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The Group A Streptococcal *virR49* Gene Controls Expression of Four Structural *vir* Regulon Genes

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Within a genomic locus termed the *vir* regulon, *virR* genes of opacity factor-nonproducing (OF^-) group A streptococci (GAS) are known to control the expression of the genes encoding M protein (*emm*) and C5a peptidase (*scpA*) and of *virR* itself. Within the corresponding genomic locus, opacity factor-producing (OF^+) GAS harbor additional *emm*-related genes encoding immunoglobulin G- and immunoglobulin A-binding proteins (*fcrA* and *enn*, respectively). The *virR* gene region of the OF^+ GAS M-type 49 strain CS101 was amplified by PCR, and 2,650 bp were directly sequenced. An open reading frame of 1,599 bp exhibited 76% overall homology to published *virR* sequences. By utilizing mRNA analysis, the 5′ ends of two specific transcripts were mapped 370 and 174 bp upstream of the start codon of this open reading frame. The deduced sequences of the corresponding promoters and their locations differed from those of previously reported *virR* promoters. Transcripts from wild-type *fcrA49*, *emm49*, *enn49*, and *scpA49* genes located downstream of *virR49* were characterized as being monocistronic. The transcripts were quantified and mapped for their 5′ ends. Subsequently, the *virR49* gene was inactivated by specific insertion of a nonreplicative pSF152 vector containing recombinant *virR49* sequences. The RNA from the resulting vir-mut strain did not contain transcripts of *virR49*, *fcrA49*, *emm49*, or *enn49* and contained reduced amounts of the *scpA49* transcript when compared with wild-type RNA. The mRNA control from the streptokinase gene was demonstrated not to be affected. When strain vir-mut was rotated in human blood, it was found to be fully sensitive to phagocytosis by human leukocytes. Thus, the present study provides evidence that *virR* genes in OF^+ GAS could be involved in the control of up to five *vir* regulon genes, and their unaffected regulatory activity is associated with features postulated as crucial for GAS virulence.

Streptococcus pyogenes, or group A streptococcus (GAS), is one of the most important bacterial pathogens. It causes purulent diseases like pharyngitis, impetigo, and erysipelas and, less frequently, severe generalized diseases like sepsis and toxic shock-like syndrome (reviewed by Stevens [53]). Although the pathogenesis of these diseases is complex, several streptococcal molecules potentially involved in attachment, colonization, and invasion of the human host have been identified. Some of these molecules are proteins attached to the streptococcal surface. Among these, a fibronectin-binding protein (50), a plasminogen receptor (31, 36), the complement-inactivating C5a peptidase (10), two types of immunoglobulin (Ig)-binding proteins, FcrA and Enn (3, 19, 21, 22, 35, 43), and the M protein (reviewed by Kehoe [27]) have been characterized on molecular and genetic levels.

The genes for M protein (*emm*) and C5a peptidase (*scpA*) and, if present, the genes encoding the M protein-related Ig-binding proteins (*fcrA* and *enn*) are clustered on the streptococcal chromosome (18, 23, 41) (Fig. 1). In a serotype M12 strain isolated from a pharyngitis patient, the expression of *emm* and *scpA* was shown to be under the control of the positive regulator gene *virR* (52). The *virR* gene was found to be located upstream of *emm* and *scpA* (51) (Fig. 1a). For consistency with the nomenclature established for other bacterial pathogens, the complete cluster was later tentatively termed the *vir* regulon (12).

Expression of virulence factors by many bacterial species as different as *Bordetella pertussis* or *Listeria monocytogenes* (reviews by Coote [15] and Mekalanos [33]) is controlled by two-component regulatory systems, which respond to environmental stimuli. GAS are exposed to a variety of atmospheric and chemical conditions as they reside on the skin and mucous membranes or in the bloodstream of the human host. Accordingly, the functional requirements for expression of the different streptococcal surface proteins should vary. In fact, in parallel experiments that either exposed GAS strains to human blood or isolated them from the spleen of animals with experimental skin infections, a variation of the expression patterns of M proteins and Ig-binding proteins could be demonstrated by Raeder and Boyle (47, 48). Because of the control of M protein expression by VirR and the association of VirR activity with environmental parameters such as anaerobic atmosphere and carbon dioxide tension (6, 34, 45), VirR is thought to be a part of a crucial regulatory system in GAS, possibly functioning as the second component in a two-component regulatory system. The one or several VirR-interacting receptors for external stimuli remain unidentified.

Traditionally, two distinct categories of GAS were discriminated by the ability of the bacteria to express serum opacity factor (OF). OF production (OF^+) or nonproduction (OF^-) was found to correlate stringently with the expression of class II and I M proteins, respectively (which are distinguished by the reactivity of their C termini with monoclonal antibodies [3]) and loosely with other clinical and molecular characteristics of the bacteria. For instance, OF^+ GAS were often isolated from skin infections and shown to express Ig-binding proteins, whereas OF^- strains were demonstrated to be associated with pharyngitis and not to express Ig-binding proteins (2, 55). With increasing data on the GAS genomic organization (18, 23, 41), it became obvious that OF^+ strains exhibit a uniform structure of their *vir* regulons, which always harbor *fcrA*, class II *emm*,

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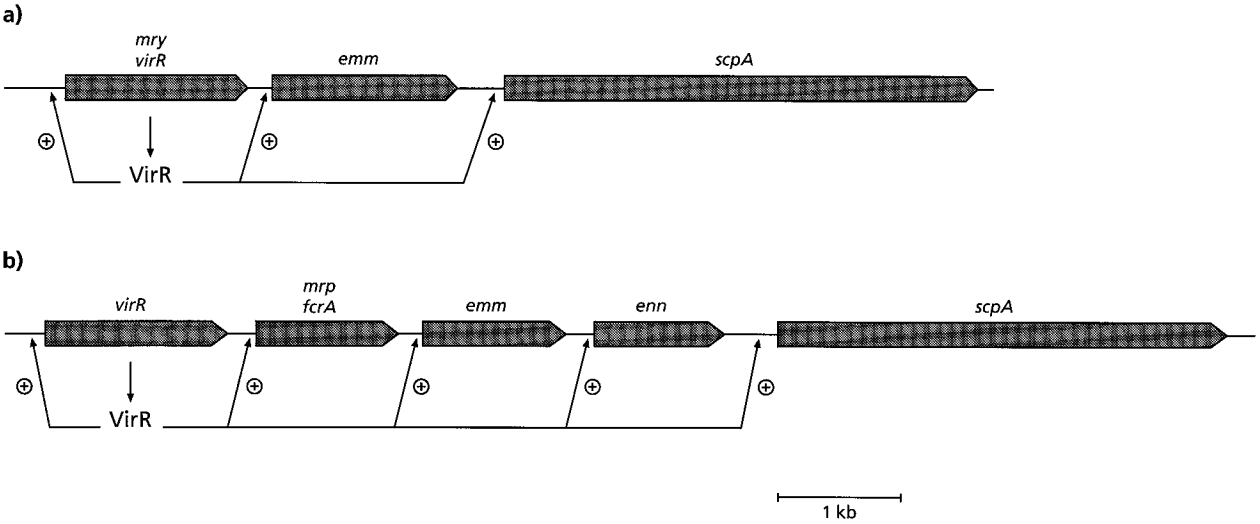

FIG. 1. Maps of representative GAS *vir* regulons. (a) Map of a *vir* regulon from selected OF^- GAS, e.g., serotypes M3, M6, and M24 (41). The product of the *virR* gene (equivalent name, *mry* [37]) regulates positively the expression of itself and of the genes encoding M protein (*emm*) and C5a peptidase (*scpA*) (52). In serotypes M1 and M12 GAS, the regulatory control is identical, but there are additional undefined sequences between *emm* and *scpA* (41). (b) Map of a *vir* regulon from OF^+ GAS. Here the product of the *virR* gene controls the expression of the genes encoding the FcrA (*fcrA*; equivalent name, *mrp* [35]) and Enn (*enn*) proteins in addition to *emm*, *scpA*, and probably *virR* (this study). Both maps were drawn to scale.

and *enn* genes (Fig. 1b). In contrast, the *vir* regulon architecture of OF^- GAS could be associated with several different forms. The loci of these strains always contain a class I *emm* gene and frequently do not include *fcrA* and/or *enn* genes.

Thus far, only *virR* genes of OF^- GAS serotypes not harboring *fcrA* or *enn* genes have been analyzed. In addition to *virR12* (51, 52), an equivalent locus termed *mry* (for *emm* mRNA yield) was identified as regulating *emm* expression in a serotype M6 GAS strain (7). Comparison of the nucleotide sequences of *mry* (37) and *virR12* (9) revealed 97% homology. Also, a partial *virR1* sequence (20) was nearly identical to the *virR12* sequence. By utilizing Southern hybridizations and functional assays subsequent to directed mutagenesis experiments, Perez-Casal et al. (38) demonstrated the presence of *virR* genes homologous to *mry* in one strain each of serotypes M1 and M3.

The locations of the *mry* promoters were deduced subsequent to the identification of the 5′ ends of the specific mRNAs by performing S1 nuclease protection assays (34). The *mry* gene was found to possess two promoters, P_1 and P_2, located approximately 410 and 120 bp, respectively, upstream of the *mry* start codon. P_1 exhibited a weak constitutive activity, whereas P_2 was demonstrated to be a comparatively strong promoter which needed induction by the Mry protein. Recently, *mry* expression was described as being potentially even more complex since under special experimental conditions the P_1 promoter showed no constitutive activity (17).

A recombinant *mry* gene was the first *virR*-type gene which was expressed in an in vitro transcription-translation experiment (37). However, only after establishing recombinant reporter plasmids containing the *emm12* and *scpA12* promoters, in the serotype M12 GAS strain CS24 could the *trans*-regulating activity of VirR be unambigously demonstrated (45). Also, in that study, potential VirR-binding boxes immediately upstream of the −35 boxes of *emm* and *scpA* promoters of OF^- strains were identified by homology comparisons. Sequences nearly identical to such boxes were also found in intergenic regions upstream of *fcrA*, *emm*, *enn*, and *scpA* genes from OF^+ strains, indicating a similar control in OF^+ strains.

In OF^+ GAS strains, the presence of genomic regions homologous to *virR* has been documented by using two different approaches, hybridization with recombinant internal *virR* fragments (18, 23) and a series of PCR assays (40). A similar conclusion of these studies was that sequences of potential *virR* genes from OF^+ strains diverged considerably from corresponding ones harbored by OF^- strains.

