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An archaebacterial RNA polymerase binding site and transcription initiation of the *his*A gene in *Methanococcus vannielii*

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ABSTRACT

Transcription initiation of the hisA gene in vivo in the archaebacterium Methanococcus vannielii, as determined by nuclease S_1 and primer extension analyses, occurs 73 base pairs (bp) upstream of the translation initiation site. Binding of M. vannielii RNA polymerase protects 43 bp of DNA, from 35 bp upstream (-35) to 8 bp downstream (+8) of the hisA mRNA initiation site, from digestion by DNase I and exonuclease III. An A+T rich region, with a sequence which conforms to the consensus sequence for promoters of stable RNA-encoding genes in methanogens, is found at the same location (-25) upstream of the polypeptide-encoding hisA gene. It appears therefore that a TATA-like sequence is also an element of promoters which direct transcription of polypeptide-encoding genes in this archaebacterium.

INTRODUCTION

Gene expression is controlled at the level of transcription initiation by the choice and frequency of use of promoters by DNA-dependent RNA polymerase (RNAP). In Escherichia coli, there are two DNA sequences conserved in most promoters, the heptanucleotide TATAATG and the hexanucleotide TTGACA, located approximately 10 and 35 bp (-10 and -35 regions), respectively, upstream of the site of transcription initiation. The -10 region was identified by comparisons of DNA sequences protected from DNase I digestion by bound E. coli RNAP (1). Exonuclease III (exoIII) protection experiments (2) and DNase I footprinting (3) revealed that RNAP also binds to the -35 region [for review, see (4)]. In eukaryotes, promoters have been identified by analyses of transcription directed by mutated DNA sequences in cell-free transcription systems and following their introduction into eukaryotic cells by transformation, transfection or injection of oocytes (5-8). Such studies have revealed that an A+T rich sequence upstream of polypeptide-encoding genes (the TATA box) and sequences within tRNA-encoding genes, transcribed by RNA polymerases II and III respectively, are necessary for accurate

initiation of transcription of these genes in eukaryotes. The evidence to date indicates that archaebacteria have only one form of RNAP (9,10), so that, as in eubacterial promoters, sequences of archaebacterial promoters for stable RNA genes and for polypeptide-encoding genes might be expected to have common elements which direct RNAP binding. The only binding regions so far determined for an archaebacterial RNAP are upstream of two clusters of stable RNA genes in M. vannielii (11). In these cases, RNAP protects the region of DNA from -31 to +18 relative to the site of transcription initiation from exoIII digestion. These protected regions contain versions of the octanucleotide, 5'TTTATATA. This highly conserved sequence, found approximately 25 bp upstream of the site of transcription initiation of stable RNA genes in the genomes of members of both phylogenetic branches of the archaebacteria (11-13), conforms to the TATA-box motif of promoters used by eukaryotic RNAP II and appears to be a consensus element of promoters of archaebacterial stable RNA genes. Suggestions for promoters for polypeptide-encoding genes in archaebacteria have been based only on S_1 analysis and the identification of conserved sequences upstream of such archaebacterial genes (14-17). The validity of these sequences as promoters will remain uncertain until their location relative to the sites of transcription initiation become known and their ability to bind RNAP is demonstrated. We report here the site of transcription initiation in vivo and the site of RNAP binding in vitro upstream of the hisA gene of M. vannielii. As anticipated, this RNAP binding site does contain, at the appropriate location, a DNA sequence which conforms to the TATA consensus sequence identified (11) as a component of promoters of genes encoding stable RNAs in this archaebacterium.

MATERIALS AND METHODS

RNA polymerase purification.

The purification was performed anaerobically. Enzyme assays and SDS-PAGE procedures have been described (18). M. vannielii cell paste (40g wet weight) was resuspended in 80ml buffer A [4M NaCl, 10mM $MgCl_2$, 50mM Tris-HCl (pH7.5)] and the cells disrupted by passage through a French pressure cell at 20,000 PSI (138 MPa). The crude extract was cleared by low-speed centrifugation and applied to a phenyl-sepharose column (3 X 15cm) equilibrated in buffer A. After washing with 3 column volumes of buffer A, the RNAP was eluted with buffer B (buffer A with the NaCl concentration decreased to 1M). Active fractions were combined, dialysed against purification

buffer (buffer A with 50mM KCl replacing NaCl) and further purified by chromatography through DEAE-cellulose and heparin-cellulose columns as previously described (19). Active fractions of RNAP from the heparin-cellulose column were concentrated by ultrafiltration, passaged through a TSK3000 molecular sieve FPLC column (LKB) and finally purified by chromatography through a MonoQ anion-exchange FPLC column (Pharmacia).

DNA probes.

