci/mol) and [α-^{32}P]UTP (760 Ci/mmol) were from New England Nuclear (Dreieich, FRG); phenyl-Sepharose was from Deutsche Pharmacia (Freiburg, FRG); DEAE-cellulose (DE32) was from Whatman (New Jersey); Seakem ME agarose was from FMC (Rockland, Maine, USA); 2-mercaptoethanol and cesium chloride from Roth (Karlsruhe, FRG); Polymin P was from BASF (Ludwigshafen, FRG); dodecyl sulfate, sodium deoxycholate, ammonium persulfate, glycerol and sodium dithionite (all of analytical grade) were from Merck (Darmstadt, FRG); bovine serum albumin ('nuclease-free') was obtained from Bethesda Research Laboratories.

Culture conditions

Methanococcus thermolithotrophicus DSM 2095 [11] was cultivated using the anaerobic technique described by Balch and Wolfe [12]. The cells were grown in a 100-l enamel-coated fermentor (HTE Bioengineering, Wald, Switzerland) at 60 °C in 'MGG' medium [12] pressurized with 500 kPa H_2/CO_2 (80:20 v/v). The cells were stored in stoppered glass bottles under nitrogen atmosphere at −80 °C.

Purification of the RNA polymerase

All procedures were performed at room temperature using an anaerobic chamber (Coy Manufacturing Company, Ann Arbor, Michigan). Oxygen was removed from the buffers as described [4].

45 g (wet weight) of frozen cells were suspended by an Ultra Turrax (Janke & Kunkel, Staufen, FRG) in 80 ml buffer A (10 mM Tris/HCl pH 7.5, 4 M NaCl, 1 mM EDTA). The cells were disrupted by a French pressure cell at 140 MPa and

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Abbreviations. kb, 10^3 base pairs; SDS, sodium dodecylsulfate.

Enzyme. RNA nucleotidyltransferase or DNA-dependent RNA polymerase, nucleosidetriphosphate:RNA nucleotidyltransferase (EC 2.7.7.6).

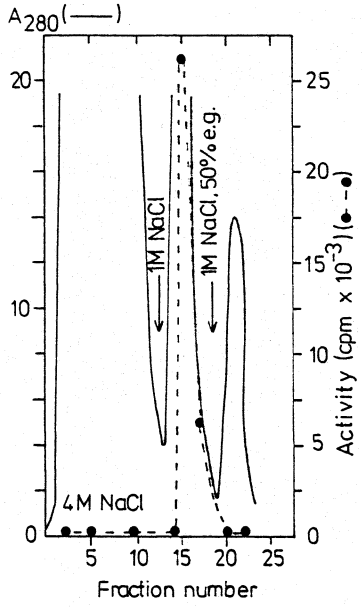

Fig. 1. *Hydrophobic interaction chromatography of a crude extract of* Mc. thermolithotrophicus. Cells were disrupted in a buffer containing 4 M NaCl. The cleared extract was applied to a phenyl-Sepharose column (3 × 8 cm) at a flow rate of 60 ml/h. Proteins adsorbed at 4 M NaCl were recovered by step elution with the same buffer containing 1 M NaCl and subsequently 1 M NaCl, 50% ethyleneglycol (e.g.). RNA polymerase activity was determined in a final volume of 200 µl (fractions eluted at 4 M NaCl) or 100 µl (other fractions) in the standard assay ([^{14}C]ATP)

