fractionation with 50 percent and 95 percent (weight to volume) ammonium sulfate. The ammonium sulfate precipitate was resuspended in distilled water and dialyzed against 10 m*M* sodium phosphate buffer, *p*H 7.2. This preparation of ECGF is designated low molecular weight-ECGF and is the starting material for the chromatographic studies with heparin-Sepharose.
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Small DNA Deletions Creating Avirulence in *Streptococcus pyogenes*

Abstract. *The M protein is the antigen on the surface of group A streptococci that allows these bacteria to resist phagocytosis. DNA encoding the M12 protein was cloned into* Escherichia coli *and used as an isotopically labeled hybridization probe to compare genomic DNA's isolated from M^+ and M^- isogenic cultures in an effort to elucidate the genetic basis of this variation. DNA's from two spontaneous, independent M^- variants contained small (approximately 50 base pairs) deletions which were mapped to identical restriction fragments within or adjacent to the M protein coding sequence. Taken together with the pleiotropic nature of these deletions, this suggests that they define a regulatory switch.*

Antigenic variation of microbial virulence has been observed in numerous pathogenic organisms. The group A streptococci (*Streptococcus pyogenes*), responsible for many human infections, have a number of characteristics that exhibit such variation—including the M protein (*1*, *2*), hyaluronic acid capsule (*2*), and streptococcal chemotactic factor inhibitor (SCFI) (*3*). The M proteins, a class of antigenically distinct but functionally similar molecules, are responsible for the ability of these streptococci to resist phagocytosis by human polymorphonuclear leukocytes (*4*–*6*). Nearly 90 different M serotypes are now recognized, although a given strain will generally have only one M protein type (*4*). Two types of variation have been observed: the inability to express an M^+ phenotype and the shift from synthesis of one M protein to a second (antigenically distinct) molecule. The phenotypic loss of M protein has been known since glossy morphological variants were first isolated (*1*) and shown to be M^- and avirulent for mice (*7*). The throats of healthy carriers gradually, over time, yield M^- streptococci (*8*). Strains with new M proteins have arisen in populations experiencing frequent infections, where initially epidemic strains were replaced by other related strains that had antigenically distinct M proteins (*9*). We now show that M^- variant streptococci have acquired a DNA rearrangement responsible for reduced synthesis of M protein by these avirulent forms.

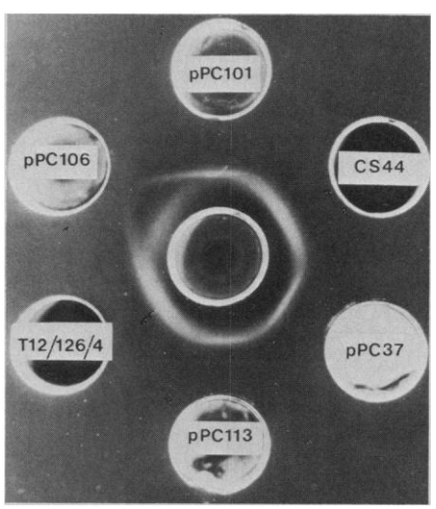

Fig. 1. Immunodiffusion analysis of *E. coli* clones that produce M protein. Center well contains antiserum to M12 prepared by inoculation of rabbits with strain CS44, a whole-cell vaccine (*23*). Peripheral wells contain acid extracts of streptococci prepared as described by Lancefield (*24*) or sonicated extracts of recombinant *E. coli* strains. Cultures of *E. coli* in the late log phase of growth were washed in phosphate-buffered saline (*p*H 7.2) and sonicated until they clarified. Cellular debris was removed by centrifugation (10,000 rev/min, 15 minutes), and the extracts were sterilized by passage through a filter (0.45 μm). The extract wells are labeled with the appropriate streptococcal strain or *E. coli* plasmid designations. Immunodiffusion agar was made as described (*15*); plates were incubated at 25°C for 15 hours.