Plasmid pET805 (Figure 1) is a derivative of pUC8 containing a 2.9Kbp insertion of M. vannielii DNA which includes the entire hisA gene, the carboxy-terminus of an upstream ORF (ORF547) and the amino-

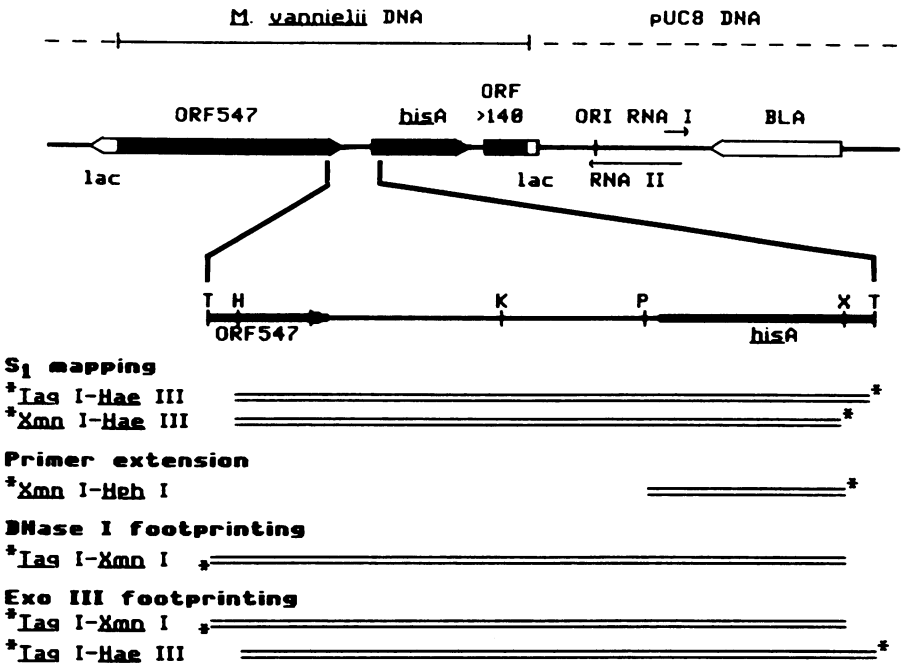

Figure 1. Probes for S_1 protection, primer extension and footprinting experiments. Double-stranded, singly end-labeled DNA probes were prepared as described (20). The probes were labeled only at the 5' end of the restriction sites marked with asterisks (*). The DNA sequence of the M. vannielii DNA in pET805 has been published (15,18,19). Boxes indicate the polypeptide-encoding regions - white boxes indicate genes within the cloning vector pUC8 DNA, and black boxes indicate genes within the M. vannielii DNA. The RNA I and RNA II transcripts of the replication origin (ORI) of pUC8 are shown. The TaqI fragment spanning the ORF547-hisA intergenic region is 462bp in length; restriction sites marked are TaqI (T), HaeIII (H), KpnI (K), HphI (P), and XmnI (X).

terminus of a downstream ORF (ORF>140) (15,20,21). Singly end-labeled DNA molecules used for S_1 protection, primer extension, DNase I and exoIII footprinting experiments were prepared from pET805 by standard methods (22). The probes used and their relationships to the ORF547-*hisA* intergenic region are shown in Figure 1.

Nuclease S_1 protection.

Nuclease S_1 protection experiments were performed as described (23). Singly 5' end-labeled probe DNAs (~3 X 10^4 dpm) were mixed with 250µg RNA (isolated from either *M. vannielii* or *E. coli*x760 (18) containing plasmid pET805 by extraction with hot phenol), precipitated by addition of ethanol, pelleted by centrifugation, dried, redissolved in 30µl hybridization buffer [40mM PIPES, 1mM Na_2EDTA, 400mM NaCl, and 80% deionized formamide (pH6.4)] and incubated at 80°C for 10min. Reaction mixtures were cooled slowly to the hybridization temperature (42°C), hybridization allowed for 16h, ice-cold nuclease S_1 solution added [100-500 units S_1 nuclease in 280mM NaCl, 4.5 mM $ZnSO_4$, 50mM Na acetate, 20µg sonicated salmon sperm DNA/ml (pH4.6)], incubation continued at 37°C for 30min and then stopped by phenol extraction. Carrier salmon sperm DNA (10µg) was added and samples prepared for electrophoresis by ethanol precipitation, centrifugation, washing with 70% ethanol and lyophilization. The dried DNAs were dissolved in 5µl electrophoresis buffer (23), heated to 90°C for 5min and visualized by autoradiography following their separation by electrophoresis through 6% polyacrylamide sequencing gels.

Primer extension analyses.

Primer extension analyses were performed as described (24). *M. vannielii* RNA (100-250µg) was mixed with singly end-labeled DNA probe (~4 X 10^4 dpm), lyophilized, dissolved in 20µg hybridization buffer, incubated at 80°C for 5min and hybridization allowed at 42°C for 16h. Nucleic acids in the mixture were then ethanol precipitated, washed with 70% ethanol, dried, redissolved in 10µl 400mM NaCl, 10mM PIPES (pH6.4), diluted with 80µl reverse transcriptase buffer [10mM dithiothreitol, 6mM $MgCl_2$, 25µg actinomycin D/ml, 0.5mM dATP, dCTP, dGTP and dTTP, and 50mM Tris-HCl (pH8.2)], 5 units of AMV reverse transcriptase added and the mixture incubated for 1h at 42°C. Samples were prepared for electrophoresis through 6% polyacrylamide sequencing gels by phenol extraction, ethanol precipitation, ethanol washing and denaturation in 5µl sample buffer at 90°C for 5min.

