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Identification of a gene that regulates expression of M protein, the major virulence determinant of group A streptococci

(conjugative transposon/insertional mutagenesis/rheumatic fever/transcription)

MICHAEL G. CAPARON AND JUNE R. SCOTT

Department of Microbiology and Immunology, Emory University Health Sciences Center, Atlanta, GA 30322

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ABSTRACT **By using Tn*916* insertional mutagenesis, we have identified *mry* (M protein RNA yield), a gene required for high-level expression of the M protein, an essential virulence determinant of the group A streptococcus. The *mry*::Tn*916* mutation causes a reduction by a factor of ≈50 in the amount of M protein produced, in the original strain and in a nonmutagenized host to which the mutation was transferred by transduction. The insertion is located ≈1.8 kilobases upstream of the structural gene for M protein (*emm*) and its promoter. The genomic region including *mry*::Tn*916* and *emm* was cloned on a cosmid vector and introduced into *Escherichia coli*. When the transposon excised precisely from the chimeric cosmid in *E. coli*, the resulting streptococcal DNA showed the same restriction pattern as the homologous chromosomal region in the parental nonmutagenized streptococcus. The sequence of the promoter for *emm* was not altered in the *mry* mutant. The reduction in protein correlates with a decrease in the amount of M protein-specific mRNA, indicating that *mry* regulates transcription of *emm*.**

It was recognized as early as 1928 that M protein is the primary virulence determinant of the group A streptococcus (1), an organism responsible for a variety of suppurative infections as well as the serious postinfective sequelae of glomerulonephritis and rheumatic fever. Subsequent investigations have shown that M protein is a fibrillar molecule composed of two α-helical protein chains in a coiled-coil conformation (2). These dimeric molecules extend from the surface of the cell (2, 3) and confer on the streptococcus resistance to phagocytosis by polymorphonuclear leukocytes (4).

Though much recent progress has been made in understanding the structure and evolution of the >80 recognized serotypes of the M molecule (5, 6), relatively little is known at the molecular level about factors that regulate its expression. Spanier *et al.* (7) described a strain with a 50-base-pair (bp) deletion located ≈0.4 (kilobase) (kb) upstream of the M12 structural gene that apparently reduced M protein expression. This suggested that the structural gene for M protein was regulated by additional genes; however, neither these genes nor their functions were further characterized.

Additional evidence for the involvement of other genes in the expression of M protein came from a study by Scott *et al.* (8), in which a strain deleted for the gene encoding the M28 protein was converted to M^+ by the introduction of a plasmid containing an M6 gene (*emm6*). The reconstituted M^+ strain produced only one-fifth the amount of M protein per cell as did the strain from which the *emm6* gene was derived, prompting Scott *et al.* (8) to hypothesize that a factor absent from the M28 host regulates expression of *emm6*.

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Further investigations of the role of additional genes in the regulation of M protein expression have been complicated by a lack of techniques for introducing defined mutations into the group A streptococcal chromosome. The identification of broad host range conjugative transposons in streptococci (9–12) has provided a method for insertional mutagenesis of a number of organisms in this genus. The conjugative transposon Tn*916* has been used to induce streptolysin S mutations in the group A streptococcus (*Streptococcus pyogenes*) (13). In this report, Tn*916* insertional mutagenesis of the *S. pyogenes* strain D471 is used to define *mry*, a gene required for the efficient transcription of the M protein structural gene.

MATERIALS AND METHODS

Bacteria, Plasmids, and Phage. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* K12 strain CG120 carries pAM120, a chimeric plasmid constructed by cloning Tn*916* into the plasmid vector pGL101 (16). Plasmid pJRS42.92 contains *emm6*, the gene encoding the type 6 M protein cloned from D471 (14, 17). The cosmid pJC74 is 15.8 kb in size and encodes resistance to ampicillin (18). A25 is a generalized transducing phage specific for group A, C, and G streptococci (19–21).

Media and Antibiotics. Unless otherwise indicated, the following media were used: brain heart infusion broth (BHI broth) (Difco) for the growth of *Streptococcus faecalis*, Luria–Bertani broth (LB broth) (22) for *E. coli*, and Todd–Hewitt yeast extract broth (THY broth) (23) for the propagation of *S. pyogenes*. In certain experiments, THY broth was supplemented with 2.0% defibrinated sheep's blood (THY-SB). Antibiotics were used at the following concentrations: ampicillin at 50 μg/ml for *E. coli*, tetracycline at 5 μg/ml for *E. coli* and streptococci, and streptomycin at 1000 μg/ml for streptococci.