the homogenate cleared by centrifugation at 20000 rpm for 20 min at 5 °C (rotor JA 20, Beckman J21 centrifuge). The pellet obtained was homogenized in 40 ml buffer A, passed again through a French pressure cell and subjected to centrifugation. The supernatants of both centrifugation steps were pooled ('high-salt extract') and pumped onto a 56-ml bed-volume phenyl-Sepharose column equilibrated with buffer A. The column was washed with buffer A until A_{280} returned to the base level. Adsorbed protein was solubilized by step-elution with buffer B (buffer A containing only 1 M NaCl) and then with buffer C (buffer B plus 50% v/v ethyleneglycol). RNA polymerase fractions, eluted with buffer B, were adjusted to 40% (v/v) glycerol by the addition of 87% (v/v) glycerol and then dialyzed against purification buffer [50 mM Tris/HCl pH 7.5, 50 mM KCl, 10 mM EDTA, 40% (v/v) glycerol]. The dialyzed fraction was then applied to a 255-ml bed-volume DEAE-cellulose column. The resin was washed with two bed-volumes of purification buffer and adsorbed protein eluted by a 5-column-volume linear gradient of 0.05–0.8 M KCl in purification buffer. The combined active fractions were desalted by dialysis and pumped onto 31-ml bed-volume single-strand-DNA–agarose (prepared according to Schaller et al. [13]). Elution was performed with a linear salt gradient from 0.05 M to 0.08 M KCl in buffer A (2 × 80 ml). Active fractions were concentrated by hollow-fiber filtration [14] and dialyzed against purification buffer containing only 10% glycerol (v/v). This fraction was then further purified by sucrose/glycerol gradient centrifugation as described by Zillig et al. [2]. The active fractions, containing the purified enzyme, were pooled and stored in 200-µl aliquots under liquid nitrogen. The yield was usually 1.5 mg RNA polymerase/45 g cells.

Isolation of DNA

Recombinant plasmids were isolated by CsCl/ethidium bromide centrifugation [15] from cleared lysates [16]. 0.9 g CsCl and 500 µg ethidium bromide were added/g cleared lysate. In order to remove contaminating material, the centrifugation was repeated three times. The ethidium bromide was extracted from the DNA with butan-1-ol and the isolated DNA further purified by phenol treatment. T7 DNA was obtained by phenol extraction of purified phages [17].

Construction of the pMV15 derivative pMV15Δ3

pMV15 DNA was cleaved with *Hin*dIII and religated with T4 DNA ligase in order to delete the *Hin*dIII fragments d, c and e (Fig. 7B) from pMV15. The buffer used for restriction contained 15 mM Tris/HCl, pH 7.5, 50 mM NaCl, 6 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and bovine serum albumin (1 mg/ml). The restriction enzyme was heat-inactivated (10 min at 65 °C) and dithiothreitol and ATP were then added to the buffer to a final concentration of 10 mM and 0.6 mM respectively. DNA (5 µg) was ligated 12 h at 5 °C with 0.2 unit T4 DNA ligase [18]. This DNA was used for transformation of *E. coli* K12 (HB101). Transformants were grown in LBO medium [19] containing 100 µg ampicillin/ml. The plasmids of ten ampicillin-resistant strains were isolated by a rapid method [20]. Most of these strains harboured a plasmid of 7.9 kb (pMV15 13.2 kb; [8]). Restriction analysis showed that these smaller plasmids (pMV15Δ3) contained only *Eco*RI/*Hin*dIII fragments a, b and f of Fig. 7B) pMV15 (data not shown). This plasmid was used for mapping the restriction enzyme cleavage sites in the *Eco*RI/*Hin*dIII fragment a (Fig. 7B) of pMV15 (the map is shown in Fig. 8B).

TRANSCRIPTION EXPERIMENTS

Standard assay

For the determination of RNA polymerase activity the incorporation of [^{14}C]AMP or [^{32}P]UMP into acid-insoluble material was measured. 1 unit of activity is defined as 1 nmol labelled nucleoside monophosphate incorporation into RNA in 1 min.

[^{14}C]AMP incorporation

Enzyme fractions were incubated for 20 min at 60 °C in 100 µl reaction mixture containing 20 mM Tris/HCl, pH 8.0, 1 mM CTP, GTP, UTP, 1 mM [8-^{14}C]ATP (1 Ci/mol), 80 mM $MgCl_2$, 20 mM KCl, DNA [poly(dA-dT)] at a concentration of 0.1 mg/ml and the enzyme fraction.

[^{32}P]UMP incorporation

The conditions were the same as with labelled ATP except that the concentration of UTP (specific activity 80 Ci/mol) was only 0.1 mM. To avoid unspecific adsorption of the labelled nucleotide, NaCl was added before the trichloroacetic acid precipitation to a final concentration of 0.3 M.

The radioactivity insoluble in 5% trichloroacetic acid was determined after filtration on glass-fiber discs (Whatman, GF/C) by liquid scintillation counting.