Filter-binding assays.

Filter-binding experiments were performed using a modification of the

published procedure (25). HaeIII and TaqI/PstI generated restriction fragments were dephosphorylated and 5' end-labeled with γ^{32}P-ATP using polynucleotide kinase. Labeled DNA fragments (~1 X 10^4 dpm) were mixed with 1-10µg RNAP in a 100µl reaction mixture containing 100mM KCl, 10mM $MgCl_2$, 0.1mM Na_2EDTA, 0.1mM dithiothreitol, 50µg bovine serum albumin (BSA)/ml, and 10mM Tris-HCl (pH8). Complexes were allowed to form at 37°C for 5min; ATP, GTP, and CTP were added (final concentrations of 167µM), incubation continued for 5min, the reaction mixtures cooled on ice and then filtered slowly through preboiled nitrocellulose filters (Scheicher and Schull; NA-45, 1cm diameter). The filters were washed with 500µl ice-cold buffer. DNA retained by the filters was eluted by incubation at 37°C for 1h in 400µl 0.2% (w/v) SDS, 20mM Tris-HCl (pH8), extracted with 500µl 50:48:2 phenol:chloroform:isoamyl alcohol, precipitated by addition of sodium acetate to 250mM and 2.5 volumes of ethanol, pelleted, lyophilized, redissolved in 20µl gel sample buffer and visualized by autoradiography following electrophoresis through 5% polyacrylamide gels. Exposures of autoradiograms, measured using a Zenith scanning laser densitometer, were quantitated using the GELSCAN and FILTER programs run on an Apple IIe computer.

DNase I footprinting

DNase I footprinting was performed using a modification of the published procedure (26,27). The amount of DNase I required for a partial digestion of each DNA probe was determined by titration in reaction mixtures identical to the footprinting reaction mixtures except that RNAP was omitted. DNase I stock solutions and dilutions were in TMK buffer [50mM KCl, 10mM $MgCl_2$, 50% (v/v) glycerol and 50mM Tris-HCl (pH8)]. Footprinting reaction mixtures (100µl) contained probe DNA (~1 X 10^4 dpm), 50mM KCl, 10mM $MgCl_2$, 0.1mM Na_2EDTA, 500µg BSA/ml and 20mM Tris-HCl (pH8). After preincubation with RNAP (0.1-10µg) at 30°C for 10min, 5µl of appropriately diluted DNase I solution was added and incubation continued for 2min. Stop solution (100µl; 100mM Na_2EDTA, 600mM NH_4 acetate, 20µg sonicated salmon sperm DNA/ml) was added, the reactions cooled on ice, nucleic acids extracted with phenol:chloroform:isoamyl alcohol, precipitated and washed with ethanol, lyophilized, redissolved in 5µl sample buffer and visualized by autoradiography following electrophoresis through 6% polyacrylamide sequencing gels.

Exonuclease III footprinting

ExoIII protection assays were performed using a modification of the published procedure (28-30). The amount of exoIII required for complete digestion of each probe was determined in reaction mixtures

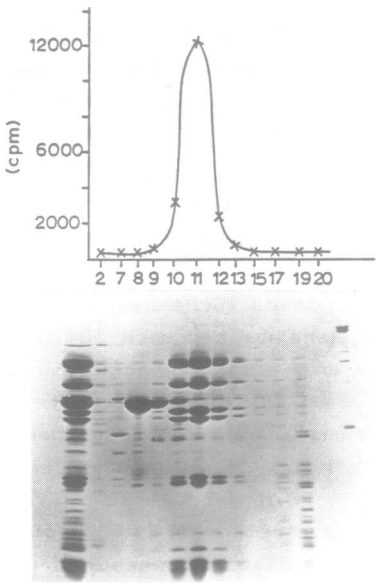

Figure 2. SDS-PAGE of purified M. vannielii RNA polymerase. M. vannielii RNAP was purified and fractions from the final chromatographic step, FPLC MonoQ, were assayed for enzyme activity by polymerization of ^{3}H-UTP into TCA precipitable material using a poly(dA:dT) template, and for purity by SDS-PAGE. The first lane of the gel is the partially purified material applied to the MonoQ column, followed by fractions eluted from the column. The rightmost lane is purified E. coli RNAP. The enzymatic activities of the material in the column fractions analyzed in the gel are shown in the graph above the corresponding gel lane.

identical to those used for footprinting, except that RNAP was omitted. Footprinting reaction mixtures (100µl) contained probe DNA (~1 X 10^{4} dpm) in the buffer used for DNase I footprinting. Serial dilutions of RNAP were prepared in TMK buffer and 1µl aliquots of RNAP solutions added to each reaction mixture. After 10min incubation at 37°C, exoIII (usually 80 units) was added and incubation continued for 10min. Reactions were stopped and the products analyzed by autoradiography following electrophoresis as described above for DNase I footprinting.

RESULTS

Purification of Methanococcus vannielii DNA-dependent RNA polymerase.