Filter Matings Between *S. faecalis* and *S. pyogenes*. Tn*916* was transferred by conjugation using the procedure of Franke and Clewell (9) with the following modifications. Cells were mixed at a ratio of 1 donor (CG110) to 30 recipients (JRS4) and the mating filter was incubated at 37°C on a THY-SB plate overnight. Transconjugants were selected by plating on THY-SB supplemented with streptomycin (to select against the donor) and tetracycline (to select for Tn*916*). Control filters containing only the donor or recipient were treated in an identical fashion. The frequency of transfer of Tn*916* was calculated as the number of transconjugants per recipient (based on the viable count of the mating mixture on medium containing streptomycin alone at the time of plating for transconjugants).

Colony Blot Radioimmunoassay. This procedure was performed as described by Scott and Fischetti (14) using a monospecific polyclonal antibody to purified M6 protein.

Abbreviations: Sm, streptomycin resistant; Tc, tetracycline resistance; Ap, ampicillin resistant.

Table 1. Bacterial strains and relevant properties

Strain	Relevant genotype	Ref.	Comments
S. pyogenes			
D471	*emm6*	14	
JRS4	*emm6 str*	8	Spontaneous Sm derivative of D471
JRS10	*emm6 str mry*::Tn*916* zzz::Tn*916*	This paper	Derivative* of JRS4
JRS11	*emm6 mry*::Tn*916*	This paper	Derivative† of D471
JRS12	*emm6 mry*::Tn*916*	This paper	Derivative† of D471
JRS13	*emm6 mry*::Tn*916*	This paper	Derivative† of D471
JRS14	*emm6 mry*::Tn*916*	This paper	Derivative† of D471
JRS15	*emm6 str* xzz::Tn*916*	This paper	Derivative* of JRS4
S. faecalis			
CG110	*rif* zzz::Tn*916*	10	Donates Tn*916* at high frequency
E. coli			
DH1	*recA1 endA1 hsdR17*	15	—
CG120	Same as DH1	16	Contains pAM120

Sm, streptomycin resistant.
*Transconjugant from mating with CG110.
†Tetracycline-resistant (Tc) transductant using JRS10 as donor.

Immunoblot Analysis. To quantitate M immunoprotein, lysin extracts (24) of *S. pyogenes* were separated on 12.5% $NaDodSO_4$/polyacrylamide gels; this was followed by transfer to nitrocellulose as described (25). M protein was detected using monoclonal antibody 10B6 (26) by the method of Blake *et al.* (27). The total amount of protein in each lysin extract was standardized by absorbance at 280 nm or by the Bio-Rad protein assay. The intensity of immunoreactive bands was monitored using a reflectance densitometer (CS-930; Shimadzu Scientific Instruments, Columbia, MD) at 650 nm. Peak heights were adjusted with lysin buffer controls and the amount of M protein in each sample was quantitated relative to a serially diluted standard D471 extract.

Southern Blot Analysis. The hybridization procedures of Meinkoth and Wahl (28) were used on DNA extracted from lysin-treated cells (17). DNA probes were labeled with ^{32}P by the method of Feinberg and Vogelstein (29) and included pAM120 to detect Tn*916* sequences and a purified *Xba* I/*Nci* I or *Nci* I/*Pvu* II fragment of pJRS42.92 (probes A and B, Fig. 3). Other probes are described in the text. Washing was performed under conditions equivalent to 34°C below the calculated melting temperature of probe B (0.03 M NaCl/0.003 M sodium citrate, pH 7.0/0.1% $NaDodSO_4$, 37°C).

Transductions. Transductions using the streptococcal phage A25 were as described by Wannamaker *et al.* (20) with the following modifications. Transducing lysates were prepared by growing the phage on the donor strain for 2 hr at 30°C and were not UV-irradiated before use. Also, recipient cells were centrifuged 30 min after infection to remove free phage, resuspended in THY broth, and incubated at 30°C for 1 hr to allow expression of the transduced antibiotic resistance marker. Transductants were selected by plating on THY-SB supplemented with the appropriate antibiotic.