Analysis of initiation of transcription

RNA synthesis was carried out in 20-µl (ternary transcription complexes) or 100-µl volumes (Southern hybridization)

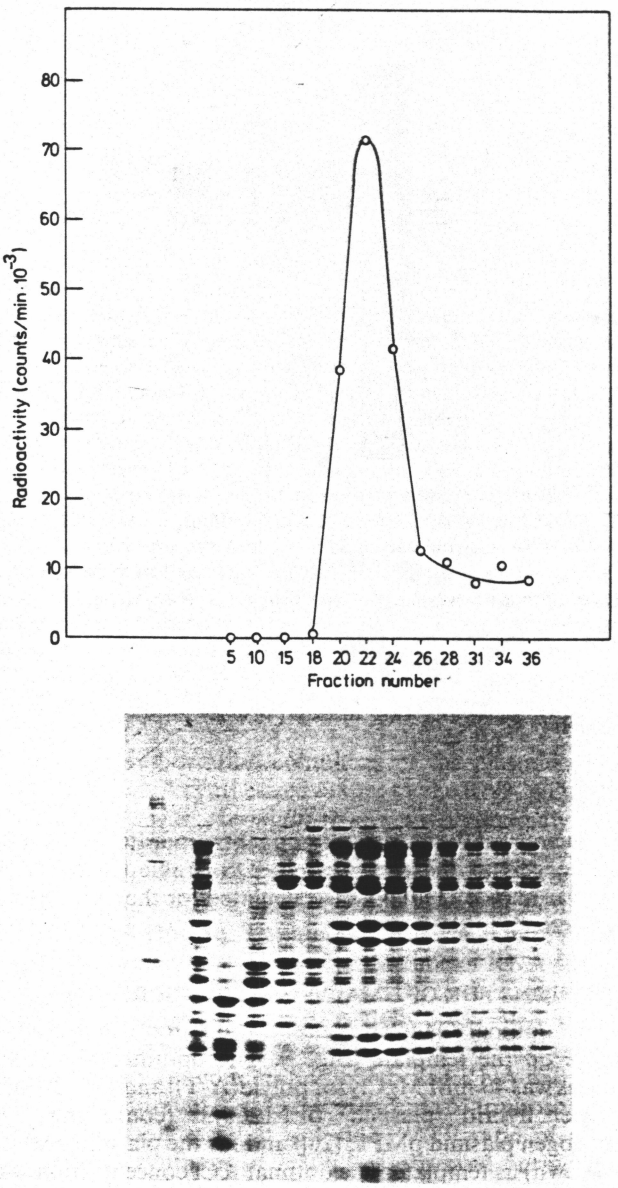

Fig. 2. *Sodium dodecylsulfate/polyacrylamide gel electrophoresis of RNA polymerase fractions after sucrose gradient centrifugation.* Equal volumes (20 µl) of each fraction were applied to the SDS/polyacrylamide gel. Left lane: RNA polymerase of *E. coli*; next lane: fraction applied on the sucrose gradient; other lanes: fractions from the sucrose gradient; assays were performed under standard conditions (^{14}C incorporation) with 5 µl of each of the fraction

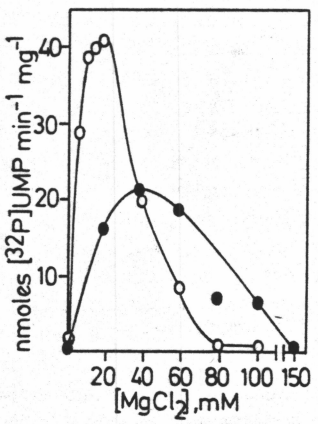

Fig. 3. *Dependence of the RNA polymerase of* Mc. thermolithotrophicus *on Mg^{2+} ions on different templates.* 0.22 µg purified RNA polymerase were assayed with 10 µg of the plasmid pPF1260-3 (○) or poly[d(A-T)] (●) as template at different $MgCl_2$ concentrations. Assays contained the other components in the usual concentrations ([^{32}P]UMP incorporation)

