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RNA Polymerase-Binding and Transcription Initiation Sites Upstream of the Methyl Reductase Operon of *Methanococcus vannielii*

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RNA polymerase, purified from *Methanococcus vannielii*, was shown by exonuclease III footprinting to bind to a 49-base-pair (bp) region of DNA in the intergenic region upstream of *mcrB*. S1 nuclease protection experiments demonstrated that transcription initiation in vivo occurs within this region at 32 or 33 bp 5′ to the ATG translation initiation codon of *mcrB* and 19 or 20 bp 3′ to a TATA box.

Although methanogens are archaebacteria, the structure and organization of their polypeptide-encoding genes appear to be the same as those in eubacteria. Methanogen genes are often arranged in what seem to be multigene transcriptional units (operons), ribosome-binding sites precede genes, and to date, introns have not been detected (5). However, methanogen-derived DNA-dependent RNA polymerases (RNAP) are very different from eubacterial RNAPs (8, 9), and a major unresolved question is therefore the structure of methanogen promoters. We have cloned and sequenced the genes (*mcrBDCGA*) which encode the subunits of component C of methyl coenzyme M reductase, the most abundant enzyme in *Methanococcus vannielii* (1, 3). Since these genes are all transcribed in the same direction and are separated only by very short intergenic regions, they appear to be organized as an operon. To account for the large amounts of methyl coenzyme M reductase synthesized, these genes must be very highly expressed and are therefore likely to be transcribed from a very strong promoter. In this report we define the DNA sequence in the intergenic region immediately upstream of *mcrB* which binds purified *M. vannielii* RNAP in vitro. We also show that this RNAP-binding site overlaps the in vivo site of transcription initiation and that it contains sequences which have been proposed as elements of promoters for transcription of stable RNA genes in *M. vannielii* (11) and in other archaebacteria (6, 12).

M. vannielii RNAP was purified and assayed as previously described for RNAP purification from *Methanococcus thermolithotrophicus* except that active fractions from the heparin-cellulose column (8, 9) were subsequently concentrated by ultrafiltration and further purified by passage through a molecular sieve (TSK3000; LKB Products, Bromma, Sweden) and anion-exchange (MonoQ; Pharmacia, Inc., Piscataway, N.J.) fast-protein liquid chromatography columns. The origin and construction of the probes used to determine the sites of RNAP binding and transcription initiation relative to the *mcrB* gene are shown in Fig. 1. Plasmid pET1400 (1) contains 1,240 base pairs (bp) of *M. vannielii* DNA cloned in pUC19 (10), including 274 bp of the intergenic region preceding *mcrB*. Plasmid pMRP5 was constructed as shown in Fig. 1. Plasmid pMRP4, obtained fortuitously from the same ligation reaction as pMRP5, was found to have suffered a deletion of 40 bp of *M. vannielii* DNA which removed all of the *mcrB* coding region and 9 bp of the adjacent intergenic region. The boundaries of the RNAP-binding sites on the DNA probes (Fig. 1) were determined by exonuclease III (*Exo*III) footprinting by using the published procedure (7, 13). Results of an experiment which located the 5′ boundary with the *Bam*HI-*Eco*RI probe (Fig. 1) derived from pMRP5 are shown in Fig. 2. Bound RNAP blocked *Exo*III digestion at a point which resulted in a DNA molecule of a length that indicated that the 5′ boundary of the RNAP-binding site must be 62 bp upstream of the ATG translation initiation codon for *mcrB*. When the same procedure was used with the *Hin*dIII-*Pvu*II probe (Fig. 1) derived from pMRP4, the 3′ boundary of the RNAP-binding site was shown to be 13 bp upstream of the ATG codon. The site of transcription initiation in vivo for the *mcrB* operon was determined by the standard S1 nuclease protection procedure (4). RNA was obtained by hot-phenol extraction of exponentially growing cells of *M. vannielii* (9). The DNA probe was derived from pET1400 (Fig. 1). Transcription initiation was found to occur primarily at one of two adjacent bases (C and T) located 33 and 32 bp upstream of the ATG translation initiation codon (Fig. 3). The in vivo transcript must therefore have a nontranslated leader region containing the sequence previously predicted to be a ribosome-binding site (1, 5).

Our results defined the *M. vannielii* RNAP-binding site as being 49 bp in length, extending 29 bp 5′ and 19 bp 3′ from the in vivo site of transcription initiation (Fig. 3). Since the deletion in pMRP4 reaches to within 4 bp of the 3′ boundary of the RNAP-binding site, sequences downstream of this point are clearly not essential for the RNAP binding. The results of this study also demonstrate that this archaebacterial RNAP does not require additional transcription factors to recognize and bind to specific DNA sequences.