RNA Transfer Blot Analysis. Total cellular RNA was prepared from *S. pyogenes* cultures as described by Hollingshead *et al.* (30). RNA was then analyzed by the method of Thomas (31). Hybridization and washing conditions were identical to the conditions used for Southern blots.

Cosmid Cloning. A library of chromosomal DNA sequences from a *S. pyogenes* strain bearing a single Tn*916* insertion (JRS14) was constructed in the cosmid pJC74 by following the procedures of Collins (18, 32). Chimeric cosmids constructed using pJC74 were packaged *in vitro* (Gigapack Plus; Stratagene Cloning Systems, San Diego, CA) and introduced into *E. coli* strain DH1 following the method suggested by the vendor.

DNA Sequence Analysis. DNA sequences were determined by the dideoxy chain-termination method of Sanger *et al.* (33) with the modifications described by Zagursky *et al.* (34) for the analysis of supercoiled plasmid DNA.

RESULTS

Mutagenesis of *S. pyogenes* with Tn*916*. Matings on membrane filters were used to transfer Tn*916* from the group D strain CG110 into JRS4, a spontaneous Sm derivative of the group A strain D471. Transconjugants that acquired the Tc determinant of Tn*916* were selected on medium containing streptomycin and tetracycline. No spontaneous Tc mutants of D471 were ever detected when cells from control filters were plated on selective medium. Under these conditions, the frequency of transfer of Tn*916* into JRS4 was $\approx 10^{-6}$ per recipient cell.

Transconjugants were screened by colony blot radioimmunoassay and those identified as potentially deficient in expression of M protein were subjected to immunoblot analysis to quantitate the amount of M protein they produced. One mutant (designated JRS10), which initially appeared to produce no M protein on the colony blot, was thus shown to produce less M protein (by a factor of 50) than the parental strain JRS4.

Determination of the Number of Tn*916* Insertions in JRS10. Preliminary studies indicated that most transconjugants acquired multiple copies of Tn*916* when CG110 was mated with JRS4. To determine the number of Tn*916* insertions in JRS10, chromosomal DNA was digested with *Hin*dIII and analyzed in Southern blots with ^{32}P-labeled pAM120 as a Tn*916*-specific probe. Since Tn*916* has a single *Hin*dIII site (10), this analysis should reveal two junction fragments for each copy of Tn*916* present in the genome. No DNA hybridizing with the Tn*916*-specific probe was observed in the parental strain JRS4 (Fig. 1*A*, lane 1). However, four hybridization fragments were observed in digests of JRS10 chromosomal DNA (Fig. 1*A*, lane 2), indicating that this strain has two copies of the transposon and that these copies are located at different sites in the JRS10 chromosome. Additional Southern blot analyses employing an *emm6*-specific probe indicated that the two Tn*916* insertions were not located in either the structural gene or promoter region of *emm6* (see below).

Transduction of Tn*916* Insertions into D471. D471 infected with phage A25 that had been grown on JRS10 was plated on medium containing tetracycline to select for Tn*916*. Tc transductants were obtained at a frequency of about 10^{-7} per plaque-forming unit of A25. Less than 10^{-9} spontaneous Tc mutants of D471 were observed in control experiments, and transductants could easily be distinguished from JRS10 by their sensitivity to streptomycin. Several transductants were screened by immunoblot analysis, and four (designated JRS11, JRS12, JRS13, and JRS14) produced M protein at a level equivalent to that produced by JRS10. The reduced amount (by a factor of 50) of M protein detected in these strains could not have been produced by a small population of revertant cells, since the frequency of loss of Tn*916* was $<10^{-3}$.

