

## Regions of *Mycoplasma pneumoniae* Cytadhesin P1 Structural Gene Exist As Multiple Copies

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The *Mycoplasma pneumoniae* cytidhesin P1 structural gene with flanking regions was labeled by nick translation and used as a probe to analyze gene copy number in *M. pneumoniae*. Multiple bands of genomic DNA were hybridized by the probe. To establish what part of the P1 gene existed as multiple copies, the P1 gene and regions adjacent to the 3' and 5' ends were divided with restriction enzymes into 14 segments ranging in size from 174 to 651 base pairs. These pieces were purified on agarose gels, subcloned into pUC19, purified, labeled by nick translation, and used to probe the entire *M. pneumoniae* genome. Several regions near the middle and carboxy end of the P1 structural gene hybridized to single copies. The remaining P1 subclones hybridized to multiple bands under stringent hybridization conditions, indicating extensive homology with other parts of the *M. pneumoniae* genome. The single- versus multiple-copy nature of P1 structural gene domains is discussed.

*Mycoplasma pneumoniae*, the etiologic agent of primary atypical pneumonia in humans, possesses a tiplike structure which mediates adherence to host cells (5, 10, 18). A 170-kilodalton protein designated P1 clusters at the tip organelle of virulent mycoplasmas and functions as the major adhesin (1, 2, 8, 9). Besides playing an essential role in the cytidherence process, protein P1 elicits a strong immune response in humans and experimental animals infected with *M. pneumoniae* (14). In an effort to understand the structural-functional properties of this important virulence determinant, we cloned the P1 gene and deduced the primary amino acid sequence (23). We identified a 13-amino-acid stretch of protein P1 that reacts with P1 cytidherence-blocking monoclonal antibodies and with acute- and convalescent-phase sera from *M. pneumoniae*-infected individuals (6). Because the spontaneous appearance of nonadherent mutants of *M. pneumoniae* occurs at a high frequency ( $7 \times 10^{-3}$  [13]) and because cells with a nonadherence phenotype typically comprise more than 50% of the population of virulent *M. pneumoniae* cultures (12), we examined the prevalence of P1 gene sequences in the *M. pneumoniae* genome. Numerous examples exist of high rates of mutation that are associated with multiple copies of partial or incomplete gene sequences of important surface proteins in procaryotic and eucaryotic parasites (15, 17, 20, 24).

To further study the P1 gene structure, the entire P1 gene was used to probe the *M. pneumoniae* genome in Southern blot analysis under stringent conditions. Because multiple bands were observed, we further subcloned the P1 gene into 14 DNA fragments which span the entire P1 sequence. When these subclones were used as probes, approximately one-third of the P1 structural gene was present in a single copy, while approximately two-thirds of the remaining sequence was present in multiple copies ranging from two to six bands. These data indicate that extensive homologies are present among specific sequences of the P1 gene and other segments of the *M. pneumoniae* genome. The implications of these observations are discussed.

### MATERIALS AND METHODS

**DNA extraction from *M. pneumoniae*.** *M. pneumoniae* M129-B16 was grown aerobically in 32-oz (907.2-g) glass prescription bottles containing 70 ml of modified Edward medium for 3 days at 37°C (14). The medium was removed, and the glass-adherent mycoplasmas were rinsed twice with PBS buffer (10 mM sodium phosphate [pH 7.2], 0.1 M NaCl) and scraped into 3 ml of PBS buffer. *M. pneumoniae* cells from 10 bottles were collected by centrifugation and lysed by suspending the pellet in 3 ml of PBS buffer containing 0.3 ml of 10% sodium dodecyl sulfate (BDH, Poole, England). The cell lysate was treated with 50 µg of RNase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml at 37°C for 30 min, and 0.3 ml of 0.5 M EDTA (pH 8.0) was added, followed by 50 µg of proteinase K (Boehringer Mannheim) per ml at 37°C for 1 h. The digested cell lysate was extracted twice with phenol, twice with phenol-chloroform (1:1), and once with chloroform-isoamyl alcohol (24:1). Sodium acetate (3 N; 0.1 volume) was added to the DNA solution, and the DNA was precipitated with ethanol.