M. vannielii RNAP was enriched anaerobically by standard column chromatographic procedures and finally purified by FPLC. Eight

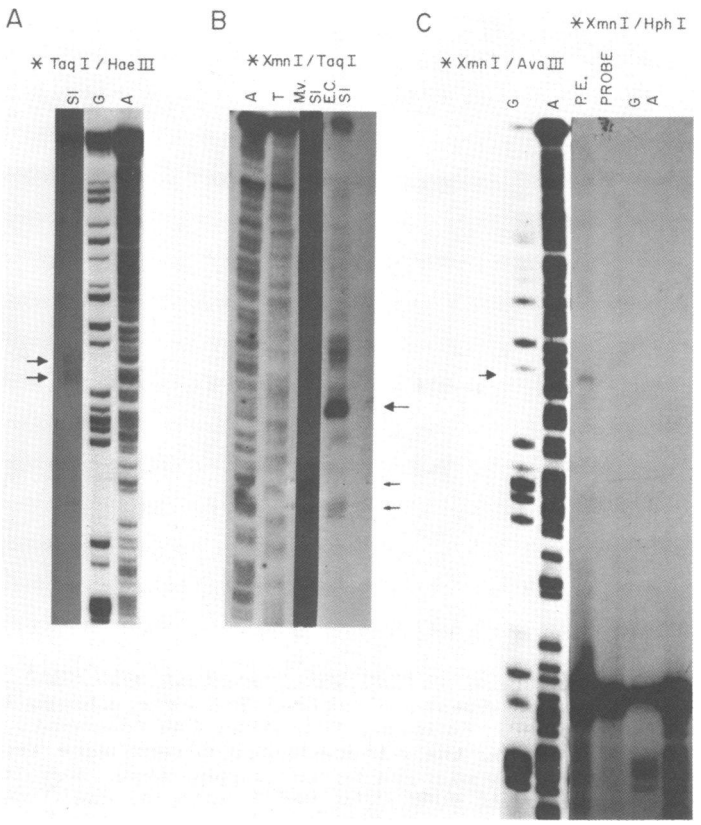

Figure 3. S_1 and primer extension analyses. A. An autoradiogram of the S_1 analysis using the *TaqI/HaeIII probe and M. vannielii RNA. S_1 resistant material is shown in lane 'S1'. G and A specific DNA sequencing reactions of the same probe are shown in lanes 'G' and 'A', respectively. Arrows indicate the positions of S_1 resistant material. B. S_1 analysis using the *XmnI/TaqI probe with either M. vannielii RNA ('Mv S1') or E. coli RNA ('Ec S1'). Arrows indicate the positions of S_1 resistant material. The upper arrow indicates the predominant band resulting from protection of the probe by RNA from E. coli containing plasmid pET805. The lower two arrows indicate the bands resulting from protection of the probe by M. vannielii RNA. A and T specific DNA sequencing reactions of the same probe are shown in lanes 'A' and 'T', respectively. C. Primer extension analysis using the *XmnI/HphI probe and M. vannielii RNA. Lanes labeled *XmnI/HphI 'G' and 'A' are G and A specific sequencing reactions of the probe DNA. Untreated probe DNA is shown under 'PROBE', and the primer extension reaction is shown under 'PE'. The arrow indicates the primer extension product in lane 'PE'. The lanes marked *XmnI/AvaIII 'G' and 'A' are G and A specific sequencing reactions of a longer *XmnI/AvaIII DNA fragment labeled at the same site as used in the *XmnI/HphI probe and serve as position standards for the primer extension product.