In this investigation, the complete sequence of the *virR* gene region from an OF^+ GAS strain is presented. The isolate chosen for analysis was serotype M49 strain CS101. The architecture of the potential *vir* regulon of this strain has been well documented. Its *fcrA49*, *emm49*, and *enn49* genes have been sequenced previously and shown to encode an IgG1-, IgG2-, and IgG4-binding protein, the M protein, and an IgA-binding protein, respectively (19, 22, 42, 43). In this study, by utilizing insertional inactivation, we provide evidence that *virR49* controls expression of *fcrA49*, *emm49*, *enn49*, and *scpA49* and that it probably regulates its own expression. Upon the loss of *virR49* function, the mutant did not express Ig-binding proteins and became sensitive to phagocytosis by human leukocytes. Finally, by quantifying the different mRNAs, we demonstrated that other factors are apparently also involved in the control of some genes of the *vir* regulon.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Serotype M49 GAS strain CS101 was provided by P. Cleary, Minneapolis, Minn. The isolate was characterized by Haanes and Cleary (19). *Escherichia coli* DH5α (Gibco BRL, Eggenstein, Federal Republic of Germany) served as the gram-negative host of recombinant plasmids.

The GAS isolates were grown in Todd-Hewitt broth (THB; Unipath, Wesel, Federal Republic of Germany) or on Todd-Hewitt agar (TH agar), which is 1.5% agar added to THB. Alternatively, the strains were cultured on blood agar base (Unipath) supplemented with 5% sheep blood. After determining the minimum inhibitory concentration of spectinomycin to be 10 mg/liter for strain CS101 according to National Committee for Clinical Laboratory Standards, TH agar was supplemented with 60 mg of spectinomycin (ICN, Meckenheim, Federal Republic of Germany) per liter for selection of recombinant GAS strains. All GAS cultures were incubated at 37°C in a 5% CO_2–20% O_2 atmosphere.

Recombinant *E. coli* strains were grown in tryptone soy (CASO) broth (Unipath) or on disk sensitivity testing agar (Unipath) supplemented with 100

TABLE 1. Sequences of oligonucleotides used for PCR, sequencing, and primer extension assays

Oligonucleotide designation[a]	Sequence (5′ to 3′)	Position no.[b]	Description of target region	T_m (°C)[c]
a)				
A1	AAA CCG TAT CTT TGA CGC ACT	337–357	Upstream of the *virR49* promoters	50.8
A3	AGG ACA ATT TGC GAG ATT AG	360–379	Upstream of the *virR49* promoters	46.4
A18	AAT TTT AGC CAT GTG ACT CTC	514–494	Upstream of the *virR49* promoters	45.8
A25	GCT CAA TAG GCG TTC ACA G	1614–1596	Central region of *virR49*	48.1
A31	CAT TAA CTT CAT GTC CTT ATC TTA	1055–1033	Upstream of *virR49*	45.2
A37	GAG CAG GCT AGT TTG TTG AC	2–21	Upstream of the *virR12* promoters	46.6
A38	TGA AGT TAA TGT ATG TAA GTA A	1046–1067	5′ End of *virR49*	42.8
A39	TCC TTA ATA TGG TTC ATA CGG	977–997	Upstream of *virR49*	46.5
A40	CGG CAA CTT CGT ATT TAT TG	2014–1995	Central region of *virR49*	48.0
b)				
B39	TTG CTC CTT ATT TTT TCA TC	1657–1638	Upstream of *emm49*	44.2
C26	AGA AAG TCC TAA AAA GCT GGC	2579–2599	Upstream of the *scpA49* promoter	49.4
C27	GAT TCT CTC CTA GCT GTC CA	2787–2768	Upstream of *scpA49*	45.7
E15	CTA AAG TTG TTT TGA GAT CTT C	1765–1744	Central region of *enn49*	42.6
E16	GTA GTC GCG TTT ACC CCT TC	1550–1531	5′ Region of *enn49*	50.7
F15	TCT TGT TGT TTC AAG TCC TCA	794–774	Central region of *fcrA49*	47.2
F16	GTT ACT CTA GGA TGT TCC TGA G	344–323	5′ Region of *fcrA49*	44.3
c)				
pyr for	GAT CCC TTT GGC GGC GA	309–315	5′ Region of the pyrase gene	56.6
pyr rev	GCG CCC CCT ACA CGT TTT A	922–904	3′ Region of the pyrase gene	55.0
slo for	TCT CGT CGC TGG GCT AC	231–247	5′ Region of the streptolysin O gene	55.5
slo rev	GGG GTC AGG GTT GAT CGT G	1899–1871	3′ Region of the streptolysin O gene	54.7
ska for	TCC AAG CTA TTG CTG GGT A	448–466	5′ Region of the streptokinase gene	48.2
ska rev	GGT GTC CCT GTA TAA CGC A	1672–1654	3′ Region of the streptokinase gene	48.1
spec for	GAT TTT CGT TCG TGA ATA CA	4–23	Region upstream of the spectinomycin resistance gene	45.2
spec rev	TTA CCA ATT AGA ATG AAT ATT TC	1124–1102	Region downstream of the spectinomycin resistance gene	43.0

[a] The oligonucleotides were designed with the aid of the OLIGO software.
[b] The nucleotide position numbers of each A primer refer to the *virR49* sequence presented in this study. The other nucleotide position numbers were published previously as follows: A37 (9); B39 (43); C26, C27, E15, and E16 (19); F15 and F16 (43); pyr for and pyr rev (13); slo for and slo rev (28); ska for and ska rev (24); and spec for and spec rev (30).
[c] The median melting temperature (T_m) for hybrids with these oligonucleotides was calculated by the OLIGO software program.

mg of spectinomycin per liter for selection of pSF-based plasmids. Cultures were grown at 37°C in ambient air.

Plasmid. Plasmid pSF152 was provided by L. Tao, Oklahoma City, Okla. This suicide vector contains a plasmid pUC19-derived origin of replication, a pUC18-derived multiple cloning site (54), and a spectinomycin resistance gene (*aad9*) active in gram-positive and gram-negative bacteria (30). The plasmid does not autonomously replicate in GAS and thus can be used as an integrational suicide vector for these bacteria (54).

Conventional DNA techniques. Chromosomal group A streptococcus DNA was prepared by the method of Martin et al. (32), and plasmid DNA from *E. coli* recombinants was prepared as described by Zhou et al. (56). Conventional techniques for DNA manipulations like restriction enzyme digests, ligation, transformation by electroporation, colony blot hybridization, 1% agarose gel electrophoresis, and Southern blotting by vacuum transfer were performed as described by Ausubel et al. (1). Transformation of GAS strain CS101 by electroporation followed the protocol of Caparon and Scott (8).

Special DNA techniques. Oligonucleotides used for primers and probes were designed by the aid of the OLIGO software (National Bioscience, Plymouth, Minn.). This program was also used to calculate the optimum annealing temperature for hybridization and sequencing assays. Sequences and target sites of the oligonucleotides are shown in Table 1 and Fig. 2, respectively.

The oligonucleotides were synthesized on an Oligo 1000 DNA synthesizer (Beckman, Munich, Federal Republic of Germany) and subsequently desalted by running them through a Sephadex G-50 (Pharmacia) minicolumn. If needed as hybridization probes, the oligonucleotides were labeled at their 3′ ends with digoxigenin (Dig)-dUTP and terminal transferase (Boehringer GmbH, Mannheim, Federal Republic of Germany) as recommended by the manufacturer.

Amplification of specific DNA fragments by PCR was based upon the optimized conditions described by Erlich et al. (16). PCR products for DNA sequencing were generated by using only 5 pmol of each primer in a 100-μl assay. Occasionally, one primer of a pair was biotinylated at its 5′ end. If necessary, PCR products were directly labeled by adding Dig-dUTP (Boehringer) at a final concentration of 500 nM to the assays.

DNA sequencing. PCR products and recombinant plasmids for sequencing

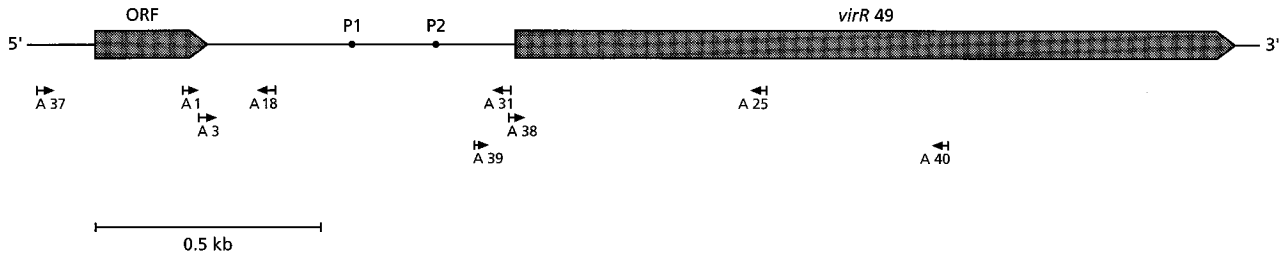

FIG. 2. Location of target sites for selected oligonucleotides within the *virR49* genomic region. On the basis of the results of the present study, the genomic region of serotype M49 GAS strain CS101 encompassing a potential ORF and the *virR49* gene is shown. Upstream of *virR49*, the sites of the two *virR* promoters P1 and P2 are shown. Below the map, arrows indicate the orientation and location of oligonucleotides used for PCR assays, RNA analysis, and insertional mutagenesis experiments with respect to their target within the genomic region. The map was drawn to scale. Sequences and descriptions of the oligonucleotides are given in Table 1a.

