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The Catabolite Control Protein CcpA Binds to P*mga* and Influences Expression of the Virulence Regulator Mga in the Group A Streptococcus[▽]†

Audry C. Almengor,[1]‡ Traci L. Kinkel,[1,2]‡ Stephanie J. Day,[1] and Kevin S. McIver[1,2]*

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9048,[1] and Department of Cell Biology and Molecular Genetics and Maryland Pathogen Research Institute, University of Maryland, College Park, Maryland 20742-4451[2]

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Carbon catabolite repression (CCR) allows bacteria to alter metabolism in response to the availability of specific sugar sources, and increasing evidence suggests that CCR is involved in regulating virulence gene expression in many pathogens. A scan of the M1 SF370 group A streptococcus (GAS) genome using a *Bacillus subtilis* consensus identified a number of potential catabolite-responsive elements (*cre*) important for binding by the catabolite control protein A (CcpA), a mediator of CCR in gram-positive bacteria. Intriguingly, a putative *cre* was identified in the promoter region of *mga* upstream of its distal P1 start of transcription. Electrophoretic mobility shift assays showed that a His-CcpA fusion protein was capable of binding specifically to the *cre* in P*mga* in vitro. Deletion analysis of P*mga* using single-copy P*mga-gusA* reporter strains found that P*mga* P1 and its upstream *cre* were not required for normal autoregulated *mga* expression from P*mga* P2 as long as Mga was produced from its native locus. In fact, the P*mga* P1 region appeared to show a negative influence on P*mga* P2 in these studies. However, deletion of the *cre* at the native P*mga* resulted in a reduction of total *mga* transcripts as determined by real-time reverse transcription-PCR, supporting a role for CcpA in initial expression. Furthermore, normal transcriptional initiation from the P*mga* P1 start site alone was dependent on the presence of the *cre*. Importantly, inactivation of *ccpA* in the M6 GAS strain JRS4 resulted in a reduction in P*mga* expression and Mga protein levels in late-logarithmic-phase cell growth. These data support a role for CcpA in the early activation of the *mga* promoter and establish a link between CCR and Mga regulation in the GAS.

Glucose is the preferred source of energy in many microorganisms, whereby the conversion of glucose into two molecules of pyruvate in the Embden-Meyerhof pathway generates two molecules of ATP. In order for another carbohydrate to be metabolized via this pathway, it first must be converted to glucose, an energy-consuming process. Therefore, bacteria have developed means to ensure that glucose is utilized before other energy sources and to ensure that enzymes necessary for the metabolism of alternative energy sources are available only when glucose is depleted (4). Carbon catabolite repression (CCR) is a process in which enzymes necessary for the metabolism of alternative sugars are inhibited in the presence of glucose.

In gram-positive organisms, regulation of carbon catabolism centers on a component of the phosphoenolpyruvate phosphotransferase system (PTS) (4). The primary purpose of the PTS is to regulate sugar uptake through phosphorylation, which is achieved by shuttling a phosphate from phosphoenolpyruvate to a cytosolic enzyme called EI, to HPr (heat-stable protein), and then to a sugar-specific membrane-bound enzyme, EII, before being attached to an incoming sugar (18). Unlike gram-negative organisms, which use cyclic AMP levels to detect the energy level of the cell, gram-positive bacteria use HPr (6). Therefore, the PTS is multifunctional in gram-positive bacteria, being involved in sugar transport as well as signal transduction in response to sugar availability.

During sugar transport, HPr is phosphorylated on a conserved histidine residue (H15). However, in the presence of glucose, an enzyme called HPr kinase phosphorylates HPr on a serine residue (S46) in response to products of glycolysis (7). This phosphorylation event allows HPr-Ser to bind to the catabolite control protein (CcpA), which belongs to the LacI/GalR family of transcription factors (5). CcpA then is capable of binding with specificity to a catabolite-responsive element (*cre*) located in promoters or coding sequences of genes with products that are involved in the metabolism of alternative sugars. Although CcpA primarily acts to repress expression of these operons, it also is known to activate transcription of genes important for growth in glucose (11, 14).

There is accumulating evidence that CCR is important for virulence in several low-G+C gram-positive pathogens. For instance, CCR has been linked to virulence in *Clostridium perfringens* (42), *Staphylococcus aureus* (29), and *Listeria monocytogenes* (26). In *Streptococcus pneumoniae*, the inactivation of CcpA, also called RegM, significantly attenuated virulence in various mouse models of pneumococcal colonization and infection (13, 16). CcpA (RegM) may be affecting systemic infection through the control of capsular gene expression (13), whereas its influence on mucosal colonization and pneumoniae could reflect alterations in basic cellular metabolism (16).

* Corresponding author. Mailing address: Department of Cell Biology and Molecular Genetics, Maryland Pathogen Research Institute MPRI, University of Maryland, College Park, MD 20742-4451. Phone: (301) 405-4136. Fax: (301) 314-9489. E-mail: kmciver@umd.edu.

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‡ These authors contributed equally to this work.

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The group A streptococcus (GAS; *Streptococcus pyogenes*) is an important gram-positive pathogen capable of eliciting a wide array of diseases in humans. Surface M protein is a major virulence determinant that is capable of such functions as inhibiting phagocytosis, binding fibrinogen, and facilitating host cell invasion. Previous studies on the GAS have shown that M protein production is affected by the sugar source (33), suggesting that CCR plays a role in its expression. Transcription of the gene encoding M protein (*emm*) is activated by the standalone response regulator Mga, which also is responsible for transcriptional activation of other virulence genes involved in adhesion, invasion, and immune evasion. Expression of *mga* is autoregulated, and its promoter (P*mga*) has two Mga-binding sites (MBSs) as well as two transcriptional start sites, named P1 (distal) and P2 (proximal) based on their proximity to the translational start site of *mga* (25, 30). Mga is known to be responsive to environmental signals such as growth phase, and the entire promoter, including 84 bp of sequence upstream of P1, has been reported to be necessary for full activity (3, 21, 23, 30, 34). Given this information, it was hypothesized that CCR could be involved in the control of M protein expression through the regulation of *mga*. In the present study, the GAS genome was scanned for *cre* based on a published *Bacillus subtilis* consensus. A functional *cre* involved in activation at the P1 start of transcription was identified within P*mga*, suggesting a direct link between carbon metabolism and Mga regulation in *S. pyogenes*.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains used in this study are listed in Table 1. The GAS was cultured static in Todd-Hewitt medium supplemented with 0.2% (wt/vol) yeast extract (THY) at 37°C except where noted, and growth was monitored by optical density (OD) using a Klett-Summerson photoelectric colorimeter with the A filter. *Escherichia coli* was grown shaking in Luria-Bertani medium (LB) at 37°C, and growth was monitored by OD. Antibiotics were used at the following concentrations: ampicillin at 100 μg/ml for *E. coli*; chloramphenicol at 5 μg/ml for *E. coli* and 1.5 μg/ml for the GAS; erythromycin at 500 μg/ml for *E. coli* and 1.0 μg/ml for the GAS; kanamycin at 50 μg/ml for *E. coli* and 300 μg/ml for the GAS; and spectinomycin at 100 μg/ml for both *E. coli* and the GAS.

DNA manipulations. Plasmid DNA was isolated from *E. coli* by alkaline lysis using either the Wizard Miniprep system (Promega) or Maxi/Midi prep purification systems (Qiagen). Chromosomal DNA from the GAS was isolated using the FastDNA kit and a FastPrep cell disruptor (Bio101, Inc.). DNA fragments were isolated from agarose gels using the QIAquick gel extraction kit (Qiagen). PCR for cloning and promoter probes was performed using Platinum Pfx high-fidelity DNA polymerase (Invitrogen), and reactions were purified by using the QIAquick PCR purification system (Qiagen). PCR for diagnostic assays was performed using *Taq* DNA polymerase (New England Biolabs). DNA sequencing was performed either using the Excel II cycle sequencing kit (Epicenter, Inc.), through the McDermott Center at the University of Texas Southwestern Medical Center, or through Genewiz, Inc.

Construction of pKSM711 and purification of GAS His-CcpA. An amino-terminal fusion of 6× His to CcpA from M6 GAS was constructed as follows. A 1,019-bp region containing the entire *ccpA* gene was PCR amplified from serotype M6 GA19681 (Table 1) genomic DNA (gDNA) using the primer pair M6ccpA_NcoI-L and M6ccpA_XhoI-R (Table 2). The resulting product was digested with NcoI/XhoI and ligated into NcoI/XhoI-digested pProEX-HTb to produce pKSM711 (Table 1). Following verification by PCR and DNA sequence analysis, pKSM711 was transformed into BL21[DE3] Gold (Stratagene) for protein expression.