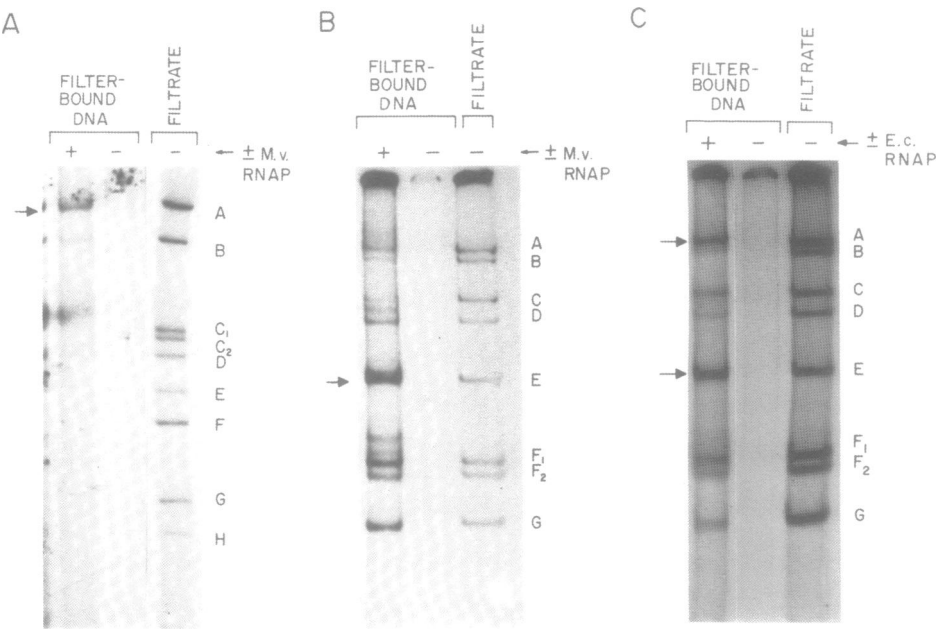

Figure 4. Filter-binding of pET805 DNA fragments with RNAP. End-labeled restriction digests of pET805 DNA were allowed to bind M. vannielii or E. coli RNAP. Following filtration, DNA fragments in the filtrates and retained by the nitrocellulose filters were separated by electrophoresis and visualized by autoradiography. Results obtained using M. vannielii RNAP ('M.v. RNAP') with (A) HaeIII digests and (B) TaqI/PstI digests of pET805 are shown. (C) Results obtained using E. coli RNAP ('E.c. RNAP') with a TaqI/PstI digest of pET805. The DNAs retained by the filters with RNAP and without RNAP added are indicated by '+' and '−', respectively, under the heading 'FILTER-BOUND DNA'. 'FILTRATE' is the DNA which passed through the filter in the absence of RNAP. Arrows to the left of the lanes indicate DNA fragments which are preferentially retained by filters in the presence of RNAP. DNA fragment designations are given to the right of the 'FILTRATE' lanes (see Figure 5 for restriction maps).

polypeptides co-purified with the enzymatic activity through all the chromatographic steps (Figure 2). These polypeptides are therefore assumed to be components of the M. vannielii RNAP.

Localization of the in vivo site of transcription initiation.

The sites of transcription initiation of the hisA gene in vivo were determined using S_1 and primer extension analyses with RNA extracted from M. vannielii and from E. coli x760 containing plasmid pET805 (Figure 3). The S_1 analyses suggested that there were two transcription start sites for the hisA gene in M. vannielii (Figure 3A),

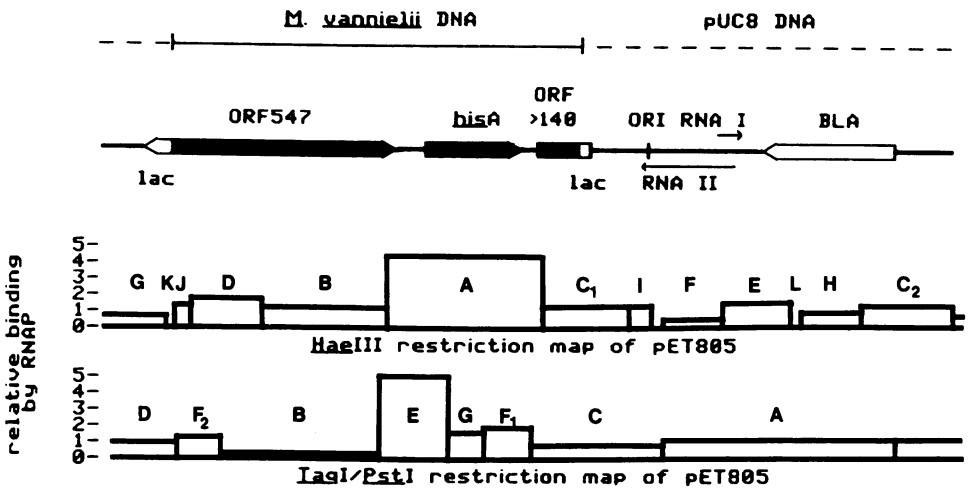

Figure 5. Quantitation of filter-binding results with M. vannielii RNAP. The amount of each DNA fragment bound figure 4 was quantitated by densitometry and is plotted as a histiogram. Relative amounts of binding are shown in arbitrary units. The organization of genes in pET805 (11,14,15) is presented above the HaeIII and IaqI/PstI restriction maps of pET805. DNA fragments are labeled as in Figure 4.

located 64 and 73 bp upstream of the ATG translation initiation codon. The relative strength of the two signals, however, varied somewhat with the RNA preparation and only the start site 73 bp upstream of the hisA ATG codon was detected in primer extension experiments (Figure 3C). Analysis of the DNA sequence provides an explanation for the detection of an apparent additional start site, using the S_1 protection procedure, at the 64 bp position. The sequence in this region is 5'GTTTTAAATGTTTTAAAT3' in which the most 5' G is the transcription initiation site identified at 73 bp relative to the ATG translation initiation codon. The second G in the sequence shown is at the 64 bp position. As the sequence is a tandem repeat, a transcript initiated at the 73 bp position could either hybridize throughout the length of this region or could form a loop and hybridize with its 5' end paired with the G at position 64. Formation of these alternative hybridization products would indicate, as seen in Figure 3A, that there are two sites for transcription initiation when investigated by S_1 protection procedures.