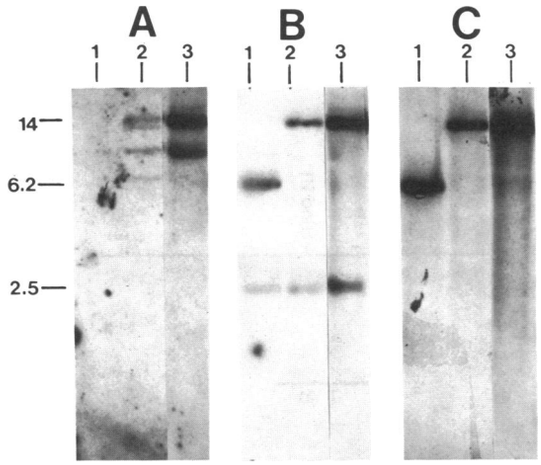

FIG. 1. Southern blot analyses of chromosomal DNA to identify Tn*916*. *Hin*dIII-digested chromosomal DNA from D471 (lanes 1), JRS10 (lanes 2), and JRS14 (lanes 3) was allowed to react with ^{32}P-labeled pAM120 to detect Tn*916* (*A*), probe A (see Fig. 3) (*B*), and probe B (see Fig. 3) (*C*). Numbers to the left indicate fragment size in kilobase pairs.

Analysis of Southern blots revealed that each of the four transductants had acquired only one copy of Tn*916* and that this copy was located at the same site in the chromosome of each transductant. This site corresponded to the location of one of the two copies of Tn*916* in JRS4. Fig. 1*A*, lane 3, shows the results obtained with JRS14.

Agarose gel electrophoresis indicated that *Hin*dIII digested the chromosome of D471 into fragments of 10 kb or less (Fig. 2, lane B). Since one of the two *Hin*dIII junction fragments of each Tn*916* insertion is a minimum of 10.9 kb in size (ref. 11; see Fig. 3), the number and location of Tn*916* insertions in each transductant could easily be determined from electrophoretic analysis (without hybridization) of chromosomal DNA digested with *Hin*dIII. The large junction fragments of Tn*916*-bearing derivatives of D471 were clearly visible in the agarose gels of *Hin*dIII digests (Fig. 2).

Mapping the Tn*916* Insertion Responsible for Decreased M Protein Expression. The gene for the M6 protein (*emm6*) contains one internal *Hin*dIII site (Fig. 3). As expected, two hybridization fragments were detected by an *emm6*-specific

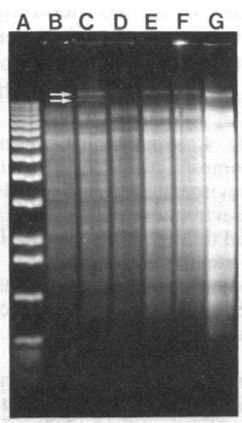

FIG. 2. Junction fragments containing the 10.9-kb arm of Tn*916* are visible following electrophoresis of chromosomal DNA digested with *Hin*dIII. Lane A, molecular size standards (1-kb ladder, Bethesda Research laboratories); lanes B–G, chromosomal DNA from D471, JRS10, JRS11, JRS12, JRS13, and JRS14, respectively. Arrows in lane C highlight the two large junction fragments between Tn*916* and the chromosome in JRS10.

probe (Fig. 3, probe A) in Southern blots of *Hin*dIII-digested D471 chromosomal DNA (Fig. 1*B*, lane 1). Probe A (Fig. 1) hybridized with a 2.5-kb fragment (containing 420 bp encoding the carboxyl-terminal end of *emm6*) in *Hin*dIII digests of chromosomal DNA from D471 and with a fragment of the same size in DNA from JRS10 and JRS14 (Fig. 1*B*, lanes 2 and 3). However, instead of reacting with a 6.2-kb fragment as observed for D471, probe A hybridized to a fragment of at least 14 kb in JRS10 and JRS14. These results were confirmed by hybridization with probe B (Fig. 3), which only recognized the 6.2-kb fragment of D471 and the high molecular weight pieces of JRS10 and JRS14 (Fig. 1*C*, lanes 1–3). As expected, this high molecular weight band also reacted with the Tn*916*-specific probe. These data indicate that the transposon insertion in JRS10 and JRS14 is located within the 6.2-kb *Hin*dIII fragment that contains the 5′ end of *emm6*. Similar results were obtained with JRS11, JRS12, and JRS13 (data not shown). Additional digests showed that this insertion was located between the *Sst* I and *Sal* I sites ≈1.8 kb upstream of the *emm6* promoter (Fig. 3) (35, 36). The Tn*916* insertion therefore defines a gene, *mry*, required for high-level expression of *emm6*.