GAS His-CcpA was purified via Ni-nitrilotriacetic acid resin (Qiagen) based on the manufacturer's protocol. Briefly, expression of protein was induced at an OD at 600 nm (OD_{600}) of 0.6 nm for 4 h with 1 mM IPTG, and cell pellets were stored at −80°C. The frozen pellet was lysed in the presence of 1 mg/ml lysozyme and 1× Complete Protease inhibitors (Roche) using a Branson sonicator (5 cycles of 30-s pulses at a 50% duty cycle, output of 7.5). His-CcpA was purified from the resulting lysate over Ni-nitrilotriacetic acid resin under native conditions, and the protein concentration was determined for each fraction using protein assay reagent (Bio-Rad) with an Ultrospec 2100 spectrophotometer (GE Healthcare). Chosen fractions were dialyzed with two buffer changes in 4 liters of TKED buffer (100 mM Tris-HCl, 150 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol), and glycerol was added to 10% prior to storage of protein aliquots at −20°C.

Electrophoretic mobility shift assay (EMSA). Double-stranded DNA probes were generated by annealing 30-bp sense and antisense oligonucleotide pairs representing P*mga*CRE, P*ccpA*CRE, a mutated P*mga*CRE, and randomly rearranged P*mga*CRE or P*ccpA*CRE, termed scrambled (see Fig. 2A). Briefly, gel-purified oligonucleotide pairs were annealed by being heated to 85°C for 5 min in 12.5 μg of each pair in 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ and slowly being cooled to room temperature for 30 min. Annealed oligonucleotides were end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs), and the resulting radiolabeled probes were separated on a 5% polyacrylamide gel and extracted by crush-and-soak elution.

EMSA was performed as described previously (20). Briefly, a constant amount of labeled double-stranded oligonucleotide probe (ca. 1 to 5 ng) and increasing amounts of purified GAS His-CcpA (5 to 20 μM) were used in each reaction. Competition assays were performed by the addition of 700 ng unlabeled double-stranded oligonucleotide probes to binding reaction mixtures. After incubating for 30 min at 30°C, reactions were mixed in 1% vol/vol Ficoll, 0.02% (wt/vol) bromophenol blue and were separated on a 5% polyacrylamide, 10% (vol/vol) glycerol gel at room temperature. Gels were dried under vacuum at 80°C for 1 h and were exposed overnight to a phosphorimaging screen. Screens were scanned using a Storm860 (Amersham Biosciences), and resulting data were analyzed with the ImageQuant analysis software (version 5.0).

Construction of the Mga$^-$ M6 GAS strain RTG229.150Lg. A chloramphenicol-resistant GAS suicide plasmid for inactivation of *mga* was constructed by ligation of a 1.5-kb *cat194* fragment of HincII-digested pLZ12 (31) into XmnI/BsaI-digested pBluescript II KS− to form pBlue-*cat194* (Table 1). A 454-bp internal fragment of *mga* was PCR amplified from M6 JRS4 gDNA using OYR-4/OYL-13 (Table 2) and was ligated into EcoRV-digested pBlue-*cat194* to form pKSM150Lg (Table 1). This plasmid was introduced into RTG229 by electroporation, and chloramphenicol-resistant insertion mutants (RTG229.150Lg) were selected and verified by PCR.

Chromosomal GusA-based transcriptional reporters at the VIT locus. A 706-bp fragment of M6 P*mga* containing sequence downstream of the putative *cre* (see Fig. 3) was PCR amplified from pP*mga*-blue using the 1201/1211 primer pair (Table 2), digested with SspI/EcoRI, and ligated into the HpaI/EcoRI-digested pP*mga-gusA* to form pKSM435 (Table 1). A 520-bp fragment of M6 P*mga* (see Fig. 3) containing sequence downstream of P1 was amplified from pP*mga*-blue (41) using the 1201/OYR-25 primer pair (Table 2), digested with EcoRI, and ligated into the HpaI/EcoRI-digested pP*mga-gusA* (41) to form pKSM427 (Table 1). A 371-bp fragment of M6 P*mga* containing sequences downstream of MBS I (see Fig. 3) was PCR amplified from pP*mga*-blue using the 1201/OYR-1 primer pair (Table 2), digested with EcoRI, and ligated into the HpaI/EcoRI-digested pP*mga-gusA* to form pKSM428 (Table 1). A 265-bp fragment of M6 P*mga* containing sequence downstream of MBS II (see Fig. 3) was PCR amplified from pP*mga*-blue using the 1201/MgaL3_Bam primer pair (Table 2), digested with EcoRI, and ligated into the HpaI/EcoRI-digested pP*mga-gusA* to form pKSM429 (Table 1). A PstI fragment of pP*mga-gusA* containing the *gusA* gene was ligated into the PstI-digested pVIT164 to form pKSM540 (Table 1).

A 381-bp fragment of P*mga* P1 (see Fig. 5) was amplified from the M6 GAS strain JRS4 using the OYR-14/OYL-14 primer pair (Table 2) and ligated into the HpaI-digested pKSM540 to form pKSM444 (Table 1). A 135-bp fragment of P*mga* P1 downstream of the putative *cre* (see Fig. 5) was PCR amplified from the M6 GAS strain JRS4 using the Δ*cre*-R_Bam/OYL-14 primer pair (Table 2) and was ligated into the HpaI-digested pKSM540 to form pKSM445 (Table 1). A promoterless GusA transcriptional reporter plasmid (see Fig. 5) was constructed by ligation of the 1.9-kb BamHI *gusA* fragment from pKSM148 (35) into BamHI-digested pVIT164 (12) to form pVIT-*gusA* (Table 1).

Plasmids pKSM310, pKSM427, pKSM428, pKSM429, pKSM435, pKSM444, and pKSM445 were linearized with PvuII and were introduced into RTG229 (Mga$^+$) and RTG229.150Lg (Mga$^-$) by electroporation. Exchange of DNA at the VIT locus was verified by the presence of kanamycin resistance and erythromycin sensitivity. Additionally, PCR amplification with the primer pair VIT-R1/*gusA*-PE was used to verify the presence of the constructs in the chromosome. Plasmid pVIT-*gusA* was linearized with PvuII and introduced into RTG229 by electroporation to form the control strain VIT-*gusA* (Table 1). Mga was inactivated in this strain by transformation with the *mga* suicide plasmid pJRS586 (25)