A genomic library of the group A streptococcal strain CS44 (M12, T12) (*2*) was constructed as follows. Partially digested (Mbo I) bacterial DNA was sized on a 10 to 40 percent sucrose gradient (*10*), and 20- to 30-kilobase (kb) fragments were ligated to the alkaline phosphatase–treated, single Bam HI restriction site of the 12.9-kb cosmid vector pSF5, a derivative of pSF1 (*11*). Recom-

binant molecules were packaged into phage λ heads in vitro (*12*) and transduced into the *Escherichia coli* lysogen LE392 (λcI_{857}) (*13*). Ampicillin-resistant colonies were tested for M antigen with M12-specific rabbit antiserum and an assay for ^{125}I-labeled protein A binding (*14*).

Of approximately 1000 colonies screened, 14 reacted with the antiserum to M12 used in this assay. Sonicated extracts of cultures of these putatively positive clones in the log phase of growth were prepared and analyzed for M protein by immunodiffusion (*15*). One colony, containing recombinant plasmid pPC37, reacted sufficiently with the antiserum to M12 to produce a precipitin line and was chosen for further study. The precipitin line formed by LE392(pPC37) extracts was identical to that produced by Lancefield acid extracts of the parental streptococcal strain CS44 and of the Lancefield M12 prototype strain T12/126/4 (Fig. 1).

Plasmid DNA preparations and digestions with various endonucleases showed that pPC37 contained a 20-kb streptococcal DNA insert. This plasmid was unstable, readily segregating deletion variants. One such variant, pPC101, was isolated and transformed into the *E. coli* strain JM83 (*16*). The precise end points of the deletion that created pPC101 have not been defined, but data to be discussed below suggest that DNA containing the M-protein structural gene sequences was not altered. Sonicated extracts of JM83(pPC101) produced a precipitin line identical to that produced by acid extracts of CS44 and T12/126/4 (Fig. 1), suggesting that it contains most, if not all, of the M-specific antigenic determinants produced by the M12 streptococci.

To define further the streptococcal sequences, we constructed a physical map of plasmid pPC101. Nine Pvu II, five Hpa I, three Ava I, two Sal I, one Bgl II, and one Eco RI sites were located; four Hae III sites within the streptococcal DNA were also mapped (Fig. 2). Since many of these sites resided in cosmid pSF5 sequences, we concluded that the 12.9-kb vector remained intact in plasmid pPC101 and that pPC101 contained approximately 4.8 kb of streptococcal DNA. Each of the four Pvu II fragments containing streptococcal sequences was subcloned into the Hinc II site of the vector pUC9 (*17*) with the use of *E. coli* strain JM83. A sonicated extract of one subclone, strain JM83-(pPC106), contained pUC9 with a 3.2-kb Pvu II insert (Fig. 2) and produced a precipitin line having partial identity to M12 streptococcal extracts of CS44 and sonicated extracts of JM83(pPC101) (Fig. 1). When separated by a well containing the JM83(pPC106) extract, the spurs produced by extracts of strains CS44 and JM83(pPC101) meet, indicating the identity of these determinants and their absence in JM83(pPC106) extracts (Fig. 1). The M-protein gene was located more precisely by construction in vitro of a deletion plasmid, pPC113. In this plasmid the 2.5-kb Ava I fragment, which contained all of the streptococcal DNA to the right of the 3.2-kb Pvu II fragment (Fig. 2), was removed. Sonicated extracts of this culture, JM83-(pPC113), produced a precipitin line identical to those lines of acid extracts of the parental streptococcal strain CS44 and sonicated extracts from *E. coli* clones JM83(pPC101) and LE392(pPC37) (Fig.

Table 1. Opsonic inhibition of M12 antiserum by sonicated extracts of *Escherichia coli* clones containing plasmids pPC101 and pPC106. The assay was performed by first rotating equivalent volumes of extract and antiserum at 4°C for 24 hours. Then log-phase streptococci (10^7 colony-forming units) were added, and the mixture was incubated at 4°C for 1 hour. Subsequently, streptococci were washed three times and diluted into freshly drawn human blood where their numbers were immediately titered. After 1 hour of rotation at 37°C, viable colony-forming units were again titered.