Table 1. *Molecular masses and relative amounts of the components of the DNA-dependent RNA polymerase from* Mc. thermolithotrophicus The molecular masses of the enzyme components were determined by comparison of their electrophoretic mobilities in SDS/polyacrylamide gel electrophoresis with those of standard proteins. Subunits of the RNA polymerase of *E. coli*, ovalbumin, chymotrypsinogen A, cytochrome *c* and aprotinin were used as standards. Molar ratios were determined by scanning the Coomassie-stained components separated on SDS/polyacrylamide slab gels. The values of the number of components per enzyme monomer have been normalized to the value for B, which was taken as 1. Components showing an immunological cross-reaction to other archaebacterial RNA polymerases are designated with large Roman letters [5, 28]

Component (designation)	$M_r \times 10^{-3}$	Molar ratio
		mol/mol
1 (A)	111	1.2
2 (B′)	80	1
3 (B″)	57	0.8
4 (C)	49.5	0.75
5 (D)	24	1.2
6	19	2
7	7.7	4

at 30 °C (RNA polymerase of *E. coli*) or 55 °C (*Methanococcus* RNA polymerase).

The RNA polymerases were preincubated with the template for 10 min at 30 °C (*E. coli*) or 2 min at 55 °C (*Mc. thermolithotrophicus*). The final concentration of the components during preincubation was 20 mM Tris/HCl, pH 8.0, 0.1 mM EDTA, 10 mM $MgCl_2$, 100 µg bovine serum albumin/ml and 50–100 µg DNA/ml. After preincubation, RNA synthesis was started by the addition of a nucleotide triphosphate mixture. The final concentration of ATP, GTP, CTP was 500 µM, that of UTP 50 µM. [α-^{32}P]UTP was added at 0.15 µCi/µl (ternary transcription complexes) or at 0.4 µCi/µl (Southern hybridization). For the analysis of ternary transcription complexes the reaction was stopped by the addition of EDTA, aurintricarboxylic acid and glycerol as described ([21]; precut) or with cordycepin 5′-triphosphate at a final concentration of 4 mM (postcut). RNA synthesized within 30 s was isolated for Southern hybridization as described [21] and hybridized to DNA bound on Genescreen™ hybridization membranes (NEN) in the presence of dextran sulfate under the conditions recommended by the manufacturer. Autoradiography was carried out with Agfa Curix RP 1 X-ray films with a Trimax 16B intensifying screen (3M, München, FRG) at −80 °C.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed according to Laemmli [22] but in 5–25% exponential polyacrylamide gels [23].

For the determination of the molar ratios of the polymerase components, Coomassie-blue-stained polyacrylamide gels

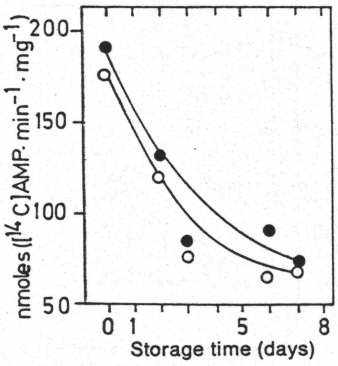

Fig. 4. *Stability of the RNA polymerases from* Mc. thermolithotrophicus *in the presence and absence of oxygen.* The purified enzymes of *Mc. thermolithotrophicus* was diluted in reduced and aerated purification buffer (Materials and Methods) to a final protein concentration of 0.04 mg/ml. After standing at room temperature in the laboratory (aerobic samples) or in the anaerobic chamber (anaerobic samples) aliquots were taken on the indicated dates and RNA polymerase activity determined in standard assays ([^{14}C]AMP) (●) anaerobically, (○) aerobically

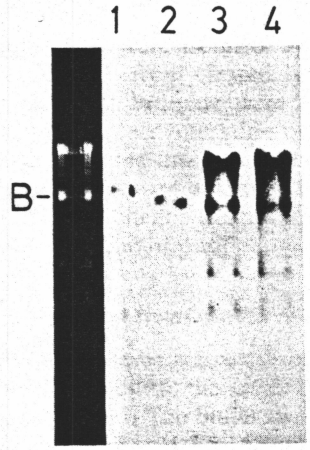

Fig. 5. *Agarose gel electrophoresis of ternary transcription complexes of T7* Mbo*I fragments.* DNA of the *E. coli* phage T7 was transcribed with the RNA polymerase of *Mc. thermolithotrophicus* (lanes 3, 4) and *E. coli* (lanes 1, 2). The DNA was hydrolyzed with *Mbo*I before (lanes 1, 3) of after RNA synthesis (lanes 2, 4). Restriction fragments were separated on 1.2% agarose gels and ternary transcription complexes detected by autoradiography. Left lane: restriction pattern of *Mbo*I-cleaved T7 DNA after staining with ethidium bromide. The *Mbo*I fragment B, containing the eubacterial promoters, is indicated on the left