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
BL21(DE3)	F$^-$ *ompT hsdSB*(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
DH5α	*hsdR17 recA1 gyrA endA1 relA1*	15
M6 GAS		
JRS4	Streptomycin-resistant derivative of D471	37
GA19681	Clinical invasive isolate	36
KSM310	P*mga* (full)-*gusA* in VIT locus, Mga$^+$	This study
KSM310.150Lg	P*mga* (full)-*gusA* in VIT locus, Mga$^-$	This study
KSM310.700	P*mga* (full)-*gusA* in VIT locus, Mga$^+$ CcpA$^-$	This study
KSM427	P*mga* (ΔP1)-*gusA* in VIT locus, Mga$^+$	This study
KSM427.150Lg	P*mga* (ΔP1)-*gusA* in VIT locus, Mga$^-$	This study
KSM428	P*mga* (ΔMBS I)-*gusA* in VIT locus, Mga$^+$	This study
KSM428.150Lg	P*mga* (ΔMBS I)-*gusA* in VIT locus, Mga$^-$	This study
KSM429	P*mga* (ΔMBS I & II)-*gusA* in VIT locus, Mga$^+$	This study
KSM429.150Lg	P*mga* (ΔMBS I & II)-*gusA* in VIT locus, Mga$^-$	This study
KSM438	P*mga* (Δ*cre*) in native locus	This study
KSM440	P*mga* (full length) in native locus	This study
KSM442	P*mga* (ΔP1) in native locus	This study
KSM435	P*mga* (Δ*cre*)-*gusA* in VIT locus, Mga$^+$	This study
KSM435.150Lg	P*mga* (Δ*cre*)-*gusA* in VIT locus, Mga$^-$	This study
KSM444	P*mga* (Δ*cre*)-*gusA* in VIT locus, Mga$^+$	This study
KSM444.150Lg	P*mga* (P1 only)-*gusA* in VIT locus, Mga$^-$	This study
KSM445	P*mga* (P1 Δ*cre*)-*gusA* in VIT locus, Mga$^+$	This study
KSM445.150Lg	P*mga* (P1 Δ*cre*)-*gusA* in VIT locus, Mga$^-$	This study
RTG229	VIT strain, Mga$^+$	12
RTG229.150Lg	VIT strain, Mga$^-$	This study
VIT-GusA	Promoterless *gusA* in VIT locus, Mga$^+$	This study
VIT-GusA-586	Promoterless *gusA* in VIT locus, Mga$^-$	This study
Plasmids		
pBluescript II KS−	ColE1 ori Amp^r *lacZ*α	Stratagene
pBlue-*cat194*	*cat194* in pBluescript II KS−	This study
pJRS233	Temperature-sensitive shuttle vector	32
pJRS586	M6 *mga* suicide vector, pUC ori	25
pKSM148	P*emm-gusA*	35
pKSM150Lg	Fragment of M6 *mga*	This study
pKSM162	*Pspac-mga* in pEU308 Δ*lacI*^{q}	22
pKSM310	Same as pP*mga-gusA*	41
pKSM427	P*mga* (ΔP1)-*gusA*	This study
pKSM428	P*mga* (ΔMBS I)-*gusA*	This study
pKSM429	P*mga* (ΔMBS I & II)-*gusA*	This study
pKSM435	P*mga* (Δ*cre*)-*gusA*	This study
pKSM436	P*mga* upstream of *cre* in pJRS233	This study
pKSM437	P*mga* downstream of *cre* cloned into pKSM436	This study
pKSM438	P*mga* Δ*cre* in pJRS233, ΩKm2	This study
pKSM439	P*mga* (full) cloned into pKSM436	This study
pKSM440	P*mga* (full) in pJRS233	This study
pKSM441	P*mga* downstream of P1 cloned into pKSM436	This study
pKSM442	P*mga* ΔP1 in pJRS233, ΩKm2	This study
pKSM444	P*mga* (P1 only)-*gusA*	This study
pKSM445	P*mga* (P1 Δ*cre*)-*gusA*	This study
pKSM540	Promoterless *gusA*, pUC ori	This study
pKSM700	Internal fragment of M6 *ccpA* in pJRS233	This study
pKSM711	M6 *his-ccpA* in pProEX-HTb	This study
pLZ12	pSH71 origin, *cat194*	31
pP*mga*-blue	P*mga* in pBluescript II KS−	41
pP*mga-gusA*	P*mga* (full)-*gusA*	41
pProEX-HTb	Expression vector N-terminal 6× His	Invitrogen
pUC4Km2	pMB1 ori, ΩKm2	31
pVIT164	Plasmid vector for integration into Tn*916*	12
pVIT-*gusA*	Promoterless *gusA*	This study

to form strain VIT-*gusA*-586 (Table 1). Complementation of the Mga$^-$ GusA reporter strains with constitutively expressed *mga* (P*spac-mga*) was performed by introduction of pKSM162 (22).

GusA assays were performed as previously described (10). Briefly, cells were grown to late logarithmic phase, lysed using a FastPrep cell disruptor (Bio101, Inc.), and assayed for GusA activity. Results are reported in GusA units, which are equivalent to the A_{420} of the lysate divided by the concentration of total lysate protein (in micrograms/microliter).

TABLE 2. PCR primers used in this study

Target and primer	Sequence[a] (5′–3′)	Reference or source
ccpA		
*ccpA*L	TTCAATGGCAACCGTTAG	This study
*ccpA*R	TCCTGACACAAAAGCGAT	This study
*ccpA*R1	CCCTAAGGCTGATTTTAGTATT	This study
M6*ccpA*_NcoI-L	catccatggCTAATACAGATGATACCAT	This study
M6*ccpA*_XhoI-R	gcgctcgagTTACTTAGTTGTCCC	This study
gusA		
gusA-PE	GTTGGGGTTTCTACAGGACG	1
Steph-*gusA*-PE	TTGTTTAAACAAATAGACGA	This study
gyrA RT		
gyrA M1 RT L	CGACTTGTCTGAACGCCAAAG	This study
gyrA M1 RT L	ATCACGTTCCAAACCAGTCAAAC	This study
M13		
1201 M13 Rev	AACAGCTATGACCATGATTACG	Clontech
1211 M13 For	GTTGTAAAACGACAACCAGT	Clontech
mga		
OYL-13	GACGGCAGAGTATCCCTTGT	23
OYR-4	GTACCATCAACATTGCG	25
mga P1 RT		
*mga*P1_L2	TAAATAATGAACAAAAAGGAATAATTGCG	This study
*mga*P1_R2	AATACCTTTCAAATTCTTTCATTAAAATCC	This study
mga RT		
mga M6 RT L	AGATGAATCCAGTTGGTCACTTTTC	This study
mga M6 RT R	AAATCGGTTATGCGTTTGATAGC	This study
P*ccpA*		
P*ccpA*-L1	GCCAATTCAGCTCCCTTT	This study
P*ccpA*-R1	CTTCACGGGCAACATCAT	This study
P*mga*		
Δ*cre*-L	TTTTTGTGAACTGGTTAA	This study
Δ*cre*-R_Bam	cgggatccAATATTGGAGTAAATTGAC	This study
ΔP*mga*_Bam	cgggatccATTTCTAATTGGTCATTAA	This study
MgaL3_Bam	caggatccGGATTTTAATGAAAGAATTT	25
OYL-1	TATGCCATTTATGCTCT	25
OYL-14	GTCACTAACTTAATTAGGT	25
OYR-1	AGAGCATAAATGGCATA	25
OYR-14	AATCTGCGAGATTAGAGTAAT	This study
OYR-17	ATATGGTAGAAGACACTATC	This study
OYR-22	TAGACCCCAAATTCCCGT	25
OYR-25	GGTTGTACCATAACAGTC	22
OYL-1	TATGCCATTTATGCTCT	25
rpsL		
GAS-*rpsL*5	GGTTGATATAGCACTTGGTGAC	This study
GAS-*rpsL*6	GTGCGCCACGAACGATATG	24
Spn-*rpsL*1	GAATGTAGATGCCTACAATTAACCA	24
VIT		
VIT-R1	TCAACGTCGCCATGAAGTAC	This study

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

Construction of P*mga* deletions at the native locus. A 1,196-bp fragment upstream of the putative P*mga cre* was amplified from M6 JRS4 gDNA using the primer pair OYR-17/Δ*cre*-L (Table 2) and was cloned blunt into EcoRV-digested pJRS233 (32) to form pKSM436 (Table 1). A fragment of P*mga* downstream of the *cre* was PCR amplified from JRS4 using the primer pair Δ*cre*-R_Bam/ΔP*mga*_Bam (Table 2), digested with BamHI, and cloned into BamHI-digested pKSM436 to form pKSM437 (Table 1). Similarly, the primer pair OYR-22/ΔP*mga*_Bam (Table 2) was used to amplify wild-type P*mga* from JRS4, digested with BamHI, and cloned into SmaI/BamHI-digested pKSM436 to form pKSM439 (Table 1). Finally, P*mga* ΔP1 was amplified from JRS4 using the primer pair OYR-25/ΔP*mga*_Bam (Table 2), digested with BamHI, and cloned into SmaI/BamHI-digested pKSM436 to form pKSM441 (Table 1). The 2.1-kb SmaI-digested ΩKm2 from pUC4ΩKm2 (31) was cloned into either SmaI-digested pKSM437 or PstI-digested and -blunted plasmids pKSM439 and pKSM441 to form pKSM438 (Δcre), pKSM440 (Full), and pKSM442 (ΔP1), respectively (Table 1). Plasmids were introduced into JRS4 by electroporation at 30°C, and plasmid integrants were selected by passage of cells at 37°C with screening for kanamycin resistance and erythromycin sensitivity as described previously (35). Strains constructed from plasmids pKSM438, pKSM440, and pKSM442 were named KSM438 (Δcre), KSM440 (Full), and KSM442 (ΔP1), respectively (Table 1; also see Fig. 4).

Primer extension analysis. Total RNA was extracted from samples in late-logarithmic phase as described previously (22) using the FastRNA kit and a FastPrep cell disruptor (Bio101, Inc.). Primer extensions were performed on 10 to 20 μg of total RNA as described previously (25) using the primers Steph-*gusA*-PE and GAS-*rpsL*5 (Table 2). Primer extension products were run on a 6% denaturing polyacrylamide gel (Amresco). Gels were dried under vacuum at 80°C for 1 h and were exposed overnight to a phosphorimaging screen. Sequence was generated using a labeled Steph-*gusA*-PE primer on the pKSM444 plasmid.

