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Non-repetitive AT-rich sequences are found in intergenic regions of *Methanococcus voltae* DNA

Claus Bollschweiler, Rainer Kühn[1] and Albrecht Klein

Molekulargenetik, Fachbereich Biologie, Philipps-Universität Marburg, PO Box 1929, D-3550 Marburg, and [1]Mikrobiologie, Universität Heidelberg, Im Neuenheimer Feld 230, D-6900 Heidelberg, FRG

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AT-rich DNA, which constitutes a distinct fraction of the cellular DNA of the archaebacterium *Methanococcus voltae*, was shown to consist of non-repetitive sequences dispersed on the chromosome and to lack continuous open reading frames in five out of six randomly analyzed cases. Upon subsequent analysis of intergenic regions, AT-rich sequences were again detected. Transcription start points were mapped in front of three open reading frames. The 5′ ends of the transcripts were found in variable positions relative to the AT-rich sequences in the different cases. Shine-Dalgarno type sequences complementary to the 3′ end of 16S rRNA were discovered at suitable distances from the 5′ ends of the genes.

Key words: archaebacteria/AT-rich sequences/intergenic regions/ribosome binding sites/transcription analysis

Introduction

Archaebacteria are a group of procaryotic organisms distinct from eubacteria (Woese *et al.*, 1978). In many respects their cellular components show unique features whereas in others they resemble either eubacteria or eucaryotic cells (Woese, 1982). The archaebacteria comprise several subgroups, which are characterized by their physiology as well as their cell architecture, namely the halophilic bacteria, the sulphur-dependent archaebacteria including extreme thermoacidophilic species, the genus *Thermoplasma* and the methanogenic bacteria. The latter group is strictly anaerobic and has a unique energy metabolism, which exploits the reduction to one-carbon units to methane for the energy production (Balch *et al.*, 1979).

We have been studying the functional organization of the genome of methanogenic bacteria. As with other archaebacteria (Moore and McCarthy, 1969b; Searcy and Doyle, 1975) the genome complexities of methanogenic bacteria resemble those of other procaryotes (Mitchell *et al.*, 1979; Klein and Schnorr, 1984). In mesophilic methanogens distinct DNA fractions have been discovered which have a low GC content compared with the average of the total cellular DNA (Klein and Schnorr, 1984). An apparently comparable situation has been described for the genome of halobacteria (Moore and McCarthy, 1969a). In these bacteria, DNA with lower than average GC content is frequently found as a large heterogeneous fraction of extrachromosomal circular DNA (Pfeifer *et al.*, 1982). However, plasmids appear to be rather rare in methanogenic bacteria. Only three cases have been reported (Thomm *et al.*, 1983; Meile *et al.*, 1983; Konisky, personal communication). The DNA of halobacteria contains many families of repetitive sequences leading to rapid rearrangements of the genomes (Sapienza and Doolittle, 1982; Sapienza *et al.*, 1982; Pfeifer *et al.*, 1982). A comparable genome instability has not been observed in methanogens.

Little is known about gene expression in archaebacteria. The best studied example is the bacterio-opsin gene of *Halobacterium halobium* (Dunn *et al.*, 1981; Betlach *et al.*, 1983). In this case the mRNA has been characterized (Dassarma *et al.*, 1984). However, transcription start signals have not been identified although the influence of insertions of transposable elements into regions upstream of the mRNA start on the gene expression has been observed (Dassarma *et al.*, 1984; Pfeifer *et al.*, 1984). In the case of a functionally related group of genes which encode the subunits of the enzyme methyl CoM reductase in the methanogenic bacterium *Methanococcus voltae*, a common transcript has been shown for at least two of the three adjacent genes but the transcriptional start point has not yet been located (Konheiser *et al.*, 1984). The RNA polymerases of archaebacteria have a strikingly different subunit composition compared with the eubacterial RNA polymerases (Zillig *et al.*, 1982; Schnabel *et al.*, 1983). Therefore, it cannot be expected that the signals on the DNA of archaebacteria regulating the transcriptional start are similar to eubacterial promoters. This is in contrast to the situation for translation start signals. The expression of genes transferred from methanogenic bacteria into eubacteria (Bollschweiler and Klein, 1982; Reeve *et al.*, 1982; Wood *et al.*, 1983; Hamilton and Reeve, 1984) implies efficient ribosome binding to the transcripts, regardless of which types of signals are used for transcription. Indeed, it has been pointed out that the 16S rRNA 3′ termini of archaebacteria and eubacteria bear strong homology (Steitz, 1978). These sequences are implicated