Cloning and Analysis of the *mry*::Tn*916* Allele in *E. coli*. To introduce the *mry*::Tn*916* allele into *E. coli*, we took advantage of the observation that the entire transposon, the intervening chromosome, and *emm6* were contained on a single 26.2-kb *Bam*HI fragment (Fig. 3). Chromosomal DNA isolated from one transductant (JRS14) was digested to completion with *Bam*HI and ligated into the *Bam*HI site of the 15.8-kb cosmid pJC74 (which encodes Ap) to produce chimeric molecules of a size (43 kb) ideal for *in vitro* packaging into bacteriophage λ particles (18, 32). *E. coli* strain DH1 was infected with the resulting cosmid library and screened for the Tc determined by Tn*916*. Tc colonies were obtained at a frequency of 1 per 120 Ap colonies.

As expected, plasmid DNA isolated from eight Tc colonies contained a 26.2-kb *Bam*HI insert in addition to the 15.8-kb cosmid vector. Derivatives lacking Tn*916* were then isolated from four of these chimeric cosmids (pJRS1001, pJRS1006, pJRS1007, and pJRS1011) following overnight culture in the absence of tetracycline. Approximately 85% of the cells became sensitive to the drug under these conditions. Excision of Tn*916*, which has been reported to be precise in *E. coli* (16), was confirmed in *Bam*HI digests of plasmid DNA isolated from 16 tetracycline-sensitive derivatives of the original four strains. In each case, the 26.2-kb *Bam*HI fragment in the original plasmid had been reduced in size by 16.4 kb, the size of Tn*916*.

To test the original *mry* mutant for the possible presence of deletions that might affect expression of *emm6*, Southern blot analysis was used to compare a chimeric plasmid from which Tn*916* had excised (pJRS1006.1) to the homologous region of the chromosome of the original wild-type strain D471. The probe for this analysis (Fig. 3, probe C) consisted of a 4.5-kb *Bam*HI/*Hin*dIII fragment purified from pJRS1006.1, which includes the promoter and 5′ region of *emm6* as well as ≈3 kb of upstream DNA. This probe recognized identical 9.8-kb *Bam*HI fragments in digests of DNA from pJRS1006.1 and D471 (Fig. 4 *Left*, lanes B and C). To visualize small deletions, digests with enzymes expected to produce multiple fragments in this region were probed. No deletions were apparent in comparisons of the hybridization patterns of *Sau*3A, *Rsa* I and *Bam*HI, or *Hae* III and *Bam*HI digests of pJRS1006.1 and D471 under standard electrophoresis conditions (Fig. 4 *Left*) or under conditions optimized to separate small (<1.3 kb) restriction fragments (Fig. 4 *Right*).

DNA Sequence Analysis of the *emm6* Promoter Contained on pJRS1006.1. The DNA sequence of a region of pJRS1006.1 containing the promoter for *emm6* was determined by the dideoxy chain-termination method (33, 34) using a synthetic