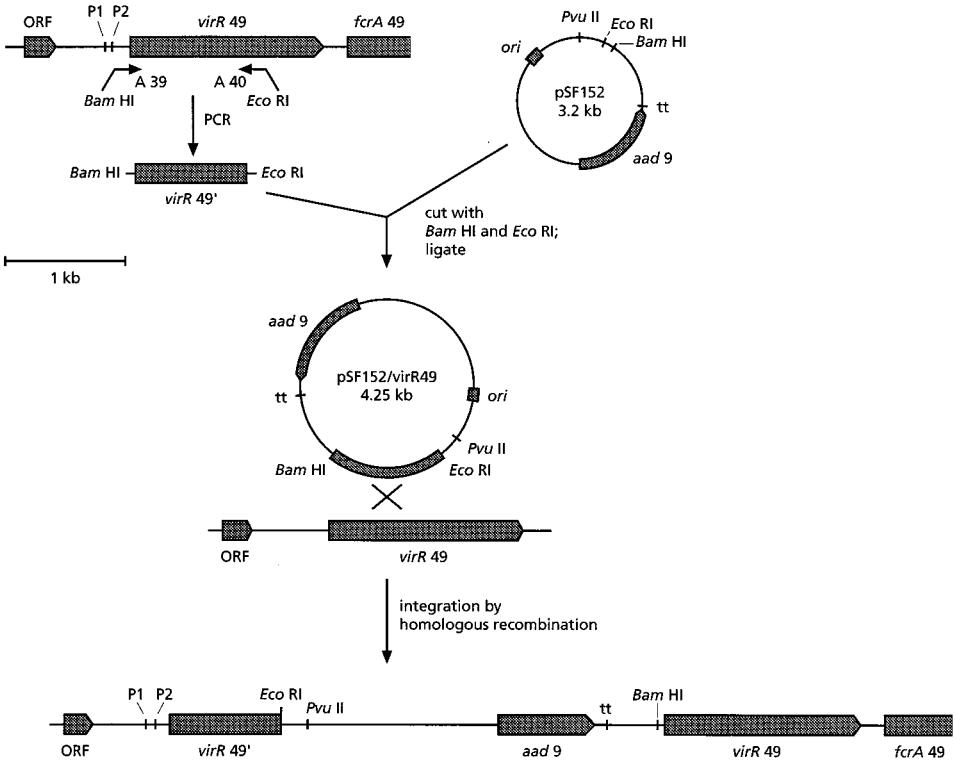

FIG. 3. Strategy for insertional mutagenesis of the *virR49* gene. An internal fragment of *virR49* was amplified by PCR (*virR49′*) and was forced-cloned via *Bam*HI and *Eco*RI 5′ tags of primers A39 and A40 (Table 1a), respectively, into the corresponding sites of plasmid pSF152 (54). This plasmid harbors a pUC19-derived origin of replication (*ori*) and thus is able to replicate in *E. coli* but not in GAS. Conversely, the spectinomycin resistance gene (*aad9*) of pSF152 includes its own transcription terminator (tt) and is active in both gram-negative and gram-positive bacteria (30). Under selective pressure in GAS, the recombinant pSF152/virR49 plasmid is specifically integrated into the genome, thereby creating a *virR49′* gene truncated by its 3′ half and another *virR49* gene without an active promoter. The map was drawn to scale. P1 and P2, regular *virR49* promoters.

were purified by using the Wizard DNA purification system (Promega, Heidelberg, Federal Republic of Germany) and sequenced with *Taq* polymerase and the Prism kit (Applied Biosystems, Weiterstadt, Federal Republic of Germany) as described in the instructions of the manufacturer. Sequencing products were analyzed on a model 373A automated DNA sequencer (Applied Biosystems); sequences were compiled and processed by using the PCGENE software (IntelliGenetics, Mountain View, Calif.). In the case of primer extension experiments, 5′ biotin-labeled PCR products to be used as templates to generate size standards upon sequencing were purified by the Streptavidin-Dynabead technique (Dynal, Hamburg, Federal Republic of Germany) and sequenced with [α-^{35}S]dCTP and the T7 polymerase sequencing kit (Pharmacia), again as described in the instructions of the manufacturer.

Insertional inactivation experiments. The specific insertional inactivation of *virR49* was performed with a recombinant integrational vector, pSF152 (54). The generation of the recombinant pSF152 plasmid and its integration into the genome of strain CS101 are shown in Fig. 3. The specific integration of the plasmid into *virR49* in the resulting strain vir-mut was confirmed by Southern blot hybridizations using *Bam*HI-, *Cla*I-, and *Xba*I-digested genomic DNAs from strains CS101 and vir-mut as targets and directly labeled PCR products generated with primers A39 and A40 and spec for and spec rev (Table 1) as probes.

RNA preparation. For RNA preparation from GAS strains, 10 ml of THB was inoculated with bacteria from fresh overnight cultures and incubated in a 5% CO_2–20% O_2 atmosphere at 37°C until the optical density at 600 nm reached 0.6. Cells were harvested by centrifugation at 4°C and washed briefly twice in ice-cold 0.2 M sodium acetate. The bacteria were resuspended in 500 μl of 100 mM Tris (pH 7.0)–1 mM EDTA–25% glucose. After adding 400 μl of lysozyme (2 mg/ml) and 10 μl of mutanolysin (5,000 U/ml of 0.1 M potassium phosphate [pH 6.2]), the suspension was incubated for 5 min at 37°C and again collected by centrifugation. The pellet was suspended in 500 μl of acetate buffer (20 mM sodium acetate [pH 5.5], 1 mM EDTA, 0.5% sodium dodecyl sulfate). After adding 1 volume of acetate buffer-saturated phenol, the cells were disrupted by keeping them at 60°C for 5 min while vortexing them for a few seconds every minute.

The supernatant was transferred to a new tube, and the phenol extraction was repeated one more time; a chloroform-isoamyl purification step followed. The RNA was sedimented by the addition of 3 volumes of ice-cold ethanol and washed twice with acetate buffer. The RNA pellet was dissolved in pure formamide (11) and stored at −20°C. For further processing, the RNA was pelleted by the addition of 4 volumes of ethanol, subsequently dissolved in diethylpyrocarbonate-water, incubated for 15 min at 37°C with RNase-free DNase and RNase inhibitor (Boehringer Mannheim), and finally extracted with phenol as described above.

RNA analysis. For quantitative Northern (RNA) blot analyses, the RNA was serially diluted in diethylpyrocarbonate-water and run on denaturing 1% agarose gels (46). RNA size markers were obtained from Gibco BRL. Transfer to nylon membranes (Biodyne B; Pall BioSupport, Dreieich, Federal Republic of Germany) was achieved by vacuum blotting using 25 mM sodium phosphate (pH 6.5) as the transfer buffer. Complete transfer was controlled by ethidium bromide stain and visual inspection of the gel for residual rRNA bands after 1.5 to 2 h of incubation under negative pressure. After UV cross-linking (Stratalinker; Stratagene, Heidelberg, Federal Republic of Germany) the RNA to the membranes, the blots were hybridized with Dig-dUTP-labeled PCR products and visualized by using the Lumiphos system (Boehringer). Specific transcripts were quantified by assessing the last dilution step of total RNA with which a band of appropriate size could be visualized. For each quantitation, at least three independent RNA preparations were analyzed. The obtained values for each kind of transcript were in the range of one dilution step.

Primer extension experiments and S1 nuclease protection assays were performed with 5 or 20 mg of total RNA per assay, respectively. The oligonucleotides necessary for primer extension experiments and for generating PCR products to be used in the S1 nuclease protection assays are shown in Table 3. The experimental procedures followed the protocols outlined by Podbielski et al. (46). Superscript2 reverse transcriptase and S1 nuclease were purchased from Gibco BRL and Boehringer, respectively. An A31 oligonucleotide (Table 1a)-primed sequencing reaction of a PCR product containing the *virR49* promoter region served as a size reference. This PCR product was generated by employing a 5′ biotinylated primer A1 (40) and a regular primer A31 (Table 1a), purified, and directly sequenced as described above.

Phagocytosis inhibition assay. A direct bactericidal assay described by Lancefield (29) was performed to measure resistance to phagocytosis by human granulocytes of M49 wild-type strain CS101 and isolate vir-mut.

Nucleotide sequence accession numbers. The *virR49* and *scpA49* sequences were submitted to the EMBL data library and have been assigned the accession numbers X68501 and X78055, respectively.

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                10        20        30        40        50        60        70        80        90
VirR49      MHVSKLFTSQQWRELKLISYLTENSNAIGVKDKELSKALNISMLTLQSCLTNMQFMKEVGGITYKDSYINIWYHQCCGLQEVYQKALRESPSLKLLELLF 100
Mry(VirR6)  .Y.................V...AD.....................T.................NG..T.....H............H.Q.F....T.. 100
VirR12      .Y.................V...AD.....................T.................NG..T.....H............H.Q.F....T.. 100

                 |-helix-turn-helix-|
VirR49      FRDFSSLEELAEELFVSLSTLKRLIKKTNTYLSHTFAISIVTSPVQVSGDERQIRLFYLKYFSEAYKISEWPFGDILNLKNCERLLSLLIKEVDVKVHFT 200
Mry(VirR6)  ....N.......................A..T...G.T.L..........H.....................E.............M........N.. 200
VirR12      ....N.......................A..T...G.T.L..........H.....................L.EM............M........N.. 200

VirR49      LFQHLKILSGVNLIRYYKGYSCSYNNKKTSHRFSQLIQTSSEIQDLSRLFYLKFGLHLDEYTIAEMFSNHLNDKLEIGCAFEIINQDPTSGGRQVTNWIH 300
Mry(VirR6)  .........S...........AV.D.............S.L..............Y...T.........V..Q....Y..DS.K..SPT.C.K..... 300
VirR12      .........S...........AV.D.............S.L..............Y...T.........V..Q....Y..DS.K..SPT.C.K....V. 300
VirR1                                                ..H....SY...T.........V..Q....Y..DS.K..SPT.C.K....V.  51

VirR49      LLDEMEIKLNLSITNKYEVAVTLHNASVLNEEDITANYLLFDYKKSYLNFYQKEHPRIYEAFVTSVEKLMQADNAQVSKELINQLTYCFFITWENSFLKV 400
Mry(VirR6)  ....L..N....V........I...TT..K.........F...........KQ...HL.K...AG.....RSEKEPI.T..T...I.A..... 400
VirR12      ....L..R....V........I...TT..K.........F...........KQ...HL.K...AG.....RSEKEPI....T...I.A..... 400
VirR1       ....L..R....V........I...TT..K.........F.....G....NKQ...IL.-...AG.....RSEKEPI.T..T...I.A..... 150

VirR49      NQKDEKVRLLVIERSYNSVGNFLKKYIGEFFSITNFDELDCLTIDLVEIEKQYDVIVTDVMVGKSEELEIFFFYKMIPEAIIDRLNEFLNVSFTDNNVMV 500
Mry(VirR6)  ......I.....A.RF...................N...A.....E.................D.................K..V...I..A.SLPLD 500
VirR12      ......I........F...................N...A.....E.................D.................K..A...I..A.SLPLD 500
VirR1       ......I........F.........V........N...A.....E.....................H.........K..A...I..A.SLPLD 250

VirR49      KNLEAPSSSKSHSDKEVQKPEKPDNSVKQATSS  533
Mry(VirR6)  .PIKN.LDFHRKELTLPTP.N.LHAPPSTT     530
VirR12      .PIKN.LDFHRKEVILPTP.N.LHAPPSTI     530
VirR1       .PI-N.LDFHRKEVILPTP.N.LHAPPSTT     279
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FIG. 4. Comparison of amino acid sequences deduced from different GAS *virR* gene sequences. The sequences were taken from Haanes-Fritz et al. (20) (VirR1), Perez-Casal et al. (37) (Mry), Chen et al. (9) (VirR12), and this study (VirR49). Positions of identical residues are marked by periods. Gaps marked by dashes were introduced into the VirR1 sequence to obtain optimum alignment. A potential helix-turn-helix motif identified by computer analysis is marked above the corresponding positions.

