Transcriptional Activation of *sclA* by Mga Requires a Distal Binding Site in *Streptococcus pyogenes*

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Streptococcus pyogenes (the group A streptococcus [GAS]) is a medically significant pathogen of humans, causing a range of diseases from pharyngitis to necrotizing fasciitis. Several important GAS virulence genes are under the control of a pleiotropic regulator called Mga, or the multiple gene regulator of GAS, including the gene encoding the streptococcal collagen-like protein, or *sclA*. Analysis of the genome sequence upstream of *sclA* revealed two potential Mga-binding sites with homology to the published Mga-binding element, which were called P*sclA*-I (distal) and P*sclA*-II (proximal) based on their location relative to a predicted start of transcription. Primer extension was used to confirm that the Mga-dependent transcriptional start site for *sclA* was located adjacent to the proximal P*sclA*-II binding site. By using overlapping P*sclA* promoter probes and purified Mga-His fusion protein, it was shown by electrophoretic mobility shift assays that, unlike other Mga-regulated promoters, Mga binds only to a distal DNA-binding site (P*sclA*-I). Binding of Mga to P*sclA*-I could be competed with cold probes corresponding to known Mga-regulated promoters (P*emm*, P*scpA*, and P*mga*) but not with a nonspecific probe or the proximal P*sclA*-II fragment. With the use of a plasmid-based green fluorescent protein transcriptional reporter system, the full-length P*sclA* was not sufficient to reproduce normal Mga-regulated activation. However, studies using a single-copy *gusA* transcriptional reporter system integrated at the native *sclA* chromosomal locus clearly demonstrated that the distal P*sclA*-I binding site is required for Mga regulation. Therefore, P*sclA* represents a new class of Mga-regulated promoters that requires a single distal binding site for activation.

A hallmark of the group A streptococcus (GAS; *Streptococcus pyogenes*) is its ability to elicit a wide array of human diseases at varied locations throughout the human body. These syndromes may include relatively minor purulent infections that target either the upper respiratory tract or the skin as well as life-threatening invasive infections of deeper tissues, bloodstream, and organs (6, 30). GAS pathogenesis involves a number of different factors that allow the organism to survive within the human host. The initial stages of infection involve cell-associated factors that contribute to colonization, cell tropism, and immune evasion, including M proteins, capsule, and extracellular matrix binding proteins. Later stages often utilize secreted bacterial factors such as proteases, toxins, and other enzymes that allow the organism to spread and invade deeper tissues (6). In order to coordinate the expression of these different determinants with the changing environments that GAS encounters during an infection, the pathogen has developed multigene networks to regulate virulence.

One such regulator is the multiple virulence gene regulator of GAS known as Mga, which activates the transcription of genes encoding primarily surface-associated proteins involved in GAS pathogenesis. These factors include the M family of proteins (*emm*, *mrp*, *arp*, and *enn*), C5a peptidase (*scpA*), fibronectin-binding proteins (*sof* and *fbx*), and the secreted inhibitor of complement (*sic*) (4, 5, 12, 20). Mga is also required for its own positive regulation or autoactivation (19, 23). Environmental growth conditions such as increased CO_2 and temperature have been shown to activate transcription of genes in the Mga regulon (3, 16, 26). Furthermore, expression of the Mga regulon is maximal during exponential phase and becomes transcriptionally silent as the bacteria enter stationary phase (18). This temporal regulation correlates with the requirement for Mga-regulated surface proteins during early stages of infection when GAS is actively growing but down regulates its expression during times of nutrient deprivation when the proteins may actually hinder the bacterium from spreading to new tissues. Thus, Mga controls its set of virulence factors in response to both environmental and temporal signals through an unknown mechanism.

Mga functions by binding directly to specific sequences found in the promoters of target genes, as demonstrated for *emm* (P*emm*) and *scpA* (P*scpA*) in the serotype M6 GAS strain JRS4 (15). An alignment of these two sites was used as the basis for a published consensus Mga-binding site (15). Binding of Mga in these two promoters occurs at similar locations consisting of a single site overlapping the −35 hexamer and just upstream of the start of transcription (15). Interestingly, autoregulation of *mga* transcription also involves binding of Mga within its own promoter (P*mga*); however, Mga interacts with two separate binding sites located more than 100 bp upstream of the major P2 start site (19). Thus, based upon the variable location of binding sites in these three promoters, the mechanisms for Mga activation (P*emm* and P*scpA*) versus autoactivation (P*mga*) may be quite different. Although all four of the identified Mga-binding sites are less than 50% identical at the DNA sequence level, recent studies have shown that the same two helix-turn-helix DNA-binding domains of Mga are responsible for recognizing each of these divergent sites (17). Furthermore, the ability of Mga to bind to these promoters

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TABLE 1. PCR primers used in this study

Target	Primer	Sequence (5′–3′)[a]	Reference
emm	OM6-16	GTTTCCTTCATTGGTGCT	17
	OM6-35	AACAGCAAATTAGCTGCTC	17
gusA	*gusA*-PE	GTTGGGGTTTCTACAGGACG	This study
mga	DivMgaL6	CTTTTAAGAAACTATTTTCC	This study
	MgaPetI_Nde	ggggcatATGTATGTAAGTAAGTTGTTT	This study
	MgaPetII_Xho	aactcgagAGTTGTGGAGGGGG	This study
	OYR-4	GTACCATCAACATTGCG	19
Nonspecific	*rpsL*-1	GAATGTAGATGCCTACAATTAACCA	17
	rpsL-2	GTGCGCCACGAACGATATG	17
nrdI	*nrdI*-PE	GGTAATCTGAGGCATAAGTG	This study
P*emm6*	P*emm*-L1	GCATGGATCCCATCGCAAAGAGCTTA	17
	P*emm*-R1	GCGGCTCGAGTAGTGTCTATTCGTGTTATT	17
P*emm1*	P*emm1*-L1	AAGCGATTATTGACAAGCTC	This study
	P*emm1*-R1	AGTCAAAGCTACCGCTACTG	This study
P*mga*	OYR-25	GGTTGTACCATAACAGTC	17
	OYL-25	TACCATAAAATACCTTTC	17
P*sclA*	P*sclA*-L	TTAAAGAAAGGATCCGGATG	This study
	P*sclA*-R	ATGGTGCTTTGATGTCAACA	This study
	P*sclA*-L2	TGGTAATCGTAATTGTCTGC	This study
	P*sclA*-R2	GGAGATCAGAGGGCTACTTT	This study
	P*sclA*-L4	AGCAGTCAAAAAAAGTAACA	This study
	P*sclA*-R4	TGTACCTTCTTGTCTGGTTG	This study
	P*sclA*-L5	ACAGCTATGATCATCTATGG	This study
	P*sclA*-R5	GATTATGGTGCTTTGATGTC	This study
	P*sclA*-L6	GTGATCCCAAGCCCCAA	This study
P*scpA*	C5-L1	AAGAATGAGATTAAGGAGGTCACA	15
	C5-R1	GCGCAATGGCAAGTTTGTC	15
sclA	*sclA*-F2	ACGTCGAGTCAAAGAGAGTCAAAAATAAA	This study
	sclA-R1	GTTTGGTTAGCTTCTTTGTCA	This study

[a] Underlined nucleotides are restriction sites, and lowercase nucleotides are anchor sequences introduced into the primer.

in vivo was shown to be essential for virulence gene activation (17). Exactly which nucleotides within a given binding site are required for Mga recognition and binding at these various promoters has not been established.

Recently, a gene encoding a cell-wall-anchored streptococcal collagen-like protein (*sclA* or *scl1*) was identified in all serotypes of GAS tested (13, 27). A mutant of the newly described surface protein was found to be defective for adherence to lung epithelial cells, but not pharyngeal cells, and was attenuated for virulence in a mouse model of soft-tissue pathology (13, 27). Expression of *sclA* was found to be Mga regulated in a serotype M1 GAS, and maximal expression occurred during exponential growth as expected (13, 14, 27). With the use of the published consensus Mga-binding site (15) to search the promoter region upstream of *sclA*, a potential Mga-binding site was found overlapping the −35 hexamer of a suspected start of transcription (13, 27) in a location similar to that observed for P*emm* and P*scpA*. In this study, we identify a second distal binding site, and we investigate whether Mga plays a direct role in transcriptional activation at the *sclA* promoter.