were scanned at 550 nm by a Gilford linear transport photometer and the peaks analysed by planimetry. The relative quantitiy of the subunits was calculated from the areas under the peaks under the assumption that the staining capacities of all components are equal. The stoichiometry was calculated from the relative quantities considering the molecular masses.

Protein determination

Protein was determined by the micromethod of Heil and Zillig [24].

RESULTS

PURIFICATION AND POLYPEPTIDE COMPOSITION OF THE ENZYME

The RNA polymerase of *Mc. thermolithotrophicus* could not be separated from its endogenous template by the usual standard procedure [1]. Therefore, the method of hydrophobic interaction chromatography on phenyl-Sepharose originally described for the isolation of histones from rat liver chromatin [25] was modified for the isolation of the RNA polymerase from *Mc. thermolithotrophicus*. The 'high-salt extract' of *Mc. thermolithotrophicus* was applied to a phenyl-Sepharose column. The flow-through contained the cellular DNA (not shown), but no RNA polymerase activity (Fig. 1). The adsorbed RNA polymerase was eluted from the column by lowering the salt concentration of the elution buffer from 4 M to 1 M NaCl (Fig. 1). The enzyme was further purified by chromatography on DEAE-cellulose and single-stranded-DNA–agarose and, finally, by sucrose gradient centrifugation.

Seven polypeptides paralleled each other and the enzymatic activity during DNA-agarose chromatography (not shown) and sucrose gradient centrifugation (Fig. 2), indicating that the enzyme consists of seven components differing in molecular mass. This finding and the immunochemical cross-reaction of these polypeptides with other archaebacterial RNA polymerases [5] are evidence for true enzyme constituents. From the molecular masses and the stoichiometry of the enzyme components separated on polyacrylamide gels in the presence of SDS (Table 1), a relative molecular mass of 390000 was calculated for the native RNA polymerase.

CONDITIONS FOR OPTIMAL *in vitro* TRANSCRIPTION

The optimal $MgCl_2$ concentration for transcription depends on the template (Fig. 3). The optimum for RNA synthesis was 40 mM $MgCl_2$ for poly[d(A-T)] and 10–20 mM for the hybrid plasmid pPF1260-3 (containing the methanogen plasmid pMP1; [26]) and for the plasmid pMV15 (not shown) as template. The optimal KCl concentration was around 50 mM, the pH optimum was found at 8.7. The temperature optimum for RNA synthesis was in the range between 55°C and 65°C (not shown), which is in agreement with the optimal growth temperature of the organism [12]. Although from a strict anaerobe, the enzyme showed no pronounced inactivation by oxygen at room temperature (Fig. 4). To prevent long-time inactivation, the enzyme was stored in portions under liquid nitrogen.

TRANSCRIPTION EXPERIMENTS

Transcription of the DNA of the E. coli *phage T7*

On the heterologous DNA of the *E. coli* phage T7 the DNA-dependent RNA polymerase of *Mc. thermolithotrophicus* formed ternary transcription complexes on all *Mbo*I restriction fragments when native T7 DNA (Fig. 5, lane 4) or their fragments (Fig. 5, lane 3) were used as a template (postcut or precut procedure of Chelm and Geiduschek [21]). Therefore, no selectivity for the *E. coli* promoters (containing *Mbo*I fragment B) exists, indicating that this enzyme cannot initiate specifically at these eubacterial promoters. With *E. coli* enzyme, however, with the same procedure ([21] Fig. 5, lanes 1, 2) fragment B of T7 DNA was recognized with high specificity.