RESULTS

Sequencing and analysis of the *virR49* gene region. On the basis of the *virR12* sequence (9), a set of PCR primers was designed to amplify the corresponding regions in OF^- and OF^+ GAS strains of 36 different serotypes (40). While regions corresponding to DNA segments approximately 200 bp upstream of *virR12* and to the 3′ end of *virR12* appeared to diverge in the different serotypes, another region at 600 to 650 bp upstream of *virR12* was found to be well conserved in all strains (40). Therefore, primer A1 directed to this region (Table 1; Fig. 2) and primer B5 directed to the 5′ end of *emm49* (41) were used to amplify the intervening *virR49* and *fcrA49* gene regions from strain CS101. Then, sequences were generated and assembled as shown in Fig. 4 and 5 by employing primers listed by Podbielski (40) as well as 12 newly designed oligonucleotides (not shown). In a separate experiment, primers A18 and A37 (Table 1a; Fig. 2) were used for PCR amplification and sequencing of a region from approximately 650 to 1,050 bp upstream of the *virR49* gene (Fig. 5).

Within the entire sequenced region, one large open reading frame (ORF) of 1,599 bp, starting at residue 1054, was identified. This ORF exhibited approximately 76% overall homology to the *virR12* and *mry* sequences. When comparing the deduced amino acid sequences of these three ORFs (Fig. 4), the first 484 residues showed 82.3% homology, whereas the 40 C-terminal residues of VirR49 differed completely from the VirR1, VirR12, and Mry (VirR6) sequences. The C-terminal 40 codons of *virR49* were found to be perfectly conserved in more than 20 other OF^+ serotypes (data not shown) and thus could be generally regarded as specific for *virR* genes of OF^+ GAS strains.

A computer analysis for signature sequences predicted a potentially DNA-binding helix-turn-helix motif at amino acid residues 107 to 126 of the VirR49 sequence. In this region, VirR12 and Mry exhibited the largest stretch of contiguous sequence identity to VirR49 (Fig. 4).

Comparison of the available sequences upstream of *virR49*, *virR12*, and *mry* demonstrated sequence divergence of the regions between the *virR* and *mry* start codons and residues approximately 400 bp further upstream (Fig. 5). Okada et al. (34) mapped the P2 promoter of *mry* within this region. Except for a small segment which contains an inverted repeat in the *virR12* sequence (9), the sequences of those regions were nearly identical in the OF^- serotypes M12 and M6 strains, whereas the OF^+ serotype M49-derived sequence differed from the others by 30.1 and 28.5%, respectively (Fig. 5). Further upstream (>400 bp) of that region, the sequences from the M49 and M12 strains were nearly identical. These highly homologous segments contained an additional 83-amino-acid residue ORF possessing a Shine-Dalgarno box and an ATG start codon. While no consensus −35 and −10 boxes could be located upstream of this ORF, an inverted repeat and a poly(T) sequence characteristic for factor-independent transcription termination sites (49) was found downstream of the ORF (Fig. 5).

Analysis of the *virR49* mRNA. To confirm that a specific mRNA was transcribed from the *virR49* ORF, total RNA was purified from strain CS101 after one passage in the presence of human blood. Forty micrograms of this RNA was subjected to denaturing agarose gel electrophoresis. Subsequent to Northern blotting, the RNA was hybridized with a directly Dig-dUTP-labeled PCR product (Table 2). Two *virR49* transcripts of 1.7 and 1.9 kb could be visualized (Fig. 6a). By utilizing a serial dilution of the CS101 RNA, the minimum amount of total RNA necessary for detection of the *virR49* transcripts was assessed as 20 μg in our experimental format (Table 2).

Considering the length of the sequenced *virR49* gene, the size of the mRNAs indicated a monocistronic transcription of *virR49*. For the localization of the *virR49* promoters and transcription termination site, the exact 5′ ends of the transcripts were mapped by an S1 nuclease protection assay.

The resulting autoradiography of the S1 nuclease protection assay demonstrated two products with differing intensities (Fig. 6b). The weaker band represented the transcript from a P1

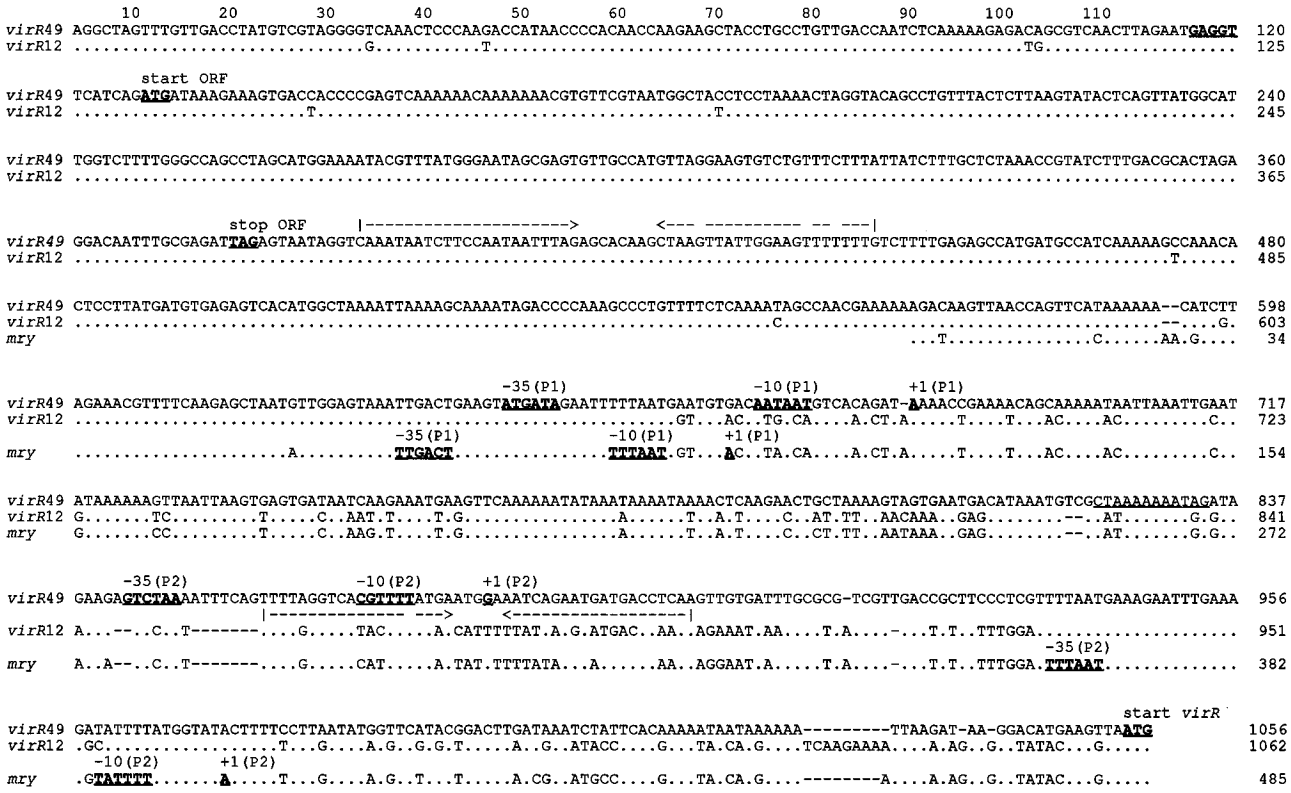

FIG. 5. Comparison of nucleotide sequences upstream of GAS *virR* genes. The sequences were taken from Perez-Casal et al. (37) and Okada et al. (34) (*mry*), Chen et al. (9) (*virR12*), and this study (*virR49*) and are aligned to the ATG start codon of *virR49* at bp 1054. Sequence numbering refers to the corresponding publications. Residues identical to those of the *virR49* sequence are marked by periods. Gaps marked by dashes were introduced for maximum alignment. Shine-Dalgarno box, start and stop codons of an upstream ORF, the −35 and −10 boxes, and the transcription start sites of the experimentally identified *virR49* and *mry* promoters P1 and P2 and the *virR* and *mry* start codons are underlined and printed in boldface type. A potential VirR49-binding site (45) upstream of the *virR49* P2 promoter is underlined. Inverted repeats present in either the *virR49* and *virR12* sequences or only the *virR12* sequence are indicated by arrows above the corresponding positions.

virR49 promoter; the stronger band represented that from a P2 *virR49* promoter. The corresponding transcription start sites are located 370 and 174 bp, respectively, upstream of the *virR49* start codon (Fig. 5).

The lack of an inverted repeat combined with a poly(T) sequence within the 3′ deduced end of the *virR49* transcripts suggested the absence of a factor-independent transcription termination site for *virR49* (49).

Analysis of transcripts from *vir* regulon genes. For the analysis of a potential control of *virR49* on the expression of other *vir* regulon genes, the transcripts from these genes had to be characterized and quantified. For a Northern blot analysis of these transcripts, 20-μg aliquots of the RNA preparation from CS101 were run on denaturing agarose gels, transferred onto nylon membranes, and hybridized with PCR-generated genomic probes (Table 2). Specific mRNAs could be detected from the four *vir* regulon genes *fcrA49*, *emm49*, *enn49*, and *scpA49*. The lengths of the transcripts were consistent with a monocistronic transcription of each gene (Table 2; Fig. 7).

Contrary to previously published results (40), the size of the *scpA49* gene resembled that of *scpA12* (3,501 bp [10]) as judged by the length of the specific transcript. The *scpA49* gene was completely sequenced (EMBL data library accession no. X78055) and found to exhibit 98% homology to *scpA12*. The main difference was located in the six 3′ terminal codons of *scpA12*. Here, the corresponding *scpA49* sequence showed no homology and would encode a protein with 3 amino acids fewer at its C terminus.