Real-time RT-PCR. gDNA was removed from total RNA by use of the MessageClean kit (GenHunter Corp.). Real-time reverse transcription-PCR (RT-PCR) was performed on 25 ng of RNA mixed with 5 pmol of each primer, 6.25 U of MultiScribe RT (Applied Biosystems), and 1× SYBR green PCR master mix (Applied Biosystems). Reaction mixtures were transferred in triplicate into a 96-well optical reaction plate (Applied Biosystems), and the plate was covered with optical adhesive covers (Applied Biosystems). An Applied Biosystems 7500 real-time PCR system was used to detect transcript levels in the absolute quantification mode with reaction conditions of 48°C for 30 min, 95°C for 10 min, and

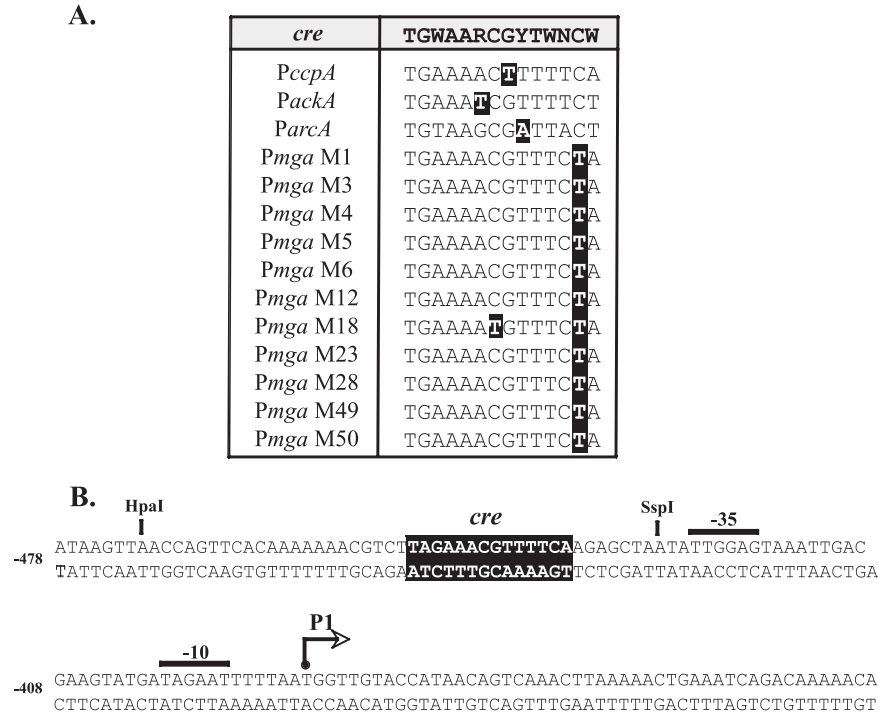

FIG. 1. Alignment of putative *cre* identified in GAS genomes. (A) Possible *cre* from the P*mga* region in 11 different serotypes of the GAS as well as those identified in the promoters of known CcpA-regulated genes P*ccpA*, P*ackA*, and P*arcA* were aligned to the consensus *B. subtilis cre* (shaded at the top) used to identify them. Nucleotides that did not match the consensus are in black boxes. (B) Location of the putative P*mga cre* (black box) relative to the P1 start of transcription in the M1 SF370 GAS genome. The P1 start of transcription (arrow), −10 and −35 hexamers (overlines), and relevant restriction sites are shown. The numbers at left reflect the position relative to the start codon for *mga*.

40 cycles of 95°C for 15 s followed by 60°C for 1 min. Analysis of data was performed using Sequence Detection Software, version 1.3 (Applied Biosystems). The *mga* RT and *mga* P1 RT primer pairs (Table 2) were used to detect levels of total *mga* and *mga* P1 transcripts, respectively, in relation to *gyrA* transcript levels (detected with the *gyrA* RT primer pair; Table 2) in RNA isolated from JRS4 (wild type), KSM440 (Full), KSM438 (Δ*cre*), and KSM442 (ΔP1). A standard curve using total RNA was used to quantify the levels of each transcript.

Inactivation of *ccpA* in the GAS. To produce the *ccpA* mutant strain KSM310.700, a 511-bp internal region of *ccpA* was amplified from JRS4 gDNA using the primer pair *ccpA*L/*ccpA*R (Table 2). The resulting fragment was ligated into EcoRV-digested pJRS233 to form pKSM700 (Table 1) and was verified by PCR using the primer pair *ccpA*L/*ccpA*R (Table 2). pKSM700 was transformed into KSM310 at 30°C, and erythromycin-resistant integrants were isolated at 37°C by following the protocol described previously (35). Mutants were verified by PCR using the primer pairs 1201/*ccpA*R1 and 1211/P*ccpA*-L1 (Table 2). GusA assays were performed as described above.

GAS protein extracts and Western blot analyses. Whole-cell GAS protein extractions, separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis, were performed as previously described (22). Blots were incubated with a 1:1,000 dilution of α-Mga-pep2 antiserum (22), incubated with a 1:25,000 dilution of α-rat (Santa Cruz Biotechnologies) horseradish peroxidase-conjugated secondary antibody, and visualized using the Western Lightning chemiluminescence system (Perkin Elmer).

RESULTS

Identification of putative *cre* in the GAS genome. As an initial step to find GAS genes under CcpA-mediated CCR, the genome of the serotype M1 strain SF370 was scanned for putative *cre* based on similarity to the published *B. subtilis* consensus sequence TGWAARCGYTWNCW (39). Allowing for a single mismatch from the consensus, 60 *cre* were identified on the direct strand, and 58 were identified on the complementary strand, resulting in 98 unique sites scattered throughout the genome (see Table S1 in the supplemental material). Many of the putative *cre* in the M1 genome were located upstream or within annotated open reading frames similar to genes regulated by CcpA in *B. subtilis* (27, 28), including *ndk*, *lctE*, and numerous sugar transport operons (Fig. 1; also see Table S1 in the supplemental material). The promoter of *ccpA*, which can be autoregulated (9), also was found to have a *cre* similar to that of the *B. subtilis* consensus, with a single mismatch (Fig. 1A). Likewise, putative *cre* were identified in the promoters of *ackA*, encoding acetate kinase, and *arcA*, encoding arginine deiminase (Fig. 1A), which are positively and negatively regulated by CcpA, respectively, in other gram-positive bacteria (8, 40). Thus, the location of *cre* identified in the GAS genome corresponds to known CcpA-regulated genes.

One such *cre* was identified within the *mga* promoter (P*mga*) upstream of the distal P1 start of transcription (Fig. 1B), and the site was highly conserved among all serotypes of the GAS for which genome sequence is available (Fig. 1A). Based on CcpA studies with *Lactococcus lactis*, the position of the P*mga* P1 *cre* centered at −54.5 bp from the start of transcription strongly suggests that it plays a role in activating P*mga* activity (44).

The catabolite control protein, CcpA, specifically binds to P*ccpA* and P*mga* in vitro. To determine if CcpA interacts with the *cre* identified in our bioinformatic screen of the GAS M1