MATERIALS AND METHODS

Bacterial strains and media. *S. pyogenes* (GAS) SF370 (31) represents an available serotype M1 GAS genome sequence (8). JRS4 is a streptomycin-resistant derivative of serotype M6 strain D471 (29). JRS519 (*mga-10*) is an Mga− derivative of JRS4 (18). MGAS166 is a streptomycin-resistant M1 strain (22). *Escherichia coli* strain DH5α (*hsdR17 recA1 gyrA endA1 relA1*) was used as the host for plasmid constructions. The T7 polymerase *E. coli* strain BL21(DE3) [F− *ompT hsdSB* (r_B^- m_B^-) *gal dcm met*] was used for protein expression and purification.

All *E. coli* strains were grown in Luria-Bertani broth. GAS was cultured in Todd-Hewitt medium supplemented with 0.2% yeast extract (THY), and growth

was assayed by optical density with a Klett-Summerson photoelectric colorimeter with the A filter. Antibiotics were used at the concentrations indicated: ampicillin at 100 μg/ml for *E. coli*, spectinomycin at 100 μg/ml for both *E. coli* and GAS, kanamycin at 50 μg/ml for *E. coli* and 300 μg/ml for GAS, and erythromycin at 500 μg/ml for *E. coli* and 1.0 μg/ml for GAS.

DNA manipulations. Plasmid DNA was isolated from *E. coli* by using either the Wizard Miniprep system (Promega) or Maxi/Midi Prep purification systems (Qiagen). DNA fragments were isolated from agarose gels by using the QIAquick gel extraction kit (Qiagen). GAS chromosomal DNA was isolated using the FastDNA kit and a FastPrep cell disruptor (Bio 101, Inc.). PCR for cloning and promoter probes was performed using *Pfx* high-fidelity DNA polymerase (Invitrogen), and reaction mixtures were purified using the QIAquick PCR purification system. PCR for diagnostic assays was performed using Vent DNA polymerase (New England Biolabs). DNA sequencing was performed either using the Excel II cycle sequencing kit (Epicentre, Inc.) or through the automated sequencing core facility in the McDermott Center at University of Texas Southwestern Medical Center.

Construction of the Mga− M1 strains KSM165-L and MGAS166.165-L. To inactivate *mga* in SF370 and MGAS166, a suicide plasmid was constructed as follows: a 983-bp PCR fragment containing an internal fragment of *mga* was amplified from M1 SF370 genomic DNA by using primers OYR-4 and DivMgaL6 (Table 1). The resulting fragment was cloned blunt into an SmaI-digested suicide vector, pCIV2 (23), to produce pKSM165-L. Serotype M1 SF370 and MGAS166 were transformed with 200 to 300 μg of pKSM165-L, and chromosomal integrations were selected for by growth on THYA plates containing kanamycin at 37°C. Mutants were verified by PCR analysis of the *mga* locus and slot blot analysis of *mga* transcripts (data not shown).

Northern blot analysis. Total RNA was isolated from samples in late exponential phase (65 Klett units) by using the FastRNA kit and a FastPrep cell disruptor as previously described (17). Northern blot assays of total RNA were performed using the NorthernMax kit (Ambion). Briefly, either 1 (*emm*) or 5 (*sclA*) μg of total RNA was separated on a 1% agarose denaturing gel (formaldehyde) and transferred to a positively charged nylon membrane. The blots were then UV cross-linked and prehybridized for 30 min at 50°C. Blots were hybridized overnight at 50°C with 5×10^6 cpm of [α^{32}P]dATP-labeled *emm* or *sclA* probe (RadPrime labeling system; Invitrogen) followed by two low-stringency washes at room temperature (RT) for 5 min and two high-stringency washes at

50°C for 15 min. Blots were visualized by exposure to a phosphorimaging cassette for 2 h. Probes were PCR amplified from a serotype M1 strain by using the primers listed in Table 1 for *emm* and *sclA*.

Primer extension analysis. Total RNA was extracted from samples in late exponential phase as described previously (17) by using the FastRNA kit and a FastPrep cell disruptor. Primer extensions were performed on 25 μg of total RNA as described previously (19) with the primers P*sclA*-R and *nrdI*-PE (Table 1). The primer extension products were run on a 6% denaturing polyacrylamide gel (Amresco), and gels were processed as described above. Sequence was generated using labeled P*sclA*-R and *nrdI*-PE primers (Table 1) on a PCR product amplified from SF370 genomic DNA (Table 1; P*sclA*-L2 and P*sclA*-R) as described above.

Expression and purification of Mga-His protein from *E. coli*. A carboxy-terminal fusion of six histidines to the serotype M6 Mga was constructed as follows: a 1.4-kb PCR fragment containing the *mga* open reading frame was amplified from GAS JRS4 genomic DNA by using primers MgaPetI_Nde and MgaPetII_Xho (Table 1). The PCR fragment was digested with NdeI/XhoI and cloned into NdeI/SalI-digested pET21a (Novagen) to produce the T7-inducible Mga-His expression vector pKSM170.

The *E. coli* strain BL21(DE3) contains the T7 polymerase under the control of P*lac* and allows induction upon addition of IPTG (isopropyl-β-D-thiogalactopyranoside). The pKSM170 *mga-his* plasmid was introduced into BL21(DE3) for expression and purification. Briefly, cultures of *E. coli* BL21(DE3) containing pKSM170 (PT7-*mga-his*) were grown at 30°C in Luria-Bertani broth plus ampicillin (100 μg/ml), and expression of the protein was induced for 1.5 h by addition of 1 mM IPTG. Cells were lysed by two passages through a prechilled French pressure cell, and Mga-His was purified over a nickel-nitrilotriacetic acid resin column (Qiagen) under native conditions. Protein concentrations were determined using the Bio-Rad protein assay kit. DNA-binding activity of purified Mga-His was verified by comparison to the published maltose binding protein (MBP)-Mga fusion protein by electrophoretic mobility shift assay (EMSA) (15, 17, 19). The stability of the purified protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis as described below.

Expression and purification of Mga-His protein from GAS. A GAS-expression plasmid possessing a carboxy-terminal fusion of six histidines to the serotype M6 Mga was constructed as follows: pKSM170 was digested with SpeI and ScaI, and the resulting 2.2-kb fragment containing *mga-his* was gel purified and cloned into SpeI/SnaBI-digested pJRS525 (18) to form pKSM163. pJRS2050 (1), possessing P*mga*, was digested with BamHI/SpeI, and the resulting 2.2-kb fragment was gel purified and cloned into BamHI/SpeI-digested pKSM163 to form pKSM164.

The P*mga-mga-his* pKSM164 plasmid was transformed into JRS519, an Mga⁻ M6 JRS4 derivative, for expression and purification. Briefly, cultures of JRS519 (pKSM170) were grown to late logarithmic phase at 37°C in THYB plus spectinomycin. Cells (20 50-ml aliquots) were lysed using five 9-s pulses at 4°C on a FastPrep cell disruptor, and Mga-His was purified over a nickel-imino acetic acid resin column (Pharmacia) under native conditions. Protein concentrations were determined using the Bio-Rad protein assay kit. The stability of the purified protein was assessed by SDS-PAGE and Western blot analysis as described below.

Western blot analysis. Anti-Mga-His immunoblot assays were performed as described previously (17). Briefly, purified Mga-His proteins were separated by SDS–10% PAGE. Proteins were transferred to nitrocellulose membranes and reacted either with the anti-Mga-pep2 antiserum (17) at a 1:250 dilution for 2 h at RT or with the anti-His monoclonal antiserum (Novagen) at a 1:2,000 dilution for 2 h at RT. Blots containing GAS whole-cell extracts were reacted with anti-rat immunoglobulin G horseradish peroxidase-conjugated secondary antibody (Sigma) or anti-mouse immunoglobulin G horseradish peroxidase-conjugated secondary antibody (Chemicon International), respectively; developed using the Renaissance chemiluminescence system (New England Nuclear); and visualized on autoradiography film (Kodak).

EMSAs. Promoter probes were generated by PCR amplification with either serotype M1 strain SF370 or serotype M6 strain JRS4 chromosomal DNA and the relevant primer pairs (P*sclA*, P*emm6*, P*mga*, and P*scpA*) listed in Table 1. PCR fragments were end labeled with [γ-³²P]ATP with the use of T4 polynucleotide kinase (New England Biolabs). Labeled fragments were excised from a 5% polyacrylamide gel, extracted by crush and soak elution, and purified using the QIAquick PCR purification system.