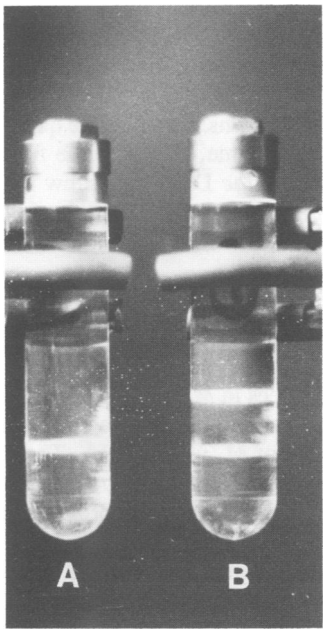

Fig. 1. Density gradient analysis of *M. voltae* DNA. **A**: 25 μg of *M. voltae* DNA were run under the conditions given in Materials and methods. **B**: 25 μg of bacteriophage λ DNA (50% GC) and 25 μg of *M. voltae* DNA (30% GC) were separated in a gradient with an initial density of 1.64 g/cm³ to show the resolution in the gradient.

Table I. Cloned AT-rich sequences from *M. voltae*

	Length (bp)	AT content[a] (%)	Continuous open reading frame
I.5	297	79.5	No
I.15	137	83.2	No
II.3	277	72.9	No
II.8	332	78.9	No
II.14	221	82.8	No
III.19	195	81.0	Yes
III.34	95	70.5	Yes
III.38	121	67.8	Yes

[a]Fractionation of AT-rich fragments from *M. voltae* DNA and cloning into pUC8 was performed as described in Materials and methods. The average AT content of *M. voltae* is 70 ± 1% (Balch *et al.*, 1979; Klein and Schnorr, 1984; Kühn, unpublished results). The sequences III.34 and III.38 were chosen as controls.

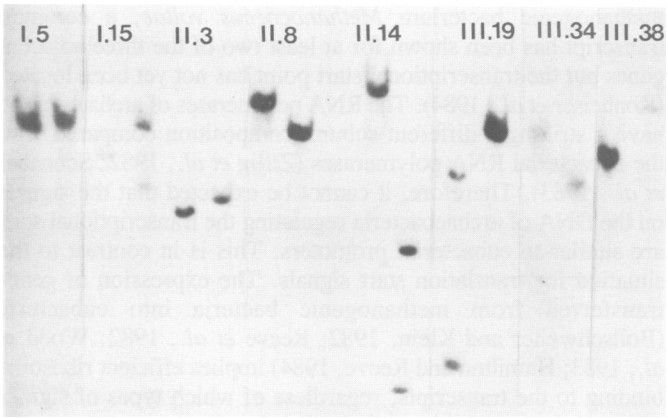

Fig. 2. Hybridization analysis of *M. voltae* AT-rich sequences against total restriction digests of *M. voltae* DNA. The numbers of the plasmids used as probes correspond to the ones given in Table I. *Eco*RI and *Hind*III digests are shown on the left or right lanes, respectively, in each case.

in the recognition of translation start signals on the mRNA molecules (Shine and Dalgarno, 1974).

Here we present data which bear on the questions of the nature, distribution on the genome, and possible functions of the AT-rich fraction of *M. voltae* DNA and show that sequences exist in front of structural genes which have the features of ribosome binding sites in eubacteria.

Results

AT-rich sequences have been found as a discrete DNA fraction both in methanogenic and halophilic archaebacteria. To find out whether such sequences in *M. voltae* are clustered in the genome as they are in the halophilic bacteria, we performed a density distribution analysis of *M. voltae* DNA. The DNA was sheared to 20 – 50 kb fragments and spun to equilibrium in a Hoechst 33258 dye CsCl gradient. This equilibrium centrifugation technique serves to separate AT- and GC-rich DNA (Redfern, 1981). Figure 1 shows a uniform distribution of the DNA with a band width comparable with that of bacteriophage λ DNA. Thus, the AT-rich DNA fraction in *M. voltae* is not contiguous, since that would have led to a satellite band in the gradient.