**Subcloning of the P1 gene.** *Escherichia coli* strains harboring plasmids containing either a 4.3-kilobase (kb) *Hind*III or 6-kb *Eco*RI fragment of the P1 gene (See Fig. 1) were grown overnight in LB broth containing 50 µg of ampicillin per ml. Cells were harvested, and plasmids were purified by cesium chloride (CsCl) gradient centrifugation (16). Inserts were released from the plasmids by digestion with appropriate restriction enzymes, followed by purification on agarose gels. These inserts were further digested with other restriction enzymes, and each DNA fragment was separated on agarose gels and purified before ligation into pUC19 as the cloning vector. When the target DNA was generated by two restriction enzyme cuts, pUC19 was digested with restriction enzymes that produced two compatible ends, and the small DNA fragment was removed by two cycles of isopropanol precipitation. When only one restriction enzyme was used, the 5' end phosphate of pUC19 was removed by treating the digested plasmid with calf intestinal alkaline phosphatase (16). The subclones of P1 DNA and the vector were mixed at a 1:1 ratio and ligated at room temperature

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TABLE 1. Restriction enzyme strategy for subcloning the P1 gene

Subclone name	Restriction site location (site no.)	Size (base pairs)	Nucleotides <sup>a</sup>
A	<i>Hind</i> III (1)→ <i>Eco</i> RI (1)	372	-528→-157
B	<i>Eco</i> RI (1)→ <i>Sma</i> I	414	-156→258
C	<i>Sma</i> I (1)→ <i>Pst</i> I	651	259→909
D	<i>Pst</i> I→ <i>Bam</i> HI	275	910→1184
E	<i>Bam</i> HI→ <i>Kpn</i> I (1)	412	1185→1596
F	<i>Kpn</i> I (1)→ <i>Kpn</i> I (2)	174	1597→1770
G	<i>Kpn</i> I (2)→ <i>Sau</i> 3A (internal)	570	1771→2340
H	<i>Sau</i> 3A (internal)→ <i>Kpn</i> I (3)	444	2341→2784
I	<i>Kpn</i> I (3)→ <i>Sma</i> I (2)	498	2785→3282
J	<i>Sma</i> I (2)→ <i>Hind</i> III (2)	539	3283→3821
K	<i>Hind</i> III (2)→ <i>Sal</i> I	281	3822→4102
L	<i>Sal</i> I→ <i>Eco</i> RV	236	4103→4338
M	<i>Eco</i> RV→ <i>Taq</i> I (1)	559	4339→4897
N	<i>Taq</i> I (1)→ <i>Taq</i> I (2)	~570	4898→?

<sup>a</sup> Numbers represent nucleotides either upstream (-) or downstream (no prefix) from the start codon (ATG), which is numbered as 1.

with T4 DNA ligase (16). The ligated DNA was used to transform competent *E. coli* DH5 $\alpha$  cells in accordance with the manufacturer's instructions (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Transformants were selected on LB agar plates containing 50  $\mu$ g of ampicillin per ml. Isopropyl- $\beta$ -D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside were added to assist in the selection of colonies containing a plasmid with an insert. Ten white colonies were identified from each subclone and grown overnight in LB broth containing 50  $\mu$ g of ampicillin per ml. Plasmid DNA was isolated from the overnight cultures (11) and analyzed on agarose gels. Colonies containing plasmids with appropriately sized inserts were further analyzed by restriction enzyme digestion and compared with the computer-generated restriction enzyme map of the P1 gene.

**Southern blot analysis of the *M. pneumoniae* genome.** To perform Southern transfers, a batch of *M. pneumoniae* DNA was digested overnight with excess amounts of appropriate restriction enzymes to ensure complete digestion. *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Sac*I, and *Sma*I were chosen because their restriction sites in the P1 gene are already established. Digested DNA (5  $\mu$ g) was loaded in each lane, and the differently sized DNA fragments were separated on a 0.7%

agarose gel. DNA was transferred to nitrocellulose filters with 10 $\times$  SSC (1 $\times$  SSC is 0.15 M sodium citrate plus 0.15 M sodium chloride [pH 7.0]) overnight as described by Southern (21). Filters were rinsed in 2 $\times$  SSC, baked at 80°C under vacuum for 2 h, and stored at room temperature until use.