EMSA was performed as described previously (15). Briefly, a constant amount of labeled promoter probe DNA (ca. 1 to 5 ng) and increasing amounts of purified Mga-His (1 to 6 μg) were used in each reaction. Competition assays were performed by addition of 750 ng of unlabeled promoter probes to binding reaction mixtures. Gels were dried under vacuum at 80°C for 1 h and exposed

overnight either to film or to a phosphorimaging screen. Phosphor screens were scanned using a Storm 820 scanner (Amersham Biosciences), and resulting data were analyzed with the ImageQuant analysis software (version 5.2).

Construction and use of a green fluorescent protein (GFP)-based transcriptional reporter plasmid. The plasmid pGreenTIR contains a promoterless *gfp* cassette modified for maximal solubility and fluorescence in bacteria (21). An 854-bp EcoRI fragment containing the promoterless *gfp* and its ribosomal binding site was cloned into the EcoRI-digested GAS shuttle vector pJRS525 (18) to produce the plasmid pKSM123. A SmaI-digested fragment of pUCΩKm2 (24), possessing ΩKm2 and T4 terminators, was gel purified and cloned at the 3′ end of *gfp* in the EcoRV-digested pKSM123 to form the transcriptional reporter plasmid pKSM410. Blunt PCR products corresponding to P*emm1* (pKSM425), a 500-bp P*sclA*-L2-R fragment (pKSM412), and a 253-bp P*sclA*-L-R fragment (pKSM414) were amplified from the serotype M1 GAS strain SF370 genomic DNA and cloned into XhoI-digested (blunted) pKSM410 (primers shown in Table 1). Plasmids were introduced into the serotype M1 strains MGAS166 (Mga⁺) and MGAS166.165-L (Mga⁻) by electroporation as described above.

GAS strains containing GFP reporter plasmids were grown to late logarithmic phase (65 Klett units), pelleted, and resuspended in phosphate-buffered saline. Aliquots (200 μl) in duplicate were transferred to a 96-well black microtiter plate with a clear bottom (Costar) along with a phosphate-buffered saline control. Samples were assayed using a FLUOStar Optima (BMG Lab Technology) fluorometer by using an excitation filter at 485 nm and an emission filter at 510 nm. Relative fluorescence units for each sample were expressed as fold above the level of the promoterless control strain containing pKSM410.

Construction and use of a chromosomal GusA-based transcriptional reporter. A 257-bp fragment of P*sclA* containing Mga-binding site II was PCR amplified from the M1 strain SF370 by using the P*sclA*-L-R5 primer pair (Table 1) and ligated into the EcoRV-digested pBluescript II KS− (Stratagene) to form pKSM417. A 619-bp fragment of P*sclA* containing sequence upstream of Mga-binding site II was PCR amplified from SF370 by using the P*sclA*-L5-R4 primer pair (Table 1) and was cloned into the SmaI-digested pKSM417 to form pKSM418. To form the plasmid pKSM422, an 890-bp fragment of pKSM418 was PCR amplified using the P*sclA*-L5-R primer pair (Table 1) and ligated into the SmaI-digested *gusA* reporter plasmid pKSM140 (28). The P*sclA*-Δ site I-*gusA* allele of pKSM422 was excised by PvuII digestion, gel purified, and cloned into the EcoRV site of the temperature-sensitive pJRS233 (25) to form the P*sclA*-Δ site I-*gusA* plasmid pKSM423.

Wild-type P*sclA* was PCR amplified from M1 SF370 by using the primer pair P*sclA*-L2-R (Table 1) and digested with HindIII prior to cloning into HindIII/HincII-digested pBluescript II KS− (Stratagene) to form pKSM419. This plasmid was then cut with KpnI/EcoRI and ligated into KpnI/EcoRI-digested *gusA* plasmid pKSM140 (28) to form pKSM403. The 2.3-kb EcoRI (blunted)/PstI P*sclA-gusA* fragment from pKSM403 was moved into PstI/SmaI-digested pVIT164 (9) to form pKSM405. The HpaI/PstI P*sclA-gusA* fragment from pKSM405 was cloned into EcoRV/PstI-digested pJRS233 (25) to form pKSM421.

The pKSM421 (P*sclA-gusA*) and pKSM423 (P*sclA*-Δ site I-*gusA*) plasmids were introduced into MGAS166 (Mga⁺) and MGAS166.165-L (Mga⁻) by electroporation. Following growth at the permissive temperature, integrants were selected by passaging the cells in THYB containing erythromycin at the nonpermissive temperature. Integration at the correct position was assessed by PCR amplification with the primer pair P*sclA*-L6 and *gusA*-PE (Table 1). The presence of the site I mutation was confirmed by digestion of this PCR product with PstI. A GusA assay on these strains was performed as previously described (7). Briefly, cells were grown to late logarithmic phase (65 Klett units), lysed with a FastPrep cell disruptor, and assayed for GusA activity. Results are reported in GusA units, which are equivalent to the optical density at 420 nm (OD_{420}) of the processed lysate divided by the concentration of total lysate protein (micrograms per microliter) as determined by the Bio-Rad protein assay kit.

RESULTS

Identification of two potential Mga-binding sites upstream of *sclA*. To identify new Mga-regulated virulence genes in GAS, a homology search of the serotype M1 SF370 genome database (AE004092) was performed using the published Mga consensus binding sequence (15). As expected, binding sites were found within promoters of known Mga-regulated genes, including *emm*, *scpA*, and *sic* (data not shown). A putative Mga-binding site was also identified centered 51 bp upstream
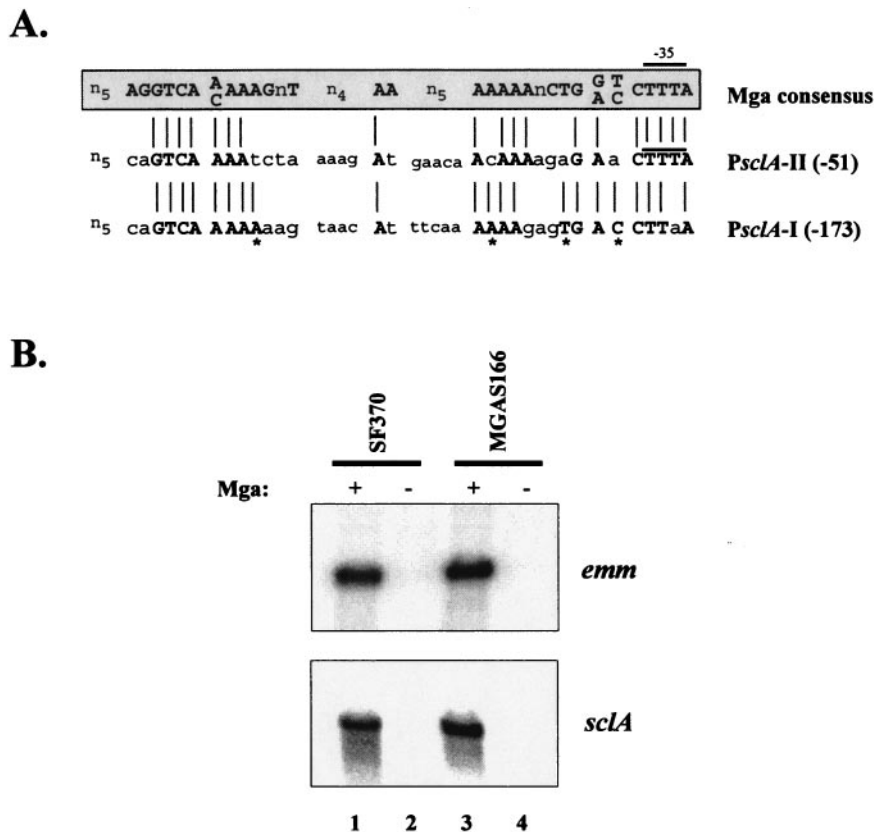
A.

B.