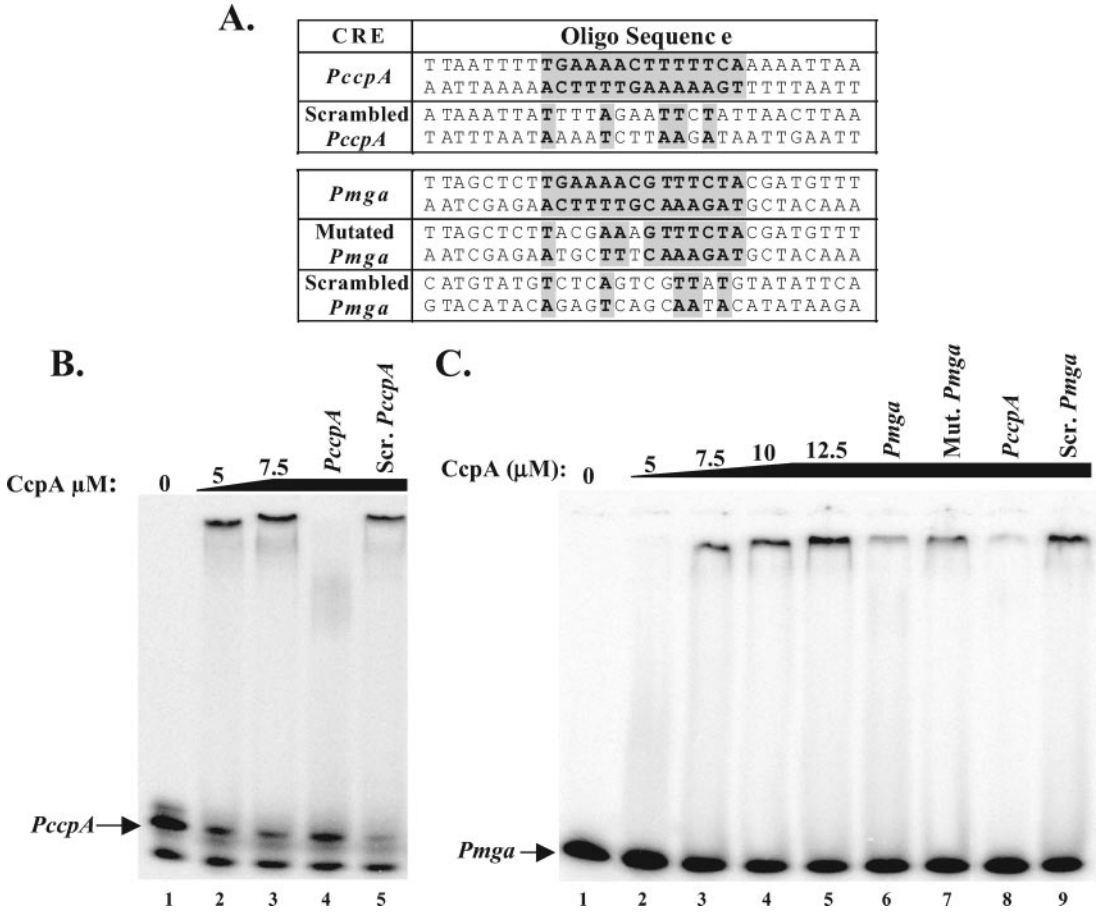

FIG. 2. EMSA of P*mga cre* using His-CcpA. (A) Sequence of annealed oligonucleotide (Oligo) probes used in EMSAs. Putative *cre* are indicated by shaded boxes. Base pairs matching P*mga cre* are shaded and in boldface. EMSA was performed on radiolabeled *PccpA*-annealed (B) or (C) P*mga*-annealed oligonucleotide probes. A constant amount (1 to 2 ng) of [γ-^{32}P]ATP-labeled probe was incubated with increasing amounts (5 to 12.5 μM) of purified GAS His-CcpA for 30 min at 30°C prior to separation on a 5% polyacrylamide, 10% glycerol gel. The specificity of His-CcpA binding to P*mga* was assayed by addition of 700 ng of unlabeled competitor annealed oligonucleotide probes corresponding to P*mga* (lane 6), mutated (Mut.) P*mga* (lane 7), P*ccpA* (lanes 4 and 8), and scrambled (Scr.) *PccpA* (lane 5) or P*mga* (lane 9) using the oligonucleotide pairs listed in Table 2.

genome, EMSAs were performed on P*ccpA* and P*mga*. Double-stranded oligonucleotide probes (30 bp) were generated that contained either the P*ccpA* (positive control) or the P*mga cre* (14 bp each) centered within the sequence (Fig. 2A). To address the specificity of CcpA binding, probes consisting of a random rearrangement of the respective nucleotides (scrambled P*ccpA* and P*mga*) or a probe containing four specific mutations in the P*mga cre* (mutated P*mga*) were generated (Fig. 2A). Since CcpA is capable of binding DNA in the absence of phosphorylated HPr-Ser in vitro (2), assays were performed using purified GAS His-CcpA alone (see Materials and Methods).

Studies with other gram-positive bacteria predict that CcpA will bind to a *cre* located within its own promoter (19, 44); therefore, we first tested the ability of purified His-CcpA to bind to the identified GAS P*ccpA cre* probe (Fig. 2A). Increasing amounts of His-CcpA (5.0 to 7.5 μM) resulted in a mobility shift of labeled P*ccpA*, indicating DNA binding (Fig. 2B, lanes 1 to 3). The addition of 700 ng of cold P*ccpA* to the reaction was able to compete for His-CcpA interaction, whereas the addition of the same amount of a cold scrambled P*ccpA* probe had no effect (Fig. 2B, lanes 4 and 5). Thus, GAS His-CcpA is able to bind specifically to the P*ccpA cre* in vitro.

The P*mga cre* probe also demonstrated slower migration upon addition of increasing amounts of purified His-CcpA (7.5 to 12.5 μM), indicating protein-DNA interaction (Fig. 2C). The 700 ng of cold P*mga* or P*ccpA cre* probe that was added to the reaction was able to compete for binding of His-CcpA to the labeled P*mga* probe to various degrees (Fig. 2C, lanes 6 and 8). In contrast, a scrambled P*mga cre* probe (9/14 mismatches) was not able to compete for His-CcpA (Fig. 2B, lane 9), suggesting that the interaction with P*mga cre* is specific. In support of this conclusion, mutation of only 4/14 nucleotides in the predicted P*mga cre* exhibited an intermediate level of competition (Fig. 2B, lane 7). Thus, GAS His-CcpA is capable of binding directly to the predicted *cre* sequences located upstream of P*ccpA* and P*mga* P1 promoters in vitro.

P1 and *cre* are not essential for P*mga* activity in the presence of existing Mga. To address whether binding of CcpA to the P*mga cre* is important for transcriptional regulation of *mga*, β-glu-