Plasmids were purified from each subclone by CsCl gradient centrifugation (16). [ $\alpha$ -<sup>32</sup>P]dATP or [ $\alpha$ -<sup>32</sup>P]dCTP was used to label the DNA in the nick translation reaction. Nick translation labeling kits were purchased from Bethesda Research Laboratories, and reactions were carried out at 15°C for 60 min and stopped by the addition of EDTA. DNA was separated from unincorporated free nucleotides by Sephadex G-50 chromatography (Boehringer Mannheim) and denatured by boiling for 5 min before use. Filters were prehybridized at 68°C overnight in 20 ml of prehybridization solution containing 6 $\times$  SSC, 0.5% sodium dodecyl sulfate, 5 $\times$  Denhardt solution (0.1% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), and 0.1 mg of denatured herring sperm DNA per ml. Hybridizations were carried out at 65°C in 10 ml of prehybridization solution plus <sup>32</sup>P-labeled probes. After hybridization, filters were rinsed twice in 2 $\times$  SSC-0.5% sodium dodecyl sulfate at room temperature, washed at 68°C in 0.1 $\times$  SSC-0.5% sodium dodecyl sulfate for 2 h with several buffer changes during the wash, dried at room temperature, and exposed to X-ray film.

To ensure that the observed multiple-band hybridization pattern was not due to incomplete digestion of restriction enzymes, each batch of digested *M. pneumoniae* DNA was blotted once with a single-copy subclone as a control. Hybridization with each subclone was performed at least twice to ensure the reproducibility of the results.

## RESULTS

**Subcloning of the P1 gene.** The strategy used to subclone the P1 gene is shown in Table 1. When the convenient restriction sites are identified based upon digestion with the 6-base-pair cutters *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Pst*I, *Sal*I, and *Sma*I (Fig. 1), the P1 gene is divided into 12 fragments, among which 2 are larger than 1 kb. A single *Sau*3A site located near the middle of *Kpn*I (site 2)→*Kpn*I (site 3) and two *Taq*I sites located near the middle and end of *Eco*RV→*Eco*RI (site 2) were used to divide these two large fragments into smaller subclones (Fig. 1). Compatible sites in pUC19 permitted ligation of the subclones into the vector. These manipulations produced 14 subclones ranging from

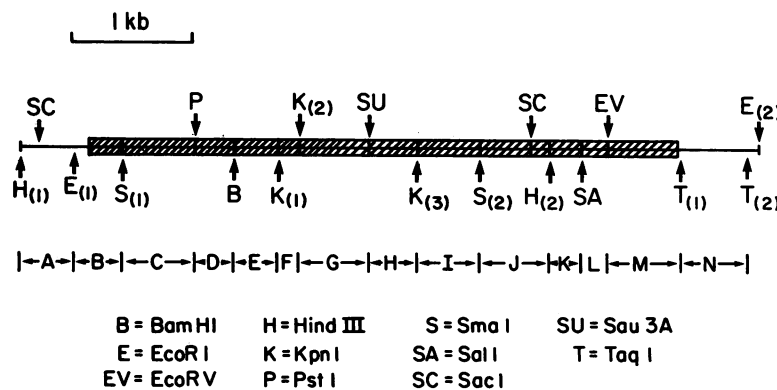


FIG. 1. Restriction enzyme map of the P1 structural gene and surrounding sequences. The boundary of each subclone from A to N is marked (see also Table 1). Restriction enzyme sites that cut more than once are numbered starting from the 5' end. *Sau*3A and *Taq*I cut many times in the P1 gene, but only the sites used for subcloning purposes are shown. Hatched bars indicate the P1 structural gene. Numbers in parentheses indicate site numbers.

651 to 174 base pairs (Table 1 and Fig. 1). Subclone A contains the sequence 5' upstream from the P1 gene. Subclone B includes the apparent P1 promoter sequence, the first 59 amino acids which comprise the putative signal peptide of P1, and the first 29 N-terminal amino acids of the mature P1 protein (23). Subclone M contains the carboxy region of the P1 protein and extends 16 nucleotides beyond the 3' end of the stop codon. The complete nucleotide sequence of subclone N has not been determined, but it contains about 570 base pairs located 3' downstream from the carboxy end of the P1 gene. Subclones C, D, E, F, G, H, I, J, K, and L represent nonoverlapping sequences of the P1 structural gene.

Each subclone of the P1 gene was purified by agarose gel electrophoresis to 80 to 90% purity before ligation and transformation. Minilysates were used to select subclones with appropriate insert sizes. Digestion of each plasmid with restriction enzymes as outlined in Table 1 released inserts of the predicted sizes. When digested with other restriction enzymes, each subclone exhibited its own unique restriction pattern (e.g., subclones A and J possess internal *SacI* sites [Fig. 1]).