There have been several reports cataloging conserved sequences upstream of archaebacterial genes (2, 5, 6, 11, 12). The sequence 5′ TATATA-(18 or 19 bases)-TGC has been proposed as a consensus sequence for promoters of stable RNA genes in both *M. vannielii* (11) and other archaebacterial species (6, 12), with transcription initiating in vivo at the G residue of this sequence (11). Within the region of *M. vannielii* DNA which we have shown to bind *M. vannielii* RNAP (Fig. 3) is the sequence 5′ ATATA, located 19 or 20 bp upstream of the in vivo sites of transcription initiation. A TATA box motif, ~19 bp upstream of the site of transcription initiation, could therefore be a common element of

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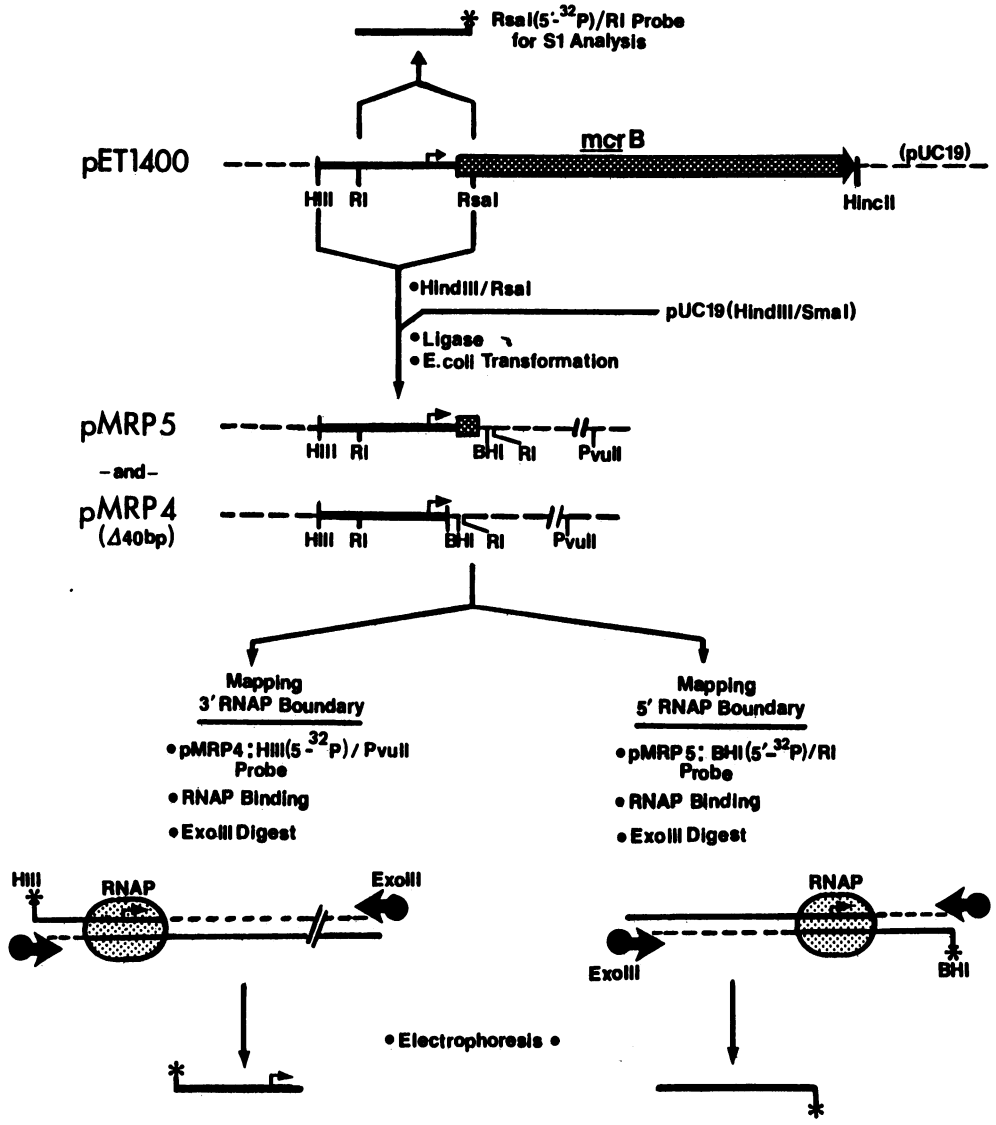

FIG. 1. Probes and experimental protocols used. Plasmid pET1400 contains part of the *mcrB* gene (▬) and the intergenic region (—) upstream of the *mcrB* gene cloned in pUC19 (1, 10). Restriction fragments, 5′ end labeled by means of polynucleotide kinase and [γ-^{32}P]ATP, were subsequently cleaved by restriction enzymes as indicated to obtain the single-end-labeled molecules used as probes. Locations of ^{32}P atoms are denoted by asterisks, and the in vivo initiation site and direction of transcription are indicated by the small rightward-pointing arrow. DNA which would be digested by *Exo*III activity in the presence of RNAP is also indicated (- - - - -). Restriction sites are indicated by the standard abbreviations except for *Hin*dIII (HIII), *Eco*RI (RI), and *Bam*HI (BHI). The sequence of the *M. vannielii* DNA shown has been published previously (1). Standard techniques were used for the in vitro DNA manipulations, S1 analysis, and *Exo*III footprinting (4, 7, 10, 13).