FIG. 1. Mga regulation of *sclA* in M1 strains. (A) A search of the serotype M1 GAS SF370 genomic sequence upstream of the known Mga-regulated *sclA* gene with the published Mga consensus binding sequence (15) revealed two potential Mga-binding sites. An alignment of P*sclA* Mga-binding site I (P*sclA*-I) and site II (P*sclA*-II) with published consensus Mga-binding sequence is shown. P*sclA*-II overlaps the predicted −35 hexamer of this promoter as described previously (13, 27). P*sclA*-I represents a novel site and is located farther upstream. Bold, capital letters and lines represent nucleotide identity to the Mga consensus binding sequence, while lowercase letters represent nonidentity. Asterisks indicate nucleotides in P*sclA*-I that are identical to the Mga-binding consensus but are not conserved in P*sclA*-II. (B) Northern blot analysis of total RNA isolated from M1 GAS strains SF370 (Mga+, lane 1), KSM165-L (Mga−, lane 2), MGAS166 (Mga+, lane 3), and MGAS166.165-L (Mga−, lane 4). Blots were hybridized with probes to *emm* and *sclA* amplified from the PCR primers listed in Table 1.

of the Mga-regulated *sclA* (Fig. 1A; P*sclA*-II) and corresponds to a site described previously (13, 27). The site shares 66% identity to the Mga consensus sequence at conserved sites and was located in P*sclA* overlapping the −35 hexamer of a predicted start of *sclA* transcription (Fig. 1A; P*sclA*-II), identical to the established Mga-binding sites for P*emm* and P*scpA* (15). Interestingly, a second site showing 72% identity to the Mga consensus sequence (Fig. 1A; P*sclA*-I) was identified centered 153 bp upstream from P*sclA*. Scanning of the 3′ sequence adjacent to P*sclA*-I did not reveal an obvious second start of transcription, suggesting that Mga bound at this potential element may target the downstream transcriptional start site next to P*sclA*-II (Fig. 2B). Furthermore, sequences that are 100% identical to the conserved consensus nucleotides for both sites (Fig. 1A) are found upstream of *sclA* in strains representing a wide array of GAS serotypes, including M2, M3, M6, M12, M18, M28, M49, M52, M56, and M77 (data not shown). Thus, P*sclA* appears to possess two potential Mga-binding elements arranged in a pattern that is unique to this Mga-regulated promoter and is conserved across GAS serotypes.

Previous studies found that expression of *sclA* is Mga regulated in the M1 strains AP1 (27) and JRS301 (14). In order to confirm that *sclA* is also Mga regulated in the M1 SF370 and MGAS166 strains used in this study, a Northern analysis was performed on wild-type and *mga*-inactivated versions of each strain on RNA isolated at the exponential phase of growth (Fig. 1B). As a control, Northern analysis was also performed on the established Mga-regulated gene *emm* (Fig. 1B). As expected, transcript levels for both *emm* and *sclA* are dramatically lower in the Mga− versions of SF370 and MGAS166 than in their wild-type counterparts, confirming Mga regulation of *sclA* in these M1 strains.

Identification of the Mga-regulated start of transcription for *sclA*. Since the predicted location of the Mga-regulated start site for *sclA* expression has not been experimentally verified, primer extension analysis was performed on total RNA isolated from both wild-type (SF370) and *mga*-inactivated (KSM165-L) serotype M1 GAS strains by using an antisense primer, P*sclA*-R (Table 1), located in the 5′ end of *sclA*. An extension product was clearly observed in a background producing Mga (Fig. 2A) that corresponded exactly to the start of transcription predicted in the literature (13, 27). However, the product was clearly absent in the *mga*-inactivated KSM165-L (Fig. 2A). As a control for the primer extension analysis, the transcriptional start for the divergently transcribed *nrdI* gene found directly upstream of *sclA* was mapped in both strains
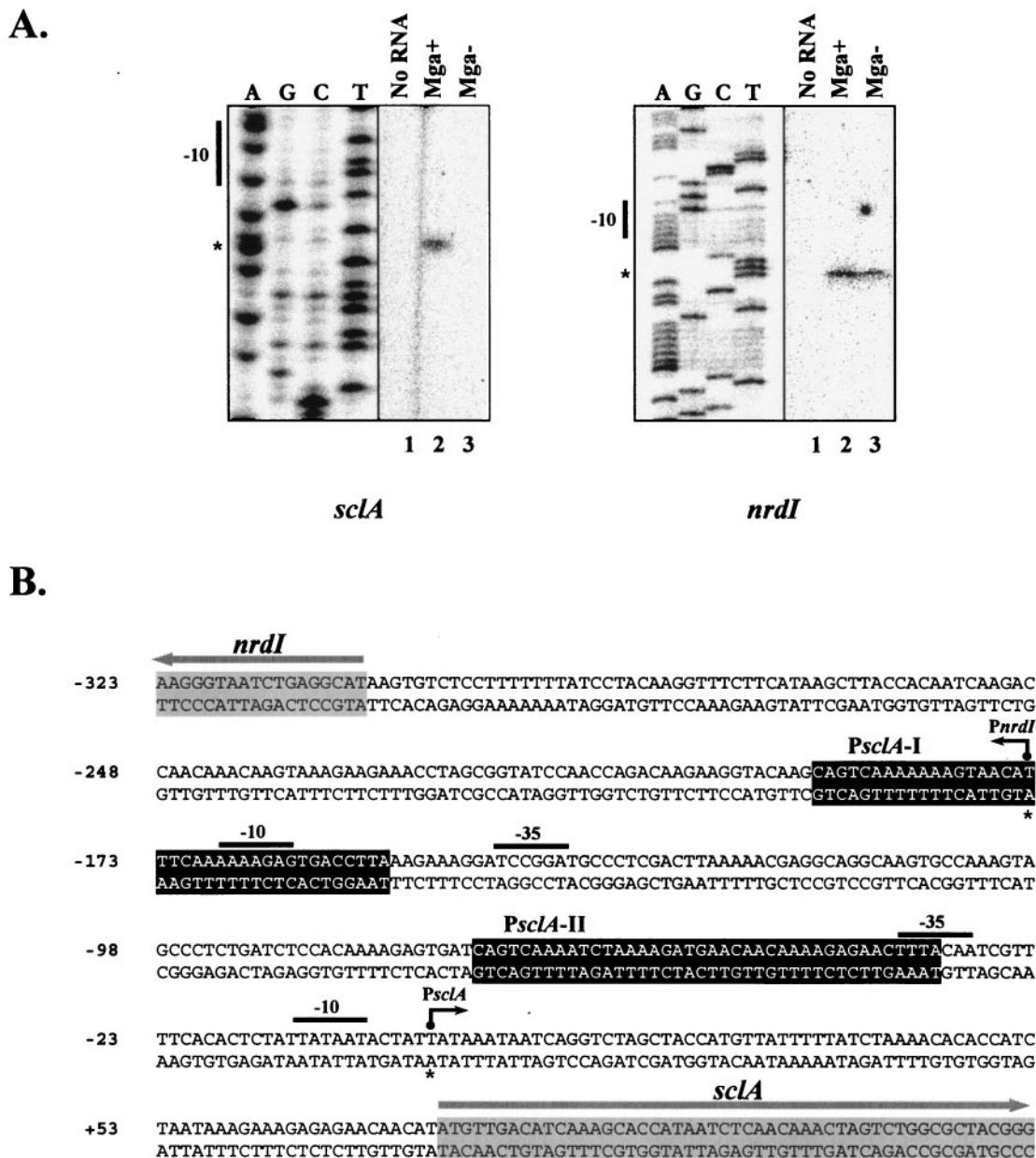
A.

sclA　　　　　　*nrdI*

B.

FIG. 2. Identification of transcriptional start sites for *sclA* and *nrdI*. (A) Primer extension analysis was performed on total RNA isolated from serotype M1 SF370 (Mga[+]) and KSM165-L (Mga[−]) by using the radiolabeled antisense primers P*sclA*-R for *sclA* and *nrdI*-PE for *nrdI* (Table 1) as described in Materials and Methods. The start of transcription for *sclA* and *nrdI* (asterisks) as well as their corresponding −10 hexamers is shown. (B) Sequence of *sclA* and *nrdI* intergenic region from serotype M1 SF370 indicating the identified start sites (asterisk and dark arrow) along with −10 and −35 regions (solid bars). Potential Mga-binding sites (black boxes) and starts of translation (gray arrows) are designated. The sequence is numbered using the P*sclA* transcriptional start site as +1.

tested (Fig. 2A). Interestingly, although the *nrdI* start site is located within the putative distal P*sclA*-I Mga-binding element, there was no alteration in *nrdI* transcription either in the presence or in the absence of Mga. These data indicate that *sclA* contains a single Mga-regulated start of transcription that is located directly downstream of the potential P*sclA*-II Mga-binding site (Fig. 2B).