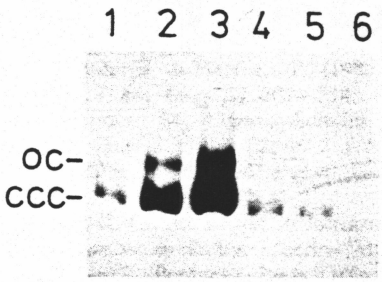

Fig. 6. *Agarose gel electrophoresis of ternary transcription complexes.* Ternary complexes, formed *in vitro* by purified RNA polymerase of *Mc. thermolithotrophicus*, were analyzed by agarose gel electrophoresis and autoradiography as described in Materials and Methods. The autoradiogram shows the amount of labelled RNA associated with the covalently circular closed (ccc) and open circular (oc) form of the plasmid pMV15 after 30 s (lanes 1, 4, 5, 6), 2 min (lane 2) and 5 min (lane 3) of RNA synthesis. RNA synthesis was stopped with sample buffer (lanes 1–3; [21]) or cordycepin 5′-triphosphate (3′-dATP) at a ratio 3′-dATP/ATP = 8 (lanes 4–5); sample 5 was then further incubated for 2 min at 55 °C, sample 4 kept in an ice bath during this time; (6) similar to (1), but the sample buffer contained in addition 1% SDS (w/v)

Transcription of the hybrid plasmid pMV15

Ternary transcription complexes of the plasmid pMV15 and labelled RNA synthesized *in vitro* by the RNA polymerase of *Methanococcus thermolithotrophicus* were analyzed by agarose gel electrophoresis. After this step, labelled RNA was associated with the DNA bands, in particular with that corresponding to the circular covalently closed form of the plasmid DNA (Fig. 6). The amount of DNA-bound radioactivity, was directly proportional to the time of RNA synthesis (Fig. 6, lanes 1–3). The addition of SDS to the sample buffer resulted in a decrease of radioactivity associated with the DNA (Fig. 6, lane 5). Therefore, the RNA polymerase seems to be necessary for comigration of labelled RNA with DNA. After the addition of 3′-dATP (cordycepin 5′-triphosphate) to the transcription assay at a 3′-dATP/ATP ratio of at least eight, no net synthesis of RNA was observed during an incubation time of 2 min (Fig. 6, lane 6). This indicates that no significant initiation or elongation of RNA chains occurred in the presence of 3′-dATP.

In order to localize initiation sites for RNA synthesis on the plasmid, it was hydrolyzed with *Eco*RI/*Hin*dIII (see map in Fig. 7B) and the resulting restriction fragments used as a template (precut). Analysis of ternary transcription complexes showed that after 20 s (Fig. 7, lane 1) and 30 s (data not shown) of RNA synthesis, fragment a, containing the DNA-sequence coding for 16S rRNA, was preferentially transcribed. This selective initiation was obtained with RNA polymerase fractions from all purification steps (data not shown). After 1 min of RNA synthesis unspecific transcription of restriction fragments occurred (Fig. 7, lane 3).

In another experiment the supercoiled plasmid was used as template. In this case RNA synthesis was stopped by the addition of 3′-dATP and the plasmid cleaved subsequently by restriction enzymes (postcut). Under these conditions the same selectivity for fragment a was observed both after 20 s (Fig. 7, lane 2) and 1 min (Fig. 7, lane 4) of RNA synthesis. This shows that RNA synthesis starts from an internal initiation site of *Methanococcus* DNA harbouring the region coding for mature 16S rRNA and around 3 kb of upstream sequences.

For a more precise localisation of initiation sites on pMV15, a derivative, pMV15Δ3, containing only the pMV15 *Eco*RI/*Hin*dIII fragments a, b and f (Fig. 7B), was constructed and used for mapping of restriction enzyme cleavage sites (see restriction map in Fig. 8B) and transcription experiments. Labelled RNA, synthesized on this template within 30 s, was hybridized to a Southern transfer of *Hpa*II/*Bgl*II/*Eco*RI-cleaved pMV15Δ3 DNA. Southern hybridization showed that pMV15Δ3 fragment a, harbouring around 390 base pairs of the sequence coding for 16S rRNA and around 1.1 kb 5′-flanking sequences, is transcribed selectively (Fig. 8A). Almost no RNA hybridizes to other parts of the molecule, suggesting that this fragment may contain a strong promoter which is recognized specifically by the purified RNA polymerase of *Mc. thermolithotrophicus*.