TABLE 2. Transcript analysis of *vir* regulon and non-*vir* regulon genes

Transcript origin	Primer pair used for generation of probe	Sequence source or reference	Transcript size(s) (10^3 bases)[a]	Minimum amt of total RNA (μg) necessary for transcript detection[b]
virR49	A38	Table 1a	1.7 and 1.9	20
	A25	Table 1a		
fcrA49	F5	43	1.5	0.6
	F15	Table 1b		
emm49	B14	43	1.5	0.13
	B18	42		
enn49	E4	43	1.4	5
	E15	Table 1b		
scpA49	C1	40	3.8	1.3
	C4	40		
pcp	pyr for	Table 1c	0.8 and 2.7	NA[c]
	pyr rev	Table 1c		
slo	slo for	Table 1c	3.0	NA
	slo rev	Table 1c		
ska	ska for	Table 1c	2.0	NA
	ska rev	Table 1c		

[a] Transcript sizes were assessed by comparison with an RNA size marker (Gibco BRL) run in parallel in Northern blot experiments.

[b] Minimum amounts of total RNA necessary for detection of specific transcripts were measured by subjecting serial dilutions of RNA to Northern blot hybridizations and subsequent visual inspection for the presence of specific bands in each dilution step.

[c] NA, not assessed.

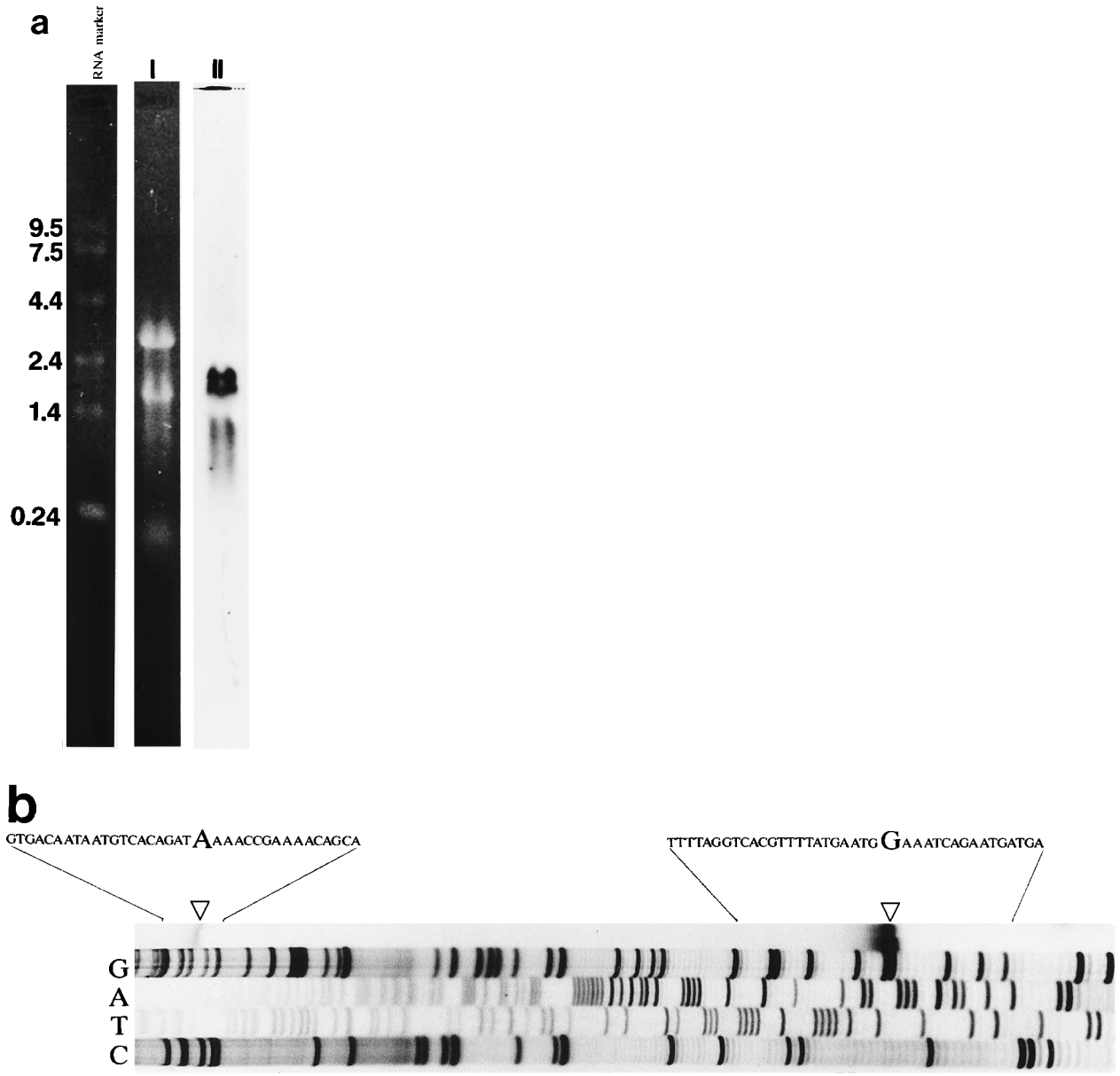

FIG. 6. Analysis of *virR49* mRNA. (a) Denaturing agarose gel electrophoresis, ethidium bromide stain (lane I), and subsequent Northern blot hybridization of total RNA from M-type 49 GAS strain CS101 with a directly labeled PCR product generated with primers A38 and A25 (Tables 1a and 2) (lane II). The sizes (in kilobases) of the bands of the ethidium bromide-stained bands of the RNA marker are indicated on the left. Two specific *virR49* transcripts of 1.7 and 1.9 kb could be distinguished in lane II. (b) ^{32}P-labeled products from an S1 nuclease protection assay on total RNA from M-type 49 GAS strain CS101 run in parallel to a corresponding sequencing reaction of the *virR49* promoter region. The primers necessary for the protection assay and the sequencing reaction are listed in Tables 1a and 3. The generation of appropriate PCR products used for both reactions is described in Materials and Methods and Results. The 5′ ends of the two transcripts correspond exactly to the nucleotide positions indicated by the sequencing products of identical size. The deduced P1 and P2 promoters are shown in Fig. 5. Of note, the intensities of the bands generated by the two protection products indicated a relatively strong P2 promoter and a weak P1 promoter.

Next, the transcripts of *vir* regulon genes were quantified by serially diluting the RNA preparation from CS101 prior to Northern blot hybridizations and then assessing the minimum amount of total RNA necessary to detect the specific mRNAs. For each of the four *vir* regulon genes, different transcript amounts were measured (Table 2; Fig. 7). Under the conditions chosen for incubation of the bacteria prior to RNA preparation, the *emm49* message exceeded the messages of *virR49*, *enn49*, and *fcrA49* by factors of 150, 16, and 4, respectively.

Since the different transcript amounts of the *vir* regulon genes could be due to different structures of their promoters, the promoters of the four genes were mapped by experimental means. The primers used for extension experiments and to generate PCR products to be employed in S1 nuclease protection assays are shown in Table 3. The sequencing reaction used for the mapping of the *virR49* promoters served as a size reference.

For the three *emm* (-related) genes, both assays worked equally well and gave consistent results (Fig. 8). Only when mapping the *scpA49* promoter did the primer extension not lead to interpretable bands, probably because of an inverted repeat located in the 5′ untranslated segment of the *scpA49*

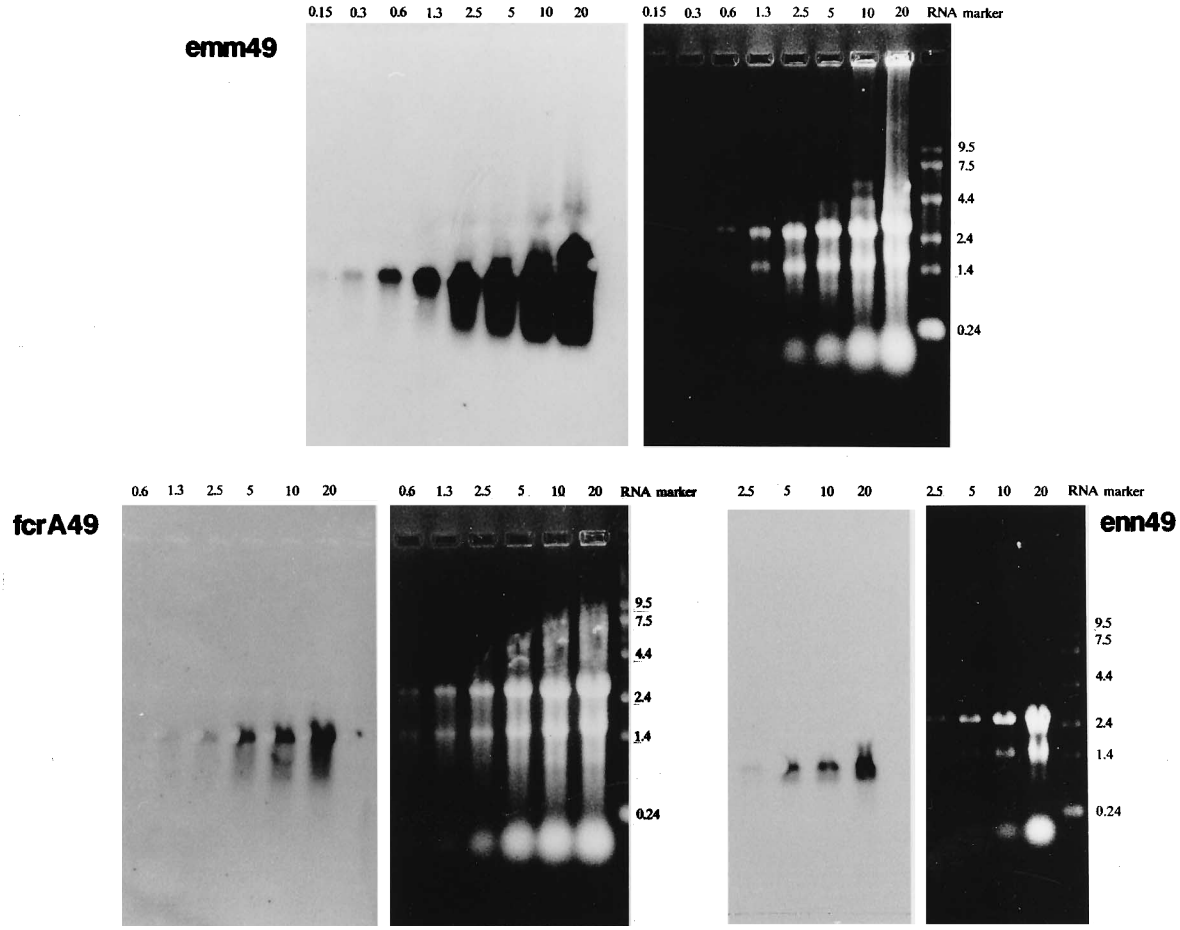

FIG. 7. Identification and quantification of transcripts from serotype M49 *emm* (-like) genes. Serial dilutions of total RNA from GAS serotype M49 strain CS101 were subjected to denaturing agarose gel electrophoresis, subsequent Northern blot transfer, and, finally, hybridization with nonradioactively labeled probes specific for the *fcrA49*, *emm49*, and *enn49* mRNAs. Photographs of ethidium bromide-stained agarose gels are shown adjacent to the corresponding Northern blots. Above each lane, the amount of total RNA (in micrograms) applied to the gel is indicated. The sizes of the fragments of the RNA marker are given in kilobases. Of note, the ratios of specific monocistronic mRNAs contained in a preparation of RNA from GAS grown to the mid-log phase indicate that *emm49* is expressed at a 4-fold-higher level than *fcrA49* and a 16- to 32-fold-higher level than *enn49*.

mRNA. A corresponding inverted repeat has been reported to prevent successful primer extension for mapping of the *scpA12* promoter (45).