Mga-His purified from *E. coli* binds to the distal P*sclA*-I site but not the promoter-proximal P*sclA*-II site. To determine whether Mga directly interacts with the two potential binding

elements within P*sclA*, EMSAs were performed on overlapping PCR-generated promoter probes encompassing the P*sclA* region (Fig. 3A). Previous EMSA experiments have successfully utilized an MBP-Mga protein generated from a serotype M6 GAS strain (15, 17, 19); however, this chimera is much larger in size than Mga alone (105 versus 62 kDa) and exhibits only 60% of wild-type Mga activity when expressed in vivo (18). In order to bypass these limitations, a carboxy-terminal fusion of six histidines to the full-length Mga from the M6 GAS strain JRS4 (Mga-His; 63 kDa) was constructed and purified
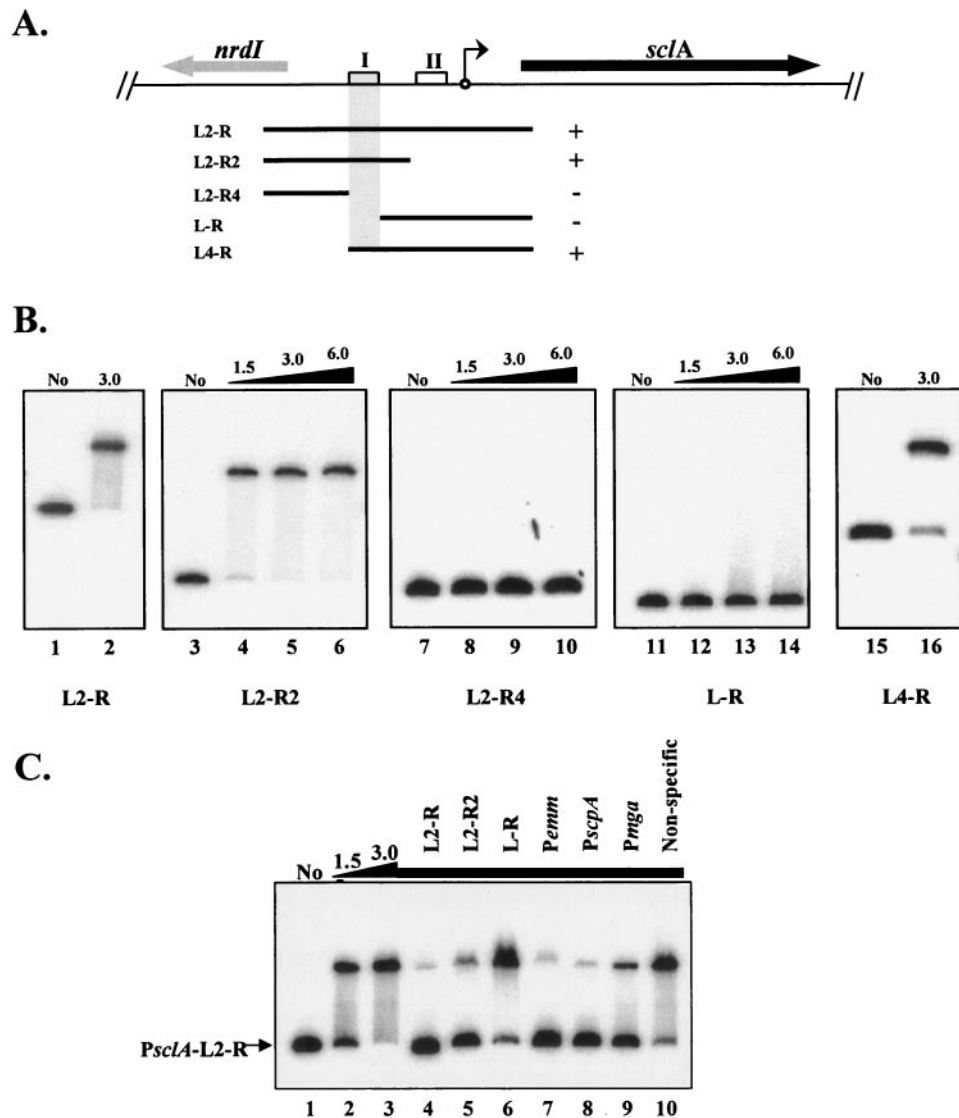
A.

B.

C.

FIG. 3. Identification of a specific Mga-binding site in the *sclA* promoter region. The ability of purified serotype M6 Mga-His to bind different PCR-generated P*sclA* promoter probes was determined by EMSA. (A) Schematic representation of the M1 GAS genomic region surrounding *sclA*, including *nrdI* encoding a putative ribonucleotide reductase (thick arrows). The start of transcription for *sclA* (circle with arrow) and potential Mga-binding sites (boxes) are shown. The ability of purified Mga-His to bind to overlapping PCR-amplified promoter probes (thin lines) in vitro is represented by either plus (+) or minus (−). The defined binding region is shaded. (B) EMSAs of P*sclA* promoter probes shown above: P*sclA*-L2-R (lanes 1 and 2), P*sclA*-L2-R2 (lanes 3 to 6), P*sclA*-L2-R4 (lanes 7 to 10), P*sclA*-L-R (lanes 11 to 14), and P*sclA*-L4-R (lanes 15 and 16). Constant amounts (1 to 2 ng) of labeled promoter probes were incubated with increasing amounts (1.5 to 6.0 μg) of Mga-His for 15 min at 16°C prior to separation on a 5% polyacrylamide gel. Shown are results representative of at least two separate experiments performed with independently purified protein preparations. (C) The specificity of Mga-His binding to P*sclA* was assayed by addition of unlabeled competitor promoter probes (Table 1). Radiolabeled P*sclA*-L2-R probe was analyzed by EMSA as described above following incubation with 0, 1.5, and 3.0 μg of Mga-His (lanes 1 to 3). To the remaining binding reaction mixtures (lanes 4 to 10, 3.0 μg of Mga-His), a constant amount of unlabeled competitor probe (750 ng) corresponding to P*sclA*, P*emm*, P*scpA*, P*mga*, and a nonspecific *rpsL* fragment was added using the PCR primer pairs listed in Table 1.

from *E. coli* lysates as described in Materials and Methods. Purified Mga-His showed DNA-binding activity to known binding sites in vitro comparable to that of MBP-Mga and was capable of wild-type transcriptional activation of Mga-regulated genes in vivo (data not shown).

All three promoter probes that contained the distal P*sclA*-I binding site demonstrated a slower migration consistent with binding when incubated in increasing amounts of the purified Mga-His protein (Fig. 3B; L2-R, L2-R2, and L4-R). In contrast, no detectable binding of Mga-His was observed to a probe that lacked the distal site I but possessed the proximal P*sclA*-II binding element and the *sclA* start of transcription (see above) at any of the concentrations tested (Fig. 3B; L-R). Furthermore, no additional binding sites were found in the region located upstream of P*sclA*-I (Fig. 3B; L2-R4). Thus, Mga appears to show in vitro DNA binding only to the distal P*sclA*-I binding site situated more than 100 bp upstream of the Mga-regulated start of transcription.