S_1 nuclease protection studies using RNA from E. coli cells containing pET805 indicated one predominant site of transcription initiation 94 bp

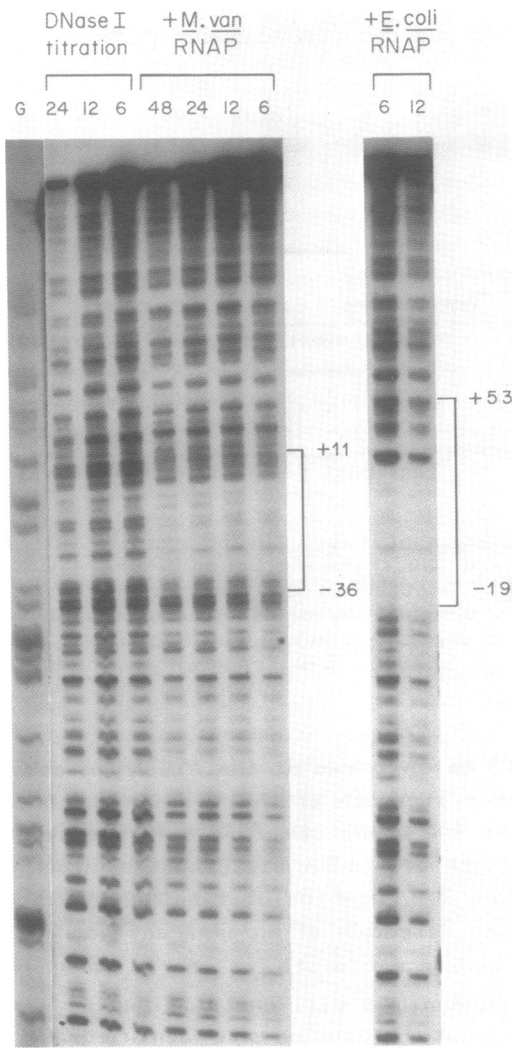

Figure 6. DNase I footprinting. RNAP:DNA complexes were prepared with the *TaqI/XmnI probe, followed by partial DNase I digestion, electrophoresis of the DNA through 6% polyacrylamide sequencing gels and autoradiography. Lane 'G' is a G specific DNA sequencing reaction of the probe DNA. Lanes under the 'DNase I titration' heading are partial DNase I digests of probe DNA in the absence of RNAP, '+M.van RNAP' lanes are reactions containing probe DNA complexed with M. vannielii RNAP and '+E. coli RNAP' lanes are reactions containing probe DNA complexed with E. coli RNAP. The numbers above each lane refer to the concentration of the stock solution of DNase I used in each reaction (μg DNase I/ml). Footprints are bracketed and the edges of the footprints are labeled to indicate their postions relative to the sites of transcription initiation.

upstream of the ATG codon of hisA and several less frequently utilized sites for transcription initiation (Figure 3B).

Identification of RNAP binding sites.

Filter-binding assays. The approximate locations of RNAP binding sites in pET805 were determined by binding DNA:RNAP complexes to nitrocellulose filters. In both HaeIII and TaqI/PstI digests of pET805, the presence of M. vannielii RNAP caused preferential retention of DNA fragments which contain the ORF547-hisA intergenic region (Figures 4 and 5).

Binding of E. coli RNAP resulted in the retention of not only the DNA fragment containing the ORF547-hisA intergenic region, but also the fragments which contain the E. coli promoters of the cloning vector pUC8 DNA (Figure 4).

DNase I footprinting experiments. RNAP binding sites in the ORF547-hisA intergenic region were determined by DNase I protection assays ('footprinting'). M. vannielii RNAP protected 44bp of DNA, 60 to 104bp upstream of the translation initiation ATG codon, from digestion by DNase I (Figure 6). This footprint spans the DNA from -36 to +11 relative to the site of in vivo transcription initiation in M. vannielii.

E. coli RNAP protected a similarly sized and overlapping region of DNA, from 48 to 112bp upstream of the translation initiation codon (Figure 6). The E. coli DNase I footprint also contains the predominant site of transcription initiation of the hisA gene in E. coliK760 containing plasmid pET805.

ExoIII footprinting experiments. The in vitro RNAP binding sites identified by DNase I footprinting were confirmed by exoIII footprinting. DNA:RNAP complexes were digested to completion with exoIII, a double-strand specific, single-strand 3' to 5' exonuclease. Blocking of digestion by bound RNAP resulted in the appearance of partial digestion products defining the 3' boundaries of the RNAP binding sites (Figure 7). At high RNAP:DNA ratios, digestion of the probe DNAs was not observed. The ends of the probe DNAs apparently were made inaccessable to exoIII by non-specific end-binding of excess M. vannielii and E. coli RNAP. Binding of M. vannielii RNAP at lower RNAP:DNA ratios resulted in partial exoIII digestion products corresponding to an upstream boundary of -35 and a downstream boundary of +8, relative to the in vivo site of transcription initiation. Binding of E. coli RNAP resulted in partial digestion products corresponding to an upstream boundary of -15, and one major and two minor downstream boundaries at +37, +38 and +47, respectively, relative to the in vivo site of transcription initiation in E. coli containing pET805. These exoIII determined