For the *fcrA49*, *enn49*, and *scpA49* transcripts, a single band for each was detected as extension and/or protection products. The deduced promoter sites (Fig. 8) corresponded exactly to the ones predicted by a homology search (45). The *emm49* message consisted of two products exhibiting a four-nucleotide difference in length.

Control of *vir* regulon genes by *virR49*. To test the influence of *virR49* on the expression of *vir* regulon genes, this gene was insertionally inactivated, resulting in strain vir-mut (Fig. 3). The amounts of *vir* regulon mRNAs and surface Ig-binding proteins as well as the resistance to phagocytosis of the CS101 wild-type and vir-mut strains were measured and compared.

At first, equal amounts (20 to 40 μg) of total RNA from strains CS101 and vir-mut were subjected to comparative Northern blot hybridizations by using PCR-generated probes (Table 2) directed to five *vir* regulon genes and three other GAS genes (Fig. 9).

Although transcripts of each of the *virR*, *fcrA*, *emm*, and *enn* genes of wild-type strain CS101 were clearly visible, no mRNA from these genes could be detected in the total RNA of strain vir-mut. While still detectable, the amount of wild-type *scpA49* message exceeded that from strain vir-mut. By utilizing quantitative Northern blot hybridization, the difference was measured to be a factor of 8 to 16 (data not shown).

To demonstrate that equal amounts of total RNA from both strains were tested and that the insertion of the recombinant pSF152 plasmid into the genome of strain vir-mut had only limited and specific effects on control of VirR-related transcription, the mRNAs from three other streptococcal genes were also compared by applying the methods described above. In both strains, the transcripts from the streptokinase (Fig. 9), streptolysin (not shown), and pyrase (not shown) genes were found to be of identical lengths and present in equal amounts within the range of one dilution step.

The FcrA and Enn proteins of wild-type CS101 were found to bind Igs at their Fc ends (22, 42, 43). As judged by the absence of specific mRNAs, these two proteins would not be expressed by strain vir-mut, and thus, the Ig-binding activity of this strain should be reduced. Therefore, the vir-mut strain was tested for expression of Ig-binding proteins. In vir-mut-derived preparations of surface proteins, no binding of polyclonal IgG or IgA could be detected (5).

The expression of M protein by GAS is currently thought to

TABLE 3. Primers and PCR products used for promoter mapping studies

Analyzed promoter	Results from[a]:			
	Primer extension analysis		S1 nuclease protection assays	
	Oligonucleotide	Sequence source	Oligonucleotide	Sequence source or reference
virR49	No result obtained		A3	40
			A31*	Present study (Table 1a)
fcrA49	F16*	Present study (Table 1b)	A11	40
			F16*	Present study (Table 1b)
emm49	B39*	Present study (Table 1b)	A11	40
			B5*	41
enn49	E16*	Present study (Table 1b)	B13	43
			E16*	Present study (Table 1b)
scpA49	No result obtained		C26	Present study (Table 1b)
			C27*	Present study (Table 1b)

[a] Primers marked with an asterisk were labeled with ^{32}P at their 5′ ends prior to further use.

be predominantly responsible for the resistance of the bacteria to phagocytosis by human leukocytes. Because of the absence of *emm49* mRNA in strain vir-mut, its M49 protein would not be expressed, and therefore, this isolate should have become sensitive to phagocytosis. In three consecutive phagocytosis assays performed with strains CS101 and vir-mut, the number of CS101 bacteria increased from 80 ± 20 to 4,000 ± 1,000 (an average factor of 50) during the entire 3 h of incubation, whereas the number of vir-mut bacteria was consistently reduced from 80 ± 20 to 0 during this period of incubation in the presence of human leukocytes.

DISCUSSION

Depending on the countries surveyed and the diseases on which the investigations were focused, about half of GAS strains isolated from human specimens exhibit an OF^+ phenotype (14, 25). Such strains were found to be predominantly associated with skin infections (55).

Contrary to many pharyngitis-associated OF^- GAS, OF^+ GAS are known to contain additional *emm*-related genes adjacent to *emm* genes on their genomes (18, 23, 41). In accordance with the control of *emm* genes from OF^- GAS, the cluster of *emm* (-related) genes from OF^+ GAS was postulated to be under transcriptional control of a potential *virR* gene (12). But, to date, neither the existence of a *virR* gene in OF^+ GAS nor its control of the expression of other genes has been shown unambiguously.

Thus, the major objectives of the present study were to analyze the *virR* gene from an OF^+ GAS strain, characterize its role in the expression of *emm* (-related) and *scpA* genes, and, finally, by proving individual control of monocistronically transcribed genes to justify the term *vir* regulon for this genomic region in OF^+ GAS strains.