Specific binding of Mga-His to P*sclA*-I in vitro. The specificity of Mga-His binding to the P*sclA*-I element was investi-
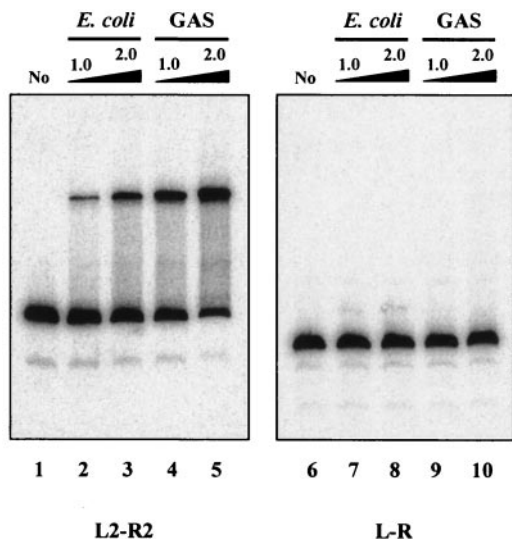
FIG. 4. Binding of native and recombinant Mga to *sclA* promoter region. M6 Mga-His proteins were purified either from *E. coli* (lanes 2, 3, 7, and 8) or directly from GAS (lanes 4, 5, 9, and 10) and compared in their abilities to bind both P*sclA* site I (L2-R2, lanes 1 to 5) and P*sclA* site II (L-R, lanes 6 to 10) in an EMSA. Radiolabeled promoter probes (1 ng) were incubated with 1.0 or 2.0 μg of each Mga-His protein for 15 min at 16°C followed by separation on a 5% polyacrylamide gel. Probes were amplified from M1 SF370 by using the primers listed in Table 1.

gated by EMSA with the large P*sclA*-L2-R probe (Fig. 3A) in the presence of different unlabeled competitor probes (Fig. 3C). The bound species was chased back to unbound only in the presence of cold probes containing P*sclA*-I (L2-R and L2-R2) and not when unlabeled P*sclA*-II probe (L-R) was added (Fig. 3C, lanes 3 to 6). Mga binding at P*sclA*-I was also competed by addition of other established Mga-binding site probes such as P*emm*, P*scpA*, and P*mga* but not a nonspecific probe internal to *rpsL* (Fig. 3C, lanes 7 to 10). In fact, an Mga mutated in the major DNA-binding domain (HTH-4), necessary for interaction at all known Mga-binding sites (17), was also incapable of binding to P*sclA* at any concentration tested (data not shown). These results verify that Mga binds in a highly specific fashion to P*sclA* and further demonstrate that this interaction occurs only at the upstream P*sclA*-I site and not at the promoter-proximal P*sclA*-II site.

Comparison of *E. coli*- and GAS-purified Mga. It is possible that Mga-His purified from a heterologous *E. coli* host lacks a modification that allows it to bind P*sclA*-II in vitro. To investigate whether Mga obtained from its native background possesses a different in vitro binding activity towards P*sclA*, Mga-His was purified directly from GAS lysates as described in Materials and Methods. EMSA was then performed on P*sclA*-I and P*sclA*-II by using Mga-His purified from both sources (Fig. 4). Interestingly, Mga purified from GAS does appear to bind P*sclA*-I more efficiently (Fig. 4, lanes 4 and 5) than does an equal quantity of Mga purified from *E. coli* (Fig. 4, lanes 2 and 3). Binding to the proximal P*sclA*-II with equivalent amounts of either protein was not observed (Fig. 4, lanes 7 to 10). However, upon significant overexposure of the image, barely detectable binding of GAS-purified Mga-His to P*sclA*-II was seen (data not shown). Thus, Mga may bind to the proximal

P*sclA*-II site but with markedly lower affinity than that observed for the distal P*sclA*-I site.

Mga-dependent regulation of P*sclA* is not observed if located on a multicopy plasmid. To investigate the role of Mga binding to the upstream P*sclA*-I site in regulation of *sclA* expression, a transcriptional reporter system based on GFP was developed. Use of GFP as a reporter allows direct quantification of promoter activity via levels of fluorescence without the need to lyse the bacteria or add reagents. Unfortunately, attempts to generate single-copy promoter fusions of the strong P*emm* promoter to *gfp* in the chromosome of GAS did not allow detection over background levels (data not shown). Therefore, a multicopy GFP transcriptional reporter plasmid called pKSM410 was constructed that is capable of replicating in GAS as well as *E. coli* (see Materials and Methods).

Promoter fragments corresponding to P*emm* (pKSM425), the entire *sclA-nrdI* intergenic region (pKSM412), and P*sclA* lacking the distal P*sclA*-I Mga-binding site (pKSM414) were cloned in front of the promoterless *gfp* in pKSM410 (Fig. 5A) and introduced into both wild-type MGAS166 and *mga*-inactivated MGAS166.165-L serotype M1 GAS. Strains were grown to late logarithmic phase, and the levels of fluorescence were quantified using a fluorometer as described in Materials and Methods. As expected for an Mga-regulated promoter, the control P*emm* construct pKSM425 showed extremely high expression in MGAS166 but was reduced 7.5-fold in MGAS166.165-L (Fig. 5B; pKSM425). However, the full-length P*sclA*-L2-R construct pKSM412, which has both the P*sclA*-I binding site and the Mga-regulated start of transcription, demonstrated very little promoter activity over background and was not affected by the presence or absence of Mga (Fig. 5B; pKSM412). Likewise, the transcriptional start site without the distal P*sclA*-I binding site showed little change in activity in the presence or absence of Mga (Fig. 5B; pKSM414). Therefore, the presence of the upstream Mga-binding site P*sclA*-I in the *sclA* promoter was not sufficient to show Mga-regulated *gfp* expression in vivo and suggests that additional *cis* sequences not included in this construct are required.

P*sclA*-I Mga-binding site is essential for Mga regulation of P*sclA* at its native chromosomal location. Due to the lack of Mga-dependent regulation of P*sclA* in a plasmid system, analysis of the promoter at its native chromosomal location was undertaken. Initial attempts to introduce a deletion of the P*sclA*-I binding site into the MGAS166 chromosome via allelic exchange were unsuccessful, possibly due to the concomitant deletion of the divergently transcribed *nrdI* promoter (Fig. 2B; data not shown). Therefore, a merodiploid approach was used to allow the temperature-sensitive integration of single-copy P*sclA*-*gusA* reporter fusions into the native chromosomal location while retaining a second wild-type promoter expressing both *nrdI* and *sclA* (Fig. 6A). Merodiploid insertion-duplication mutants containing either wild-type P*sclA* (KSM421) or the P*sclA*-I deletion allele (KSM423) fused to a promoterless *gusA* were constructed in MGAS166 (Mga⁺) as well as MGAS166.165-L (Mga⁻), and GusA liquid assays were performed on exponential-phase cultures as described in Materials and Methods. The wild-type KSM421 exhibited strong Mga-regulated GusA activity, whereas the P*sclA*-I deletion strain KSM423 showed GusA activity at background levels regardless of the presence or absence of Mga (Fig. 6B). These
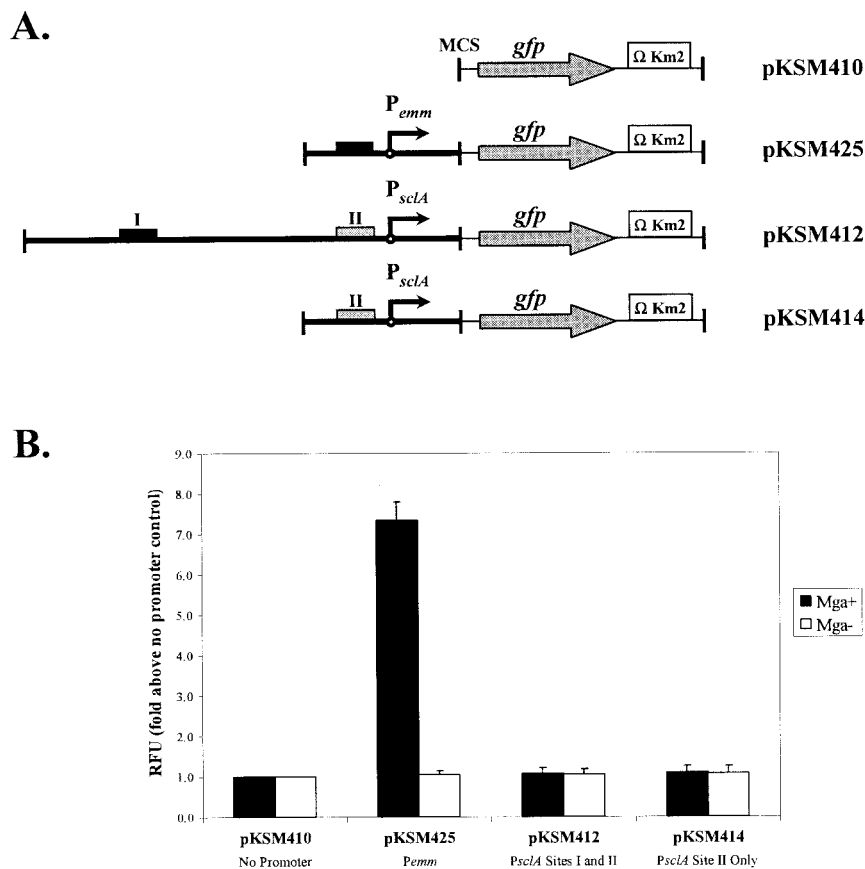
A.