Origin of bacterial extract*	M type of extract	Anti-serum† to M12	Survival (percent)‡	
			Experiment 1	Experiment 2
None		−	500.0	
None		+	7.4	
CS44	12	+	500.0	
CS130	1	+	14.8	
T12/126/4	12	+	600.0	
JM83(pPC101)	12	+	500.0	228
JM83(pUC9)		+	20.0	13
JM83(pPC106)	12	+		192

*Sonicated extracts were used for *E. coli* strains, and Lancefield acid extracts were used for streptococcal strains. †The (+) notation indicates that M12-specific rabbit antiserum at a final dilution of 1:10 or 1:20 was added; the (−) indicates that phosphate-buffered saline was added. ‡Survival refers to the number of CS44, M12 streptococcal colony-forming units remaining in blood after 1 hour of rotation at 37°C compared to the number of colony-forming units initially added to blood.

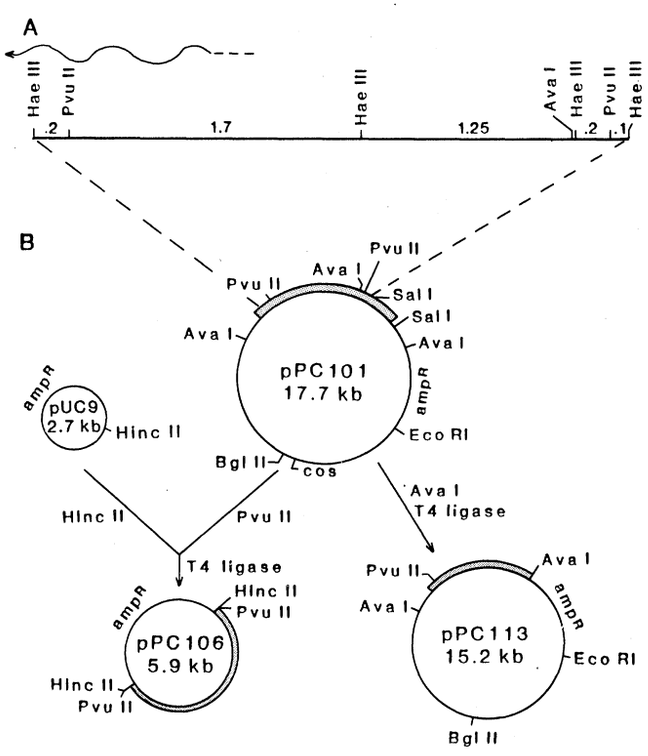

Fig. 2. (A) Expanded physical map of the 3.4-kb region of streptococcal DNA delineated by Hae III restriction sites. Numbers denote fragment size in kilobase pairs. The wavy line represents the approximate location and direction of transcription of the M-protein gene. (B) Construction of recombinant plasmids pPC106 and pPC113 from pPC101. Shaded areas denote streptococcal DNA (4.8 kb). Cos refers to the phage λ cos sites. Plasmid pPC106 was constructed by blunt-end ligation of the 3.2-kb Pvu II fragment from pPC101 to the single Hinc II site in pUC9. Plasmid pPC113 was constructed by Ava I digestion of plasmid pPC101 and subsequent self-ligation of the three cleavage products.

1). We infer from these data that the amino-terminal end of the M-protein gene lies within the 3.2-kb Pvu II fragment and that transcription proceeds from right to left beyond the Pvu II site (see Fig. 2).