**Southern blot analysis of the *M. pneumoniae* genome.** Genomic DNA isolated from *M. pneumoniae* was digested to completion with *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, and *Sma*I. Different sizes of DNA were separated by agarose gel electrophoresis and transferred to nitrocellulose filters. When the entire 6-kb *Eco*RI fragment of the P1 gene (23) was used to probe the *M. pneumoniae* genome, besides hybridizing to the predicted parental P1 sequence, the DNA fragment hybridized to many other sequences in the *M. pneumoniae* genome under stringent hybridization conditions (Fig. 2). To further characterize the multiple-copy nature of the P1 gene, we used the 14 subclones (Table 1) as probes to hybridize back to the entire *M. pneumoniae*

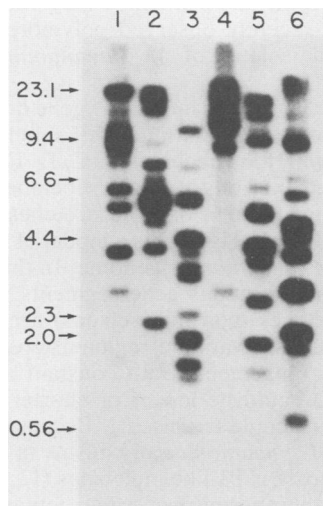


FIG. 2. Southern blot analysis of the *M. pneumoniae* genome with the entire P1 gene as a probe. *M. pneumoniae* DNA was digested with different restriction enzymes, separated by electrophoresis, and transferred to nitrocellulose filters as described in Materials and Methods. The 6-kb *Eco*RI fragment of the P1 gene was labeled by nick translation and used as a probe. Lanes: 1, *Bam*HI digest of *M. pneumoniae* DNA; 2, *Eco*RI; 3, *Hind*III; 4, *Pst*I; 5, *Sac*I; 6, *Sma*I. The numbers on the left are the positions of the *Hind*III-digested  $\lambda$  phage DNA used as molecular weight markers (in kilobases).

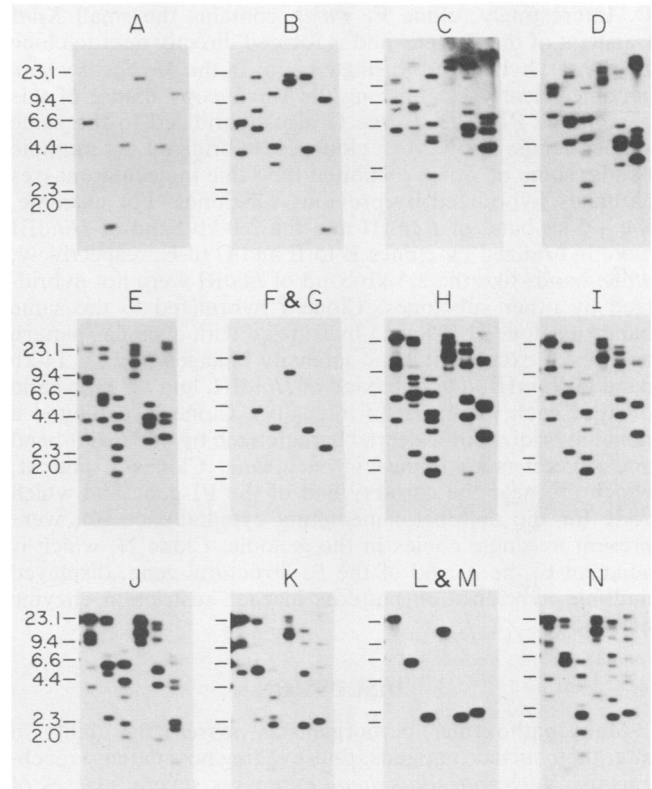


FIG. 3. Southern blot analysis of *M. pneumoniae* genomic DNA with different subclones as probes. Experimental conditions are identical to those in Fig. 2, except that different P1 subclones were substituted for the entire 6-kb P1 gene. Lanes in each panel from left to right are *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Sac*I, and *Sma*I digests of *M. pneumoniae* DNA. Letters A to N on top of each group of profiles coincide with the restriction map presented in Fig. 1, and horizontal bars on the left indicate DNA molecular weight standards (in kilobases) (note that the migration of standards varied slightly between experiments). Because subclones F and G and subclones L and M produced the same hybridization pattern, only one representative picture of each is shown.