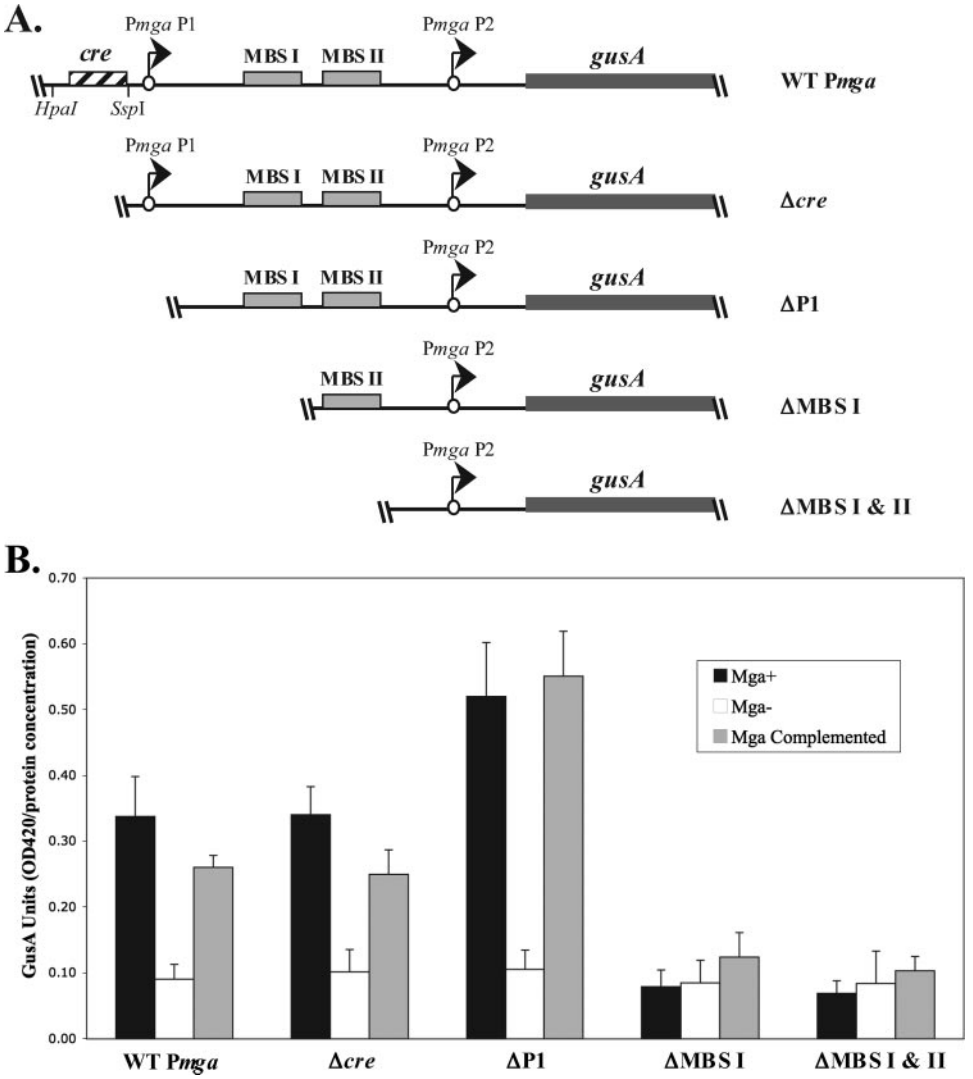

FIG. 3. VIT GusA transcriptional reporter assay for deletion analysis of P*mga*. (A) Schematic representation of *gusA* transcriptional reporters in the chromosomal VIT locus of the M6 GAS corresponding to wild-type P*mga* (KSM310), Δ*cre* (KSM435), ΔP1 (KSM427), ΔMBS I (KSM428), and ΔMBS I & II (KSM429). The starts of transcription for *mga* (circles with arrows), the *gusA* gene (thick line), MBSs (solid boxes), putative *cre* (striped box), and relevant restriction sites are shown. (B) GusA assays for the P*mga-gusA* constructs depicted in panel A inserted into the VIT locus of the wild-type JRS4-derived M6 GAS strain RTG229 (black bars), isogenic *mga*-inactivated strain KSM150Lg (open bars), and *mga*-inactivated KSM150Lg complemented with the P*spac-mga* plasmid pKSM162 (shaded bars). Data are reported in GusA units (OD_{420}/concentration of total protein [in micrograms per microliter]) and represent an average of the results from at least three independent experiments. The error bars express the standard deviations for each strain measured.

curonidase (GusA) transcriptional reporter assays were performed. Various single-copy P*mga-gusA* alleles were constructed in the JRS4-derived M6 GAS strain RTG229 (12) genome at an ectopic site (VIT) separate from the native *mga* locus. Promoter fragments corresponding to the full-length (wild type; KSM310) P*mga*, a deletion of the *cre* only (Δ*cre*; KSM435), a deletion of the entire P1 with *cre* (ΔP1; KSM427), a deletion of P1/*cre* and Mga-binding site 1 (ΔMBS I; KSM428), and a deletion of P1/*cre* and both Mga-binding sites (ΔMBS I & II; KSM429) were introduced into the VIT locus of both a wild-type and Mga^- M6 GAS (Fig. 3A and Table 1). The Mga^- strains then were complemented by introduction of a multicopy P*spac-mga* plasmid for constitutive expression of *mga* (22). Strains were grown to late logarithmic phase, and the levels of GusA activity were quantified (see Materials and Methods).

The wild-type P*mga*, Δ*cre*, and ΔP1 strains all showed Mga-regulated GusA activity, which could be restored upon complementation (Fig. 3B). As predicted from previous studies (25), deletion of either MBS I (ΔMBS I) or both MBSs (ΔMBS I & II), in addition to the upstream P1 promoter and *cre*, eliminated all Mga-dependent regulation of P*mga* (Fig. 3B). Thus, deletion of the *cre* alone or the entire P1/*cre* had little effect on wild-type activity (Fig. 3B), suggesting that the Mga produced from its native locus was sufficient to activate P*mga* at the VIT locus from the P2 promoter alone. Of note, GusA levels appeared to increase slightly upon deletion of P1 (Fig. 3B), in-

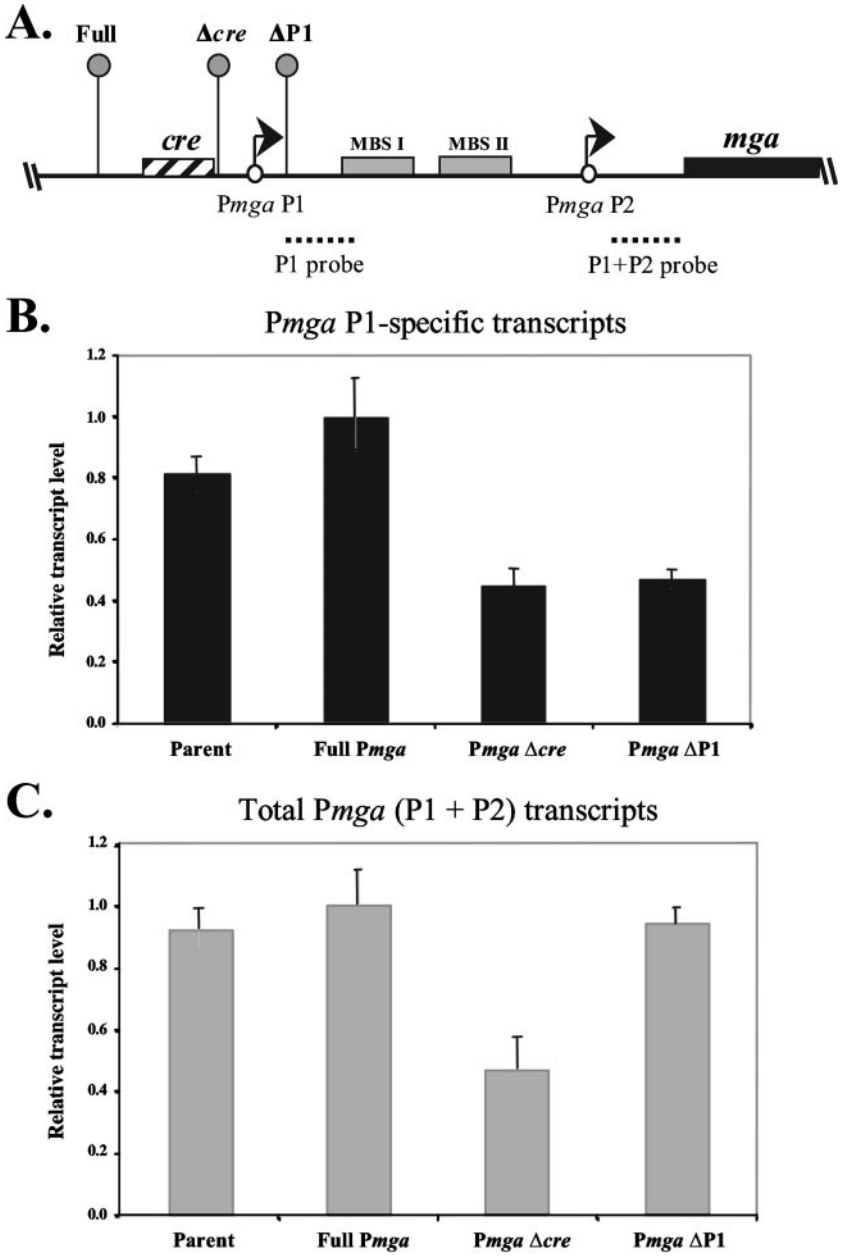

FIG. 4. Real-time RT-PCR analysis of native P*mga* P1 and *mga* transcripts. (A) Real-time RT-PCR was performed on total RNA isolated from JRS4-derived strains KSM440 (Full), KSM438 (Δ*cre*), and KSM442 (ΔP1) as shown in the schematic. The ΩKm2 cassette (lollipops) and all relevant P*mga* elements are shown. The location of the P1 (*mga* P1 RT) and the P1 plus P2 (*mga* RT) probes are indicated (dotted lines). (B) Levels of *mga* transcribed from the P*mga* P1 start site only (black bars) were assessed using the P1 probe. (C) Total P*mga* transcript levels (gray bars) were assessed using the P1 plus P2 probe. Transcript levels are shown as the fold transcript level above that of the full-length promoter (KSM440) that had been normalized to levels of the *gyrA* control. Reactions were performed in triplicate for three independent experiments. Error bars represent the minimum and maximum relative transcript levels based on the standard errors for the samples.

dicating that the P*mga* P1 region also may contain sequences that repress *mga* expression.

The P*mga cre* is necessary for full transcriptional activity of P*mga* at its native locus. To determine whether the *cre* is important for P*mga* activity in the absence of exogenously produced Mga, P*mga* mutations were constructed using the ΩKm2 cassette at the native *mga* locus in the serotype M6 strain JRS4 chromosome. The ΩKm2 cassette has T4 terminators that flank the antibiotic resistance marker to prevent upstream transcription through the element (31). As a wild-type control, the ΩKm2 cassette was inserted by allelic exchange upstream of the wild-type P*mga* promoter (Fig. 4A). To generate P*mga* alleles lacking either *cre* or the entire P1 promoter, the ΩKm2 cassette was inserted either immediately downstream of *cre* (Δ*cre*) or downstream of the P1 transcriptional start site (ΔP1), respectively (Fig. 4A) (see Materials and Methods).