DISCUSSION

The first step of RNA polymerase purification, the removal of the endogenous DNA by Polymin P [1, 27], had to be replaced by another procedure as the enzyme of *Mc. thermolithotrophicus* was inactivated. A highly active and DNA-free RNA polymerase fraction could be obtained from the crude extract by hydrophobic interaction chromatography on phenyl-Sepharose. This method turned out to be suitable also for the isolation of other prokaryotic DNA-dependent RNA polymerases (data not shown) and of DNA-binding proteins, e.g. restriction enzymes (Thomm and Stetter, unpublished). The purified RNA polymerase from *Mc. thermolithotrophicus* shows the polypeptide composition AB′B″C, typical for the methanogenic-halophilic branch of the archaebacteria [5, 28].

Similar to the DNA-dependent RNA polymerase from sulfur-dependent archaebacteria [6, 7] the enzyme of *Methanococcus thermolithotrophicus* is unable to initiate transcription specifically on the T7 DNA fragment containing the promoters of 'early' genes. Since the RNA polymerase of many phylogenetically distant eubacteria are able to recognize at least some of these promoters [29], the negative result with the archaebacterial enzymes could be explained by a principal structural difference of the archaebacterial and eubacterial promoters caused by the large phylogenetic distance between the two kingdoms [30].

In spite of the lack of specificity on T7 DNA, the enzyme can transcribe *Methanococcus* DNA selectively as could be shown both by analyses of ternary transcription complexes and Southern hybridization with *in vitro* RNA. As this selectivity was obtained with crude enzyme fractions and the purified enzyme, additional factors seem not to be necessary for specificity in contrast to the enzymes known from eukaryotic organisms [31–33].

While transcription of the uncleaved methanogen DNA in the plasmid pMV15 is highly specific, transcription of the restriction fragments is less selective (Fig. 7, lanes 3 and 4). The diminished specificity may be due to additional initiation sites created by the restriction enzymes [34] or to the lack of superhelicity of the cleaved template.

The restriction fragment harbouring the strong specific initiation signal for the *Methanococcus* RNA polymerase comprises around 10% of the pMV15 plasmid. It contains the region upstream from the 16S rRNA and the first 390 nucleotides of the sequence coding for 16S rRNA [8]. The most probable interpretation for initiation within this section is the recognition of a promoter(s) for rRNA synthesis by this enzyme. This result and the absence of significant initiation