genome. Figure 3 demonstrates the hybridization patterns of individual subclones. No internal restriction enzyme sites were used to cut genomic DNA in the subclones except for the use of an internal *Sac*I site in subclones A and J as previously described. Therefore, only single bands should hybridize to the probes if those domains of the P1 gene are present in single copies. Clone A, which is 5' to the P1 gene, hybridized to single bands, demonstrating the single-copy nature of this sequence (the *Sac*I cut revealed an extra-light band). Clone B, which contains the promoter sequence as well as the initial coding region of the P1 gene (23), hybridized to one or two additional DNA fragments from each digestion mixture in addition to the predicted pattern which was seen in the hybridization profile of clone A. Clone C displayed an even more complex pattern, with one or two additional bands in each profile beyond those identified by clone B. Clone D displayed the same pattern as clone C except that the intensities of specific bands increased and those of others decreased (e.g., hybridization to a 4.1-kb *Eco*RI-generated DNA fragment increased in intensity, while there was a decrease in hybridization to a 4-kb *Bam*HI-cut DNA fragment). Clone E exhibited a hybridization pattern that was similar but not identical to that of clone

D. Interestingly, clone F, which contains the small *KpnI* fragment of the P1 gene and is located directly next to clone E (Fig. 1), hybridized to single bands in the *M. pneumoniae* genome, clearly establishing the single-copy nature of this part of the P1 gene. Clone G also hybridized to the same single bands. However, clone H hybridized to multiple bands, some of which exhibited the same molecular masses as bands hybridized by previous subclones. For example, the 4.0-kb band of *BamHI* and the 2.0-kb band of *HindIII* were hybridized by clones B to E and C to E, respectively, while bands like the 2.5-kb band of *EcoRI* were not hybridized by other subclones. Clone I hybridized to the same bands as clone H. Clone J hybridized with a similar pattern as clone I except for band intensity changes (e.g., a 14-kb band of *BamHI*, a 6.1-kb band of *HindIII*, and a 5.8-kb band of *SacI* each increased in intensity). Clone K exhibited a novel hybridization pattern characterized by one major band and several minor bands in each lane. Clones L and M, which are near the carboxy end of the P1 gene and which code for the epitope(s) mediating cytoadherence (6), were present as single copies in the genome. Clone N, which is adjacent to the 3' end of the P1 structural gene, displayed multiple hybridization patterns in each restriction enzyme digestion.

## DISCUSSION

Many pathogenic microorganisms possess the ability to alter their surface antigens, thus evading host defense mechanisms. *Neisseria gonorrhoeae* expresses several classes of major outer membrane proteins, such as the pilus and opacity proteins (7, 22). Genes coding for these two types of proteins exist as multiple gene families, and their expression regulates bacterial adhesion, colonization, and survival (3, 4, 19, 22). Gene conversion among different members of these gene families alters structural and functional properties of the proteins (20, 22). Similar mechanisms operate in *Borrelia hermsii*, an arthropod-borne pathogen that causes relapsing fever in humans and similar diseases in other mammals (17). It is believed that genes coding for the serotype antigens exist as multiple copies and that antigenic variation is associated with DNA rearrangements and differential expression of these surface proteins. In trypanosomes, the vector-borne eucaryotic parasites which cause sleeping sickness, repertoires of more than 100 different surface antigens have been found to be sequentially expressed starting from a single clone as a result of gene conversion among these gene families (15).

Protein P1 of *M. pneumoniae* is a strongly immunogenic surface protein and plays an essential role in cytoadherence and virulence (1, 2, 8, 9). Our results indicate that the P1 structural gene can be divided into several domains, some of which exist as single genomic copies (subclones F, G, L, and M) and others of which exist as multiple copies or share homology with other genes (subclones B, C, D, E, H, I, J, and K).

Recently, we determined that an immunodominant P1 epitope located near the carboxy terminus of the P1 protein (near regions L and M [6]) mediates cytoadherence. This region exists as a single copy. We suggest that the unique nature of the sequence reflects the critical function of this domain in mycoplasma virulence. We have not yet determined a specific function for any of the remaining portions of the P1 gene. From subclone B to subclone E the complexity of the hybridization pattern increases until subclones F and G, which appear as single-copy regions. Subclones H to K

also hybridize to multiple bands, but their patterns differ from those of subclones B to E. Subclones L and M occur as single copies, while subclone N occurs as multiple copies. Because the hybridization and washing procedures were performed under highly stringent conditions, only stretches of DNA which share extensive homology would have been detected. The possibility exists that other regions of the *M. pneumoniae* genome which share less homology with the P1 gene may have gone undetected.