To find out whether the AT-rich sequences are repetitive. *M. voltae* DNA was sheared to fragments <500 bp. It was then fractionated on hydroxyapatite and the AT-rich fractions cloned in pUC8 after linker attachment. Fragments with average AT content served as controls. Table I gives a survey of length distribution and AT content of the cloned fragments used in the subsequent experiments. The plasmids were labelled with ^{32}P and used as probes for hybridization against nitrocellulose filter bound total digests of *M. voltae* total DNA (Figure 2). In most cases only one band of the digest is probed by an AT-rich sequence. In only one case (plasmid III.19, *Eco*RI digest) two fragments of the digest clearly bear homology to the cloned fragment. In the case of plasmid II.14 an internal *Hind*III restriction site was found upon sequence analysis of the cloned fragment, explaining the probing of two *Hind*III fragments of the genomic restriction digest (data not shown). Thus, the experiment yields no indication of highly repetitive AT-rich DNA in the *M. voltae* genome.

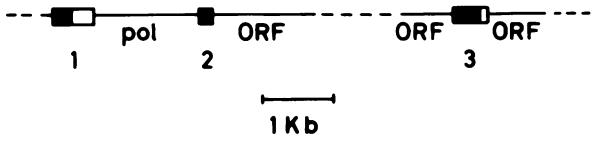

Fig. 3. Schematic representation of the relative positions of open reading frames (ORF) and AT-rich regions on a continuous fragment of *M. voltae* DNA. The boxes show the intergenic regions. The filled parts represent AT-rich sequences (compare Figure 4).

Upon sequence analysis, an interesting feature was discovered with one of the cloned DNA fragments (I.5, Table I). It contained the beginning of an apparent open reading frame starting with an ATG codon and preceded by a possible ribosome binding site (see below) and a highly AT-rich region of 120 nucleotides with 87.5% AT content. This made us wonder whether such an arrangement might have a more general significance. To test this notion we started from a known structural gene on the *M. voltae* DNA: this gene had been found to code for a subunit of the DNA-dependent RNA polymerase of ~50 kd, using for its identification an experession vector with *M. voltae* DNA inserted such that fusion polypeptides synthesized by the resulting clones could be screened with antiserum (see Konheiser *et al.*, 1984, for details of the method) against purified RNA polymerase of the closely related *Methanococcus thermolitotrophicus* (Thomm and Klein, unpublished results). With the plasmid from a positive clone as a probe, DNA sequences in two *M. voltae* genomic libraries constructed using replacement vectors (Loenen and Brammar, 1980; Frischauf *et al.*, 1983) could be found, which contained the gene on a DNA fragment of ~10 kb. The gene is flanked by AT-rich sequences which do not contain open reading frames. In addition, the next open reading frame follows immediately after the AT-rich region terminating the polymerase gene. Approximately 3.5 kb further downstream another AT-rich intergenic region is located. Figure 3 summarizes the results schematically. The sequences of the three AT-rich segments are given in Figure 4.

To look for a possible function of these regions besides the separation of structural genes, we attempted to localize transcription starts in front of the identified open reading frames. RNA was prepared from exponentially growing *M. voltae* cells and used in experiments mapping the 5′ ends of RNAs on the DNA next to the 5′ ends of the open reading frames using the nuclease S1 mapping technique. A typical result is shown in Figure 5. Similar results were obtained not only for the polymerase gene and the two open reading frames downstream but also for the insert DNA of plasmid I.5 (described above) which had been obtained in the course of random cloning of AT-rich sequences. The location of the 5′ ends relative to the AT-rich regions and