Given that transcript levels from P*mga* P1 often are quite

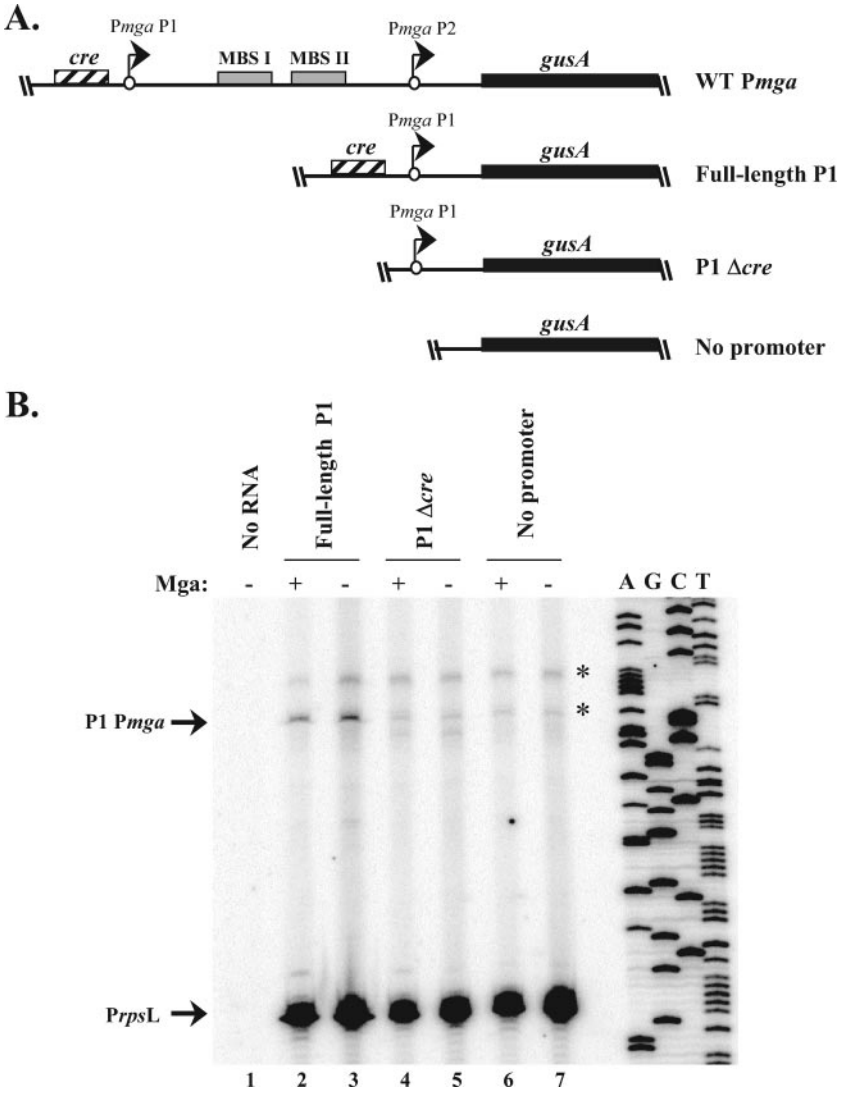

FIG. 5. Deletion analysis of the P*mga* P1 promoter region. (A) Schematic representation of *gusA* transcriptional reporters in the chromosomal VIT locus of the M6 GAS strains KSM310 (WT P*mga*), KSM444 (full-length P1), KSM445 (P1 Δ*cre*), and VIT-GusA (no promoter). The starts of transcription for *mga* (circles with arrows), the *gusA* gene (thick line), MBSs (solid boxes), and the putative *cre* (striped box) are shown. (B) Semiquantitative primer extension analysis was performed on total RNA from the full-length P1, P1 Δ*cre*, and no-promoter reporter strains using the radiolabeled antisense primers Steph_*gusA*-PE for *gusA* and GAS-*rpsL5* for *rpsL* (Table 2) in the same reaction. The starts of transcription for *gusA* (P1 P*mga*) and *rpsL* (P*rpsL*) are shown at the left, and a P*mga* P1 sequence ladder is provided at the right. Nonspecific background bands are indicated with an asterisk.

low (25), quantitative real-time RT-PCR was utilized to assess the effects of promoter deletions on the expression of *mga*. Total RNA was isolated from the wild-type JRS4, full P*mga*, Δ*cre*, and ΔP1 strains grown to late logarithmic phase. Transcript levels of both P*mga* P1 alone (Fig. 4B) and total P*mga* (Fig. 4C) were detected using the *mga* P1 RT and *mga* RT primer pairs (Table 2), respectively, and results were normalized to transcript levels of the housekeeping gene *gyrA*.

As expected, relative levels of *mga* transcribed from P*mga* P1 alone and total P*mga* were similar between the parent strain, JRS4, and the full P*mga* control strain (Fig. 4B and C), indicating that the ΩKm2 cassette inserted upstream of P*mga* had little effect on the transcription of *mga* (Fig. 4B and C). Importantly, deletion of the *cre* in P*mga* caused an approximately twofold reduction in the levels of both P*mga* P1 and total P*mga*, suggesting that the *cre* is important in the activation of *mga* transcription. Unexpectedly, the deletion of the P1 promoter resulted in increased transcript levels from P2 (*mga* P1-specific transcript levels remained low) (Fig. 4B and C). As seen with the GusA reporter assays described above (Fig. 3B), this result supports previous reports of a repressor region in P*mga* near P1 (25).

***cre* is necessary for activation of P*mga* P1.** In order to further assess the effect of the *cre* on the activity of P*mga* P1 alone, a *gusA* transcriptional fusion was made to P*mga* P1 with and without the *cre* in the VIT locus of wild-type and Mga$^-$ M6 GAS to form full-length (*cre*$^+$) and P1Δ*cre* (*cre* mutant) strains, respectively (Fig. 5A). Since the level of GusA activity

from the reporter strains was too low to detect, transcript levels were assessed directly using semiquantitative primer extension. Total RNA was extracted from late-logarithmic-phase cells representing the full-length P*mga* P1, the P1 Δ*cre*, and the no-promoter control *gusA* reporter strains either in the presence or absence of a functional *mga*. Primer extensions were performed simultaneously for both *gusA* and the constitutive *rpsL* for each of the strains.

Promoter-specific products were not observed in the absence of RNA (Fig. 5B, lane 1) or in the no-promoter control strains (Fig. 5B, lanes 6 and 7), with the exception of two light background bands (Fig. 5B). However, a product of the predicted size for P*mga* P1 was detected in the *cre*$^+$ strains (Fig. 5B, lanes 2 and 3), while it was reduced approximately 4.2-fold in the *cre* mutant strains, as determined by densitometry (Fig. 5B, lanes 4 and 5). This correlates with the Δ*cre* results from the real-time RT-PCR analysis of P*mga* P1 at its native locus (Fig. 4B). As expected from the absence of Mga-binding sites in P1, Mga had no detectable effect on transcription from P*mga* P1 (Fig. 5B, lanes 2 to 5) when its transcript levels were normalized to *rpsL*. Thus, the *cre* is necessary for transcriptional activation from the P*mga* P1 start site.

Inactivation of *ccpA* affects *mga* expression. The role of CcpA-mediated activation of P*mga* P1 on *mga* expression in vivo was assessed using an insertion-inactivation mutant of *ccpA* in the M6 P*mga-gusA* VIT reporter strain KSM310 (KSM310.700; Table 2). The *ccpA*-defective strain did not exhibit any significant growth defects compared to growth of the wild-type KSM310 when grown in rich THY medium (data not shown). Inactivation of *ccpA* resulted in a greater than threefold reduction in P*mga*-specific GusA activity compared to the activity of the parental KSM310 samples grown to late-logarithmic phase in THY medium (Fig. 6A). The resulting GusA activity was slightly higher than background levels observed in the Mga$^-$ control strain KSM310.150 (Fig. 6A). In addition, Western analysis of whole-cell extracts found that steady-state levels of Mga also were reduced in the CcpA$^-$ mutant at the same point in growth (Fig. 6B). These data indicate that CcpA is necessary for wild-type production of Mga during logarithmic phase, a point in growth when the Mga virulence regulon shows maximal expression (23).