B.

FIG. 5. Analysis of P*sclA* activity in vivo using a GFP reporter plasmid. (A) Schematic representation of different promoter fusions in the GFP transcriptional reporter plasmid pKSM410 as described in Materials and Methods. Plasmids pKSM425 (P*emm1*), pKSM412 (P*sclA*-L2-R), and pKSM414 (P*sclA*-L-R) are shown. The multiple cloning site (MCS), starts of transcription (circles with arrows), modified promoterless *gfp* (gray arrows), kanamycin resistance cassettes containing T4 transcriptional terminators (open boxes), and functional (dark boxes) or nonfunctional (gray boxes) Mga-binding sites are indicated. (B) GFP fluorescence assay on M1 GAS strain MGAS166 (Mga$^+$, dark bars) or MGAS166.165-L (Mga$^-$, white bars) containing the reporter plasmids described above (shown on bottom). Equal numbers of cells taken from late-logarithmic growth were used for each assay. Results are presented as fold increase above the promoterless control strain containing pKSM410 (shown as 1.0). Data represent at least two independent experiments, and standard error bars are provided. RFU, relative fluorescence units.

results support in vivo the results of the in vitro EMSAs and indicate an essential role for the distal P*sclA*-I binding site in the Mga-dependent expression of *sclA*.

DISCUSSION

What constitutes an Mga-binding site? Since every Mga-regulated gene described to date encodes a protein involved in GAS pathogenesis, the ability to identify potential Mga-regulated genes through an Mga-binding site in their promoter would be highly advantageous. The published consensus Mga-binding element was constructed using known binding sites (P*emm* and P*scpA* from serotype M6) as well as predicted sites from sequenced promoters corresponding to *emm*, *mrp*, *arp*, *enn*, and *scpA* from various other GAS serotypes (15). The resulting 45-bp consensus element contains three regions of conserved nucleotides totaling 29 nucleotides separated by several stretches of irrelevant nucleotides (Fig. 1A). However, this consensus was not able to predict the location of the two Mga-binding sites found in P*mga* (19), indicating that Mga can interact with diverse sequences and that any true consensus must reflect these various sites. Since Mga appears to use the

same two helix-turn-helix domains of the protein for binding to all of the known promoter sites (17), there likely exists a core consensus binding element that is shared by all sites.

Although the two sites identified within P*sclA* shared significant identity (72 and 66%, respectively) to the nucleotides in the published consensus, we have shown here that Mga was able to bind only the distal P*sclA*-I site in vitro (Fig. 2 to 4). In fact, P*sclA*-I contains only four nucleotides identical to conserved consensus sequences that are lacking in P*sclA*-II (Fig. 1A, asterisks). Thus, it is likely that one or more of these nucleotides are critical for the in vitro interaction of Mga and provide excellent targets for mutational analysis of Mga-binding sites. Attempts to introduce both P*mga* and the P*sclA*-I binding site into the Mga-binding consensus resulted in a very nonspecific sequence consisting of two short stretches of primarily adenine nucleotides (data not shown). Therefore, future experiments will focus on changing the nonbinding P*sclA*-II sequence to resemble P*sclA*-I to allow for a gain-of-function phenotype and identification of those nucleotides that are essential for binding. These types of directed biochemical and mutational analyses of Mga-binding sites should provide a
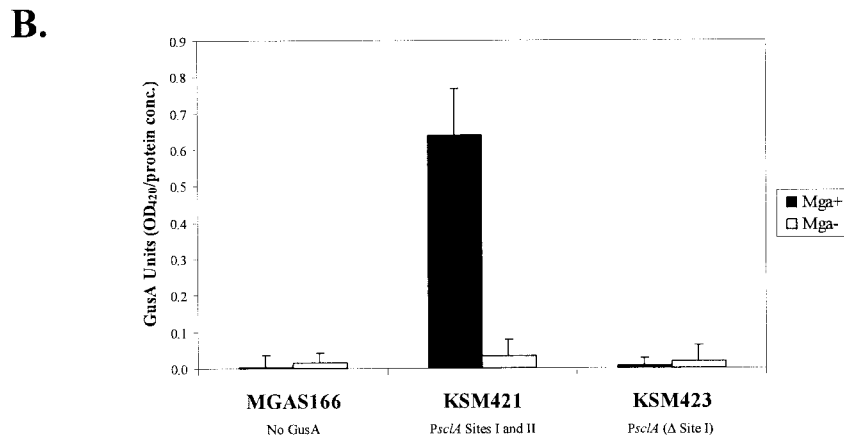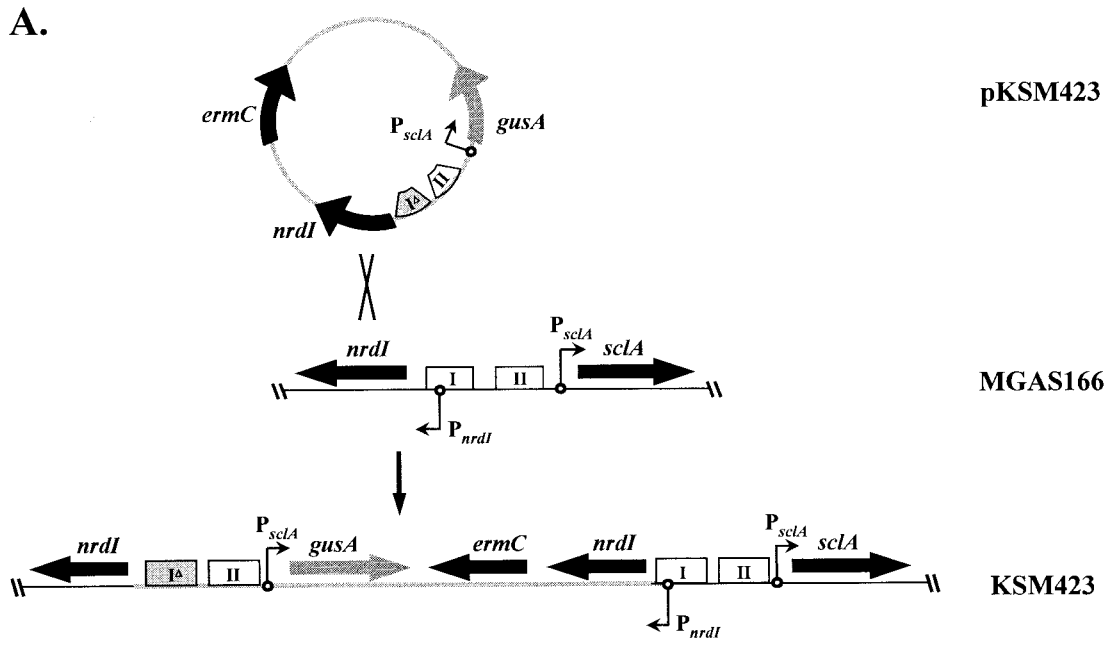
FIG. 6. Analysis of P*sclA* activity in vivo with a chromosomal GusA reporter. (A) Construction of an insertion-duplication allele that produces a chromosomal P*sclA* fused to a *gusA* transcriptional reporter while allowing the preservation of the *nrdI* promoter. Briefly, the P*sclA-gusA* transcriptional fusion was cloned into a temperature-sensitive plasmid (pKSM423). Integrants into the chromosome of MGAS166 were isolated after growth at the nonpermissive temperature, which created the strain KSM423. Genes (thick black arrows), *gusA* (thick gray arrows), starts of transcription (circles with arrows), and wild-type (open boxes) and mutated (gray boxes) Mga-binding sites are indicated. (B) GusA reporter assay on M1 GAS strains MGAS166 (Mga$^+$), MGAS166.165-L (Mga$^-$), KSM421 (Mga$^+$, P*sclA-gusA*), KSM421.165-L (Mga$^-$, P*sclA-gusA*), KSM423 (Mga$^+$, P*sclA-Δ* site I-*gusA*), and KSM423.165-L (Mga$^-$, P*sclA-Δ* site I-*gusA*). Data are reported in GusA units (OD$_{420}$/concentration of total protein [micrograms per microliter]) and represent an average of the results of three independent experiments. The error bars express the standard deviation for each strain measured.

more reliable way than sequence alignment to establish the essential core Mga-binding sequence.