In previous studies, sequences exhibiting some homology to *virR* genes were found to be located upstream of the *emm*-related *fcrA* genes in OF^+ GAS (18, 23, 40). After sequencing 2,650 bp of this genomic region from M-type 49 GAS strain CS101, we could show the existence of an ORF encoding 533 amino acids and exhibiting the same transcriptional orientation as the *emm*-related genes located downstream of it. Comparison of the length and sequence homologies of the ORF with those of other GAS sequences indicated the *virR6*-equivalent *mry* gene to be its closest relative. However, a 24% sequence difference would place this potential *virR49* gene in a

```
        10         20         30         40         50         60         70         80         90
TAGGATTTCAGACGTCATGGTAAGAAAAAAAGAATCATTTACAGCCCATAGAGGTAAGGTCAAAAGCTGAAAACAGCTCAAAAAAACTGACCTTTACCTT  100
TTGGCTTTTTTTTATTTAGAATAATTTTATTGGAGAGATGCTTAATAATTTAAGCACAATTCTTAGAAAGTTAAGAATAAGGAGTAAACAATG (fcrA49) 192

TAAGCCCAACCCACACTATCTTTTCTAGCCCAAGAAAAAAACAAAAAAAGAGGAAGACCATTCCTCTTTTTTTGAACGGTTAAACAGCAAAAAGGTCAAA  100
AAGGTACTAAAGTCTCAAAACCTGGTCTTTACCTTTTACCGCTTATTATTTAGAATAGAATTATTAGAGAGAAGTCTTAGAAAAATGAGGCTAATTCCCT  200
AAAGATGAAAAAATAAGGAGCAAATAATG (emm49)                                                                  229

TAAGCTATTAGACTGATGCTAAAGCTAAGAGAGAATCAAATGATTCTCTCTTTTTGAGTGGCTAAGTAACTAACAATCTCAGTTAGACCAAAAAATGGGA  100
ATGGTTCAAAAAGCTGGCCTTTACTCCTTTTGATTAACCGTATATAATAAAAACATTAGGAAAATAATAGTAATATTAAGTTTGTTTCCTCAATAAAATC  200
AAGGAGTAGATAATG (enn49)                                                                                215

TAAGCCTTTAAAACTTGGTTTTTGTAACGGTGCAATAGACAAAAGCAAGCAAGGCCAAAAACTGAGAAAGTCCTAAAAAGCTGGCCTTTACCCCTCAAAA  100
TTAATGTTTTATAATAAAGATGTTAGTAATATAATTGATAAATGAGATACATTTAATCATTATGGCAAAAGCAAGAAAAATAGCTGTATCATATGCAAAT  200
AACCCCTGTTTGCTCTTTAAAAAAGACGTTATCCTTATTTCTCCACGCACAGATGGACAGCTAGGAGAGAATCGTTTGATTCTCTCTTTTCTTAATGGTC  300
ATAAAGACAAAGTCTCTCATCATGAAAGGACGACACATTG (scpA49)                                                      340
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FIG. 8. Localization of the transcript start sites and deduced promoters of structural *vir* regulon genes. On the basis of transcript analysis performed with total RNA from M-type 49 GAS strain CS101, the transcription start sites of the *fcrA49*, *emm49*, *enn49*, and *scpA49* genes were mapped. The primers and PCR products employed in primer extension experiments and S1 nuclease protection assays are listed in Tables 1b and 3. The stop codons of upstream genes, the start codons of the structural genes analyzed, the −35 and the −10 boxes, and the transcription start sites are printed in boldface type and are underlined. Of note, a second location of the 5′ end of the *emm49* mRNA was mapped 4 nucleotide positions downstream of the 5′ end of the major product, indicating the potential existence of a second promoter for *emm49*. Potential VirR-binding boxes (45) are underlined. The sequences were taken from Podbielski et al. (43) (*fcrA49* promoter region), Haanes et al. (18) (*emm49* promoter region), and Haanes and Cleary (19) (*enn49* and *scpA49* promoter regions).

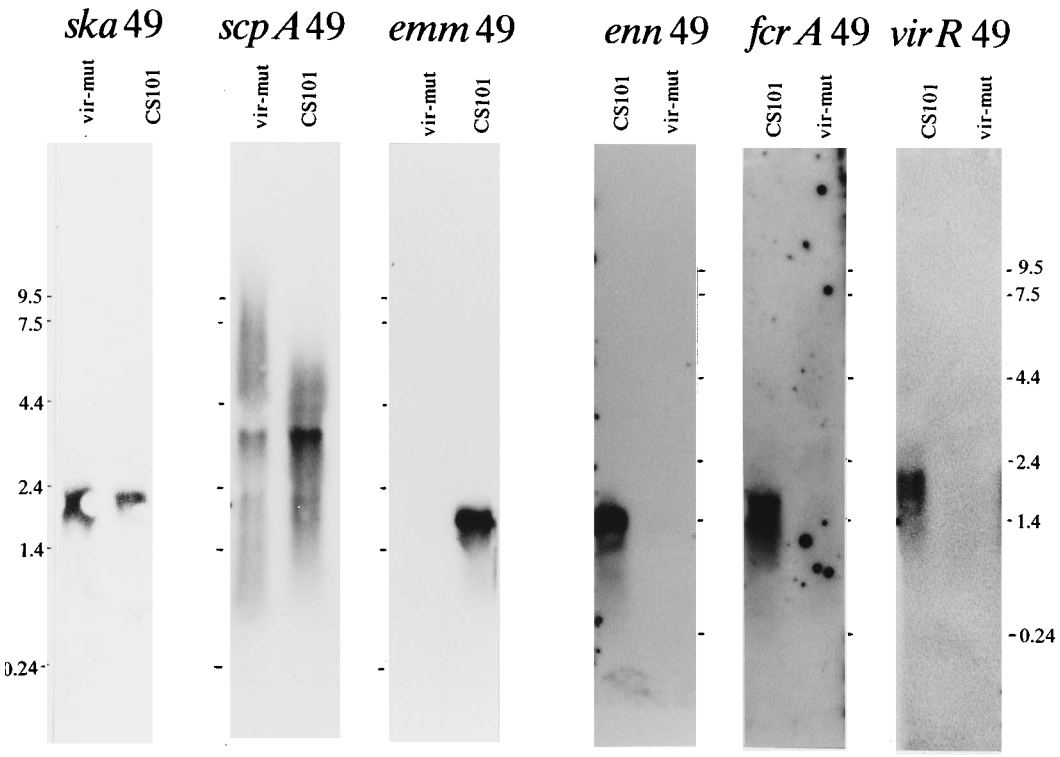

FIG. 9. Effect of insertional inactivation of *virR49* on transcript amounts from *vir* regulon genes. Equal amounts of total RNA from the wild-type M49 GAS strain CS101 and the isogenic strain vir-mut were subjected to denaturing agarose gel electrophoresis and Northern blotting on nylon membranes. Subsequently, the blots were hybridized with six different specific probes generated by directly labeling PCR products. The sequences of primers used for PCR amplification are listed in Table 1, and the sets of primers directed to the different genes are listed in Table 2. As a control, the amounts of message from the streptokinase genes (*ska*) from the isogenic GAS pair were in the range of one dilution step. In the total RNA from mutant strain vir-mut, there were no bands detectable resulting from a *virR49*, *fcrA49*, *emm49*, or *enn49* transcript. As judged by the diminished band intensity, the amount of *scpA49* mRNA was decreased in this strain. The sizes of the fragments of the RNA marker (in kilobases) are shown on the right and left.

separate group from the *virR* sequences of OF^- strains, which do not differ from each other for more than 3% in most portions. The complete dissimilarity of 40 codons of the *virR49* 3′ end from the corresponding portions of other known *virR* sequences confirmed the results from a previous PCR-based study on *virR* sequences from OF^+ GAS (40). Provided that the different *virR* genes function identically, the variability of this region suggests that it is dispensable for specific function. This is in good agreement with the finding that this *virR* portion could be involved in recombination and gene fusion events apparently without affecting the pathogenicity of the bacteria (44). The assumption does not contradict the experimental finding of Perez-Casal et al. (37) that a recombinant *mry* gene deleted for 87 codons at its 3′ end could not complement the function of an inactivated chromosomal *mry* gene.

The differences between *virR* genes from OF^+ and OF^- GAS were paralleled by the diversity of their promoter regions, which accounted for about 30% of the reported sequences. These differences could reflect the fact that *virR* genes from the two GAS groups apparently control partially different arrays of genes. Therefore, their regulation of *virR* expression itself could need different ways of fine-tuning. The deviating localization of the *virR49* (Fig. 5 and 6b) and *mry* (34) transcription start sites could also result from this necessity. Conversely, the similar presence of two differently active promoters in both serotypes and the autoregulation of the P2 promoter not only by Mry (34) but potentially also by VirR49 may indicate that the promoter locations are artifactual in one of the strains analyzed. Since S1 nuclease protection was used for mapping of both the *mry* and *virR49* promoters, promoter locations could be confirmed by applying different and RNA-independent methods such as in vivo or in vitro footprinting assays.

Sequencing of the *virR49* genomic region demonstrated the presence of an ORF homologous to *virR* genes, and mRNA analysis confirmed that the potential *virR49* gene was actually transcribed. The insertional inactivation of *virR49* indicated that a functional *virR49* gene was involved in the positive control of up to five GAS genes. Still, it is possible that the insertion had a polar effect on the expression of the four downstream genes. However, since the five genes are monocistronically transcribed, the insertion in *virR49* carries a transcription terminator at its 3′ end, and a residual transcription of one gene (*scpA49*) in the vir-mut strain was detected, a polar effect of the insertion is unlikely. The establishment of an additional functional *virR49* gene at a different genomic site in the mutant and the resultant expression of the *virR49*-controlled genes could confirm these results.

Another formal possibility is that the decreased transcription of all five genes results from a phase variation as reported for OF^- M12 strains (51). However, phase variation, which would also include an altered colony morphology in CS101, was never observed in the course of these studies. In addition, phase variation was reported to affect *emm* and *scpA* expression to the same extent, which is not the case in the vir-mut strain. Furthermore, phase variation was found to be a reversible process leading to the simultaneous presence of both phenotypes in one culture. Such a reversion was not found in the vir-mut strain, even when passaging it on several occasions in

human blood, which would strongly select for the wild-type phenotype.

The completely abolished transcription of *emm* (-related) genes in strain vir-mut compared with only a decreased amount of *scpA49* mRNA in this isolate indicated a differential effect of *virR49* on the control of *vir* regulon genes. To some extent, the different amount of mRNAs from *vir* regulon genes also observed in the wild-type strain at any given point in time supports the idea of superimposed regulatory mechanisms.

The characterization of the promoter regions of the five query genes presented in this study could provide clues to mechanisms of differential control. Except for the *virR49* promoters, the −35 and −10 boxes of the *vir* regulon genes from strain CS101 exhibited a high degree of conservation. The sequence homologies included the potential VirR-binding boxes (45) upstream of and adjacent to the promoters. Such homologies would be a prerequisite for a synonymous activity of a specific DNA-binding regulator such as the *virR49* gene product. A genomic segment exhibiting some homology to a potential VirR-binding box was also localized upstream of the P2 promoter of *virR49* (Fig. 5). This finding would correspond to the autoregulation of *virR49* expression indicated to occur by our data. In addition, it was in good agreement with the results of Okada et al. (34) regarding the P2 promoter of their *mry* gene: these authors could show that the P2 promoter was upregulated upon induction with appropriate stimuli. The deviation of the *virR49* P2 promoter sequences from the other identified *virR* promoter consensus sequences (45) could explain its comparably weak activity even after being appropriately induced.

The *fcrA49*, *enn49*, and *scpA49* genes were all transcribed only from their *virR* consensus promoters. However, their expression differed by up to a factor of 16 when transcript amounts of *fcrA49* and *enn49* were compared (Table 2; Fig. 4). That the *scpA49* promoter region harbored an inverted repeat like that already identified to decrease *scpA12* transcription could explain the difference between transcript amounts from *fcrA49* and *scpA49*. No such structural element was contained in the promoter region of *enn49*. Still, transcripts from this gene were less abundant than those from any other structural gene of the *vir* regulon, which corresponded to experimental data on *emmL2.1* and *emmL2.2* transcript ratios reported by Bessen and Fischetti (4). These low amounts of *enn* transcripts could indicate the existence of a negative regulator specifically affecting transcription from the *enn* gene. However, a possible role of message stability in producing different levels of mRNA currently cannot be ruled out.

Overall, the results presented in this investigation provide evidence that the OF^+ GAS strain CS101 contains a *virR49* regulator gene which synergistically controls the expression of four genes involved in GAS virulence and thus, at least indirectly, the phenotype of resistance to phagocytosis. On the basis of the data presented here, it should become feasible to further characterize the *virR49* gene product and its interaction with specific DNA sequences as well as its similarities to and differences from *virR* gene products from OF^- GAS, which control a smaller number of virulence genes. Furthermore, it will be of interest to elucidate *virR* involvement in control of genomic regions downstream of *scpA49*, which are typical for OF^+ GAS (39), as well as to investigate the conserved region upstream of *virR49*, which could harbor genes for signal receptors interacting with *virR*. Together, these data could provide a better understanding for the largest GAS regulon known to date.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1990. Current protocols in molecular biology. Wiley-Interscience, New York.