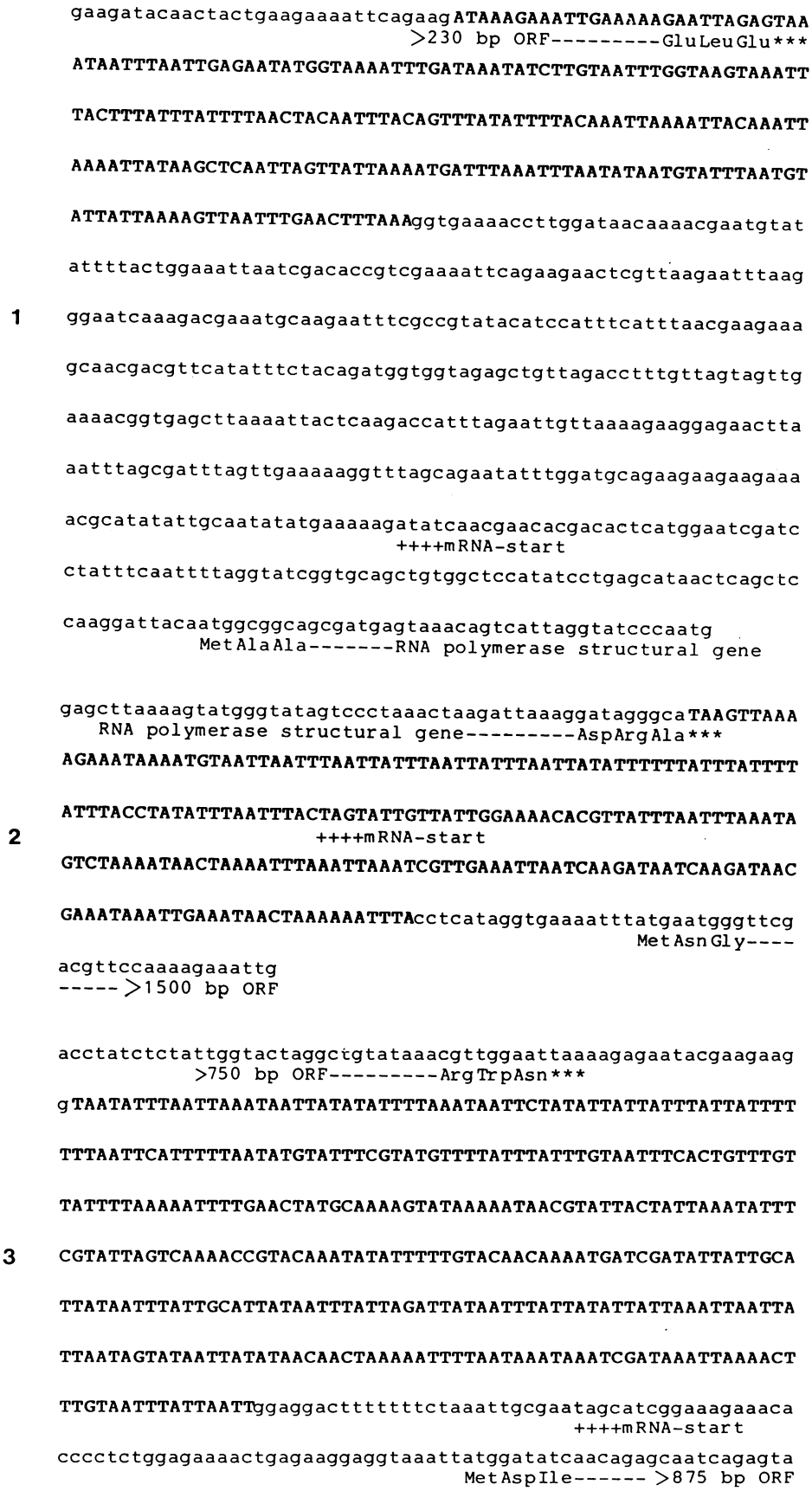

Fig. 4. Sequences of the intergenic regions represented in Figure 3. The C-terminal and N-terminal ends of the presumptive polypeptides encoded by the open reading frames next to the respective intergenic regions are indicated by three amino acids. The transcription starts are given with an estimated accuracy of ±2 nucleotides.