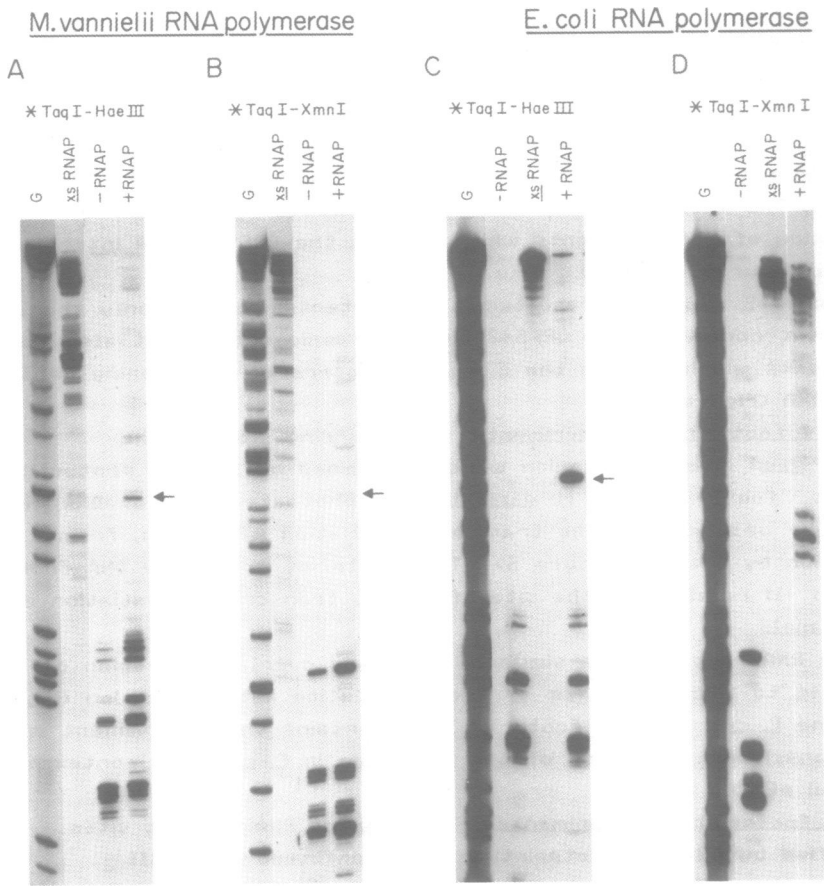

Figure 7. Exo III footprinting. RNAP:DNA complexes prepared using the *TaqI/HaeIII probe (A and C) or the *TaqI/XmnI probe (B and D) were digested with exoIII and analyzed by autoradiography following electrophoresis through 6% polyacrylamide sequencing gels. In (A) and (B), complexes were formed with M. vannielii RNAP. In (C) and (D), complexes were formed with E. coli RNAP. Lanes labeled 'G' are G specific DNA sequencing reactions of the probe. Lanes labeled 'xs RNAP' are from reactions in which a 25:1 molar excess of RNAP was added. Lanes labeled '+RNAP' are from reactions in which an approximately equimolar ratio of RNAP:DNA was used. Lanes labeled '-RNAP' are from reactions without RNAP. Arrows indicate bands in '+RNAP' lanes resulting from blockage of exoIII digestion by bound RNAP.

boundaries for the binding sites of M. vannielii and E. coli RNAP to the M. vannielii DNA between ORF547 and hisA coincide almost exactly to the limits of the DNase I footprints obtained in this region after binding of the RNAPs.