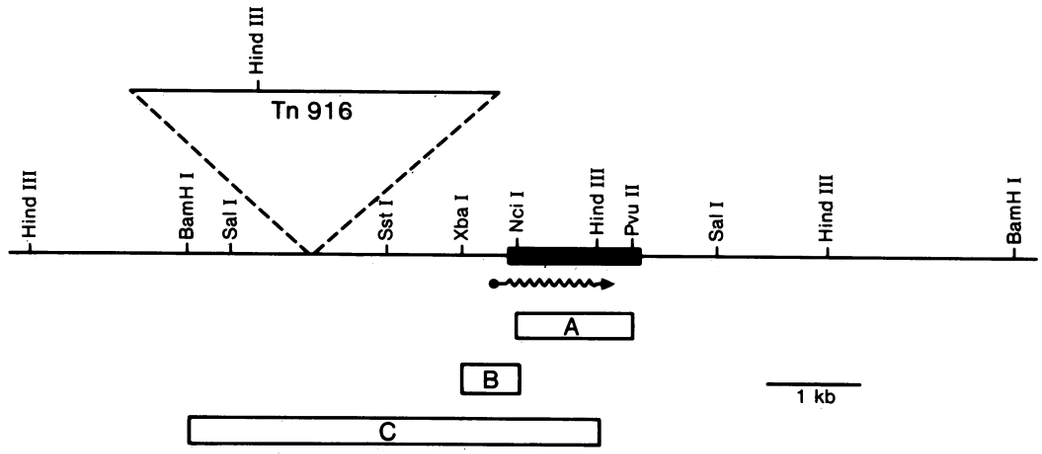

FIG. 3. Restriction map of the chromosomal region surrounding *emm6* in D471 and *mry* mutant strains. The location of *emm6* is indicated by the heavy line. The circle and wavy line directly below the map show the location of the promoter and direction of transcription of *emm6*. Open boxes (A, B, and C) represent probes used in Southern blot analyses. The dashed lines indicate the orientation and the approximate location of the Tn*916* insertion in *mry*. Tn*916* is shown at one-fourth scale relative to the rest of the map.

oligonucleotide primer that hybridized to the leader sequence of the coding region of *emm6*. Comparison with the promoter sequence of *emm6* in D471 as defined by primer extension (36) indicated that there were no changes in the spacing or nucleotide sequence of the −10 or −35 regions in the *mry* mutant strain.

Analysis of *emm6* Transcription in *mry* Mutant Strains. To determine the relative amount of the *emm6*-specific transcript in the *mry* mutant strains, total cellular RNA isolated from D471, JRS10, and JRS14 was subjected to RNA transfer blot analysis. Comparisons between strains were made from equivalent amounts of RNA as judged from the intensity of rRNA bands visualized by staining with ethidium bromide. Hybridization with probe A revealed that each strain contained the 1.57-kb RNA species identified (36) as the *emm6* transcript (Fig. 5). An additional control strain JRS15 (a derivative of D471 with a Tn*916* insertion that does not affect expression of M6) contained about the same amount of the 1.57-kb *emm6* transcript as strain D471 (not shown). However, the amount of *emm6*-specific RNA in the *mry* mutant strains JRS10 and JRS14, as well as JRS11, JRS12, and JRS13 (data not shown), was much lower than that in the wild-type strain D471.

DISCUSSION

Using Tn*916* insertional mutagenesis, we have defined *mry* (M protein RNA yield), a gene required for efficient transcription of *emm6*. The transposon insertion responsible for the M-deficient phenotype is located ≈1.8 kb upstream of the structural gene for *emm6* (Fig. 3) and of its promoter (35, 36), demonstrating that the transposon did not interrupt the coding sequence of the protein or cause a polar effect on *emm6* expression. This indicates that an additional gene required for high-level production of M protein was inactivated.

In any study using mutagenesis, it is critical to establish that the mutant phenotype results from a single genomic lesion. Linkage of Tn*916* to the mutation responsible for the reduction in expression of M protein was demonstrated by transduction to a nonmutagenized D471 strain. This process separated the two Tn*916* insertions in JRS10 and identified the one responsible for the mutant phenotype. However, because reduction of M protein expression can result from spontaneous 50-bp deletions upstream from the structural gene for *emm12* (7), it seemed possible that a similar deletion occurred near *emm6* in JRS10. Such a deletion could have been cotransduced with the transposon by phage A25, since the Tn*916* insertion was located only 1.8 kb from *emm6*. No deletions were found when the D471 chromosome upstream of *emm6* and the homologous region of an *mry*::Tn*916* mutant were compared. Furthermore, the DNA sequence of the promoter region of one strain (JRS14) was identical to the sequence of the wild-type promoter, eliminating the possibility of an independent second mutation there.

In the course of this work, we found that the locations of the various Tn*916* insertions could be determined directly by restriction fragment analysis of the streptococcal chromosomal DNA on an agarose gel, since the high molecular