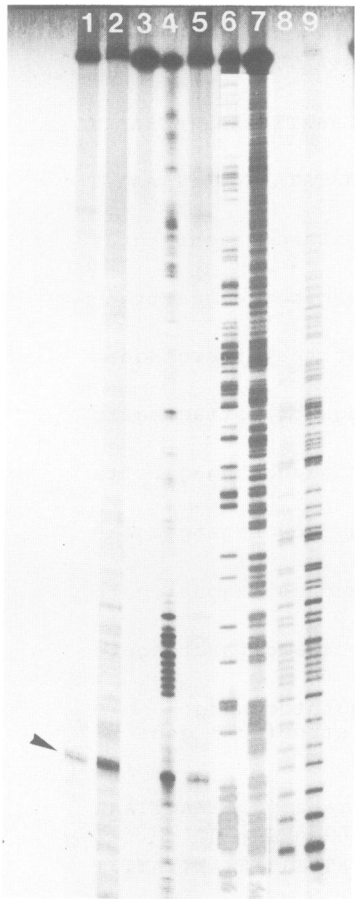

Fig. 5. S1 mapping of the 5′ end of the RNA in front of the RNA polymerase 50-kd subunits structural gene (compare Figures 3 and 4). Two independent RNA preparations were used. A 1.2-kb *XbaI*/*ClaI* restriction fragment terminally labelled with ^{32}P at the *ClaI* restriction site located within the polymerase gene, 234 bp downstream of its 5′ end was employed. The autoradiogram of a denaturing polyacrylamide gel is shown. **Lanes 1, 2**, DNA/RNA hybrids (RNA preparation I), digestion times 30 or 15 min, respectively; **3**, control without RNA, digestion time 30 min; **Lanes 4, 5**, DNA/RNA hybrid (RNA preparation II), digestion times 2 or 30 min, respectively; all digestions were done at 37°C; **6–9** sequence of a known DNA fragment used as a size marker, G, A+G, T+C and C tracks, respectively.

the presumptive starts of the genes is indicated in the sequences shown in Figure 4. In all cases, the identified transcripts hybridized to only one strand, which had been identified to be the one whose transcription would lead to a translatable mRNA. Thus all four open reading frames can be taken as structural genes. One additional finding strengthens the view. The region immediately upstream of the genes shows strong homology to the 3′ end of 16S rRNA, whose sequence is known to be identical in all methanogenic bacteria (Fox *et al.*, 1977), with the last nucleotide missing in *Methanococcus vannielii* (Jarsch and Böck, 1983). This situation is shown in Figure 6. We consider it very likely that the detected sequence homology indicates Shine-Dalgarno type ribosome binding sites on the mRNAs.

Discussion

Our finding that mesophilic methanogenic bacteria contain DNA fractions with unusually high AT contents (Klein and Schnorr, 1984) was the starting point for the investigation presented here. In another archaebacterial group, the halophilic bacteria, studies

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3'c c u c c a c u a g g . . . . . .    16s rRNA
. . 5' A G G A T T A C A A T G          1a
5' G G C G G C A G C G A T G . . . . .  1b
. . 5' A G G T A T C C C A A T G . . . .  1c
. . 5' A G G T G A A A A T T T A T G . .  2
5' G G A G G T A A A T T A T G . . . . .  3
. . 5' A G G T C A T G A T A T T A T G .  I.5
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Fig. 6. Homology of sequences in front of open reading frames with the 3′ end of 16S rRNA from *M. voltae* (Jarsch and Böck, 1983). The 16S rRNA sequence is shown on top. The bottom line represents the beginning of the open reading frame found on the *M. voltae* DNA insert of plasmid I.5 (compare Table I). Sequences 1, 2 and 3 are taken from Figure 4. In the case of sequence 1 alternative possibilities for the start of the RNA polymerase subunit structural gene are indicated (1a, b, c). Bases complementary to the rRNA are indicated in bold type.

on the physical organization of the genome had led to the discovery of families of repetitive DNA (Sapienza and Doolittle, 1982). Sequences cloned from the AT-rich DNA fraction of halobacteria appeared to belong to such families (Pfeifer *et al.*, 1982). The data presented here rule out that the AT-rich DNA fraction of *M. voltae* represents repetitive DNA. This view is supported by the fact that intergenic AT-rich sequences were found on unique restriction fragments of the cellular DNA of *M. voltae* which are not clustered.

What is the function of this DNA? The simplest assumption is that the AT-rich sequences serve as spacers between genes. This is borne out by our results since we have found that five out of six randomly cloned AT-rich sequences do not have any continuous open reading frame (Table I) and that, indeed, AT-rich sequences are found in between genes (compare Figure 4 and Figure 5). Similar observations have been made in other laboratories, where it was found that AT-rich DNA sequences occur close to structural genes in *M. voltae* and *M. vannielii* (Wich *et al.*, 1984; Konisky and Reeve, personal communication).

Our findings that transcription starts occur in or close to the AT-rich regions make us wonder whether they might play a role in transcription initiation. Comparing these sequences we have not been able to detect distinct signals reminiscent of promoter elements of either the procaryotic or the eucaryotic type in the area upstream of the apparent transcription starts. More independent sequence information is needed to identify such elements. Nevertheless it is worth noting that AT-rich sequences have been found to be characteristic constituents of eubacterial promoter regions (Siebenlist, 1979).