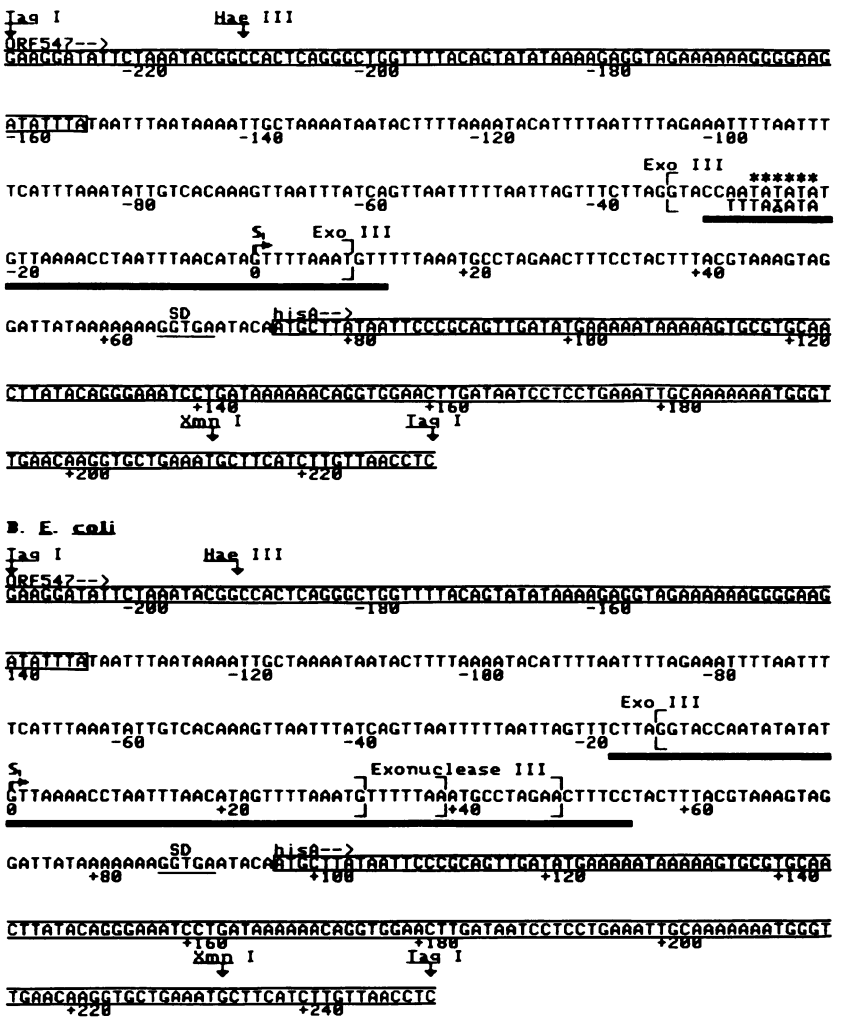

Figure 8. Summary of S_1 protection and footprinting results. The results obtained (A) with M. vannielii RNAP and (B) with E. coli RNAP in S_1, DNase I and exoIII protection experiments are summarized. The sequence is that of the pET805 TaqI fragment spanning the ORF547-hisA intergenic region (Figure 1). The starred sequence shows the homology with the octanucleotide 5'TTTAAATA (11-13). Transcription initiation sites are marked by arrows under the designation 'S_1', DNase I footprints by heavy bars and exoIII footprint boundaries by brackets across the sequence. The ORF547 and hisA polypeptide-encoding sequences are boxed. The sequence proposed as a ribosome binding site (SD) for the hisA gene is underlined (15). The sequences are numbered relative to the sites of transcription initiation, which are designated as '0'.

DISCUSSION

The results described here define the sites of transcription initiation and the sites of RNAP binding upstream of the polypeptide-encoding hisA gene of the methanogen M. vannielii. The location of this binding site and the DNA sequence protected by M. vannielii RNAP are in excellent agreement with the RNAP binding sites, determined by exoIII footprinting procedures, for the promoters of two stable RNA-encoding genes in M. vannielii (11). RNAP purifications (9,10) have indicated that archaebacterial cells, like eubacterial cells, contain only one major form of RNAP. These results are now supported by the observation that RNAP from M. vannielii binds at a similar location relative to the sites of transcription initiation of both the hisA and stable RNA genes and that, in both cases, the binding site contains an A+T rich TATA sequence. For promoters of stable RNA genes in archaebacteria, this sequence has been shown to have the consensus of an octanucleotide, namely 5'TTTATATA (11-13). A survey of intergenic sequences upstream of published and unpublished polypeptide-encoding genes from M. vannielii (G. S. Beckler, Ph.D thesis, The Ohio State University, 1987) has shown that in every case there is a sequence which matches this consensus octanucleotide in at least 6 positions. Such a sequence has, in fact, also been suggested as likely to be the promoter for transcription initiation of three polypeptide-encoding genes in the virus-like particle SSV1 of Sulfolobus B12 (17). The most highly conserved feature upstream of polypeptide-encoding genes in M. vannielii is the alternating palindromic TATATA sequence. The consistent occurrence of this TATA sequence and its demonstration here as a part of the RNAP binding site for the hisA gene strengthen the conclusion that this is, in fact, a general component of M. vannielii promoters. If additional sequence elements exist which regulate expression of specific genes or gene-types by controlling transcription initiation, they have yet to be identified.

As sequence specific, DNA-binding activity was obtained with purified archaebacterial RNAP, it appears that trans-acting transcription factors are not required in vitro for recognition and binding to specific DNA sequences by this enzyme. This is usual for eubacterial RNAPs, but not for eukaryotic RNAPs which require auxiliary DNA-binding transcription factors to facilitate their recognition and binding to promoter sequences.

E. coli RNAP was found to bind to almost the same region of DNA upstream of the M. vannielii hisA gene as bound by the M. vannielii RNAP (Figure 8). This was a surprise and it seems likely that the overlapping of RNAP binding sites is a coincidence. M. vannielii RNAP

does not recognize the classical rrnB P1 eubacterial promoter (11) and E. coli RNAP has been shown previously not to bind to the promoters of the genes encoding stable RNAs in M. vannielii (G. Wich and M. T., unpublished results), nor to the intergenic DNA between the divergent eurE and ORFC genes of Methanobacterium thermoautotrophicum ΔH (J. W. B. and J. N. R., unpublished results). Transcription of the hisA gene in E. coli is, in fact, initiated at several sites both upstream and downstream of the site at which the E. coli RNAP binds to this DNA in vitro (Figure 3). Transcription initiation upstream of the methanogen hisA gene in E. coli was, however, expected as this gene was originally cloned by complementation of a hisA mutant of E. coli x760 and complementation was obtained with the methanogen DNA cloned in either orientation relative to the vector DNA (15).

ACKNOWLEDGEMENTS

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