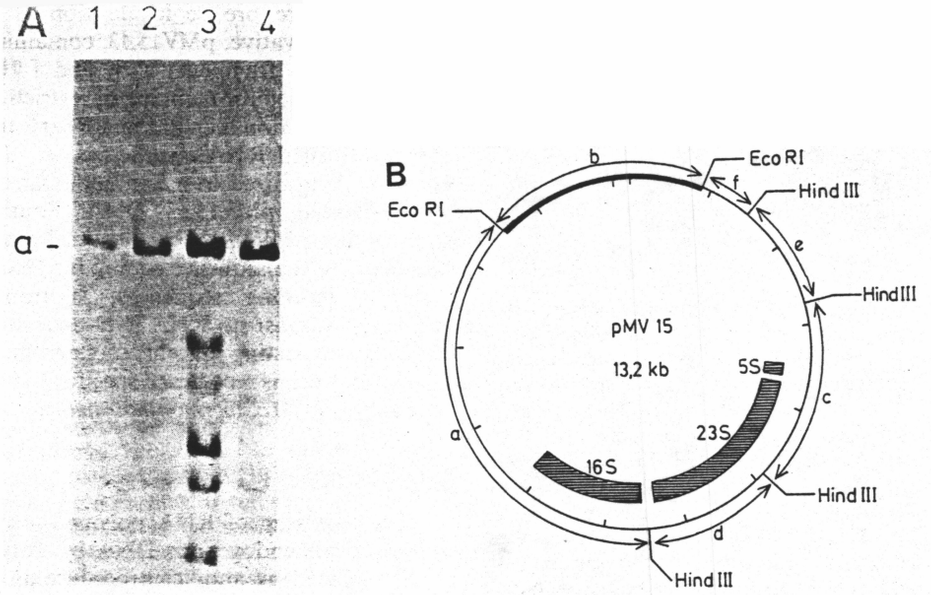

Fig. 7. *Comparison of ternary complexes formed on restriction fragments and supercoiled pMV15 DNA.* (A) The plasmid pMV15 was treated with the restriction enzymes *Eco*RI/*Hin*dIII before (1, 3) or after RNA synthesis (2, 4). Ternary transcription complexes were separated on 1.2% agarose gels as described [21]. RNA was synthesized by the purified enzyme for 20 s (lanes 1, 2) and 1 min (lanes 3, 4). The ratio enzyme/DNA = 1 mol/mol. The position of the *Eco*RI/*Hin*dIII restriction fragment a is indicated on the left side of the column (see also restriction map below). (B) Restriction enzyme cleavage map of pMV15. The heavy line indicates the vector plasmid, the thin line represents methanogenic DNA sequences. The region coding for ribosomal genes of *Mc. vannielii* is indicated inside the figure. The *Eco*RI/*Hin*dIII restriction fragments are designated with small Roman letters according to size. One scaling unit corresponds to 1000 base pairs (1 kb)

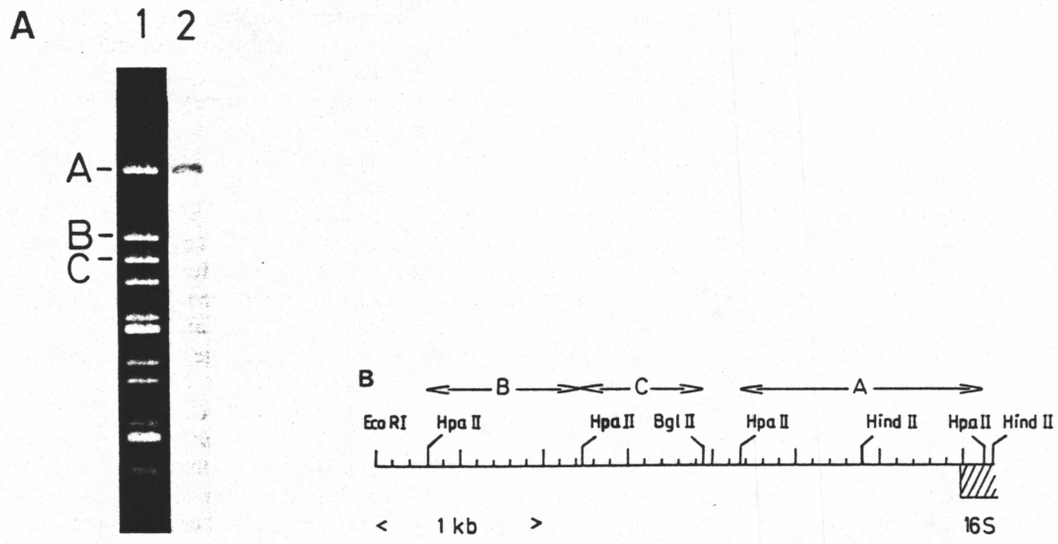

Fig. 8. *Southern hybridization of* in vitro *RNA on restriction fragments of pMV15Δ3 DNA.* (A) RNA, synthesized on pMV15Δ3 DNA as template, was hybridized to *Hpa*II/*Bgl*II/*Eco*RI restriction fragments of pMV15Δ3 immobilized on a membrane. Lane (1) *Hpa*II, *Bgl*II, *Eco*RI restriction fragments of pMV15Δ3 DNA separated on a 1.75% agarose gel; Lane (2) hybrids obtained after 30 s transcription of pMV15Δ3 DNA. The restriction fragments upstream from the 16S rRNA sequence are indicated on the left side. The ratio enzyme:DNA = 1 mol/mol. (B) Restriction enzyme cleavage map of the 16S rRNA 'upstream region' of pMV15. The beginning of the sequence coding for mature 16S rRNA is indicated inside the figure

of transcription on other parts of the molecule suggest that the ribosomal genes in *Methanococcus* may also be cotranscribed in the order 16S, 23S, 5S, like the ribosomal genes in eubacteria [35]. Owing to its specificity, the *Mc. thermolithotrophicus* RNA polymerase may be well suited for further identification of promoters in methanogens.

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