The RNA transcripts of the genes detected in our studies start well in front of respective open reading frames (compare Figure 4). This is an apparent contrast to the situation found in the only other case studied in archaebacteria, the bacterio-opsin gene. In this case the mRNA starts only 2 bp upstream of the 5′ end of the gene. However, it is known from the eubacterium *Escherichia coli* that even within one organism both situations can occur (Ptashne *et al.*, 1976; Kozak, 1983). Therefore we do not consider the two archaebacterial cases to be principally different.

As mentioned above, DNA from methanogens can be transcribed and translated in eubacterial cells. We have identified the sequences on methanobacterial DNA which are involved in the transcription promotion in *E. coli*. They are apparently different from the start signals used in the methanobacteria themselves

(Bollschweiler and Klein, in preparation). The translation signals, however, appear to be simliar. This is concluded from the data in Figure 6 and similar findings by J.Konisky and J.Reeve (Personal communication) in *M. voltae* and *M. vannielii*, and explains the efficient heterologous expression of the archaebacterial genes in eubacteria in the cases in which they are transcribed.

Materials and methods

DNA preparation

DNA from *M. voltae* (DSM 744, German Collection of Microorganisms, Göttingen) was extracted after lysis of the cells by the addition of 0.1% SDS and phenol extraction as previously described (Klein and Schnorr, 1984). The DNA was sheared if necessary by sonication and sized by agarose gel electrophoresis using size makers. λcI857S7 DNA was obtained from W.Reiser (Heidelberg).

Gradient centrifugation (Redfern, 1981)

For the separation according to different GC contents the DNA was dissolved in 10 mM Tris pH 8 at 10−100 μg/ml and mixed at a 1:1 mass ratio with Hoechst 33258 dye (Calbiochem) from an aqueous stock solution of 50 μg/ml, brought to a final concentration of 20−50 μg of DNA, 5 ml of 0.16 M NaCl, 0.01 M sodium phosphate pH 7.4, and CsCl was added to give an initial density of 1.60 g/cm^3 unless stated otherwise. The solution was centrifuged to equilibrium at 40 000 r.p.m. for 20 h in a 75 Ti Beckman rotor at 20°C. The DNA was visible as fluorescent bands in 366 nm incident light.

Hydroxyapatite chromatography

The method of Bernardi (1971) was used employing thermochromatography (Miyazawa and Thomas, 1965). The DNA was applied to a hydroxyapatite (Bio-Gel HTP, Bio-Rad, Munich) column in 30 mM sodium phosphate pH 6.8 and eluted at 120 mM of the same buffer raising the temperature stepwise from 65°C to 95°C. The AT contents of the fractions were determined after renaturation by monitoring the temperature-dependent hyperchromicity as previously described (Klein and Schnorr, 1984).

Cloning of M. voltae DNA fragments

DNA fragments were inserted into the *Eco*RI restriction site of plasmid pUC8 (Vieira and Messing, 1982) obtained from E.Beck (Heidelberg). For that purpose blunt ends were generated in a gap filling reaction using the *E. coli* DNA polymerase I Klenow fragment (Boehringer, Mannheim) and *Eco*RI dodecamer linkers (Collaborative Research Inc.) were attached as described (Maniatis *et al.*, 1982).

DNA/DNA hybridization

Hybridization of DNA probes labelled with ^{32}P by nick translation (Rigby *et al.*, 1977) using [^{32}P]dATP (Amersham Buchler) to restriction fragments generated by *Hind*III or *Eco*RI endonuclease (Boehringer, Mannheim) digestion and bound to BA 85 nitrocellulose filters (Schleicher and Schuell) was performed according to Southern (1975).

DNA sequencing

Sequence analysis of DNA followed the procedure devised by Maxam and Gilbert (1980).

RNA extraction from M. voltae cells

RNA was prepared from exponentially growing cells using precautions to avoid RNase action as described earlier (Konheiser *et al.*, 1984).

S1 mapping

The 5′ ends of the transcripts were mapped using nuclease S1 (type III, Sigma) according to the described methods (Berk and Sharp, 1977; Maniatis *et al.*, 1982), using salt concentrations minimizing the difference in melting behavior of AT- and GC-rich sequences (Shenk *et al.*, 1975).

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