Since domains of the P1 gene exist as multiple copies, different P1 proteins could exist if DNA conversion occurred between homologous families. However, no detectable variation of the P1 protein has been observed. Vu et al. (25) monitored the antigenic variation of *M. pneumoniae* over a 10-year period and found that five major peptide antigens (including P1) remained stable in terms of their molecular mass. However, these conclusions were based upon immunoblotting of clinical isolates with acute- and convalescent-phase sera, and variations in specific protein domains and epitopes could go undetected. Recently, Watson et al. (26) found structural and antigenic heterogeneity in a major surface antigen complex of *Mycoplasma pulmonis*. They also observed a high rate of variation of the antigen complex ( $2 \times 10^{-3}$  per cell per generation) within the same strain. This variation is very similar to the antigenic variation of other parasites (7, 15, 17, 20, 22) and points to the possibility of gene conversion in *Mycoplasma* species.

There may be several explanations for the multiple- versus single-copy nature of P1 gene domains. The P1 gene may consist of a mosaic of DNA segments which code for different functions. Some domains, such as the L and M regions associated with cytoadherence, may be highly specific and conserved. Other P1 gene segments may code for structural-functional domains which are common among classes of mycoplasma proteins (e.g., transmembrane domains, signal sequences, etc.), and gene recombination among these segments may occur. Therefore, the P1 gene may be polymorphic and protein P1 may exhibit amino acid variability. We recently observed polymorphism in the P1 gene in clinical isolates of *M. pneumoniae* (unpublished data).

Since specific P1-related multicopy gene domains (PMGD) hybridize to stretches of continuous P1 subclones (e.g., 4-kb fragments of *BamHI* in subclones B, C, D, E, H, I, J, and N and 4.1-kb fragments of *EcoRI* in subclones C, D, and E [Fig. 3]), there appear to be ordered stretches of gene regions similar to the P1 gene. Figure 4 is a model of the P1 gene and PMGD in the *M. pneumoniae* genome. In this scheme some PMGD exist as continuous gene segments in which multicopy DNA stretches (constant regions) are interrupted by single-copy domains (variable regions), creating a nonrandom physical arrangement. Both constant and variable regions in PMGD could be longer or shorter than in the P1 structural gene or could be missing. Because a predominant population of *M. pneumoniae* organisms in cultures do not cytoadhere yet possess P1-like molecules (12, 13), the PMGD may represent a repertoire of gene copies which, under appropriate conditions, regulate the structural and functional properties of P1. Mechanisms of phase and antigenic variations may be operable in which P1 occurs as a family of adhesin-related molecules with altered specificities and affinities, as determined by the organization of constant and variable gene sequences.

Many interesting questions arise based upon these data. Are the homologous sequences clustered around the P1 gene or scattered throughout the entire *M. pneumoniae* genome?

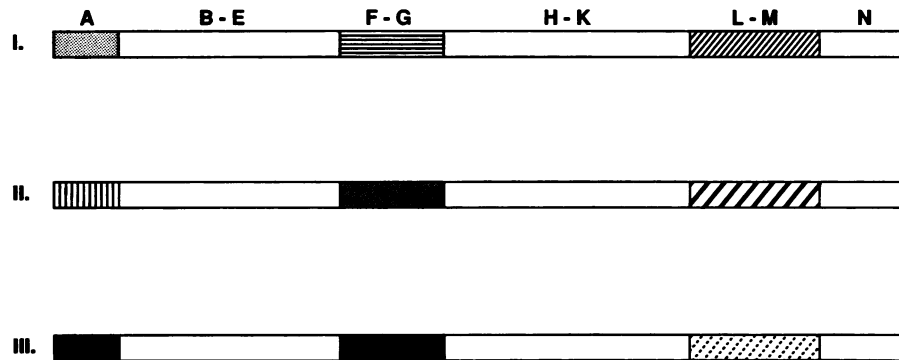


FIG. 4. Model for the P1 gene and PMGD in *M. pneumoniae*. I, P1 gene; II and III, P1-related sequences in which multicopy gene domains (open [constant] regions) are interrupted by non-P1-related single-copy domains (patterned [variable] regions).

Are these multiple-copy sequences expressed and translated, and if so, what classes of proteins share these sequences? Does recombination occur among the homologous gene segments in *M. pneumoniae*? Is the gene that codes for an analogous protein (140 kilodaltons) of *Mycoplasma genitalium* also multiply copied? Experiments designed to clarify these issues are currently under way.

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