The M proteins are defined by their antiphagocytic function; in vitro, this function is experimentally evaluated by the ability of extracted M protein to bind and remove opsonic antibody from type-specific M antiserum (*18*). In the presence of type-specific M antibodies and complement, M^+ bacteria are opsonized and efficiently phagocytized (*19*). To ensure that our clones produced M12 protein rather than some other cross-reacting protein, we performed experiments to test whether *E. coli* extracts specifically reduced the opsonic capacity of the M12 serum. The M12 streptococcal extracts specifically removed M12 opsonic antibodies resulting in the growth (absence of phagocytosis) of M12 bacteria (strain CS44). However, an extract from an M1 culture did not remove M12 opsonic antibodies; therefore, the M12 bacteria were fully opsonized and phagocytized when exposed to blood (Table 1). Similarly, extracts of *E. coli* clones JM83(pPC101) and JM83(pPC106) removed M12 opsonic antibodies so that the resulting antiserum did not opsonize M12 streptococci, which subsequently grew in blood. An extract of strain JM83 containing the pUC9 plasmid without streptococcal DNA had no effect on opsonization or phagocytosis of CS44 cells. These data indicate that both the 3.2- and 4.8-kb streptococcal DNA fragments of plasmids pPC106 and pPC101, respectively, contain sufficient information to encode antiphagocytic determinants.

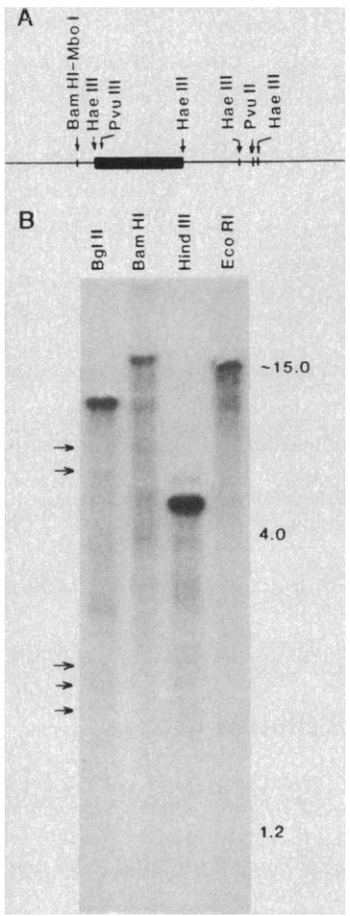

Fig. 3. Autoradiograph of strain CS44 DNA digested with enzymes that do not cleave cloned M protein DNA sequences. The 1.9-kb Hae III fragment of streptococcal DNA was used as a ^{32}P-labeled probe (solid heavy line). Molecular weight markers are in kilobases. Some of the bands that hybridize weakly to the probe are indicated by arrows. Genomic DNA from strain CS44 was prepared as described (*25*) except that protoplasts were made by digestion of streptococci with mutanolysin (100 μg per 100 ml of overnight culture) at 37°C for 1 hour. The streptococcal DNA (5 μg) was digested with Bam HI, Bgl II, Hind III, or Eco RI, subjected to electrophoresis on a 0.7 percent agarose gel at 40 V for 15 hours, transferred to nitrocellulose, and hybridized under stringent conditions (*25*). The probe was labeled with ^{32}P by nick translation as described (*25*) except that the DNA was taken directly from agarose having a low melting temperature (Bethesda Research Laboratories). Therefore, the reaction mixture contained approximately 0.18 percent agarose. X-ray film (XAR-5, Kodak) was exposed to the blot for 4 days at −70°C.