2. **Bessen, D., and V. A. Fischetti.** 1990. A human IgG receptor of group A streptococci is associated with tissue site of infection and streptococcal class. J. Infect. Dis. **161:**747–754.
3. **Bessen, D. E., and V. A. Fischetti.** 1990. Differentiation between two biologically distinct classes of group A streptococci by limited substitutions of amino acids within the shared region of M protein-like molecules. J. Exp. Med. **172:**1757–1764.
4. **Bessen, D. E., and V. A. Fischetti.** 1992. Nucleotide sequences of two adjacent M or M-like protein genes of group A streptococci: different RNA transcript levels and identification of a unique immunoglobulin A-binding protein. Infect. Immun. **60:**124–135.
5. **Boyle, M. D. P.** Personal communication.
6. **Caparon, M. G., R. T. Geist, J. Perez-Casal, and J. R. Scott.** 1992. Environmental regulation of virulence in group A streptococci: transcription of the gene encoding M protein is stimulated by carbon dioxide. J. Bacteriol. **174:**5693–5701.
7. **Caparon, M. G., and J. R. Scott.** 1987. Identification of a gene that regulates expression of M protein, the major virulence determinant of group A streptococci. Proc. Natl. Acad. Sci. USA **84:**8677–8681.
8. **Caparon, M. G., and J. R. Scott.** 1991. Genetic manipulation of pathogenic streptococci. Methods Enzymol. **204:**556–586.
9. **Chen, C., N. Bormann, and P. P. Cleary.** 1993. VirR and Mry are homologous *trans*-acting regulators of M protein and C5a peptidase expression in group A streptococci. Mol. Gen. Genet. **241:**685–693.
10. **Chen, C. C., and P. P. Cleary.** 1990. Complete nucleotide sequence of the streptococcal C5a peptidase gene of *Streptococcus pyogenes*. J. Biol. Chem. **265:**3161–3167.
11. **Chomczynski, P.** 1992. Solubilization in formamide protects RNA from degradation. Nucleic Acids Res. **20:**3791–3792.
12. **Cleary, P. P., D. LaPenta, D. Heath, E. J. Haanes, and C. Chen.** 1991. A virulence regulon in *Streptococcus pyogenes*, p. 147–151. *In* G. M. Dunny, P. P. Cleary, and L. L. McKay (ed.), Genetics and molecular biology of streptococci, lactococci, and enterococci. American Society for Microbiology, Washington, D.C.
13. **Cleuziat, P., A. Awadé, and J. Robert-Baudouy.** 1992. Molecular characterization of *pcp*, the structural gene encoding the pyrrolidone carboxylyl peptidase from *Streptococcus pyogenes*. Mol. Microbiol. **6:**2051–2063.
14. **Colman, G., A. Tanna, A. Efstratiou, and E. T. Gaworzewska.** 1993. The serotypes of *Streptococcus pyogenes* present in Britain during 1980–1990 and their association with disease. J. Med. Microbiol. **39:**165–178.
15. **Coote, J. G.** 1991. Antigenic switching and pathogenicity: environmental effects on virulence gene expression in *Bordetella pertussis*. J. Gen. Microbiol. **137:**2493–2503.
16. **Erlich, H. A., D. Gelfand, and J. J. Sninsky.** 1991. Recent advances in the polymerase chain reaction. Science **252:**1643–1651.
17. **Geist, R. T., N. Okada, and M. G. Caparon.** 1993. Analysis of *Streptococcus pyogenes* promoters by using novel Tn*916*-based shuttle vectors for the construction of transcriptional fusions to chloramphenicol acetyltransferase. J. Bacteriol. **175:**7561–7570.
18. **Haanes, E. J., D. G. Heath, and P. P. Cleary.** 1992. Architecture of the *vir* regulons of group A streptococci parallel opacity factor phenotype and M protein class. J. Bacteriol. **174:**4967–4976.
19. **Haanes, E. J., and P. P. Cleary.** 1989. Identification of a divergent M protein gene and an M protein-related gene family in *Streptococcus pyogenes* serotype 49. J. Bacteriol. **171:**6397–6408.
20. **Haanes-Fritz, E., W. Kraus, V. Burdett, J. B. Dale, E. H. Beachey, and P. Cleary.** 1988. Comparison of the leader sequences of four group A streptococcal M protein genes. Nucleic Acids Res. **16:**4667–4677.
21. **Heath, D. G., M. D. P. Boyle, and P. P. Cleary.** 1990. Isolated DNA repeat region from *fcrA76*, the Fc-binding protein gene from an M-type 76 strain of group-A streptococci, encodes a protein with Fc-binding activity. Mol. Microbiol. **4:**2071–2079.
22. **Heath, D. G., and P. P. Cleary.** 1989. Fc-receptor and M-protein genes of group A streptococci are products of gene duplication. Proc. Natl. Acad. Sci. USA **86:**4741–4745.
23. **Hollingshead, S. K., T. L. Readdy, D. L. Yung, and D. E. Bessen.** 1993. Structural heterogeneity of the *emm* gene cluster in group A streptococci. Mol. Microbiol. **8:**707–717.
24. **Huang, T. T., H. Malke, and J. J. Ferretti.** 1989. Heterogeneity of the streptokinase gene in group A streptococci. Infect. Immun. **57:**502–506.
25. **Johnson, D. R., D. L. Stevens, and E. L. Kaplan.** 1992. Epidemiologic

analysis of group A streptococcal serotypes associated with severe systemic infections, rheumatic fever, or uncomplicated pharyngitis. J. Infect. Dis. **166:**374–382.
26. **Kaufhold, A., A. Podbielski, D. R. Johnson, E. L. Kaplan, and R. Lütticken.** 1992. M protein gene typing of *Streptococcus pyogenes* by nonradioactively labeled oligonucleotide probes. J. Clin. Microbiol. **30:**2391–2397.
27. **Kehoe, M. A.** 1994. Cell-wall-associated proteins in gram-positive bacteria. New Compr. Biochem. **27:**217–261.
28. **Kehoe, M. A., L. Miller, J. A. Walker, and G. J. Boulnois.** 1987. Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging, thiol-activated toxins. Infect. Immun. **55:**3228–3232.
29. **Lancefield, R. C.** 1957. Differentiation of group A streptococci with a common R antigen into three serological types, with special reference to the bactericidal test. J. Exp. Med. **106:**525–544.
30. **LeBlanc, D. J., L. N. Lee, and J. M. Inamine.** 1991. Cloning and nucleotide base sequence analysis of a spectinomycin adenyltransferase AAD(9) determinant from *Enterococcus faecalis*. Antimicrob. Agents Chemother. **35:** 1804–1810.
31. **Lottenberg, R., C. C. Broder, M. D. P. Boyle, S. J. Kain, B. O. Schroeder, and R. Curtiss III.** 1992. Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmin receptor. J. Bacteriol. **174:**5204–5210.
32. **Martin, N. J., E. L. Kaplan, M. A. Gerber, M. A. Menegus, M. Randolph, K. Bell, and P. P. Cleary.** 1990. Comparison of epidemic and endemic group G streptococci by restriction enzyme analysis. J. Clin. Microbiol. **28:**1881–1886.
33. **Mekalanos, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1–7.
34. **Okada, N., R. T. Geist, and M. G. Caparon.** 1993. Positive transcriptional control of *mry* regulates virulence in the group A streptococcus. Mol. Microbiol. **7:**893–903.
35. **O'Toole, P., L. Stenberg, M. Rissler, and G. Lindahl.** 1992. Two major classes in the M protein family in group A streptococci. Proc. Natl. Acad. Sci. USA **89:**8661–8665.
36. **Pancholi, V., and V. A. Fischetti.** 1992. A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. J. Exp. Med. **176:**415–426.
37. **Perez-Casal, J., M. G. Caparon, and J. R. Scott.** 1991. Mry, a *trans*-acting positive regulator of the M protein gene of *Streptococcus pyogenes* with similarity to the receptor proteins of two-component regulatory systems. J. Bacteriol. **173:**2617–2624.
38. **Perez-Casal, J. F., H. F. Dillon, L. K. Husmann, B. Graham, and J. R. Scott.** 1993. Virulence of two *Streptococcus pyogenes* strains (types M1 and M3) associated with toxic-shock-like syndrome depends on an intact *mry*-like gene. Infect. Immun. **61:**5426–5430.
39. **Podbielski, A.** Unpublished results.
40. **Podbielski, A.** 1992. Ubiquitous occurrence of *virR* and *scpA* genes in group A streptococci. Med. Microbiol. Immunol. **181:**227–240.
41. **Podbielski, A.** 1993. Three different types of organization of the *vir* regulon in group A streptococci. Mol. Gen. Genet. **237:**287–300.
42. **Podbielski, A., J. Hawlitzky, T. D. Pack, A. Flosdorff, and M. D. P. Boyle.** 1994. A group A streptococcal Enn protein potentially resulting from intergenomic recombination exhibits atypical immunoglobulin-binding characteristics. Mol. Microbiol. **12:**725–736.
43. **Podbielski, A., A. Kaufhold, and P. P. Cleary.** 1993. PCR-mediated amplification of group A streptococcal genes encoding immunoglobulin-binding proteins. ImmunoMethods **2:**55–64.
44. **Podbielski, A., B. Krebs, and A. Kaufhold.** 1994. Genetic variability of the *emm*-related genes of the *vir* regulon of group A streptococci: potential intra- and intergenomic recombination events. Mol. Gen. Genet. **243:**691–698.
45. **Podbielski, A., J. A. Peterson, and P. Cleary.** 1992. Surface protein-CAT reporter fusions demonstrate differential gene expression in the *vir* regulon of *Streptococcus pyogenes*. Mol. Microbiol. **6:**2253–2265.
46. **Podbielski, A., J. Schönling, B. Melzer, and G. Haase.** 1991. Different promoters of SHV-2 and SHV-2a β-lactamase lead to diverse levels of cefotaxime resistance in their bacterial producers. J. Gen. Microbiol. **137:**1667–1675.
47. **Raeder, R., and M. D. P. Boyle.** 1993. Association between expression of immunoglobulin G-binding proteins by group A streptococci and virulence in a mouse skin infection model. Infect. Immun. **61:**1378–1384.
48. **Raeder, R., and M. D. P. Boyle.** 1993. Association of type II IgG-binding protein expression and survival of group A organisms in human blood. Infect. Immun. **61:**3696–3702.
49. **Richardson, J. P.** 1993. Transcription termination. Crit. Rev. Biochem. Mol. Biol. **28:**1–30.
50. **Sela, S., A. Aviv, A. Tovi, I. Burstein, M. G. Caparon, and E. Hanski.** 1993. Protein F: an adhesin of *Streptococcus pyogenes* binds fibronectin via two distinct domains. Mol. Microbiol. **10:**1049–1055.
51. **Simpson, W. J., and P. P. Cleary.** 1987. Expression of M type 12 protein by a group A streptococcus exhibits phaselike variation: evidence for coregulation of colony opacity determinants and M protein. Infect. Immun. **55:** 2448–2455.
52. **Simpson, W. J., D. LaPenta, C. Chen, and P. P. Cleary.** 1990. Coregulation of type 12 M protein and streptococcal C5a peptidase genes in group A streptococci: evidence for a virulence regulon controlled by the *virR* locus. J. Bacteriol. **172:**696–700.
53. **Stevens, D. L.** 1992. Invasive group A streptococcus infections. Clin. Infect. Dis. **14:**2–13.
54. **Tao, L., D. J. LeBlanc, and J. J. Ferretti.** 1992. Novel streptococcal-integration shuttle vectors for gene cloning and inactivation. Gene **120:**105–110.
55. **Wannamaker, L. W.** 1970. Differences between streptococcal infections of the throat and of the skin. N. Engl. J. Med. **282:**78–85.
56. **Zhou, C. Y., Y. Yang, and A. Y. Jong.** 1990. Miniprep in ten minutes. BioTechniques **8:**172–173.