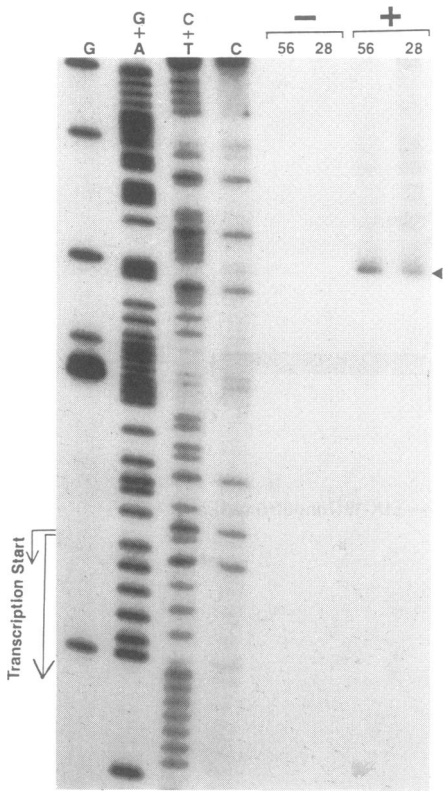

FIG. 2. *Exo*III determination of the 5′ boundary of the *M. vannielii* RNAP-binding site. RNAP-DNA complexes (+) prepared by the pMRP5-derived *Bam*HI-*Eco*RI probe (Fig. 1) were digested with *Exo*III (28 and 56 U of *Exo*III-reaction mixture), and the products were visualized by autoradiography following electrophoresis through an 8% polyacrylamide sequencing gel. Labeled probe DNA in control experiments without added RNAP (−) was completely digested by the added *Exo*III (28 and 56 U). DNA sequencing ladders produced from the probe DNA molecule, resolved in the adjacent tracks, were used to determine the exact size of the RNAP-protected fragment. The in vivo sites of transcription initiation are indicated to the left of the figure.

promoters for archaebacterial genes encoding both stable RNA (6, 11, 12) and polypeptides (2, 5). The results presented here show that this conserved sequence is part of a sequence specifically bound by *M. vannielii* RNAP.

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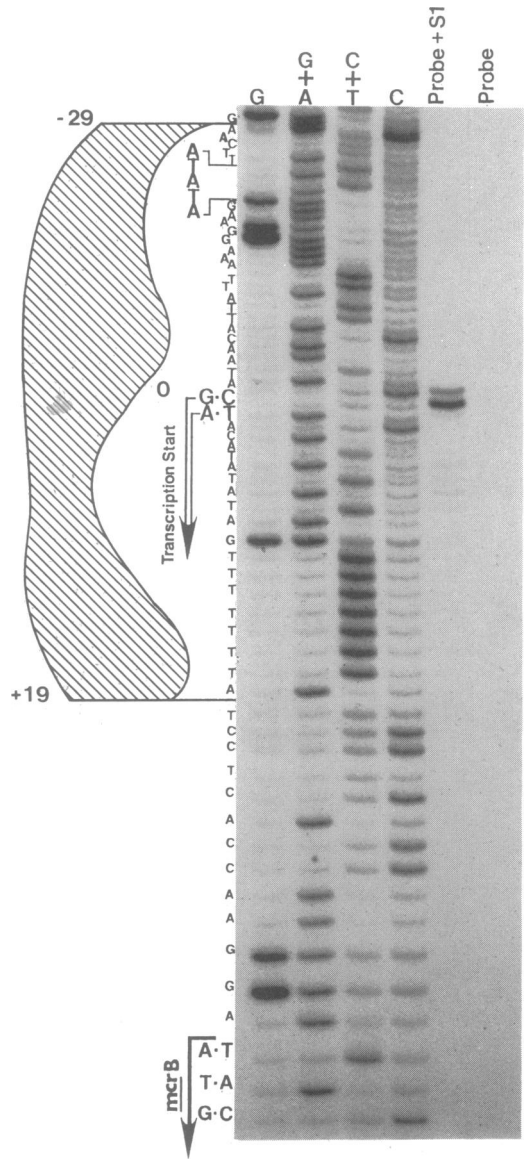

FIG. 3. In vitro site of RNAP binding and in vivo sites of transcription initiation. The sequence of the DNA strand which serves as the template for transcription is shown. The ATG translation initiation codon for *mcrB* is at the bottom of the figure. DNA molecules protected from S1 nuclease digestion (probe + S1) (4) by hybridization of the *Rsa*I-*Eco*RI probe obtained from pET1400 [Fig. 1]) to RNA extracted from growing cells of *M. vannielii* are shown adjacent to the sequencing ladders. DNA molecules in control experiments containing no RNA (probe) were completely digested by the S1 nuclease. The boundaries of the RNAP-binding site as determined by the *Exo*III footprinting are shown at −29 and +19 relative to the C residue (designated 0) at which transcription initiation occurs. The ATATA motif within the region of *M. vannielii* DNA protected from *Exo*III digestion by bound RNAP (▨) is highlighted.

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