The segregation of M^- cells from M^+ streptococci has been described in studies performed both in vitro (*1*, *2*, *7*) and in vivo (*8*). The following experiments tested whether alteration of DNA within or adjacent to the M protein structural gene was the molecular basis for this variation. We chose the group A streptococcal strain CS24 because of its known unstable M phenotype: generally, CS24 exists as a mixed population of cells [the content of which can be altered by manipulations in vitro (*2*)]. Strain CS44 is a spontaneous mutant of strain CS24 with a more stable M^+ phenotype (*2*). Both strains produce large matt or mucoid colonies on blood agar plates, whereas their M^- counterparts produce small, glossy colonies (*1*, *2*). The M^- strains CS46 and CS64 are spontaneous variants isolated from CS24 and CS44, respectively (*2*). These variants do not revert to the M^+ state, even after extensive selection by numerous passages through phagocytically active human blood. Using cloned M12 sequences as probe, we compared DNA's from these strains by DNA–DNA hybridization. Hae III digestion products of DNA from strains CS24, CS44, CS46, and CS64 were subjected to electrophoresis, transferred to

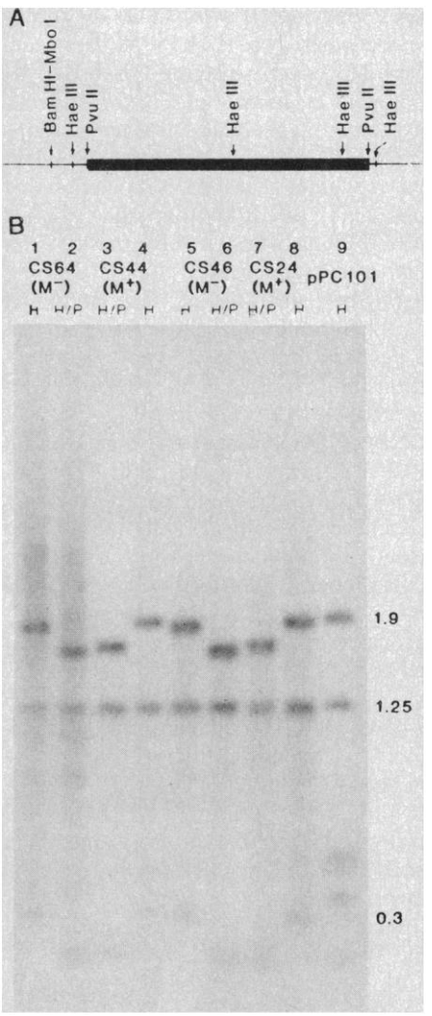

Fig. 4. Autoradiograph of Hae III– and Hae III-Pvu II–digested DNA's from M^+ strains CS24 and CS44 and M^- strains CS46 and CS64. Plasmid pPC106, including the 3.2-kb Pvu II fragment of streptococcal DNA, was used as a ^{32}P-labeled probe. Molecular size markers are in kilobases. The letter H refers to single Hae III digestions, and H/P refers to Hae III and Pvu II double digestions. All conditions were as described in legend to Fig. 3 except that electrophoresis was on a 1 percent agarose gel at 40 V for 18 hours and the probe was made from CsCl gradient-purified plasmid. X-ray film (Cronex, Dupont) was exposed to the blot for 48 hours at −70°C.

nitrocellulose by the Southern blot method, and probed with nick-translated plasmid pPC106 (which contains the 3.2-kb Pvu II fragment of streptococcal DNA from plasmid pPC101) (Fig. 4). This probe hybridized to three Hae III fragments in the four genomic streptococcal DNA's examined (Fig. 4, lanes 1, 4, 5, and 8). The two smaller fragments (1.25 and 0.3 kb) appeared identical; however, the 1.9-kb Hae III fragment from M^+ cells was slightly, but reproducibly, larger than that from M^- streptococci (Fig. 4, lanes 4 and 5). Analysis of Pvu II digests also revealed a shortened Pvu II fragment (the 3.2-kb fragment from M^+ cells and pPC101) in DNA's from M^- cells (data not shown). This difference was estimated to be about 50 base pairs (bp). Moreover, since the 1.9-kb Hae III fragment of plasmid pPC101 was identical to that in genomic DNA from strains CS24 and CS44 (Fig. 4, lanes 4, 8, and 9), we concluded that the DNA deletion that produced pPC101 from the original pPC37 clone did not involve these particular DNA sequences.