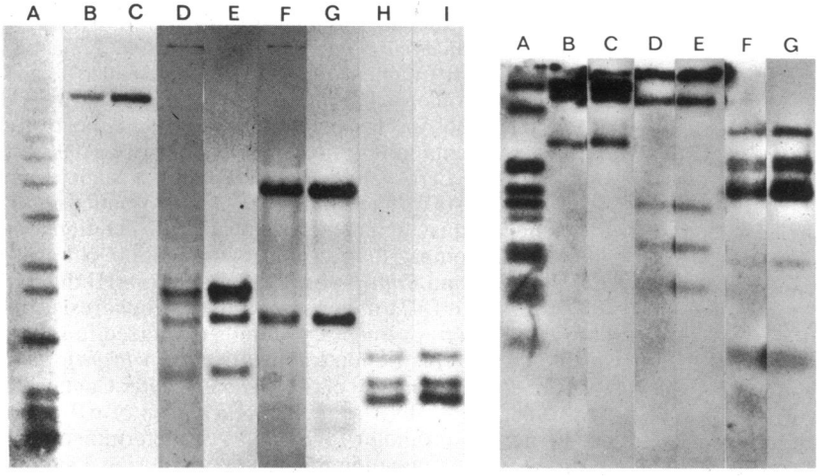

FIG. 4. Southern blots using probe C (Fig. 3) to compare restriction fragment sizes of D471 chromosomal DNA and pJRS1006.1 under different electrophoresis conditions. (*Left*) Digests separated through 0.8% agarose. Lane A, molecular size standards; lanes B, D, F, and H, D471 chromosomal DNA; and lanes C, E, G, and I, pJRS1006.1. Digests are as follows: lanes B and C, *Bam*HI; lanes D and E, *Sau*3A; lanes F and G, *Hae* III + *Bam*HI; and lanes H and I, *Rsa* I + *Bam*HI. (*Right*) Digests separated through 4.0% Nusieve agarose (FMC, Rockland, ME). Lane A, molecular size standards (see Fig. 2); lanes B, D, and F, D471 chromosomal DNA; and lanes C, E, and G, pJRS1006.1. Digests are as follows: lanes B and C, *Sau*3A; lanes D and E, *Hae* III + *Bam*HI; and lanes F and G, *Rsa* I + *Bam*HI. This gel does not resolve fragments larger than about 1.3 kb. The lowest band is 75 bp and the one above it is 142 bp.

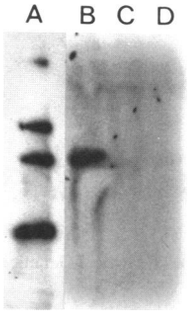

FIG. 5. Comparison of the levels of the *emm6* transcript in D471 and *mry* mutant strains in a RNA transfer blot using probe A (Fig. 3). Lane A, molecular size standards; lane B, D471; lane C, JRS10; and lane D, JRS14. Equivalent amounts of total cellular RNA (5 μg) were analyzed for each strain.

weight junction fragments containing the 10.9-kb arm of Tn*916* were visible in *Hin*dIII digests. Studies involving *Hin*dIII digests of chromosomal DNA from other group A streptococci have also identified very few high molecular weight bands. These differ in size between strains of the same serotype isolated from geographically diverse locations (30, 44). It is possible that in these cases the bands observed result from the presence of conjugative transposons, which may therefore be widespread among group A strains in nature.

The identification of *mry* indicates that expression of the M protein gene is regulated by at least one additional gene. It is possible that *mry* encodes the trans-acting regulatory factor suggested by the observation that an M^- strain into which a plasmid encoding M6 was introduced produced less M protein than did the M^+ strain from which the *emm6* gene had been cloned (8). Preliminary DNA hybridization experiments suggest that the M^- strain used in that experiment is not only deleted for the M protein gene but for *mry* as well (data not shown). Further work is necessary to test whether the addition of the *mry* gene to the M^- strain increases the amount of M protein produced from a subsequently introduced plasmid encoding M6.

The amount of *emm6*-specific mRNA is drastically reduced by the *mry*::Tn*916* mutation. This indicates that *mry* regulates production of M protein at the level of transcription. The *mry* gene may, therefore, encode a factor required for transcription or for inactivation of a transcriptional repressor or encode a factor that alters stability of *emm6* mRNA. In many species of pathogenic bacteria, regulatory genes that simultaneously control synthesis of several different virulence proteins have been identified (37–40). Like *mry*, some of these genes have been shown to exert their regulatory effect at the level of transcription (38–40). Whether *mry* affects expression of other streptococcal virulence determinants in addition to the M protein remains to be determined.

Development of an M protein vaccine to control group A streptococcal disease has proved difficult, because of the extreme antigenic diversity of M proteins in infectious strains (5) and because of the cardiac reactivity of some anti-M protein antibodies (41–43). The identification of *mry*, a gene required for efficient expression of the M protein, suggests that an alternative approach to disease intervention might be based on the regulation of this essential virulence determinant.

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