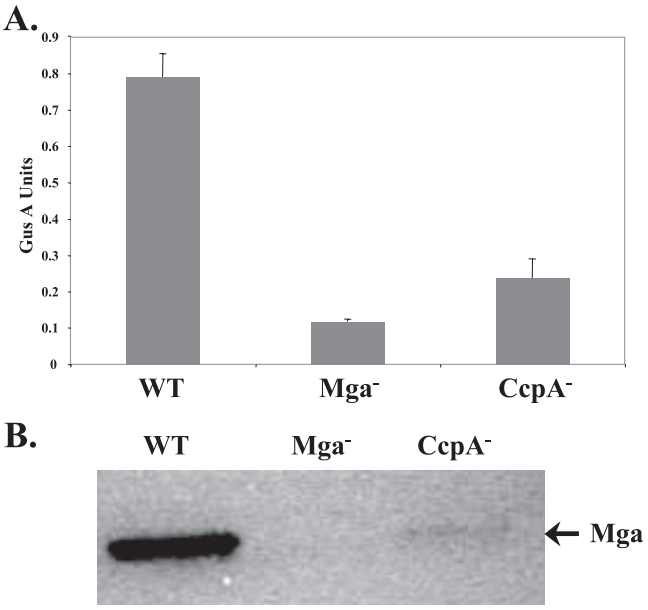

FIG. 6. Effect of a CcpA$^-$ mutant on *mga* expression. (A) GusA reporter assay for the M6 GAS P*mga-gusA* reporter strain KSM310 (WT), the *mga*-inactivated derivative KSM310.150Lg (Mga$^-$), and the *ccpA*-inactivated derivative KSM310.700 (CcpA$^-$). Data are reported in GusA units (OD_{420}/concentration total protein [in micrograms per microliter]) and represent an average of the results from at least three independent experiments. The error bars express the standard deviations for each strain measured. (B) Mga protein production was assessed using Western analysis on whole-cell protein extracts derived from the same samples used for panel A and probed with an anti-M6 Mga antibody.

DISCUSSION

CcpA and *cre* in the GAS. CcpA-mediated CCR of metabolic gene expression through direct binding to catabolite control elements (*cre*) is a highly conserved process in low-G+C gram-positive bacteria (4, 38, 43). Much of our knowledge on this topic comes from extensive studies on *B. subtilis* that have resulted in the determination of a consensus *cre*-binding site as well as the residues of CcpA essential for interaction with *cre* (17, 39). Using the *B. subtilis* consensus *cre* (TGWAARCGY TWNCW) to scan the genome of the serotype M1 SF370 with one mismatch, 98 potential *cre* were identified (Fig. 1; also see Table S1 in the supplemental material). This number is in accordance with the 126 putative *cre* found in a recent survey of the larger *B. subtilis* genome using a slightly more stringent version of the same consensus sequence (27) and with the 82 *cre* found in the *L. lactis* genome based on an *L. lactis* consensus (44). The fact that the majority of the potential GAS *cre* were found near genes regulated by CCR in other gram-positive organisms (data not shown) provides confidence that the overall approach identifies such genes in a heterologous genome. Of particular interest, several *cre* were found associated with genes encoding established virulence factors (e.g., SpeB and Ska) as well as the virulence regulator Mga (Fig. 1A; also see Table S1 in the supplemental material).

In the case of two identified GAS *cre* located in P*mga* and P*ccpA*, specific binding by a purified GAS His-CcpA was demonstrated in vitro (Fig. 2), demonstrating that these are functional CcpA-binding sites. An alignment of a subset of GAS *cre* found that the single-nucleotide mismatches to the *B. subtilis* consensus were located in variable positions in the site (Fig. 1A), suggesting that a GAS-derived consensus *cre* might be somewhat divergent. Furthermore, this would strongly indicate that these nucleotides, although important for *B. subtilis*, likely fall in positions that are not essential for CcpA-*cre* interactions in GAS. Regardless, a more comprehensive analysis utilizing CcpA-binding studies and growth in defined sugar sources will be required to determine whether these *cre* are important for CCR in the GAS.

What is the role of P*mga* P1 in transcription of *mga*? The P*mga* region is quite complex and contains two starts of transcription, a strong gene-proximal P2 start site that is autoactivated by Mga and a weaker distal P1 start site that has been thought to be constitutive. Our studies show that CcpA was able to bind specifically to the putative *cre* located upstream of the P1 −35 region (Fig. 1B and 2) in a position that suggests an

activating function based on recent studies on CcpA in *L. lactis* (44). Furthermore, deletion of the *cre* at the native *mga* locus or in the context of P*mga* P1 alone leads to decreased *mga* transcription (Fig. 4 and 5B). Inactivation of *ccpA* in the serotype M6 JRS4 also shows a reduction in both P*mga* activity and Mga levels in the cell (Fig. 6). Taken together, these results indicate that CcpA can activate the P*mga* P1 start site through the upstream *cre* and that this interaction is important for normal expression of *mga* in the GAS during exponential growth. Previously, P*mga* P1 was thought to provide low-level constitutive expression of *mga* early in growth that, when translated, would amplify its expression from P*mga* P2 (30). Our results suggest a modification of this model, whereby CcpA may activate P*mga* P1 in response to glucose levels in the cell early in growth and provide the initial trigger that leads to high production of Mga and autoactivation at P*mga* P2. Experiments are currently under way to test whether P*mga* P1 expression can be influenced by glucose and CCR via its *cre*.

Earlier P*mga* deletion studies indicated that P*mga* P1 was essential for the expression of *mga* (30). However, deletion of the entire P*mga* P1 and *cre* did not prevent Mga-regulated expression from P*mga* P2 as long as Mga was produced from its native locus (Fig. 3B). Although these data appear to contradict the published findings, the P*mga* P1 deletion constructs used in this study included approximately 20 bp of extra sequence not found in the previous study and used a different reporter gene (30). Therefore, it is possible that this extra sequence is necessary for the normal expression of *mga*.

Rather than being essential to the activity of *mga*, P*mga* P1 may actually play a role in repression. An increase in P*mga* P2 expression is observed upon deletion of the P1 promoter at both its native locus (Fig. 4C) and at an ectopic locus (Fig. 3), suggesting that sequences in this region actually function to repress transcription initiated from P*mga* P2. A repressor has been suggested to function at the MBS (MBS I) in P*mga* (25); however, no such regulator has been identified to date. One possible model is that P*mga* P1 repression is important for fine-tuning *mga* expression later in growth. However, this repression would be neutralized early on in the presence of CcpA and P*mga* P1 *cre* (Fig. 4B). In theory, P*mga* P1 functions to activate *mga* expression levels in conjunction with CCR and suppresses its activity later in growth in the absence of a preferred energy source. Overall, the regulation of *mga* requires a complex interplay between P1, P2, Mga, and other unknown regulators.

CcpA and Mga virulence regulation. In this study, a link between carbon catabolite repression and *mga* regulation in the GAS has been demonstrated. The significance of this regulation may be that Mga virulence gene expression is influenced by the availability of a preferred carbon source during infection. Presumably, this would enable the bacterium to sense its environment and express the genes it requires to survive during early time points in growth. The importance of CCR in the regulation of *mga* may be observed by the strict conservation of the *cre* in an activating position in the *mga* promoter in all sequenced serotypes of the GAS (Fig. 1A). However, this may be unique to *S. pyogenes*, since an examination of the promoters of *mga* orthologs found that this conservation is not consistent across other pathogenic streptococcal species (data not shown). In the GAS, Mga originally may have functioned to regulate genes important in carbon metabolism and later evolved to regulate virulence genes. This hypothesis is supported by a recent microarray analysis of Mga-regulated genes from different serotypes of GAS showing that inactivation of *mga* alters the ability of the GAS to grow in different sugars (36).

It has been known for some time that the expression of Mga is regulated by growth phase, with maximal expression occurring during exponential phase (23). The results presented here may provide a mechanism for this connection. As the GAS colonize a new tissue site with available glucose, the pathogen would enter into exponential-phase growth. CCR would be active at this time, and the expression of *mga* from P*mga* P1 is activated. Once the *mga* transcripts are translated, Mga autoactivates itself from P*mga* P2. Upon depletion of glucose, the bacteria enter stationary-phase growth, and CcpA would no longer activate expression of P*mga* P1. However, since Mga still may be able to activate transcription from P*mga* P2, an unidentified factor that suppresses Mga activity during stationary phase must come into play, either by modifying Mga to inactivate it or through competition for binding at P*mga*. A complete understanding of the events that occur at P*mga* may provide insights into how the GAS is able to coordinate Mga regulation in response to growth-phase-dependent cues.

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