Interestingly, when Mga is purified from GAS compared to being purified from *E. coli*, binding to P*sclA*-I is noticeably enhanced (Fig. 4). In addition, Mga purified from *E. coli* is still sufficient to activate transcription of Mga-regulated promoters in an in vitro transcription assay (A. C. Almengor and K. S. McIver, unpublished results), but not as effectively as Mga purified from GAS. This difference may indicate several possibilities: that an additional factor present only in GAS copurifies with Mga and augments its activity, that a specific mod-

ification of Mga occurs in GAS and not *E. coli*, or simply that the recombinant expression of Mga in a heterologous host is not optimal for its activity (e.g., improper folding). Regardless of the mechanisms involved, these results support the importance of using Mga purified from GAS in future experiments dealing with its activity.

P*sclA* represents a new category of Mga-regulated promoters. Mga is a DNA-binding protein that functions through direct interaction at binding sites within its target promoters. Based upon the biochemical analysis of three Mga-regulated promoters from the serotype M6 strain JRS4 (15, 19), the

location of Mga binding at these promoters falls into two types. In the case of P*emm* and P*scpA*, Mga binds to a single site centered at −51 bp from the start of transcription and juxtaposed with the −35 hexamer. This places Mga in a position similar to class I transcriptional activators that stabilize RNA polymerase through direct contact with the carboxy-terminal domain of the α subunit and increase initiation (11, 15). In contrast, P*mga* contains two Mga-binding sites situated further upstream (−100 bp and −181 bp) from the P2 start of transcription, and both of these sites are required for the autoactivation of the downstream start site (19). Currently, it is not clear by what mechanism Mga is able to activate transcription initiation at such a distance.

In this study, we have shown that Mga binds specifically within the promoter of the Mga-regulated *sclA*, which encodes a streptococcal collagen-like protein involved in GAS virulence. Although the *sclA* promoter (P*sclA*) contains two potential binding sites based upon homology to the published consensus sequence, Mga interacts only with a single binding site (P*sclA*-I) located upstream (−168 bp) of the Mga-regulated start of transcription (Fig. 2 to 4). Additionally, the distal P*sclA*-I was found to be necessary for activation of *sclA* transcription in vivo (Fig. 6). The location of P*sclA*-I is somewhat similar to the most distal P*mga* Mga-binding site (−181 bp) and may suggest some commonality of activation between these two promoters. Activators that bind at a distance will often use DNA looping or bending to bring the regulator into physical contact with the transcriptional machinery (2, 10). On the other hand, the fact that P*sclA* uses only a single bound Mga, while autoactivation at P*mga* requires two binding sites, suggests that P*sclA* represents a unique type of Mga-regulated promoter. Unpublished work from our laboratory indicates that an Mga-regulated promoter (P*sof*) from a class II strain of GAS also possesses a single distal Mga-binding site of this type.

Based on the differences observed in the location of Mga-binding sites with respect to the start of transcription in Mga-regulated promoters, we propose that the promoters be grouped into three categories: a single proximal binding site (category A, P*emm* and P*scpA*), a single distal binding site (category B, P*sclA* and P*sof*), and two distal binding sites (category C, P*mga*). At some time in the past, the second putative P*sclA* binding site (P*sclA*-II) may have been fully functional but is no longer utilized to regulate *sclA* expression. Alternatively, P*sclA*-II may be functional for binding only under very specific temporal or environmental conditions in vivo. Evidence of the possible functionality of P*sclA*-II comes from our observation of barely detectable Mga binding to this site upon significant overexposure of the EMSA, several orders of magnitude below that seen at P*sclA*-I (data not shown). Therefore, P*sclA* may have once represented an Mga-regulated promoter containing both a proximal (category A) and a distal (category B) binding site. However, P*sclA* has evolved such that it no longer requires the proximal site for Mga-specific activation.

Mga regulation of P*sclA* occurs at its native locus but is not observed on a multicopy plasmid. Expression of *sclA* has been shown previously to be strongly activated at the level of transcription in wild-type M1 GAS strains but not in isogenic strains lacking Mga (Fig. 1B) (14, 27). Our primer extension data confirm these results, showing a strong start of transcription in total RNA isolated from wild-type M1 strain SF370 but

not in the *mga*-inactivated KSM165-L (Fig. 2A). Thus, Mga appears to control *sclA* by activating transcriptional initiation at P*sclA*. The ability of Mga to bind to the promoter-distal P*sclA*-I suggests strongly that interaction at this site is directly involved in the observed activation of the downstream transcriptional start site. Further, the presence of P*sclA*-I at its native position in the GAS chromosome is essential to the activation of *sclA* transcription in vivo with a GusA reporter system (Fig. 6). However, when a large (500-bp) region of P*sclA*, including the P*sclA*-I binding site and the downstream start site, was inserted in a transcriptional reporter plasmid based on a promoterless *gfp*, it did not exhibit detectable Mga-specific activation, nor did it demonstrate significant promoter activity over background levels of the system (Fig. 5). In contrast, a control plasmid containing the category A P*emm*-GFP produced very strong Mga-specific activation in the GFP reporter system. There are several possible explanations for why Mga regulation of *sclA* occurs only in a single copy at its native locus and not on a multicopy plasmid. First, one or more *cis*-acting elements located either further upstream or within *sclA* itself may be required for Mga regulation. Alternatively, there may be a gene dosage effect observed in the plasmid system, resulting in the titration of a factor necessary for Mga regulation to occur. Finally, DNA looping may be required to bring Mga in contact with the RNA polymerase, and this event may not be occurring properly in our plasmid system.

Upstream of *sclA* in the SF370 M1 genome is the divergently transcribed *nrdI* gene, which is predicted to encode a putative ribonucleotide reductase (Fig. 2B). As an internal control for primer extension of P*sclA*, we mapped the start of transcription for *nrdI* and found that it was located in the center of the P*sclA*-I Mga-binding site on the antisense strand. Although this might be expected to result in the Mga-specific repression of *nrdI* transcription, a primer extension product was clearly observed in SF370, an Mga⁺ strain (Fig. 2A). In fact, the presence of the *nrdI* promoter in the middle of the distal Mga-binding site may have hindered our attempts to delete P*sclA*-I at its native site in the chromosome (data not shown), suggesting the importance of *nrdI* in growth under the conditions used in this study. This suggests an even more complex interplay among Mga, P*sclA*, and the overlapping P*nrdI* regions that must be further explored.

In order to assess the importance of the distal Mga-binding site of P*sclA* while avoiding the deletion of P*nrdI*, single-copy P*sclA*-*gusA* reporter fusions were integrated into the native chromosomal location such that a second wild-type promoter expressing both *nrdI* and *sclA* was preserved in each case. These integrations clearly showed that Mga-regulated activity requires the distal Mga-binding site (Fig. 6). As stated above, several reasons for Mga regulation of *sclA* only at its native locus can be proposed, including DNA topology and gene dosage from a multicopy plasmid. These possibilities might explain why a category A Mga-regulated promoter (P*emm*), which presumably does not require DNA looping, shows significant Mga-specific activity in the plasmid-based GFP reporter whereas a category B P*sclA* does not function in the same way. However, it is clear that the ability of Mga to regulate P*sclA* from a distal binding site is more complex than activation from a proximal site and may require additional factors.

In conclusion, we have demonstrated that Mga binds to a

single distal binding site within the *sclA* promoter but not to a second putative site located proximal to the Mga-regulated start of transcription. Further, P*sclA*-I is required for in vivo transcriptional activation of *sclA*. Notably, an alignment of sequences for P*sclA*-I found in multiple serotypes of GAS showed no differences in key conserved nucleotides. Therefore, P*sclA* and the role of Mga in its regulation appear to be conserved among most serotypes of GAS. Analysis of this novel Mga-regulated promoter should help build our overall knowledge of the molecular mechanisms by which Mga controls virulence in GAS.

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