Further analysis of Hae III–Pvu II double-digestion products revealed that a deletion of DNA was most likely responsible for the 50-bp difference observed between M^+ and M^- genomic DNA's. Since both the Hae III and Pvu II fragments were shorter in M^- DNA's, we reasoned that an inversion should change the orientation of one Hae III site relative to one of the two Pvu II sites demarking the 3.2-kb Pvu II fragment (Fig. 2). If an inversion had occurred, then Hae III–Pvu II digests of DNA from M^- and M^+ cells would differ, and Pvu II would not cleave the 1.85-kb Hae III fragment from M^- cells. Pvu II cleaved that fragment identically in both M^- and M^+ DNA (Fig. 4, lanes 2, 3, 6, and 7). Therefore, we concluded that the M^- phenotype in strains CS46 and CS64 did not arise from a DNA inversion but was probably the result of similar deletions within or adjacent to the M-protein structural gene.

Numerous traits of hemolytic streptococci exhibit phenotypic variation concomitant with changes in M protein synthesis. Capsule formation (*2*), SCFI activity (*3*), immunoglobulin G Fc receptor expression (*20*), and serum opacity activity (*2, 20*) are a few examples. Strains CS46 and CS64, in contrast to their M^+ parent strains, lack hyaluronic acid capsules and SCFI activity (*21*). The pleiotropic nature of the event that created these M^- mutants suggests that the genes responsible for these phenotypes might be under coordinate control, possibly as a virulence operon. This hypothesis is consistent with data showing that the M^- variants contain a deletion in the restriction fragment that includes the amino-terminal end of the M-protein gene. Since two independent M^- variants harbor these small deletions, which are pleiotropic and map in a common 355-bp segment of DNA (*21*), we postulate that they identify a programmed regulatory switch. Comparisons of the nucleotide sequences of the M12 gene and adjacent DNA with those from M^- variants and the M6 gene (*22*) should help clarify the mechanisms of antigenic variation exhibited by this important human pathogen.

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Scintigraphy of Normal Mouse Ovaries with Monoclonal Antibodies to ZP-2, the Major Zona Pellucida Protein

Abstract. *The zona pellucida is an extracellular glycocalyx, made of three sulfated glycoproteins, that surrounds mammalian oocytes. Parenterally administered monoclonal antibodies specific for ZP-2, the most abundant zona protein, localize in the zona pellucida. When labeled with iodine-125, these monoclonal antibodies demonstrate a remarkably high target-to-nontarget tissue ratio and provide clear external radioimaging of ovarian tissue.*

The advent of monoclonal antibodies has markedly improved the ability of external radioimaging techniques to locate tumors (*1, 2*) and to identify certain noncancerous diseases (*3*). However, the success of these studies is highly dependent on the concentration and distribution of the antigen recognized by the antibody. Many monoclonal antibodies detect antigens only when the latter are present in at least 10^4 or 10^5 copies per cell, and most cancer-associated antigens are present in targeted as well as nontargeted tissue. This lack of specificity has hampered the usefulness of such antibodies for immunoscintigraphy. Much better radioimaging might be expected for tumor-specific antigens, as has been reported with anti-idiotypic antibodies in B-cell leukemias (*4*), or for normal tissue antigens that are found exclusively in a particular organ (*5, 6*).

During growth and development, mammalian oocytes synthesize and secrete an extracellular zona pellucida (ZP). Each murine ZP is composed of three sulfated glycoproteins known as ZP-1, ZP-2, and ZP-3, which have molecular weights of 185,000, 140,000 and 83,000, respectively (*7, 8*). The ZP has three apparent functions: (i) mediation of species-specific sperm binding at the time of fertilization, (ii) blockage of postfertilization polyspermy, and (iii) protection of the preimplantation embryo as it passes down the oviduct (*9*). ZP-3 composes 19 percent of the ZP mass and may be the species-specific sperm receptor (*10*). ZP-2, which makes up almost 50 percent of the ZP, is biochemically modi-